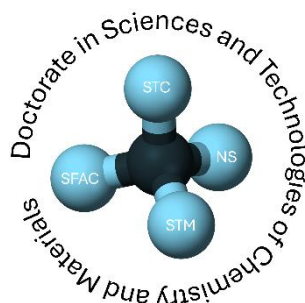


UNIVERSITY OF GENOA



XXXVIII Cycle

**Doctorate in Sciences and Technologies of Chemistry and
Materials**

Pharmaceutical, Food and Cosmetic Sciences

Ph.D. Thesis

**Design, synthesis and biological evaluation of heterocyclic
compounds as potential anticancer agents**

Ph.D. Candidate: Marta Falesiedi

Advisor: Prof. Silvia Schenone

“There is so much more to be done; the patients are waiting”

Dr. Paul Janssen

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PREFACE

This doctoral thesis is the final product of the Ph.D. program in Sciences and Technologies of Chemistry and Materials, curriculum of Pharmaceutical, Food, and Cosmetic Sciences at the Department of Chemistry and Industrial Chemistry (DCCI) of the University of Genoa. The synthetic work was conducted at the Department of Pharmacy under the supervision of Prof. Silvia Schenone and at the Department of Drug Design and Pharmacology at the University of Copenhagen under the supervision of Prof. Bente Frølund. The computational studies presented in this thesis were carried out by Prof. Elena Cichero (University of Genoa) and Dr. Francesco Bavo (University of Copenhagen). *In vitro* and biological data were collected by Prof. Santina Bruzzone (University of Genoa), Dr. Vago (San Raffaele Institute in Milan), Dr. Previtali (University of Copenhagen) and Prof. Bogdanov's group (University of Southampton).

The thesis is organized into three chapters, each corresponding to one of the research lines that have defined the following doctoral activity over the past three years:

1. Structure-based design of pyrazolo[3,4-*d*]pyrimidines as SIRT2 inhibitors and their molecular simplification to diamino-1,3,5-triazines.
2. Pyrazolo[3,4-*d*]pyrimidine derivatives as potential anticancer agents for prostate and bladder cancer: SAR expansion, halloysite nanotube conjugation, and Src inhibition profile.
3. Development of GQX analogues coupled with a ROS-sensitive moiety to afford ROS-responsive prodrugs active on GABA receptors.

Chapters 1 and 2 focus on pyrazolo[3,4-*d*]pyrimidine derivatives, since their scaffold constitutes a privileged chemotype in medicinal chemistry, functioning as an isostere of the adenine ring in ATP, enabling it to emulate the binding interactions at the kinase active site hinge region. This scaffold possesses substantial pharmacological and biological significance, including notable potential as an anticancer agent. The synthesis of amino-substituted pyrazolo[3,4-*d*]pyrimidines SIRT2 inhibitors, guided by structure-based design, and its molecular simplification into diamino-1,3,5-triazines, is discussed in Chapter 1. Moreover, in Chapter 2 pyrazolo[3,4-*d*]pyrimidine derivatives were also explored for potential applications in uro-genital cancer. Diversifying the substituents, a broader structure-activity relationship (SAR) analysis was performed, also enabling both covalent and adsorptive attachment to halloysite nanotubes, a delivery system aimed at improving solubility and therapeutic administration. Current research efforts include Src kinase inhibition profiling, chemical stability evaluation, and studies of carrier conjugation to elucidate factors influencing cellular efficacy and to inform the selection of lead candidates.

Motivated by the emerging evidence for an immunosuppressive role of GABA signalling within the tumour microenvironment, Chapter 3 focuses on the synthesis of GQX (guanidinium–quinoxaline) antagonists, followed by their conversion into ROS-responsive prodrugs through installation of a boron-containing, oxidatively cleavable promoiety. This prodrug strategy is intended to enable site-selective unmasking in ROS-enriched malignant tissues, thereby coupling on-target GABA-receptor antagonism with improved delivery characteristics.

Taken together, the development of diversity-oriented routes to pyrazolo[3,4-*d*]pyrimidines, 1,3,5-triazines, and GQX-based chemotypes yielded previously unreported notable compounds spanning SIRT2 inhibition, c-Src–associated antiproliferative activity, and ROS-triggered GABA-receptor antagonism. These results substantiate the pharmaceutical promise of these heterocyclic frameworks and delineate a coherent platform for subsequent optimization, translational delivery studies, and mechanism-driven biological assessment in oncology-relevant settings.

CHAPTER 1

Research for new heterocyclic compounds active as Sirtuin inhibitors

1. Introduction

1.1. Overview of the Sirtuin family

Sirtuins (SIRT) are evolutionarily conserved class III histone deacetylases comprising seven members in mammals.¹ All SIRTs have a catalytic core that binds nicotinamide adenine dinucleotide (NAD⁺) and, depending on biological context, display distinct substrate preferences. Divergence in the sequence and length of their N- and C-terminal regions underlies differences in localization and function.² An expanding literature links SIRTs to multiple diseases, including cancer and cardiovascular disorders, and highlights the therapeutic promise of SIRT modulators across indications.^{3–6}

The sirtuin field originated with Sir2, identified nearly four decades ago in *Saccharomyces cerevisiae* as a mating-type regulator.⁷ Sir2 was subsequently implicated in transcriptional silencing at rDNA, mating-type loci, and telomeres.⁸ In the late 1990s, Sir2 was shown to extend yeast lifespan by limiting genomic instability.⁹ The discovery that Sir2 is an NAD⁺-dependent histone deacetylase provided a mechanistic framework connecting NAD-driven deacetylation to chromatin silencing, aging, and metabolism, with analogous roles later demonstrated in *Caenorhabditis elegans*.^{10,11} Sir2 homologs have since been identified across bacteria, plants, and mammals, and are collectively termed sirtuins.^{12,13}

Over the years, several members of the sirtuin family have been identified and their physiological roles elucidated. Specifically, the sirtuin family comprises seven isoforms, SIRT1–SIRT7. Differences among these isoforms lead to distinct patterns of intracellular distribution: nucleolus (SIRT7), nucleus (SIRT6), mitochondria (SIRT3/5), cytoplasm and nucleus during the G₂/M transition, associated with chromatin and deacetylates H4K16 (SIRT2), and nucleus with subsequent cytoplasmic shuttling (SIRT1) (Figure 1). This diversity in localization influences biological properties, protein substrates, cellular binding partners, and enzymatic activities.^{14,15} Localization can vary by cell type and cell-cycle phase.¹⁶

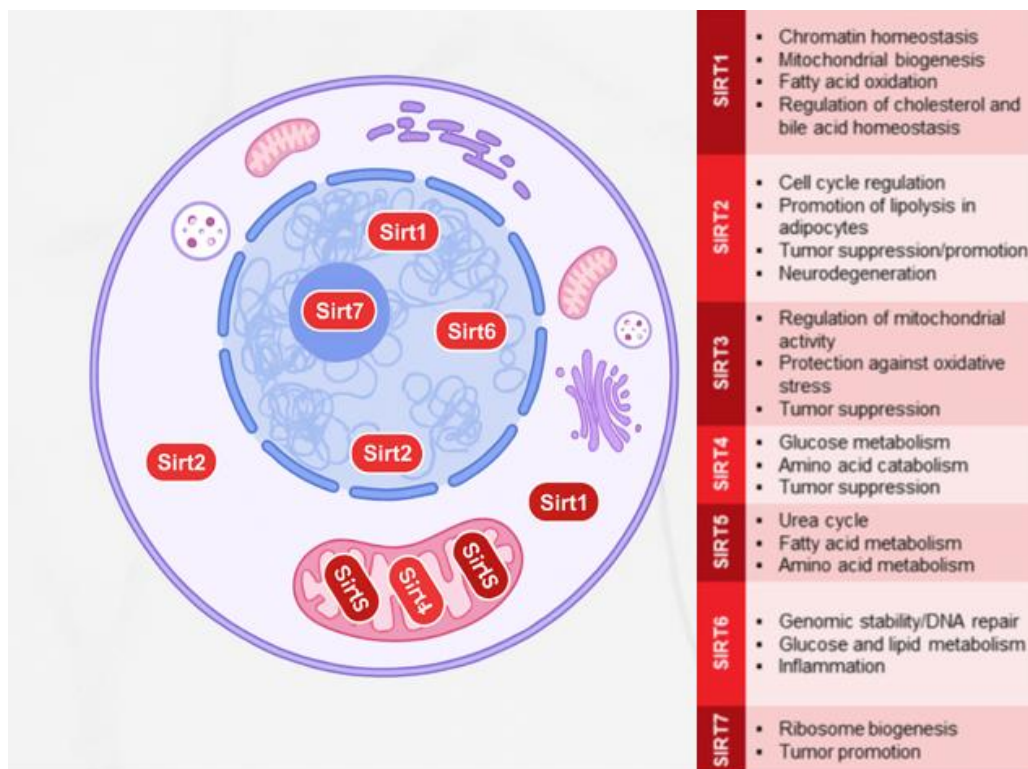


Figure 1. Subcellular sites and biological roles of mammalian sirtuins. SIRT1 resides mainly in the nucleus but can also localize to the cytosol. SIRT2 is chiefly cytosolic with nucleocytoplasmic shuttling. SIRT3, SIRT4, and SIRT5 are mitochondrial, whereas SIRT6 and SIRT7 are nuclear and nucleolar, respectively. Their principal cellular functions are summarized in the accompanying boxes.¹⁷

Despite a conserved catalytic core, subtle differences exist across active-site architectures.¹⁷ X-ray structures show a bilobal core (~275 residues) comprising an α/β Rossmann-like domain for NAD^+ binding and a smaller zinc-ribbon domain; together they form a pocket that accommodates NAD^+ and acetyl-lysine substrates. N- and C-terminal segments vary markedly in length, composition, and susceptibility to post-translational modifications (notably phosphorylation), thereby modulating substrate engagement and regulation.^{2,18–20} Catalysis also differentiates family members. The deacetylation cycle involves (i) binding of NAD^+ and acetyl-lysine, (ii) glycosidic bond cleavage, (iii) acetyl transfer, and (iv) formation of O-acetyl-ADP-ribose, nicotinamide, and deacetylated lysine. Beyond deacetylation, mammalian SIRTs catalyze diverse NAD^+ -coupled reactions: SIRT1–SIRT3 are strong deacetylases; SIRT4 ADP-ribosylates targets (e.g., glutamate dehydrogenase) and suppresses insulin secretion in β cells;¹⁸ SIRT5 removes succinyl, malonyl, and glutaryl groups from lysine residues;^{19,20} SIRT6 exhibits mono-ADP-ribosyltransferase and long-chain deacylase activities;^{21,22} SIRT7, localized to nucleoli, was first characterized as a $\beta\text{-NAD}^+$ -dependent deacetylase that influences RNA

*inflammatory cytokines. (C) SIRT6 also restrains the nuclear factor (NF)- κ B signalling cascade.*²⁸

SIRT6, particularly SIRT1 and SIRT6, modulate dendritic-cell differentiation and macrophage activation by controlling inflammatory mediator output. In dendritic cells, SIRT1 tunes the Th1/Treg balance; SIRT1 deletion reduces Treg induction, biases toward Th1 responses, and heightens T-cell-driven inflammation. In macrophages, SIRT6 loss increases FoxO1 acetylation and stability, amplifying inflammatory tone; re-expression of SIRT6 dampens this response. SIRT3 overexpression favours M2 polarization and attenuates inflammation *in vivo*.^{29–31} SIRT1–SIRT3 generally modulate pro-inflammatory signalling and cytokine release. SIRT1 deacetylates NF- κ B p65 (RelA), reducing NF- κ B activity and TNF- α production in a SIRT1-dependent manner; knockdown elevates, while activation lowers, TNF- α secretion.^{32,33} SIRT6 likewise suppresses NF- κ B-driven IL-6 and TNF- α expression and mitigates reactive oxygen species (ROS)/Akt-dependent inflammatory responses in vascular fibroblasts; SIRT1 exerts similar effects via Toll-like receptor 2 (TLR2)/SIRT1/NF- κ B. In obese septic mice, resveratrol increases SIRT1, reduces leukocyte/platelet adhesion and E-selectin/ICAM-1 expression, and improves survival.^{34–36} SIRT3 restrains IL-1 β and IL-18, limits inflammasome activity, and reduces oxidative stress; its deficiency enhances lipopolysaccharide (LPS)-evoked TNF- α and increases inflammatory cell infiltration.^{37–39}

Although SIRT6s are predominantly anti-inflammatory, context-dependent exceptions exist: early SIRT2 inhibition reduced certain neuroinflammatory markers without reversing cognitive deficits, and combined SIRT2/3 deficiency increased secretion of both pro- and anti-inflammatory cytokines by macrophages.^{40,41}

NF- κ B (classically a p65/p50 heterodimer) is a central inflammatory hub.^{42,43} Upon cytokine stimulation (e.g., IL-1 β , IL-6, TNF- α), NF- κ B translocates to the nucleus; its activity is modulated by post-translational modifications (PTMs) including acetylation.^{44–46} SIRT1 deacetylates p65 at Lys310, suppressing NF- κ B-dependent transcription.^{47,48} SIRT6 attenuates NF- κ B by deacetylating H3K9 at promoters of NF- κ B target genes and by supporting RelA/p65 degradation; decreased SIRT1/6 activity amplifies chronic inflammatory gene expression.^{49,50} SIRT7 shows model-dependent effects on p65 localization and cytokine output, with reports supporting both anti-inflammatory and pro-inflammatory roles.⁵¹ SIRT2 deacetylates p65, thereby limiting cytokines and ameliorating arthritis severity; conversely, SIRT2 inhibition

enhances microglial activation by increasing NF- κ B transcriptional activity.⁵² Overall, SIRT6 dampens inflammation by restricting NF- κ B nuclear translocation, activity, or chromatin access.

Activation of nucleotide-binding oligomerization domain-like receptors containing pyrin domain 3 (NLRP3) follows priming and subsequent assembly, culminating in IL-1 β /IL-18 maturation and inflammatory amplification.^{53–55} SIRT1 negatively regulates NLRP3 activation in several models, including acute liver injury.⁵⁶ SIRT3 reduces ROS accumulation, often a consequence of impaired mitophagy/autophagy, and in turn limits NLRP3 activity, oxidative damage, and cytokine release.⁵⁷ Whether SIRT3 acts directly on NLRP3 remains to be clarified.

Interplay between SIRT6s and noncoding RNAs (ncRNAs) shapes inflammatory signalling. Multiple miRNAs downregulate SIRT1, thereby promoting inflammation; conversely, lowering miR-217 or miR-543 mitigates inflammation through the SIRT1/AMP-activated protein kinase (AMPK)/NF- κ B axis.⁵⁸ miR-378 diminishes SIRT1 and facilitates NF- κ B–TNF- α signalling by targeting AMPK γ 2.⁵⁹ Monocyte chemoattractant protein-induced protein 1 (MCP1) eases inflammation by inducing SIRT1 via miR-9, and SIRT1 suppresses inflammation through the p53/miR-22 pathway, reducing COX-2 and inducible nitric oxide synthase (iNOS).^{60,61} Targeting SIRT6–ncRNA circuits may thus offer therapeutic leverage.

Most SIRT6s exert anti-inflammatory effects, despite their impact can diverge with disease stage and context. Open questions include whether SIRT6 depletion represents a shared pathogenic feature across inflammatory diseases and how to reconcile conflicting observations (e.g., SIRT7, SIRT2). Defining context-specific mechanisms should inform translational strategies.

1.2.2. SIRT6s in metabolism

Metabolism encompasses ordered biochemical reactions that sustain growth, structural integrity, and environmental adaptation. While SIRT3–SIRT5 act primarily in mitochondria, nuclear SIRT6s also influence metabolic gene networks (Figure 3).⁶²

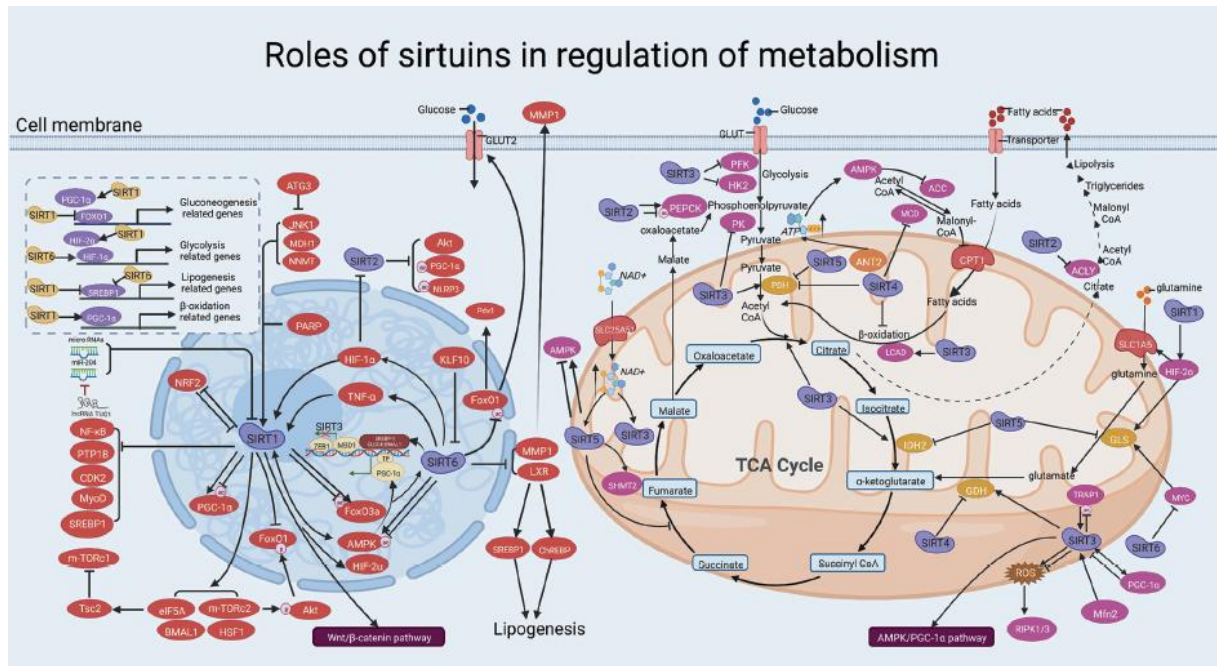


Figure 3. Sirtuin control of cellular metabolism across compartments. SIRTs coordinate glucose, lipid, and amino-acid metabolism by acting on transcription factors and metabolic enzymes in the nucleus, cytoplasm, and mitochondria. (i) In the nucleus, SIRT1 and SIRT6 promote glycolysis by activating HIF-2α and HIF-1α, respectively, via distinct mechanisms. SIRT1 stimulates gluconeogenesis by activating receptor gamma coactivator-1 alpha (PGC-1α) and inhibiting FoxO1, thereby modulating transcription of their target genes. SIRT1 also promotes fatty-acid β-oxidation through PGC-1α-dependent target gene induction. Conversely, SIRT1 and SIRT6 suppress Sterol Regulatory Element-Binding Protein 1 (SREBP1) to transcriptionally repress lipogenesis. (ii) In the cytoplasm, under low-nutrient conditions, SIRT2 deacetylates and activates the rate-limiting gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) 1, enhancing gluconeogenesis. SIRT2 also inhibits ATP citrate lyase (ACLY), deterring lipid synthesis. (iii) in the mitochondria, SIRT4 and SIRT5 reduce pyruvate dehydrogenase (PDH) activity. SIRT3 and SIRT4 oppositely regulate Glycerol-3-Phosphate Dehydrogenase (GDH) (SIRT3 activates; SIRT4 inhibits). Beyond GDH, SIRT3 activates isocitrate dehydrogenase (IDH) 2 and acyl-CoA dehydrogenase (LCAD), enhancing cellular respiration and stimulating fatty-acid β-oxidation. SIRT5 represses IDH2 and may disrupt glutamine metabolism via glutaminase (GLS). Activation and inhibition are indicated by arrows and blunt-ended lines, respectively.²⁸

Glucose homeostasis integrates glycolysis (anaerobic), oxidative phosphorylation (aerobic), glycogen turnover, and gluconeogenesis.⁶³ Its dysregulation contributes to insulin resistance

and diabetes.⁶⁴ SIRT1 enhances systemic insulin sensitivity and modulates pancreatic insulin secretion, promoting glucose uptake and utilization.⁶⁵ Mechanistically, SIRT1 interfaces with AMPK to correct glucose imbalance; upregulating SIRT1 can reverse diabetic phenotypes via the AMPK/acetyl-CoA carboxylase axis, whereas reduced SIRT1 compromises AMPK activity and glucose tolerance.⁶⁶ SIRT1 also improves insulin signalling by suppressing Protein Tyrosine Phosphatase 1B (PTP1B), a negative regulator of insulin receptor pathways.⁶⁷ Interdependencies among SIRT1, AMPK, and PTP1B merit further investigation. Other isoforms contribute as well. SIRT3 constrains aerobic glycolysis by inhibiting Hypoxia-Inducible Factor (HIF)-1 α and confers limited whole-body benefits when increased in liver during diet-induced insulin resistance.^{68,69} SIRT6 supports global and tissue-specific glucose control: in β cells, SIRT6 deacetylates protein Forkhead box O (FoxO) 1, elevates glucose transporter (GLUT) 2, and preserves glucose sensing and tolerance; exercise increases hepatic SIRT6 and improves insulin signalling in obese rats; modest SIRT6 overexpression enhances insulin sensitivity in skeletal muscle and liver.^{70–72} Collectively, SIRTs directly and indirectly shape glucose metabolism and may provide targets for earlier intervention in metabolic disease.

1.2.3. SIRTs in oxidative stress

Oxidative stress arises when the cellular production of ROS exceeds the capacity of endogenous antioxidant systems to neutralize them. Under homeostatic conditions, ROS are generated at low levels and efficiently scavenged; when this balance is lost, ROS-driven damage ensues and contributes to the pathogenesis of many disorders.^{73,74} Accumulating evidence implicates the SIRTs as key modulators of this process. By governing the expression and activity of diverse genes and signalling nodes (Figure 4), SIRTs enhance cellular resilience to oxidative stress.

AMPK is a master regulator of metabolic homeostasis and is commonly engaged during ischemic or hypoxic stress.⁷⁵ SIRT1 interfaces with this pathway at several levels. Liver kinase B1 (LKB1), an upstream AMPK activator, promotes AMPK signalling; in turn, AMPK lessens oxidative injury by improving insulin sensitivity, stimulating fatty-acid oxidation, and driving mitochondrial biogenesis to sustain ATP production.⁷⁶ Overexpression of SIRT1 deacetylates LKB1, facilitates its translocation from the nucleus to the cytoplasm, and thereby augments AMPK activity, collectively mitigating oxidative stress.⁷⁷ By contrast, in severe acute hepatic hypoxia SIRT1 has been reported to dampen LKB1 activation, reducing AMPK α Thr172 phosphorylation and exacerbating oxidative stress.⁷⁸ Thus, SIRT1 can either potentiate or restrain AMPK depending on context but typically supports stress resistance via LKB1 regulation.

SIRT3 and SIRT6 also engage AMPK, with beneficial effects. Loss of AMPK α elevates SIRT3 expression, which in turn modulates oxidative stress in cardiac tissue *in vitro* and *in vivo*.⁷⁹ Conversely, AMPK can activate SIRT3, limiting oxidative burden and preserving mitochondrial DNA integrity and function.⁸⁰ SIRT3 further lessens ROS and lipid peroxidation by improving mitochondrial performance; mechanistically, SIRT3 deacetylates LKB1 and secondarily activates AMPK, reinforcing this protective loop.⁸¹ SIRT6 has likewise been shown to enhance AMPK expression, leading to increased transcription of antioxidant enzymes, like manganese superoxide dismutase (MnSOD) and catalase (CAT), and consequent suppression of oxidative stress.⁸² In summary, SIRT1, SIRT3, and SIRT6 counter oxidative damage through direct or indirect reinforcement of AMPK signalling, although additional work is needed to define how other SIRTs intersect with this pathway under oxidative stress.

Nuclear factor erythroid 2-related factor 2 (Nrf2) functions as the central transcriptional regulator of the antioxidant response, orchestrating antioxidant response element (ARE) driven programs once released from its repressor Keap1 and translocated to the nucleus.⁸³ Within this framework, sirtuins, especially SIRT1, SIRT2, and SIRT6, serve as upstream enablers of Nrf2 signalling. SIRT1 promotes Nrf2 activation by remodelling Keap1, thereby facilitating Nrf2 nuclear accumulation and the induction of detoxifying enzymes such as glutathione S-transferases and UDP-glucuronosyltransferases.^{80,84} SIRT2 appears necessary to sustain basal Nrf2 activity in injury contexts; its downregulation after peripheral nerve damage attenuates Nrf2 signalling and aggravates oxidative stress.⁸⁵ In parallel, SIRT6 reinforces the Nrf2/ARE axis *in vivo* and reduces oxidative injury, protects human lens epithelial cells via Nrf2 activation, and confers resistance to hydrogen peroxide through further enhancement of Nrf2-dependent transcription.^{71,86} Collectively, these findings position SIRT1, SIRT2, and SIRT6 as convergent modulators that strengthen Nrf2-driven antioxidant defences, thereby improving cellular capacity to neutralize reactive oxygen species and limit oxidative damage.

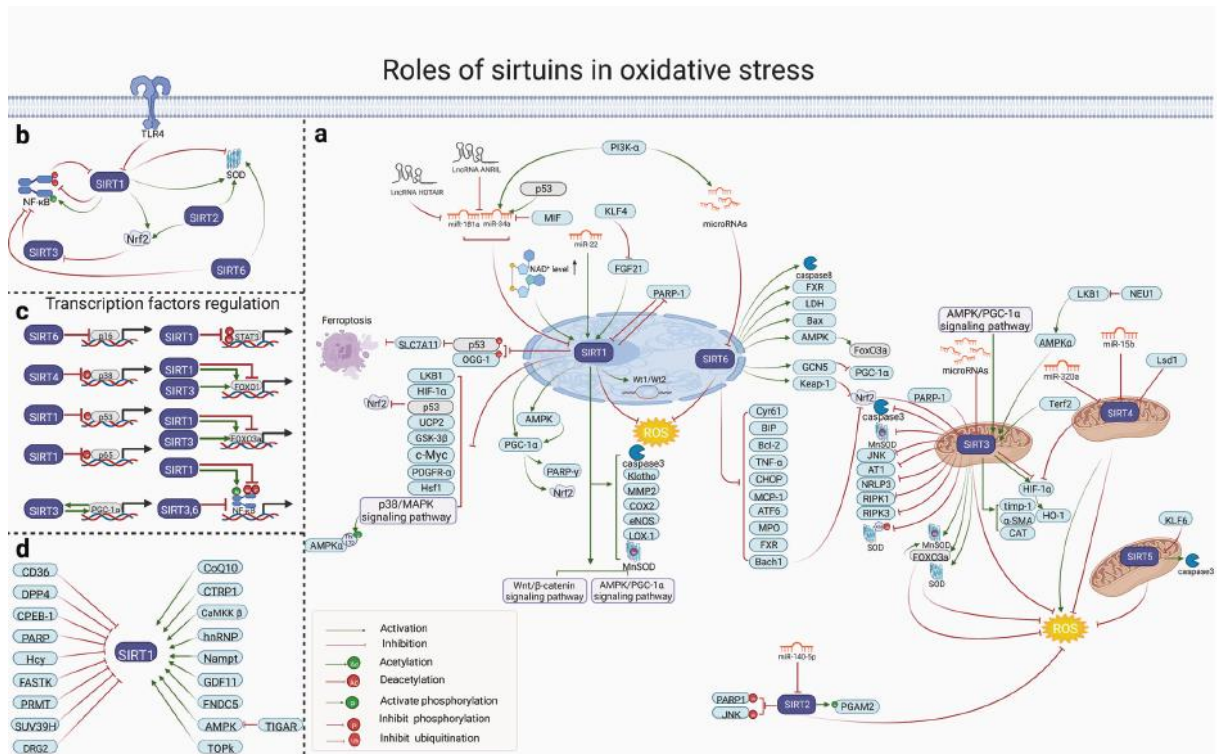


Figure 4. Sirtuins orchestrate antioxidant defences at multiple regulatory levels. (A) Global control of cellular oxidative stress. Sirtuins modulate ROS homeostasis chiefly through the Nrf2, FoxO factors, and SODs. SIRT1 and SIRT6 indirectly tune Nrf2 signalling, thereby shaping antioxidant gene expression. SIRT3 activates FoxO3, which elevates MnSOD and enhances ROS clearance. In addition, SIRT1, SIRT2, and SIRT6 can increase SOD expression, lowering intracellular ROS and limiting oxidative damage. (B) Regulation of NF-κB-dependent stress responses. Reduced SIRT1 (e.g., via NF-κB-driven repression) promotes oxidative stress. By reducing mitochondrial ROS, SIRT3 and SIRT6 dampen NF-κB activation: both actions collectively restrain oxidative injury. (C) Transcription-factor targeting. SIRT1 increases FoxO1 expression and deacetylates p53, actions that suppress ROS production and oxidative stress. SIRT1 also activates PGC-1α, supporting mitochondrial defences and mitigating oxidative damage. (D) Emerging modulators of SIRT1. Less-studied proteins reported to influence SIRT1 activity. Green arrows denote activation; red arrows denote inhibition.²⁸

1.2.4. SIRT1 in cell apoptosis

Among the various biological roles of sirtuins, the regulation of apoptosis is one of the most extensively studied. SIRT1s influence apoptotic processes by modulating histone acetylation and by interacting with key apoptosis-related proteins and signalling pathways (Figure 5).^{87,88}

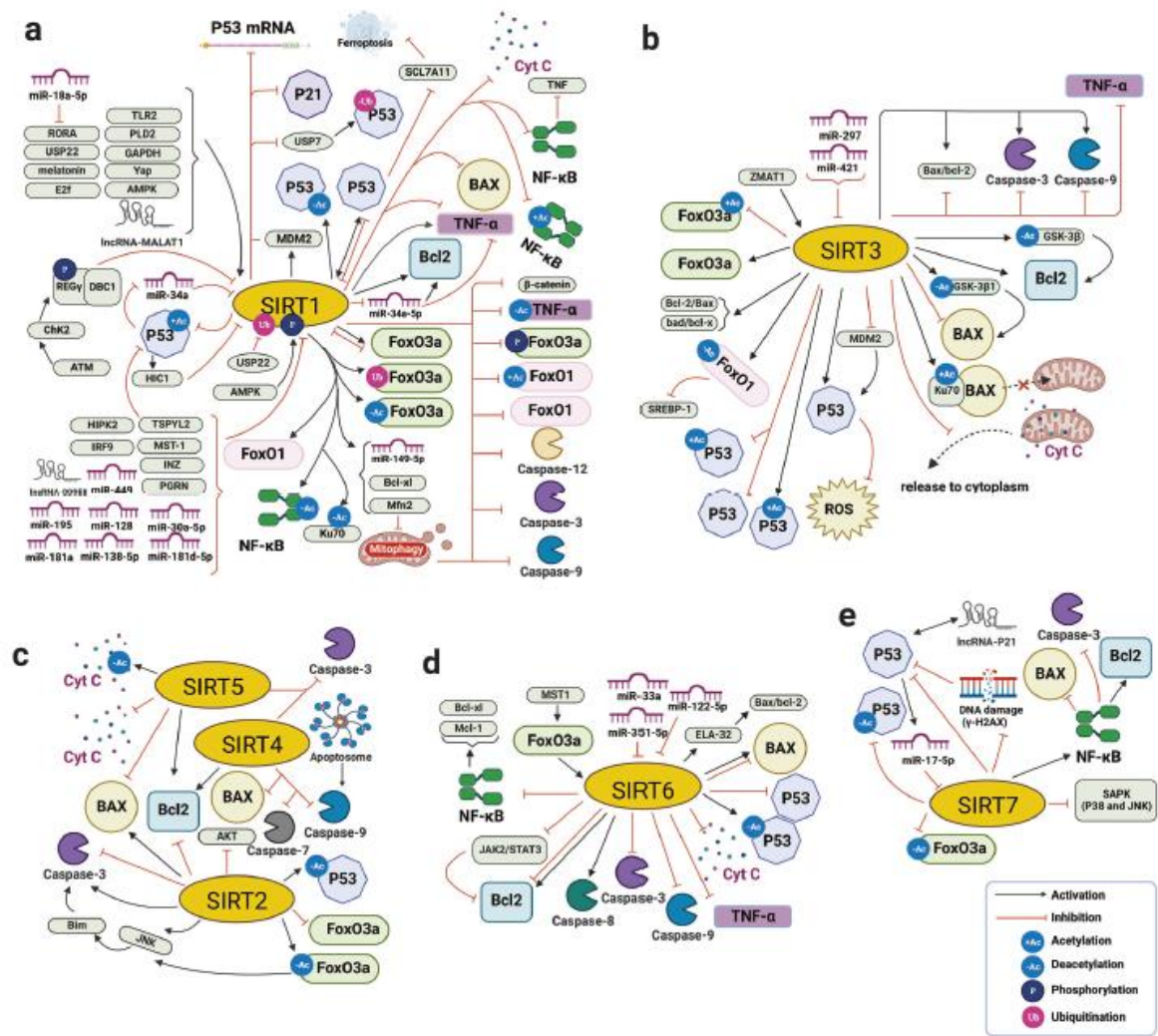


Figure 5. Sirtuins shape apoptotic competence through lysine acylation control and pathway crosstalk. In turn, SIRTs are themselves tuned by upstream regulators. (A) SIRT1 targets key apoptosis nodes, integrates inflammatory and stress cues, and coordinates downstream mitochondrial events such as BCL-2 family balance and Cyt c release. Noncoding RNAs and transcription factors further refine its pro- or anti-apoptotic output. (B) As a mitochondrial deacetylase, SIRT3 calibrates intrinsic apoptosis by restraining oxidative stress and regulating permeability of the outer mitochondrial membrane, thereby influencing Cyt c efflux and caspase activation. It also intersects with stress- and growth-responsive modules. (C) SIRT2 interfaces with p53, FoxO3a, and NF- κ B, affecting oxidative-stress tolerance and the caspase cascade. SIRT4 and SIRT5 adjust mitochondrial and metabolic checkpoints that gate life-versus-death decisions, shaping Cyt c release and executioner caspase activation. (D) A nuclear chromatin-associated SIRT that links the ATM DNA-damage response to transcriptional programs governing apoptosis, with additional coupling to NF- κ B and epigenetic regulators that set survival thresholds. (E) SIRT7. A nucleolar SIRT that integrates cellular stress and the p53

*axis; its effects on apoptotic susceptibility are context-dependent and shaped by the surrounding transcriptional landscape.*²⁸

As NAD⁺-dependent histone deacetylases, SIRT6 affect chromatin structure and gene expression to control apoptotic responses. For instance, SIRT1 reduces histone acetylation at promoter regions of genes such as *AR*, *BRCA1*, *ERS1*, *ERS2*, *EZH2*, and *EP300*, thereby modulating cancer cell apoptosis.⁸⁹ SIRT6 deacetylates histone H3K9, repressing *ERK1/2* transcription and linking histone regulation to NF-κB signalling and lifespan extension in animal models.⁴⁸ It also enhances GATA5 expression by deacetylating H3K9 at the *Nkx3.2* promoter, protecting endothelial cells from injury.⁸⁸ Emerging data indicate that SIRT6 may also modify histones via sumoylation or ubiquitination, though the role of these modifications in apoptosis remains uncertain.^{90,91}

Among SIRT6s, SIRT1 is the most comprehensively characterized regulator of apoptosis, acting through multiple transcription factors including p53, NF-κB, and FoxO: inhibits apoptosis by deacetylating p53, particularly at Lys379, thereby suppressing p53-mediated transcription of pro-apoptotic genes, and caspases.^{92–97} SIRT1 also deacetylates the p65 subunit of NF-κB, attenuating inflammatory apoptosis. Overexpression of SIRT1 suppresses inflammasome activation and promotes cell survival, though in some contexts NF-κB activation can also induce apoptosis, indicating a dual regulatory role.^{98–100} Moreover, SIRT1 enhances FoxO activity by deacetylation, increasing the expression of antioxidant and anti-apoptotic genes, thus reducing oxidative stress–induced apoptosis.⁹⁴ Molecules such as microRNAs, growth factors, and small compounds (e.g., melatonin, cambinol, ginsenoside Rc) regulate SIRT1 activity, impacting p53 deacetylation and apoptosis suppression.^{85,101,102}

The role of SIRT2 appears context dependent. It can inhibit apoptosis by deacetylating cytoplasmic substrates such as p53, NF-κB, and FoxO3, but under other conditions, its activation promotes apoptosis via the caspase and Akt/FoxO3a pathways.^{103–105} Hence, SIRT2's dual behavior likely depends on cellular context and stress conditions.

Mitochondrial SIRT6s (SIRT3–5) regulate intrinsic apoptosis through effects on mitochondrial integrity and energy metabolism. SIRT3 modulates GSK-3β/BAX, BAX/BCL-2, and BAD/BCL-xL ratios to influence caspase-9 activation and cell death.^{70,106,107} However, its function is disease-specific, pro-apoptotic in cancer but anti-apoptotic under stress injury models. It also interacts with FoxO1 and FoxO3a to modulate apoptosis through deacetylation and dephosphorylation mechanisms.^{108,109}

SIRT4 inhibits apoptosis by regulating the ratios of pro- and active caspases and preventing BAX translocation, while SIRT5 suppresses apoptosis via deacetylation of cytochrome c and inhibition of caspase-3 activation.^{110,111}

Nuclear SIRT6 and SIRT7 participate in p53-mediated apoptosis regulation.^{30,112} SIRT7 enhances cell survival after genotoxic stress by attenuating DNA damage and the p53 response.¹¹³ However, research on their roles remains limited, representing an emerging area for investigation.

Overall, the SIRT family functions as a bidirectional regulator of apoptosis, capable of either promoting or suppressing programmed cell death depending on cellular context. Despite significant progress, the molecular mechanisms underlying SIRT-mediated apoptotic regulation remain incompletely understood, warranting further exploration of their pathophysiological relevance.

1.2.5. SIRT6 in cell proliferation

Cell proliferation is a fundamental biological process through which cells grow and divide to maintain tissue homeostasis and enable development, repair, and reproduction. It relies on the coordinated regulation of cell growth and division, ensuring controlled tissue expansion and regeneration.¹¹⁴ Emerging evidence indicates that sirtuins participate in multiple aspects of cell proliferation by modulating transcription factors, acetylation status, and metabolic pathways.

SIRT1 exhibits dual functions in cell proliferation, acting as both a promoter and inhibitor depending on the cellular context. It stimulates proliferation by regulating microtubule-associated proteins 1A/1B light chain 3B (LC3) in retinoblastoma (Rb) acetylation; in endometrial cancer cells, SIRT1 reduces LC3 acetylation to enhance proliferation.^{101,115} SIRT1 can also drive macrophage and hepatocellular carcinoma (HCC) cell growth by modulating E2F transcription factor 1 (E2F1) and p53 expression.^{98,116} Conversely, SIRT1 may inhibit proliferation through AMPK/mTOR and STAT3/MMP-13 pathways, suppressing HCC and gastric cancer cell growth.^{117,118}

SIRT2 also exhibits context-dependent effects. It promotes proliferation by stabilizing Myc oncoproteins (N-Myc, c-Myc) and supports cell proliferation in leukaemia models, whereas it suppresses growth in neuroblastoma (NB) and renal podocytes by acting as a HDAC and reducing autophagy levels.^{110,119}

SIRT3, the main mitochondrial deacetylase, regulates proliferation through metabolic remodelling. It promotes proliferation by deacetylating serine hydroxymethyltransferase 2 (SHMT2), which increases one-carbon metabolism and supports mitochondrial function. However, under certain conditions, SIRT3 suppresses proliferation by downregulating HIF-1 α through the Profilin-1/SIRT3 axis.^{120,121}

SIRT4 generally acts as an antiproliferative factor, inhibiting cancer cell growth via repression of glutamine metabolism. The mTORC1 pathway can repress SIRT4 to sustain proliferation, while SIRT4 suppression activates glutaminase and triggers the Akt/GSK3 β /Cyclin D1 pathway, enhancing tumour progression in colorectal cancer (CRC).^{51,122}

SIRT5 displays both proliferative and inhibitory activities. It enhances proliferation by activating the MAPK pathway via acetyl-CoA acetyltransferase 1 and by desuccinylating enzymes such as citrate synthase and SHMT2, facilitating metabolic flux in cancer cells. SIRT5 also targets transcription factors such as E2F1 and pancreatic and duodenal homeobox 1 (PDX1), promoting HCC proliferation, though it suppresses pancreatic β -cell proliferation by deacetylating H4K16 and downregulating PDX1 transcription.^{123–128}

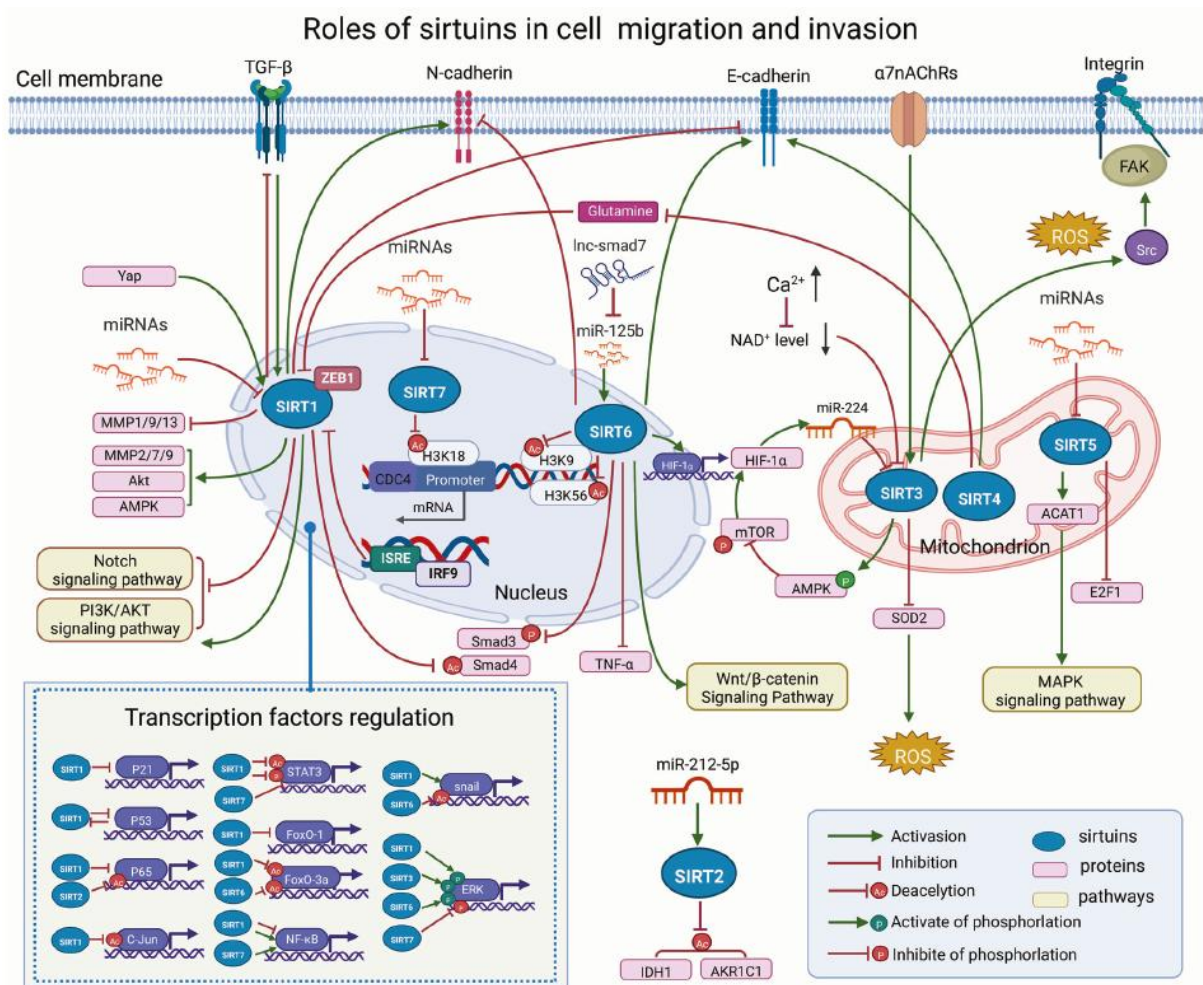
SIRT6 plays a bidirectional role, regulating downstream molecules such as AMPK, ERK, and MAPK. It can stimulate COX-2 expression by repressing AMPK or enhance ERK phosphorylation to drive proliferation.^{129,130} Conversely, its deletion in hematopoietic stem cells causes excessive proliferation. In multiple myeloma, SIRT6 depletion increases proliferation through MAPK upregulation, further highlighting its context-dependent behaviour.^{82,131}

SIRT7 has primarily proliferative roles. Its upregulation promotes vascular smooth muscle (VSM) cell proliferation in atherosclerosis, whereas SIRT7 deficiency attenuates VSMC , growth and neointimal formation after vascular injury.^{132,133} In cancer models, SIRT7 depletion suppresses proliferation by downregulating androgen receptor signalling and activating p38 MAPK pathways.¹³⁴

Overall, SIRTs constitute a complex regulatory network that influences cell proliferation through acetylation-dependent modulation of transcription factors and metabolic pathways. Their effects vary between cell types and conditions, underscoring the importance of context in determining whether SIRTs act as proliferative or antiproliferative regulators. Understanding these mechanisms could provide valuable therapeutic insights for diseases characterized by aberrant cell growth.

1.2.6. SIRT6 in cell migration and invasion

Cell migration and invasion are essential biological processes during embryogenesis, wound healing, and immune-cell trafficking. In pathological contexts such as cancer, they enable tumour cells to infiltrate surrounding tissues, enter the vasculature, and establish metastases at distant sites.^{135,136} Accumulating evidence highlights the involvement of the sirtuin family in these processes, particularly through regulation of transforming growth factor (TGF) β signalling and epithelial–mesenchymal transition (EMT), two key pathways driving tumour progression (Figure 6).^{137,138}



*and invasion. SIRT2 limits invasiveness through deacetylation of cytosolic targets, notably AKR1C1 and IDH1, thereby dampening downstream pro-migratory signalling and metabolic rewiring that support motility. Mitochondrial SIRT3, SIRT4, SIRT5 couple bioenergetics and redox balance to adhesive and cytoskeletal behaviors. By tuning oxidative metabolism and ROS handling, they influence integrin-dependent adhesion and EMT effectors, with consequences for directional movement and matrix invasion.*²⁸

SIRT1 influences migration and invasion by deacetylating several EMT-related transcription factors and adhesion proteins. In melanoma, SIRT1 deacetylates Beclin-1, promoting autophagic degradation of E-cadherin and facilitating EMT.¹³⁹ In chondrosarcoma, SIRT1 enhances Twist expression, a key EMT transcription factor, thereby increasing metastatic potential.¹³⁸ Moreover, a positive feedback loop between SIRT1 and zinc finger E-Box binding homeobox (ZEB1) amplifies EMT in osteosarcoma cells.⁸⁶ SIRT1 downregulation is associated with reduced invasiveness in cancers and autoimmune disorders, indicating its regulatory role in cell motility.^{17,140} Functionally, SIRT1 knockdown suppresses EMT, migration, and TGF- β signalling in keratinocytes, impairing wound repair.¹⁴¹ In hypoxia models, SIRT1 promotes migration via phosphoinositide 3-kinase/protein kinase B (PI3K)/AKT and Notch signalling.¹⁴² In non-small cell lung cancer (NSCLC), SIRT1-mediated AMPK/mTOR signalling enhances proliferation and invasion.¹⁴³ Regulation of SIRT1 by noncoding RNAs is also well established: miR-34a represses SIRT1 and inhibits migration in colorectal and HCC cells, whereas long non-coding RNA (lncRNA) MALAT1 and circular RNA circ_0088036 upregulate SIRT1, promoting migration in HCC and rheumatoid arthritis fibroblast-like synoviocytes.^{144–146}

SIRT2 modulates invasion through deacetylation of cytosolic enzymes that interact with signal transducer and activator of transcription 3 (STAT3) and metabolic pathways. It deacetylates aldo-keto reductase family 1 member C1 (AKR1C1), suppressing STAT3 transcriptional activity and limiting migration in NSCLC models.¹⁴⁷ In CRC, SIRT2-dependent IDH1 deacetylation inhibits cell migration and liver metastasis *in vitro* and *in vivo*.¹⁴⁸

Mitochondrial SIRT3–SIRT5 generally act as suppressors of migration and EMT. Loss of SIRT3 in tumour cells lowers E-cadherin and accelerates EMT-driven growth, whereas its presence inhibits migration by maintaining epithelial characteristics.¹⁴⁹ SIRT4 similarly restrains EMT through upregulation of E-cadherin in gastric cancer (GC) cells.¹³⁹ Additionally, SIRT3 mediates the antimigratory effects of $\alpha 7$ nicotinic acetylcholine receptors in vascular smooth muscle cells, highlighting a link between mitochondrial signalling and motility

regulation.¹⁵⁰ Conversely, SIRT5 exhibits context-dependent functions: it promotes migration in HCC, but in hypoxic conditions, its inhibition can enhance invasion, suggesting environment-specific roles.^{151,152}

Among nuclear sirtuins, SIRT6 and SIRT7 regulate migration and invasion via EMT and matrix metalloproteinase (MMP) expression. SIRT6 promotes N-cadherin and vimentin through FoxO3a deacetylation in HCC and enhances MMP9 expression via MAPK/ERK1/2 signalling in osteosarcoma cells.^{60,153} However, in other contexts, SIRT6 suppresses EMT by inhibiting TGF- β 1/Smad3 and c-Jun activation, as observed in airway remodelling models.¹³⁷ SIRT7, on the other hand, fosters invasion by repressing E-cadherin in CRC cells and is overexpressed in endometrial carcinoma, where its silencing reduces invasiveness.^{154,155}

Collectively, SIRTs form a multifaceted regulatory network modulating cell migration and invasion across cellular compartments. Nuclear SIRTs (SIRT1, SIRT6, SIRT7) chiefly orchestrate transcriptional control of EMT and matrix remodelling, cytosolic SIRT2 acts through metabolic and STAT3-linked pathways, and mitochondrial SIRTs (SIRT3–SIRT5) modulate energy metabolism and epithelial integrity. These findings underscore the context- and tissue-specific nature of SIRT-mediated control of cell motility, providing insight into their dual roles as both suppressors and facilitators of metastasis.

1.3. The dual role of SIRTs in cancer

SIRTs regulate chromatin structure, DNA repair, metabolism and cell cycle. Their pervasive involvement in pathways that are frequently altered in tumours has placed them at the crossroads between cancer and aging biology.^{3,28,156,157} Interestingly, almost every member of the family has been reported to behave either as a tumour suppressor or as an oncogenic factor depending on tissue type, genetic background and disease stage (Figure 7).^{156,158,159}

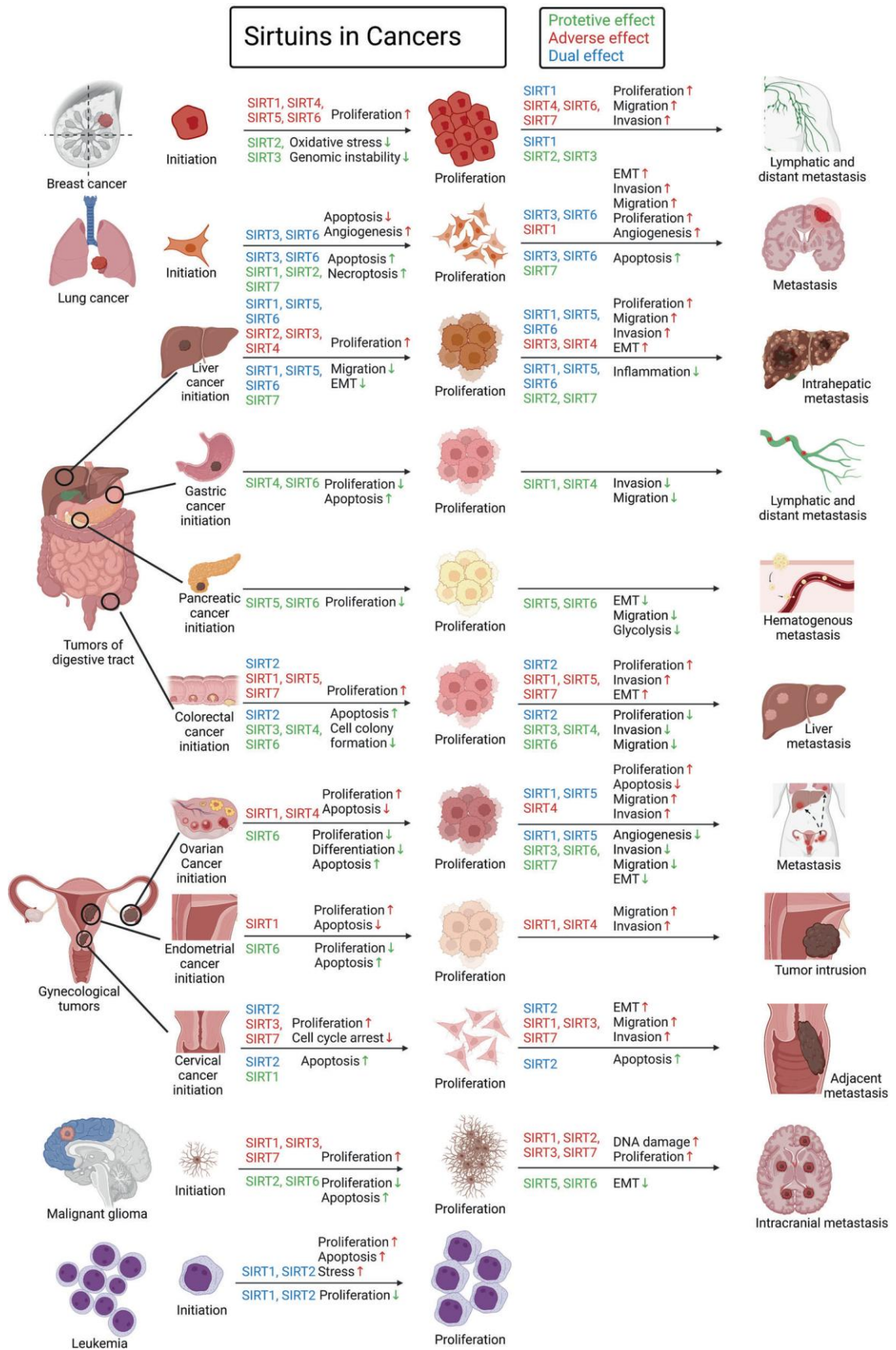


Figure 7. The dual role of SIRT in cancers. SIRTs have been implicated in a broad spectrum of human malignancies, including BC, lung cancer (LC), HCC, GC, pancreatic cancer (PC),

CRC, ovarian cancer (OC), endometrial cancer (EC), cervical cancer (CC), malignant glioma and leukaemia. Across these entities, individual SIRTs can function as oncogenic drivers (in red), tumour-suppressive factors (in green), or exhibit a context-dependent dual behaviour (in blue), acting as either promoters or suppressors of tumorigenesis. In solid tumours, their activities intersect with the key stages of cancer evolution: tumour initiation, expansion of the primary lesion and metastatic dissemination. Along this trajectory, SIRTs modulate a range of biological processes, including cell proliferation, oxidative stress responses, apoptosis, angiogenesis, EMT, as well as cell migration and invasion, which can be either enhanced or restrained depending on the isoform and context. Consequently, the patterns of metastatic spread vary according to the primary tumour site and may involve lymph nodes, distant organs, the liver or adjacent structures.²⁸

This context dependence is now recognized as a core feature of sirtuin biology in cancer. Indeed, SIRTs control several cancer-relevant processes. For example, SIRT1, SIRT6 and SIRT7 regulate double-strand break repair, base-excision repair and chromatin compaction, thereby limiting genomic instability, a hallmark of cancer.^{3,28,157} Nuclear sirtuins deacetylate histones (e.g., H3K9 and H4K16) and transcription factors such as p53, NF- κ B and FOXO proteins, reshaping transcriptional programs that govern proliferation and apoptosis. Mitochondrial sirtuins (SIRT3–5) remodel oxidative phosphorylation, glycolysis, fatty-acid oxidation and glutamine metabolism, whereas SIRT1 and SIRT6 also influence metabolic gene expression in the nucleus.^{28,157,160} Finally, multiple sirtuins modulate apoptotic and autophagic pathways through direct control of BCL-2 family proteins key kinases such as mTOR, AMPK and c-Jun N-terminal kinases (JNKs).^{28,157}

Because these pathways can be either protective or harmful depending on the oncogenic landscape, the same sirtuin may support tumour suppression in one scenario and tumour progression in another.^{156,158,159}

SIRT1 is the most extensively studied sirtuin in cancer and also the best example of functional ambivalence.^{3,28,156,159} SIRT1 safeguards genome integrity by promoting DNA repair and maintaining chromatin stability. It deacetylates histones at sites of DNA damage and facilitates recruitment of repair factors to double-strand breaks, contributing to both homologous recombination and non-homologous end joining.^{3,157} SIRT1 also deacetylates and activates FOXO transcription factors and PGC-1 α , thereby enhancing antioxidant defences and limiting ROS-induced mutagenesis.^{28,157} In several models, SIRT1 overexpression reduces spontaneous

tumour formation and suppresses intestinal or hepatic carcinogenesis, consistent with a tumour-suppressive role driven by improved genome maintenance and controlled inflammation.^{3,28,156,157} In CRC, the dose-dependent nature of SIRT1's effects is particularly clear: heterozygous deletion enhances c-Myc-driven glutamine metabolism and tumour formation, whereas complete loss of SIRT1 triggers apoptotic pathways, reduces autophagy and ultimately limits tumour burden, illustrating that both insufficient and excessive SIRT1 activity can be deleterious in different ways.²⁸ Conversely, SIRT1 can promote tumorigenesis by repressing classic tumour suppressors. Deacetylation of p53 weakens its transcriptional activity on pro-apoptotic genes (for example *PUMA*, *NOXA* and *BAX*), dampening DNA-damage-induced apoptosis and facilitating survival of genomically unstable cells.^{3,156,157} SIRT1 can also antagonize RB signalling, stimulate cell-cycle progression and contribute to EMT and metastasis by modulating EMT transcription factors and TGF- β / β -catenin pathways in BC and LC models.²⁸ Clinically, SIRT1 is overexpressed in several malignancies, including subsets of breast, HCC and CRC, and often correlates with poor prognosis or chemoresistance; yet in other cohorts, high SIRT1 expression accompanies better outcomes.^{3,28,158,159} These discrepancies are consistent with the idea that SIRT1's net effect reflects the balance between its genome-stabilizing and pro-survival outputs, modulated by tumour type, mutation status (notably p53) and tumour stage.^{3,156,159}

SIRT2, largely cytosolic, regulates microtubule dynamics, mitotic progression and metabolic enzymes.^{28,157} By deacetylating α -tubulin and mitotic regulators, SIRT2 contributes to proper chromosome segregation and prevents aneuploidy. Loss of SIRT2 has been associated with chromosomal instability and spontaneous tumour formation in mouse models.^{156,157} In breast cancer (BC), SIRT2 promotes BC type-1 susceptibility protein (BRCA1) and BRCA1-associated RING domain protein 1 (BARD1) heterodimerization via deacetylation, thereby facilitating homologous recombination and protecting against DNA-damage-driven tumorigenesis.²⁸ SIRT2 can also restrict migration and metastasis, exemplified by the deacetylation of AKR1C1 and IDH1, which limits pro-metastatic STAT3 signalling and CRC liver metastases.^{28,161} In other contexts, SIRT2 supports cancer cell proliferation and aggressiveness. It stabilizes c-Myc and N-Myc proteins, enhancing transcription of proliferative and metabolic genes. In basal-like BC, SIRT2 deacetylates and stabilizes the EMT transcription factor Slug, fostering invasive behaviour and metastatic competence. SIRT2-dependent deacetylation of glycolytic enzymes can also reinforce anabolic metabolism and tumour growth in NSCLC.²⁸ Thus, SIRT2 opposes tumour initiation by preserving mitotic fidelity yet may

become pro-tumorigenic once a transformed clone is established, particularly in aggressive, EMT-prone subtypes.

SIRT3 is the major mitochondrial deacetylase and a key regulator of oxidative metabolism and redox homeostasis, and possesses tumour-suppressive roles.^{157,160} By deacetylating and activating enzymes of the tricarboxylic-acid (TCA) cycle and electron-transport chain, SIRT3 promotes mitochondrial respiration and limits reliance on aerobic glycolysis (the Warburg effect).[3,7] It also enhances antioxidant defences via activation of MnSOD and IDH2, thereby reducing ROS-induced DNA damage and transformation.^{28,157} Loss of SIRT3 has been linked to increased genomic instability, heightened ROS and a higher incidence of mammary and haematologic tumours in mice.¹⁶⁰ In several human cancers, including HCC and lung adenocarcinoma, SIRT3 expression is reduced and its restoration suppresses proliferation, induces apoptosis and increases sensitivity to chemotherapeutic agents.²⁸ Conversely, SIRT3 can support tumour growth when its metabolic rewiring favours biosynthesis. Deacetylation of serine SHMT2 by SIRT3 in CRC enhances serine–glycine one-carbon metabolism and nucleotide synthesis, thereby driving carcinogenesis. In some tumour types, elevated SIRT3 promotes survival under metabolic stress and confers resistance to oxidative damage or drugs that exploit.^{28,160} These observations exemplify how the same mitochondrial adaptation, optimizing oxidative metabolism and redox balance, can either prevent transformation in normal tissues or sustain the fitness of established tumours, depending on timing and context.¹⁶⁰

SIRT4 and SIRT5 show particularly interesting dual roles due to their specialized activities on metabolic enzymes. SIRT4 exerts ADP-ribosyltransferase and deacylase activities on mitochondrial proteins involved in glutamine metabolism, such as GDH. By inhibiting GDH, SIRT4 restrains anaplerotic entry of glutamine into the TCA cycle, thereby limiting proliferation of glutamine-addicted tumour cells.^{28,157} Reduced SIRT4 expression has been reported in several cancers, and its overexpression typically suppresses proliferation, migration and invasion, for example in colorectal and thyroid cancer, partly through inhibition of glutamine metabolism and upregulation of E-cadherin.²⁸ These findings support a predominantly tumour-suppressive function. However, under severe stress conditions, SIRT4 can also promote survival by modulating metabolic checkpoints and DNA-damage responses, and some studies in transformed cell lines indicate that SIRT4 may sustain viability after genotoxic insults.¹⁶¹ This suggests that SIRT4 may switch from growth brake to stress-tolerance factor depending on nutrient availability and genotoxic burden.

SIRT5 demalonylates, desuccinylates and deglutarylates mitochondrial enzymes, thereby impacting glycolysis, the TCA cycle and ammonia detoxification.^{28,157} In multiple cancers, SIRT5 promotes proliferation and migration by activating metabolic pathways: desuccinylation of citrate synthase or SHMT2 enhances flux through the TCA cycle and one-carbon metabolism, while regulation of glutaminolysis supports biomass production. High SIRT5 expression often associates with poor prognosis and aggressive phenotypes. On the other hand, in specific contexts such as HCC, microRNAs that target SIRT5 have been described as tumour suppressors, and loss of SIRT5 can sensitise cells to ferroptosis and oxidative damage.²⁸ These data highlight that also SIRT5 can act either as a facilitator of metabolic adaptation or as a vulnerability when excessively suppressed.

SIRT6 is a chromatin-associated deacetylase and mono-ADP-ribosyltransferase that regulates telomere maintenance, base-excision repair and transcription of metabolic genes.^{28,157} As a tumour suppressor, SIRT6 represses glycolytic gene expression and oncogenic signalling, thereby limiting the Warburg effect and tumour growth in several models, including colorectal, pancreatic and BC.^{3,157,162} It also promotes DNA repair and protects against genomic instability, consistent with tumour-preventive functions.^{3,157} Yet SIRT6 can also act as an oncogene. In HCC, SIRT6 promotes EMT by stimulating autophagic degradation of E-cadherin, thereby enhancing migration and invasion. Increased SIRT6 expression correlates with aggressive behaviour in osteosarcoma, where it drives ERK1/2–MMP9 signalling and metastatic potential. In some skin cancer models, SIRT6 upregulates COX-2 expression and fosters inflammation-driven tumorigenesis.²⁸ Thus, the impact of SIRT6 on cancer appears to hinge on whether its dominant output is genome and metabolic restraint or pro-invasive transcriptional reprogramming.

SIRT7, mainly nucleolar, regulates ribosomal RNA transcription and chromatin structure.^{28,157} It is frequently overexpressed in solid tumours and haematological malignancies, where it promotes proliferation, EMT and invasion, for example by repressing E-cadherin in CRC and activating oncogenic signalling pathways in breast and endometrial cancer.²⁸ In these settings, SIRT7 behaves clearly as an oncogene. Nonetheless, SIRT7 can also support stress resistance and maintain genome integrity in normal tissues, and its deficiency in non-tumour models has been associated with features of premature ageing and impaired DNA repair.^{157,162}

Across isoforms, several recurring principles help explain why sirtuins oscillate between oncogenic and tumour-suppressive functions: (i) expression level and dosage are critical: as

shown for SIRT1 in CRC, partial reduction versus complete loss of a sirtuin can have opposite consequences on proliferation and apoptosis, reflecting non-linear control of metabolism and cell-death pathways;^{3,28,156} (ii) tumour type and genetic context matter; the downstream consequences of deacetylating p53, RB, c-Myc, HIF-1 α or NF- κ B differ dramatically depending on whether those pathways are wild-type, mutated or rewired by other oncogenes;^{3,156,159} (iii) disease stage is important: during early tumorigenesis, sirtuins that preserve genome stability (for example SIRT1, SIRT2, SIRT3 and SIRT6) tend to be protective, whereas in established tumours the same activities may instead maintain viability under stress, support metabolic flexibility and favour therapy resistance;^{3,160} (iv) subcellular localisation and post-translational modifications influence substrate selection; shuttling between nucleus, cytosol and mitochondria, as well as phosphorylation or auto-acylation of sirtuins, can reconfigure their substrate repertoire and flip their biological output;^{28,157} (v) the tumour microenvironment and systemic metabolism shape sirtuin activity. Hypoxia, nutrient availability and NAD⁺ levels determine whether sirtuin-driven metabolic rewiring supports or antagonises tumour progression.^{157,158,160} Understanding these determinants is essential to harness sirtuins therapeutically without inadvertently promoting malignant traits.

The context-dependent activities of sirtuins pose both opportunities and pitfalls for development of both inhibitors, to block pro-survival, pro-proliferative or EMT-promoting activities of SIRT1, SIRT2, SIRT5 or SIRT7 in cancers where these isoforms are oncogenic, and activators, to exploit the tumour-suppressive functions of SIRT1, SIRT3, SIRT4 and SIRT6, particularly their roles in genome maintenance and metabolic restraint. However, given the dual roles outlined above, indiscriminate systemic activation or inhibition of a given sirtuin is unlikely to be universally beneficial. Recent reviews emphasise the need for isoform-selective modulators combined with patient stratification based on SIRT expression, mutation profiles and metabolic signatures. An emerging concept is to use sirtuin status as part of biomarker panels that predict whether a particular tumour will respond better to an inhibitor (for example SIRT1-high, p53-wild-type BC) or to an activator (for example SIRT3-low, ROS-high HCC). Future work should aim to map sirtuin interactomes and modification states in well-annotated patient cohorts, define stage-specific effects of sirtuin modulation *in vivo* and integrate sirtuins into combination strategies with DNA-damaging agents, PARP inhibitors, metabolic drugs or immunotherapies.^{158,159,162}

1.4. SIRT2

1.4.1. Overall structure of SIRT2

SIRT2 is a member of the sirtuin family of NAD⁺-dependent deacetylases and deacylases, originally identified as the major cytoplasmic sirtuin but now known to shuttle between cytoplasm and nucleus in a cell-cycle-dependent manner.¹⁶³ SIRT2 participates in multiple biological processes, including metabolic regulation, oxidative stress response, genome stability, mitotic control, autophagy, apoptosis, and tumorigenesis. Its catalytic activity removes acetyl and other acyl modifications (propionyl, butyryl, succinyl, malonyl) from lysine residues of target proteins, thereby modulating their stability and function.¹⁰³ Although SIRT2 shares structural similarity with other sirtuins, it has distinct subcellular localization and substrate specificity. It acts on cytosolic proteins such as tubulin, FoxO transcription factors, p53, and NF-κB, influencing redox balance and cell survival.^{105,164} In the nucleus, SIRT2 regulates chromatin condensation and genome integrity by deacetylating histone H4K16 and H3K56, promoting orderly mitosis and DNA repair.¹⁶⁵

The human gene encoding SIRT2 is located on the long arm of chromosome 19 at position 19q13.2 and consists of 17 exons that undergo alternative splicing, giving rise to five isoforms. Among these, only isoforms 1, 2 and 3 are translated into proteins.¹⁶⁶

Isoform 1 consists of 389 amino acid residues and is generated by transcription and subsequent splicing of all exons of the gene. It contains an N-terminal domain (residues 1–38), a catalytic domain (residues 84–268) and a C-terminal domain (residues 351–389) (Figure 8). On the other hand, isoform 2 is composed of 352 residues and differs from isoform 1 by the absence of the N-terminal domain, which is lost owing to exclusion of the second exon during splicing. Isoform 2 is expressed predominantly in the central nervous system, whereas isoform 1 is more abundant in most other tissues and is therefore regarded as the canonical isoform.^{166–168} Notably, although the various isoforms show preferential localization to specific subcellular compartments, their distribution can vary in a tissue-specific manner and during the cell cycle.^{163,166,169,170} Apart from the N-terminal sequence, the two isoforms share the same overall structure (Figure 8). Between residues 41 and 51, SIRT2 harbours a nuclear export sequence (NES) that mediates export of the protein from the nucleus to the cytoplasm via the CRM1 (Chromosome Region Maintenance 1)–dependent export pathway.^{168,171} By contrast, the mechanism of nuclear import of SIRT2 has not yet been fully elucidated. Recent proteomic studies indicate that SIRT2 interacts with several proteins classified as nuclear importins and that the C-terminal tail can restrict importin–SIRT2 interactions, thereby acting as a negative regulator of nuclear import.¹⁷²

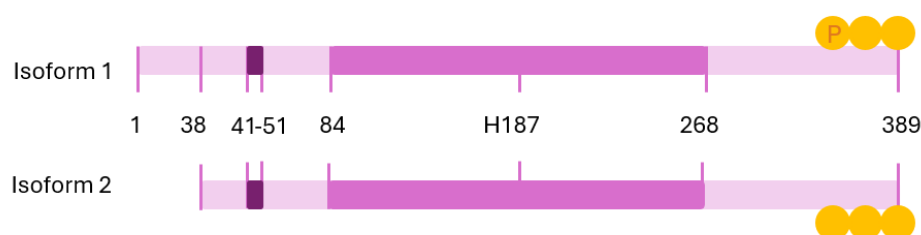


Figure 8. Primary structure of SIRT2 isoforms 1 and 2. The catalytic domain is shown in purple. The NES sequence is depicted in deep purple. In the C-terminal region, the yellow dots indicate phosphorylation sites.

The central portion of the protein, which is responsible for deacetylase activity and is highly conserved among HDAC family members, contains the amino acid sequence required for binding the cofactor NAD^+ , the residues that coordinate the Zn^{2+} ion, and the key histidine residue His187, which is essential for catalysis. His187 functions as a proton acceptor and activates the 2'-hydroxyl group of the ribose moiety of NAD^+ .¹⁶⁸ Finally, the C-terminal region contains several phosphorylation sites that play a role in regulating enzymatic activity. In particular, phosphorylation of Ser331 and Ser335 by cyclin-dependent kinase (CDK) 5 is required for nuclear translocation, whereas phosphorylation of Ser368 by CDK1 slows cell-cycle progression.^{168,173}

Because of its dual localization, SIRT2 links metabolic and epigenetic regulation, integrating energy status with cell-cycle checkpoints and stress responses. Nevertheless, a canonical nuclear localization signal (NLS) has not been detected in the SIRT2 primary sequence, whereas a functional NES is present in both known human protein-coding isoforms. This combination suggests that nuclear entry is largely passive, while export is actively driven.^{167,168} The key question therefore became how sufficient nuclear SIRT2 levels are achieved to support chromatin-related functions. In the mouse, an additional SIRT2 splice variant lacking the NES-encoding segment had been reported.^{174,175} The predicted protein would be refractory to NES-driven export, providing a simple explanation for nuclear enrichment. These observations raised the possibility that alternative splicing of the human SIRT2 transcript might likewise generate isoforms with distinct localization and functions.¹⁶⁷

Building on this hypothesis, a novel human SIRT2 splice variant generated by skipping exons 2–4 was identified. The resulting transcript, composed of exon 1 followed directly by exons 5–

16, encodes a protein referred to as isoform 5. Isoform 5 lacks the N-terminal region that harbours the NES, and, in cellular experiments, it displays predominant nuclear localization, in clear contrast to the largely cytosolic isoforms 1 and 2. At the same time, isoform 5 is catalytically inactive.^{176,177}

In addition, the absence of exons 2–4 in isoform 5 alters the N-terminal architecture of the protein: isoform 2 lacks residues 1–37 compared to isoform 1, whereas isoform 5 is characterized by the replacement of amino acids 6–76 with a single arginine. Consequently, the N-terminal portion of the catalytic domain (defined by alignment and structural analysis as residues 64–336) is truncated. A phylogeny-based conservation analysis showed that these splicing events do not remove highly conserved catalytic residues or residues directly involved in NAD⁺ binding.¹⁷⁸ Indeed, homology and threading-based modelling further indicated that isoform 5 adopts a fold very similar to that of isoform 1, with only subtle changes. The most parsimonious interpretation is therefore that isoform 5 is structurally intact, and that its catalytic inactivity reflects more nuanced alterations in substrate recognition or active-site dynamics rather than global misfolding. Because isoform 5 is nuclear and catalytically inactive, it is likely to act as a non-enzymatic regulator, for instance by organizing or modulating nuclear protein complexes. The study also highlights that alternative splicing is not the only mechanism generating SIRT2 diversity: ribosomes can initiate translation at an internal AUG within the isoform 1 transcript, giving rise to isoform 2.^{168,179} This translational mechanism probably contributes to isoform-specific expression patterns observed *in vivo*.¹⁷⁵

Finally, the notion that a structurally intact but catalytically inactive isoform might perform regulatory functions is consistent with observations in other enzyme systems. For SIRT1, proteolytic cleavage in the C-terminal region generates a catalytically inactive fragment that relocates to mitochondria and protects against apoptosis by interfering with apoptosome assembly.¹⁸⁰ By analogy, SIRT2 isoform 5 may function as a nuclear scaffold or modulator whose activity does not rely on lysine deacetylation per se but rather on protein–protein interactions, possibly in a NAD⁺-dependent manner. Further work will be required to define its binding partners, clarify whether it can act on non-canonical substrates, and elucidate its contribution to SIRT2-dependent pathways in chromatin regulation and gene expression.¹⁶⁷

The catalytic core of SIRT2 is an elongated assembly composed of two structurally distinct domains. The larger domain, formed by residues 53–89, 146–186 and 241–356, adopts a modified Rossmann-like architecture characteristic of many NAD⁺/NADP⁺-dependent

enzymes.¹⁸¹ The smaller domain, comprising residues 90–145 and 187–240, harbours a structural Zn^{2+} ion. These two domains are interlinked by four polypeptide crossovers that create, at their interface, a pronounced groove defined by the crossovers and three loops emanating from the large domain. Within the larger domain, six β -strands ($\beta 1$ – $\beta 3$ and $\beta 7$ – $\beta 9$) form a parallel β -sheet that is flanked by six α -helices ($\alpha 1$, $\alpha 7$, $\alpha 8$ and $\alpha 10$ – $\alpha 12$) packing against the sheet (Figure 9).

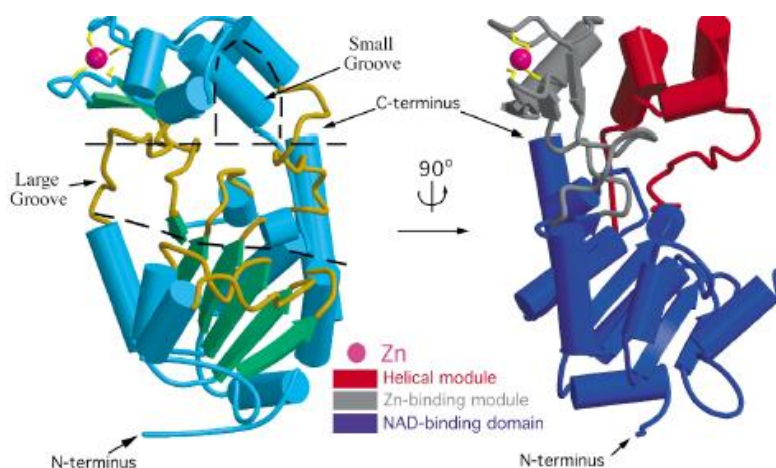


Figure 9. Structure of SIRT2.

This Rossmann-like region displays the expected structural motifs of a canonical NAD-binding site, including a Gly–X–Gly motif crucial for interaction with the phosphate groups of NAD^+ , a small pocket accommodating the adenine moiety, and charged residues appropriately positioned to contact the ribose hydroxyls.¹⁸¹ The smaller domain is built from two insertions in the Rossmann fold (Figure 9). The first insertion gives rise to a helical module consisting of four α -helices ($\alpha 3$ – $\alpha 6$) that assemble into an open bundle. This module contains a hydrophobic pocket that opens into the main interdomain groove; its size, shape and conservation pattern suggest it may serve as a class I-specific docking site for protein segments of the substrate. The second insertion forms a zinc-binding module composed of a three-stranded antiparallel β -sheets ($\beta 4$, $\beta 5$, $\beta 6$), an α -helix ($\alpha 9$) oriented at roughly 45° to the sheet, and a Zn^{2+} ion coordinated in a tetrahedral geometry by four cysteine residues (Cys195, Cys200, Cys221 and Cys224). This zinc-binding motif is topologically reminiscent of a RING finger domain, known to mediate protein–protein interactions in many proteins, but differs by lacking the second Zn^{2+} ion and by the altered packing of its helix relative to the β -sheet.¹⁸²

The interdomain groove that emerges between the Rossmann fold, and the small domain encompasses the NAD^+ -binding site and contains residues that are strictly conserved across all

five SIRT2 classes. Mutagenesis of these conserved residues abolishes histone deacetylase activity, indicating that this groove houses the catalytic machinery. The N-terminal extension of SIRT2 (approximately 19 residues) folds into an amphipathic helix ($\alpha 0$) that does not contact the remainder of the SIRT2 molecule but participates instead in crystal packing interactions. Consistent with this, removal of this N-terminal stretch does not impair *in vitro* deacetylase activity, implying that $\alpha 0$ is dispensable for catalysis but may act as a protein–protein interaction motif, in analogy with other amphipathic helices involved in transcriptional regulation.¹⁸³

The helical and zinc-binding modules interact extensively across a predominantly hydrophobic interface formed largely by $\alpha 5$ and $\alpha 6$ of the helical module and $\alpha 9$ together with loops L3 and L4 of the zinc module. The helical module contains two long helices ($\alpha 3$ and $\alpha 6$) and two shorter helices ($\alpha 4$ and $\alpha 5$) that adopt an open configuration (Figure 9). This arrangement creates a hydrophobic pocket approximately 13 Å in length and 10 Å in width, lined by solvent-exposed nonpolar residues (Figure 10) that are highly conserved within SIRT2 isoforms.¹⁸³

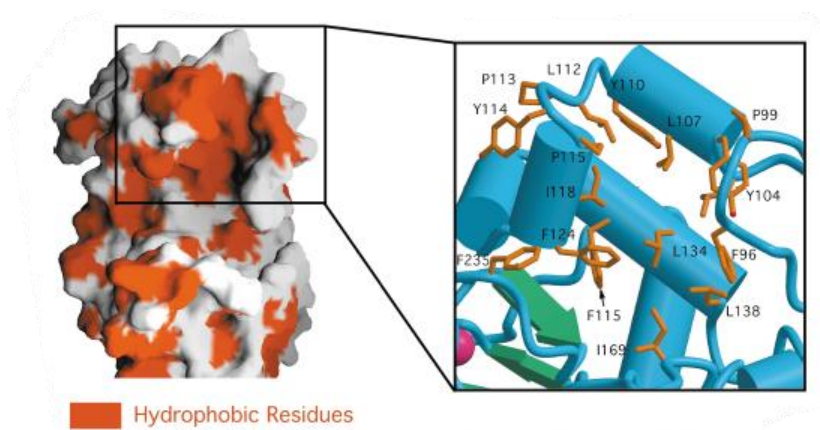


Figure 10. An expanded surface representation of the hydrophobic residues of SIRT2.¹⁸³

The zinc-binding module itself consists of the three-stranded antiparallel β -sheet and $\alpha 9$, with the four zinc-ligating cysteines conserved throughout the SIRT2 isoforms. The small domain packs against one side of the Rossmann fold, generating a deep groove that runs roughly perpendicular to the long axis of the protein (Figure 9). This groove is bounded by large, well-ordered loops stabilized by secondary-structure elements.¹⁸³

Crystallographic analyses have shown that the SIRT2 active site can be subdivided into multiple predominantly hydrophobic regions, including pockets A–C, a selectivity pocket, and the acetyl-lysine channel (Figure 11). Pockets A and B are responsible for binding the ADP-ribose (ADPR) portion of NAD⁺, while pocket C hosts the nicotinamide group. The channel that

accommodates the acetyl-lysine substrate is formed by several phenylalanine residues, which provide a continuous connection between the NAD⁺-binding region and the acetyl-lysine site. Co-crystal structures with diverse SIRT2 inhibitors reveal a range of hydrophobic and electrostatic contacts, and some ligands adopt distinct binding poses that are associated with different conformational rearrangements of the active site. The hydrophobic pocket in the helical module is thought to contribute to substrate recognition.¹⁸³

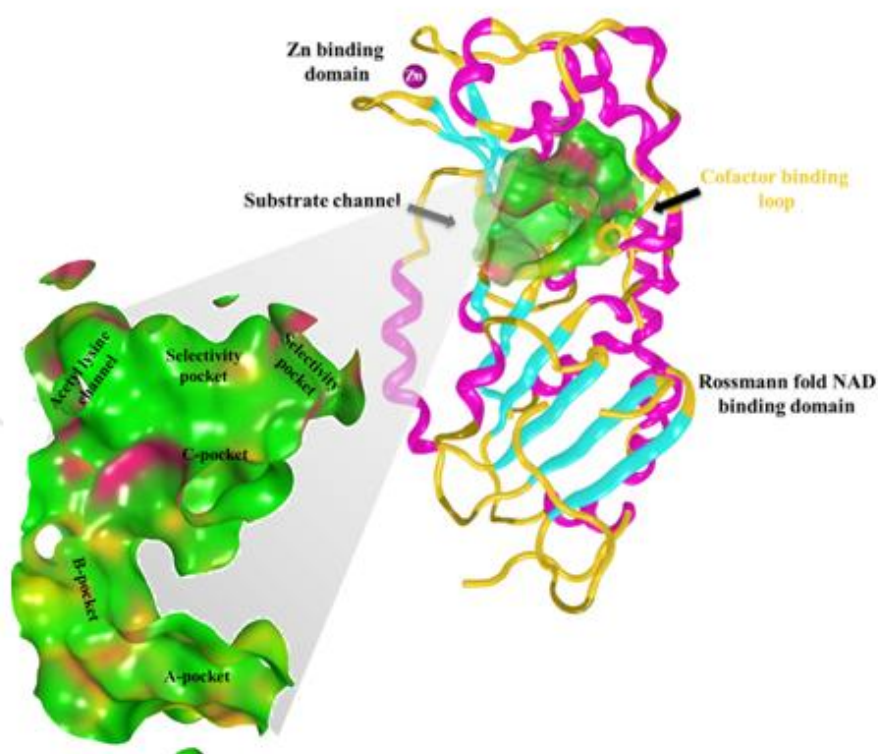


Figure 11. Overall structure of human SIRT2 (PDB: 5Y0Z) showing its active site and surface representation of the active site of SIRT2.¹⁸⁴

Finally, mechanistic proposals for NAD⁺ cleavage implicate the C site serine (Ser88 in SIRT2) as a nucleophile that attacks the glycosidic bond, generating an enzyme–ADP-ribose intermediate.² The intermediate would then be resolved to produce an ADP-ribose species, potentially an oxocarbenium ion, which participates in the deacetylation reaction.

1.4.2. SIRT2 in cell-cycle regulation and proliferation

SIRT2 is tightly associated with cell-cycle progression. Its protein levels rise in G₂/M phase, during which deacetylates α -tubulin and histones to facilitate chromosome segregation.¹¹⁰ In

cancer contexts, SIRT2 exhibits context-dependent functions, acting as either a tumour suppressor or promoter.

In several malignancies, SIRT2 enhances proliferation through stabilization of Myc oncoproteins. Deacetylation of N-Myc and c-Myc by SIRT2 increases their stability and transcriptional activity, leading to the up-regulation of cell-cycle genes such as Cyclin D1 and CDK4.^{110,185} Similar effects have been documented in leukemic cells, where SIRT2 supports glycolytic flux and nucleotide synthesis, sustaining rapid proliferation.^{186,187} Conversely, in NB cells and renal podocytes, SIRT2 behaves as a growth suppressor. Its HDAC and delactylase activities repress proliferative gene expression, leading to growth arrest and reduced autophagy.^{119,188} These findings underscore the dual regulatory capacity of SIRT2, which depends on tissue context, metabolic status, and interacting pathways.

1.4.3. SIRT2 in apoptosis regulation

SIRT2 modulates apoptosis through both p53-dependent and p53-independent mechanisms. By deacetylating p53, SIRT2 limits its transcriptional activation of pro-apoptotic genes such as *BAX* and *PUMA*, thereby favouring cell survival.¹⁰³ However, under stress conditions, excessive SIRT2 activity can enhance apoptosis by promoting cleaved caspase-3 and *BAX* expression while downregulating *BCL-2*.^{165,189}

Experimental models demonstrate that SIRT2 inhibition (e.g., with AGK2) reduces neuronal apoptosis in ischemia/reperfusion injury, indicating that the direction of its effect depends on cell type and injury context. In cancer cells, the balance between SIRT2-mediated survival and death signals may determine its tumour-suppressive versus oncogenic role.

1.4.4. SIRT2 in autophagy and cellular homeostasis

Autophagy maintains proteostasis and organelle quality control, and SIRT2 contributes to this system by modulating acetylation of autophagy-related proteins. It can regulate transcription factor EB (TFEB) mRNA stability and control autophagic flux.¹⁹⁰ Loss of SIRT2 alters α -synuclein acetylation and aggregation, leading to autophagy dysfunction in neuronal models.¹⁹¹ Moreover, SIRT2 interacts with the AMPK/mTOR pathway, where it can either activate or suppress autophagic processes depending on nutrient status.¹⁹² In NSCLC, downregulation of SIRT2 disrupts argininosuccinate-synthetase (ASS)–induced apoptosis through exosomal autophagy signalling.¹⁹³ Thus, SIRT2 maintains redox and metabolic equilibrium via a fine-tuned autophagy regulation.

1.4.5. SIRT2 in metabolic regulation

Beyond protein acetylation, SIRT2 orchestrates metabolic adaptation in cancer. It deacetylates and activates phosphoglycerate mutase 1 (PGAM1), an enzyme essential for glycolysis, thereby facilitating NADPH production and redox balance in NSCLC cells.¹⁹⁴ This enhances tumour growth and survival under oxidative stress. SIRT2 also participates in lipid metabolism by deacetylating acetyl-CoA carboxylase and ATP-citrate lyase, contributing to lipid biosynthesis when needed for membrane formation during proliferation. These findings emphasize SIRT2's role as a metabolic integrator connecting glycolytic and lipogenic pathways to cell-cycle control.

1.4.6. SIRT2 in DNA damage response and genome stability

In the nucleus, SIRT2 preserves genomic integrity through chromatin remodelling and DNA-repair facilitation. It promotes heterodimerization via deacetylation, a crucial step in homologous recombination repair.¹⁹⁵ Loss of SIRT2 results in chromosomal instability and aneuploidy. Moreover, SIRT2 regulates checkpoint kinases and mitotic spindle assembly, underscoring its role in mitotic fidelity. Such genome-protective effects align with its tumour-suppressive function in certain cancers, including breast and colorectal carcinomas.

1.4.7. SIRT2 in cell migration, invasion, and EMT

SIRT2 significantly influences cell motility and metastatic dissemination through modulation of EMT and cytoskeletal dynamics. In NSCLC, SIRT2 deacetylates AKR1C1, suppressing STAT3 transcriptional activation and limiting migration and invasion.¹⁹⁶ In CRC, SIRT2 deacetylates IDH1, decreasing metastatic potential both *in vitro* and in mouse models.¹⁹⁷ These findings indicate a tumour-suppressive role for SIRT2 in metastasis.

However, in basal-like BC, SIRT2 displays pro-metastatic activity by stabilizing Slug, a transcription factor that drives EMT and invasiveness.¹⁹⁸ Therefore, SIRT2's influence on migration and invasion is highly context-specific, governed by tissue type, oncogenic mutations, and microenvironmental cues.

1.4.8. SIRT2 in specific cancer types

1.4.8.1. Breast cancer

SIRT2 expression is often reduced in breast tumours compared with normal tissue, and higher SIRT2 levels correlate with better overall survival.¹⁹⁹ SIRT2 contributes to DNA repair through

the BRCA1–BARD1 pathway, linking its deficiency to genomic instability and tumorigenesis.¹⁹⁵ Yet in basal-like BC, SIRT2 may enhance malignancy via Slug stabilization, promoting EMT and invasiveness.¹⁹⁸ Thus, SIRT2 behaves as a tumour suppressor in most breast-cancer subtypes but may switch to a pro-oncogenic role in aggressive basal phenotypes.

1.4.8.2. Lung cancer

SIRT2 has dual and sometimes opposing functions in LC. On one hand, it inhibits metastasis by deacetylating AKR1C1 and repressing STAT3-dependent transcription, reducing migratory ability.¹⁹⁶ On the other hand, it can promote proliferation by enhancing glycolytic flux through PGAM1 deacetylation, increasing NADPH production and antioxidant capacity.²⁰⁰ Clinically, high SIRT2 expression has been associated with poor prognosis and drug resistance in NSCLC patients.²⁰¹ Modulating SIRT2 expression or activity has therefore been proposed as a potential therapeutic strategy in LC.¹⁰⁴

1.4.8.3. Colorectal Cancer

In CRC, SIRT2 expression is frequently downregulated, and its loss correlates with advanced tumour grade and metastasis. Mechanistically, SIRT2 deacetylates IDH1, restoring normal mitochondrial metabolism and suppressing migration and liver metastasis.¹⁹⁷ Conversely, miRNA miR-212-5p directly targets SIRT2 mRNA, lowering its expression and promoting CRC proliferation and invasion.²⁰² These findings position SIRT2 as a tumour suppressor that maintains metabolic homeostasis and limits EMT in colorectal malignancies.

1.4.8.4. Gynaecologic cancers

In endometrial carcinoma, SIRT2 is often upregulated, where it drives proliferation and stemness through mitogen-activated protein kinase (MEK)/ERK signalling while reducing chemosensitivity.^{203,204} Silencing SIRT2 decreases cancer-cell viability and enhances response to cytotoxic drugs. In ovarian cancer, SIRT2 shows the opposite behaviour: its overexpression restores cisplatin sensitivity, suggesting a protective role against therapy resistance.²⁰⁵ These contrasting outcomes again illustrate the cell-context dependency of SIRT2 activity.

1.4.8.5. Hepatocellular carcinoma and other tumours

Although less studied, SIRT2 can affect HCC progression via modulation of the AMPK/mTOR axis and mitochondrial biogenesis. It may enhance survival under metabolic stress, even if it suppresses metastasis by restricting EMT. Similarly, in glioblastoma and pancreatic cancer,

SIRT2's contribution to oxidative stress adaptation and cell-cycle control is emerging, indicating broader significance across tumour types.^{28,206}

1.4.8.6. SIRT2 in skin cancers

In the context of cutaneous biology, sirtuins have been linked to responses to ultraviolet (UV) damage, oxidative stress, wound healing, and carcinogenesis.²⁰⁷ Among these, SIRT2 has emerged as a significant player with complex, sometimes contradictory, roles in skin health and cancer progression. Its activity has been implicated in maintaining cellular redox balance, mediating autophagic and apoptotic signalling, and regulating tumour suppressive or oncogenic processes depending on tissue type and microenvironmental context.^{18,209}

The skin, constantly exposed to environmental insults such as UV radiation and pollution, relies heavily on redox-sensitive enzymes like sirtuins to preserve its homeostatic integrity.^{208,209} SIRT2, through its deacetylation of FoxO3a, modulates oxidative stress signalling and apoptosis.²¹⁰ Specifically, SIRT2 deacetylates FoxO3a in response to caloric restriction and oxidative challenge, thereby enhancing the transcription of MnSOD, a key antioxidant enzyme. This pathway decreases H₂O₂-induced ROS accumulation and promotes survival under stress.²¹⁰

Within dermal cells, the antioxidant defence orchestrated by SIRT2 complements that of SIRT1, which modulates FoxO3a and p53 under similar conditions.^{211,212} However, while SIRT1 often promotes cell survival, SIRT2's activation may induce apoptosis under excessive stress, an example of its dual role as both a protector and an executor of cell fate decisions. This duality becomes particularly relevant in the tumour microenvironment, where oxidative stress functions as both a trigger for carcinogenesis and a mechanism for tumour suppression. In cutaneous wound repair, SIRT2 contributes to keratinocyte proliferation and migration, processes essential for re-epithelialization.²¹³ The deacetylase's function in cytoskeletal organization via α -tubulin deacetylation supports keratinocyte motility and structural remodelling during tissue regeneration.²¹⁴ These findings suggest that SIRT2's regulation of cytoskeletal and metabolic targets contributes to cutaneous repair, although direct mechanistic studies in skin are still limited. The capacity of SIRT2 to enhance wound closure highlights its physiological importance in maintaining epidermal integrity, which, when dysregulated, could contribute to neoplastic transformation.

1.4.8.7. *SIRT2 in melanoma*

The most detailed insights into SIRT2's oncological role in skin come from melanoma, a malignancy derived from melanocytes that is characterized by aggressive metastatic behaviour and high resistance to therapy.²¹⁵

In melanoma, SIRT2 appears to act primarily as a negative regulator of tumour progression. Indeed, a pharmacologic activation of SIRT2 downregulated melanoma progression markers, including Cyclin D1 and Bcl-2, thereby suppressing proliferation. This modulation sensitized melanoma cells to the chemotherapeutic agent doxorubicin, reducing the effective dose required for cytotoxicity.²¹⁶ These findings suggest a synergistic therapeutic potential of SIRT2 activation in combination regimens, enhancing drug efficacy while minimizing toxicity.

However, later findings complicated this interpretation. It was demonstrated that systemic SIRT2 promotes melanoma progression by selectively suppressing NK-cell infiltration, proliferation, and cytotoxic programming within the tumour microenvironment (TME), while tumour-intrinsic SIRT2 status in melanoma cells does not alter growth in immunocompetent hosts. Pharmacologic SIRT2 inhibition restores NK infiltration and retards tumour growth in a genetically on-target fashion.²¹⁷ Indeed, the SIRT2-selective inhibitor **SirReal2** increased α -tubulin K40 acetylation (a canonical pharmacodynamic readout) in mouse liver lysates, reduced tumour growth in wild type (WT) mice and increased tumour-infiltrating NK cells by percentage and absolute number. Importantly, **SirReal2** had no effect in SIRT2-KO mice, establishing on-target specificity. Collectively, the genetic and pharmacologic datasets converge on a model: systemic SIRT2 suppresses NK recruitment and function, and its inhibition reverses that deficit, thereby slowing melanoma progression.

These results are consistent with SIRT2's context dependent roles in cancer. On one hand, SIRT2 preserves genome integrity and can suppress tumorigenesis during initiation;^{218,219} on the other, it can be a progression promoter by reprogramming immunity or cytoskeletal dynamics.^{220,221}

Mechanistically, several threads are suggestive though not fully resolved. In cytoskeletal control, SIRT2 inhibition raises α -tubulin K40 acetylation, a modification linked to microtubule stability, immune-cell motility, and immune synapse formation.²¹⁷ Thus, excess of SIRT2 activity may depress NK migration/infiltration, matching the transwell migration deficit in SIRT2-overexpressing NK-92MI cells.

In transcriptional/effector programming, down-regulation of TNF- α , programmed death-ligand 1 (PD-L1), Fas ligand, granzyme, and FoxO1 in SIRT2-knock-in cells suggests a broad restraint of activation, development, and lytic machinery consistent with reduced *ex vivo* cytotoxicity and in-tumour proliferation.^{82,217,222–224}

Prior work links SIRT2 and Nrf2-driven surveillance,^{225,226} such axes could modulate redox-metabolic fitness required for NK antitumor responses. Two immediate implications emerge: SIRT2 as an immunomodulatory target and its potential as biomarker.

Although SIRT2's individual functions are distinct, its activity intersects with that of other sirtuins, forming a complex regulatory network. For example, SIRT1, frequently overexpressed in melanoma, promotes proliferation and inhibits senescence via deacetylation of p53 and downstream regulators.^{227,228} SIRT3, a mitochondrial isoform, has been shown to exert a pro-proliferative function in melanoma, enhancing growth and migration by promoting oxidative metabolism.²²⁹

At a mechanistic level, SIRT2's contribution to melanoma pathobiology stems from its gatekeeping control of α -tubulin acetylation and microtubule dynamics, a nexus that programs cell motility and thereby enables invasion and metastatic dissemination.²¹⁴ In skin cells, proper cytoskeletal organization determines not only cell migration during wound healing but also the invasive potential of cancer cells. Loss of SIRT2-mediated deacetylation results in hyperacetylated tubulin, which can alter cellular motility and adhesion, promoting invasive phenotypes seen in squamous-cell carcinoma (SCC) and metastatic melanoma. Moreover, SIRT2 connects aging, acetylome balance, and carcinogenesis through deacetylation of histone H4K16 and other mitotic regulators. In keratinocytes, dysregulation of this function can lead to genomic instability, a hallmark of cancer. By preserving chromosomal segregation fidelity, SIRT2 acts as a guardian against aneuploidy and malignant transformation.²⁰⁷

SIRT2's involvement in gluconeogenesis and metabolic flux may also influence cancer metabolism. Cancer cells often display enhanced glycolytic activity, known as the Warburg effect, and SIRT2's regulation of NAD⁺-dependent deacetylation could reprogram metabolic pathways toward oxidative phosphorylation, thereby reducing tumour aggressiveness. Its cooperation with FoxO3a in antioxidant gene transcription further supports its role in maintaining a non-oncogenic redox balance.²¹⁰

The link between SIRT2 and drug resistance in melanoma highlights its regulatory control over the MAPK signalling cascade, which is frequently activated by gene BRAF mutations. SIRT2 loss leads to hyperactivation of MEK, sustaining proliferation despite inhibitor therapy. Thus, pharmacologic restoration of SIRT2 activity could enhance the efficacy of BRAF/MEK inhibitors and counteract adaptive resistance mechanisms.²³⁰

1.4.9. Molecular pathways linking SIRT2 to cancer hallmarks

Several convergent pathways underlie SIRT2's diverse oncogenic and tumour-suppressive functions. In the PI3K/Akt/mTOR pathway, SIRT2 interacts with AKT signalling, affecting proliferation and autophagy. Inhibition of SIRT2 can block AKT phosphorylation, leading to reduced tumour growth. In the AMPK–mTOR axis, SIRT2 cross-talks with AMPK in controlling energy metabolism; depending on nutrient status, it may either activate or repress autophagy and biosynthetic programs. Moreover, Deacetylation of AKR1C1 by SIRT2 dampens STAT3 activity and metastatic behaviour, particularly in LC.¹⁹⁶ Through deacetylation of IDH1 and stabilization of Slug, SIRT2 alternately represses or promotes EMT across cancer types.^{197,198} By stabilizing BRCA1–BARD1, SIRT2 maintains genome stability and suppresses transformation.¹⁹⁵ SIRT2 also acts as a histone deacetylase/delactylase, influencing chromatin accessibility and gene transcription related to proliferation and differentiation.^{119,188}

Collectively, these pathways illustrate SIRT2's integration of metabolic sensing, transcriptional control, and DNA repair, situating it at a pivotal junction between metabolism and oncogenesis.

1.4.10. Therapeutic implications and pharmacological modulation of SIRT2

Given its context-dependent roles, targeting SIRT2 requires precision strategies. Small-molecule inhibitors such as **AGK2**, **SirReal2**, and thiomristoyl lysine analogues selectively inhibit SIRT2 deacetylase activity, displaying antitumor efficacy in preclinical models of NB, BC, and leukaemia. Conversely, SIRT2 activators (e.g., resveratrol derivatives) have shown benefit in models where SIRT2 acts as a tumour suppressor (CRC, NSCLC metastasis). Combination therapies exploiting SIRT2 modulation alongside metabolic inhibitors or DNA-damaging agents are an emerging area of translational research. From a biomarker standpoint, differential SIRT2 expression may help stratify patients: low SIRT2 predicts aggressive and metastatic disease in colorectal and breast cancers, whereas high SIRT2 associates with poor prognosis and therapy resistance in lung and endometrial cancers.^{199,201,203} This dualistic pattern reinforces the necessity of tumour-type-specific evaluation before therapeutic targeting.

Given its dualistic behaviour, tumour-suppressive in SCC and BCC, yet complex in melanoma, SIRT2 represents a context-sensitive therapeutic target.^{231–233} Enhancing SIRT2 activity may restore differentiation, suppress proliferation, and re-sensitize melanoma cells to kinase inhibitors. Conversely, uncontrolled activation could interfere with homeostatic redox signalling or normal keratinocyte function. Emerging pharmacologic tools, including selective SIRT2 activators and inhibitors (such as AGK2 derivatives and MC2562), enable more precise modulation.²¹³ Clinical translation will depend on determining the tumour-type-specific threshold where SIRT2 shifts from protective to deleterious.²³⁰ Expression profiling across tumour stages, combined with *in vivo* genetic models, will be essential to elucidate these dynamics. Moreover, SIRT2 expression patterns may serve as diagnostic or prognostic markers.²³³ For instance, reduced SIRT2 expression could indicate poor differentiation or high metastatic potential, particularly in SCC, while high SIRT2 levels might predict better response to BRAF-targeted therapies in melanoma.²³⁴

Collectively, the evidence portrays SIRT2 as a multifaceted guardian of skin integrity, whose dysfunction contributes to tumorigenesis and therapy resistance. Future research should aim to integrate molecular, histological, and therapeutic perspectives to fully harness SIRT2's potential as both a biomarker and a therapeutic modulator in skin cancers.

1.5. SIRT2 inhibitors

SIRT2's physiological plasticity links it to numerous diseases. Consequently, SIRT2 has emerged as both a drug target and a biological marker. In the past decade, the field of SIRT2 inhibitors has progressed from early, moderately selective compounds (AGK2, cambinol) to highly optimized scaffolds such as **SirReal2** and thiomyrystoyl lysine analogues, with improved selectivity, potency, and mechanistic diversity.²³⁵

As already mentioned, SIRT2 catalyses lysine diacylation using NAD⁺ as a cofactor: nicotinamide cleavage yields an ADP-ribosyl intermediate that accepts the acyl group from the substrate lysine.²³⁶ The catalytic site comprises a Rossmann-fold NAD⁺-binding domain and a zinc-binding domain, bridged by a flexible loop that shapes the substrate pocket.

Crystallographic data (e.g., 4RMH, 5MAT) revealed that SIRT2 possesses a unique selectivity pocket that opens only upon inhibitor binding, absent in SIRT1/3, allowing design of isoform-selective molecules.²³⁶ This discovery, made through co-crystallization with **SirReal2**, revolutionized structure-guided drug design.

Unlike SIRT1, SIRT2 can remove both acetyl and long-chain fatty acyl groups (e.g., myristoyl, palmitoyl).²³⁵ This dual function complicates the discovery of selective inhibitors because most scaffolds show different potency toward deacetylation and demyristoylation reactions. Selectivity requires careful tuning of chemical geometry to target either the catalytic or the hydrophobic acyl-binding pocket.

As a growing body of work has clarified SIRT2's involvement across diverse pathologies, small-molecule SIRT2 inhibitors have come to the fore as promising therapeutic leads for related diseases.²³⁷ SIRT2 inhibitors are mechanistically diverse, generally classified as: (i) competitive inhibitors (**AGK2**, **SirReal2**) that directly block NAD⁺ or substrate access; (ii) mechanism-based covalent inhibitors (thiomyristoyl lysine derivatives) that trap the substrate intermediate of the enzyme; (iii) allosteric inhibitors (pyrazolopyrimidines) that stabilize inactive conformations. Inhibitory potency is strongly influenced by substrate kinetics. Thiomyristoyl lysine derivatives, for example, inhibit both acetyl- and myristoyl-lysine reactions by forming a covalent adduct, while **SirReal2** selectively interferes with acetyl-peptide turnover.²³⁸ Allosteric modulation has emerged as an attractive approach, leveraging SIRT2's conformational flexibility to improve selectivity and minimize toxicity.²³⁶

1.5.1. AGK2

AGK2 (Figure 12) was the first selective SIRT2 inhibitor, discovered through phenotypic screening.²³⁵ It inhibits SIRT2 with an IC₅₀ $\approx$ 3.5 μ M, sparing SIRT1/3. In neurodegenerative models, **AGK2** rescues α -synuclein-induced cytotoxicity, while in cancer cells it reduces proliferation via increased acetyl- α -tubulin. However, limited solubility, metabolic instability, and off-target effects prohibit its *in vivo* translation.

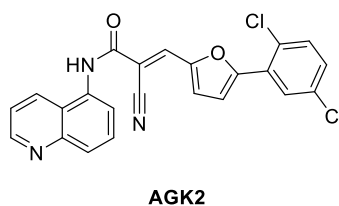


Figure 12. Structure of the first selective SIRT2 inhibitor **AGK2**.

1.5.2. *SirReal2* and analogues

In 2015 **SirReal** chemotype, exemplified by **SirReal2** (Figure 13), was reported as an allosteric class of SIRT2 inhibitors, showing notable potency and selectivity.²³⁹

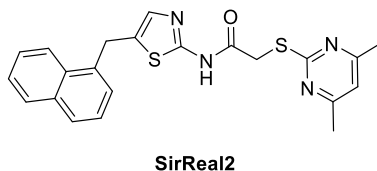


Figure 13. Structure of the allosteric SIRT2 inhibitor **SirReal2**.

SirReal2 achieves this by inducing a rearrangement of the SIRT2 active site, creating an induced hydrophobic pocket that confers isoform selectivity. The first co-crystal structure of SIRT2 with **SirReal2** (PDB: 4RMG) demonstrated that the ligand primarily engages hydrophobic residues within this pocket (Figure 14), thereby rationalizing both potency and selectivity and highlighting the deep hydrophobic cavity as a privileged site for SIRT2-focused drug design.²³⁹

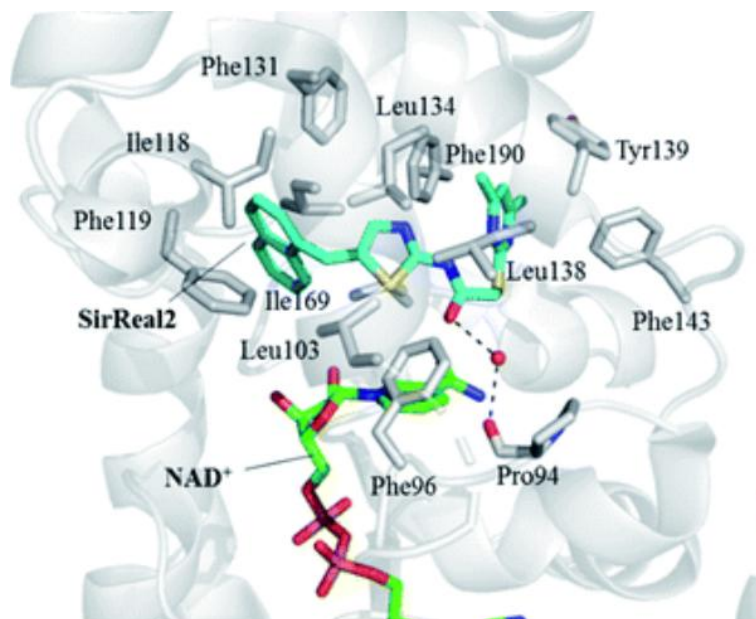


Figure 14. Crystallographic analyses of **SirReal2** with SIRT2 (PDB: 4RMG). **SirReal2** opens and occupies an induced hydrophobic pocket adjacent to the SIRT2 active site, stabilizing via extensive hydrophobic contacts (Leu/Phe/Ile residues) and a few backbone hydrogen bonds (e.g., Pro94/loop 92–96). It does not displace NAD⁺ but instead decouples the catalytic conformational dynamics, which collectively account for its high potency and isoform selectivity toward SIRT2.²⁴⁰

Building on these interactions, the same group designed compounds **1** and **2** with improved potency, aqueous solubility, and cellular activity (Figure 15).²⁴¹ They further advanced a SIRT2-directed proteolysis targeting chimera (PROTAC) **3** by linking a thalidomide warhead to SirReal-based ligands, providing a chemical knockdown approach to interrogate SIRT2 pharmacology.²⁴²

Substitution of **SirReal2**'s thiazole ring with a phenyl ring produced *N*-(3-(phoxymethyl)phenyl)acetamide derivatives (e.g., **4**), while systematic SAR around the 2-((4,6-dimethylpyrimidin-2-yl)thio)-*N*-phenylacetamide core yielded more potent analogues **5** and **6** (Figure 15).²⁴³

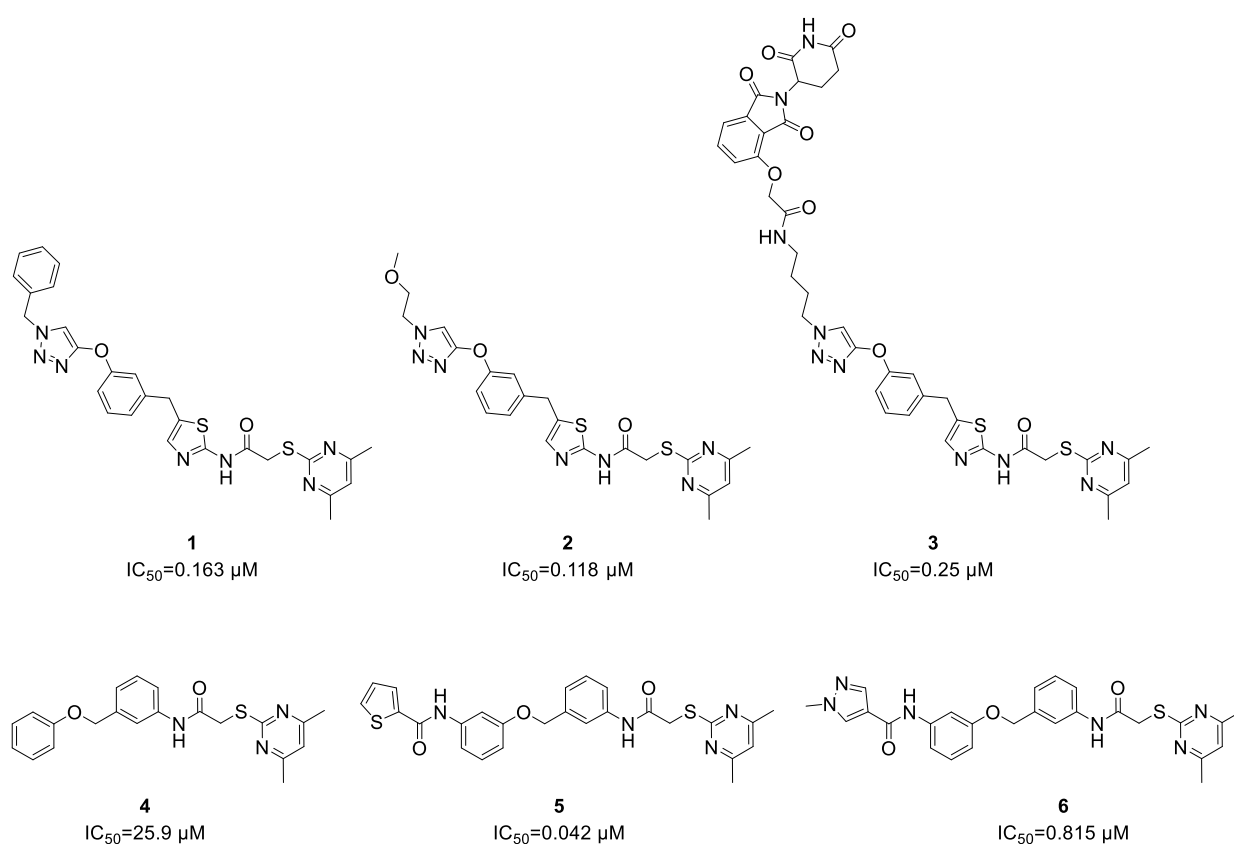


Figure 15. *SirReal*-derived SIRT2 inhibitors: structures and fluorescence-based inhibition profiles.^{240,242–244}

The co-crystal structure of **6** revealed distinct hydrophobic contacts within SIRT2 (Figure 16), plausibly mimicking long-chain acyl-lysine binding.²³⁹

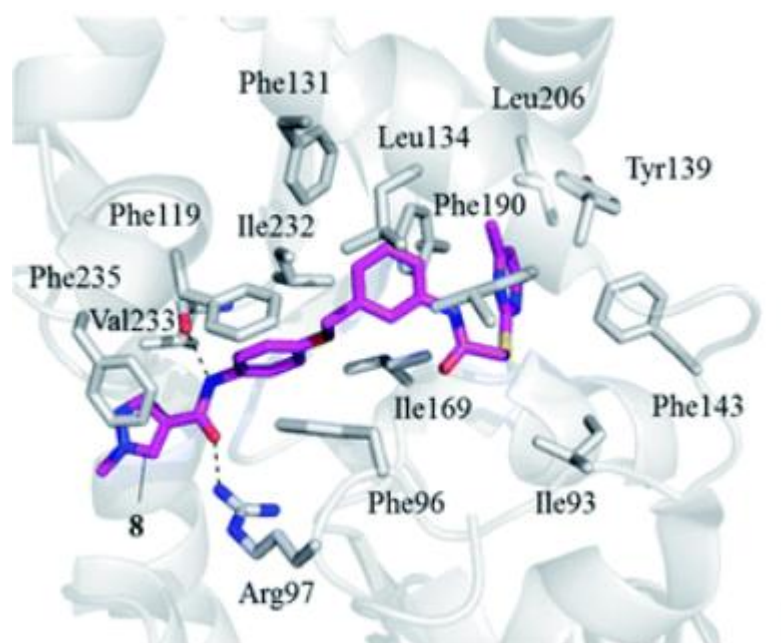


Figure 16. Crystallographic analyses **6** with SIRT2 (PDB: 5YQO). The ligand is deeply buried by hydrophobics on both faces: Phe96, Ile93, Ile169, Phe190 on one side; Phe119, Phe131, Leu134, Tyr139, Phe143 on the other. Stabilization arises from edge-to-face π interactions (notably Phe96/Phe190/Phe235) plus van der Waals contacts. Two polar anchors orient the ligand: an H-bond to Arg97 at the entrance and a backbone H-bond near Val233. NAD^+ remains bound, indicating allosteric inhibition rather than displacement. This hydrophobic burial with sparse H-bonds plausibly mimics long acyl-lysine binding, explaining the compound's potency and SIRT2 selectivity.²⁴⁰

Functionally, **5** increased α -tubulin acetylation in a dose-dependent manner and inhibited BC cells proliferation, while **6** curtailed growth, invasion, and migration in NSCLC models, expanding the toolbox for SIRT2-oriented cancer therapeutics.²⁴³

1.5.3. Nicotinamide and analogues

Nicotinamide (Figure 17), the reaction product of sirtuin-catalysed deacetylation, is a weak, non-selective inhibitor that primarily interferes with deacetylation.²⁴⁵ To improve both potency and selectivity, a fragment-based SAR combining the naphthylamide sulfonic acid motif from suramin with the nicotinamide core was performed, identifying aromatic formamide naphthalene–nicotinamide derivatives. This effort furnished compound **7** (Figure 17), a potent and selective SIRT2 inhibitor with an IC_{50} of 48.3 nM and significant selectivity over SIRT1/SIRT3.²⁴⁵ In cells, **7** increased α -tubulin acetylation in a concentration and time dependent manner. Kinetics indicated acetyl-lysine substrate competition, rather than NAD^+

competition. The same group elaborated the 5-aminonaphthalen-1-yloxy nicotinamide series to generate 5-((3-amidobenzyl)oxy)-nicotinamides (e.g., **8** and **9**, Figure 17), where **8** may compete with both the substrate and NAD⁺; notably, **8** and **9** mitigated α -synuclein-induced cytotoxicity in SH-SY5Y cells, supporting exploration of SIRT2 inhibitors in Parkinson's disease.²⁴⁶

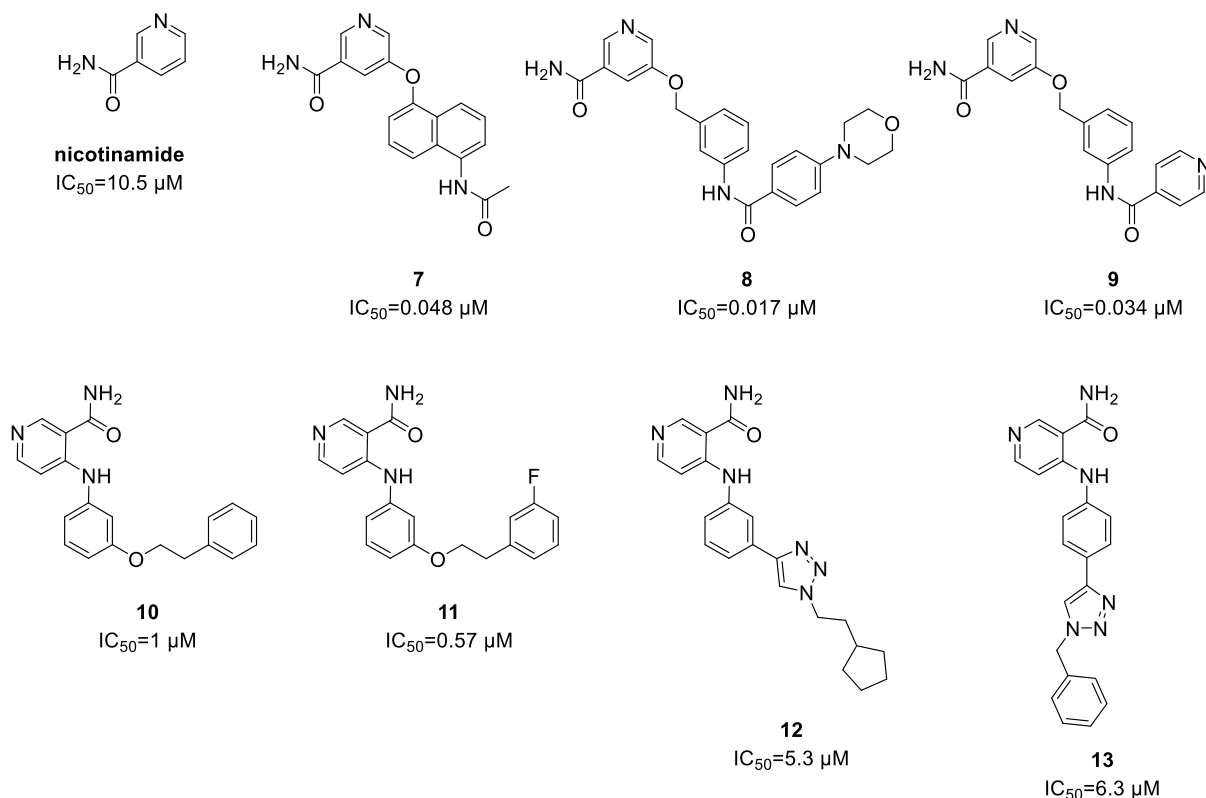


Figure 17. Nicotinamide-based SIRT2 inhibitors: structures and fluorescence-based inhibition profiles.^{240,242–244}

In 2012, 2-anilinobenzamide analogues were introduced as SIRT2 inhibitors and identified **10** and **11** (Figure 17) as potent SIRT2-selective agents outperforming **AGK2** on enzyme selectivity. The cocrystal of SIRT2 and **10** (PDB: 5Y5N) revealed hydrophobic contacts within the induced pocket and a nicotinamide-like moiety engaging water-mediated H-bonds to Phe96, Gln167, Asp95, His187 (Figure 18), underscoring the selectivity pocket in SIRT2-focused design.²⁴⁷ Subsequent click-chemistry libraries of *m/p*-substituted 2-anilinobenzamides uncovered **12** and **13** (Figure 17) as additional SIRT2-selective inhibitors with AGK2-surpassing selectivity.²⁴⁸ Guided by the cocrystal of SIRT2 and **10**, the group later developed **14**, which spans both the substrate “selectivity” site and the NAD⁺ site (Figure 18) and exhibits anti-proliferative activity in MDA-MB-231 and MCF-7 cells.²⁴⁷

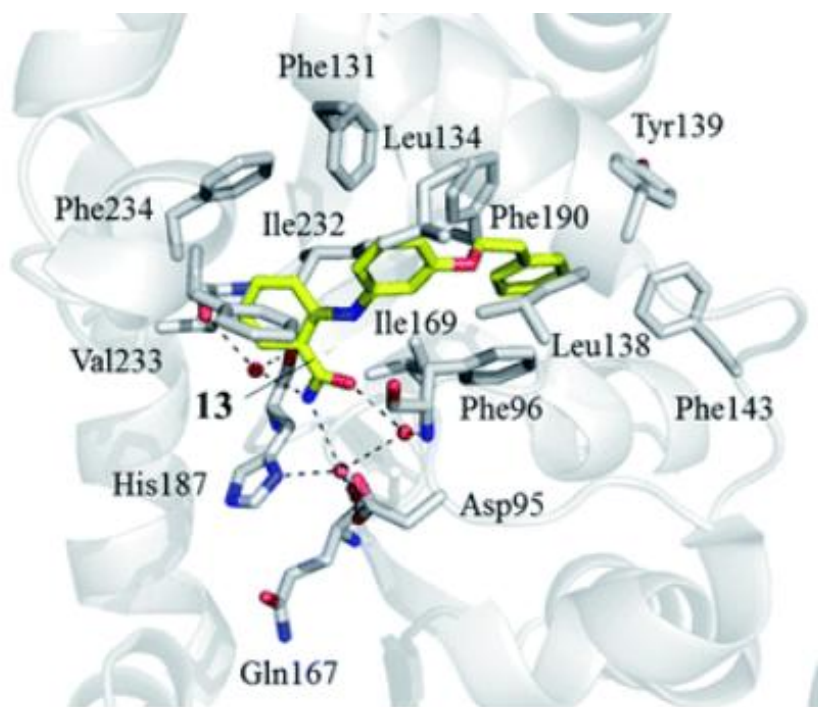


Figure 18. Crystallographic analyses **10** with SIRT2 (PDB: 5Y5N). The ligand adopts an extended pose along the SIRT2 selectivity pocket, adjacent to the catalytic channel. The distal aryl segment is deeply buried by a hydrophobic clamp formed by Phe190, Ile169, Leu134, Phe131, Tyr139, Phe143, and Leu138, while additional apolar contacts with Ile232, Val233, Phe234 and Leu206 cap the pocket. Near the pocket entrance, the nicotinamide-like headgroup engages a polar network: water-bridged hydrogen bonds to the backbone around Asp95/Phe96, and side-chain contacts with Gln167 and His187 orient the amide.²⁴⁰

1.5.4. Hydroxynaphthaldehyde derivatives

Sirtinol (Figure 19), among this class of SIRT inhibitors, displays modest SIRT2 and substantial SIRT1 inhibition but nonetheless elicits antitumor effects *in vitro*, for example, senescence-like arrest in MCF-7 and H1299 cells. Linker optimization led to **salermide**, which maintained SIRT2 inhibition while improving anti-proliferative and pro-apoptotic effects across cancer lines.²⁴⁹ Replacing **sirtinol**'s aniline with 6-phenyl-2-thiouracil yielded **cambinol**, the first SIRT2 inhibitor with *in vivo* antitumor activity, which triggers apoptosis in BCL-6-positive Burkitt lymphoma and is effective in xenografts.¹⁰² Further heterocycle swaps in the pyrimidinedione core generated **14** and **15**, with enhanced SIRT2 selectivity; tetracyclic pyrimidinediones were built by cyclizing the 2-hydroxynaphthalene substituent of **cambinol** and devised tricyclic analogues by ring opening, affording **16** and **17**, with improved SIRT2 inhibition and potent SIRT1 activity.²⁴⁹

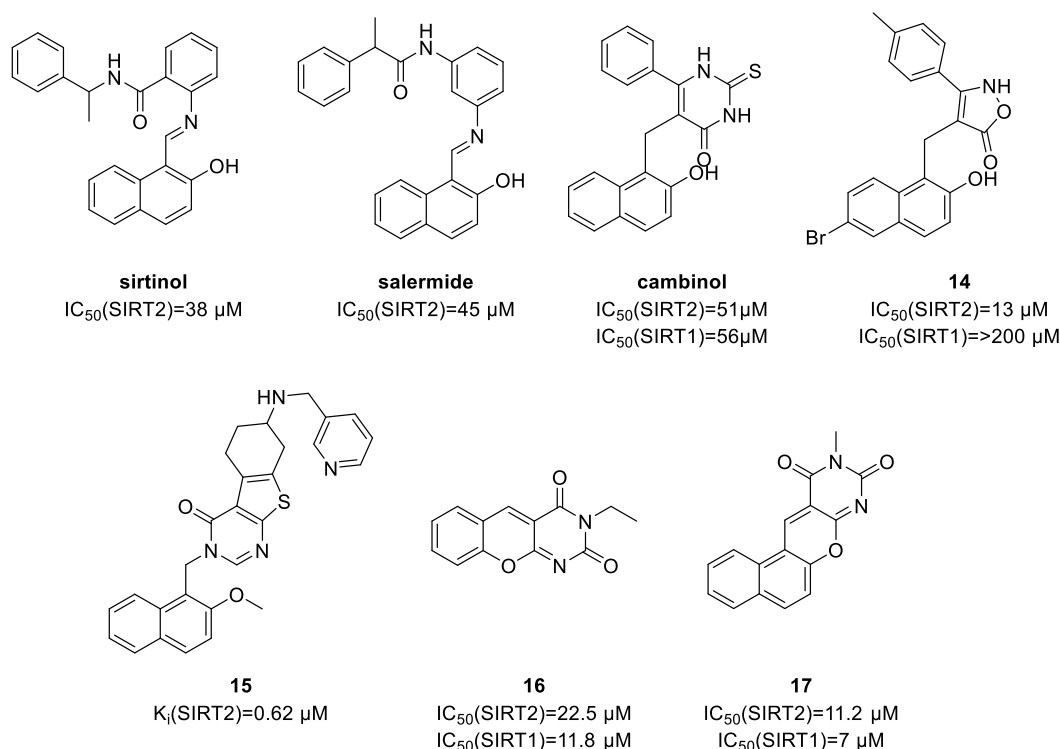


Figure 19. Hydroxynaphthaldehyde-derived SIRT2 inhibitors: structures and inhibition profiles (fluorescence-based and SIRT-Glo).

1.5.5. 3-Benzenesulfonylaminobenzamide derivatives

AK-7 (Figure 20) is the most representative member of this class: despite modest SIRT2 potency ($IC_{50}=15.5\ \mu M$), it exhibits neuroprotection in Huntington's disease models, likely aided by brain permeability.²⁵⁰ Structure-guided SAR tuned sulfonamide/benzoyl substituents and amide orientation, identifying higher-potency inhibitors, notably **18** and **19**.²⁵¹ **AGK2**, a chemically distinct SIRT2 blocker, also confers neuroprotection in Parkinson's disease model.^{6,252} While **AGK2** is active, its α,β -unsaturated carbonyl raises concerns of off-targets/toxicity; replacing this Michael acceptor furnished compound **20** with 6–7-fold higher potency ($IC_{50}=17.75\ \mu M \rightarrow 2.47\ \mu M$).²⁵³ Compound **21** also improved inhibitory activity and selectivity for SIRT2.²⁵⁴

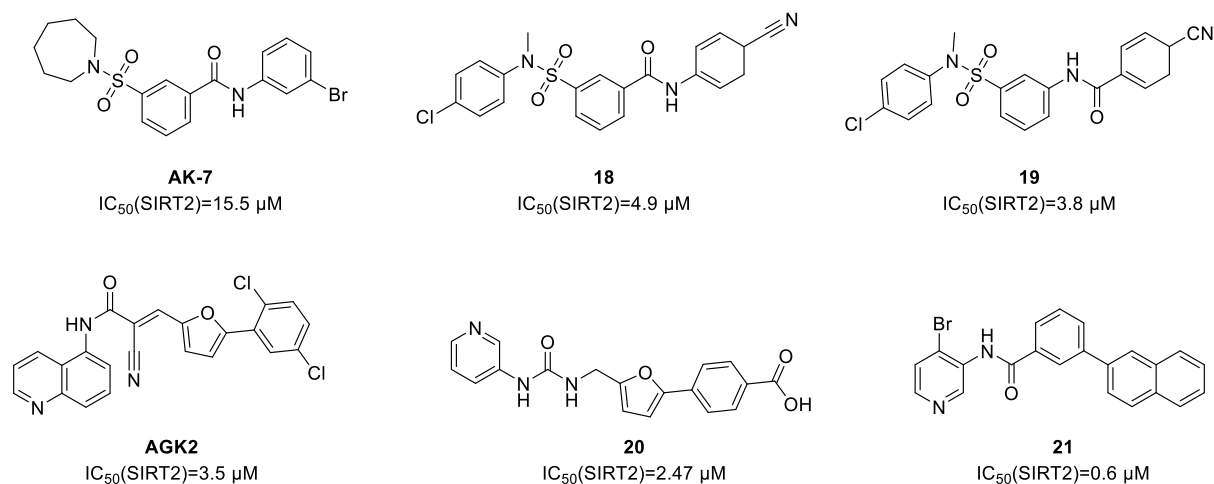


Figure 20. 3-Benzenesulfonylaminobenzamide SIRT2 inhibitors: structures and inhibition profiles.

1.5.6. Indole derivatives and analogues

Indole-based SIRT inhibitors (**22**, **23**, Figure 21) were identified by high-throughput screening; these preferentially inhibit SIRT1 over SIRT2, likely by blocking SIRT1-catalyzed deacetylation.²⁵⁵ To advance the scaffold, 2,3,4,9-tetrahydro-carbazole of **23** was replaced with 2,3,4,5-tetrahydro-pyrido[4,3-*b*]indole, generating compound **24** and **25**, which showed enhanced potency/selectivity and increased p53 and α -tubulin acetylation in HepG2 and MDA-MB-231 cells.²⁵⁶ In parallel, opening the benzene of the carbazole and swapping from a pyrrole to a pyrazole core, led to obtain 4,5,6,7-tetrahydro-1*H*-indazoles, among which **26** (Figure 21) most potently inhibited SIRT2 (with moderate SIRT1 activity).²⁵⁷ Adenosine-mimetic indoles such as **27** were reported to inhibit SIRT1 and SIRT2 with a IC₅₀=3.5 μM and 0.8 μM, respectively.²⁵⁸ More recently, a triazole anchor was added to yield indoles like **29**, that engage the SIRT1/2 cavity.²⁵⁹

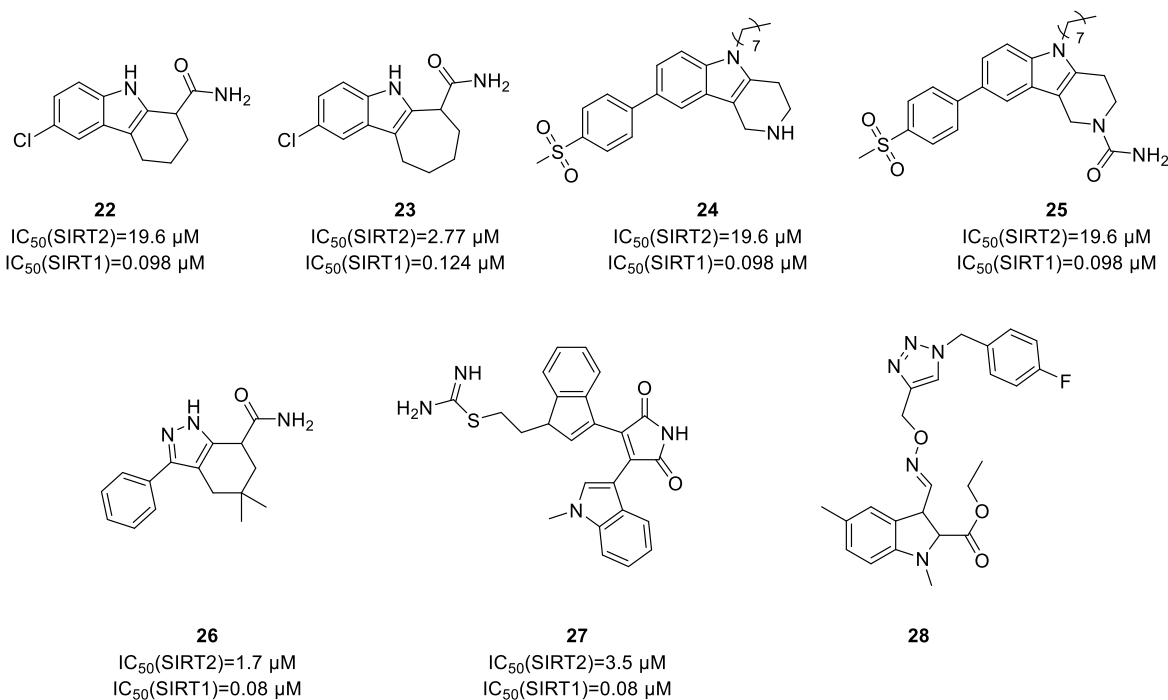


Figure 21. Indole-based SIRT2 inhibitors: chemotypes and inhibition profiles across sirtuin isoforms.

1.5.7. Tenovins and analogues

Tenovin-1 (Figure 22) was discovered in 2008 through low-toxicity phenotypic screenings. It activates p53 and suppresses tumour growth.²⁶⁰ Subsequently, the discovery of the water-soluble **Tenovin-6** and the following target validation established SIRT1/SIRT2 inhibition as its primary mode of action. SAR on the A-ring produced **29**, with markedly improved potency and selectivity over **Tenovin-1/-6**. In recent years, 1-(2,2-dimethyl-dihydrobenzopiperan-4-yl)-3-phenylureas (e.g., **30**) were unveiled as SIRT1/2 inhibitors with anti-proliferative activity in U373, T98G, Hs683 glioma lines and zebrafish xenografts.²⁶¹ This urea scaffold can be viewed as a cyclized Tenovin A-ring analogue and is therefore classified here as a Tenovin analogue.

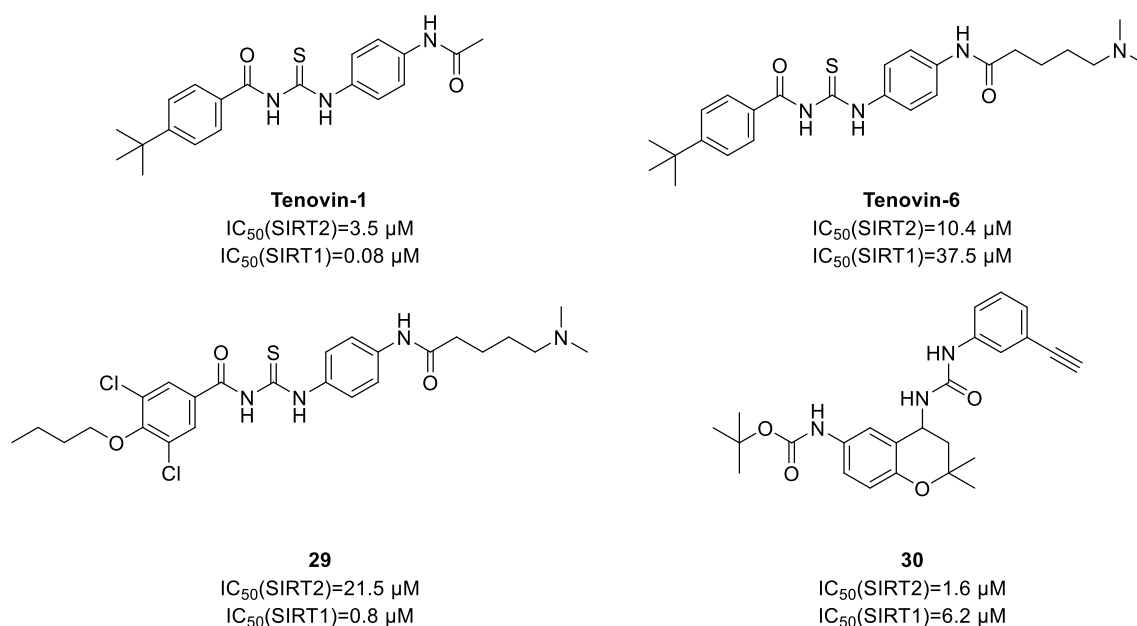


Figure 22. Tenovin based SIRT2 inhibitors: chemotypes and inhibition profiles.

1.5.8. 3,5-Disubstituted-1,2,4-oxadiazoles

In 2008, **31** (Figure 23) was identified through virtual screening as a dual SIRT1/2 inhibitor. SAR studies (ring substitutions, linker edits) yielded SIRT1-improved analogues, with no significant improvement on SIRT2 affinity.²⁶² A separate virtual screen targeting the pocket of SIRT2/3/5 found **32**, bearing the 3,5-disubstituted-1,2,4-oxadiazole core.¹¹¹ Subsequent SAR delivered SIRT2-selective inhibitors **33** and **34** with enhanced potency.

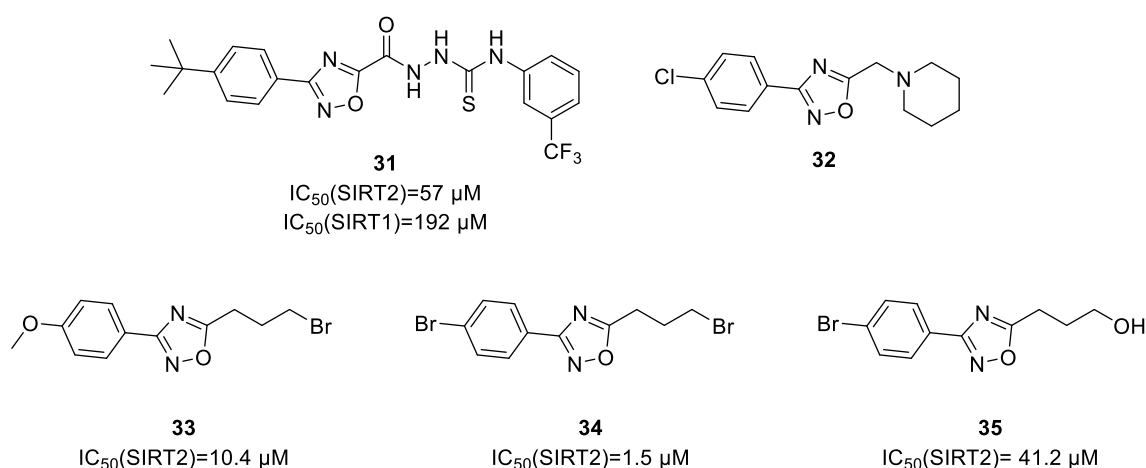


Figure 23. Oxadiazoles based SIRT2 inhibitors: chemotypes and inhibition profiles.

Crystallography of **35** bound to SIRT2 located the ligand in a previously unexplored hydrophobic site bordered by Leu138, Leu134, Ile69 (Figure 24), reinforcing the concept of

SIRT2-specific hydrophobic pockets for selective inhibitor design. Kinetic data were consistent with uncompetitive inhibition versus both substrate and NAD^+ .²⁶³

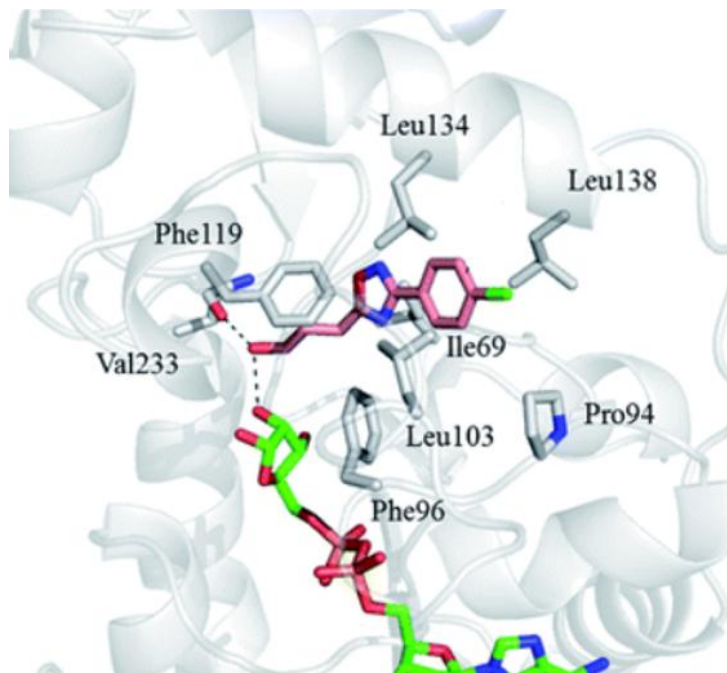


Figure 24. Crystallographic view of **35** (in pink) bound in the selectivity pocket adjacent to the catalytic cleft of SIRT2. The ligand lays beneath the 92–96 loop (Pro94–Phe96) while NAD^+ (green) remains co-bound, indicating co-occupancy rather than cofactor displacement. Binding is dominated by a hydrophobic pocket formed by Ile69, Leu103, Leu134, Leu138, Phe96, and Phe119, with additional enclosure toward Val233. A linker amide donates a backbone hydrogen bond in the Phe119/Val233 region (dashed line), anchoring the pose.

1.5.9. Benzopyrone derivatives

Benzopyrones as SIRT2-selective inhibitors, including **36** (Figure 25), were synthesized via microwave-assisted, base-mediated aldol condensation in a single step.²⁶⁴ Subsequent SAR on 2-position substitution produced **37** and **38** with comparable or slightly reduced activity compared to racemic **36**.²⁶⁵ Biologically, **37** and **38** inhibited MCF-7 and A549 cells proliferation in a dose-dependent manner and increased α -tubulin acetylation in MCF-7. By photoaffinity labelling, either a diazirine or an azide moiety was added at the 6-position of **36**, affording photoactivatable probes **39** and **40**. Particularly, **39** retained superior SIRT2 potency and enabled binding-site mapping.²⁶⁶ Most recently, bioisosteric replacement of the benzopyrone core with quinolin-4(1*H*)-ones, benzothiazine-1,1-dioxides, benzothiadiazine-

1,1-dioxides, and saccharins yielded multiple series, and benzothiadiazine-1,1-dioxides emerged as the most potent SIRT2 inhibitors, exemplified by **41**.²⁶⁷

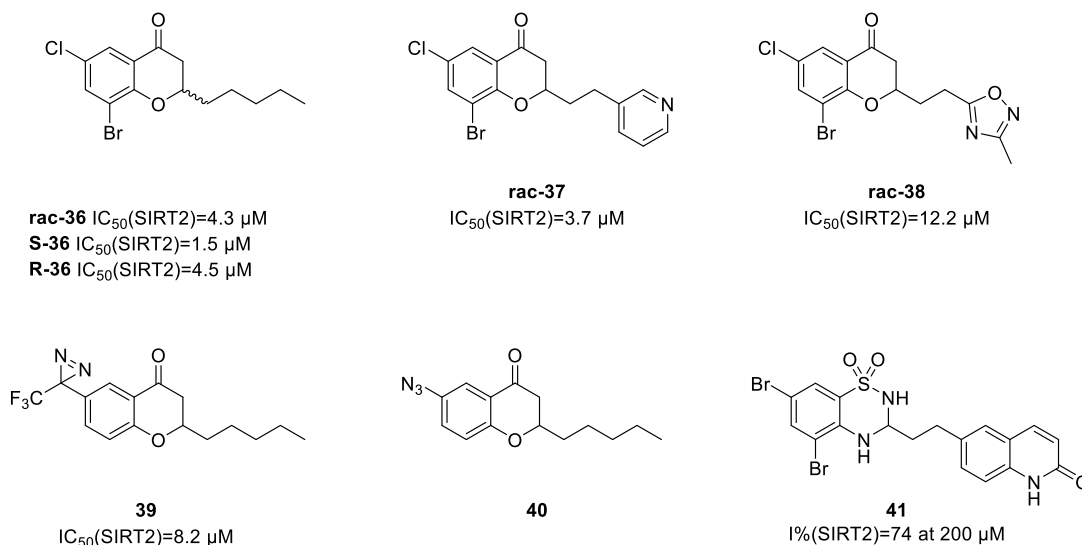


Figure 25. Benzopyrone based SIRT2 inhibitors: chemotypes and inhibition profiles.

1.5.10. Thiomyristoyl lysine derivatives

Thiomyristoyl lysine derivatives (Figure 26) are mechanism-based covalent inhibitors that mimic myristoylated lysine substrates. They form a stable thioimide intermediate with NAD^+ , blocking both deacetylation and demyristoylation.²³⁵ Thiomyristoyl lysine derivatives displays a 650-fold selectivity for SIRT2 over SIRT1 and induces c-Myc degradation, suppressing tumorigenic growth in HCT116 colon carcinoma cells. SIRT2 overexpression rescues these effects, confirming on-target inhibition.

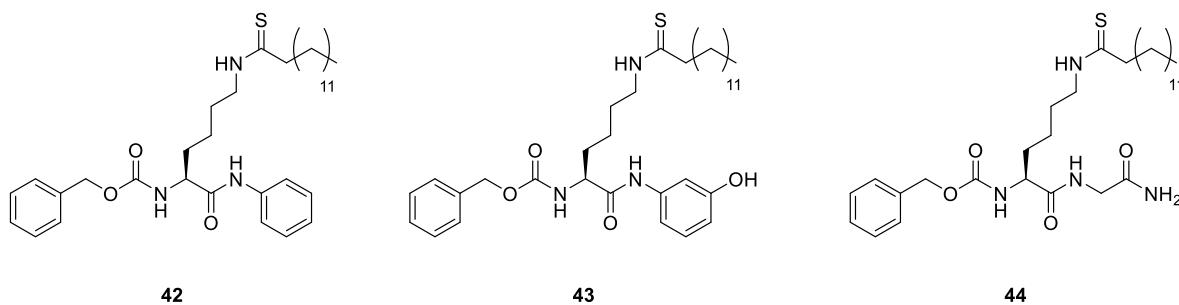


Figure 26. Thiomyristoyl lysine based SIRT2 inhibitors.

1.5.11. Other inhibitors

Suramin sodium salt (Figure 27), a large hydrophilic molecule, was reported in 2007 as a dual SIRT1/2 inhibitor, the same year, it was identified as a SIRT5 binder and structurally

characterized in complex with SIRT5.^{268,269} In 2009, **45**, a benzothiazole quaternary ammonium compound, was disclosed as a SIRT2-selective inhibitor that raises α -tubulin, p53, and H4 acetylation and selectively kills cancer cells via apoptosis.²⁷⁰ Pyrimidothiophenes (e.g., **46**) were discovered using Encoded Library Technology (ELT) as pan-SIRT1/2/3 inhibitors with nanomolar potency. The triazole **47** was reported to have a neuroprotective activity in a *Drosophila* Huntington's model.²⁷¹ More recently, benzothieno[3,2-*d*]pyrimidines (e.g., **48**) showed good cytotoxicity against MCF-7 and UO-31 cell lines and selective SIRT2 inhibition.²⁷² Finally, benzimidazoles (e.g., **49**) have also been described as SIRT2 inhibitors, collectively expanding the chemical space and providing probes to interrogate SIRT2 biology across disease contexts.^{273,274}

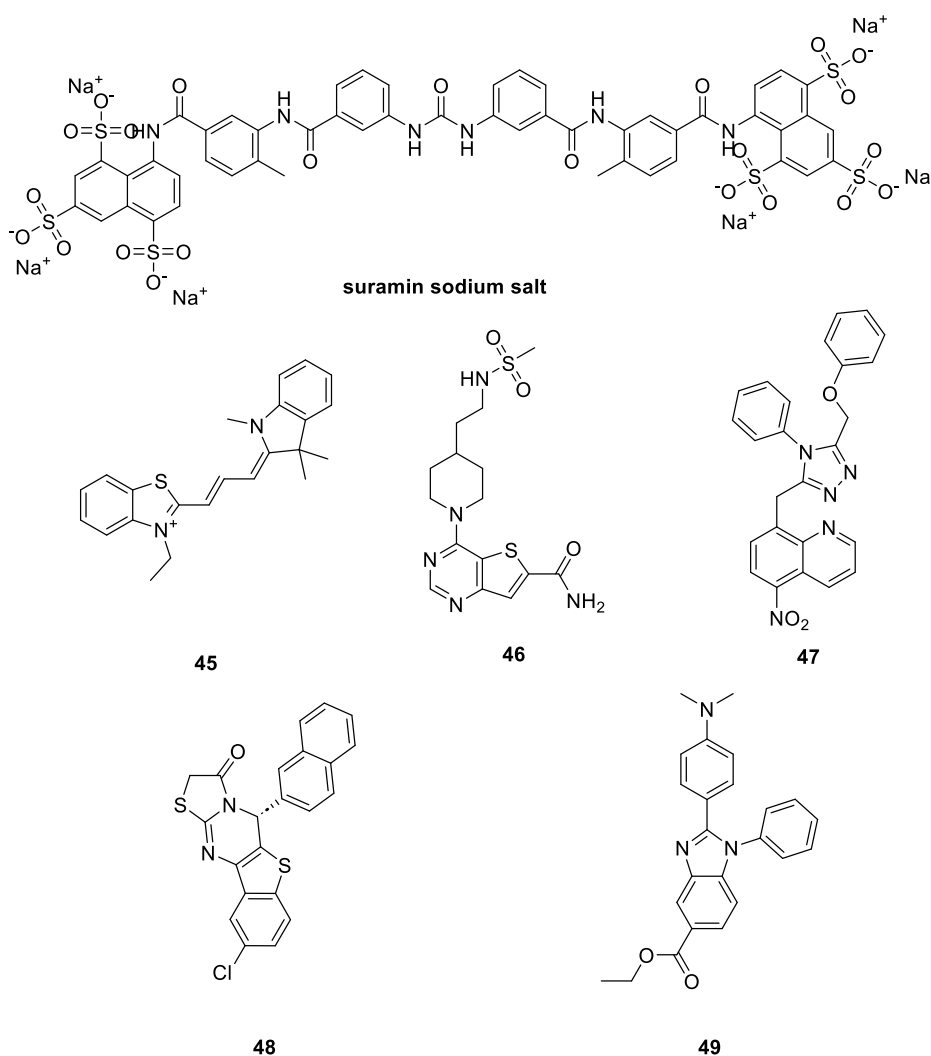


Figure 27. Chemical structures of other SIRT2 inhibitors.

1.5.12. Pyrazolopyrimidine derivatives

A survey of SIRT2 co-crystal structures indicates that high-affinity ligands frequently display a central bicyclic heteroaromatic core flanked by two hydrophobic terminal substituents, with at least one terminal ring being heteroaromatic or bearing H-bonding functionality. Consistent with this pattern, preliminary screenings suggested that incorporation of a bicyclic heteroaromatic scaffold supports SIRT2 inhibition, as exemplified by **50** (Figure 28).

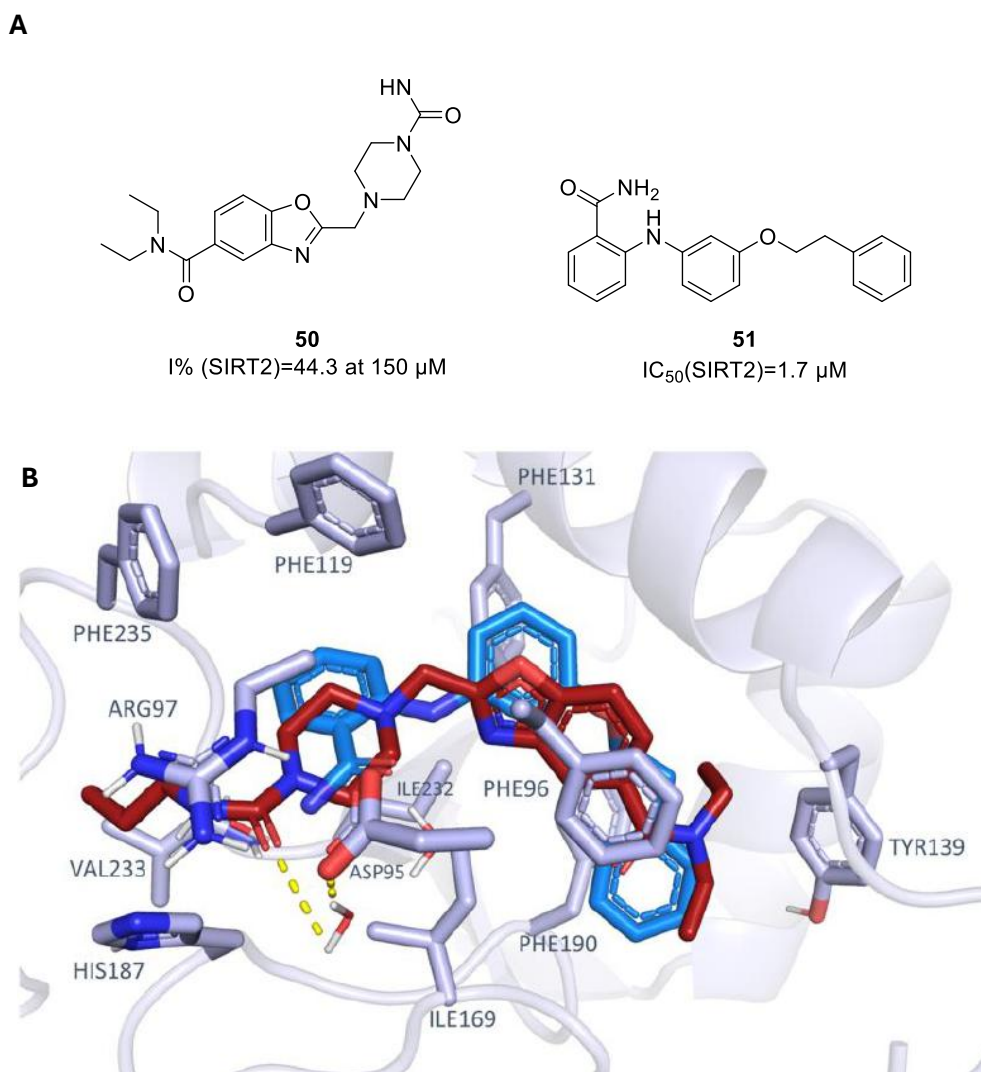


Figure 28. (A) Chemical structure and inhibition profiles of SIRT2 inhibitors **50** and **51**; (B) Docking pose of **50** (in red) overlaid with the SIRT2 co-crystallized with the selective nicotinamide derivative **51** (PDB: 5Y5N, in blue). Hydrogen bonds are depicted as yellow dashed lines.²³⁶

These observations motivated a focus on pyrazolo[3,4-*d*]pyrimidines as a main chemotype for further study, given their planarity, tractable synthesis, and capacity to project two termini into the SIRT2 pocket. Hence, an initial set of five derivatives (**52-56**) was selected from an *in-house* library to represent the structural requirements deduced from crystallography and virtual screening (Figure 29).

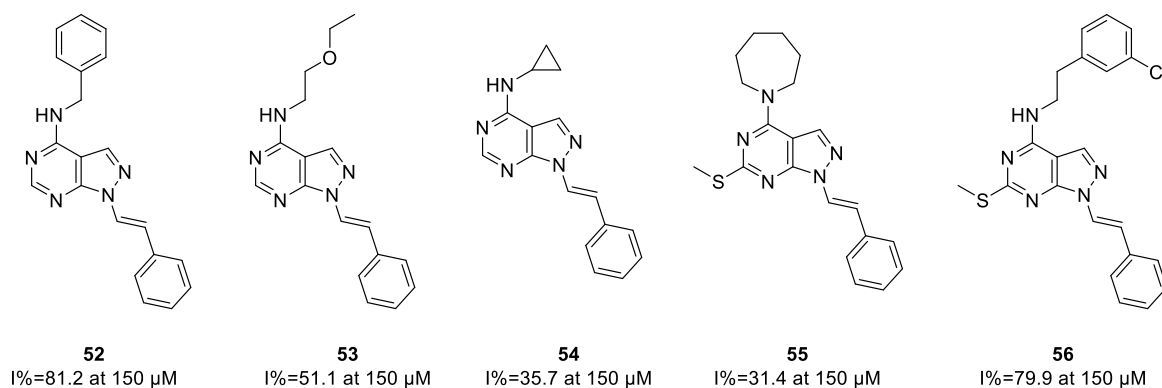


Figure 29. Chemical structure of the *in-house* pyrazolo[3,4-*d*]pyrimidine derivatives **52-56** selected as SIRT2 inhibitors and their SIRT2 enzymatic activity inhibition profile. Each compound was tested at the final concentration of 150 μ M.

Compound **52** was designated the reference, bearing two unsubstituted phenyl rings tethered to the central pyrazolo-pyrimidine core, whereas analogues **53-56** explored the replacement of one or both phenyl termini with additional aromatic or flexible H-bonding groups, thereby probing whether polar vectors could be accommodated without compromising shape complementarity. Prior synthetic access to **52-56** facilitated immediate re-profiling in the SIRT2 context.^{275,276}

Binding hypotheses were developed using induced-fit docking to allow local side-chain adaptation in the selectivity pocket and adjacent groove. In the preferred poses, the pyrazolo-pyrimidine nucleus aligns with the planar pharmacophore, that in **51** corresponds to an amide-substituted aniline. The two termini projected into a hydrophobic corridor culminating in the induced selectivity pocket, with recurrent contacts to Val233, His187, Phe96, and Phe235 (Figure 29). For **52** and **56**, a phenylalkyl-amine substituent oriented toward His187/Phe235, enabling π - π and cation- π interactions, and formed a hydrogen bond to Val233 that was often retained via a tightly bound water molecule; additional stabilization arose from a water-mediated H-bond to Phe96 in **52** or a direct H-bond to His187 in **56** (Figure 30). The vinyl moiety in **52** and **56** reproduced, respectively, the phenoxy and aniline vectors of **51**,

rationalizing favourable geometries. In contrast, bulky polar termini increased three-dimensionality and reduced planarity near Val233, disrupting the polar pin and diminishing π -stacking opportunities.²³⁶

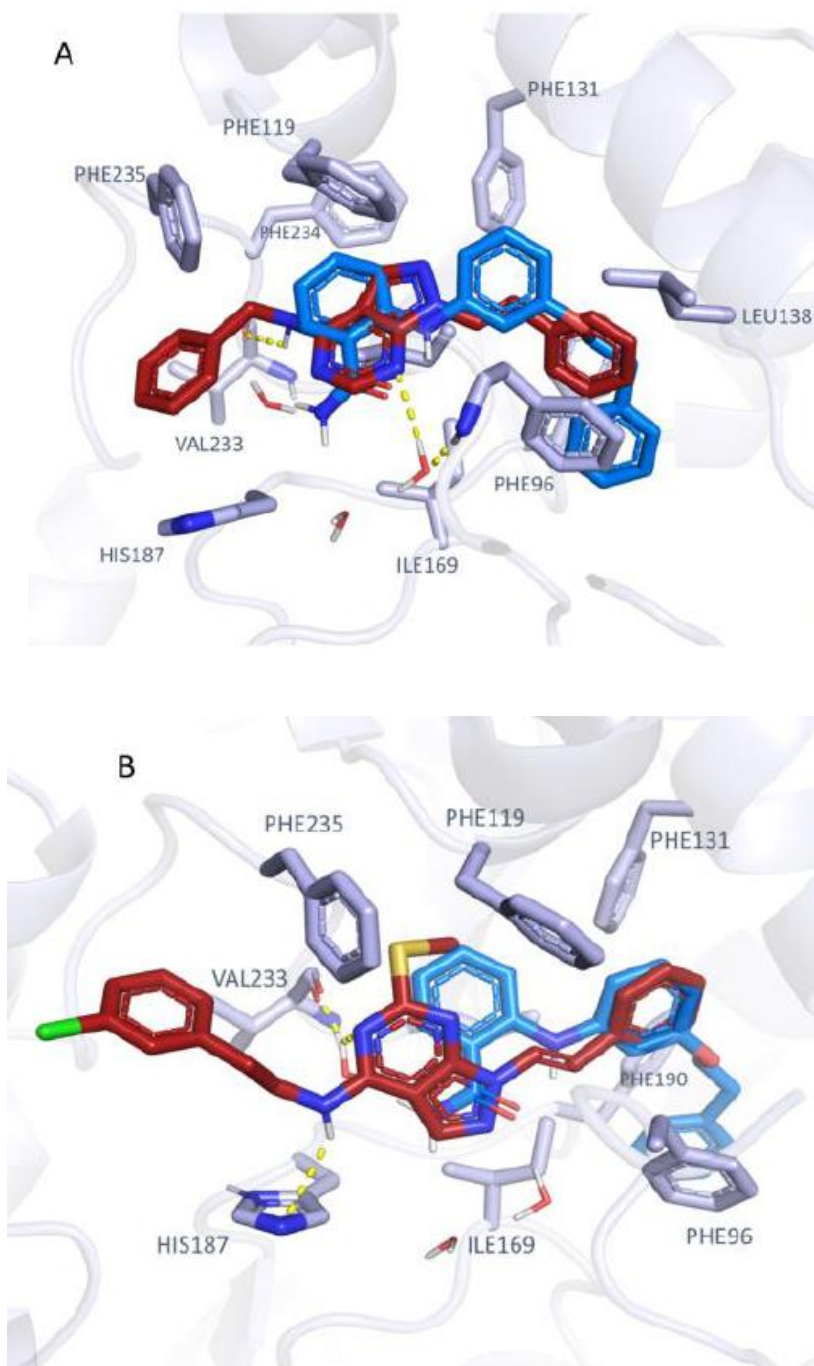


Figure 30. Docking pose of 52 (A) and 56 (B) (in red) overlaid with the SIRT2 co-crystallized with the selective nicotinamide derivative 51 (PDB: 5Y5N, in blue). Hydrogen bonds are depicted as yellow dashed lines.²³⁶

The docking models and co-crystal analysis point to three recurrent elements that appear to govern productive engagement: (i) two hydrophobic termini remain generally favourable, aligning with the X-ray survey; one terminal substituent can be either aromatic or heteroaromatic while the other may incorporate a flexible H-bonding moiety, retaining global planarity and vector geometry; (ii) aromatic recognition at His187/Phe235, supporting π -stacking/cation- π interactions from one terminal group; (iii) a polar anchor at Val233, often realized as a direct or water-bridged H-bond from an amide/amine vector; (iv) hydrophobic enclosure beneath the 92–96 loop (Pro94–Phe96), with Phe96 frequently contributing to shape complementarity (Figure 30). These interactions are allowed with SIRT2's selectivity pocket, which can open adjacent to the catalytic cleft while NAD⁺ remains co-bound, enabling cofactor retention with concomitant allosteric occupancy. Under these conditions, compact and planar termini tend to be better accommodated than bulky polar groups, which can perturb water networks and compromise the alignment of donor/acceptor vectors toward Val233.

These findings prompted a second modelling cycle on an internal library previously disclosed as kinase inhibitors, yielding thirteen SIRT2 candidate inhibitors that were designed to probe (i) flexible aryl-alkyl substituents at N1 to optimize hydrophobic complementarity along the Phe96/Phe131/Leu137 shelf, (ii) length/electronics at C4 to address the His187–Val233–Phe235 recognition zone, and (iii) lipophilic handles at C6 to bias the core toward Ile169 within the SIRT2 cleft.^{236,277,278} Among these, compounds **57–59** showed the most pronounced target inhibition in biochemical assays (Figure 31). Since compounds **57–59** exceeded 85% inhibition of SIRT2 in a single-point screening at 150 μ M, these analogues were titrated to IC₅₀.

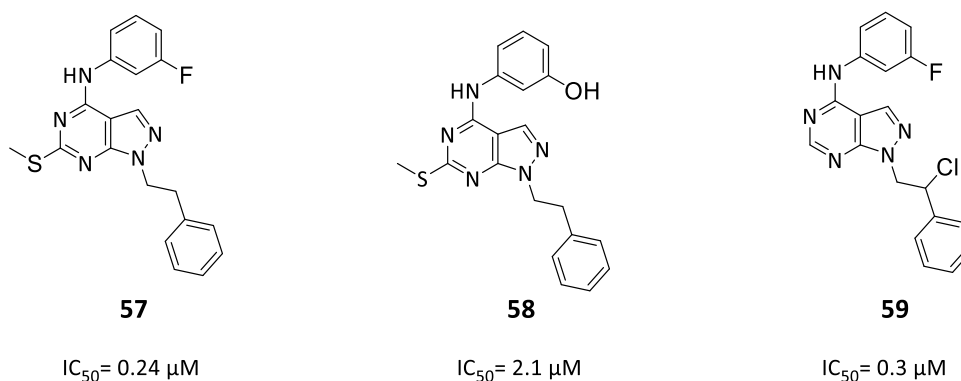
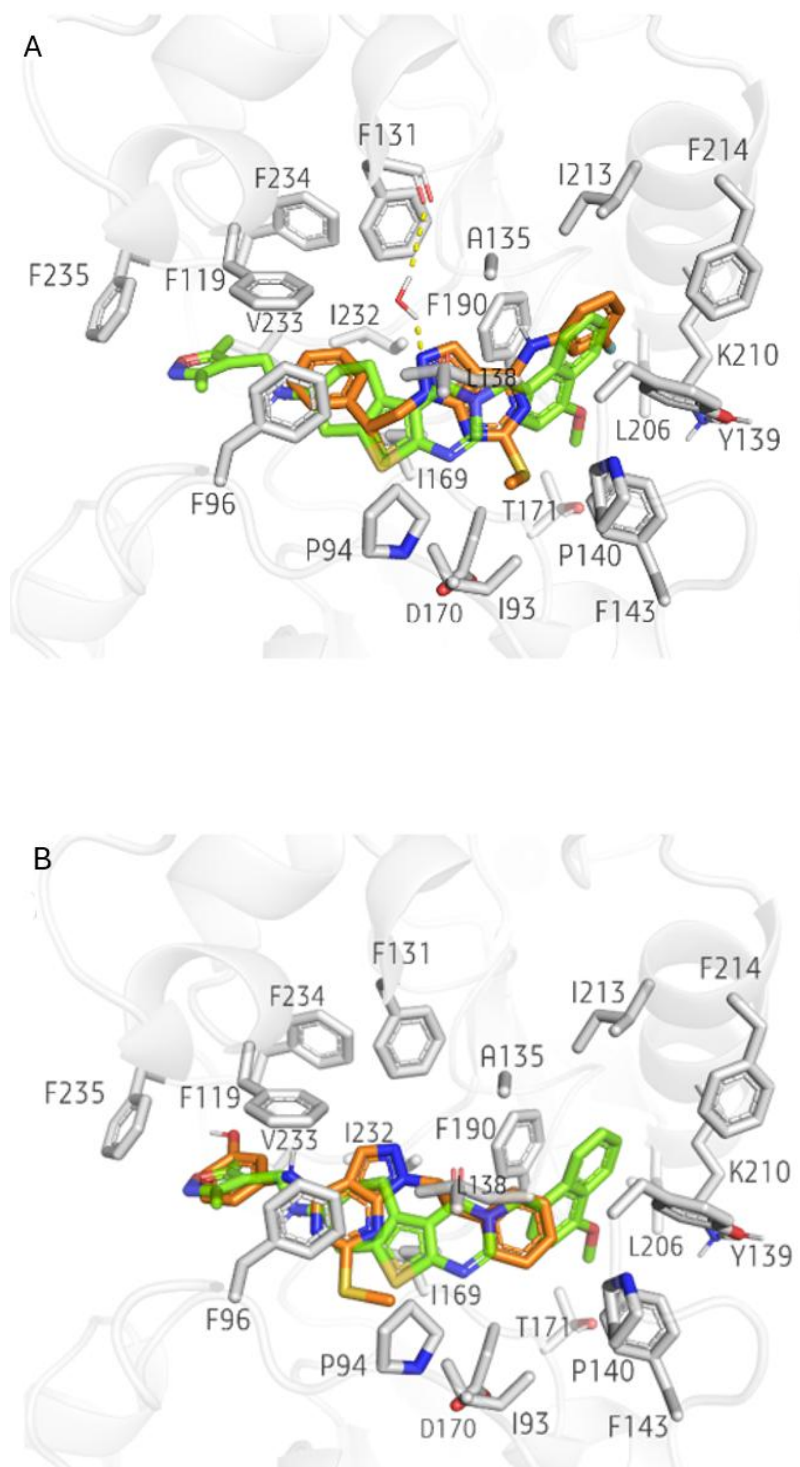


Figure 31. Chemical structure of the in-house pyrazolo[3,4-*d*]pyrimidine derivatives **57–59** selected as SIRT2 inhibitors and their IC₅₀ values.

For the three depicted leads, the N1 phenylalkyl amine orients toward Phe235/His187, enabling π - π /cation- π interactions, and establishes a hydrogen bond to Val233 (direct or water-mediated), frequently complemented by auxiliary H-bonding to Phe96 or His187 (Figure 32).



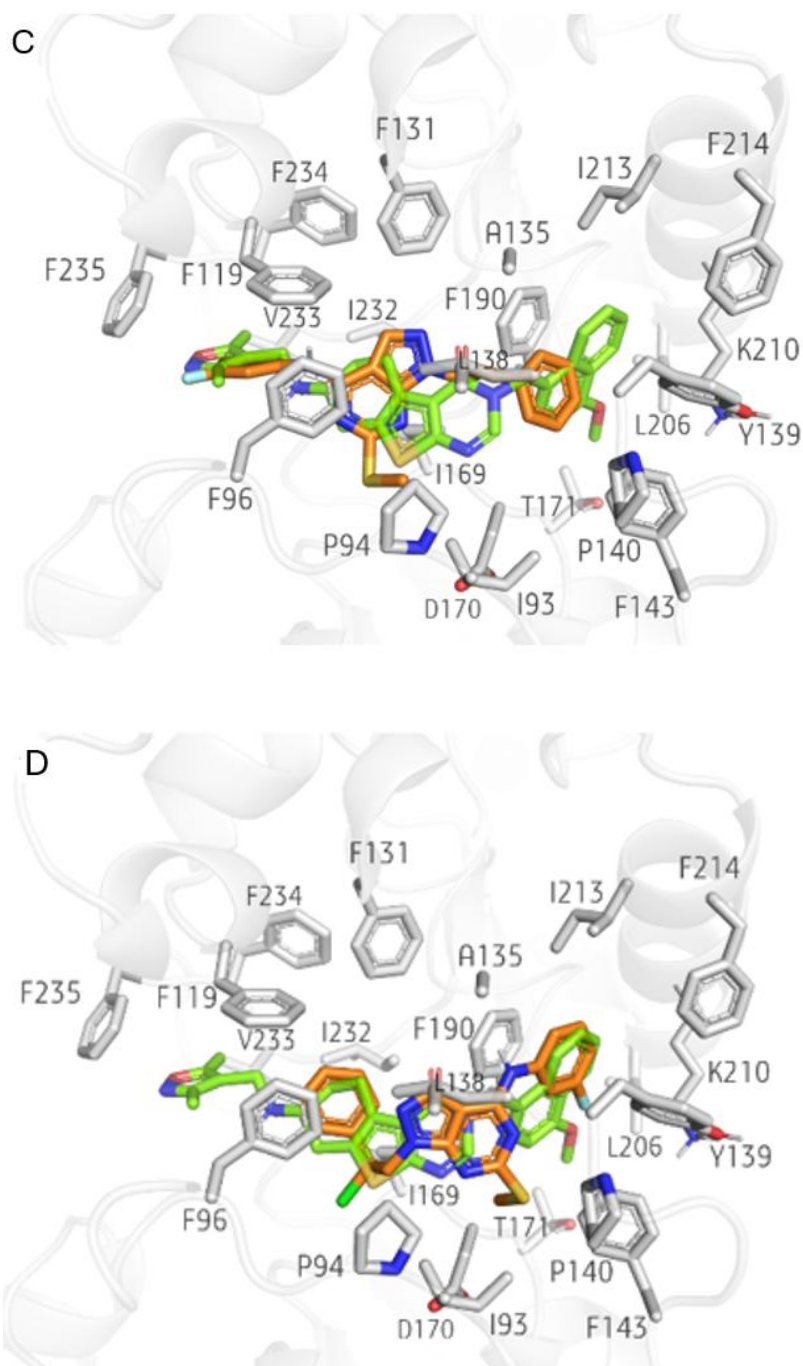


Figure 32. Docking pose of **57** (A), **58** (B), **59(R)** (C) and **59(S)** (D) (in orange) overlaid with the SIRT2 co-crystallized with a thienopyrimidinone derivative (PDB: 5MAT, in green).

Enantiomeric docking for compound **59** revealed two recurring solutions that exchange the roles of the N1 and C4 aromatics on opposite sides of the crevice; for example, **59(R)** forms a water-mediated contact to Phe131 while directing the phenethyl arm toward

Phe96/Val233/Phe235, and **59(S)** adopts the inverted arrangement with the C4 aryl aimed at Tyr139/Phe190/Phe214 (Figure 32, panels C and D). N1 phenethyl functions as the principal aromatic handle for Phe235/His187 recognition and for Val233 anchoring through a strategically placed donor/acceptor. The C4 anilide provides a second vector capable of either reinforcing Val233 H-bonding or extending toward Tyr139/Phe190/Phe214, thereby improving enclosure upon partial closure of the 92–96 loop. The C6 lipophile biases the bicyclic plane toward Ile169, improving van der Waals complementarity along the bottom of the pocket. In contrast, bulky polar termini that increase three-dimensionality tend to disrupt the Val233 water network and compromise π -stacking.

The design of **57-59** is coherent with the earlier virtual screening already seen for **51** (Figures 28 and 30), which engaged Phe96 through π -stacking and established water-mediated contacts involving Asp95/Gln167, while anchoring the polar functionality near Val233. These features are mirrored by the pyrazolo[3,4-*d*]pyrimidine series. The present leads thus represent a chemically tractable translation of those binding heuristics into a compact, planar heteroaromatic core capable of exploiting the same residue triad.

The data collectively suggest three immediate directions for SAR optimization: (i) fine-tuning the Val233 H-bond; (ii) enhancing π -stacking/cation- π with His187/Phe235 through vinyl/ethynyl-linked aromatics at either N1 or C4 to maintain the pharmacophore and (iii) controlling planarity and compactness at the end proximal to the 92–96 loop to favour lid closure without steric clashes.

Collectively, these evidences validate the pyrazolo-pyrimidine scaffold as a productive starting point for SIRT2 inhibition.

2. Rationale and aims of the project

The identification of selective SIRT2 inhibitors has become an important objective in medicinal chemistry, owing to the biological relevance of sirtuins in cancer biology, metabolism, cell-cycle regulation, and neurodegenerative processes. The availability of numerous X-ray structures of SIRT2–ligand complexes has provided a rich structural foundation for rational drug design, enabling detailed interrogation of key molecular determinants involved in ligand recognition and selectivity. This structural information prompted our group, in collaboration with Prof. Cichero and Prof. Bruzzone of the University of Genoa, to undertake a structure-

based investigation aimed at discovering new SIRT2 inhibitors within an *in-house* library of pyrazolo[3,4-*d*]pyrimidines.

The structure-based investigation revealed several recurring features among high-affinity SIRT2 ligands. Potent inhibitors frequently possess a planar bicyclic heteroaromatic core flanked by two terminal hydrophobic groups, one of which may incorporate heteroatoms or a hydrogen-bond donor/acceptor. This architecture positions the central scaffold within the catalytic cleft while projecting the termini into a hydrophobic channel that culminates in the inducible selectivity pocket. Hydrogen bonding, direct or water-mediated to Val233, as well as π - π or cation- π interactions involving His187 and Phe235, emerged as consistent contributors to productive binding (Figures 28 and 30).²³⁶

These insights motivated the selection of five pyrazolo[3,4-*d*]pyrimidine derivatives (**52–56**) from our internal collection as representatives of the binding features identified in crystallographic surveys (Figure 29). As previously reported, compound **52**, bearing two unsubstituted phenyl rings, was chosen as the reference structure, while analogues **53–56** explored the introduction of flexible or polar substituents to probe the tolerance of the SIRT2 pocket to different terminal vectors. Their prior synthetic accessibility facilitated rapid profiling and comparison within the SIRT2 context.^{275,276} Docking studies supported the hypothesis that the pyrazolo-pyrimidine core effectively reproduces the essential pharmacophore observed in known inhibitors such as compound **51** (Figure 28). Preferred docking poses indicated that both aromatic termini align along the hydrophobic corridor shaped by Phe96, Phe131 and Leu137, while also engaging the His187–Val233–Phe235 triad. For compounds **52** and **56**, which displayed 81.2% and 79.9% SIRT2 inhibition at 150 μ M, respectively, N1 phenylalkyl-amines adopted favourable orientations toward His187/Phe235, enabling π - π or cation- π interactions and forming a polar contact with Val233, often mediated by a tightly conserved water molecule (Figures 28 and 30). The vinyl moieties in these compounds reproduced the positioning of pharmacophores present in **51**, supporting their compatibility with the binding pocket.²³⁶ In contrast, derivatives bearing bulkier polar groups displayed reduced planarity near Val233, which disrupted the polar anchoring network and interfered with key aromatic interactions.

These observations reinforced the notion that maintaining compactness and overall planarity is crucial for productive engagement within the SIRT2 active site. Such conclusions informed the design of a second generation of analogues, leading to the synthesis of thirteen new pyrazolo-

pyrimidines with strategic variations at N1, C4 and C6. The rationale guiding these modifications included: (i) optimization of hydrophobic complementarity near Phe96/Phe131/Leu137; (ii) reinforcement of interactions within the His187–Val233–Phe235 recognition zone; and (iii) fine-tuning of lipophilic positioning toward Ile169 to improve van der Waals enclosure.^{236,277,279,278}

Among these new analogues, compounds **57–59** exhibited the most potent inhibitory activity (Figure 31). Their preferred docking conformations showed that the N1 phenethyl group served as the principal aromatic handle for Phe235/His187 recognition while contributing a key donor/acceptor vector for anchoring to Val233. Meanwhile, substituents at C4 provided secondary interactions either reinforcing the Val233 hydrogen bond or extending toward Tyr139, Phe190 or Phe214. The C6 lipophilic substituent ensured productive hydrophobic complementarity at the base of the cleft (Figure 31).²³⁶

These modelling cycles informed the design of two virtual libraries of analogues (Figure 33). The first series, derived from the activity profiles of compounds **52** and **56**, incorporates a phenylvinyl group at the N1 position. The second series, inspired by compounds **57–59**, is defined by a substituted or unsubstituted phenethyl chain at N1. In both libraries, additional diversity was introduced at positions 4 and 6 to further probe the SAR.

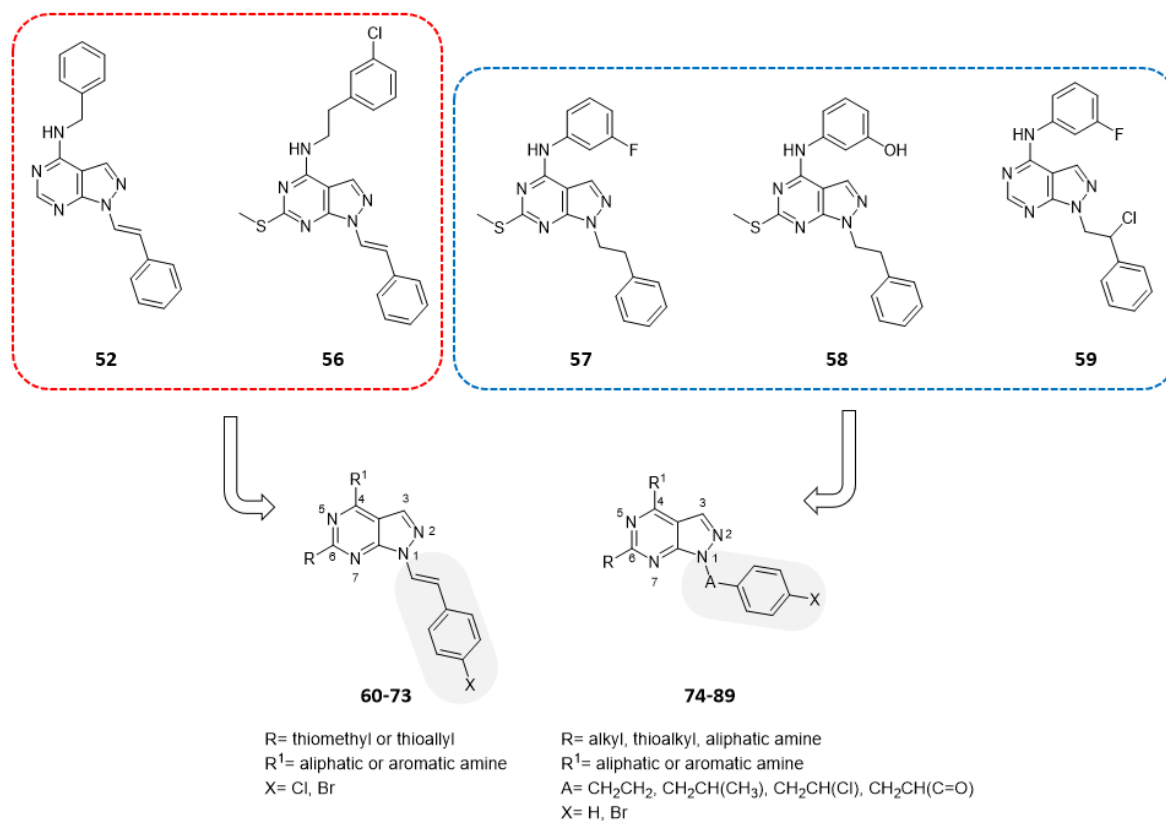


Figure 33. General structure of the synthesised pyrazolo[3,4-*d*]pyrimidine derivatives 60-89.

The collective evidence derived from crystallographic surveys, modelling, and biochemical data strongly supports the pyrazolo[3,4-*d*]pyrimidine scaffold as a promising foundation for the development of selective and potent SIRT2 inhibitors. Moreover, the SAR trends obtained to date highlight several concrete directions for future optimization. These include refining the hydrogen-bond interaction with Val233, strengthening the aromatic contacts with His187 and Phe235, and maintaining sufficient scaffold planarity to ensure efficient accommodation beneath the 92–96 loop.

On the basis of the most promising pyrazolo[3,4-*d*]pyrimidine derivatives, a second design phase was undertaken in which a molecular simplification strategy was applied. Guided by docking studies performed in collaboration with Prof. Cichero of the University of Genoa, the N2 pyrazole nitrogen was formally incorporated into the pyrimidine ring, affording a new heterocyclic scaffold with a triazine core (Figure 34).

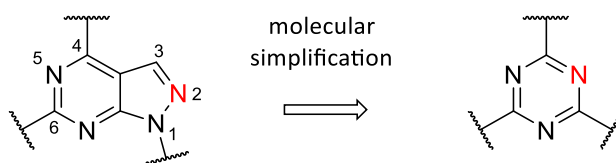


Figure 34. Rationale for the synthesis of a new family of triazine derivatives potentially active as SIRT2 inhibitor.

Following a rational design approach, the first series of triazine derivatives was conceived to retain the substitution pattern of compound **57** (Figure 31), which had emerged as the most active analogue within the previous pyrazolo[3,4-*d*]pyrimidine series. The synthetic work described in this project therefore focused on the preparation of compounds **90–93** (Figure 35). All members of this series share a 3-fluoroaniline moiety and a phenethyl side chain. In compounds **90** and **91**, the phenethyl chain is *p*-brominated, a modification inspired by the biological profile of pyrazolo[3,4-*d*]pyrimidine **61** (see section 3.2, Biology). In **90** and **91**, position 6 of the triazine ring is unsubstituted (bearing a hydrogen), whereas in **92** and **93** a thiomethyl group has been introduced, in analogy to compounds **88** and **89**.

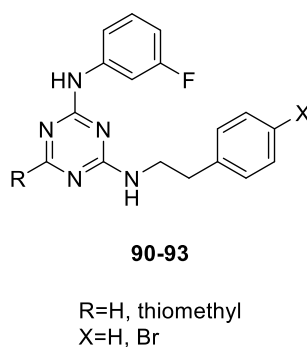


Figure 35. General structure of the synthesised triazines derivatives **90-93**.

The triazine ring is able to occupy the SIRT2 binding pocket in a manner closely resembling that of the pyrazolo[3,4-*d*]pyrimidine nucleus (Figure 36). In particular, there is a marked overlap between the benzene rings of the two scaffolds, suggesting preservation of the key binding interactions.

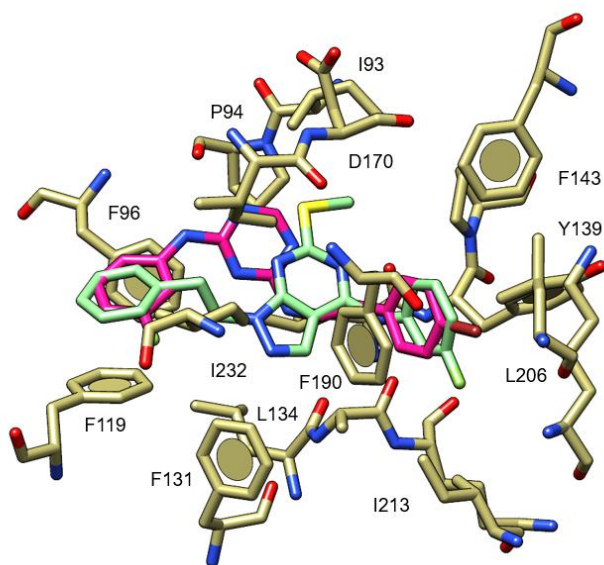


Figure 36. Docking pose of the pyrazolo[3,4-*d*]pyrimidine **57** (light green) and the triazine derivative **90** (magenta) within the SIRT2 binding site.

From a practical and strategic standpoint, the non-thiomethylated derivatives were synthesised first. This choice was made both to broaden the available SAR around the new triazine scaffold and to account for the high cost of the thiomethyl triazine starting material. Overall, by focusing on essential pharmacophoric features and reducing structural complexity, the triazine series was designed to maintain SIRT2 inhibitory activity while improving synthetic accessibility and offering new opportunities for systematic SAR exploration.

Based on this rationale, the present project is designed to: (i) synthesise and characterise a new series of pyrazolo[3,4-*d*]pyrimidine derivatives (**60–89**), guided by structure-based insights and focusing on systematic modifications at N1, C4 and C6 to explore SAR and enhance SIRT2 inhibitory potency; (ii) design and prepare a second, simplified series of triazine derivatives (**90–93**) by incorporating the N2 pyrazole nitrogen into a triazine core, with the aim of retaining the key pharmacophoric features of the pyrazolo[3,4-*d*]pyrimidine scaffold while improving synthetic accessibility; (iii) evaluate all synthesised compounds in biochemical assays, first in single-point screening and subsequently by determining IC₅₀ values for the most active inhibitors, in order to define robust SAR trends and to providing a solid basis for future optimisation toward more drug-like SIRT2 inhibitors.

3. Results and Discussion

3.1. Chemistry

On the basis of the structure-based rationale previously described, the pyrazolo[3,4-*d*]pyrimidine scaffold was selected as a privileged core for the development of new SIRT2 inhibitors. Crystallographic and modelling data had indicated that potent ligands typically consist of a planar bicyclic heteroaromatic nucleus flanked by two hydrophobic termini, one of which can donate or accept a hydrogen bond to Val233, while both ends engage in π - π or cation- π contacts with the His187-Phe235 triad along the hydrophobic corridor defined by Phe96, Phe131 and Leu137 (Figure 30).

A first screening of an *in-house* collection (compounds **52–56**) confirmed that the pyrazolo[3,4-*d*]pyrimidine core is able to reproduce this interaction pattern and tolerate phenylalkyl appendages at N1 (Figure 29). Docking and enzymatic assays highlighted **57–59** (Figure 31) as particularly promising, with the N1 phenethyl group acting as a primary aromatic handle toward His187/Phe235 and the C4 substituent reinforcing the Val233 contact. The C6 lipophilic residue was found to contribute mainly to Van der Waals complementarity at the base of the cleft.

These observations guided the design of two virtual libraries in which the N1 position bears either a phenylvinyl (styryl) or a phenethyl vector, while positions C4 and C6 were diversified to probe hydrophobicity, electronics and steric constraints. From these libraries, thirteen pyrazolo[3,4-*d*]pyrimidines were selected for synthesis, numbered **60–89** in the present work (Figure 33).

The synthesis of the designed compounds **60–89** was performed through a multi-step approach (Schemes 1, 7, 8) and the exact nature of the N1, C4 and C6 substituents for each individual analogue is reported in Table 1.

All intermediates and final compounds were fully characterised by ^1H and ^{13}C NMR spectroscopy and elemental analysis, confirming their structures and indicating a chemical purity exceeding 95 %.

3.1.1. Synthesis of compounds **60–86**, **88**, **89**.

The synthetic sequence (Scheme 1) started from differently substituted hydrazines **94a–e** chosen to predefine the nature of the N1 side chain (phenylvinyl or phenethyl). Condensation of **94a–e** with ethyl (ethoxymethylene)cyanoacetate or afforded the corresponding 5-amino-1-

substituted pyrazole-4-carboxamides **95a–e** in good yield. Intermediate **95c** was oxidized via sulfur trioxide–pyridine complex to obtain derivative **95d**. Subsequent reaction of **95a–f** with benzoyl isothiocyanate in refluxing tetrahydrofuran (THF) gave the thiourea intermediates **96a–f**, which, upon treatment with aqueous NaOH (2 M, 100 °C), underwent cyclisation to furnish the 6-thioxo-1,5,6,7-tetrahydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-ones **97a–f**. Precipitation from acetic acid followed by filtration provided these key bicyclic intermediates in analytically pure form.

This sequence thus established the pyrazolo[3,4-*d*]pyrimidine core with the desired N¹ substitution already in place and a synthetically versatile C6 thioxo group available for further functionalisation in line with the design hypotheses.

To modulate the hydrophobic complementarity at the bottom of the SIRT2 catalytic cleft, the C6 thioxo moiety of **97a–f** was alkylated with different alkyl halides (Scheme 1). Reaction with methyl iodide or ethyl iodide in refluxing THF furnished the 6-methylthio and 6-ethylthio derivatives **98a–i**, whereas treatment with allyl bromide in acetone/K₂CO₃/pyridine or in refluxing ethanol yielded the 6-allylthio analogues **98a–i**.

The resulting set of intermediates **98a–i** therefore encompassed compact (thiomethyl), slightly bulkier (thioethyl) and more extended, conformationally flexible (thioallyl) thioalkyl groups at C6. These substituents were selected to explore the steric and lipophilic tolerance of the narrow subpocket proximal to Ile169 and Phe96, identified in the modelling study as a secondary hot spot for Van der Waals interactions (Figures 30 and 32).

Intermediates **98a–i** were next converted into the corresponding 4-chloro derivatives **99a–i** via a Vilsmeier–Haack reaction using the Vilsmeier reagent in CHCl₃. This transformation activated the C4 position toward nucleophilic aromatic substitution by replacing the 4-oxo group with a chlorine atom. After aqueous work-up and chromatographic purification, compounds **99a–i** were obtained in good yields and served as the branching point for the preparation of both phenylvinyl and phenethyl/aminoalkyl final analogues.

Displacement of the C4 chlorine was performed with a panel of aliphatic and aryl amines designed to test different polar and aromatic environments in the Val233 region (Figure 30 and 32). Aliphatic amines (propylamine, butylamine, cyclopropylamine, benzylamine, phenethylamine, 1-naphthylmethylamine) reacted smoothly with **99a–i** in dry toluene at room temperature, affording the corresponding 4-amino derivatives in good to excellent yields. In

contrast, aryl amines such as 3-fluoroaniline, 3-methoxyaniline, 4-amino-3-methylphenol, 4-methoxy-2-methylaniline, 3-amino-4-methylphenol, 1-naphthylamine and 1-amino-5-hydroxynaphthalene required refluxing ethanol to achieve efficient substitution.

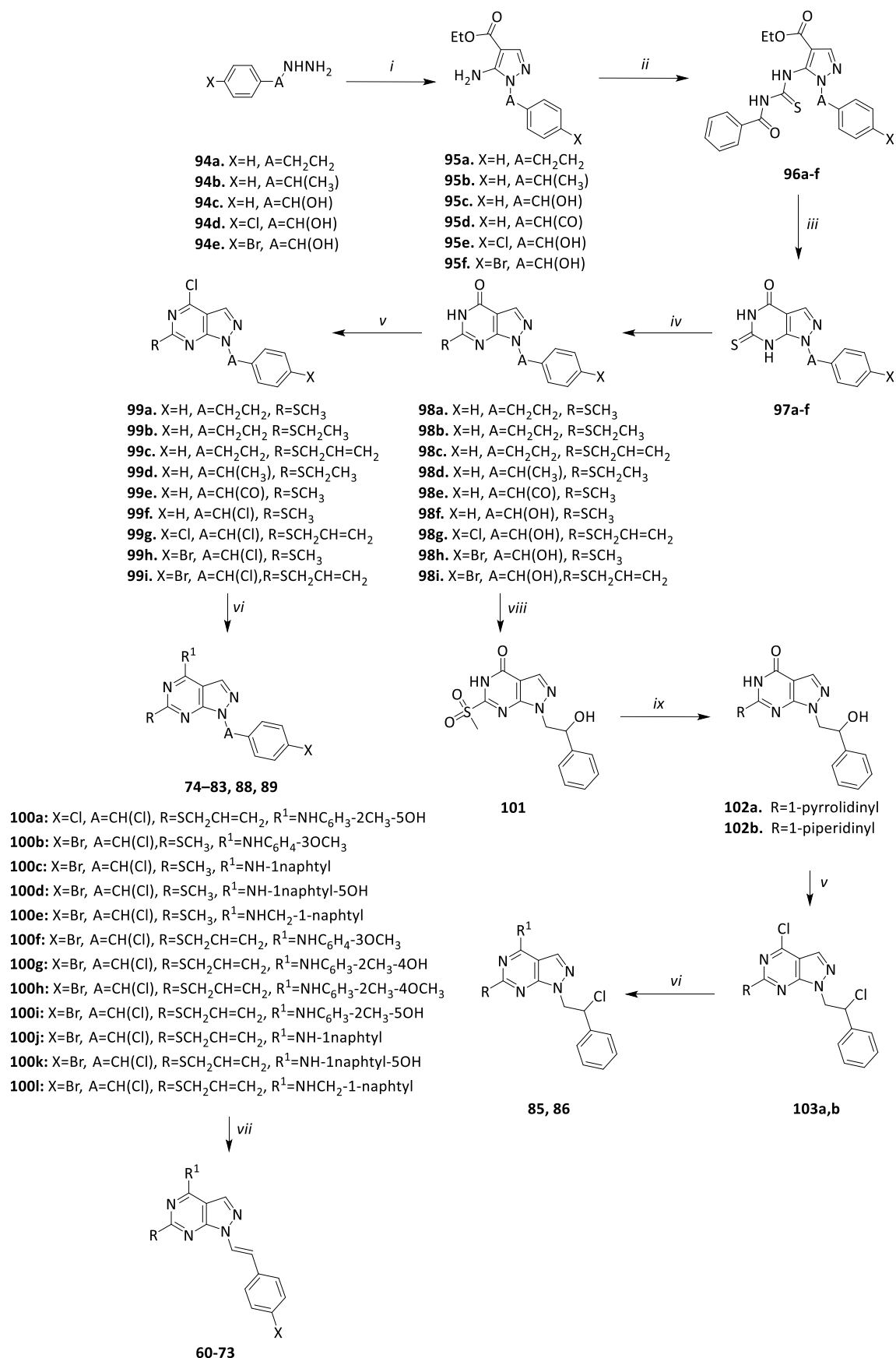
For the phenylvinyl series (compounds **60–73**), the saturated benzylic chloride intermediates were subjected to base-induced β -elimination. Treatment with 4 M NaOH in ethanol under reflux promoted elimination of HCl from the 2-chloro-2-phenylethyl fragment, generating a styryl (2-phenylvinyl) side chain at N1. This transformation furnished a family of pyrazolo[3,4-*d*]pyrimidines in which the central heterocycle is flanked by a phenylvinyl group and a C4 anilide or amino substituent, while C6 bears one of the previously introduced thioalkyl groups.

According to the design rationale, the phenylvinyl group was expected to align along the hydrophobic channel towards the selectivity pocket and to overlay with the vinyl pharmacophore observed in known SIRT2 ligands (e.g., compound **51**), thereby reinforcing π - π contacts with His187 and Phe235 and maintaining a favourable hydrogen bond to Val233.

A second branch of the synthetic plan was devoted to more polar analogues bearing aminoalkyl chains at N1 (compounds **74–86**, **88**, **99**), conceived to probe the impact of increased basicity and solvation on SIRT2 recognition and potential off-target profiles. Starting from **98f**, which carries a 2-hydroxy-2-phenylethyl group at N1 and a thiomethyl substituent at C6, oxidation with *m*-chloroperbenzoic acid (*m*-CPBA) afforded the corresponding sulfone. Nucleophilic substitution of this activated sulfone with pyrrolidine or piperidine gave intermediates **102a–b**, in which the pyrazolo[3,4-*d*]pyrimidine core is tethered to a flexible aminoalkyl chain via a sulfonyl linkage.

Subsequent Vilsmeier–Haack chlorination of **102a–b** produced the 4-chloro derivatives **103a–b**, which were finally converted into the saturated targets **74–86**, **88**, **89** by nucleophilic aromatic substitution with selected anilines under the same conditions used for the previous series.

These aminoalkyl derivatives complement the phenylvinyl subset by systematically increasing polarity at N1 while preserving the C4 pharmacophore and C6 lipophilic handle identified as crucial in the earlier SAR.

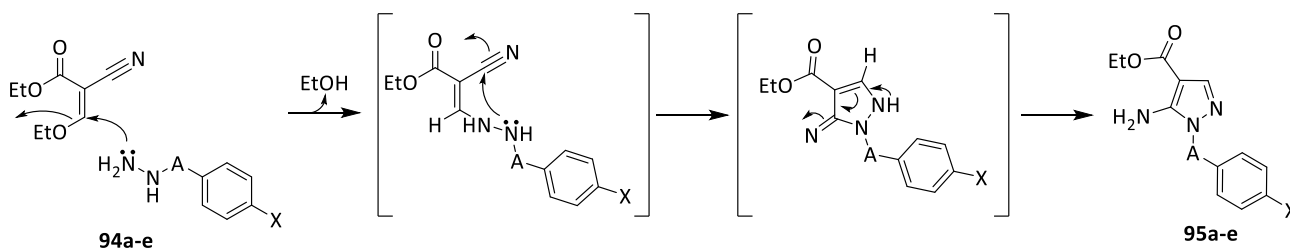


Scheme 1. Synthesis of compounds 60–86, 88, 89. Reaction conditions: (i)

ethyl(ethoxymethylene)cyanoacetate, toluene, 100°C, 8h; then sulphur trioxide pyridine complex in DMSO, TEA, rt, 30 min (to obtain **95d**); (ii) benzoyl isothiocyanate, dry THF, 66°C, 8h; (iii) 2 M NaOH, 100°C, 1h; (iv) Method A: iodomethane or iodoethane, dry THF, 65°C, 12h (to obtain compounds **98a**, **98e**, **98f**, **98h** and **98b**, **98d**); Method B: allyl bromide, pyridine, K₂CO₃, acetone, rt, 2h (to obtain compounds **98g** and **98i**) or allyl bromide, EtOH, 95°C, 1h (to obtain compound **98c**); (v) POCl₃/DMF, CHCl₃, 60°C, 12h; (vi) Method A: the proper aliphatic amine, dry toluene, rt, 24-48h (to obtain compounds **100c-e**, **100j-l**, **74-83**, **85** and **86**); Method B: the proper aniline, EtOH, 95°C, 8h (to obtain compounds **100a**, **100b**, and **100f-i**); (vii) 4 M NaOH, EtOH, 95°C, 4h; (viii) *m*-CPBA, CHCl₃, rt, 12 h; (ix) the proper aliphatic amine, DMSO, 100°C, 12h.

3.1.1.1. Cyclocondensation of a substituted hydrazine

In step (i) the substituted phenethylhydrazine intermediates **94a-e** react with ethyl 2-cyano-3-ethoxyacrylate through a cyclocondensation that builds the pyrazole ring *in situ* (Scheme 2).



Scheme 2. Proposed mechanism for the cyclocondensation of substituted phenethylhydrazines **94a-f** with ethyl 2-cyano-3-ethoxyacrylate to give **95a-f**. The reaction proceeds via initial nucleophilic addition of the terminal hydrazinic nitrogen, ethanol elimination and intramolecular attack on the nitrile, followed by ring closure and aromatization of the pyrazole core.

The terminal –NH₂ of the hydrazine performs a conjugate (Michael-type) addition to the activated C=C bond of the cyanoacrylate. This gives a β-hydrazino ester in which the hydrazine is now attached to the former double bond. Proton transfers within this adduct then facilitate the departure of ethanol from the ethoxyacetal moiety, generating an imidate / iminium-type intermediate that is still bearing the cyano group. Next, the internal nitrogen of the hydrazine attacks the electrophilic carbon of the nitrile intramolecularly. This nucleophilic addition to the cyano group closes the five-membered ring and produces a pyrazoline intermediate. Finally,

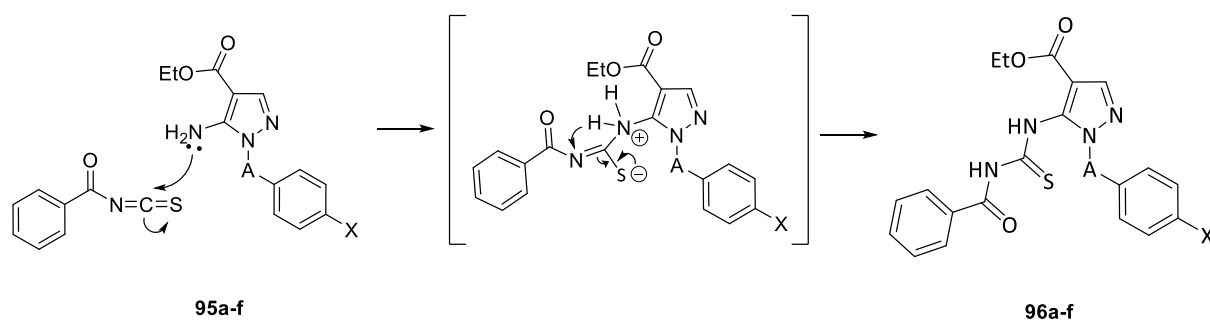
further proton transfers and rearomatisation of the ring lead to the formation of the aromatic 5-aminopyrazole-4-carboxylate intermediates **95a–e**. Overall, the reaction can thus be viewed as a Michael addition of a substituted hydrazine to an activated cyanoacrylate, followed by intramolecular cyclisation onto the nitrile and elimination of ethanol to furnish the pyrazole.²⁸⁰

3.1.1.2. Oxidation of **95c**

A solution of sulphur trioxide–pyridine complex in DMSO was added to **95c** in the presence of TEA at room temperature. Under these conditions, the benzylic alcohol undergoes a oxidation to the corresponding ketone: the sulphur trioxide–pyridine complex activates DMSO to form an electrophilic dimethylsulfonium species, which is attacked by the alcohol to give an alkoxydimethylsulfonium intermediate. Deprotonation at the α -carbon by triethylamine then promotes the elimination of the dimethylsulfide-based leaving group, affording **95d**.

3.1.1.3. Formation of benzoylthioureas

In step (ii) the 5-aminopyrazoles **95a–f** are treated with benzoyl isothiocyanate in dry THF (Scheme 3). The nucleophile in this case is the exocyclic amino group at C5 of the pyrazole, which attacks the electrophilic carbon of the N=C=S unit of benzoyl isothiocyanate. This addition generates a zwitterionic thiocarbamidate in which the negative charge is located on sulfur and the positive charge on nitrogen. The collapse of this intermediate, followed by the reformation of the C=N bond, gives the benzoylthiourea derivatives **96a–f**, where the pyrazole attacking nitrogen is now linked to the C(=S)–NH–COPh fragment. Overall, step (ii) is an acylthiourea formation by nucleophilic addition of the 5-amino group to an isothiocyanate.

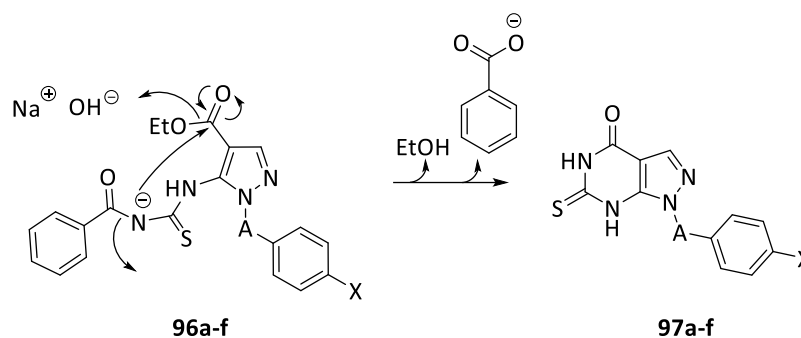


Scheme 3. Proposed mechanism for the formation of benzoylthioureas **96a–f** from ethyl 5-amino-1-substituted-1H-pyrazole-4-carboxylates **95a–f** and benzoyl isothiocyanate. The exocyclic amino group attacks the electrophilic carbon of the N=C=S moiety, generating a

zwitterionic intermediate that collapses with re-formation of the C=N bond to give the corresponding *N*-acyl-*N*1-(pyrazolylcarbonyl)thiourea derivatives.

3.1.1.4. Base-promoted cyclisation to 6-thioxopyrazolo[3,4-*d*]pyrimidin-4-ones

Step (iii) is a base-promoted intramolecular cyclocondensation of a 5-aminopyrazole benzoylthioureas **96a–f** to a 6-thioxopyrazolo[3,4-*d*]pyrimidin-4-one, with concomitant loss of benzamide (Scheme 4). Under strongly basic conditions (2 M NaOH, 100 °C), the amide nitrogen is deprotonated and able to attack intramolecularly the carbonyl carbon of the ester moiety. This intramolecular nucleophilic addition forms a tetrahedral intermediate in which the former C=S carbon is now tetra-coordinated. The collapse of this intermediate occurs with expulsion of benzamide anion as a leaving group and formation of a new N–C bond, thereby closing a six-membered ring fused to the pyrazole. After proton transfers and tautomerization, the pyrazolo[3,4-*d*]pyrimidin-4-one intermediates **97a–f**, bearing a thioxo group at C6, are obtained. Subsequent protonation during work-up with acetic acid affords the neutral thioxopyrazolopyrimidinone products.



Scheme 4. Proposed mechanism for the base-promoted cyclisation of benzoylthioureas **96a–f** to give **97a–f**. Deprotonation of the amide nitrogen generates an anionic intermediate that attacks the carbonyl carbon intramolecularly, forming the fused pyrimidinone ring and expelling benzoate.

3.1.1.5. S-alkylation at C6

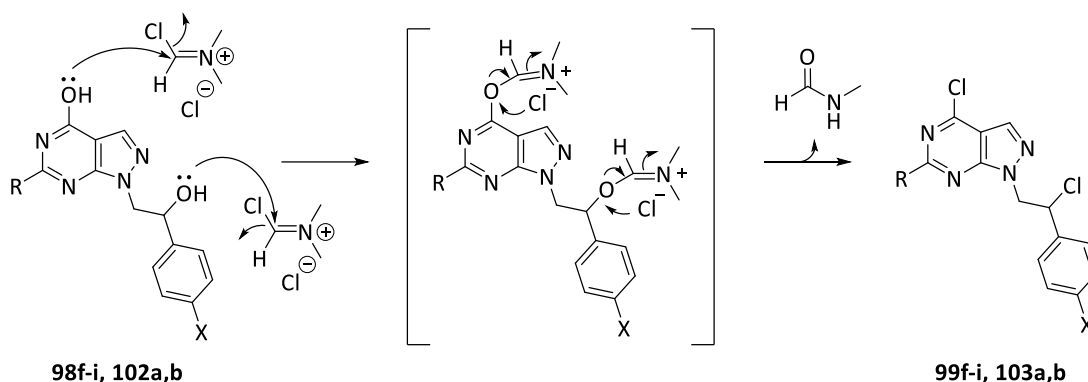
In step (iv) the thioxo group at C6 of intermediates **97a–f** is converted to give the S-alkylthio derivatives **98a–i** by reaction with methyl iodide, ethyl iodide or allyl bromide. The 6-thioxo fragment exists in equilibrium with its thiol tautomer; in the presence of base or excess alkyl halide, deprotonation of this thiol gives a soft thiolate anion located at sulfur. This thiolate

undergoes bimolecular nucleophilic substitution (S_N2) on the alkyl halide, displacing iodide or bromide and furnishing the corresponding 6-methylthio, 6-ethylthio or 6-allylthio pyrazolo[3,4-*d*]pyrimidin-4-one. N-alkylation is disfavoured because, in the thione/thiol tautomeric system, deprotonation occurs predominantly at sulfur to give a thiolate, which is a softer and more polarizable nucleophile than the corresponding anionic nitrogen. According to HSAB principles, this soft S⁻ centre matches better the relatively soft alkyl iodide/bromide electrophiles, so the S_N2 attack takes place preferentially at sulfur, and S-alkylation predominates, whereas the “harder” and resonance-stabilised amide-type nitrogen is much less competitive for alkylation.²⁸¹

For compound **98c**, the general S-alkylation protocol in acetone proved inefficient and had to be modified by performing the reaction in refluxing ethanol. This behaviour can be rationalised by considering that in **98c** the N1 chain is a neutral phenethyl group, whereas in several analogues that react smoothly under the standard conditions the N1 substituent is a 2-chloro-2-phenylethyl fragment. A benzylic chloride exerts a significantly stronger σ-withdrawing effect than an unsubstituted phenethyl chain and, through conjugation along the N1–C5–C6 system, stabilises the negative charge developing on the C6 thioamide/thiolate, effectively increasing the acidity of the thione. For those substrates, the relatively mild and poorly ionising medium (K₂CO₃ in acetone) is sufficient to generate a detectable amount of thiolate and to promote clean S-allylation. Indeed, it is the benzylic chlorine which, through an inductive effect, increases the acidity of the thioamide and therefore facilitates the formation of the C6 thiolate. In **97c**, where the N1 substituent lacks the benzylic chlorine and is electronically more neutral, the C6 thione is less acidic and under the same conditions only a small fraction of thiolate is formed; in addition, the non-halogenated scaffold is more poorly soluble in acetone. As a consequence, S-alkylation in acetone is sluggish. Switching to refluxing ethanol, which solvates the heterocycle and provides a more polar, better ionising medium, facilitates the thione/thiol equilibrium and allows the sulfur nucleophile to react directly with allyl bromide, so that S-allylation can proceed to completion. Thus, the absence of the benzylic chloride on the N1 side chain, combined with solubility issues in acetone, offers a reasonable mechanistic explanation for the need to modify the conditions in the case of **98c**.

3.1.1.6. Vilsmeier–Haack chlorodehydration at C4

Step (v) is a Vilsmeier–Haack-type chlorodehydration at C4 (Scheme 5). Treatment of intermediates **98a–i** with POCl₃ and DMF in chloroform generates *in situ* the classical Vilsmeier reagent, formally [Cl–P(O)Cl₂·DMF]⁺, which behaves as a chloroiminium electrophile. The carbonyl oxygen of the 4-one attacks this electrophilic iminium carbon, forming an O-alkylated Vilsmeier adduct in which the carbonyl carbon has developed cationic character. Subsequent attack by chloride at this carbon and collapse of the intermediate eliminate the DMF-derived fragment and water, replacing the 4-oxo group with chlorine. The outcome is a set of 4-chloro-6-thioalkyl pyrazolo[3,4-*d*]pyrimidines (**99a–i** and **103a,b**) in which the C4 carbon is strongly activated toward nucleophilic aromatic substitution by the adjacent ring nitrogens.



*Scheme 5. Proposed mechanism for the Vilsmeier–Haack chlorodehydration of 4-oxo derivatives **98f–i** and **102a,b** to give **99f–i** and **103a,b**. The oxygens first attack the chloroiminium species generated from POCl₃/DMF, affording a Vilsmeier adduct in which the C4 carbon is activated. Subsequent intramolecular proton transfers and collapse of this intermediate lead to formation of the C4–Cl bond and restoration of aromaticity.*

3.1.1.7. Nucleophilic aromatic substitution at C4

In step (vi), the previously obtained 4-chloro derivatives **99a–i** and **103a,b** undergo nucleophilic aromatic substitution with a variety of amines. The amine, either aliphatic or aryl, adds to the electron-poor C4 bearing the chlorine, forming a σ -complex (Meisenheimer intermediate) in which the negative charge is delocalised over the pyrazolo[3,4-*d*]pyrimidine ring and especially onto the ring nitrogens. Because the heterobicycle is highly electron-deficient, this addition is facilitated. The intermediate then collapses by expulsion of chloride,

restoring aromaticity and giving the 4-amino or 4-anilido pyrazolo[3,4-*d*]pyrimidin-4-one derivatives **100a–l** or final compounds **74–83**, **85**, **86**, **88**, and **89**. When the nucleophile is a less reactive aniline, elevated temperature in ethanol is required, whereas aliphatic amines react at room temperature in toluene. Notably, under these conditions the benzylic chloride on the N1 side chain remains unchanged, most likely because its sterically congested environment renders direct displacement by the amines kinetically disfavoured compared with the more accessible and strongly activated C4–Cl bond.

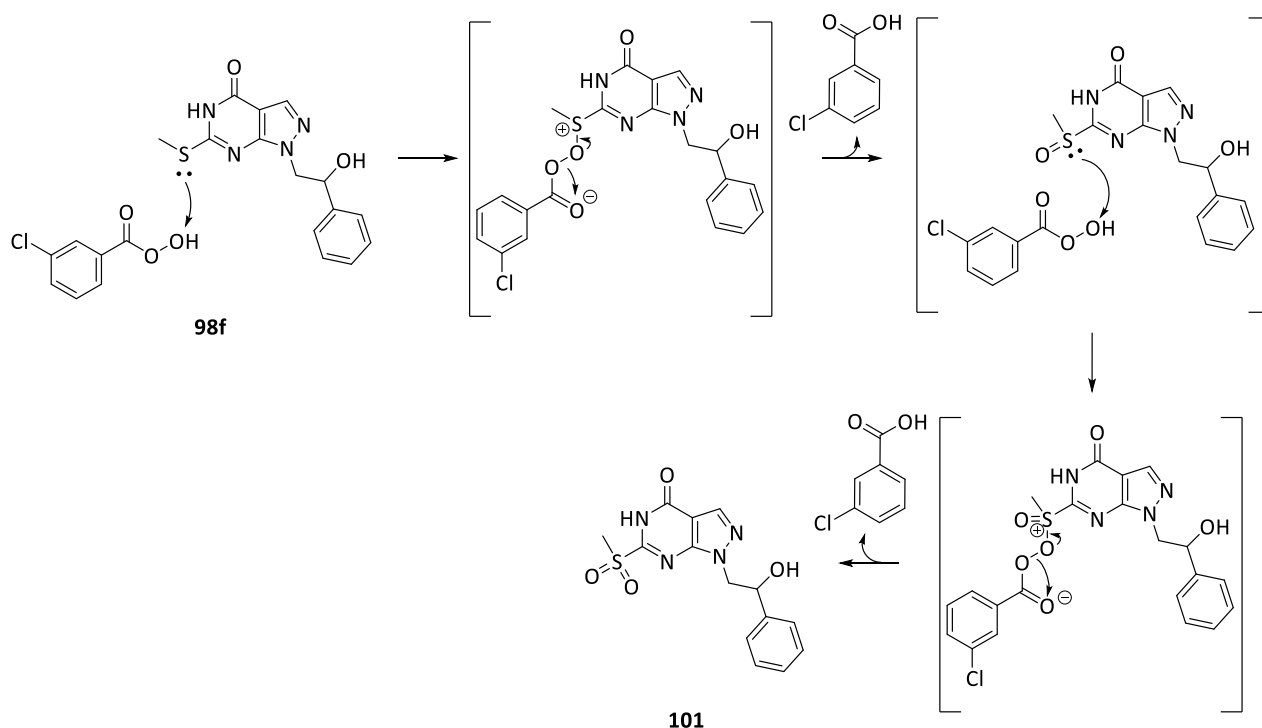
3.1.1.8. *β-Elimination to form the styryl side chain*

Step (vii) concerns the conversion of the 2-chloro-2-phenylethyl substituents at N1 of derivatives **100a–l** or final compounds **88** and **89** into styryl groups, to obtain the final compounds **60–73**. Under 4 M NaOH in ethanol at 95 °C, hydroxide acts as a strong base and deprotonates the β-carbon adjacent to the benzylic chloride. In a concerted E₂ dehydrohalogenation, removal of this proton occurs in an anti-periplanar arrangement with respect to the C–Cl bond, which simultaneously breaks to give chloride. The result is the formation of a C=C double bond between the benzylic carbon and the adjacent methylene, producing an N1-styryl (2-phenylvinyl) side chain. In the relevant staggered conformation of the N–CH₂–CH(Cl)Ph fragment, this anti-periplanar geometry is achieved in the rotamer that, upon C–H/C–Cl bond cleavage and π-bond formation, leads selectively to the E alkene, whereas the syn-periplanar pathway to the Z isomer is both sterically and stereoelectronically disfavoured. In addition, under strongly basic, high-temperature conditions any minor amount of Z product can isomerise to the thermodynamically more stable E isomer. Overall, this step converts a flexible, saturated fragment into a planar vinyl–aryl vector that can better occupy the SIRT2 selectivity pocket envisaged in the docking studies.

3.1.1.9. *Oxidation of thioether to sulfone*

In step (viii) the thioether intermediate **98f** is oxidised with *m*-CPBA to the corresponding sulfone **101** (Scheme 6). The peracid first transfers an oxygen atom to the sulfur, giving a sulfoxide via a tetrahedral “persulfonium” intermediate that collapses with release of *m*-chlorobenzoate. A second equivalent of the peracid then oxidises the sulfoxide further to the sulfone, now containing two S=O bonds. Throughout this process the S–C framework is preserved, but the sulfur centre changes from S(II) in the thioether to S(IV) in the sulfoxide and

finally to S(VI) in the sulfone. The newly formed sulfone strongly withdraws electron density and activates the adjacent carbon for nucleophilic substitution.

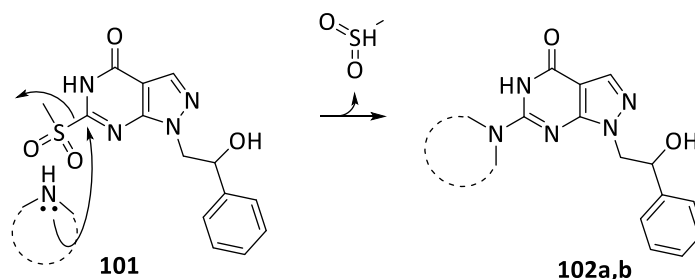


Scheme 6. Proposed mechanism for the oxidation of thioether **98f** to sulfone **101** by *m*-CPBA. The sulfur atom first attacks the electrophilic terminal oxygen of the peracid, forming a peroxysulfonium intermediate that collapses with O–O bond cleavage to give the sulfoxide and *m*-CPBA. A second *m*-CPBA-mediated oxygen transfer to sulfur via an analogous peroxysulfonium intermediate affords the final sulfone **101**.

3.1.1.10. Nucleophilic substitution on the activated sulfone

Finally, step (ix) involves sulfone **101** as an electrophilic platform to introduce aminoalkyl chains (Scheme 7). In DMSO at 100 °C the α -CH₂ next to the sulfone is relatively acidic and can be deprotonated, generating a carbanion stabilised by the two S=O groups. Nucleophilic attack of this carbanion on a suitable electrophilic carbon of the incoming aliphatic amine (or, alternatively, direct S_N2 displacement of an activated leaving group attached to the sulfone's α -carbon by the amine) leads to substitution of the original group by a pyrrolidine or piperidine fragment, yielding aminoalkyl sulfones **102a,b**. These intermediates then re-enter the same

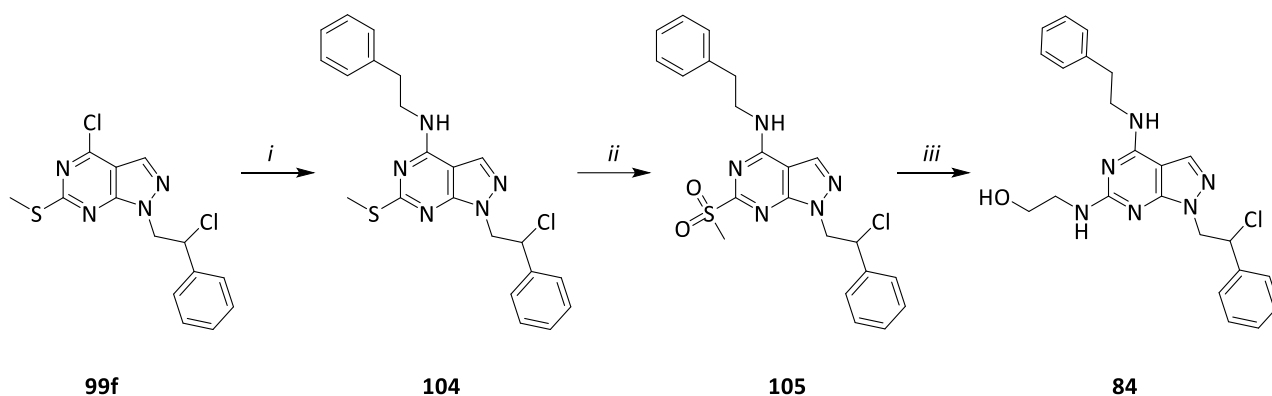
Vilsmeier–Haack (**103a,b**) and nucleophilic aromatic substitution ($\text{S}_{\text{N}}\text{Ar}$) sequence at C4 as described above, ultimately providing the pyrazolo[3,4-*d*]pyrimidine analogues **85** and **86**.



*Scheme 7. Proposed mechanism for the nucleophilic substitution of sulfone **101** to give aminoalkyl derivatives **102a,b**.*

3.1.2. Synthesis of compound **84**

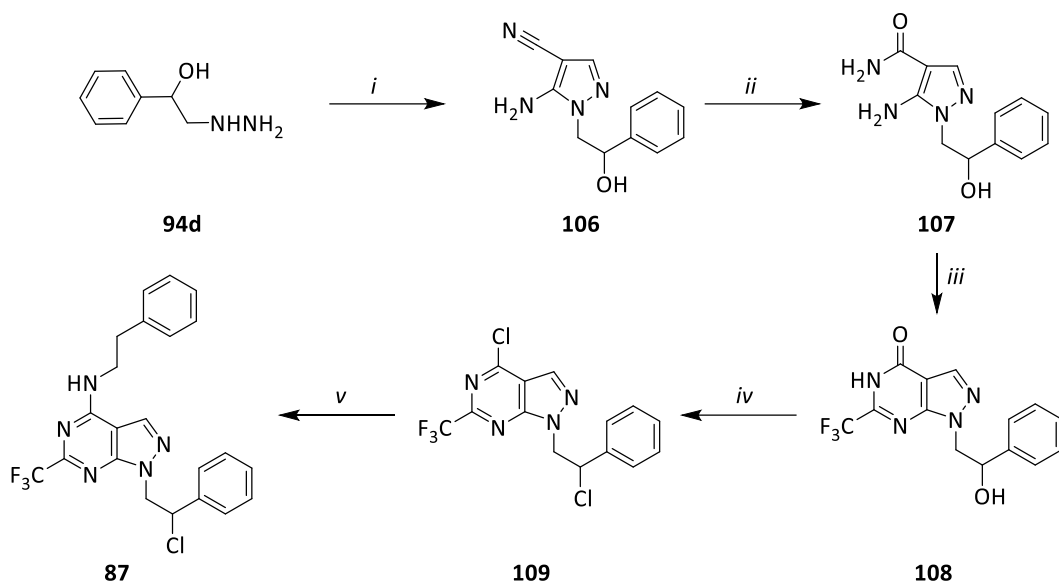
The target analogue **84** was obtained from the 6-methylthio precursor **99f** through a short three-step sequence designed to replace the lipophilic thioether with a polar sulfonamide side chain while preserving the N1 benzylic chloride (Scheme 8). In the first step, 4-chloro pyrazolopyrimidine **99f** was converted into the corresponding 4-aminoderivative **104** by nucleophilic aromatic substitution at C4, in which the chlorine atom is displaced by phenethylamine to give the exocyclic 4-(phenethylamino) group. The resulting thioether **104** was then oxidised with *m*-CPBA in chloroform at room temperature to afford sulfone **105**; this transformation increases the electron-withdrawing character of the C6 substituent and activates it towards nucleophilic displacement, in full analogy with the strategy previously reported for compound **101**. Importantly, installation of the C6 aminoethoxy fragment was deliberately postponed to this late stage: introducing 2-aminoethanol before the Vilsmeier–Haack chlorination used upstream to generate **99f** would likely result in undesired chlorination of the amino alcohol by the POCl_3/DMF complex. Finally, heating **105** with 2-aminoethanol in DMSO promotes nucleophilic substitution of the methylsulfonyl group by the amino alcohol, yielding compound **84**. This sequence thus converts the original neutral thioether into a more polar, hydrogen-bond-competent handle while maintaining the overall pyrazolo[3,4-*d*]pyrimidine scaffold required for SIRT2 recognition.



Scheme 8. Synthesis of compound **84**. Reaction conditions: (i) POCl_3/DMF , CHCl_3 , 60°C , 12h; (ii) *m*-CPBA, CHCl_3 , rt, 12 h; (iii) phenethylamine, DMSO, 100°C , 12h.

3.1.3. Synthesis of compound **87**

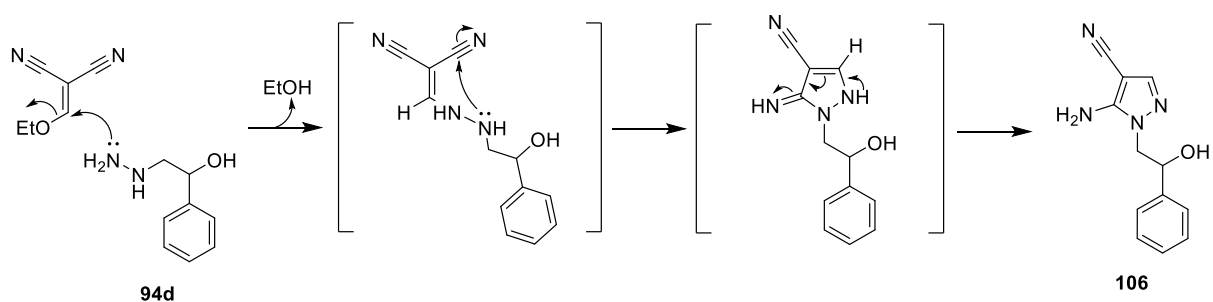
The synthetic route to compound **87** (Scheme 9) started from 2-hydroxybenzyl hydrazine **94d**, a substituted hydrazine chosen to predefine the N1 2-hydroxybenzyl side chain. Condensation of **94d** with 2-(ethoxymethylene)malononitrile in refluxing ethanol afforded the 3-aminopyrazole-4-carbonitrile **106** in good yield. Subsequent treatment of **106** with 2 M NaOH in ethanol at 95°C promoted nucleophilic addition of hydroxide to the cyano group, followed by rearrangement/hydrolysis, to furnish the corresponding 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carboxamide **107**. In step (iii), **107** was converted into the fused pyrazolo[3,4-*d*]pyrimidin-4-one by N-acylation with methyl trifluoroacetate under basic conditions (EtONa/EtOH), followed by intramolecular cyclocondensation, giving the 3-trifluoromethyl bicyclic intermediate **108**, still bearing the 2-hydroxybenzyl substituent at N1. Vilsmeier–Haack chlorination with POCl_3/DMF in CHCl_3 at 60°C then replaced the 4-oxo group with chlorine and, at the same time, converted the benzylic hydroxyl into a benzylic chloride, affording the activated dichloro derivative **109**. Finally, nucleophilic aromatic substitution of the C4–Cl bond with phenethylamine in dry toluene at room temperature provided the target compound **87**, featuring a 4-(phenethylamino) substituent on the 3-trifluoromethyl pyrazolo[3,4-*d*]pyrimidine scaffold while preserving the benzylic chloride handle.



Scheme 9. Synthesis of compound **87**. Reaction conditions: (i) 2-(ethoxymethylene)malononitrile, EtOH, 95°C, 6h; (ii) 2 M NaOH, EtOH, 95°C, 3h; (iii) methyl trifluoroacetate, EtONa, EtOH, 95°C, 12h; (iv) POCl₃/DMF, CHCl₃, 60°C, 12h; (v) phenethylamine, dry toluene, rt, 48h.

3.1.3.1. Cyclocondensation to 3-aminopyrazole-4-carbonitrile

Step (i) involves the cyclocondensation of 2-hydroxybenzyl hydrazine **94d** with 2-(ethoxymethylene)malononitrile in refluxing ethanol, giving the 3-aminopyrazole-4-carbonitrile **106** (Scheme 10). Mechanistically, the terminal hydrazinic nitrogen adds to the activated C=C bond of the ethoxymethylenemalononitrile, the adduct eliminates ethanol, and the internal nitrogen then attacks one of the cyano groups intramolecularly. Ring closure and subsequent tautomerization furnish the 1-(2-hydroxybenzyl)-3-aminopyrazole-4-carbonitrile.

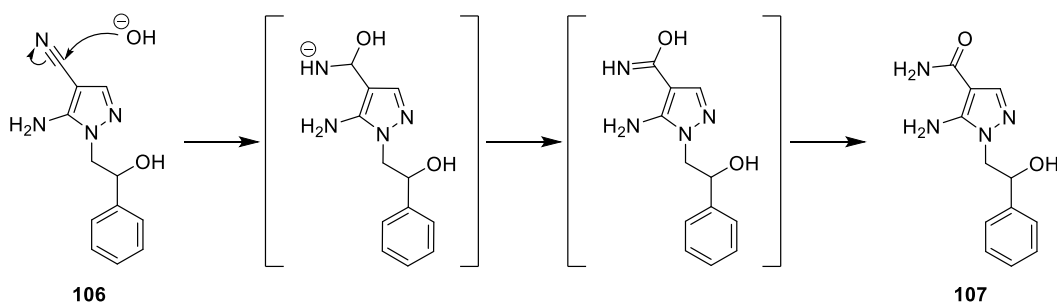


Scheme 10. Proposed mechanism for the cyclocondensation of 2-hydroxybenzyl hydrazine **94d** with 2-(ethoxymethylene)malononitrile to give **106**. The terminal hydrazinic nitrogen first adds

to the activated C=C bond of the ethoxymethylene reagent, and the resulting adduct eliminates ethanol to form an imidoyl intermediate. Intramolecular nucleophilic attack of the internal nitrogen on one cyano group, followed by ring closure and tautomerization, furnishes 1-(2-hydroxybenzyl)-3-aminopyrazole-4-carbonitrile **106**.

3.1.3.2. Hydrolysis of the nitrile to carboxamide

In step (ii), treatment of **106** with 2 M NaOH in ethanol at 95 °C promotes nucleophilic addition of hydroxide to the nitrile carbon, generating a tetrahedral anionic imidate intermediate in which the former nitrile carbon bears both OH and NH substituents (Scheme 11). Proton transfer from solvent to this anion affords the neutral imidic acid, which then undergoes base-catalysed tautomerization: deprotonation at nitrogen followed by reprotonation at oxygen shifts the C=N–OH system to the thermodynamically more stable carboxamide motif –C(=O)–NH₂. After work-up, this sequence furnishes 5-amino-1-(2-hydroxybenzyl)-1*H*-pyrazole-4-carboxamide **107**, while the pyrazole ring and the 2-hydroxybenzyl substituent remain unchanged.

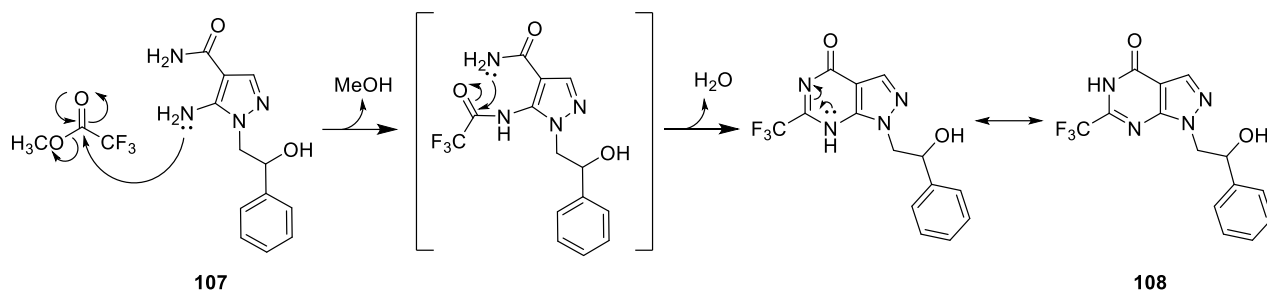


Scheme 11. Proposed mechanism for the base-catalysed hydration of nitrile **106** to give carboxamide **107**. Hydroxide adds to the nitrile carbon to form a tetrahedral imidate anion, which after protonation furnishes the corresponding imidic acid. Subsequent imidic acid–amide tautomerization affords **107**.

3.1.3.3. Construction of the 3-trifluoromethyl pyrazolo[3,4-*d*]pyrimidin-4-one

Step (iii) converts this monocyclic pyrazole into the fused pyrazolo[3,4-*d*]pyrimidin-4-one **108** (Scheme 12). Under basic conditions (EtONa in EtOH), the exocyclic amino group of **107** is acylated by methyl trifluoroacetate, giving a trifluoroacetamide intermediate. Basic conditions enable intramolecular nucleophilic attack on the newly formed carbonyl, closing the six-membered ring with elimination of methoxide. Tautomerization then affords the 3-

trifluoromethyl pyrazolo[3,4-*d*]pyrimidin-4-one **108**, which still bears the 2-hydroxybenzyl substituent at N1.



Scheme 12. Proposed mechanism for the construction of the 3-trifluoromethyl pyrazolo[3,4-*d*]pyrimidin-4-one **108**. Under basic conditions, the exocyclic amino group is acylated by methyl trifluoroacetate to give a trifluoroacetamide intermediate, which then undergoes intramolecular nucleophilic attack thus closing the six-membered ring with elimination of methanol. Subsequent proton transfers and tautomerization afford **108**.

3.1.3.4. Vilsmeier–Haack chlorodehydration at C4

In step (iv), compound **108** is subjected to POCl₃/DMF in CHCl₃, undergoing a Vilsmeier–Haack chlorination, undergoing a Vilsmeier–Haack chlorination according to the same protocol already described for the conversion of **98a–f** into **99a–f**: the 4-oxo group is activated by the Vilsmeier reagent and transformed into a 4-chloro function, while the benzylic hydroxyl is also converted into a benzylic chloride. The outcome is the dichloro derivative **109**.

3.1.3.5. Nucleophilic aromatic substitution

Intermediate **109** is activated at C4 toward nucleophilic aromatic substitution to obtain final compound **87**, carrying a benzylic chloride handle on the N1 side chain. Also in this case, the benzylic chloride on the N1 side chain remains unchanged.

3.1.3.6. Dehydrohalogenation

For the phenylvinyl series (compounds **60–73**), the saturated benzylic chloride intermediates were subjected to base-induced β-elimination. The transformation proceeds through a base-promoted β-elimination of the saturated benzylic chloride intermediate. Under reflux in ethanolic 4 M NaOH, the hydroxide acts as a strong base and abstracts the β-proton from the

methylene group adjacent to N1. In a concerted E₂ step, the C–H bond electrons form the new C=C double bond while the chloride leaves from the benzylic carbon, resulting in the corresponding styryl (2-phenylvinyl) substituent at N1. The reaction is favoured by the formation of a conjugated alkene and typically provides predominantly the E- isomer.

3.1.4. Molecular simplification strategy: from pyrazolopyrimidines to triazines

On the basis of the most promising pyrazolo[3,4-*d*]pyrimidine derivatives, a second design phase was undertaken in which a molecular simplification strategy was applied (Figure 34). In this approach, the N2 pyrazole nitrogen of the original pyrazolo[3,4-*d*]pyrimidine scaffold was formally incorporated into the triazine core to generate a new, more compact heterocycle. Docking studies (Figure 36) indicated that this triazine framework is able to occupy the SIRT2 binding pocket in a binding mode closely resembling that of the parent pyrazolo[3,4-*d*]pyrimidines, with substantial overlap of the aromatic systems and preservation of the key pharmacophoric interactions.

Guided by these considerations, a first series of triazine derivatives (**90–93**) was designed as a simplified mimic of the lead pyrazolo[3,4-*d*]pyrimidine **57**, retaining its 3-fluoroaniline fragment and phenethyl side chain while progressively reducing structural complexity (Figure 35). In **91** and **93** the phenethyl chain is para-brominated, in analogy to pyrazolopyrimidine **61**, whereas in **92** and **93** a thiomethyl group is introduced at position C6, mirroring the substitution pattern of compounds **88** and **89**.

From a synthetic point of view, the triazine series was deliberately conceived as a molecularly simplified and synthetically accessible platform. The general route to compounds **90–93** relies on nucleophilic substitutions on a prefunctionalised 1,3,5-triazin-2-one core. In the first stage, the phenethylamine fragment is introduced at one of the triazinic positions through S_NAr on an activated chloro-triazine precursor. Under appropriate conditions, displacement of a chloro substituent by phenethylamine affords an N1-phenethyltriazine intermediate. A subsequent S_NAr step with 3-fluoroaniline installs the second aniline-type substituent, yielding the non-thiomethylated triazines **90** and **92** (Scheme 14). In the thiomethyl analogues **91** and **93**, the methylthio group at position C6 is present from the starting triazine building block, providing the desired thiomethyl handle (Scheme 15).

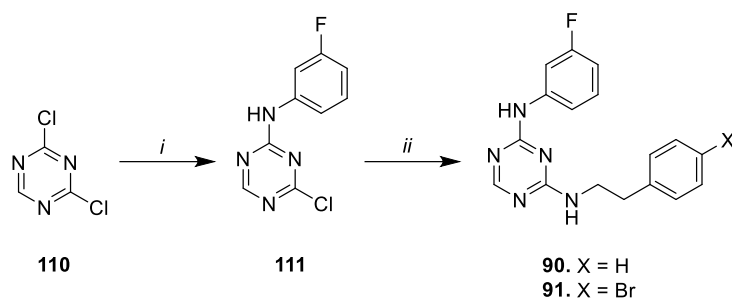
The synthetic plan was further refined by taking into account practical and economic considerations. In particular, the thiomethyl triazine starting materials required for **92** and **93** are relatively expensive and less readily available. For this reason, the initial experimental effort focused on the non-thiomethylated derivatives **90** and **92**. This choice not only reduced costs and synthetic complexity at the outset but also allowed expansion of the SAR around the new triazine scaffold without the confounding influence of a C6 lipophilic substituent. Once the feasibility and activity of the simplified triazine core were established, the more elaborate thiomethyl analogues **91** and **93** were prepared to probe the contribution of the C6 lipophilic vector to SIRT2 binding.

The exact nature of the substituents for each individual analogue is reported in Table 2.

3.1.4.1. *Synthesis of compounds 90 and 91*

The synthesis of the triazine derivatives **90** and **91** was accomplished through a sequential nucleophilic aromatic substitution on 2,4-dichloro-1,3,5-triazine (Scheme 13). In the first step, 2,4-dichloro-1,3,5-triazine (**110**) was reacted with 3-fluoroaniline in anhydrous THF in the presence of *N,N*-diisopropylethylamine (DIPEA) under nitrogen atmosphere. The arylamine selectively displaced the more activated chlorine at C2 of the triazine ring, affording the mono-substituted intermediate 4-chloro-*N*-(3-fluorophenyl)-1,3,5-triazin-2-amine **111** in good yield.

In the second step, intermediate **111** was subjected to a further $\text{S}_{\text{N}}\text{Ar}$ reaction with the appropriate phenethylamine (unsubstituted or 4-bromophenethylamine) in *i*-propanol at 100 °C, again using DIPEA as base. Under these conditions, the remaining C4–Cl bond is displaced by the benzylic amine to give the corresponding *N*2-(3-fluorophenyl)-*N*4-phenethyl-1,3,5-triazine-2,4-diamines **90** and **91**. This two-step sequence thus installs the 3-fluoroaniline and phenethyl fragments in a regioselective manner on the 1,3,5-triazine core, providing the desired molecularly simplified analogues for biological evaluation.



Scheme 13. Synthesis of compounds **90** and **91**. Reaction conditions: (i) 3-fluoroaniline, DIPEA, dry THF, 0°C, 30 min, then rt, 2 h; (ii) phenethylamine or p-bromophenethylamine, DIPEA, iPrOH, 100°C, 2 h.

3.1.4.2. Characterization of the tautomeric mixture

The analysis of the ^1H and ^{13}C NMR spectra of compounds **90** and **91** raised the possibility that the isolated products might consist of a mixture of two tautomers. In principle, a diamino-1,3,5-triazine can exist in seven distinct tautomeric forms: one τ - τ tautomer, which retains both exocyclic amino groups; four π - τ tautomers, in which one of the two amino groups has undergone tautomerization to an imine functionality; and two π - π tautomers, in which both exocyclic groups are present in the imine form (Figure 37).

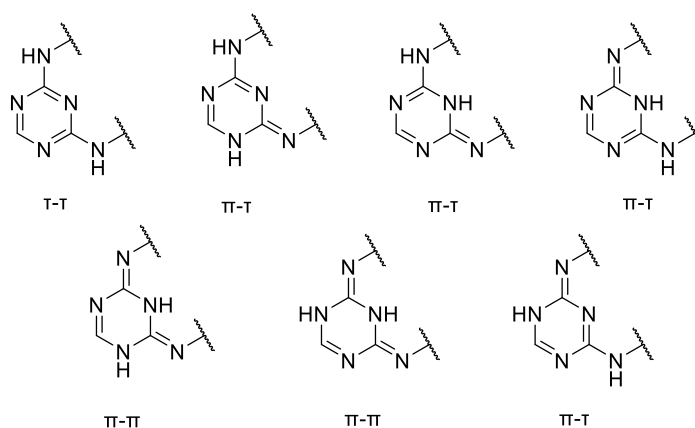


Figure 37. Possible tautomeric forms of a generic diamino-1,3,5-triazine.

Detailed characterisation of compound **90** led to the conclusion that, among the possible tautomers, the species actually present are the τ - τ tautomer and one π - τ tautomer in which imine formation occurs on the phenethylamino side chain (Figure 38). The tautomeric ratio determined for **90** and **91** is 2:1 (τ - τ : π - τ) in DMSO- d_6 .

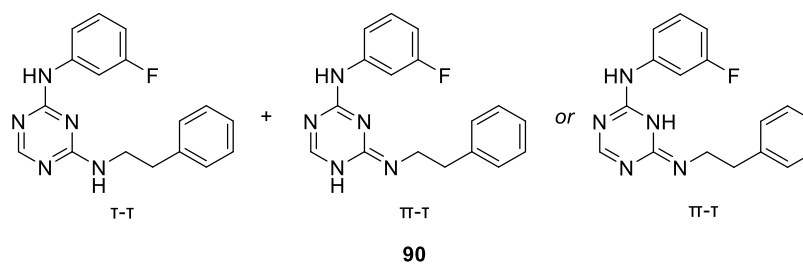


Figure 38. Possible tautomeric forms of **90**.

The ^1H NMR spectrum of **90** (Figure 39) is consistent with a mixture of two tautomers; indeed, both the total number of observed protons and the signal multiplicities support this hypothesis. Two distinct resonances are detected at δ 9.78 and 9.69 ppm, which can be assigned to the two aniline NH protons, one for each tautomer. This interpretation is in line with the observation that, in the monosubstituted 3-fluoroaniline derivative, the aniline NH appears at δ 10.33 ppm. Two singlets are further observed at δ 8.23 and 8.12 ppm and can be attributed to the two triazine H-6 protons, again one for each tautomer. In the aromatic region, a total of 20 protons is detected. This pattern can be rationalised as follows: eighteen aromatic protons arise from the two phenyl rings present in the two tautomers (4 + 5 H per tautomer), while two additional signals are plausibly due to one phenethylamino NH and one triazine NH that resonate in the same region.

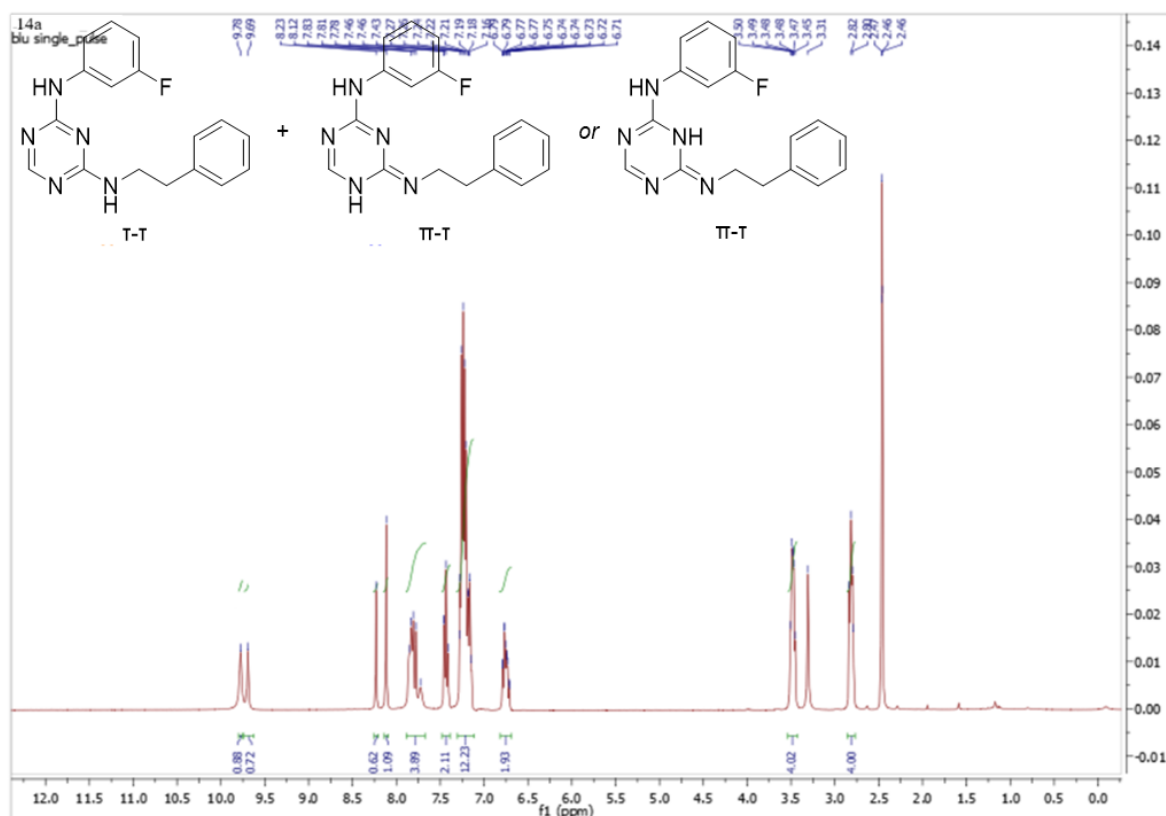


Figure 39. ^1H NMR spectrum ($\text{DMSO-}d_6$) of triazine **90**, showing duplication of the aniline NH and triazine H-6 signals consistent with the presence of two tautomers, assigned to the τ - τ form and one π - τ tautomer involving imine formation on the phenethylamino side chain.

This assignment is supported by the 2D COSY spectrum, in which a cross-peak is observed between a signal at δ 3.48 ppm (aliphatic CH_2) and a resonance at δ 7.82 ppm; this correlation justifies the assignment of the δ 7.82 signal to the NH of the phenethyl side chain. In the aliphatic region, the signals integrate for a total of four protons, corresponding to two CH_2 groups, one from each of the two phenethyl chains (Figure 40).

Additional evidence that the tautomerism involves exclusively the phenethylamino NH is provided by the following observations: (i) the monosubstituted intermediate **110**, bearing only the 3-fluoroaniline fragment, does not display any detectable tautomerism; (ii) the reaction of 2,4-dichloro-1,3,5-triazine with phenethylamine gives an NMR spectrum clearly indicative of a mixture of tautomers. However, on the basis of the available data it was not possible to unambiguously establish which of the two π - τ tautomers is formed.

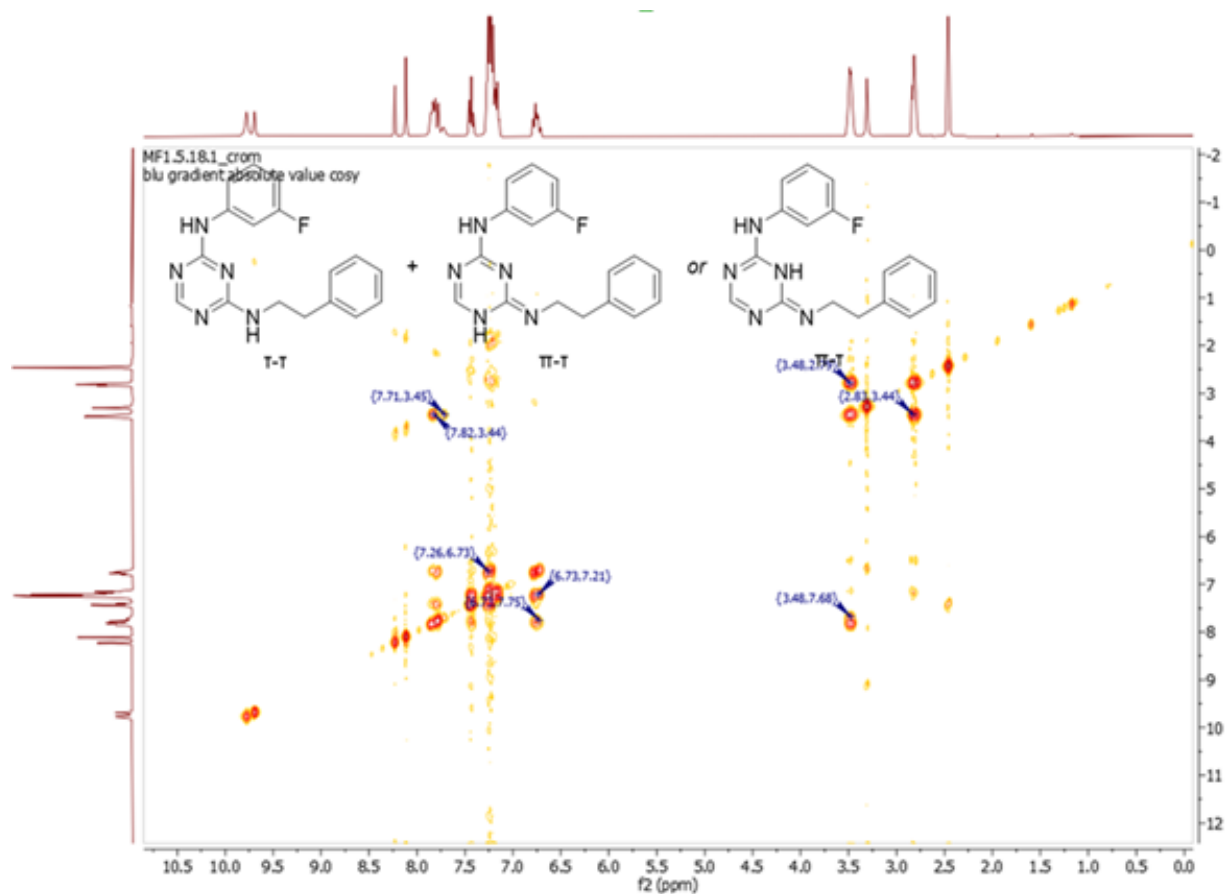


Figure 40. COSY spectrum (DMSO- d_6) of triazine **90**, showing the presence of two tautomeric forms (τ - τ and π - τ) and the key cross-peak between the aliphatic CH_2 signal (δ 3.48 ppm) and the resonance at δ 7.82 ppm, which supports the assignment of this signal to the phenethylamino NH.

Consistently with this interpretation, the mass and UV–visible spectra of **90** (Figure 41) are also compatible with a tautomeric mixture. In both cases only a single signal is observed, as expected for two rapidly interconverting species with identical elemental composition. In the mass spectrum this peak corresponds to the pseudomolecular ion at m/z $[\text{M} + \text{H}]^+$.

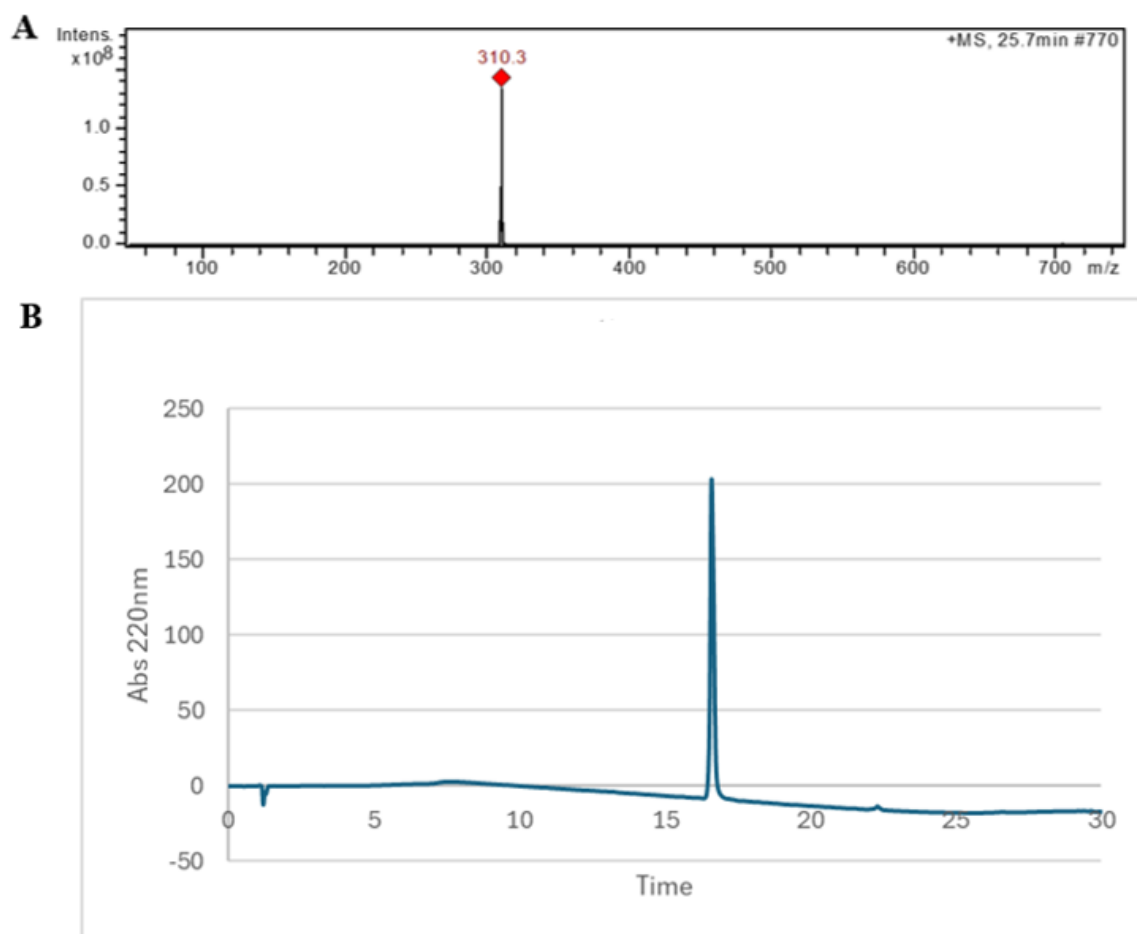
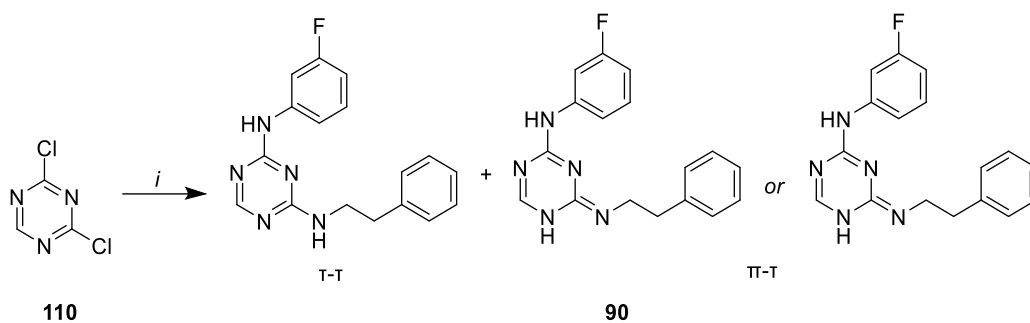


Figure 41. (A) Positive-ion mass spectrum of **90** showing a single peak corresponding to the pseudomolecular ion at m/z 310.3 $[M + H]^+$; (B) HPLC–UV trace of **90** recorded at 220 nm, displaying a single chromatographic peak, consistent with a single chemical species (tautomeric mixture).

3.1.4.3. One-pot synthesis of **90** and attempts to isolate a single tautomer

In an attempt to obtain exclusively the τ – τ tautomer, a one-pot protocol was investigated. 2,4-dichloro-1,3,5-triazine was first reacted with 3-fluoroaniline in the presence of NaHCO_3 at 0°C in a H_2O /acetone mixture and the progress of the reaction was monitored by thin layer chromatography (TLC). Once complete consumption of the starting material was confirmed, 2-phenethylamine and an additional portion of NaHCO_3 were added, and the mixture was stirred at room temperature for 12 h (Scheme 14).



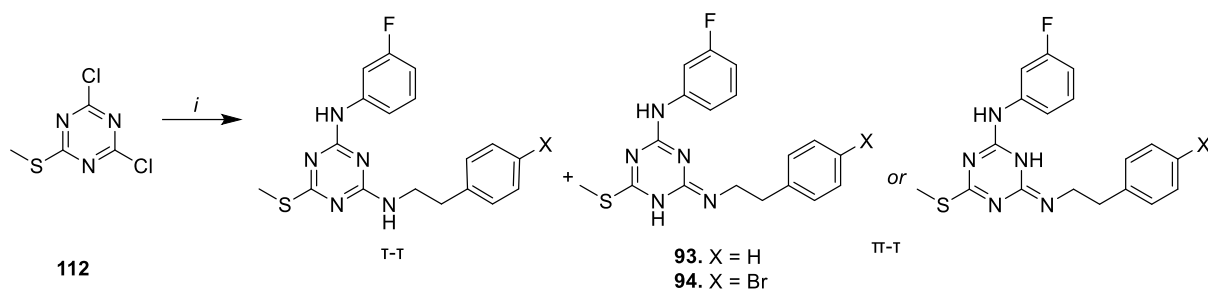
Scheme 14. Synthesis of compound **90** and **91**. Reaction conditions: (i) 3-fluoroaniline, NaHCO_3 , $\text{H}_2\text{O}/\text{acetone}$, 0°C , 1 h, then phenethylamine, NaHCO_3 , $\text{H}_2\text{O}/\text{acetone}$, rt, 12 h.

Under these conditions, however, **90** was again obtained as a tautomeric mixture with a τ - τ : π - τ ratio of 2:1 in $\text{DMSO}-d_6$.

Reactions were also performed under conditions designed to kinetically trap a single tautomer, by running the substitution at 0°C in polar aprotic solvents (EtOH , $i\text{PrOH}$) meant to be able to hydrogen bond to the phenethylamino NH and thereby disfavour tautomerisation. Unfortunately, a tautomeric mixture was again obtained.

3.1.5. Synthesis of compounds **93** and **94**

For the synthesis of compounds **93** and **94**, a one-pot approach was adopted, since the reaction between commercially available 2,4-dichloro-6-(methylthio)-1,3,5-triazine (compound **112**), and 3-fluoroaniline led to an unstable intermediate. Thus, 2,4-dichloro-6-(methylthio)-1,3,5-triazine was first reacted with 3-fluoroaniline at 0°C in the presence of NaHCO_3 and, after completion of this step, phenethylamine was added to afford the final *N*2-(3-fluorophenyl)-6-(methylthio)-*N*4-phenethyl-1,3,5-triazine-2,4-diamine **93**, or 2-(4-bromophenyl)ethan-1-amine was introduced to give *N*2-(3-fluorophenyl)-6-(methylthio)-*N*4-(4-bromophenethyl)-1,3,5-triazine-2,4-diamine **94**. Also in this case, the products were obtained as mixtures of tautomers (Scheme 15). The tautomeric ratio (τ - τ : π - τ) determined for **93** and **94** was again 2:1.



Scheme 15. Synthesis of compound **93** and **94**. Reaction conditions: (i) 3-fluoroaniline, NaHCO_3 , $\text{H}_2\text{O}/\text{acetone}$, 0°C , 1 h, then phenethylamine or *p*-bromophenethylamine,, NaHCO_3 , $\text{H}_2\text{O}/\text{acetone}$, rt, 12 h.

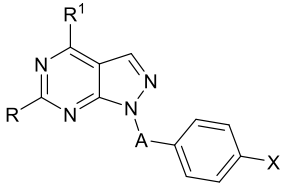
3.2. Biology

The inhibitory activity of the new pyrazolo[3,4-*d*]pyrimidine derivatives **60–90** toward SIRT2 was evaluated using a recombinant human SIRT2 (hSIRT2) deacetylase assay based on an acetylated peptide substrate and NAD^+ , under the same experimental conditions previously described for this chemotype. All compounds were first screened at 150 μM , and the percentage of SIRT2 inhibition was determined relative to a DMSO control (Table 1). Although this may appear a relatively high concentration, it is actually in line with many literature reports on SIRT2 inhibitors and can be rationalized by the peculiar topology of the SIRT2 active site. The enzyme features a long, narrow access channel leading to a deep catalytic pocket, so that ligands must adopt specific, extended conformations to reach a productive binding mode. Screening at 150 μM increases the probability of populating the binding site and allows moderate-affinity ligands to be reliably detected in the initial phase of the biological evaluation.

Compounds that produced a high level of inhibition in this primary screen ($\geq 85\%$ at 150 μM) were subsequently tested directly at 10 μM , in order to obtain a rapid indication of potency at a lower concentration. On the basis of the results obtained for the first set of compounds tested at 150 μM , a second round of experiments was carried out in which selected analogues were assayed directly at 10 μM , in order to obtain a more stringent evaluation of their potency. For the most promising analogues, full dose–response curves were then recorded to determine IC_{50} values.

3.2.1. Biological evaluation of pyrazolo[3,4-d]pyrimidines **60–89** as SIRT2 inhibitors

The exact nature of the N1, C4 and C6 substituents for each individual pyrazolo[3,4-d]pyrimidine derivative and its biological activity is reported in Table 1.

							
Cpd	X	A	R	R ¹	I% at 150 μM ^a	I% at 10 μM ^a	IC ₅₀ μM ^a
60	Cl	CH=CH	SCH ₂ CH=CH ₂	NHC ₆ H ₃ -2CH ₃ -5OH	100	36±6	13.9±2.3
61	Br	CH=CH	SCH ₃	NHC ₆ H ₄ -3F	-	100	-
62	Br	CH=CH	SCH ₃	NHC ₆ H ₄ -3OCH ₃	-	13.9±2.3	0.6±0.1
63	Br	CH=CH	SCH ₃	NH-1-naphtyl	-	60,34	-
64	Br	CH=CH	SCH ₃	NH-1-naphtyl-5OH	-	36.64	-
65	Br	CH=CH	SCH ₃	NHCH ₂ -1-naphtyl	-	45.26	-
66	Br	CH=CH	SCH ₂ CH=CH ₂	NHC ₆ H ₄ -3F	-	84	-
67	Br	CH=CH	SCH ₂ CH=CH ₂	NHC ₆ H ₄ -3OCH ₃	-	24.15	-
68	Br	CH=CH	SCH ₂ CH=CH ₂	NHC ₆ H ₃ -2CH ₃ -4OH	-	70.26	pending
69	Br	CH=CH	SCH ₂ CH=CH ₂	NHC ₆ H ₃ -2CH ₃ -4OCH ₃	-	47.84	-
70	Br	CH=CH	SCH ₂ CH=CH ₂	NHC ₆ H ₃ -2CH ₃ -5OH	-	79.6±13	6±0.8
71	Br	CH=CH	SCH ₂ CH=CH ₂	NH-1-naphtyl	-	27.59	-
72	Br	CH=CH	SCH ₂ CH=CH ₂	NH-1-naphtyl-5OH	-	73.27±7	pending
73	Br	CH=CH	SCH ₂ CH=CH ₂	NHCH ₂ -1-naphtyl	-	15.52	-
74	H	CH ₂ CH ₂	SCH ₃	NHCH ₂ C ₆ H ₄ -3F	100	100	-
75	H	CH ₂ CH ₂	SCH ₃	NHC ₆ H ₃ -2CH ₃ -5OH	100	100	-
76	H	CH ₂ CH ₂	SCH ₂ CH ₃	NHC ₄ H ₉	85.28±7	-	-
77	H	CH ₂ CH ₂	SCH ₂ CH=CH ₂	NHC ₆ H ₃ -2CH ₃ -5OH	-	28.8	-
78	H	CH ₂ CH ₂	SCH ₂ CH=CH ₂	NHC ₆ H ₄ -3F	-	60	-
79	H	CH ₂ CH(CH ₃)	SCH ₂ CH ₃	NHC ₄ H ₉	58.4	-	-
80	H	CH ₂ CO	SCH ₃	NHCH ₂ C ₆ H ₄ -2F	67.88	-	-
81	H	CH ₂ CO	SCH ₃	NHCH ₂ C ₆ H ₄ -4F	93.69	-	43

82	H	CH ₂ CO	SCH ₃	NHC ₆ H ₅	70.69	-	-
83	H	CH ₂ CO	SCH ₃	NHC ₆ H ₄ -3F	73.98	-	-
84	H	CH ₂ CH(Cl)	NHCH ₂ CH ₂ OH	NHCH ₂ CH ₂ C ₆ H ₅	47.22	-	-
85	H	CH ₂ CH(Cl)	1-pyrrolidinyl	NHC ₃ H ₇	64.17	-	-
86	H	CH ₂ CH(Cl)	1-piperidinyl	NHCH ₂ C ₆ H ₅	2.14	-	-
87	H	CH ₂ CH(Cl)	CF ₃	NHCH ₂ C ₆ H ₅	79.7428	-	-
88	Br	CH ₂ CH(Cl)	SCH ₃	NHC ₆ H ₄ -3F	-	100	-
89	Br	CH ₂ CH(Cl)	SCH ₂ CH=CH ₂	NHC ₆ H ₄ -3F	-	82.4	-

Table 1. Structure and biological activity of the synthesized pyrazolo[3,4-*d*]pyrimidine derivatives **60–89**. ^aThe percentage of SIRT-2 activity inhibition is obtained by the mean of three independent experiments.

Overall, the majority of the new derivatives showed appreciable SIRT2 inhibition at 150 μ M, confirming that the pyrazolo[3,4-*d*]pyrimidine core bearing a phenethyl or styryl-type side chain at N1 is a suitable scaffold for SIRT2 targeting. Several molecules (e.g., **74**, **75**, **88** and **90**) produced essentially complete inhibition in the primary screen and maintained high inhibition at 10 μ M, underscoring their promising potency. A second group of compounds exhibited 60–80 % inhibition at 150 μ M but more modest activity at 10 μ M, whereas only a few analogues showed poor inhibition.

3.2.1.1. Effect of the N1 side chain

Within the H series (X = H), three main types of N1 substituent were explored: a phenethyl chain (CH₂CH₂Ph), benzylcarbonyl chain (e.g. CH₂COPh), and 2-chloro-2-phenylethyl chain (CH₂CH(Cl)Ph). Phenethyl derivatives are among the most active; in particular, compounds **74** and **75** inhibit SIRT2 completely both at 150 μ M and at 10 μ M, indicating that a flexible, purely hydrophobic phenethyl unit at N1 is highly favourable. Benzylcarbonyl substituents lead to a slight decrease in activity: these analogues still display substantial inhibition at 150 μ M, but lose part of their potency at 10 μ M, suggesting that introduction of the additional carbonyl does not bring a clear binding advantage. The more electron-withdrawing and sterically encumbered 2-chloro-2-phenylethyl chains generally further erode activity, in line with the idea that excessive polarisation or steric hindrance close to N1 may perturb the optimal positioning of the ligand in the catalytic cleft.

In the halogenated series ($X = \text{Cl}$ or Br), the N1 side chain is either a *p*-halo-styryl group ($\text{CH}=\text{CH}-\text{C}_6\text{H}_4-p\text{X}$) or a *p*-halo-phenethyl chain ($\text{CH}_2\text{CH}_2-\text{C}_6\text{H}_4-p\text{X}$). The *p*-chlorostyryl analogue shows full inhibition in the primary screen but only partial inhibition at $10\ \mu\text{M}$, indicative of medium potency. By contrast, several *p*-bromophenyl derivatives (e.g. **88** and **90**) remain very active at $10\ \mu\text{M}$, often retaining complete inhibition. These data point to a beneficial effect of a *p*-bromophenyl unit at N1, either in the styryl or in the phenethyl series, probably due to the increased polarizability and enhanced hydrophobic contacts of the bromophenyl ring within the long lipophilic channel adjacent to the selectivity pocket.

3.2.1.2. *Effect of the C6 substituent*

Most compounds bear a thioalkyl group at C6 ($\text{R} = \text{SCH}_3$, SCH_2CH_3 or $\text{SCH}_2\text{CH}=\text{CH}_2$), with a few analogues incorporating more unusual substituents (e.g., CF_3 or amines). For the H series with N1 = phenethyl, keeping $\text{R} = \text{SCH}_3$ generally affords the highest levels of inhibition: analogues with a methylthio group at C6 include some of the most potent members of the set, whereas elongation of the thioalkyl chain is often associated with a decrease in activity at $10\ \mu\text{M}$. This behaviour supports the design hypothesis that a compact, neutral thioether at C6 is optimal, efficiently filling the narrow hydrophobic subpocket near Ile169/Phe96 without introducing unnecessary flexibility.

Replacing the thioether with more strongly perturbing functionalities is poorly tolerated. For instance, when the thioalkyl group is replaced by bulky, non-aromatic amines, the resulting compounds are weak inhibitors, suggesting that a basic centre at this position is disfavoured. Conversely, a CF_3 group can still sustain measurable inhibition, indicating that an electron-withdrawing but non-basic substituent is compatible with binding, although it does not clearly outperform the thioether.

3.2.1.3. *Effect of the C4 substituent*

The C4 position, bearing various benzylic or arylamines, exerts a strong influence on SIRT2 inhibition. For compound **74**, bearing a phenethyl chain at N1 and a thiomethyl at C6, the 3-fluorobenzylamino substituent is clearly optimal, delivering full inhibition at both concentrations tested. Electron-rich anilines and benzylamines bearing phenolic or methoxy groups are also well tolerated: several analogues incorporating hydroxy- or alkoxy-substituted phenyl rings at C4 still show high inhibition at $150\ \mu\text{M}$ and retain significant activity at $10\ \mu\text{M}$.

These data indicate that the SIRT2 pocket can accommodate hydrogen-bond donors and acceptors at this position, and that polarity per se is not detrimental, provided that the aromatic character is preserved.

By contrast, a marked loss of activity is observed when the benzyl/aryl unit is replaced by saturated aminoalkyl fragments such as pyrrolidinyl or piperidinyl, or when the C4 substituent becomes bulky and non-planar. In these cases, inhibition drops sharply, suggesting that a flat aromatic surface at C4 is a key requirement, likely to maintain π - π stacking with residues lining the substrate channel and to preserve the overall planarity of the ligand.

In summary, the SAR emerging from compounds **60–89** can be rationalised as follows: a phenethyl or *p*-bromophenyl side chain at N1, a small neutral thioether at C6, and an aromatic C4 substituent (ideally a 3-fluorobenzyl or suitably substituted phenyl/benzyl) constitute the most favourable combination for potent SIRT2 inhibition. Deviations from this pattern, in particular loss of aromatic character at C4, excessive steric encumbrance at N1, or disruption of the compact hydrophobic environment at C6, are generally associated with a reduction in activity.

3.2.1.4. Preliminary biological evaluation of triazines derivatives **90–93** as SIRT2 inhibitors

The inhibitory activity of triazine derivatives (Table 2) toward SIRT2 was evaluated in the same recombinant human SIRT2 deacetylase assay used for the pyrazolo[3,4-*d*]pyrimidine series. The preliminary data indicate that these compounds retain a measurable ability to inhibit SIRT2.

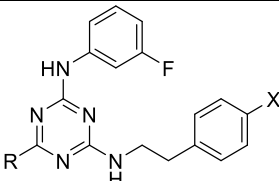
					
	R	X	^a I% at 150 μ M	^a I% at 20 μ M	^a I% at 10 μ M
90	H	H	85.2 $\pm$ 6.4	-	-
91	H	Br	100 $\pm$ 8.9	46.4 $\pm$ 7.2	-
92	SCH ₃	H	86.7 $\pm$ 6.6	-	-
93	SCH ₃	H	-	-	-

Table 2. Structure and biological activity of the synthesized triazine derivatives **90–93**.

^aThe percentage of SIRT-2 activity inhibition is obtained by the mean of three independent experiments.

At 150 μ M, all three tested analogues (**90–92**) display substantial inhibition (≈ 85 – 100 %), and the most promising member of the series, compound **91**, still inhibits SIRT2 by 46.4 ± 7.2 % at 20 μ M. This derivative is characterised by an unsubstituted triazinic C6 position ($R = H$) and a *p*-bromophenethyl side chain at N4, suggesting that introduction of the *p*-bromophenyl vector, already beneficial in the pyrazolo[3,4-*d*]pyrimidine series, may also enhance the SIRT2 affinity of the triazine scaffold.

For practical reasons, the triazine derivatives were profiled at 20 μ M rather than the 10 μ M concentration used for most pyrazolopyrimidines: these compounds were available only in limited amounts and, on the basis of docking studies and physicochemical properties, were expected to exhibit a more moderate potency. An intermediate single-point concentration was therefore chosen to maintain assay sensitivity while preserving material for further investigations.

Given the very small number of analogues and the limited concentration range explored, these results can only be considered indicative. More robust conclusions on the SAR within the triazine series will require additional biological data and the synthesis of a broader panel of derivatives.

4. Conclusions

Taken together, the work described in this chapter confirms the pyrazolo[3,4-*d*]pyrimidine scaffold as a privileged platform for the development of novel SIRT2 inhibitors and demonstrates that a rational, structure-based approach can be successfully combined with systematic synthetic optimisation. Starting from previously identified leads, a focused library of derivatives (**60–89**) was prepared, in which the N1, C4 and C6 positions were varied according to precise hypotheses derived from docking and available crystallographic data. The synthetic routes developed are robust and modular provide a solid foundation for further expansion of this chemotype.

Biological evaluation on recombinant human SIRT2 highlighted several members of the series as promising inhibitors in the low-micromolar range. A clear SAR could be delineated: at N1,

either a phenethyl chain or a *p*-bromophenyl vector (in styryl or phenethyl form) emerged as optimal, consistently associated with high levels of inhibition at 150 μ M and, for selected analogues, at 10 μ M as well. These findings are in line with the docking models, which indicated that such hydrophobic handles are well suited to fill the long lipophilic channel leading to the selectivity pocket and to sustain π - π contacts with the His187/Phe235 pair. At C6, compact thioethers, particularly the methylthio group, proved superior to more extended or polar substituents, supporting the idea that efficient, but not excessive, occupation of the narrow subpocket close to Ile169/Phe96 is required. Finally, the C4 position was shown to be highly sensitive to the nature of the substituent: aromatic benzyl or anilide fragments, especially those bearing a 3-fluoro group or appropriately placed hydroxy/alkoxy substituents, were compatible with potent inhibition, whereas replacement by saturated aminoalkyl moieties generally led to a loss of activity.

On this basis, a molecular simplification strategy was implemented to access a second family of inhibitors in which the pyrazolo[3,4-*d*]pyrimidine core was reduced to a 1,3,5-triazine ring while preserving the key terminal vectors. The first triazine subset (**90–93**) was designed as a minimal mimic of lead compound **57** and was synthesised through straightforward nucleophilic aromatic substitution on pre-functionalised chloro-triazines. Despite the additional complexity introduced by triazine tautomerism, the spectroscopic characterisation allowed the identification of a well-defined τ - τ/π - τ equilibrium and confirmed the structural integrity of the new scaffold. From a biological standpoint, the preliminary screening demonstrated that the simplified triazines retain the capacity to inhibit recombinant SIRT2, with compounds **90–92** displaying 85–100 % inhibition at 150 μ M and the *p*-bromophenethyl analogue **91** still inhibiting SIRT2 by ~46 % at 20 μ M. Although less potent than the best pyrazolopyrimidines, these data validate the triazine core as a viable, synthetically more accessible surrogate capable of reproducing the main binding interactions.

In conclusion, this project has delivered two complementary chemotypes: pyrazolo[3,4-*d*]pyrimidines and triazines, that satisfy the structural requirements for SIRT2 recognition and provide multiple points of entry for further optimisation. Within the pyrazolopyrimidine series, several analogues already achieve robust inhibition in the low-micromolar range and display coherent SAR trends that can guide the next cycles of design. The triazine derivatives, although at an earlier stage of development, demonstrate that substantial molecular simplification is

possible without a complete loss of activity, thereby opening the way to leaner, potentially more drug-like SIRT2 inhibitors.

Future work will focus on translating these findings into more potent and selective candidates. From a biochemical perspective, the triazine series requires completion of its profiling: all members (**90–93** and subsequent analogues) will be systematically tested under the standard conditions used for the pyrazolopyrimidines, including inhibition at 10 μ M and full IC_{50} determination for the most promising compounds. In parallel, the triazine library will be expanded by diversifying the phenethylamino side chain, varying the length/functionalisation of the spacer. This will allow a more refined exploration of the interactions within the hydrophobic tunnel and around the selectivity pocket and may help to mitigate the tautomeric complexity by favouring specific electronic environments at the exocyclic nitrogens.

Ultimately, once improved SIRT2 potency and selectivity have been achieved, the most active pyrazolopyrimidines and triazines will be advanced to cellular assays to evaluate their ability to modulate α -tubulin acetylation and other SIRT2-regulated pathways, and to preliminary ADME studies to assess their drug-like properties. In this way, the work presented here lays a solid conceptual and experimental foundation for the continued development of pyrazolopyrimidine- and triazine-based SIRT2 inhibitors as potential pharmacological tools and, in the longer term, as leads for therapeutic intervention in SIRT2-driven pathologies.

CHAPTER 2

Pyrazolo[3,4-*d*]pyrimidine derivatives as potential anticancer agents for prostate and bladder cancer

1. Introduction

1.1. Tyrosine kinases

Tyrosine kinases (TKs) form a subgroup of protein kinases that transfer the γ -phosphate of ATP onto specific tyrosine residues of protein substrates. These enzymes are widely conserved in eukaryotes and operate in both unicellular and multicellular organisms, where they control numerous cellular functions.²⁸²

Tyrosine kinases are typically modular proteins in which an N-terminal regulatory region is coupled to a C-terminal catalytic domain. The regulatory portion often contains Src homology (SH) 3 and SH2 domains that mediate docking to partner proteins and thereby control localization and activation of the kinase. SH3 domains (≈ 60 amino acids) recognize proline-rich segments that adopt a left-handed polyproline type II helix; preferred ligands frequently conform to short consensus motifs such as RPLPLP or Φ PPLPXR (Φ = hydrophobic residue, X = any residue), although sequence specificity is generally modest and single amino-acid substitutions usually cause only limited changes in affinity.^{283,284} SH2 domains (≈ 100 amino acids) bind phosphotyrosine-containing motifs, discriminating primarily on the residues C-terminal to the phosphorylated tyrosine. Combinatorial phosphopeptide library studies have defined optimal recognition sequences for many SH2 domains, illustrating how distinct tyrosine kinases are recruited to different phosphorylated adaptors and receptors.²⁸⁵ In this way, SH3 and SH2 domains position the catalytic core within specific signalling complexes and regulate access to substrates (Figure 42).

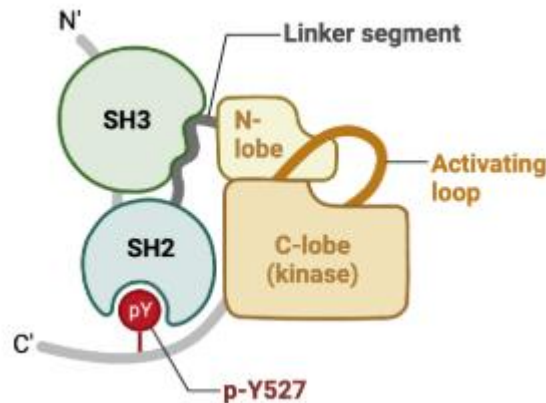


Figure 42. Schematic representation of the modular organization of *c-Src* in its inactive, closed conformation, as a representation of the architecture of a generic tyrosine kinase. The N-terminal SH3 and SH2 domains are connected to the C-terminal kinase domain via a proline-rich linker; intramolecular engagement of the SH2 domain with the phosphorylated C-terminal tyrosine (p-Y527) and of the SH3 domain with the linker stabilizes the N-lobe/C-lobe arrangement and activation loop in a catalytically non-permissive state.

Within the catalytic domain, the tyrosine kinase reaction requires a divalent metal ion and proceeds by aligning the hydroxyl group of the substrate tyrosine with the γ -phosphate of ATP. The substrate binds on a platform on the C-terminal lobe of the kinase, a surface formed in part by residues of the activation loop (A-loop). In many tyrosine kinases, phosphorylation of one or more tyrosine residues within the A-loop stabilizes an active conformation of this segment, thereby enhancing catalysis (Figure 42). Key residues that support catalysis and maintain the geometry of the active site are contributed by several conserved structural elements: in the N-lobe, the glycine-rich P-loop (nucleotide-binding loop), β -strand 3 and the α C-helix; in the C-lobe, the catalytic loop (β -strands 6 and 7) and the A-loop, a stretch of ~ 20 residues located between strand 8 and the α C-helix. These elements encompass highly conserved sequence motifs that are characteristic of the protein tyrosine kinase family and are essential for efficient phosphotransfer.²⁸⁶

Despite differences in substrate selectivity and regulatory mechanisms, TKs in their active state adopt very similar 3D structures. This convergence reflects the strict spatial constraints required to bring catalytic residues into the proper arrangement. Consequently, the structural elements that carry these residues assume comparable relative orientations.

In resting cells, most TKs are maintained in inactive conformations that either disrupt the catalytic geometry or prevent binding of substrates and cofactors. Unlike active states, which

are structurally alike across kinases, inactive conformations are highly heterogeneous. Structural studies have revealed a variety of auto-inhibitory strategies. Frequently, repositioning of the α C-helix and/or the A-loop plays a central role, and in many cases segments outside the kinase domain fold back to block functional sites or induce inhibitory conformational changes. Several of these mechanisms are conserved not only among TK subfamilies, but also in non-tyrosine kinases.²⁸⁷

Activation of TKs governs essential cellular processes, including proliferation, survival, tissue morphogenesis, neovascularization, and tissue repair and regeneration.²⁸⁸ Under physiological conditions, TK activity is tightly controlled; persistent or inappropriate kinase activation has been reported in many pathologies, especially cancer. Aberrant activation may arise from gain-of-function mutations, chromosomal rearrangements, gene amplification, overexpression, or abnormal autocrine/paracrine/endocrine stimulation of the receptor–ligand system. In numerous human malignancies, deregulated TK signalling has been linked to tumour onset and progression.

Because TKs contribute to multiple aspects of the malignant phenotype, they have become attractive drug targets. Targeted TK therapy, however, is effective only when the kinase is a key survival driver for the tumour, whereas cancer cells often harbour numerous genetic and epigenetic alterations. Even so, in many cases the inactivation of a single oncogene is sufficient to compromise tumour cell viability or proliferation. This concept, known as “oncogene addiction”, underpins the rationale for molecularly targeted treatments. Support for oncogene addiction comes from the growing list of successful therapies based on antibodies or small molecules directed against specific oncogenic drivers. Examples include **imatinib**, which inhibits Bcr-Abl in chronic myeloid leukaemia and c-Kit in gastrointestinal stromal tumours, and **gefitinib** and **erlotinib**, which target epidermal growth factor receptor (EGFR) in non-small cell lung cancer, pancreatic tumours, and glioblastoma.²⁸⁹

In human cells, TKs are subdivided into two main classes: receptor tyrosine kinases (RTKs), which are transmembrane receptors, and non-receptor tyrosine kinases (NRTKs), which are intracellular enzymes.

1.1.1. Receptor tyrosine kinases

The RTK family comprises 20 subfamilies (ALK, AXL, DDR, EGFR, EPH, FGFR, INSR, MET, MUSK, PDGFR, PTK7, RET, ROS, ROR, RYK, TIE, TRK, VEGFR, AATYK and the

less characterized DKFZp761P1010). All RTKs are single-pass transmembrane glycoproteins containing: (i) an extracellular domain, which binds ligands; (ii) a single transmembrane helix; (iii) an intracellular segment that includes the tyrosine kinase domain. Upon binding their ligands, RTKs become activated and transmit signals into the cell primarily by:

1. phosphorylation (autophosphorylation) of tyrosines in their own cytoplasmic tails;
2. phosphorylation of tyrosine residues on downstream signalling proteins (Figure 43).

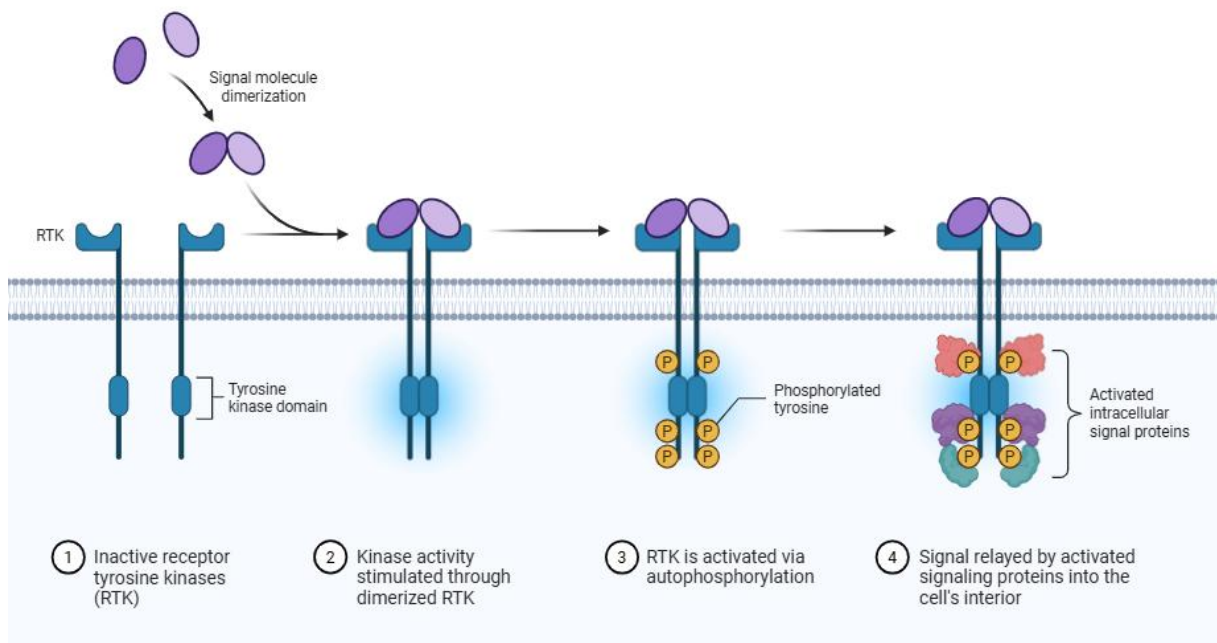


Figure 43. Schematic representation of RTK activation and downstream signalling. In the resting state, RTKs are present as inactive monomeric receptors in the plasma membrane (1). Binding and dimerization of an extracellular ligand bring two RTK molecules into proximity, stimulating kinase activity within the cytoplasmic tyrosine kinase domains (2). The juxtaposed kinases then undergo trans-autophosphorylation on specific tyrosine residues in their intracellular tails (3). These phosphotyrosine sites serve as docking platforms for cytoplasmic signalling proteins, which become activated upon recruitment and propagate the signal to intracellular pathways to elicit appropriate cellular responses (4).

Through these events, RTKs trigger multiple intracellular pathways that control proliferation, differentiation, migration and metabolism.²⁹⁰

RTK activation typically involves two key outcomes: the increase in intrinsic catalytic activity and the formation of docking sites for downstream effectors. For most RTKs, both effects are achieved via ligand-induced receptor oligomerization, which promotes tyrosine

autophosphorylation. In the absence of ligand, RTKs may exist as monomers or pre-assembled oligomers in inactive conformations. Ligand binding stabilizes specific relative orientations of receptor subunits, driving non-covalent dimerization/oligomerization of monomeric receptors or conformational rearrangements in preformed complexes (e.g., the insulin receptor heterotetramer), which favours trans-autophosphorylation of the intracellular domains.²⁹¹

1.1.2. Non receptor tyrosine kinases

Non-receptor tyrosine kinases comprise 10 subfamilies (Abl, Ack, Csk, Fak, Fes, Frk, Jak, Src, Tec, Syn) that are activated indirectly by extracellular signals.

Some NRTKs are tethered to cellular membranes via N-terminal lipid modifications such as myristoylation or palmitoylation. As in other kinases, their catalytic domain is positioned between an N-lobe and C-lobe and features the typical bilobal architecture. Besides the kinase core, NRTKs carry additional modules that mediate interactions with proteins, lipids, or DNA.

The most prevalent protein–protein interaction domains in NRTKs are SH2 domains (~100 residues), which recognize phosphotyrosine-containing motifs in a sequence-specific manner, and SH3 domains (~60 residues), which bind to proline-rich sequences that adopt a polyproline type II (PPII) helical conformation.

Some NRTKs lack SH2/SH3 modules but instead harbour alternative, subfamily-specific interaction domains.

Regulation of NRTKs is dominated by tyrosine phosphorylation. With some exceptions, phosphorylation of tyrosines within the activation loop enhances catalytic activity, via trans-autophosphorylation or phosphorylation by another NRTK. Conversely, phosphorylation of tyrosines outside the activation loop can suppress kinase activity.²⁸⁸ A comprehensive overview of cytoplasmic TKs has been provided by Tsygankov.²⁸⁴

1.2. Src family

The Src family of protein tyrosine kinases (SFKs) includes nine members: Src, Fyn, Yes, Blk, Yrk, Fgr, Hck, Lck and Lyn. These kinases integrate signals from a wide variety of cell surface receptors in many cellular contexts. They participate in fundamental processes such as proliferation, differentiation, migration, survival, cell morphology, and specialized signalling events.²⁹²

Several SFK members have been identified as cellular proto-oncogenes, and their pleiotropic functions highlight their importance in both physiological and pathological states.²⁹³

1.3. Structure and activation of c-Src

Human c-Src is a 536-amino acid protein whose organization is representative of SFKs. From N- to C-terminus, Src comprises an N-terminal SH4 domain, a short unique domain, an SH3 domain, a SH2 domain, a PPII region, a catalytic kinase domain (SH1), and a short C-terminal regulatory tail (Figure 44).²⁹⁴

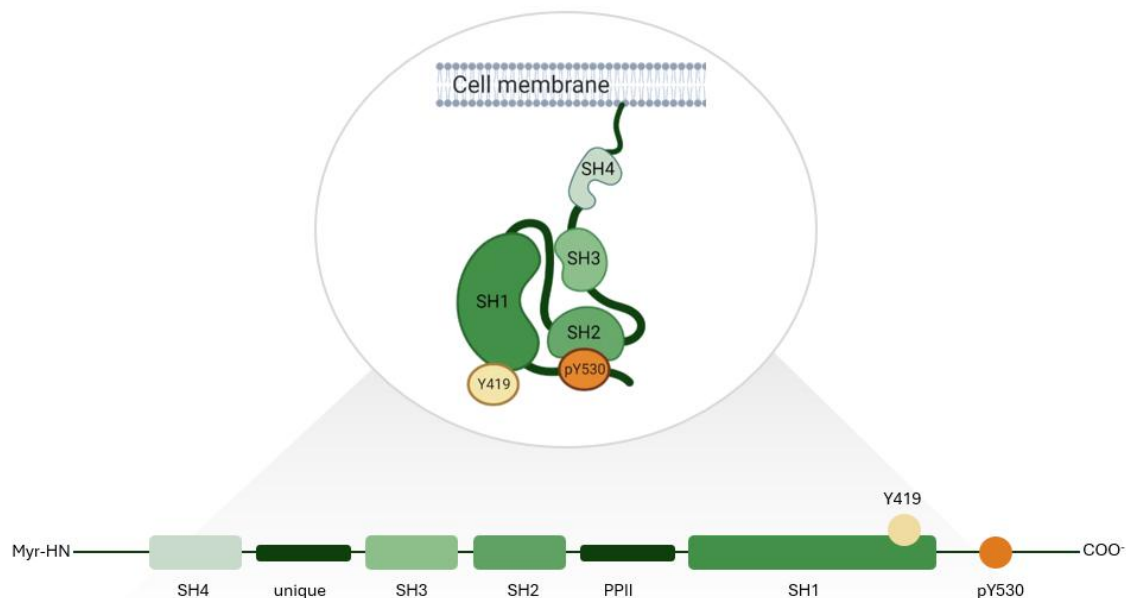


Figure 44. Schematic representation of the modular organization of c-Src. The linear domain architecture is represented in the bottom, comprising an N-terminal myristoylated SH4 region, the unique domain, SH3 and SH2 domains, a PPII linker, and the catalytic SH1 tyrosine kinase domain, followed by the C-terminal regulatory tail bearing Tyr530. The approximate three-dimensional arrangement of these modules is also depicted, with the SH4 region mediating membrane association and the SH3/SH2 tandem packed against the kinase domain. The activating tyrosine in the activation loop (Tyr419) and the inhibitory C-terminal phosphotyrosine (pTyr530) are highlighted as key regulatory sites controlling Src kinase activity.

The SH4 segment contains an N-terminal glycine that is always myristoylated and in some cases palmitoylated.²⁹⁵ These lipid modifications anchor Src to the inner leaflet of the plasma membrane.

The unique domain (50–70 residues) differs among SFK members and is thought to contribute to specific protein–protein interactions.

SH2 and SH3 are highly conserved in SFKs and are key regulators of Src activity. The SH2 domain (~100 residues) has a central three-stranded β -sheets flanked by α -helices; this architecture generates two binding pockets for phosphotyrosine peptides. The SH3 domain (~60 residues) forms a β -barrel of five antiparallel β -strands plus two loops and recognizes PXXP motifs in the PPII region, a proline-rich linker between SH2 and the kinase domain. PPII adopts a helical conformation that fits onto a hydrophobic/aromatic surface on SH3.

The SH1 kinase domain is responsible for catalytic activity and displays the typical bilobal organization of protein kinases, with a smaller N-lobe, largely β -strands plus the α C-helix, and a larger C-lobe, mainly α -helical. The ATP and substrate binding site lies in the cleft between lobes. The N-lobe hosts five β -strands and the α C-helix, as well as the glycine-rich G-loop (P-loop), which positions ATP for γ -phosphate transfer. The C-lobe contains the activation loop (A-loop), which includes the key regulatory tyrosine Tyr419 (human Src). The flexible linker joining N- and C-lobes is known as the hinge region (Figure 45).²⁹⁶

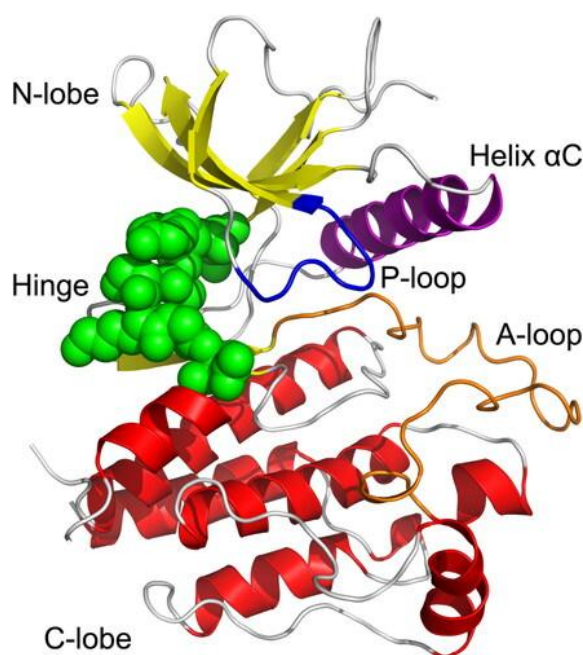


Figure 45. Representation of the SH1 catalytic domain. The small N-terminal lobe (N-lobe, yellow) and the larger C-terminal lobe (C-lobe, red) form a bilobal architecture that creates the ATP/substrate binding cleft. The α C-helix (magenta) and glycine-rich P-loop (blue) within the N-lobe, together with the activation loop (A-loop, orange) in the C-lobe, are key regulatory elements that control catalytic activity. The hinge region (green spheres) connects the two lobes

and provides the backbone hydrogen-bonding platform for ATP and ATP-competitive inhibitors.

Src cycles between two major conformations: a closed, inactive state, and an open, active state. Two tyrosines are central to this regulation: Tyr419 and Tyr530.

In the inactive state, Tyr419 is dephosphorylated and Tyr530 is phosphorylated. Intramolecular contacts between SH2 and SH3 and the kinase domain lock the protein in a closed conformation. SH3 interacts with the PPII segment, and SH2 binds the phosphorylated C-terminal tail (pTyr530). SH3 and SH2 pack against the N- and C-lobes on the side opposite to the catalytic cleft. Although SH2 and SH3 do not physically obstruct the ATP/substrate site, this arrangement inactivates the enzyme because of the displacement of the α C-helix, that removes a catalytically critical residue from the active site; in addition, the A-loop adopts a conformation that prevents phosphorylation of Tyr419 and hinders substrate access. Finally, the relative orientation of the two lobes is suboptimal for catalysis (Figure 46).²⁹³

Activation is achieved by dephosphorylation of Tyr530 and autophosphorylation of Tyr419, which disrupt intramolecular interactions between SH2 and SH3, reorganize domain contacts and reposition the A-loop to permit substrate entry and Tyr419 phosphorylation. Under basal conditions *in vivo*, ~90–95 % of Src is in the inactive, pTyr530-phosphorylated form. C-terminal Src kinase (Csk) and Csk-homologous kinase (Chk) are the main enzymes known to phosphorylate Tyr530 and maintain this inhibitory state.

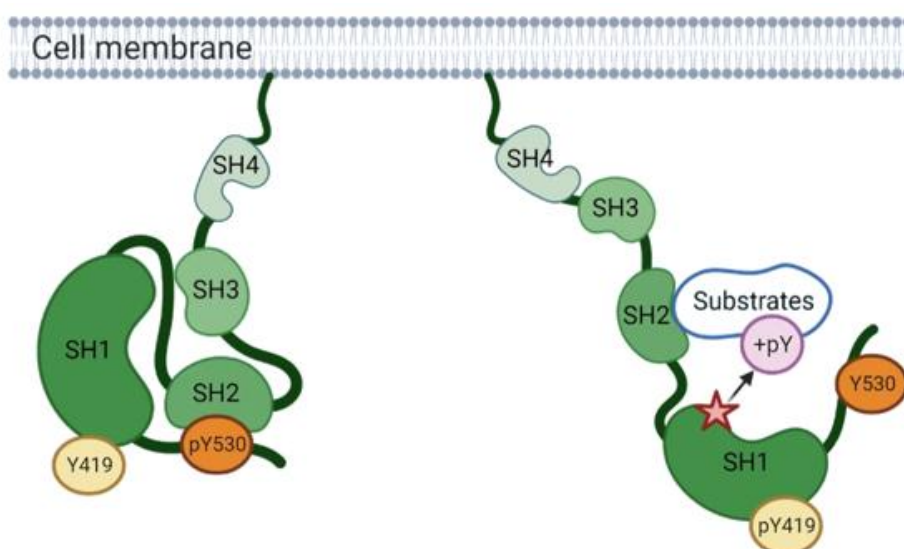


Figure 46. Schematic representation of Src regulation. In the inactive conformation (left), the intramolecular engagement of both the SH2 domain with the C-terminal phosphotyrosine

(pY530) and the SH3 domain with the linker constrains the kinase (SH1) in a closed, catalytically repressed state, with Tyr419 unphosphorylated. Right, dephosphorylation of Y530 and subsequent autophosphorylation of Tyr419 in the activation loop promote an open conformation in which the SH2/SH3 tandem no longer clamps the kinase domain, allowing access of protein substrates to SH1 and phosphorylation of tyrosine residues.

Several phosphatases can remove the phosphate from Tyr530 and thereby activate Src. These include transmembrane tyrosine phosphatases and the cytosolic enzymes protein tyrosine phosphatase 1B (PTP1B), Src homology region 2 domain-containing phosphatase (Shp) 1 and 2.²⁹⁷ In addition, ligand-occupied cell-surface receptors or cytosolic proteins can bind SH2/SH3 and displace them from the kinase domain, facilitating Tyr419 autophosphorylation.

A wide array of extracellular stimuli activates Src by engaging growth factor receptors, integrins and other adhesion receptors, G-protein-coupled receptors (GPCRs), cytokine receptors, and ion channels. Once activated, Src phosphorylates numerous downstream proteins and integrates multiple signalling cascades, including Ras/Raf, Rho GTPase-activating proteins (RhoGAPs), PI3K/AKT and others (Figure 47).

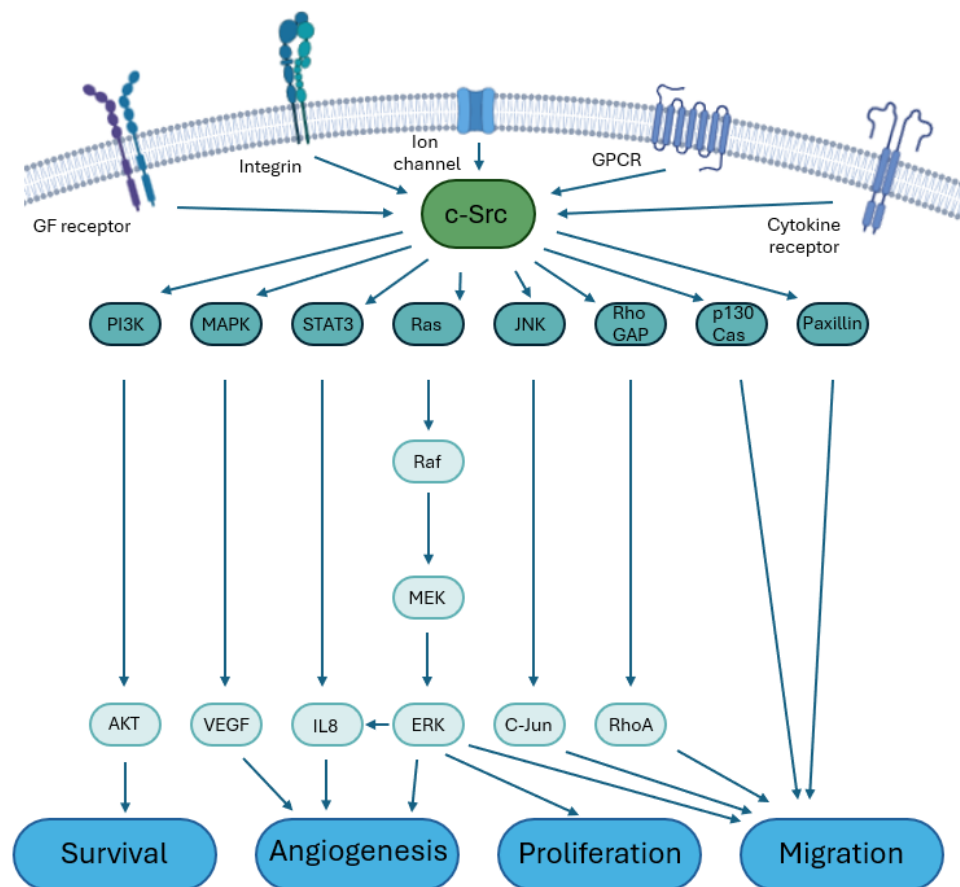


Figure 47. Schematic representation of c-Src signalling pathway.

1.3.1. *c-Src and cancer*

SFKs occupy central positions in many signalling networks and are implicated in the pathogenesis of various diseases, particularly cancer.^{298,299} Src was the first SFK member linked to cancer biology and remains one of the best characterized in tumour progression.

In normal cells, Src activity is transiently elevated in response to diverse physiological stimuli, including mitogenic and survival signals, adhesion and motility inputs, all processes that become dysregulated during malignant transformation.³⁰⁰ Oncogenic activation of Src is associated with the inability of cells to switch off its kinase activity. Four of the six classic hallmarks of cancer (self-sufficiency in growth signals, resistance to apoptosis, sustained angiogenesis, and tissue invasion/metastasis) depend heavily on tyrosine kinase signalling pathways, that include Src.³⁰¹

Early work on Src in oncogenesis traced back to the discovery of v-Src, the viral homolog of c-Src lacking the inhibitory C-terminal region, which induces sarcomas in chickens and other animals.³⁰² In human tumours, deregulated SFK activity is often due to overexpression or hyperactivation rather than obvious mutations, although activating Src mutations have been detected in advanced colon cancer.³⁰³ For instance, a point mutation introducing a stop codon at position 531 yields a truncated Src-531 protein with increased activity.

Elevated Src levels or kinase activity correlate with tumour stage and aggressiveness in many cancers. Constitutive SFK activation has been reported in breast, gastric, colon, pancreatic, ovarian, prostate, head and neck, lung, and bladder cancers, as well as in neuronal tumours and haematological malignancies (leukaemia, lymphoma, myeloma).³⁰³

1.3.1.1. *c-Src in glioblastoma*

Glioblastoma (GB) is the most frequent and aggressive primary malignancy of the central nervous system. According to WHO, it is classified as grade IV astrocytoma and may arise *de novo* or via progression from lower-grade astrocytomas.³⁰⁴ Prognosis is poor: about half of patients die within one year of diagnosis and ~90 % within three years.³⁰⁵ GB features high proliferative activity and increased metabolic/oxygen demands, reflected histologically by microvascular proliferation and necrosis.³⁰⁶

Standard of care includes maximal safe surgical resection followed by radiotherapy combined with chemotherapy, and then adjuvant chemotherapy. Despite this multimodal treatment,

outcome remains dismal; virtually all patients relapse, and current salvage therapies provide limited survival benefit.

Consequently, intense efforts focus on new therapeutic targets in GB, including growth factor receptors and other TKs involved in intracellular signalling.^{307,308} Members of the Src family are among the kinases proposed as drivers of GB development and progression.³⁰⁹

Initial studies documented increased Src activity in GB relative to normal brain and demonstrated its oncogenic potential in brain tumours.³¹⁰ Src acts downstream of overexpressed growth factor receptors commonly found in GB, such as EGFR and platelet-derived growth factor receptor (PDGFR), and cooperates with focal adhesion kinase (FAK) to manipulate cytoskeleton-linked survival and migration pathways.^{311,312} In preclinical GB models, genetic or pharmacological inhibition of Src decreases cell proliferation and invasion.^{313–315}

1.3.1.2. c-Src and neuroblastoma

NB is a tumour of the sympathetic nervous system and the most common extracranial solid neoplasm in children, particularly during infancy.³¹⁶ Prognosis varies with age: patients younger than one year generally fare better and their tumours may differentiate or even regress spontaneously, whereas older patients often have poor outcomes.³¹⁷

Clinically, NB shows highly heterogeneous behaviour. Despite progress in treating specific, well-defined risk groups, long-term survival for children with high-risk NB remains below 40%, and the outcome has improved only modestly.

Current management relies on multimodal strategies: surgery, chemotherapy, radiotherapy, differentiation agents, immunotherapy, and, in selected low-risk cases, careful observation alone.³¹⁸ High-risk NB therapy typically uses dose-intensive regimens of cisplatin plus etoposide alternating with vincristine, doxorubicin and cyclophosphamide.³¹⁶ Isotretinoin is administered during first remission. Nonetheless, prognosis for advanced NB is still unsatisfactory, underscoring the need for innovative therapies.

c-Src has been implicated in NB cell differentiation, adhesion and survival, primarily via hyperactivation rather than overexpression.³¹⁹ c-Src may contribute to the progression of aggressive NB forms.³²⁰ Targeting Src with small-molecule inhibitors to suppress its kinase activity has been proposed as a potential therapeutic approach in NB and has shown encouraging preclinical results.²⁷⁶

1.3.1.3. c-Src and medulloblastoma

Medulloblastoma (MB) is the most prevalent malignant brain tumour in children, accounting for 15–20 % of paediatric brain neoplasms. Overall cure rates range from ~40 % to 90 %, depending on molecular subtype.³²¹ Standard treatment consists of surgical resection followed by craniospinal irradiation and chemotherapy.

Because central nervous system (CNS) irradiation can severely affect the developing brain, radiotherapy is often avoided in children under three years of age, which, however, may compromise disease control and survival.³²² Combining radiotherapy with agents such as ifosfamide, cisplatin and etoposide has improved survival and reduced some of the cognitive and endocrine sequelae associated with radiation.³²³

Despite these advances, a fraction of patients still relapses or shows progressive disease. Efforts to further decrease morbidity and mortality are hampered by toxicity of conventional therapies and by the blood brain barrier (BBB), which limits penetration of hydrophilic or large lipophilic drugs.^{321,324} Thus, there is a strong need for new agents that more readily cross the BBB and provide better disease control with lower neurocognitive toxicity.

Recent discoveries have shed light on MB pathogenesis and identified key proteins, including several RTKs and telomerase. Elevated SFK activity, evidenced by increased tyrosine phosphorylation, has been reported in paediatric brain tumours, including medulloblastoma, astrocytoma and ependymoma, when compared with normal brain tissue.³²⁵ This observation supports a contributory role of Src in MB development.

Certain pyrazolo[3,4-*d*]pyrimidine derivatives have been shown to slow medulloblastoma growth in mouse models by inhibiting Src.³²⁶

1.3.1.4. c-Src and colon cancer

Colorectal cancer ranks among the most common malignancies worldwide, together with lung, prostate and breast cancer.³²⁷ Early-stage disease can often be cured surgically, but many patients present with metastatic disease at diagnosis and require systemic chemotherapy. In this setting, conventional chemotherapy yields modest results, with survival rates below 10 % in the presence of metastases.

Major advances have nevertheless occurred, including the establishment of adjuvant chemotherapy, especially beneficial in stage III, and improvements in surgical techniques to reduce morbidity and improve outcomes.³²⁸

Increased Src activity is observed in about 80 % of colon cancer patients, underscoring its importance in this tumour type.^{329,330} Src activity tends to increase progressively from premalignant conditions (ulcerative colitis) through polyp formation to invasive tumours and metastases.³³¹ An activating mutation in the Src gene is present in ~12% of patients, but accounts for only a fraction of Src hyperactivation in colon cancer.³⁰³

Other studies did not find recurrent Src mutations, suggesting that upstream regulatory defects or altered Src regulation are more relevant.³³²

Work on the Src negative regulators Csk and Chk revealed that altered localization of Csk at the membrane contributes to SFK-driven invasion in colon cancer cells, providing a novel mechanism for oncogenic SFK activation.³³³ Overexpression of Src in colon cancer cells enhances adhesion, invasion and migration without significantly affecting proliferation, pointing to a primary role in tumour progression rather than initiation.³³⁴ Consequently, pharmacological Src inhibition is considered a promising strategy to improve survival in colon cancer.³³⁵

1.3.1.5. c-Src and breast cancer

BC is the most frequently diagnosed life-threatening malignancy in women and a leading cause of cancer-related death in this population. Numerous SFK members are expressed in BC tissues and cell lines, but Src is the most extensively studied. Extensive data implicate Src in BC progression.³³⁶

Src regulates several signalling pathways that collectively drive tumour growth. Through interactions with RTKs, especially EGFR family members, Src contributes to the control of cell proliferation. Up to ~60% of breast cancers overexpress one or more EGFR family receptors, often accompanied by elevated Src levels.^{337–339} Functional studies show that Src and EGFR act synergistically to promote neoplastic transformation of mammary epithelial cells. Pharmacologic Src inhibition can interfere with EGFR-mediated proliferation.^{340,341}

Src also participates in heregulin-driven growth and survival of BC cells, via both Her2-dependent and independent mechanisms.³⁴² Furthermore, Src contributes to Her2–Her3 heterodimer formation and augments their signalling capacity.³⁴³

In addition, Src may indirectly promote c-Met signalling by enhancing transcription of its ligand, hepatocyte growth factor (HGF).³⁴⁴ These observations reinforce the notion that Src

promotes aggressive BC phenotypes by modulating signalling via receptors associated with poor prognosis.^{345,346}

BC cell lines with high Her1 and Src expression exhibit enhanced Src homology 2 domain-containing (Shc) phosphorylation, stronger MAPK activation and greater tumorigenic potential compared with lines lacking EGFR/Src overexpression.³⁴⁷

Tumours with elevated Src activity often express the progesterone receptor, suggesting that SFKs may also support hormone-dependent growth signalling.³⁴⁸

Some studies indicate that Src can be activated in BC through PTP1B-mediated dephosphorylation of the inhibitory C-terminal tyrosine.³⁴⁹ A clinical study has evaluated **dasatinib** in combination with **trastuzumab** (anti-Her2 antibody) and paclitaxel as first-line therapy for Her2-positive advanced BC.³⁵⁰

1.3.1.6. c-Src and pancreatic cancer

Pancreatic cancer is among the most lethal solid tumours. Src overexpression and hyperactivation are observed in pancreatic carcinoma cell lines compared with normal pancreatic cells.³⁵¹ Preclinical studies suggest that Src inhibition could be an effective strategy in metastatic pancreatic cancer.^{352,353} c-Src is also subject to tyrosine nitration in pancreatic carcinoma cells, which may contribute to its activation.³⁵⁴

Rationales for combining Src inhibitors with cytotoxic drugs include the assumptions that (i) constitutively active Src is linked to chemotherapy resistance;^{355–357} (ii) inhibition of Src leads to downregulation of thymidylate synthase, the molecular target of 5-fluorouracil. Reduced thymidylate synthase expression is associated with restoration of 5-FU sensitivity and with decreased tumour growth and metastatic spread *in vivo*;³⁵⁸ (iii) **dasatinib** can block oxaliplatin-induced Src activation, thereby enhancing oxaliplatin efficacy *in vitro* and *in vivo*.³⁵⁹

Collectively, these data support Src inhibition as a promising therapeutic strategy for pancreatic cancer.³⁶⁰

1.3.1.7. c-Src and prostate cancer

Prostate cancer is one of the most frequently diagnosed malignancies in men and a major cause of cancer-related mortality worldwide. A substantial body of experimental and clinical evidence indicates that Src and other SFKs contribute to prostate carcinogenesis and, in particular, to disease progression toward castration-resistant and metastatic stages.^{361–363} Immunohistochemical and transcriptomic analyses have shown increased expression and/or

activation of Src, Fyn and Lyn in high-grade primary tumours and in metastatic lesions compared with benign prostatic tissue, and higher SFK activity has been associated with adverse pathological features and poorer outcome.^{362,363}

Mechanistically, Src integrates signals from a variety of upstream inputs that are central to prostate cancer biology. These include the androgen receptor (AR), multiple RTKs e.g., EGFR, insulin-like growth factor 1 (IGF-1R), human epidermal growth factor receptor 2 (HER2), integrins and cytokine receptors; together they form a network in which Src regulates proliferation, survival, motility, invasion and epithelial–mesenchymal transition through effectors such as FAK, STAT3, PI3K/AKT and MAPK pathways.^{364–366} Bidirectional crosstalk between Src and AR is particularly relevant in castration-resistant prostate cancer (CRPC): Src can phosphorylate and activate AR under low-androgen conditions, while AR signalling can in turn modulate Src expression and activity, providing a mechanism for bypassing androgen-deprivation therapies.^{365,366} Interactions with the bone microenvironment further amplify Src signalling, promoting osteotropic metastasis and resistance to AR-targeted agents.^{364,367}

Preclinical studies using small-molecule Src inhibitors or genetic approaches have consistently shown reduced proliferation, migration and invasion of prostate cancer cells, as well as impaired formation and growth of bone metastases.^{364,368} For example, inhibition of Src myristoylation, required for membrane localization, effectively suppresses Src kinase activity and slows prostate tumour growth *in vivo*.³⁶⁸ These data supported clinical evaluation of Src inhibitors such as **dasatinib** and **saracatinib** in men with metastatic CRPC, particularly those with bone involvement. Although early-phase trials documented biological activity, e.g., effects on bone turnover markers and PSA dynamics, randomized studies have so far shown limited impact on overall survival, highlighting the need for better patient selection and for combination strategies that co-target AR and Src-dependent pathways.^{361,369,370}

1.3.1.8. *c-Src and bladder cancer*

Bladder cancer (BCa) is a common urothelial malignancy characterized by high recurrence rates and a significant risk of progression from non-muscle-invasive to muscle-invasive disease. Tyrosine kinases, including SFKs, are increasingly recognized as key regulators of BCa initiation and progression.^{371,372} Elevated Src expression and kinase activity have been documented in several bladder carcinoma cell lines and tumour specimens, and correlate with more aggressive phenotypes in some series.^{373,374}

Functionally, Src participates in signalling downstream of RTKs, integrins and TGF β in urothelial cells, where it cooperates with FAK and adaptor proteins to control cytoskeletal dynamics, migration and invasion. Inhibition of SFKs with the classical inhibitor **PP2** reduces proliferation and invasiveness of bladder carcinoma cells *in vitro*, partly through modulation of AKT activation and plakoglobin expression.^{373,375} Likewise, pharmacological or genetic suppression of Src attenuates TGF β -induced migration and invasion of T24 and 5637 cells by downregulating FAK phosphorylation, supporting a central role for the Src–FAK axis in BCa cell motility and survival.^{375,376} The oral Src inhibitor **saracatinib** also inhibits migration and promotes more stable cell–cell contacts in NBT-II bladder cancer cells, consistent with anti-invasive activity.³⁷⁷

However, the contribution of Src to bladder cancer biology appears context dependent. In some cohorts, high levels of caveolin-1 combined with reduced Src expression and activity correlate with advanced disease and poor prognosis, suggesting that loss of Src signalling may cooperate with other alterations to drive metastasis in specific molecular subtypes.^{378,379} Large-scale multi-omics analyses nevertheless identify SRC as a potential prognostic biomarker in BCa, with increased SRC expression associated with unfavourable clinical features and shorter survival in certain datasets.³⁷⁴ Recent work also points to subtype-specific roles, where Src kinase activity more strongly promotes invasion in luminal than in basal BCa models, and pharmacologic Src inhibition yields heterogeneous effects on metastatic traits.³⁸⁰

Based on these preclinical observations, Src inhibitors such as **dasatinib** and **saracatinib** have been explored in early-phase trials or preclinical development for advanced or metastatic BCa, primarily aiming to limit invasion and metastasis rather than to shrink primary tumours.^{381,382} While clinical benefit has been modest so far, ongoing efforts to define Src-addicted molecular subsets and to combine Src inhibition with chemotherapy or immunotherapy may better clarify the therapeutic potential of targeting c-Src in bladder cancer.

1.3.1.9. c-Src and angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is controlled by a balance of pro-angiogenic and anti-angiogenic factors. Several lines of evidence implicate Src in the regulation of angiogenic processes.³⁸³

The viral oncogene v-Src induces expression of vascular endothelial growth factor (VEGF), a key mediator of vasculogenesis and angiogenesis that helps restore oxygen supply to hypoxic

tissues.³⁸⁴ VEGF induction by Src involves activation of STAT3, and c-Src is required for hypoxia-driven VEGF production in multiple cell types.³⁸⁵

SFKs are activated by pro-angiogenic growth factors such as VEGF and fibroblast growth factor (FGF). In endothelial cells, TNF-related activation-induced cytokine (TRANCE) stimulates angiogenesis via Src and phospholipase C, further underscoring the role of Src in vascular growth.³⁸⁶

1.4. *c-Src inhibitors*

TK inhibitors are commonly classified according to how and where they engage the kinase structure within the enzyme–inhibitor complex. Within the kinase domain, three conformational states are particularly amenable to small-molecule modulation: the catalytically competent DFG-in active conformation and the two inactive conformations DFG-out and α C-helix-out.³⁸⁷ Most approved agents are ATP-competitive kinase-domain inhibitors that target one of these states and are usually categorized as type I or type II inhibitors. Type I inhibitors, which represent the majority of marketed TKIs, bind to the ATP pocket when the kinase is in the active, DFG-in conformation. They closely mimic the binding mode of ATP, typically reproducing the adenine interactions and forming one to three hydrogen bonds with the hinge region, thereby engaging a conformation poised for catalysis. Structural analyses further subdivide the ATP-binding cleft into several subpockets, hydrophobic regions I and II, the adenine pocket, the ribose pocket and the phosphate-binding region (Figure 48).³⁸⁸ Among these, the deep hydrophobic region I is particularly important for potency and selectivity, because its shape and size vary across kinases and are sensitive to the activation state.²⁹³

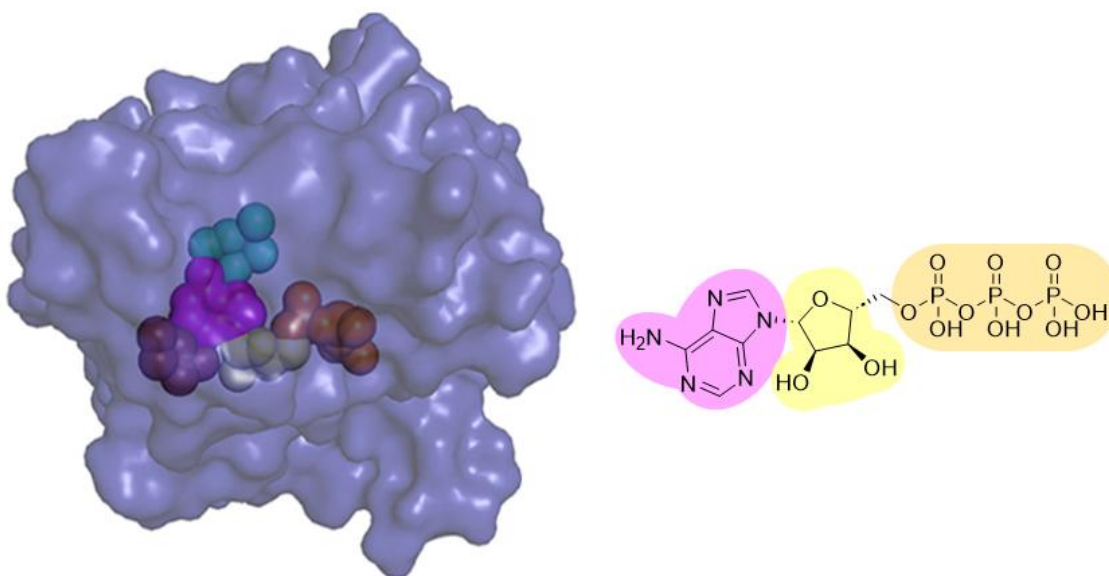


Figure 48. The kinase-domain ATP-binding pocket, shown as a surface representation, with the individual subregions highlighted by space-filling atoms (left) and color-matched on the ATP molecule (right). The binding site can be divided into an adenine region (purple), a sugar region (yellow), a phosphate region (orange), and two hydrophobic regions (I and II, green and red). Notably, hydrophobic regions I and II are not directly occupied by ATP itself.

Type II inhibitors are also ATP-competitive but preferentially recognize the kinase when it adopts an inactive DFG-out conformation. In this state, the Asp residue of the DFG motif swings away from the active site and can no longer coordinate Mg^{2+}/ATP , and the inhibitors, in addition to occupying the adenine region, extend into an adjacent hydrophobic pocket, often termed an allosteric pocket, that becomes accessible only in the DFG-out state.^{388,389} A third conformational class comprises αC -helix-out inhibitors, which stabilize another inactive state by shifting or rotating the αC -helix, thereby disrupting the conserved Lys–Glu salt bridge that is essential for catalysis.^{387,390}

A second category of TKIs comprises allosteric inhibitors, which are generally grouped as type III and type IV compounds. Type III inhibitors bind to an allosteric pocket located adjacent to the catalytic site, whereas type IV inhibitors interact with a distinct allosteric site that is remote from the active site. By stabilizing alternative conformations or altering the conformational dynamics of the protein, these molecules modulate enzymatic activity and are particularly attractive for overcoming resistance to first-generation ATP-competitive drugs.³⁹¹ Finally, covalent inhibitors, sometimes designated as type V, form an irreversible bond, most often with a nucleophilic residue such as cysteine, within the catalytic site or in neighbouring regions. This covalent attachment results in prolonged target engagement and can translate into sustained pharmacological effects.

1.4.1. Progress in Src kinase inhibitor research

Src inhibitors were initially developed primarily for hematologic malignancies but soon showed activity in solid tumours as well. Accordingly, Src is now widely regarded as an attractive pharmacological target in oncology. However, designing selective Src inhibitors has proven challenging for several reasons: (i) the catalytic domains of SFKs are highly conserved, facilitating off-target inhibition within the family; (ii) the ATP-binding site is strongly conserved across many kinase families, limiting selectivity for Src over other kinases; and (iii) Src undergoes substantial conformational transitions between active and inactive states, so inhibitors must recognize and stabilize specific conformations.³⁹² In addition to these selectivity

issues, the emergence of resistance mutations poses a major hurdle. Mutations frequently occur at gatekeeper residues in the hinge region, weakening or abolishing key inhibitor–protein contacts and necessitating the design of next-generation agents.³⁹³ Classical Src inhibitors therefore often display narrow selectivity windows and may lose clinical efficacy over time as resistance develops.

In response, recent medicinal chemistry efforts have prioritized strategies to enhance selectivity, overcome resistance (including gatekeeper mutations), and optimize drug-like properties and delivery (e.g., solubility, stability and pharmacokinetics). To date, three “generations” of small-molecule kinase inhibitors (SMKIs) can be distinguished. **Imatinib** (Figure 49), the prototype SMKI for chronic myeloid leukaemia (CML), markedly improved 5-year survival in CML patients.³⁹⁴ However, resistance driven by Abl kinase mutations prompted the development of second-generation Src/Abl inhibitors such as **dasatinib** and **bosutinib**, which retain activity against many imatinib-resistant Abl mutants but not against the gatekeeper T315I variant.^{395–397} This mutation enhances Abl oncogenicity and is strongly associated with treatment failure (Figure 49).

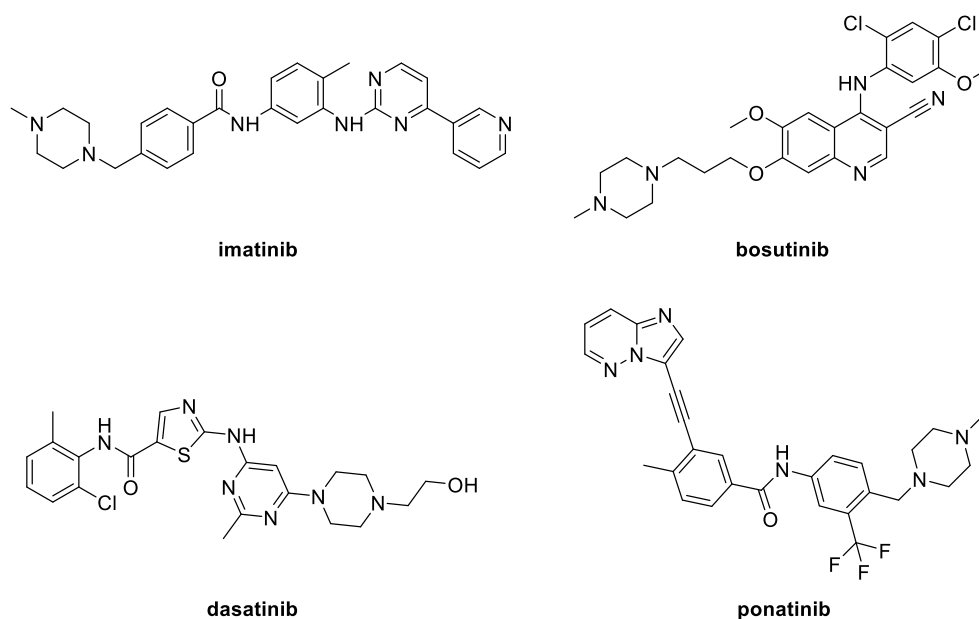


Figure 49. Chemical structures of the first, second and third generation Src/Abl kinase inhibitors *imatinib*, *bosutinib*, *dasatinib* e *ponatinib*.

Ponatinib, a third-generation Src/Abl inhibitor, was rationally designed to inhibit both wild-type Abl and the T315I mutant, while also targeting Src.³⁹⁸ Despite its potency, **ponatinib** was temporarily withdrawn in October 2013 because of serious adverse events, including arterial occlusion, heart failure and hepatotoxicity.³⁹⁹ Its use was later reinstated under restricted

indications due to the lack of alternative options, but toxicity and the emergence of resistance remain major concerns (Figure 49).^{400,401}

Tirbanibulin is the first dual Src-signalling/microtubule polymerization inhibitor approved for human use. Granted Food and Drugs Administration (FDA) approval in 2020 for the topical treatment of actinic keratosis, it is now being investigated in other dermatologic indications such as basal cell carcinoma and plaque psoriasis.^{402,403} Ongoing SAR studies aim to optimize tirbanibulin-derived scaffolds and identify more effective anticancer analogues (Figure 50).^{115,404,405}

Saracatinib, another Src/Abl inhibitor, was evaluated extensively from around 2006 to 2024 in a range of cancer indications. Its modest efficacy and relatively high incidence of adverse events prevented approval in oncology, but interest has shifted toward non-oncological uses including Alzheimer's disease, pulmonary fibrosis, chronic pain and allergy (Figure 50).^{406,407}

TPX-0022, a macrocyclic inhibitor with comparable potency against Met and Src, received FDA fast-track and orphan-drug designations for gastric cancer in 2021 (Figure 50).⁴⁰⁸

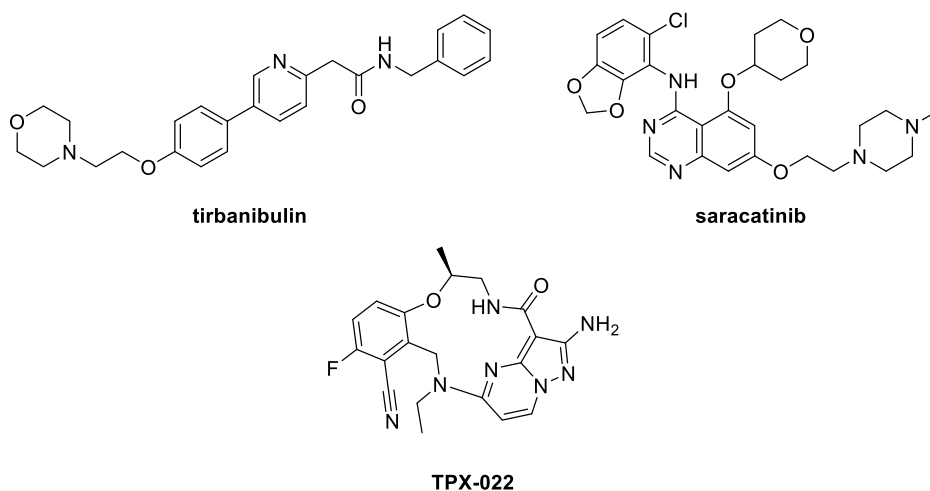


Figure 50. Chemical structures of the dual Src–signalling/microtubule inhibitor **tirbanibulin**, the Src/Abl kinase inhibitor **saracatinib** and the macrocyclic Met/Src inhibitor **TPX-0022**.

Historically, most Src inhibitors have been ATP-competitive and, as a consequence of the strong conservation of the ATP-binding site across the kinome, this approach carries an inherent risk of target promiscuity.⁴⁰⁹ While multi-target profiles can be advantageous by addressing pathway redundancy, tumour heterogeneity and resistance,^{409–411} they become problematic when they interfere with protective pathways or induce unacceptable toxicities.^{412–415} As a result, current research has diverged into two broad directions: the development of highly

selective Src inhibitors, and the rational design of dual or multi-kinase inhibitors in which Src is one of the intended targets.

Importantly, the type of inhibitor (I and II) does not automatically guarantee selectivity. Type II inhibitors often lack a preference for a particular activation state in biochemical assays and cannot be reliably classified by activity-based readouts alone.⁴¹⁶ Transforming a Type I scaffold into Type II frequently results in only marginal changes, or even loss, in selectivity. In practice, the most selective compounds tend to exploit unique structural or conformational features of the target kinase, rather than relying solely on ATP-site occupancy.³⁹⁰

Over the last decade, classical Type I ATP-competitive Src inhibitors have served as starting points for more innovative designs. For instance, covalent inhibitors that bind outside the canonical ATP site were synthesised.⁴¹⁷ Peptide and peptide-like inhibitors with high specificity for Src have been reported, and Breen et al. described the first substrate-competitive Src inhibitor with activity in both biochemical and cellular assays.^{418–420} These efforts have not only generated promising candidates but also deepened our understanding of Src structure and kinase–drug interaction mechanisms.

Recognizing that Src functions within a complex signalling matrix rather than acting in isolation, and that monotherapies targeting a single node often fail to achieve durable control, several groups have explored dual/multi-kinase inhibitors. In 2017 the first rationally designed dual EGFR/Src inhibitor was reported and later IGF-1R/Src dual inhibitors were developed through pharmacophore coupling strategies.^{372,421–424}

Throughout the last decade, numerous new chemical backbones have been introduced for Src-directed drug design, with growing emphasis on Type II and non-ATP-competitive mechanisms. In parallel, greater attention has been given to drug-like properties (solubility, stability, ADME) to facilitate clinical translation.³⁹²

1.4.2. *Phenylaminopyrimidine derivatives*

The phenylaminopyrimidine (PAP) core is another key scaffold in kinase inhibitor chemistry. **Imatinib** (Figure 49), a prototypical PAP, is a Type II inhibitor that differentially targets Abl and Src. Subsequent SAR work dissected how substitutions on the pyrimidine and phenyl rings influence kinase selectivity and potency.⁴²⁵

In a series of PAP derivatives, the terminal pyridine ring at C4 was systematically varied. The resulting SAR indicated that 3-pyridyl termini confer superior Src selectivity and that

preserving a methoxy substituent on the aryl cap is crucial for maintaining high Src inhibitory activity. Compound **113** (Figure 51), bearing a 3-pyridyl on the terminal nitrogen, achieved an EC_{50} of 1.3 μ M against Src. Docking showed that the terminal pyridine orients toward the protein surface and forms a hydrogen bond between its ring nitrogen and Glu353. Excessive bulk in this region decreased potency, probably by introducing steric clash with Glu353. A bis-3,5-(trifluoromethyl)phenyl group in the phenyl ring enhanced affinity via improved hydrophobic pocket occupation. In cellular assays, compound **113** showed notable activity in HT-29 cells (IC_{50} = 11.3 μ M) but limited effects in LS-174T colorectal cells, suggesting the need for further optimization to broaden its antitumor scope.⁴²⁶

Covalent kinase inhibitors offer several advantages, including high potency, prolonged target engagement, and the ability to counter resistance.⁴²⁷ Compound **114** (Figure 51), a PAP-based covalent inhibitor, demonstrated that targeting Cys280 in the Src P-loop is feasible, but its broad kinase reactivity limited its applicability.^{428,429}

A subsequent study addressed this limitation by designing compound **115** (Figure 51), obtained by combining structural elements of **dasatinib** (Figure 49) and compound **114**. Compound **115** is a highly potent Src inhibitor (IC_{50} = 0.003 μ M) with strong selectivity and robust antiproliferative activity in MDA-MB-231, H1975 and HCC827 cells.⁴²⁹ The key SAR insight was that optimization of the segment engaging hydrophobic pocket I had the greatest impact on selectivity, whereas warhead tuning or modifications in solvent-exposed regions were comparatively less beneficial.

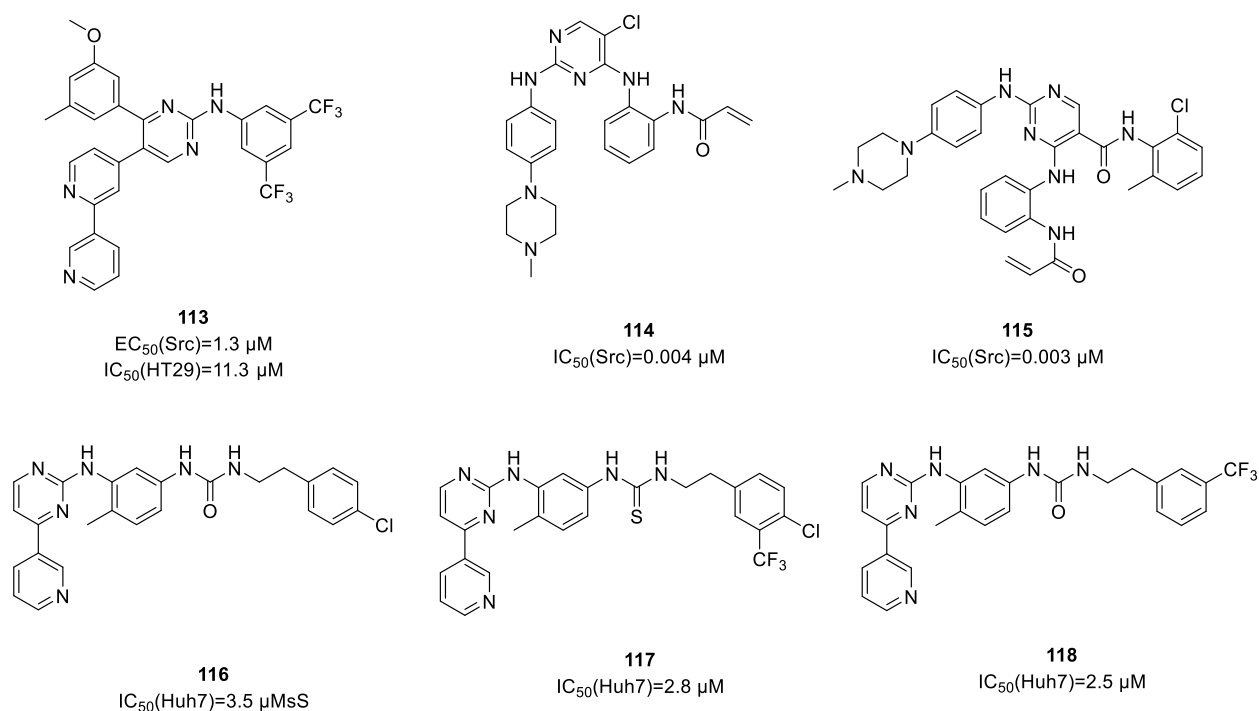


Figure 51. Chemical structures of PAP-derived Src inhibitors **113–118**. Compounds **113–116** illustrate the optimization from the initial lead **1** to highly potent analogues with submicromolar Src inhibition, while compounds **117** and **118** represent further structural modifications aimed at enhancing antiproliferative activity in Huh7 cells.

Crystallography with Src (PDB: 6E6E) confirmed covalent attachment of **115** to Cys280 via continuous electron density (Figure 51). The anilino-pyrimidine core forms two hydrogen bonds with Met341 in the hinge. A chloromethyl phenyl ring engages Ile297 and Leu396, and a methylpiperazine tail contacts Val284 and Gly347. The dual mechanism, P-loop covalent binding and occupation of the backside hydrophobic pocket, explains its potency and selectivity. Due to high sequence similarity within SFKs, off-target inhibition remains possible, and further refinement of covalent linkers is being pursued to optimize inactivation kinetics and specificity.⁴²⁹

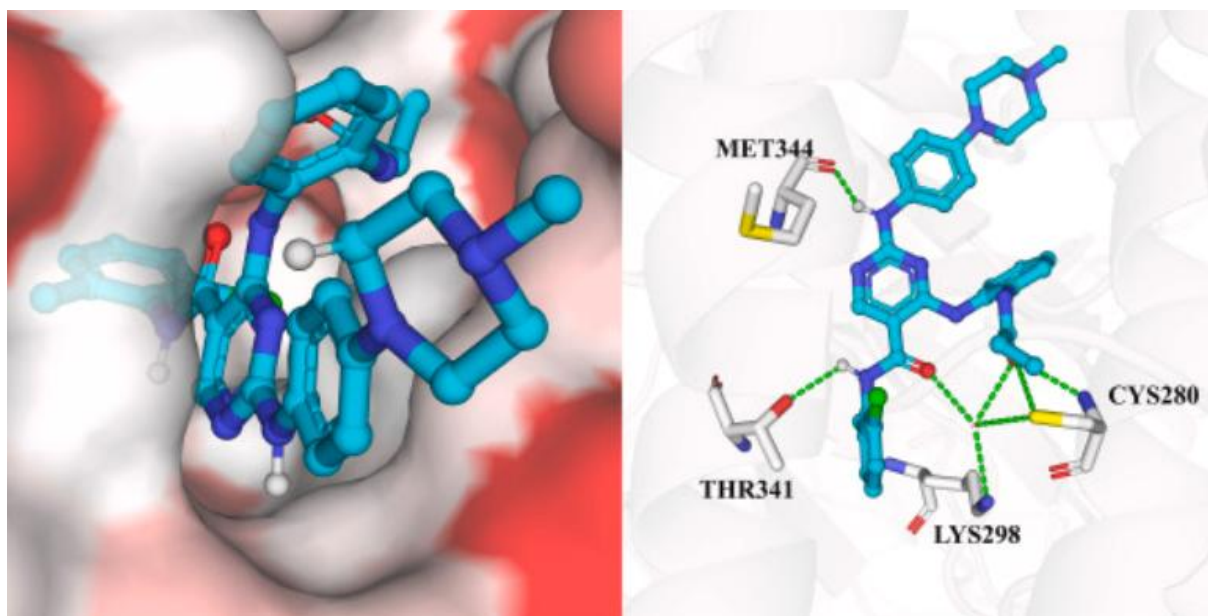
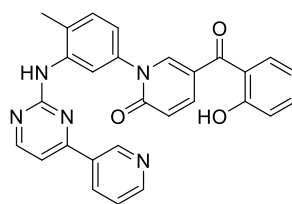


Figure 52. Schematic representation of the binding mode of compound **155** to Src (PDB: 6E6E). The lighter shades of white indicate progressively higher hydrophobic character of the hydrophobic contacts between a single Src subunit and the ligand (left). In the binding pose of **115** in the Src active site (right), hydrogen bonds are highlighted as green dashed lines. Nitrogen, oxygen, chlorine and hydrogen atoms are shown in blue, red, green and white, respectively.³⁹²

PAP chemistry was further expanded by introducing urea or thiourea substituents.⁴³⁰ SAR indicated that chloro and meta-trifluoromethyl groups on the terminal phenyl or benzoyl ring enhance antitumor activity. Docking of compounds **116–118** into vascular endothelial growth factor receptor (VEGFR) and Src suggested a binding mode closely resembling that of the PAP core in **imatinib**. In addition, the fluorine atoms present in compounds **117** and **118** create extra contacts with the binding site. Consistent with this binding model, these derivatives showed promising activity in Huh7 hepatocellular carcinoma cells.⁴³⁰

In a separate study, a hybridization strategy was used to link a pyridylpyrimidinylaminophenyl (PPAP) fragment derived from **imatinib** to various lactam or amide units, including 2-pyridone and benzopyran motifs. Compound **7** (Figure 53), a PPAP amide incorporating a pyridin-2(1*H*)-one unit, displayed balanced dual Src/Abl inhibition (IC_{50} = 2.8 and 2.9 μ M, respectively) and induced apoptosis in CCRF-CEM cells.⁴³¹



119

$IC_{50}(\text{Src})=2.8 \mu\text{M}$

$IC_{50}(\text{Abl})=2.9 \mu\text{M}$

Figure 53. Chemical structures of PPAP–lactam hybrid **119**, a pyridin-2(1H)-one-containing analogue that shows balanced dual Src/Abl inhibition ($IC_{50} \approx 2.8$ and $2.9 \mu\text{M}$, respectively).

1.4.3. Aminoimidazole and aminothiazole derivatives

Dasatinib is a nanomolar Src inhibitor and has long been used as a template to build second-generation molecules with improved selectivity or better safety profiles.⁴³²

A panel of novel kinase inhibitors incorporating the aminothiazole hinge fragment present in **dasatinib** synthesized. SAR work showed that removing, or replacing with an alkyl chain, the amide bridge between the thiazole and phenyl rings substantially reduced Src inhibition, consistent with the loss of key hydrogen bonds to Thr338.⁴³² The (2-hydroxyethyl)piperazinyl side chain was identified as optimal for improving solubility and engaging in electrostatic interactions with the binding site. Conversely, small and highly flexible side chains tended to disrupt stable binding and resulted in weaker kinase inhibition. Among these analogues, compound **120** (Figure 54) inhibited Src with an IC_{50} of $0.097 \mu\text{M}$ and targeted Yes, Lyn and Fyn ($IC_{50} = 220\text{--}433 \mu\text{M}$), displaying antiproliferative activity in SH-SY5Y and K562 cells, although overall less potent than **dasatinib** (Figure 49).⁴³²

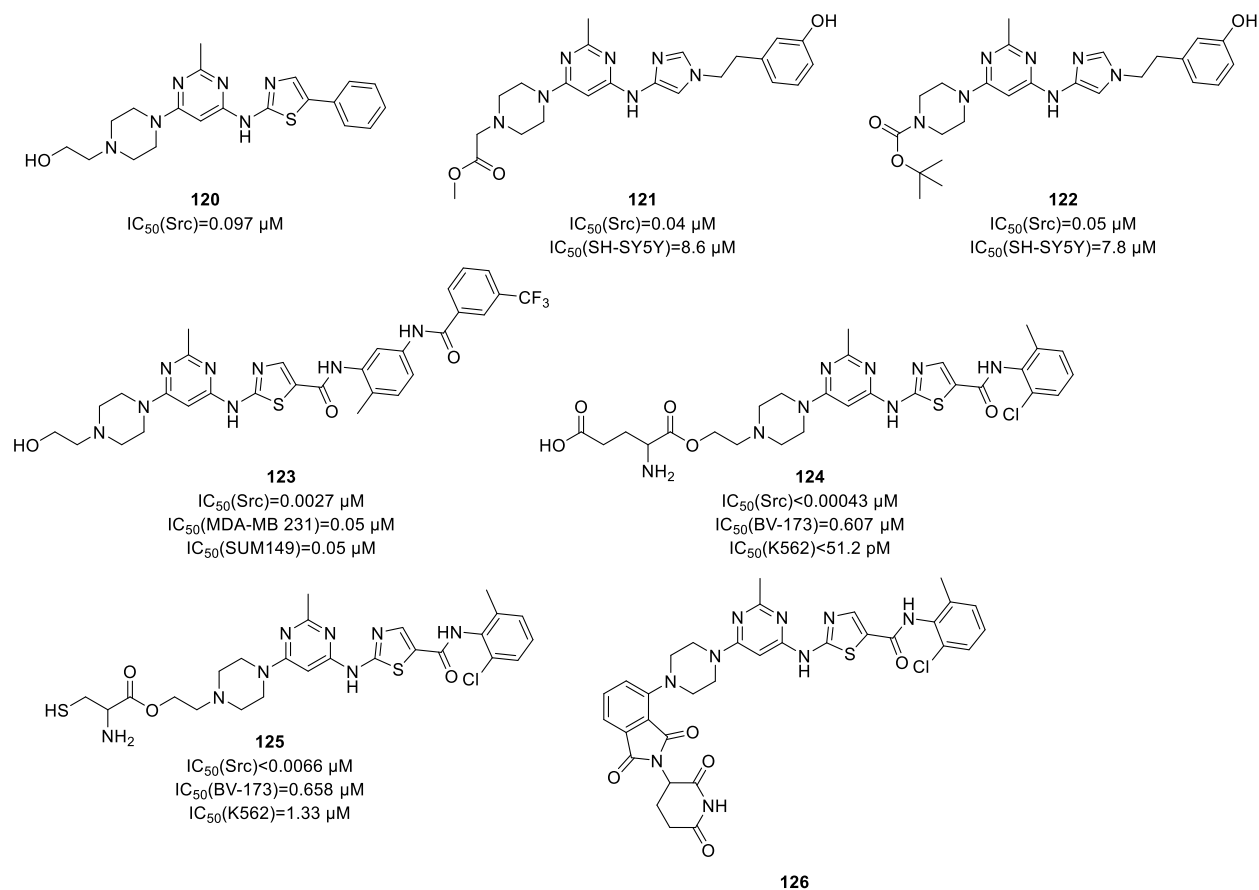


Figure 54. Chemical structures of derivatives 120–126 and their biological profiles, illustrating Src kinase inhibitory potency together with antiproliferative activities in selected tumour cell lines.

Substitution of the aminothiazole ring with a 4-aminoimidazole core created an alternative hinge-binding scaffold. Hydroxyl, amide or methyl ester groups placed in *ortho* or *meta* positions improved Src inhibition. Compounds **121** and **122** (Figure 54) exemplified this trend, with IC_{50} values of 0.04 and 0.05 μM for Src respectively, and both outperformed **dasatinib** against SH-SY5Y cells.⁴³²

Aminoimidazole and aminothiazole cores introduce nitrogen and sulfur atoms that increase overall polarity, which can favour aqueous solubility and generate new interaction patterns with the kinase surface. These features may be leveraged to fine-tune Src selectivity and pharmacokinetics.

Compound **123** (Figure 54) was obtained by introducing a meta-trifluoromethyl benzamide substituent on the distal phenyl ring of **dasatinib**. This analogue binds Src in a DFG-out conformation and behaves as a dual Src/p38 inhibitor, displaying a higher affinity for inactive Src than **dasatinib** itself.^{416,433}

The hydroxyl group of **dasatinib**, positioned outside the ATP pocket and close to the substrate docking site for Csk, was exploited as a handle for extending the binding footprint into more variable regions without perturbing ATP-site interactions.⁴³⁴ Conjugation of amino acids or fatty acids to this hydroxyl afforded derivatives with improved selectivity, with amino acid conjugates clearly outperforming their fatty acyl counterparts. Compounds **124** and **125** (Figure 54) displayed sub-nanomolar to low-nanomolar Src inhibition ($IC_{50} < 0.00043$ and $0.0066 \mu M$) and strong antiproliferative effects in BV-173 and K562 cells, while maintaining at least tenfold selectivity over Abl and Csk. This gain in specificity is likely attributable to additional contacts formed with regions outside the ATP cleft, particularly in the vicinity of the αD -helix.⁴³⁵ These findings underscore that future Src inhibitor design should not focus exclusively on the canonical ATP-binding cavity, but also explore other structural elements, such as the P-loop, substrate docking regions, and small allosteric pockets, that may confer enhanced selectivity and lower off-target toxicity.

Because kinases have important non-catalytic roles in scaffolding and signalling networks, full functional inhibition often requires more than blockade of catalytic activity alone. Genetically knocking down Src (e.g., by siRNA) has different consequences than pharmacologic inhibition, which motivates the exploration of targeted protein degradation strategies.^{436–438}

PROTACs offer a way to remove kinases altogether.⁴³⁹ Compound **126** (Figure 54), the first dual Csk/Src PROTAC degrader/inhibitor, was designed by tethering **dasatinib** to **thalidomide**. Short alkyl linkers conferred higher degradation selectivity for Src over Abl. Compound **126** induces ubiquitination and proteasomal degradation of Src and shows antiproliferative effects in Src-dependent cancer cells that resemble those achieved by Src knockdown via siRNA.⁴⁴⁰

1.4.4. Indole-based derivatives

Indole scaffolds are widely used in medicinal chemistry and have more recently been explored as Src kinase inhibitors. SAR studies have focused mainly on substitutions at positions 3 and 5 of the indole nucleus (Figure 55).

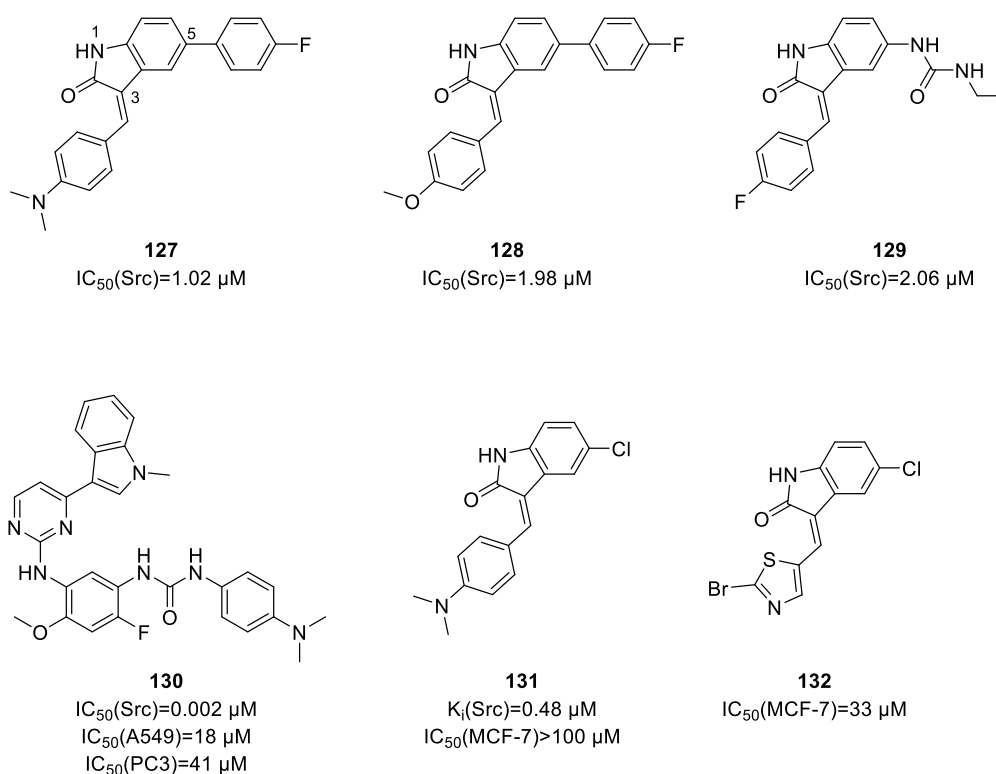


Figure 55. Chemical structures of indole-based compounds **127–132** and their biological activities.

In 2015, 1,3,5-substituted indolin-2-one derivatives were designed and identified as Src inhibitors.⁴⁴¹ Comparative evaluation of imine versus benzylidene derivatives indicated that replacing the benzylidene CH by nitrogen generally reduced Src inhibition. Derivatives bearing a *p*-fluorophenyl at position 5 of the indolin-2-one ring tended to be more potent than those with ethyl urea or benzyl thiourea substituents. Further SAR analysis revealed that electron-withdrawing cyano and nitro groups, as well as electron-donating dimethylamino substituents, on the benzylidene phenyl ring improved activity. Compound **127** (Figure 55), which carries a *p*-fluorophenyl at C5 and a *p*-dimethylaminobenzylidene substituent at C3, emerged as the most potent of the series, with an IC_{50} of 1.02 μM for Src.⁴⁴¹ Docking into Src (PDB: 3GEQ) showed that the *p*-dimethylaminobenzylidene moiety reaches into a hydrophobic pocket formed by Val281, Lys295, Ile336, Ala403 and Phe405, while simultaneously forming hydrogen bonds with Met341 (Figure 56).

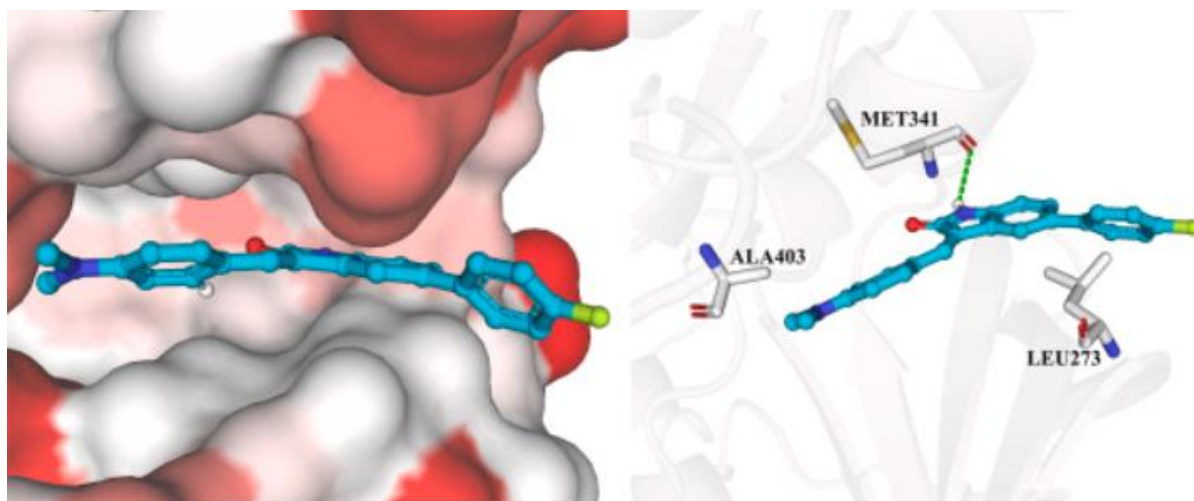


Figure 56. Binding of compound **127** to Src (PDB ID: 3GEQ). The lighter shades of white indicate progressively higher hydrophobic character of the hydrophobic contacts between a single Src subunit and the ligand (left). In the binding pose of **127** in the Src active site (right), hydrogen bonds are highlighted as green dashed lines. Nitrogen, oxygen and fluorine atoms are shown in blue, red and light green.³⁹²

Further work compared compound **127** with compounds **128** and **129** (Figure 55), which also inhibit Src ($IC_{50} = 1.98$ and $2.06 \mu M$, respectively). Their interaction patterns with Src and Abl resembled those of **dasatinib**, suggesting potential dual Src/Abl activity relevant to leukaemia. Both **128** and **129** exhibited good aqueous solubility and favourable gastrointestinal absorption profiles; notably, compound **128** was effective in both imatinib-sensitive and imatinib-resistant K562 cells. By contrast, compound **127** violated Lipinski's rules ($MlogP > 4.15$).⁴⁴² Strategies such as introducing polar substituents, applying bioisosteric replacements, or developing prodrugs could help correct its physicochemical profile and increase its oral bioavailability.

In 2023, a combination of *in silico* and experimental methods to design indole-based molecules as dual EGFR/Src inhibitors applied, aiming to generate alternatives to **osimertinib** with a broader mechanism of action.⁴⁴³ They found that urea derivatives were generally more active than analogous non-urea compounds, likely because the urea unit helps lock the molecule into conformations that fit the ATP pocket and improve overall drug-like properties. Within this series, compound **130** (Figure 55), featuring a urea substituent at the 3-position of the aniline ring, displayed potent dual inhibition of EGFR and Src ($IC_{50} = 1.026 \mu M$ and $0.002 \mu M$, respectively). Docking suggested hydrogen bonding with Met793 in EGFR and Met341 in Src, consistent with dual occupancy of their ATP sites (Figure 56).⁴⁴³ Importantly, compound **130**

was selectively cytotoxic in lung and prostate cancer cells but showed minimal effects on normal cells, making it an attractive candidate for further *in vivo* and preclinical evaluation.

In 2022, 6-chlorohydroxyindole derivatives were explored by varying aromatic groups linked to indole C3 through methylene or conjugated vinyl tethers, and *p*-substituted phenyl rings bearing cyano, nitro or dimethylamino groups provided strong enzyme inhibition, whereas acidic or hydrophobic substituents were detrimental.⁴⁴⁴ The presence of the aniline nitrogen was identified as a key requirement for activity. Compound **131** (Figure 55), with a *p*-dimethylamino group on the C3 phenyl ring, inhibited isolated Src kinase by 85 % at 10 μ M (IC_{50} = 0.71 μ M, K_i = 0.48 μ M).⁴⁴⁴ Docking suggested hydrophobic interactions between the dimethylaniline moiety and Ala403 and standard hinge interactions between the indole NH and Met341. Despite its strong enzyme inhibition, compound **131** was essentially inactive in MCF-7 cells (IC_{50} > 100 μ M), indicating poor cell penetration or inadequate intracellular distribution. By contrast, compound **132** (Figure 55), which incorporates a thiazole ring in the C3 side chain, produced only moderate Src inhibition (53 % at 10 μ M) but showed significant cytotoxicity in MCF-7 cells (IC_{50} = 33 μ M).⁴⁴⁴ The discrepancy between enzymatic and cellular data implies that **132** may act through additional targets or benefit from better cell uptake. Approaches such as conjugation to cell-penetrating peptides (CPPs), active transport exploitation, or protein–drug conjugates could further enhance the pharmacological properties of these indole-based inhibitors.

1.4.5. Quinazoline derivatives

Quinazoline frameworks are well known in kinase inhibitor design and several derivatives have been shown to interact specifically with Src.^{445,446} The emergence of gatekeeper mutations as a frequent mechanism of resistance has motivated the design of inhibitors able to retain efficacy against such variants.

4-aminoquinazoline headgroup was combined with a type III allosteric pyrazole–urea fragment to generate hybrid molecules.⁴⁴⁷ The most successful construct, compound **133** (Figure 57), links these moieties in a 1,4-*para* orientation and strongly inhibits both wild-type Src and the gatekeeper mutant Src-Thr338M (IC_{50} = 0.021 μ M and 0.034 μ M, respectively).⁴⁴⁷

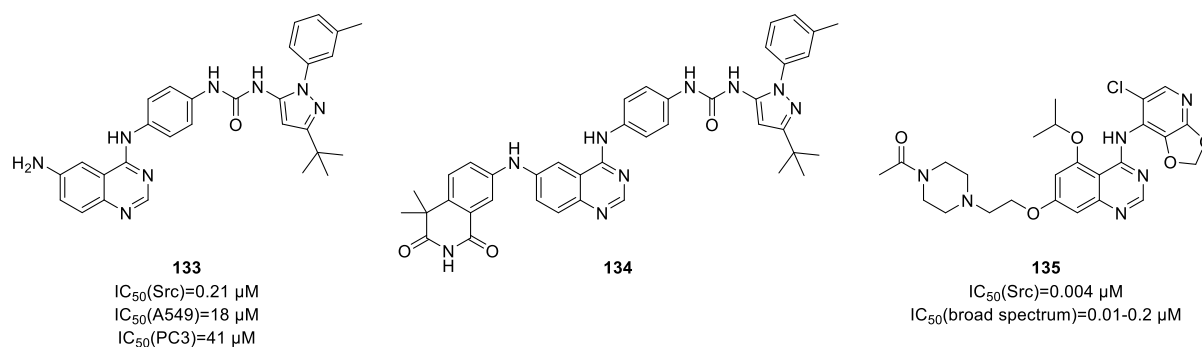


Figure 57. Chemical structures and biological activities of quinazoline derivatives **133**–**135**.

SAR analysis indicated that 1,4-linked hybrids provided the best compromise between potency and selectivity against wild-type and mutant Src, whereas 1,3-linked analogues showed weaker affinity for Src-Thr338M. Substituents at the 6-position of the quinazoline ring were particularly important for Src inhibition (Figure 58).

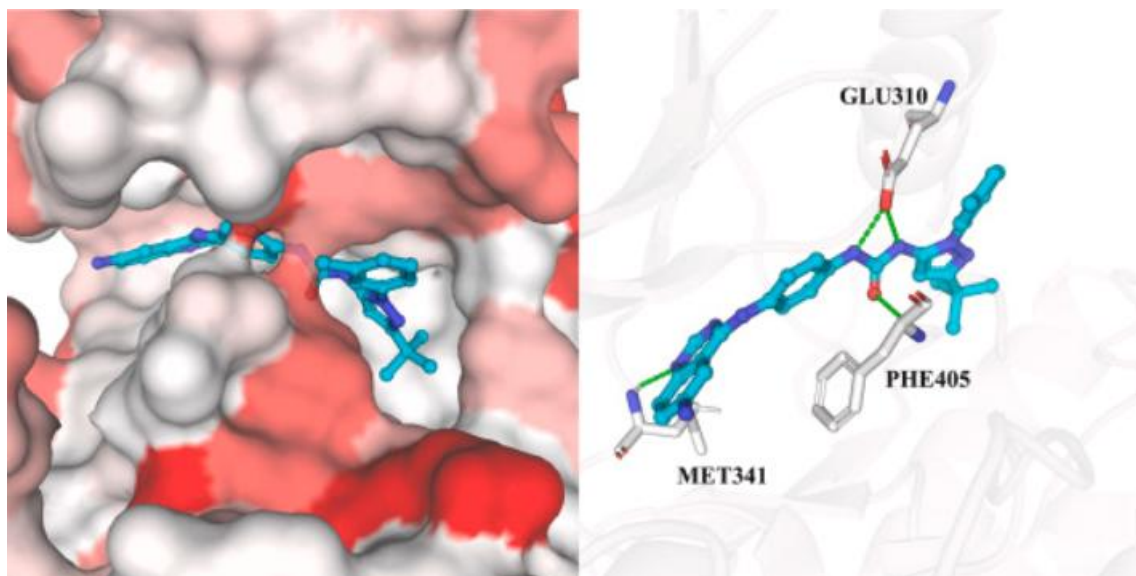


Figure 58. Binding of compound **133** to Src (PDB ID: 3F3W). The lighter shades of white indicate progressively higher hydrophobic character of the hydrophobic contacts between a single Src subunit and the ligand (left). In the binding pose of **133** in the Src active site (right), hydrogen bonds are highlighted as green dashed lines. Nitrogen and oxygen atoms are shown in blue and red.³⁹²

Crystallographic structures with wild-type and mutant Src (PDB: 3F3W) revealed that compound **133** stabilizes a DFG-out inactive conformation and behaves as a Type II inhibitor.⁴⁴⁸ The quinazoline nitrogen forms a hinge hydrogen bond, while the pyrazole–urea fragment extends into the allosteric region, generating hydrogen bonds with residues in the C-helix and at the N-terminus of the DFG motif. The central phenyl spacer connecting the two

pharmacophores provides sufficient flexibility to accommodate the altered gatekeeper residue without losing critical contacts.⁴⁴⁸

Computational optimization produced compound **134** (Figure 57), which features a 4,4-dimethylisoquinoline-1,3(2*H*,4*H*)-dione fragment in place of the original linker. Simulations suggested that LIG-1346 preserves strong hydrogen bonds to Met341, Glu310 and Asp404 while gaining new elastic hydrogen bonds to Gly274 and π - π interactions with Phe405, potentially enhancing binding affinity and residence time.⁴⁴⁹ Experimental confirmation for this redesigned ligand is still needed.

This optimization culminated in compound **135** (Figure 57), a dual Src/Abl inhibitor with low-nanomolar potency against both targets ($IC_{50} \approx 0.004 \mu M$) and good oral bioavailability.⁴⁵⁰ A Phase I trial in solid tumour patients assessed its tolerability and pharmacodynamics. Frequently observed treatment-related adverse events included nausea, fatigue, anorexia and alopecia. Signs of Src inhibition were evident at daily doses above 20 mg, and disease stabilization lasting ≥ 6 weeks occurred in $\sim 17\%$ of patients, though no objective responses were seen in monotherapy.⁴⁵¹ Compound **135** was subsequently tested in combination with the MEK inhibitor **trametinib** in KRAS-mutant colorectal cancer models. In HCT116 xenografts, **trametinib** alone increased Src Tyr419 phosphorylation, whereas **trametinib** plus **135** produced greater tumour growth inhibition than **trametinib** alone.⁴⁵² However, compound **135** failed to fully reverse trametinib-acquired resistance *in vitro*, likely due to activation of multiple compensatory pathways.⁴⁵²

1.4.6. Chromene derivatives

Chromenes and benzochromenes exhibit a broad spectrum of biological activities, including anticancer, vascular-disrupting, and antioxidant effects, thanks to their lipophilic core, which facilitates membrane permeation, and a versatile scaffold amenable to extensive functionalization.^{453–456}

8-substituted 1*H*-benzo[*f*]chromene derivatives were synthesized and evaluated as Src inhibitors and antiproliferative agents.⁴⁵⁷ Their SAR suggested that halogenation at the *para* position of the phenyl ring at C1 generally improved activity over electron-donating substituents, and that a lipophilic electron-donating methoxy group at position 8 was crucial for cytotoxicity. In the 8-methoxy series, compound **136** (Figure 59), bearing a 4-methylphenyl at position 1, showed the strongest Src inhibition ($IC_{50} = 0.009 \mu M$), whereas in the 8-bromo series, compound **137** with a 4-chlorophenyl group achieved the best cytotoxicity profile (IC_{50}

= 0.089 μM).⁴⁵⁷ Both were active against MCF-7, HCT-116 and HepG-2 cells and inhibited migration of MDA-MB-231 cells, while sparing normal fibroblasts.

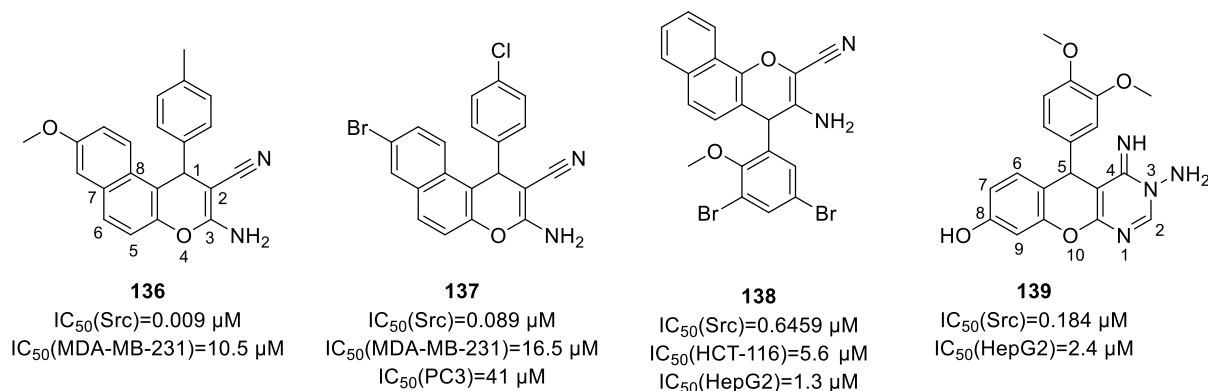


Figure 59. Chemical structures and biological activities of Src inhibitors **136–139**.

Docking into Src (PDB: 2SRC) indicated that the nitrile groups of these molecules form hydrogen bonds with the backbone NH of Met341, whereas the naphthalene moiety nestles into a hydrophobic pocket. For compound **136**, its methoxy group additionally hydrogen-bonded with Asp404 and Lys295, accounting for its superior potency (Figure 60).⁴⁵⁷

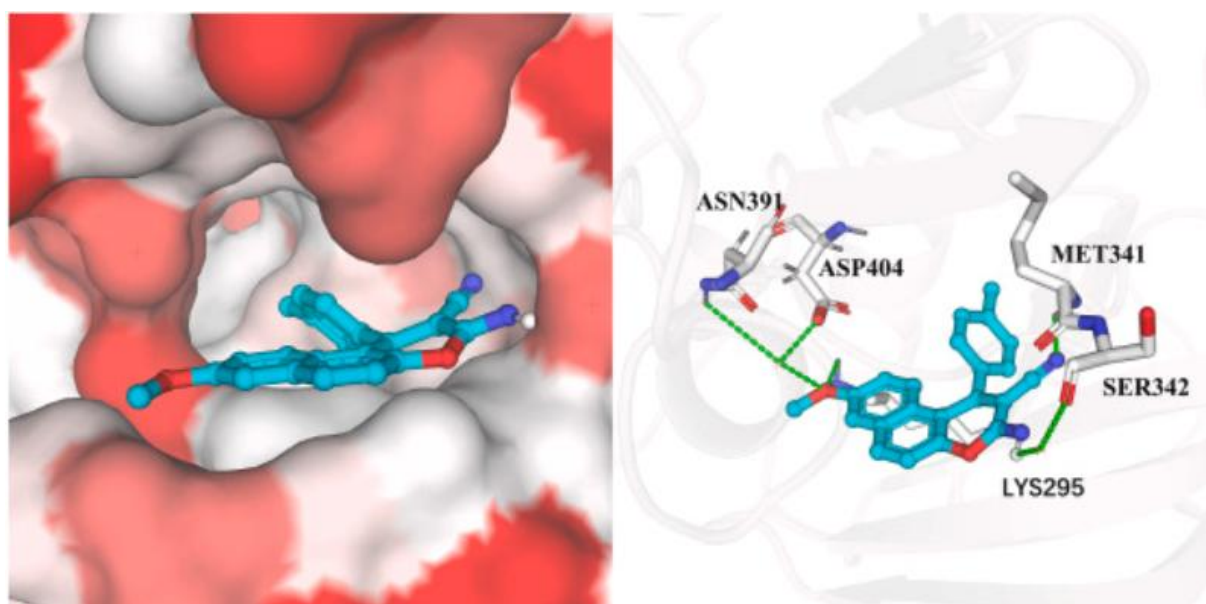


Figure 60. Binding of compound **136** to Src (PDB ID: 2SRC). The lighter shades of white indicate progressively higher hydrophobic character of the hydrophobic contacts between a single Src subunit and the ligand (left). In the binding pose of **136** in the Src active site (right), hydrogen bonds are highlighted as green dashed lines. Nitrogen, oxygen and hydrogen atoms are shown in blue, red and white.³⁹²

Work on 4*H*-benzopyran isomers confirmed the importance of phenyl substitution patterns: compound **138** (Figure 59), featuring a 3,5-dibromo-2-methoxyphenyl at position 4, showed marked Src inhibition ($IC_{50} = 0.6459 \mu M$) and broad antiproliferative activity.^{456,458}

More recently, chromene was fused with pyrimidine or triazolopyrimidine rings to obtain compound **139** (Figure 59) as a particularly potent chromenopyrimidine.⁴⁵⁵ Compound **139** inhibited Src with an IC_{50} of $0.184 \mu M$ and selectively killed HepG2 cells ($IC_{50} = 2.40 \mu M$) while displaying low toxicity in normal cells. Docking analyses suggested that **139** interacts with Met341 and Asp348 through hydrogen bonds and engages Leu273 via π -interactions.⁴⁵⁵

Future optimization of chromene-based Src inhibitors could explore varying the fusion site and orientation of additional heterocycles, increasing conjugation length, and adding π -rich groups to strengthen stacking and hydrophobic interactions with the target protein.

1.4.7. *Acridine derivatives*

Acridine motifs are well established in anticancer research and some members of this family have been reported to inhibit Src.⁴⁵⁹ The scaffold is particularly attractive for multi-target strategies that include EGFR, MEK and Src inhibition.⁴⁵⁹

Acridine compounds were initially described bearing anilinoacridine and phenylurea groups, which showed modest Src and MEK inhibition.⁴⁵⁹ The limited potency was attributed to the formation of only a single hydrogen bond with the Src hinge. Replacing acridine with an azaacridine core markedly increased activity, highlighting the importance of properly positioning a nitrogen atom to act as a hydrogen bond donor/acceptor within the active site.

SAR studies demonstrated that the nature and position of the linker, as well as the substitution pattern on the phenyl ring, critically shape the cytotoxic profile. *p*-positioned amide linkers were more effective than meta ones, and amide bonds generally outperformed urea linkers. Phenyl rings bearing fluorine substituents yielded better antiproliferative effects than unsubstituted, methylated or methoxylated analogues.

Among these, compound **140** (Figure 61) stood out as a dual EGFR/Src inhibitor with a 75.42 % inhibition rate toward Src at $1 \mu M$.⁴²³ Docking into Src revealed three hydrogen bonds involving the azaacridine NH and nitro groups with Met340, Met341 and Lys295, plus two hydrogen bonds between the amide moiety and Glu310/Asp404 in the DFG-out pocket. Three π - π stacking interactions (with Leu273, Phe405 and Tyr382) and a cation- π contact with Lys295 further stabilized binding. In EGFR (PDB: 1M17), the azaacridine core formed three

hydrogen bonds with Thr766, Gln767 and Lys721 in the hinge region, while the amide linker extended into a hydrophobic cavity, reinforcing affinity.⁴²³

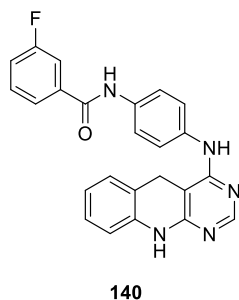


Figure 61. Structures of the acridine derivative **140**.

Compound **140** showed robust antiproliferative activity against K562 and A549 cells, reduced tumour cell invasion, and triggered apoptosis. Ongoing optimization efforts aim to refine its kinase inhibition profile and anticancer potency.⁴²³

1.4.8. Pyrrolopyrimidine derivatives

Pyrrolopyrimidines are recognized as privileged scaffolds in kinase inhibitor chemistry because they mimic the adenine portion of ATP and offer multiple points for substitution.^{460,461}

Ruxolitinib (Figure 62), a pyrrolopyrimidine approved for Janus kinases (JAKs) 1/2-driven diseases, inhibits these kinases with IC₅₀ values of 0.0033 and 0.0028 μ M, respectively.^{462,463}

Ruxolitinib is also a weak Type I Src inhibitor (IC₅₀ = 2.8 μ M). Structural inspection revealed substantial unoccupied space between the drug and the binding pocket, especially near the α C-helix, suggesting that modifications extending into this region could improve Src potency.⁴⁶³

Modifying pyrrolopyrimidines at C4 and C6 modulated EGFR activity, and several compounds displayed activity against SFKs, albeit without particular specificity for Src. Nonetheless, these data confirm the value of pyrrolopyrimidine as a platform for Src-focused optimization.⁴⁶¹

Musumeci and colleagues synthesized pyrrolo[2,3-*d*]pyrimidines as deazaisosteres of their Src/Abl-targeting pyrazolo[3,4-*d*]pyrimidines.²⁷⁶ SAR showed that the length and type of linker at C4 strongly influenced Src inhibition and that smaller substituents at N7 enhanced the activity of analogues bearing longer C4 side chains. Compound **141** (Figure 62), which carries a 4-fluorobenzyl group at N7 and a benzylamino chain at C4, emerged as the most potent derivative with a K_i of 0.5 μ M for Src and an IC₅₀ of 7.1 μ M in U87 glioblastoma cells. Docking with Src suggested cation- π interactions between Lys295 and the aromatic system as well as hydrophobic contacts within the hydrophobic region I. The smaller 4-fluorobenzyl group,

lacking the bulk of a 2-chloro-2-phenethyl side chain, likely accommodates longer C4 substituents more efficiently in the ATP cleft, allowing deeper penetration into the hydrophobic region I.²⁷⁶

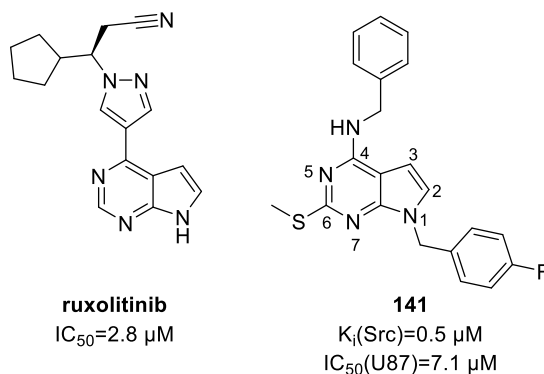


Figure 62. Chemical structures and biological activities of pyrrolopyrimidine derivatives *ruxolitinib* and **141**.

Pyrrolo[2,3-*d*]pyrimidines lack the ring nitrogen that, in pyrazolo[3,4-*d*]pyrimidines, usually interacts with the backbone of Met341. This structural difference generally makes them less potent than their pyrazolo counterparts.⁴⁶³ Nonetheless, their promising activity in glioblastoma models justifies further optimization regarding potency, selectivity and pharmacokinetic behaviour.^{464,465}

1.4.9. Other inhibitors

A variety of additional Src inhibitors have emerged through scaffold hybridization, virtual screening and large-scale docking campaigns. These include tetrafluorophenol diphenylamines, benzofuran–pyrazole hybrids, peptide-mimetic oxadiazinones, triazoles, 2-amino-3-cyanopyridines, oxadiazole/chalcone hybrids, pyrano[3,2-*c*]quinolines, pyrrolobenzodiazepine dimers, imidazole-based cores and indazole derivatives.^{420,460,466–470}

Although many of these chemotypes have been only partially characterized, they expand the accessible chemical space for Src inhibition and may help overcome resistance to current drugs by introducing new binding modes and structural motifs.

Linking a benzofuran moiety to various aromatic or heteroaromatic rings via a pyrrole-*N*-acetylpyrazoline unit led to the identification of compound **142** (Figure 63) as a dual Src/ZAP-70 inhibitor.⁴⁷¹

2-aryl-1,3,4-oxadiazin-5-ones are structurally novel tyrosine kinase inhibitors.⁴⁷² Compound **143** displayed both enzymatic and cellular Src inhibition, antiproliferative activity in several tumour lines, and compliance with Lipinski's rules and predicted ADME requirements. Docking suggested occupation of the ATP pocket of Src.⁴⁷¹

Compound **144** (Figure 63), based on a 2-amino-3-cyanopyridine core, inhibited VEGFR2 and Src with IC₅₀ values of 1.50 and 9.32 μ M, respectively, and exhibited promising antiproliferative effects.⁴⁶⁶ Systematic expansion of this scaffold series could clarify SAR trends and improve potency.

1,3,4-oxadiazole/chalcone hybrids can multi-target agents that inhibit EGFR, Src and the pro-inflammatory cytokine IL-6.⁴⁷³ Compound **145** (Figure 63) was the most potent member, with IC₅₀ values of 0.24 μ M for EGFR and 0.96 μ M for Src, and it reduced IL-6 levels to 20 % of baseline, showing particular efficacy in leukaemia models.⁴⁷²

2*H*-indazole derivatives were studied as selective kinase inhibitors. A 5-hydroxy-6-aza-2*H*-indazole motif with *m*-substitution on the phenyl linker and an amide bond to a halogenated phenyl ring were required for the activity of this family of compounds. Compound **146** (Figure 63), with 2,3-dihalo substitution, inhibited Src with an IC₅₀ of 0.498 μ M.⁴⁷⁴

Two imidazole-containing Src inhibitors were identified as Src inhibitors: **147** and **148**. Compound **147** (Figure 63), was modelled on SFK inhibitor **PP2**, with IC₅₀ values of 0.15 and 0.21 μ M against Src and Fyn respectively and promising activity in GBM cell lines; compound **148** (Figure 63), is a benzo[*d*]imidazole-based inhibitor. Docking suggested ATP-site engagement, and ADME predictions indicated that compound 49 could be suitable as a CNS drug.^{65,470}

Compound **149** (Figure 64) is a ponatinib-derived Src inhibitor with reduced cardiotoxic risk. Structural modifications include insertion of a piperidine ring into the pyrazole, increasing flexibility and steric bulk, and an ethynyl chain on the phenyl ring, altering the electronic environment and potentially decreasing binding to cardiac kinases, while retaining affinity for Src.⁴⁶⁹

In 2024, benzenesulfonamide derivatives were reported as Src/Abl inhibitors.⁴⁷⁵ Compound **150** (Figure 64) inhibited Src and Abl with IC₅₀ values of 0.25 and 0.08 μ M, respectively, and displayed antiproliferative activity in HL-60 cells. Docking indicated that its guanidine group resides in the adenine pocket, the benzenesulfonamide sits in hydrophobic region II, and the

aryl-substituted pyrimidine occupies hydrophobic region I, with overall favourable oral bioavailability predicted.⁴⁷⁵

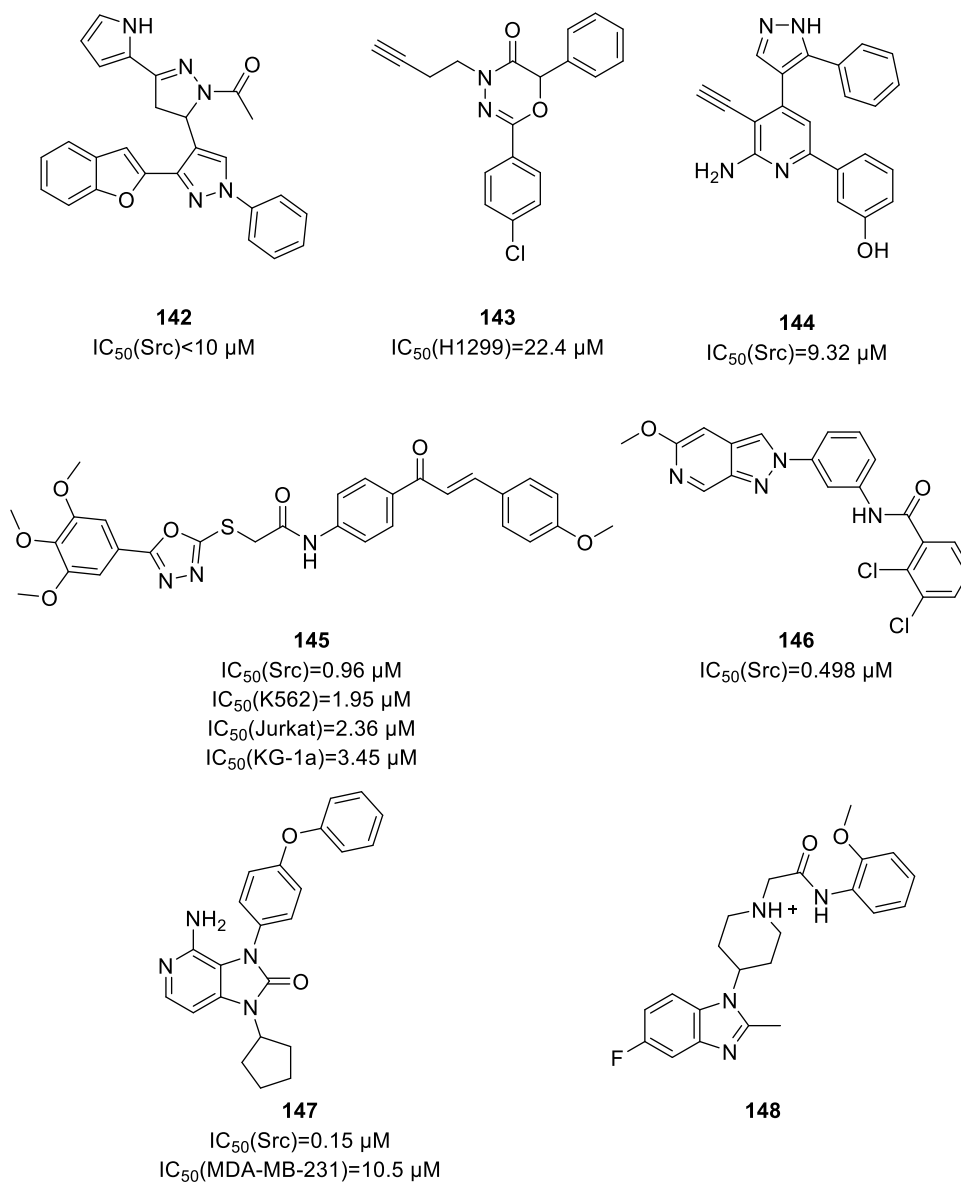


Figure 63. Chemical structures and biological activities of Src inhibitors **142**–**148**.

A distinct mechanistic approach aims to inhibit substrate-kinase interactions rather than ATP binding. These interfaces are typically large and flat, complicating small-molecule design.⁴⁷⁶ A substrate-activity screening led to identify weak non-peptide substrates and then converted them into inhibitors.⁴²⁰ Among them, compound **151** (Figure 64), featuring a tetrafluorophenol diphenylamine core, is one of the most potent and selective Src substrate-competitive inhibitors. Its biochemical K_i (16 μM) closely matched the IC_{50} for Src autophosphorylation in cells (15

μM), and among closely related SFKs, it maintained >5-fold selectivity for Src over Yes despite 95% overall sequence identity in the kinase domain.⁴⁷⁷ The same group described a broadly applicable screening platform for discovering substrate-competitive tyrosine kinase inhibitors, highlighting how this mechanism can yield exceptional selectivity.⁴²⁰

A series of pyrano[3,2-*c*]quinoline analogues was fused to three different spirocycles to identify compound **152** (Figure 64) as a non-ATP-competitive Src inhibitor ($\text{IC}_{50} = 0.87 \mu\text{M}$).⁴⁶⁸ Steady-state kinetics confirmed a non-competitive mechanism, and compound **152** inhibited migration, growth and proliferation while inducing apoptosis in MCF7 and MDA-MB-231 cells.⁴⁶⁸ Incorporation of spiro rings introduces conformational restriction, reducing the entropic cost of binding and offering a useful strategy for designing non-ATP-competitive inhibitors.

Allosteric kinase inhibitors in general offer more varied binding modes and mechanisms than orthosteric ATP-competitive agents, with potential advantages in selectivity and reduced resistance. However, identifying and validating allosteric sites remains challenging, and confirming allosteric mechanisms is often complicated by subtle conformational and dynamical changes that propagate through multi-protein networks.

Natural products are another underexploited source of Src inhibitors. Their structural diversity can provide new options in patients who are resistant or intolerant to existing drugs. **Erybraedin A (EBA)**, a natural product, suppresses Src activation in NSCLC cells by disrupting interactions between Src and integrin $\beta 1/\beta 3$ (Figure 64).⁴⁷⁸ Additional natural inhibitors of Src have been identified from plants such as *Aralia chinensis*, *Salvia castanea*, and *Aronia melanocarpa*.⁴⁷⁹

Drug repurposing has also yielded new Src inhibitors. The benzimidazole anthelmintic **oxfendazole**, that has been used for years in veterinary medicine, has recently been identified as a novel c-Src inhibitor in NSCLC cells. **Oxfendazole** suppresses c-Src activation, induces G0/G1 cell-cycle arrest with modulation of CDK4/6–Rb–E2F1 and p53/p21 pathways, and enhances the cytotoxicity of cisplatin, supporting its potential repositioning as an anticancer agent.^{480–482}

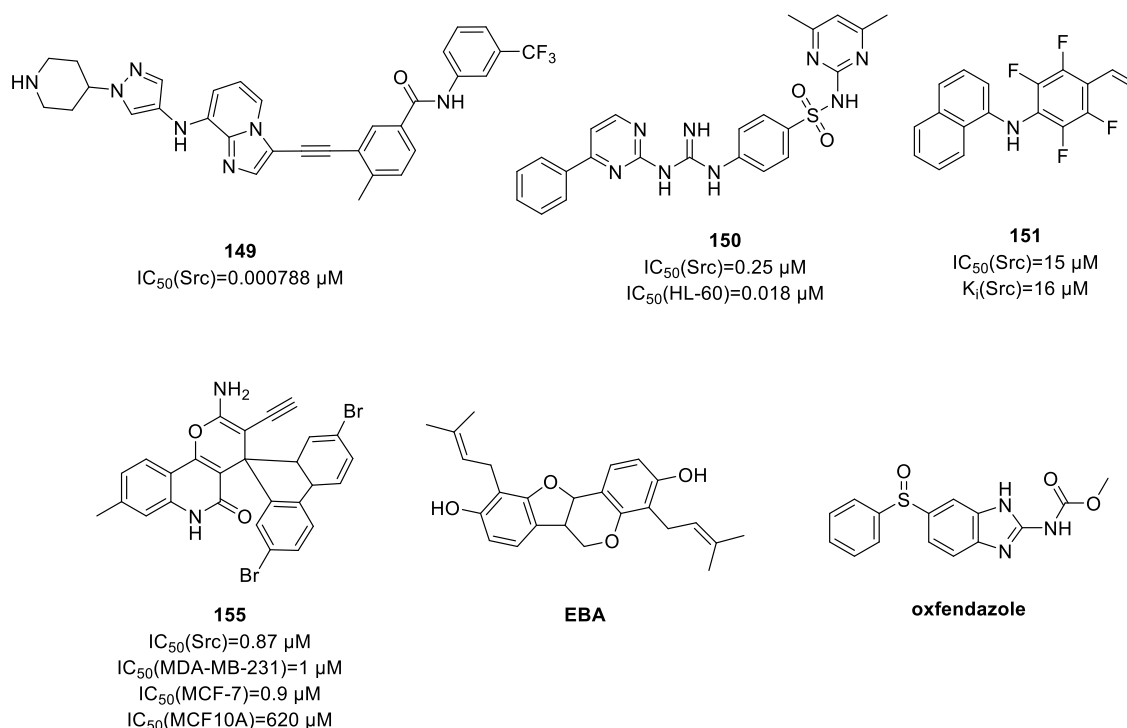


Figure 64. Chemical structures and biological activities of Src inhibitors **149–152**, **EBA** and **oxfendazole**.

1.4.10. Pyrazolopyrimidine derivatives

The pyrazolo[3,4-*d*]pyrimidine scaffold combines pyrazole and pyrimidine rings and it is structurally reminiscent of purines. This structural analogy makes it an excellent surrogate for ATP's adenine moiety and has led to its extensive use in ATP-competitive Src inhibitor development.⁴⁸³ Multiple SAR studies have explored substitutions at positions 1, 3, 4, and 6 of this core (Figure 65).

A key achievement in research for pyrazolo[3,4-*d*]pyrimidine-based Src inhibitors was the identification of compound **153** (Figure 65). Modelling studies showed that substituents at C6 point toward the solvent-exposed region of the binding site, suggesting that polar groups at this position could enhance aqueous solubility without compromising activity. Indeed, introducing a polar moiety at C6 improved water solubility (1.7 $\mu g/mL$) while preserving high metabolic stability (95 %) and strong Src inhibition ($K_i = 0.21\ \mu M$). Free energy perturbation (FEP) and molecular dynamics simulations were then used to refine binding to both DFG-in and DFG-out Src conformations.⁴⁸⁴

The systematic variation of halogen (Cl, Br, F) and hydroxyl substituents on the C4-aniline ring led to the identification of **154** (Figure 65), bearing a *m*-bromine and displaying dual Src/Abl

inhibition with K_i values of 0.13 and 0.12 μM , respectively, along with marked antiproliferative potency in NB cells and efficacy in SH-SY5Y xenografts ($\text{IC}_{50} = 0.34 \mu\text{M}$). This study underscored the functional impact of halogens as “toxicophores” in fine-tuning activity. The presence of a *m*-hydroxyl group on the C4 aniline ring was found to improve solubility and Src binding, and to favour interactions with the DFG-in conformation, notably through a hydrogen bond with Glu310.⁴⁸⁴

Subsequent optimization targeted the *o*-position of the C4 aminophenyl ring, introducing chloro or hydrogen atoms. SAR suggested that *o*-chloro substitution increased Src affinity regardless of C6 modifications, and that N1-chlorinated side chains were generally superior to methyl analogues. Compound **155** (Figure 65) with an aminochlorophenyl at C4 exhibited a K_i of 0.074 μM against Src and strong activity in SH-SY5Y cells ($\text{IC}_{50} = 3.1 \mu\text{M}$). However, parallel artificial membrane permeability assay (PAMPA) data indicated poor passive permeability, suggesting reliance on active transport.⁴⁸⁵ Although more potent than compound **154** at the enzymatic level, compound **155** did not show proportionally enhanced cellular efficacy, likely due to limited permeability.

In 2022, new pyrazolo[3,4-*d*]pyrimidine derivatives were reported targeting both Src and Abl, including the T315I mutation relevant in CML.⁴⁸⁵ Poor aqueous solubility was a recurrent problem for derivatives lacking C6 substitution or bearing thioalkyl groups. Introducing an ethanolamine at C6 markedly increased solubility while maintaining good activity and ADME profiles. Two particularly promising analogues were the 2-chloro-5-hydroxyphenylamine derivative **156** and the 3-hydroxyphenylamine derivative **157** (Figure 65), both displaying nanomolar potency in enzymatic and cellular assays and favourable ADME characteristics.

Adding a vinylbenzyl chain at N1 generated dual Src/Abl inhibitors **158** and **159** (Figure 65) capable of overcoming T315I resistance. However, compound **159** showed a three-log drop in potency from enzymatic to cell-based assays due to poor solubility. Interestingly, both compounds **153** and **154** (earlier series) and compound **159** carry polar C6 substituents, whereas compound **158** does not, suggesting that simply adding a polar group at C6 is not sufficient to guarantee improved solubility. Additional structural or formulation strategies would be required to enhance compound **159**'s solubility.

Docking with Abl showed that the chloro substituent on compound **156**'s aminobenzene ring optimally occupies a pocket involving Ala269, Val270, Lys271, Ile313, Ile314 and Thr315, a

pattern shared with compound **158**. This distinct pocket occupation explains the superior Abl inhibition of compounds **156** and **158** compared to compound **157**.⁴⁸⁶

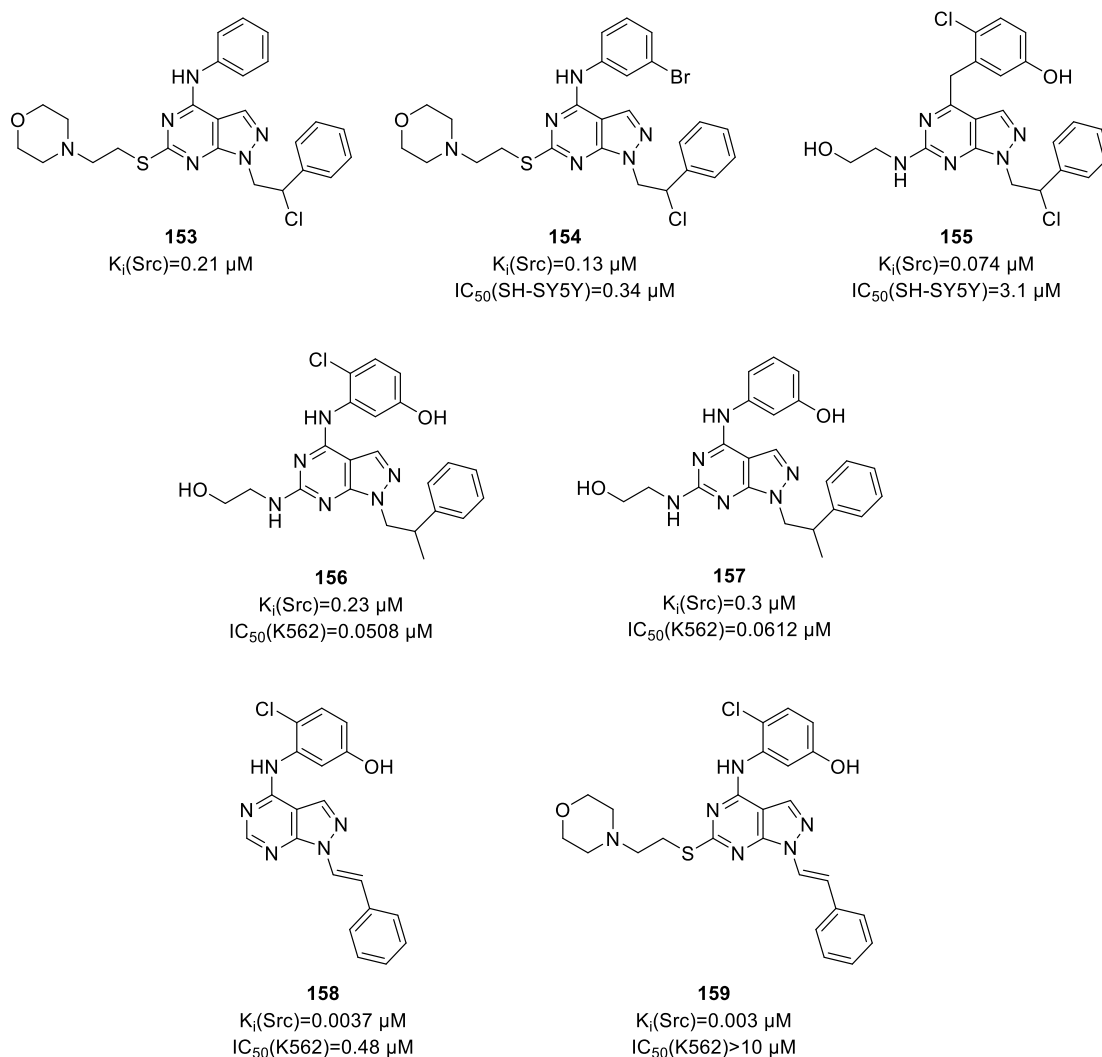


Figure 65. Chemical structures and biological activities of pyrazolo[3,4-d]pyrimidine derivatives **153**–**159**.

Earlier work attempted to address T315I resistance via modifications at C6 (including benzene ring substitution and triazole incorporation). Although these analogues displayed modest kinase and cellular potencies, they retained activity against T315I, indicating that such scaffolds are promising starting points for further optimization.⁴⁸⁷

In 2023, the University of Siena group further explored C6 substitutions with small alkyl chains (methyl, ethyl, propyl) but these changes did not yield optimal Src inhibitors, likely due to insufficient polar contacts. Likewise, a polar thioethylmorpholinyl chain at C6, predicted in silico to improve features, failed to deliver high-potency Src/Abl inhibitors. However, a specific combination of 2-chloro-5-hydroxyphenylamino at C4 and thioethylmorpholinyl at C6

produced a marked increase in Src inhibition, highlighting a finely tuned cooperative SAR that was lost upon alteration of the C4 hydroxyl group.^{485,486}

A key outcome of this work was compound **160** (Figure 66), bearing a methylthio at C6 and an *o*-chloro anilino group at C4. This analogue showed dual Src/Abl inhibition with K_i values of 0.423 and 0.45 μM , respectively, and effectively targeted Src signalling in T98G and U251 cell lines and GBMSC83 glioblastoma stem-like neurospheres. Notably, SI388 potentiated radiotherapy responses, supporting its further preclinical evaluation.⁴⁸⁸

N- or thioalkyl substitutions at C4 were subsequently evaluated. SAR indicated that the binding pocket tolerates chains of 3–5 carbon atoms (e.g., propyl, sec-butyl, cyclopentyl), whereas shortening or extending the chain diminishes activity. Among these, compound **161** (Figure 66), an N-propyl derivative, was particularly promising, inhibiting Src with an IC_{50} of 0.148 μM and showing strong cytotoxicity against triple-negative breast cancer MDA-MB-231 cells (IC_{50} = 1.13 μM). Docking with Src revealed a robust interaction network: the pyrazole nitrogen forms a hydrogen bond with the backbone NH of Met341, and the aromatic system participates in hydrophobic contacts with this residue. The propyl NH hydrogen-bonds to the carbonyl of Glu339, while the pyrimidine nitrogen interacts with Thr338, stabilizing the compound within the binding pocket.⁴⁸⁹

From a broader perspective, hinge binding remains a central strategy for designing selective kinase inhibitors. Variations in the hinge-binding motif can have large effects on both potency and selectivity. A crucial SAR finding was that 1-methylpiperidin-4-yl substituents at N1 of a pyrazolo[1,5-*a*]pyridine scaffold were associated with hERG channel inhibition and cardiotoxicity, likely due to interactions between the basic nitrogen and aromatic residues in the hERG binding site.⁴⁹⁰

To address this, the team replaced these with nitrogen-free basic groups at N1, thereby reducing hERG liabilities. Among the resulting derivatives, compound **162** (Figure 66) emerged as a standout: it showed negligible hERG inhibition, low acute toxicity in rats (oral doses up to 200 mg/kg), and potent Src and KDR inhibition (IC_{50} = 0.003 and 0.032 μM , respectively). Structural studies with Src indicated a Type II binding mode: the pyrazolo[3,4-*d*]pyrimidine core forms key hydrogen bonds with Glu339 and Met341 in the hinge, while the trans-4-hydroxycyclohexyl group interacts with Asp348. The amide linkage interacts with Glu310, and van der Waals contacts with Leu322, Val402, Val377, and Leu317 stabilize the 3-trifluoromethylphenyl group in the allosteric pocket.

Functionally, compound **162** shows strong activity in triple-negative breast cancer (TNBC) and PDAC models, both *in vitro* and *in vivo*, marking it as a compelling candidate for further development.^{491,492}

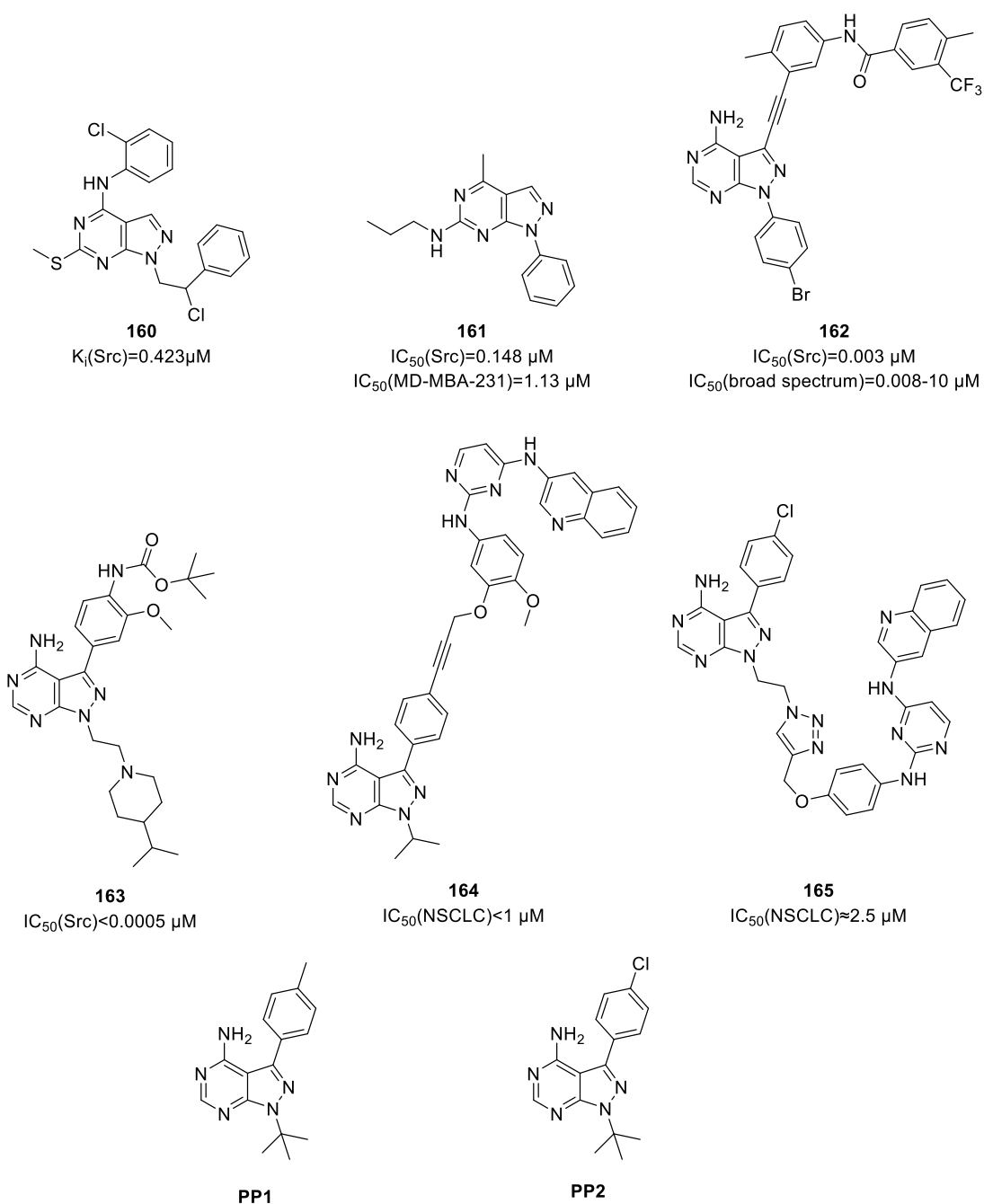


Figure 66. Chemical structures and biological activities of pyrazolo[3,4-*d*]pyrimidine derivatives **160–165** and **PP1–2**.

A different strategy focused on **PP1** and **PP2** (Figure 66), early SFK inhibitors based on a 3-phenylpyrazolo[3,4-*d*]pyrimidine scaffold.^{493,494} These molecules suffer from poor solubility and limited selectivity.⁴³¹ Using a phenotypic screening approach, a library of analogues with

discrete modifications at N1 and C3 was generated to improve physicochemical and pharmacological properties. This led to compound **163** (Figure 66), an exceptionally selective Src inhibitor with sub-nanomolar potency ($IC_{50} < 0.0005 \mu M$) and ~1000-fold selectivity over Abl. Compound **163** outperformed **dasatinib** while avoiding its prominent cardiotoxicity, possibly by sparing Abl, which plays roles in cardiac development and repair.^{495,496} Interestingly, despite having a 2-[4-(dimethylamino)-1-piperidinyl]ethyl at N1, compound **163** does not cause marked hERG-related cardiotoxicity. This may again reflect differences in overall kinase selectivity compared to **ponatinib** analogues. Docking with Src indicates that the dimethylamino group engages Asp404, whereas docking with Abl (suggests steric conflict between the N1 substituent and Tyr253, with the flexible polyamine linker further disfavoring Abl binding.⁴⁹⁶

Crystallography with full-length Src (PDB: 7N7G) later revealed that compound **163** stabilizes a native-like inactive conformation of the kinase (Figure 67). It forms three hydrogen bonds in the ATP pocket and additional interactions with Met344, Asp407, and Ser348. Moreover, it induces a global conformational change that prevents the SH2 domain from docking onto FAK, thereby inhibiting both catalytic and scaffolding roles of Src. Since inactive conformations differ more across kinases than active ones, stabilizing such states can significantly enhance selectivity.

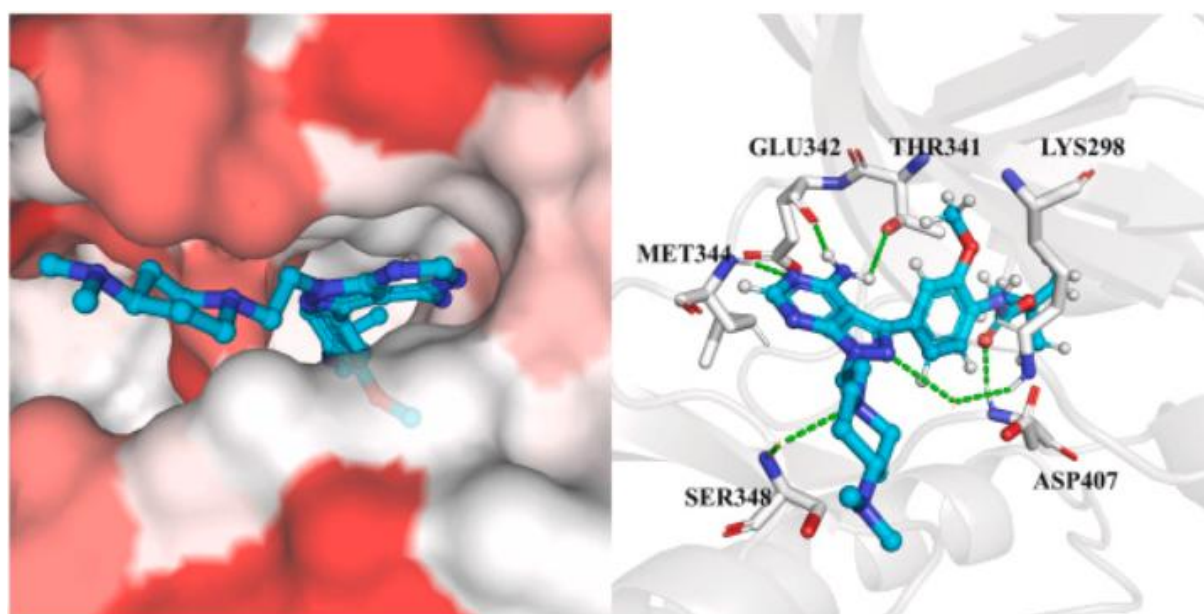


Figure 67. Binding of compound **163** to Src (PDB ID: 7N7G). The lighter shades of white indicate progressively higher hydrophobic character of the hydrophobic contacts between a single Src subunit and the ligand (left). In the binding pose of **163** in the Src active site (right),

*hydrogen bonds are highlighted as green dashed lines. Nitrogen, oxygen and hydrogen atoms are shown in blue, red and white.*³⁹²

In preclinical tumour models, compound **163** acts as a “pan-Src” inhibitor and displays excellent activity against Src-dependent BC cell lines, with good tolerability in animals.⁴⁹⁷

Pharmacophore combination has also been applied to multi-target inhibitor design. Since Src contributes to resistance against IGF-1R inhibitors,^{498–500} a 4-aminopyrazolo[3,4-*d*]pyrimidine was linked to a 2,4-bis-arylamino-1,3-pyrimidine via an acetylene spacer. SAR showed that a *p*-methoxy group on the aminophenol ring and a *m*-positioned alkyne linker on the aminophenyl ring optimize activity. The resulting dual IGF-1R/Src inhibitor compound **164** (Figure 66) showed IC₅₀<1 μM across several NSCLC cell lines and maintained inhibition of IGF-1R and Src phosphorylation for up to 5 days at 1 μM in A549, H1299 and H460 cells. In preclinical models, compound **164** displayed superior efficacy and safety compared with combining **linsitinib** and **dasatinib**.^{424,490,501}

In 2021, a related strategy produced compound **165** (Figure 66), in which a pyrazolo[3,4-*d*]pyrimidine was conjugated with a 2,4-bis-arylamino-1,3-pyrimidine via a triazole linker. Ether linkers were inactive; triazole linkers yielded potent anti-tumour activity. Steric effects near the triazole strongly influenced potency. Compound **165** exhibited triple inhibition of Src, IGF-1R and AXL with K_d values of 3.4, 5 and 1.6 μM, respectively, and broad activity across NSCLC models. Importantly, **165** outperformed combinations of individual IGF-1R/Src/AXL inhibitors with reduced toxicity.³⁷² However, its oral bioavailability remains suboptimal, prompting consideration of solubility enhancement, absorption improvement, bypassing first-pass metabolism, or alternative delivery systems.

One persistent challenge with pyrazolo[3,4-*d*]pyrimidines is poor water solubility, largely due to their rigid, planar aromatic core that favors π–π stacking. Researchers at Siena addressed this with a prodrug approach, attaching N-methylpiperazine at C4 via an O-alkyl carbamate linker.⁵⁰² These prodrugs showed much improved solubility and metabolic stability. The prodrug of compound **154**, in particular, demonstrated strong anti-neuroblastoma and anti-glioblastoma activity *in vitro* and *in vivo*, with higher plasma exposure and longer circulation times than the parent compound.^{502–505}

Other solubilization strategies include cyclodextrin complexation, liposomal delivery, and functionalized gold nanoparticles.^{315,506,507} Sanna and co-workers introduced a high-throughput screening platform using inkjet-printed polymer–drug microarrays to generate amorphous solid

dispersions, which markedly improved the apparent solubility of pyrazolo[3,4-*d*]pyrimidines.^{508,509}

1.5. *Halloysite nanotubes*

Nanomaterials have attracted increasing interest because their dimensions in the nanometre range confer chemical and physical properties that differ markedly from those of the corresponding bulk materials, with potential advantages.⁵¹⁰ Among sustainable, naturally derived nanomaterials, clay-based systems are particularly appealing due to their abundance, low cost and inherent biocompatibility.^{511–514} Halloysite, commonly used in the form of halloysite nanotubes (HNTs), is a kaolin-group aluminosilicate characterised by a hollow tubular morphology and alternating silica and alumina layers, which provide a high adsorption capacity and substantial internal volume for guest loading.⁵¹⁵ The chemically distinct inner lumen and outer surface can be addressed separately by supramolecular or covalent modification, enabling encapsulation and controlled release of a broad range of molecules.^{516–518} This review focuses on the structure, physicochemical properties, biocompatibility and biomedical applications of HNTs as natural nanocarriers for drug delivery.

1.5.1. *Structure of halloysite nanotubes*

Halloysite is a 1:1 phyllosilicate, $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4 \cdot n\text{H}_2\text{O}$, structurally related to kaolinite but organised in rolled bilayers that form submicrometric tubes.^{519–521} It is available worldwide as a relatively inexpensive nanomaterial produced on a large scale, with major deposits in New Zealand, Belgium, Brazil, France, China, Australia, Turkey and the USA. Depending on the geological environment, halloysite can exhibit tubular, spheroidal or plate-like morphologies, and trace metal ions may affect colour and purity.^{522–524} Transmission electron microscopy analyses indicate that many deposits contain predominantly tubular particles with heterogeneous size distributions.⁵²⁵

The inner and outer surfaces of HNTs display different functional groups and, consequently, different charge characteristics. The lumen is enriched in aluminolic Al–OH groups, typically positively charged, whereas the outer surface mainly exposes negatively charged siloxane Si–O–Si groups.⁵²⁶ This anisotropic charge distribution, which is pH-dependent, allows selective electrostatic interactions with oppositely charged species. Several types of Al–OH groups participate in acid–base equilibria, particularly those on the surface and at tube edges, and strongly influence the reactivity of halloysite.⁵²⁷ Typical HNTs have internal diameters of approximately 10–30 nm, external diameters of 40–70 nm, and lengths ranging from a few

hundred nanometres to several micrometres, although shorter tubes are generally preferred for biomedical applications (Figure 67).^{528,529} Halloysite occurs in hydrated “10 Å” and dehydrated “7 Å” polymorphs, depending on the presence of a monolayer of interlayer water exhibiting different degrees of mobility and hydrogen bonding.^{515,522,530,531}

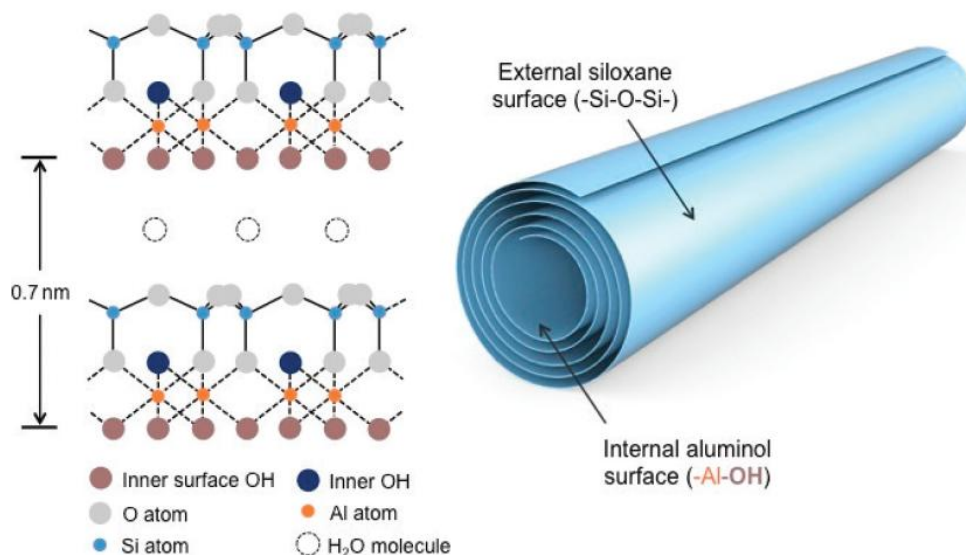


*Figure 67. Structure of halloysite nanotube.*⁵³²

From a surface-engineering perspective, three regions can be targeted: (i) the positively charged lumen, where Al–OH groups can undergo covalent functionalisation; (ii) the negatively charged outer siloxane surface, which can be modified with organosilanes or coated with cationic polymers, biopolymers or surfactants; and (iii) the interlayer space, where intercalation of small organic molecules or salts can expand the gallery spacing and provide additional loading sites.^{533,534}

1.5.2. Effects of nanomaterial on human health

Despite the advantages of nanomaterials, their size-dependent properties may lead to unforeseen biological responses.^{535–538} Nanoparticles can enter the organism via inhalation, ingestion, injection or dermal exposure and subsequently distribute to various organs via the bloodstream.^{539–541} Biodistribution is strongly influenced by particle size: particles larger than roughly 200 nm tend to accumulate in the liver and spleen, whereas very small particles (<5 nm) are rapidly cleared by renal filtration.^{542,543} Once internalised, nanomaterials may induce oxidative stress, inflammation, apoptosis or DNA damage through chemical mechanisms (e.g. generation of reactive oxygen species, membrane disruption, lipid peroxidation) and physical

effects (e.g. membrane rupture, interference with transport pathways, protein misfolding).^{544–547} Comprehensive characterisation of composition, size, solubility and surface chemistry is therefore essential to anticipate toxicity and to support the rational design of safer nanomaterials.^{548–550}

1.5.3. *Halloysite Biocompatibility*

Clay minerals have long been exploited in pharmaceutical technology as excipients, active ingredients and drug adsorbents, and their interactions with drugs can be utilised to modulate release profiles.^{551,552} For HNTs, the increasing levels of purification and surface modification mandate systematic biological evaluation. Interactions with cells involve electrostatic forces, van der Waals interactions and ion exchange, all of which affect uptake and downstream responses.⁵⁵³ *In vitro* and *in vivo* studies generally indicate that HNTs possess acceptable biocompatibility, but toxicity outcomes depend on cell type, concentration and exposure time. For example, a dose- and time-dependent decrease in viability of A549 and BEAS-2B lung epithelial cells exposed to 10–200 µg/mL HNTs was observed, with lower IC₅₀ values at longer incubation, highlighting the variability among cellular models.⁵⁵⁴

1.5.4. *Halloysite nanotubes applications in drug delivery*

Because pristine HNTs interact weakly with certain drugs, several loading strategies have been proposed, including filling of the lumen, adsorption on the external surface and intercalation between layers.^{555,556} A frequently used method disperses dry HNTs in a saturated solution of the cargo, followed by sonication or stirring and repeated vacuum application to promote penetration of the solution into the lumen and maximise loading.⁵⁵⁷ The high specific surface area (up to ~185 m²/g), large pore volume and the presence of Si–OH and Al–OH groups enable binding of both cationic and anionic species through electrostatic interactions, hydrogen bonding and van der Waals forces.^{517,558,559} Drug molecules can thus be encapsulated within the cavity or adsorbed on the external surface, while the tubular morphology also enhances mechanical properties such as tensile strength and bending resistance.^{560,561}

One major application of HNTs is in nanomedicine, particularly for the controlled delivery of anticancer agents. Unmodified HNTs, however, display limited loading capacity for highly hydrophobic drugs such as **curcumin** (CUR), **doxorubicin** (DXR) and **paclitaxel** (PTX).^{562–564} To address this limitation, a variety of surface-engineered systems has been developed. For instance, curcumin has been encapsulated in the lumen by vacuum-assisted loading and the resulting particles coated with chitosan, yielding DLHNTs-CH, which showed promising

cytotoxicity against MCF-7 BC cells.⁵⁶⁵ Paclitaxel has been incorporated into HNTs modified with distearoylphosphoethanolamine (DSPE), producing DSPE-HNTs-PTX, a formulation that significantly inhibited tumour growth in MDA-MB-231 xenograft mice.⁵⁶⁶ A hybrid HNT platform containing curcumin–gold nanoparticles and a chitosan coating (HNT-CUR-Au/CS) afforded pH-responsive release and enhanced anticancer activity under acidic, tumour-like conditions.⁵⁶⁷ Furthermore, the multifunctional construct HNT-DTX/Au-SORT, combining docetaxel-loaded HNTs, plasmonic gold nanoparticles and a targeting monoclonal antibody, exhibited high selectivity for ovarian cancer cells over normal fibroblasts.⁵⁶⁸ These examples collectively demonstrate that suitable HNT functionalisation can substantially improve loading, targeting and therapeutic performance of anticancer drugs.

Surface modification is also crucial for tuning hydrophilicity, colloidal stability and compatibility with biological media. Beyond small molecules, HNT lumens can accommodate proteins, nucleic acids and antimicrobial agents, with the tailored lumen chemistry enabling control of binding affinity and release kinetics.^{558,569,570} Release may occur by diffusion from the lumen or desorption from the outer surface and can be further modulated through adjustment of tube diameter or by adding “stoppers” at the tube ends.⁵⁷¹ Incorporation of stimuli-responsive polymers, including thermosensitive chains, provides an additional level of control over the release profile.⁵⁷²

The applicability of HNTs extends beyond oncology. In cartilage repair, halloysite was employed to deliver **kartogenin** (KGN) in a laponite-based hydrogel (HNT/KGN/Lap), affording sustained drug release and good cytocompatibility in HepG2 cells, with slower liberation in synovial fluid than in buffer, a desirable feature for intra-articular therapy.⁵⁷³ Functionalisation with (3-aminopropyl)triethoxysilane (APTES) has been used to enhance the loading and sustained release of ibuprofen via electrostatic attraction between aminopropyl groups and the carboxylate of the drug.⁵⁷⁴ In other systems, external modification with triazolium salts improved curcumin encapsulation and yielded HNT hybrids exhibiting strong cytotoxic activity against several tumour cell lines.⁵⁷⁵ Ciprofloxacin has likewise been efficiently immobilised on APTES-modified HNTs, with significantly higher adsorption than on pristine tubes.⁵⁷⁶

Multifunctional HNT-based constructs have been explored for antibacterial and regenerative therapies. A multilayer polylactic acid (PLA)-HNT porous membrane loaded with **gentamicin** exhibited pronounced activity against both Gram-positive and Gram-negative bacteria, supporting its use as an antibacterial barrier in bone regeneration.⁵⁷⁷ Clay-based composites

containing HNTs and magnetic nanoparticles were proposed for pH-responsive pulmonary delivery of dexamethasone in the context of COVID-19 therapy, while an injectable GelMA hydrogel incorporating DEX-loaded HNTs showed suitable mechanical properties, biodegradability, cytocompatibility with mesenchymal stem cells and *in vivo* bone regeneration in animal models.^{578,579}

HNTs have also been used as carriers for phototherapeutic agents. Systems in which the lumen is loaded with the photosensitiser indocyanine green and the surface is decorated with poly(sodium *p*-styrenesulfonate) or red blood cell membranes, sometimes combined with targeting ligands and fluorescent probes, have demonstrated efficient photothermal or photodynamic ablation of breast tumours in preclinical models.^{580,581} In addition, the tubular shape, mechanical robustness and interfacial activity of HNTs make them suitable solid stabilisers for Pickering emulsions, expanding their potential in advanced formulations and delivery platforms.⁵⁸²

Taken together, halloysite nanotubes emerge as versatile, naturally occurring nanocontainers with tunable surface chemistry, generally favourable biocompatibility and broad applicability in drug delivery and other biomedical technologies. Ongoing research on their functionalisation, safety assessment and integration into complex hybrid architectures is expected to consolidate their role as key building blocks for next-generation nanomedicines.

2. Rationale and aims of the project

Prostate cancer is the second most frequently diagnosed malignancy in men worldwide, whereas bladder cancer ranks among the ten most common cancers and shows a higher incidence in men than in women. Despite the availability of surgery, radiotherapy and systemic treatments, many patients eventually develop recurrent or metastatic disease, underscoring the need for more effective and better-tolerated targeted therapies.^{302,372}

The proto-oncogene c-Src is a non-receptor tyrosine kinase whose aberrant expression and activation are critically involved in tumour growth, invasion, epithelial–mesenchymal transition, and resistance to therapy in several human cancers, including prostate and bladder carcinoma. In this context, small-molecule kinase inhibitors based on heterocyclic scaffolds have gained considerable attention, as they can selectively interfere with oncogenic signalling pathways that drive proliferation, survival, and metastatic dissemination. Among these, pyrazolo[3,4-*d*]pyrimidine derivatives can act as adenine bioisosteres and effectively mimic ATP within the catalytic cleft of Src family kinases, thereby blocking downstream

signalling.^{275,276,484} Clinical approval of pyrazolopyrimidine-based kinase inhibitors and the advancement of other analogues into clinical trials highlight the translational potential of this chemotype. In particular, as previously reported, derivative **154** has been identified as a potent and selective c-Src inhibitor ($K_i = 0.13 \mu\text{M}$), showing relevant antiproliferative and antimigratory effects on diverse tumour cell lines while sparing non-transformed cells. However, **154** and related pyrazolo[3,4-*d*]pyrimidines display extremely low aqueous solubility (for **154**, $3.7 \mu\text{g L}^{-1}$), which severely limits their *in vivo* bioavailability and has prompted the exploration of suitable carrier systems to enable clinical translation.²⁷⁶

Halloysite is a naturally occurring aluminosilicate clay that has attracted growing interest as a drug delivery platform. Owing to its tubular morphology, high aspect ratio and the presence of an empty internal lumen, halloysite nanotubes (HNTs) can encapsulate significant amounts of active molecules, particularly poorly soluble drugs (Figure 68).^{573,573} The material combines several advantageous features: biocompatibility, high purity, chemical and mechanical stability, and low cost, which make it suitable for large-scale pharmaceutical applications. In addition, the different chemistry of the inner lumen and outer surface enables orthogonal functionalisation, allowing both supramolecular loading and covalent grafting of molecules, as demonstrated in previous studies from Prof. Riela's group from the University of Catania on photoluminescent hybrids and halloysite–curcumin conjugates.^{560,583}

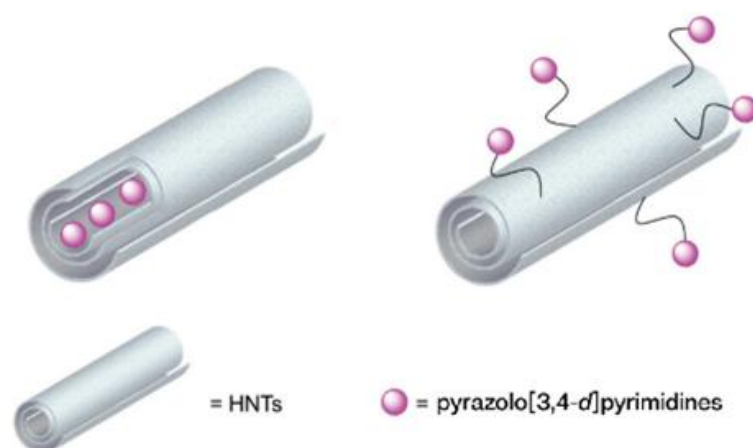


Figure 68. Cartoon representation of HNT-based pyrazolo[3,4-*d*]pyrimidine nanomaterials.⁵⁸³

Building on this background, the design and development of halloysite/pyrazolo[3,4-*d*]pyrimidine nanomaterials as potential therapeutics for prostate and bladder cancer were recently carried out. In this work, **154** and its closely related analogue **166**, already known as Fyn inhibitor (Figure 69), were loaded into the HNT lumen. Upon supramolecular loading, three main outcomes can be distinguished: (i) efficient incorporation and proper localisation of the

drugs were achieved, with a loading degrees of approximately 4 wt% and preserving the hydrodynamic size and tubular morphology; (ii) the loaded systems exhibited sustained, diffusion-controlled release in physiological media; (iii) the antiproliferative activity was retained: in RT112, UMUC3 and PC3 cancer cell lines, **HNT/154** and **HNT/166** displayed IC₅₀ values in the low micromolar range, comparable to or in some cases slightly improved compared to the corresponding free compounds. At the same time, halloysite loading markedly enhanced the apparent aqueous dispersibility of these poorly soluble inhibitors and reduced the reliance on organic co-solvents, without compromising their biological efficacy. The nanomaterials revealed promising antiproliferative profiles and confirming the suitability of HNTs as carriers for pyrazolo[3,4-*d*]pyrimidines.⁵⁸³

Within this framework, this project, performed in collaboration with Prof. Riela of the University of Catania, aims to develop a new library of halloysite-based nanomedicines for the targeted delivery of pyrazolo[3,4-*d*]pyrimidine kinase inhibitors to prostate and bladder cancer. Building on the preliminary data obtained for **154** and **166** (Figure 69), the work focused on (i) the rational design and synthesis of additional allyl-functionalised pyrazolo[3,4-*d*]pyrimidines to optimise potency, selectivity and physicochemical properties; (ii) the fine-tuning of HNT surface chemistry to control drug loading, spatial distribution and release kinetics and (iii) the comprehensive biological evaluation of the resulting nanomaterials in prostate and bladder cancer models, with particular attention to Src pathway inhibition, effects on cell proliferation, migration and invasion, and potential off-target toxicity.

In particular, **154** and **166** were ad hoc modified with allyl groups to generate a library of derivatives (**167-176**) bearing functional handles suitable for the coupling to HNTs.⁵⁸³ These molecules were then incorporated into halloysite by covalent grafting onto the external surface of pre-functionalised HNT-SH.

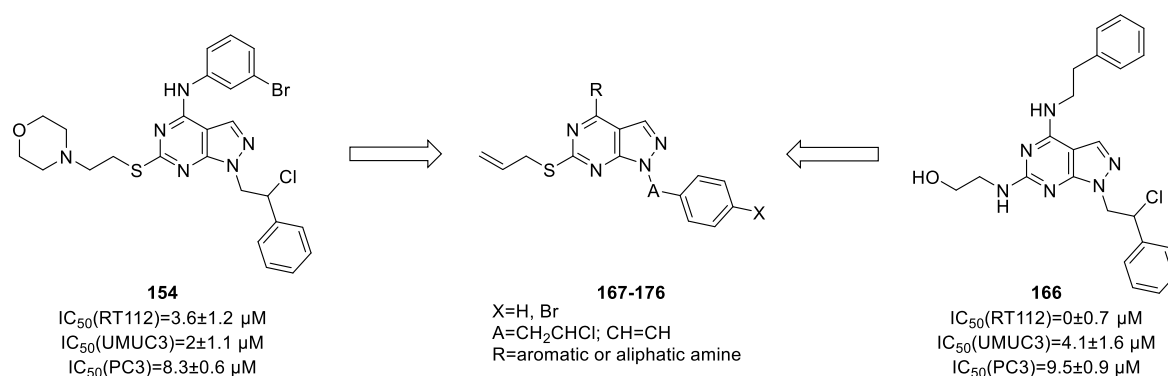


Figure 69. Rationale, general structure and biological activity of the synthesised pyrazolo[3,4-*d*]pyrimidine analogues.

While **154** and **166** themselves were loaded into the halloysite lumen, as they correspond to the original non-functionalised Src-family kinase inhibitors, their allylated analogues **167–176** were specifically designed to introduce a terminal double bond compatible with thiol–ene chemistry and were therefore employed for covalent grafting onto the outer HNT surface. This approach enabled the preparation of stable HNT–drug conjugates and allowed a direct comparison between supramolecular encapsulation and covalent anchoring in terms of physicochemical properties and biological performance.

All experiments on halloysite functionalisation, physicochemical characterisation and biological evaluation of the nanoconjugates were carried out in collaboration with, and experimentally performed by, the research group of Prof. Riela at the University of Catania. At present, compounds **167–170** are the only members of the allyl-functionalised pyrazolo[3,4-*d*]pyrimidine series that have been fully characterised within this collaborative framework.

The presented results provide a proof-of-concept for halloysite-based delivery of Src inhibitors. Nevertheless, several key questions remain unanswered: only a limited set of pyrazolo[3,4-*d*]pyrimidine analogues has been investigated so far, and a systematic exploration of the SAR governing both kinase inhibition and nanocarrier loading/release is still lacking. Moreover, the influence of different parameters e.g. loading modalities (lumen encapsulation versus covalent grafting), surface densities, stability, and biological performance of HNT formulations has not been fully investigated. In addition, most of the available data derive from 2D monolayer cultures and short-term viability assays: deeper mechanistic studies on Src signalling modulation, cell migration and invasion, as well as validation in 3D models and *in vivo*, are required to properly assess the therapeutic potential of these systems.

Overall, this project is conceived to bridge medicinal chemistry and nanotechnology in order to translate potent but poorly soluble pyrazolo[3,4-*d*]pyrimidine kinase inhibitors into potential candidates for urogenital cancers. By integrating ligand design, nanocarrier engineering and biological studies, the proposed research is expected to generate a coherent body of knowledge on halloysite-based drug delivery systems and to provide novel nanotherapeutic prototypes suitable for further preclinical development.

3. Results and discussion

3.1. Chemistry

The design of the new pyrazolo[3,4-*d*]pyrimidine series **167–176** was driven by two parallel objectives: (i) to preserve the structural features of the lead Src/Fyn inhibitors **154** and **166** that are critical for kinase recognition, and (ii) to introduce a terminal alkene suitable for the coupling to HNT, thereby enabling stable covalent anchoring of the drug to the halloysite surface. The N1 and C4 vectors, crucial for kinase recognition, were therefore preserved as far as possible, whereas the C6 position was modified to bear an allylthio handle, providing the chemical functionality required for covalent grafting onto halloysite without drastically altering the binding pharmacophore (Figure 69). The synthesis of the designed compounds **167–176** was performed through a multi-step approach (Scheme 16) and the exact nature of the N1 and C4 substituents for each individual analogue is reported in Table 4.

3.1.1. Synthesis of allyl-functionalized pyrazolo[3,4-*d*]pyrimidines **167–176**

From a synthetic standpoint, the route to **167–176** closely parallels the one already developed in Chapter 1 for compounds **60**, **65–73**, **77**, **78** e **89** (Schemes 1 and 16). To obtain the desired derivatives, differently substituted phenylalkyl hydrazines (intermediates **94d,f**), selected to reproduce the N1 side chains of **154** and **166**, were first condensed with ethyl(ethoxymethylene)cyanoacetate in refluxing toluene, giving the 5-amino-1-substituted pyrazole-4-carboxamides (intermediates **95d,f**) in good yield. The latter ones were then reacted with benzoyl isothiocyanate in THF under reflux to afford the corresponding benzoylthioureas **96d,f**. Subsequent treatment with 2 M NaOH at 100 °C promoted intramolecular cyclisation, with nucleophilic attack of the pyrazole amino group on the thiocarbonyl centre and concomitant loss of benzoate, furnishing the 6-thioxo-1,5,6,7-tetrahydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-ones (**97d,f**), bearing the required N1 substitution (Scheme 16).

In analogy with the S-alkylation chemistry used in Scheme 1, the thioxo group at C6 was selectively alkylated with allyl bromide in acetone in the presence of K₂CO₃ and a catalytic amount of pyridine, affording the 6-allylthio derivatives **98i** and **177**. The allyl fragment is compact and lipophilic, and its terminal C=C bond provides the reactive handle required for the coupling to HNTs.

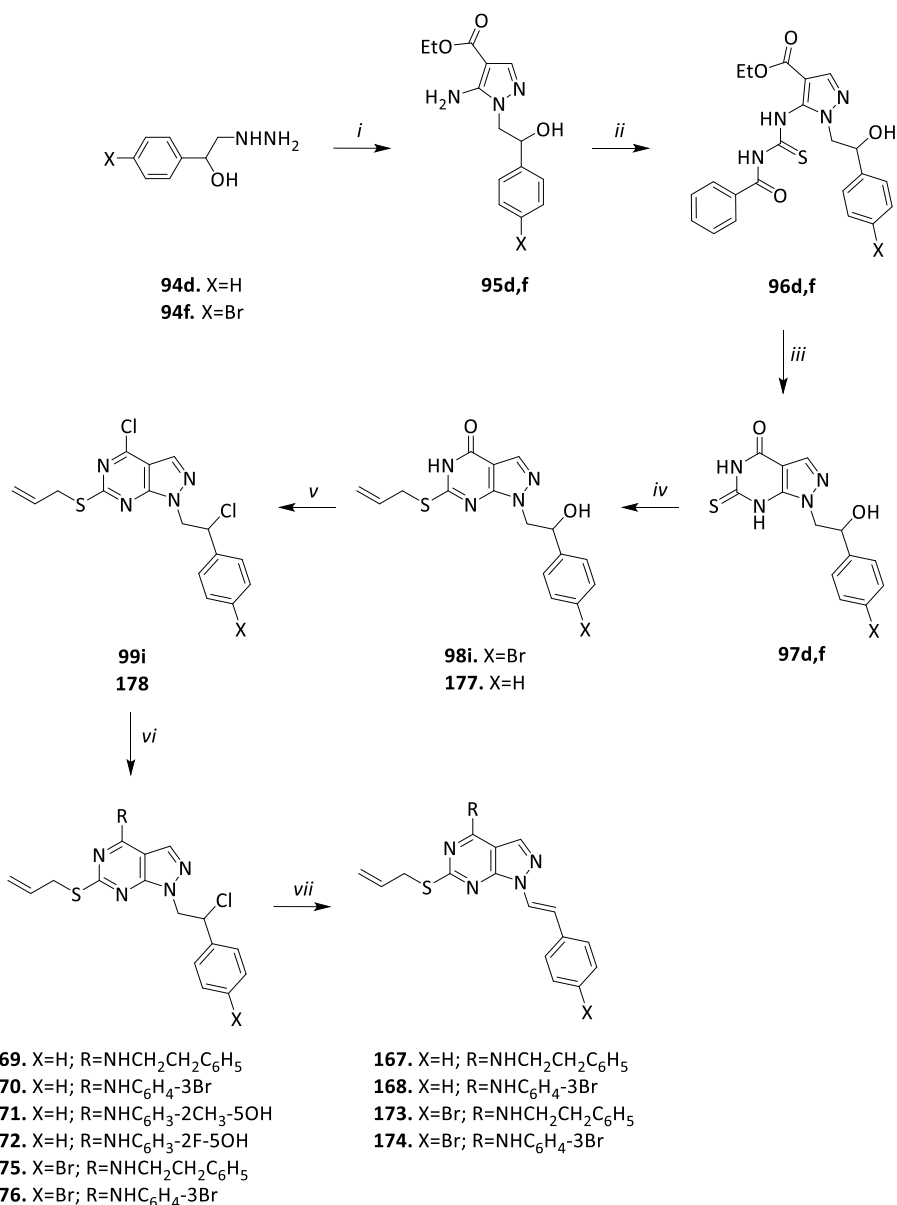
Conversion of the 4-oxo function into a good leaving group was then achieved via a Vilsmeier–Haack chlorodehydration using the Vilsmeier reagent in CHCl₃, as previously optimised for the SIRT2-directed library. This step replaces the 4-oxo group with chlorine, activating C4 towards nucleophilic aromatic substitution (intermediates **99i** and **178**).

Further substitution of the C4 chlorine atom was carried out by S_NAr under two sets of conditions, again mirroring the strategy used in Scheme 1. Reaction with an excess of phenethylamine in dry toluene at room temperature (Method A) furnished the 4-(phenethylamino) analogues (compounds **169** and **175**), which reconstruct the C4 pharmacophore of **166**. Alternatively, coupling with appropriately substituted anilines in refluxing ethanol (Method B) afforded 4-anilino derivatives designed to mimic the C4 environment of **154** and to probe the effect of subtle electronic and steric changes in this region.

For those members of the series carrying a 2-chloro-2-phenylethyl fragment at N1, a final base-induced β-elimination with 4 M NaOH in refluxing ethanol converted the saturated side chain into a styryl (2-phenylvinyl) unit (compounds **156**, **168**, **175** and **176**).

The final library **167–176** comprises both phenethyl and styryl derivatives, directly comparable to the reference inhibitors **154** and **166** and suitable for systematic SAR evaluation once grafted onto HNTs.

All target compounds **167–176** were obtained in good overall yields after chromatographic purification and recrystallisation, and their structures were confirmed by ¹H and ¹³C NMR spectroscopy and elemental analysis.

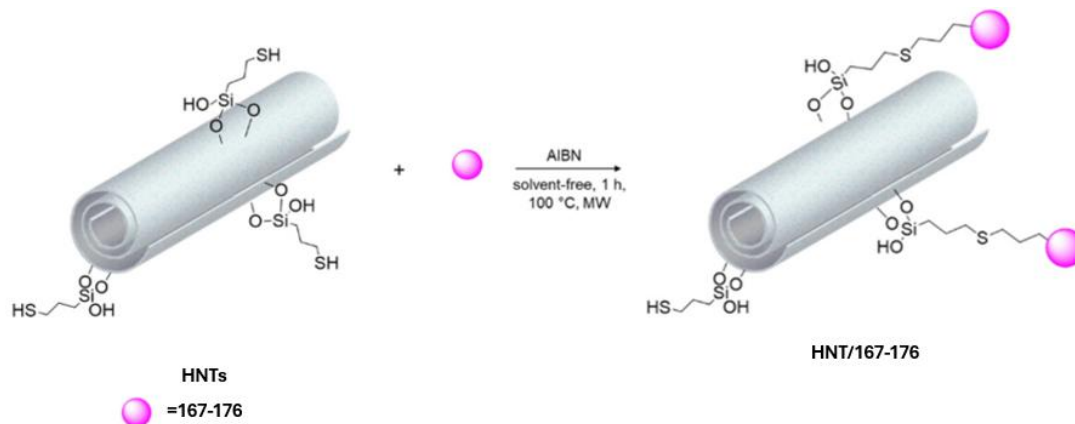


Scheme 16. Synthesis of compounds **167–176**. Reaction conditions: (i) ethyl(ethoxymethylene)cyanoacetate, toluene, 100°C, 8h; (ii) benzoyl isothiocyanate, dry THF, 66°C, 8h; (iii) 2 M NaOH, 100°C, 1h; (iv) allyl bromide, pyridine, K₂CO₃, acetone, rt, 2h; (v) POCl₃/DMF, CHCl₃, 60°C, 12h; (vi) Method A: phenethylamine, dry toluene, rt, 24–48h (to obtain compound **169** and **175**); Method B: the proper aniline, EtOH, 95°C, 8h (to obtain compounds **170–172** and **176**); (vii) 4 M NaOH, EtOH, 95°C, 4h.

3.1.2. Grafting of compounds **167–176** onto HNTs.

The allyl derivatives **167–176** were covalently attached to the outer surface of the nanotubes by a microwave-assisted thiol–ene reaction in the presence of azo(bis)isobutyronitrile (AIBN), employing thiol-functionalized HNTs under solvent-free conditions at 100 °C, as depicted in

Scheme 17. After purification, thermogravimetric analysis indicated drug loadings of about 1-2 wt%. Comparing these values with the initial thiol density on HNTs shows that only a fraction of the available –SH groups participated in the thiol–ene process, most likely because of steric constraints at the nanotube surface.



Scheme 17. Thiol–ene coupling between HNTs and allyl-functionalised pyrazolo[3,4-*d*]pyrimidines **167–176**.

3.1.3. *In silico* profiling and *c*-Src binding analysis

To assess whether introduction of the allyl handle was compatible with conservation of the Src-directed pharmacophore, compounds **167**, **168** and **170** were first examined by computational methods. Basic physicochemical descriptors (molecular weight, calculated logP, hydrogen-bond donors/acceptors, polar surface area) were within ranges compatible with oral small-molecule drugs and comparable to those of the parent inhibitors.

Structure-based modelling was then performed using crystal structures of *c*-Src in complex with ATP-competitive ligands. Docking into the ATP-binding cleft showed that all three compounds can reproduce the canonical binding mode of pyrazolo[3,4-*d*]pyrimidines: the heterobicycle anchors in the hinge region through a pair of hydrogen bonds, while the anilide side chain projects into the selectivity pocket. Among the series, compound **170** displayed the closest overlap with the reference inhibitor, maintaining most of the key contacts in the catalytic pocket and giving the best overall docking score. Compounds **167** and **168** showed slightly less favourable interaction networks, consistent with a modest predicted drop in potency relative to the lead.

3.1.4. Physicochemical characterization of **HNT-167**, **HNT-168** and **HNT-170**

The nanoconjugates were analysed with a combination of spectroscopic and colloidal techniques.⁵⁸³

FT-IR spectroscopy confirmed the presence of the organic ligands on the nanotubes: new bands in the aromatic C=C/C–N region, absent in pristine HNTs, appear in spectra of **HNT-167**, **HNT-168** and **HNT-170** and are consistent with the pyrazolopyrimidine and anilide moieties.⁵⁸³

Thermogravimetric analysis provided quantitative estimation of the organic content and showed distinct mass-loss events associated with degradation of the grafted inhibitors, in addition to the dehydration steps typical of halloysite. This shift of the drug-related mass-loss events to higher temperatures compared with the free inhibitors indicates that covalent anchoring on the halloysite surface confers additional thermal stability to the organic payload, which is favourable from a formulation and storage standpoint.⁵⁸³

Dynamic light scattering and ζ -potential measurements revealed that covalent grafting markedly increases the hydrodynamic size (Z-Average Size) compared with native HNTs, suggesting the formation of larger aggregates in aqueous media, likely mediated by π – π stacking and hydrophobic interactions between surface-exposed ligands, data expressed as polydispersity index (PDI). All nanoconjugates retained a negative ζ -potential, though less negative than that of bare HNTs, consistent with partial masking of surface silanols by organic coverage (Table 3). Although the increase in hydrodynamic size points to a tendency toward aggregation, the persistence of a net negative ζ -potential suggests that the colloidal stability of the nanoconjugates in aqueous media is at least partially preserved, which is acceptable for subsequent biological assays.⁵⁸³

Nanomaterial	Z-Average Size (nm)	PDI	Z potential (mV)
HNT	295±16	0.300	-19
HNT-154	330±20	0.481	-16.9
HNT-166	299±32	0.350	-12.6
HNT-167	654±80	0.368	-15.4
HNT-168	1110±70	0.895	-12.3
HNT-169	-	-	-
HNT-170	1026±170	0.744	-13.3

HNT-171	-	-	-
HNT-172	-	-	-
HNT-173	-	-	-
HNT-174	-	-	-
HNT-175	-	-	-
HNT-176	-	-	-

Table 3. Average size, PDI and ζ -potential values of the synthesized nanomaterials. Reported are the mean values of three independent experiments run in triplicate.

Electron microscopy showed that the tubular morphology of halloysite is maintained after functionalisation, but the nanotubes tend to associate into more compact clusters, in line with the DLS data. The lumen remains visible, confirming that these systems are true surface conjugates rather than lumen-loaded hybrids. This behaviour is fully consistent with a surface-grafted architecture in which the inorganic scaffold preserves its native morphology while the organic corona promotes inter-tubular contacts, without obstructing the inner cavity that could in principle be exploited for additional loading strategies (Figure 70).⁵⁸³

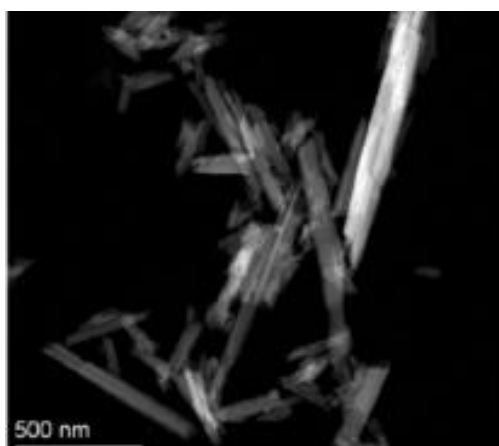


Figure 70. STEM image of HNTs-170.

3.2. Biology

3.2.1. Cytotoxicity of the free molecules

The antiproliferative activity of the free compounds was evaluated on three human cancer cell lines relevant to the project aims: RT112 and UMUC3 (bladder carcinoma) and PC3 (prostate carcinoma). Cells were exposed to increasing concentrations of **167**, **168** or **170** for 72 h and viability was quantified using an MTT assay (Table 4). All three derivatives reduced cell

viability in a dose-dependent fashion, with IC₅₀ values in the low- to mid-micromolar range. Compound **170** emerged as the most active member of the set, with IC₅₀ values close to those of compounds **154** and **166**, used as internal reference (Figure 69), whereas **167** and **168** were slightly less potent. These data confirm that the allyl modification does not abolish the antiproliferative profile of the scaffold and supports the use of **167**, **168** or **170** as covalent payloads for nanoconjugation.

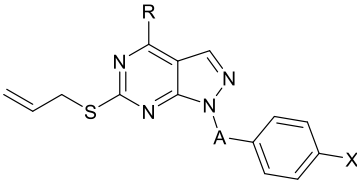
						
Cpd	X	A	R	IC ₅₀ ^a μM RT112	IC ₅₀ ^a μM UMUC3	IC ₅₀ ^a μM PC3
167	H	CH=CH	NHCH ₂ CH ₂ C ₆ H ₅	25.0±5.0	9.9±0.1	9.3±2.1
168	H	CH=CH	NHC ₆ H ₄ -3Br	13.6±4.3	9.0±1.7	16.3±2.1
169	H	CH(Cl)	NHCH ₂ CH ₂ C ₆ H ₅	-	-	-
170	H	CH(Cl)	NHC ₆ H ₄ -3Br	10.4±2.9	5.0±1.3	10.7±1.5
171	H	CH(Cl)	NHC ₆ H ₃ -2CH ₃ -5OH	-	-	-
172	H	CH(Cl)	NHC ₆ H ₃ -2F-5OH	-	-	-
173	Br	CH=CH	NHCH ₂ CH ₂ C ₆ H ₅	-	-	-
174	Br	CH=CH	NHC ₆ H ₄ -3Br	-	-	-
175	Br	CH(Cl)	NHCH ₂ CH ₂ C ₆ H ₅	-	-	-
176	Br	CH(Cl)	NHC ₆ H ₄ -3Br	-	-	-

Table 4. Structures and antiproliferative activity of **167–176**. ^aIC₅₀ values were determined on RT112 and UMUC3 bladder cancer cells and PC3 prostate cancer cells after 72 h of treatment and are reported as mean of at least three independent experiments performed in quadruplicate.

3.2.2. Biological evaluation of the nanoconjugates

The antiproliferative activity of **HNT-167**, **HNT-168** and **HNT-170** was assessed on the same tumour cell lines used for the free molecules. Cells were incubated for 72 h with increasing concentrations of nanoconjugates; concentrations were normalised to the amount of pyrazolopyrimidine present (Table 5). Halloysite alone was used as a control and did not significantly affect cell viability, confirming the intrinsic biocompatibility of the carrier.

Nanomaterial	IC ₅₀ ^a μ M RT112	IC ₅₀ ^a μ M UMUC3	IC ₅₀ ^a μ M PC3
HNT-154	4.3 $\pm$ 0.6	3.4 $\pm$ 1.8	>10
HNT-166	4.6 $\pm$ 0.4	6.3 $\pm$ 1.5	8.5 $\pm$ 1.6
HNT-167	>10	>10	>10
HNT-168	>10	>10	>10
HNT-169	-	-	-
HNT-170	2.8 $\pm$ 0.8	>10	>10
HNT-171	-	-	-
HNT-172	-	-	-
HNT-173	-	-	-
HNT-174	-	-	-
HNT-175	-	-	-
HNT-176	-	-	-

Table 5. Antiproliferative activity of **HNT-154**, **HNT-166**, **HNT-167**, **HNT-168** and **HNT-170**.

^aIC₅₀ values were determined on RT112 and UMUC3 bladder cancer cells and PC3 prostate cancer cells after 72 h of treatment with increasing concentrations of each formulation, normalised to the amount of pyrazolo[3,4-d]pyrimidine present, and are reported as mean of at least three independent experiments performed in quadruplicate. “>10” indicates that 50% inhibition was not reached at the highest concentration tested.

All three nanomaterials induced a concentration-dependent reduction in cell viability, indicating that the covalently bound inhibitors retain a degree of biological activity when displayed on the nanotube surface. Among them, **HNT-170** showed the most pronounced effect, reaching half-maximal inhibition at low micromolar concentrations in RT112 cells, whereas its activity on UMUC3 and PC3 remained weak (IC₅₀ > 10 μ M). **HNT-167** and **HNT-168** produced only modest viability reductions within the tested range, but still exerted detectable antiproliferative effects, suggesting that the halloysite platform can present different analogues to the biological target, although with variable efficiency.

4. Conclusions

Taken together, the results presented in this chapter provide a first validation of halloysite nanotubes as modular carriers for Src-directed pyrazolo[3,4-*d*]pyrimidine inhibitors and illustrate how they can be combined with nanotechnology to face issues of poor aqueous solubility and delivery. Building on the potent but poorly soluble leads **154** and **166**, a small library of allyl-functionalised analogues **167–176** was conceived to preserve the hinge-binding pyrazolopyrimidine core and the key N1 and C4 vectors for kinase recognition, while introducing at C6 a compact allylthio handle compatible with thiol–ene coupling to HNTs. The multistep synthetic route afforded the target molecules in good overall yields and confirmed that the pyrazolo[3,4-*d*]pyrimidine scaffold is chemically robust and liable to further diversification.

Biological profiling of the free compounds on RT112 and UMUC3 bladder cancer cells and PC3 prostate cancer cells showed that the introduction of the allylthio group is compatible with retention of antiproliferative activity in the low- to mid-micromolar range. Within the limited series tested, compound **170** emerged as the most promising derivative, with IC₅₀ values close to those of **154** and **166** and a docking pose that closely reproduces the canonical Src binding mode. These data support the original design hypothesis that the allyl fragment can function primarily as a conjugation handle, leaving the pharmacophore responsible for kinase inhibition substantially unperturbed.

Covalent grafting of selected analogues (**167**, **168** and **170**) onto thiol-functionalised HNTs confirmed the practical feasibility of this coupling strategy. Physicochemical characterisation, performed in collaboration with the group of Prof. Riela, demonstrated successful surface functionalisation and preservation of the tubular halloysite morphology, together with increased thermal stability of the grafted drugs and acceptable colloidal behaviour in aqueous media. From a pharmacological standpoint, the nanoconjugates retained a measurable, though generally attenuated, antiproliferative effect compared with the corresponding free inhibitors. **HNT-170** showed the most favourable profile, achieving low-micromolar IC₅₀ values in RT112 cells, whereas **HNT-167** and **HNT-168** were less active but still capable of reducing cell viability in a concentration-dependent fashion. These findings indicate that halloysite can effectively present covalently bound pyrazolopyrimidines to tumour cells, even though the

efficiency of delivery and target engagement is highly dependent on the nature of the grafted analogue.

It should be emphasized, however, that not all compounds and nanoconjugates in the **167–176** series have been fully characterized to date, and some biological data are still incomplete or limited to a single cell line. Future work will therefore focus on completing the physicochemical and antiproliferative profiling of the remaining analogues (both as free molecules and as HNT conjugates) and on validating the most promising candidates in more advanced biological models. In parallel, the library will be expanded by systematic variation of the N1 chain and of the C4 aniline moiety, with the aim of refining the SAR and optimizing both Src inhibition and nanocarrier compatibility. Moreover, the tendency of nanoconjugates to aggregate, together with partial utilisation of surface thiols, suggests that optimisation of loading density, linker architecture and surface coverage will be crucial to maximise biological performance.

In conclusion, this chapter establishes a coherent proof-of-concept for the use of halloysite nanotubes as carriers for Src-targeted pyrazolo[3,4-*d*]pyrimidines. The work demonstrates that allyl-functionalised derivatives can be synthesised efficiently, maintain significant antiproliferative activity, and be covalently displayed on HNTs without loss of structural integrity. At the same time, it underscores the need for broader libraries and more systematic SAR exploration. In a broader perspective, the combination of medicinal chemistry and clay-based nanotechnology developed here provides a versatile platform that could be extended to other kinase targets, ultimately contributing to the translation of potent but formulation-challenged inhibitors into viable candidates for the treatment of urogenital cancers.

CHAPTER 3

Development of GQX analogues coupled with a ROS-sensitive moiety to afford ROS-responsive prodrugs active on GABA_A receptors

1. Introduction

1.1. GABA

γ -Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the mammalian CNS. Although GABA was first identified in biological tissues in 1910, its neuromodulatory role in mammals was not established until the late 1950s.⁵⁸⁴ Subsequent electrophysiological studies on cortical neurons in the late 1960s provided definitive evidence that GABAergic signalling is inhibitory, prompting extensive investigations into the molecular mechanisms underlying GABA-mediated inhibition and its contribution to diseases. GABA is synthesized in the cytosol of presynaptic neurons from the amino acid glutamate (Glu), one of the prevalent excitatory neurotransmitters in CNS, via an enzymatic decarboxylation carried out by glutamic acid decarboxylase (GAD).^{585,586} GABA and Glu are metabolized and exchanged between neurons and astrocytes (AS) through the Glu/GABA-glutamine (Gln) cycle.⁵⁸⁷ In the cycle, GABA in the synaptic cleft is mainly taken up in neurons by GABA transporters (GATs) located on AS, where it is converted in Glu and, further, in Gln, which is released in the extra-synaptic space. Then Gln, which is taken up in neurons, is subsequently deamidated to obtain Glu and, finally, GABA is synthesized out from Glu via decarboxylation performed by GAD.⁵⁸⁸ Vesicular inhibitory amino acid transporters (VIAAT/VGAT) load cytosolic GABA into synaptic vesicles, from which it is released into the synaptic cleft to act on pre-, post-, and extra-synaptic GABA receptors. Plasma-membrane GATs, expressed on presynaptic terminals and neighbouring glial cells, finally remove GABA from the extracellular space. Reuptake allows recycling (in presynaptic neurons) or discharge (in AS) of transmitter, whereas cytosolic GABA can be metabolized by mitochondrial GABA transaminase (GABA-T) to succinic semialdehyde, an inactive intermediate of the TCA, thereby contributing to clearance and homeostasis (Figure 71).⁵⁸⁹

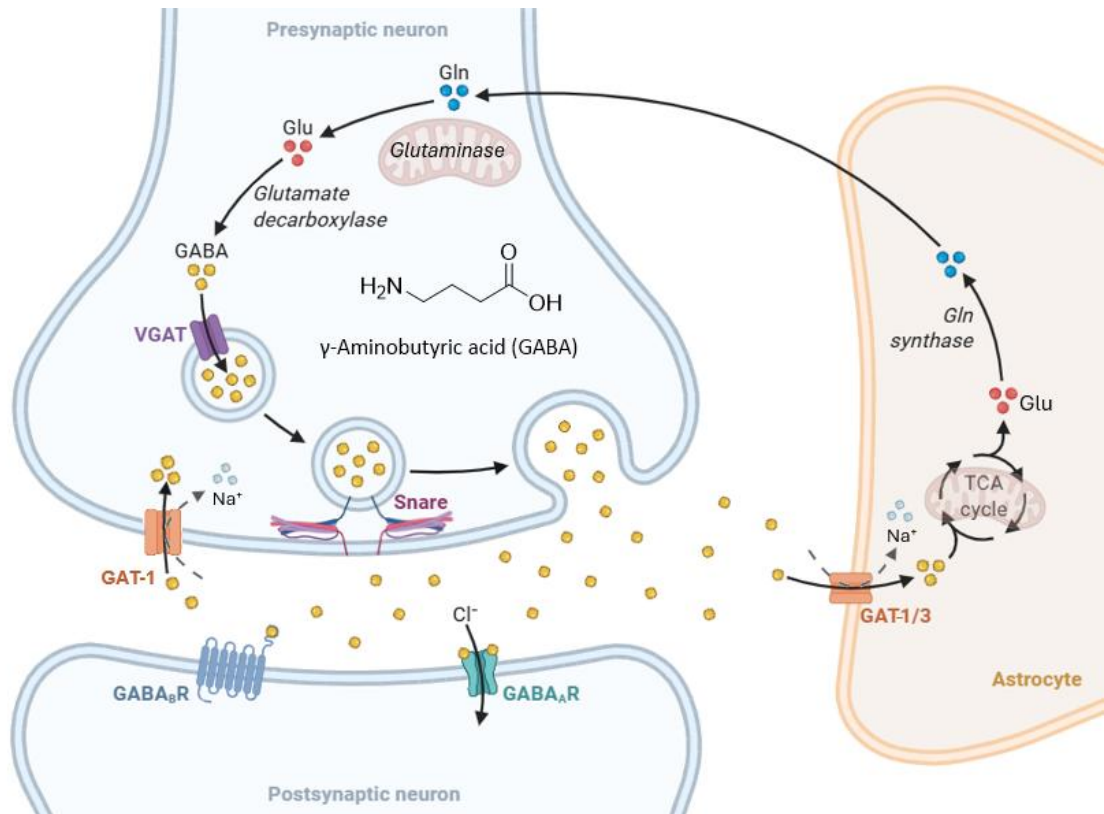


Figure 71. A schematic overview of GABA metabolism. Glu, obtained by the transamination of α -Ketoglutarate, generated in the tricarboxylic acid cycle of both presynaptic neurons and AS, is the immediate precursor for GABA biosynthesis. GAD then converts Glu in cytosol of neurons to GABA, which is packaged into synaptic vesicles and released upon neuronal activity. Following release, extracellular GABA is rapidly cleared by high-affinity GATs located on both neurons and glial cells, thereby supporting transmitter recycling and maintaining synaptic homeostasis.

GABA typically suppresses neuronal excitability by hyperpolarizing postsynaptic membranes, principally via GABA_A receptor-mediated Cl^- currents and GABA_B receptor-activated K^+ conductance, and by producing presynaptic inhibition through the reduction of voltage-gated Ca^{2+} entry and transmitter release. The net effect is a decrease in the activation of the affected neurons, manifesting as reduced motor drive in spinal and brainstem circuits and attenuated cortical activity.^{590,591}

1.2. GABA Transporters

The magnitude and duration of GABA's action are governed by high-affinity transporters expressed on neurons and glia. GATs are Na^+/Cl^- coupled plasma-membrane transporters that clear GABA from the synaptic cleft and surrounding extracellular space after release. When an

action potential reaches the presynaptic terminal of an inhibitory interneuron, the ensuing depolarization opens voltage-gated Ca^{2+} channels (VGCCs). The resulting Ca^{2+} influx drives soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent docking, priming, and fusion of GABA-containing synaptic vesicles at the active zone. This exocytotic fusion releases GABA into the synaptic cleft, thereby terminating inhibitory transmission, constraining spillovers, and setting ambient GABA levels that tonically activate GABA receptors.^{592,593} Although reuptake generally lowers extracellular GABA, a reversal transport can occur under appropriate ionic and voltage conditions, allowing GATs to release GABA and help stabilize baseline extracellular levels⁵⁹⁴. Through these mechanisms, reuptake is a key determinant of synaptic time course and, by shaping receptor activation, contributes to the network rhythms governed by GABAergic transmission. Four GABA transporters have been cloned – GAT-1, GAT-2, GAT-3, and GAT-4 – distinguished by their amino-acid sequences and pharmacological profiles.^{595,595} Of these transporters, GAT-1 and GAT-3 are largely restricted to the brain. GAT-1 is enriched in neurons, while GAT-3 is expressed predominantly by astrocytes.^{595,596}

1.3. GABA Receptors

In the synaptic cleft, GABA binds to the α and β subunits on GABA receptors, usually found on the postsynaptic neuron or other presynaptic axon terminals.⁵⁹⁷ Two principal classes of GABA receptors are recognized: ionotropic receptors (GABA_ARs) which are ligand-gated ion channels, and metabotropic receptors (GABA_BRs), which are G-protein–coupled receptors.⁵⁹⁸ Activation of GABA_ARs, mostly located on the postsynaptic neuron, opens ligand-gated Cl^- channels, increasing chloride influx and typically hyperpolarizing the postsynaptic membrane to generate inhibitory postsynaptic potentials. By contrast, GABA_BRs, common also in presynaptic axon terminals, suppress voltage-gated Ca^{2+} channels and enhance G protein–gated inwardly rectifying K^+ (GIRK) currents on both pre- and postsynaptic elements. In presynaptic neurons, GABA_BRs activation reduces Ca^{2+} entry and thereby lowers the probability of transmitter release, particularly glutamate; in postsynaptic neurons, it promotes K^+ efflux and membrane hyperpolarization, further decreasing neuronal.⁵⁹⁹

1.3.1. GABA_ARs

GABA_ARs are a part of the bigger family of gating chloride channels (LGICs), encompassing several families of receptors including nicotinic acetylcholine receptors, 5-HT₃ receptors, strychnine-sensitive glycine receptors, and some invertebrate anionic glutamate receptors.⁶⁰⁰

LGICs mediate fast synaptic transmission via the movement of ions through channels gated by neurotransmitters such as acetylcholine and GABA. As members of the nicotinoid superfamily, GABA_ARs are composed of 5 proteins. Nineteen distinct subunits have been identified (α 1–6, β 1–3, γ 1–3, δ , ϵ , θ , π , and ρ 1–3), with the most common pentameric arrangement being 2 α –2 β –1 γ (Figure 72) and a suggested maximum of the order of 800 combinations.^{601,602} In some GABA_ARs, the γ subunit is replaced by δ . Subunit expression is not uniform across the CNS: broadly distributed arrangements of subunits include α 1, β 2/ β 3, and γ 2, whereas α 2– α 6, β 1, and δ show more region-restricted patterns. Notably, δ commonly co-assembles with α 4, with prominent expression in thalamus and striatum.^{603,604}

Each subunit bears a large extracellular N-terminal domain which incorporates part of the agonist-/antagonist-binding site, followed by three membrane-spanning domains (M1-3), an intracellular loop of variable length, and a fourth membrane-spanning domain (M4), and the C-terminal end being extracellular. Each subunit arranges itself such that the second membrane-spanning domain (M2) forms the wall of the channel pore and the overall charge of the domain determines whether the channel conducts anions or cations (Figure 72).⁶⁰⁵

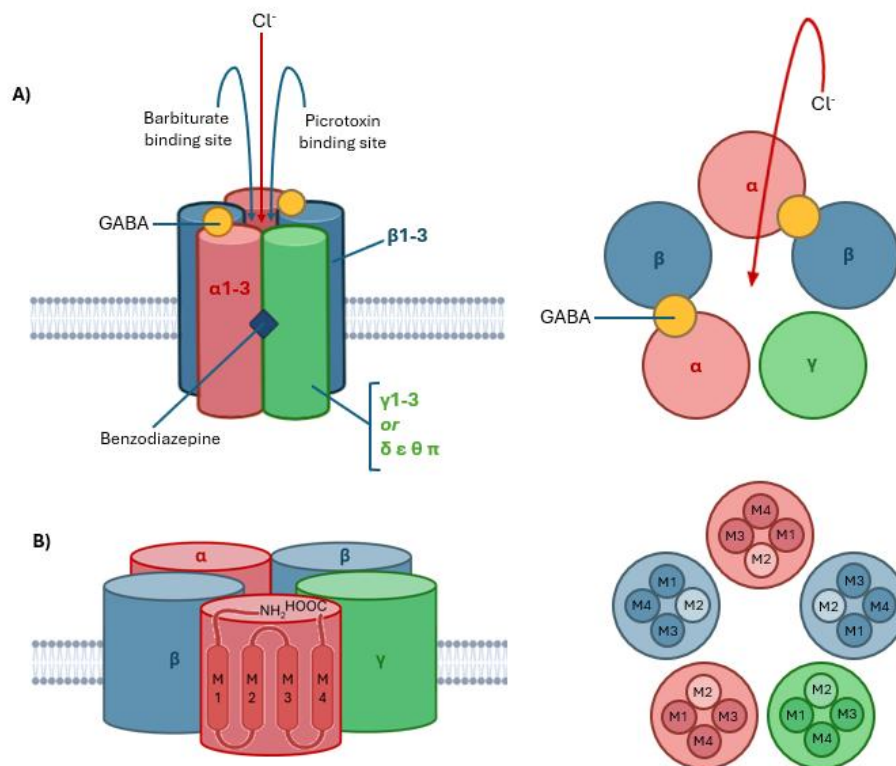


Figure 72. GABA_A receptor architecture and subunit organization within the LGIC superfamily. (A) Side and extracellular top view of a canonical pentameric GABA_AAR embedded

in the membrane, illustrating the frequent $2\alpha-2\beta-1\gamma$ stoichiometry. GABA-binding sites lie at the $\alpha\beta$ interfaces (yellow), whereas the BZD site is located at the $\alpha-\gamma$ interface (blue diamond); the central pore conducts anions during activation. (B) Single-subunit topology: a large extracellular N-terminal domain contributing to agonist/antagonist binding, four transmembrane segments (M1–M4), and an extracellular C-terminus. Transmembrane helical bundle highlighting that M2 from each subunit lines the pore; residues within M2 govern ion selectivity.

At least 11 structurally specific sites on GABA_ARs have been identified: 1) agonist sites that also bind competitive antagonists; 2) picrotoxin sites, which also interact with γ -butyrolactones, caprolactams, and some insecticides; 3) sedative–hypnotic barbiturate sites; 4) neuroactive steroid sites; 5) benzodiazepine sites; 6) ethanol sites; 7) stereoselective sites for inhaled anesthetics; 8) furosemide sites; 9) Zn²⁺ sites; 10) sites for other divalent cations; and 11) La³⁺ sites.⁶⁰⁶ The chemical diversity of ligands that act at GABA_ARs supports the presence of multiple recognition sites tuned to specific structural motifs. Some sites appear to be subunit-associated: for example, the diuretic furosemide preferentially targets sites linked to $\alpha 6$ -containing receptors, and residues in transmembrane domains (TMD) M1 and M2 critically determine the antagonist specificity of furosemide at GABA_ARs.^{606–608}

The orthosteric binding sites are located between α and β subunits. An allosteric site exists between subunits α and γ .⁶⁰¹ The GABA_ARs presents two equivalent neurotransmitter pockets in the extracellular domain, each located at an interface (Figure 73). On the β side (+), the pocket is formed largely by aromatic residues (Y97, Y157, F200, Y205) that pack around the ligand's amino group; the position of F200 and Y205 creates cation– π interactions. E155 lies ~ 4.2 Å from the amino nitrogen and may further stabilize the end of the ligand. T202 is placed to hydrogen-bond with carboxylate. On the complementary (–) face provided by the α -subunit, F65 forms the bottom of the pocket and contributes to hydrophobic interactions. T130 may donate a hydrogen bond to the carboxylate, while R67 (conserved in α but absent in β/γ subunits) appears crucial for recognizing the anionic headgroup.⁶⁰⁹

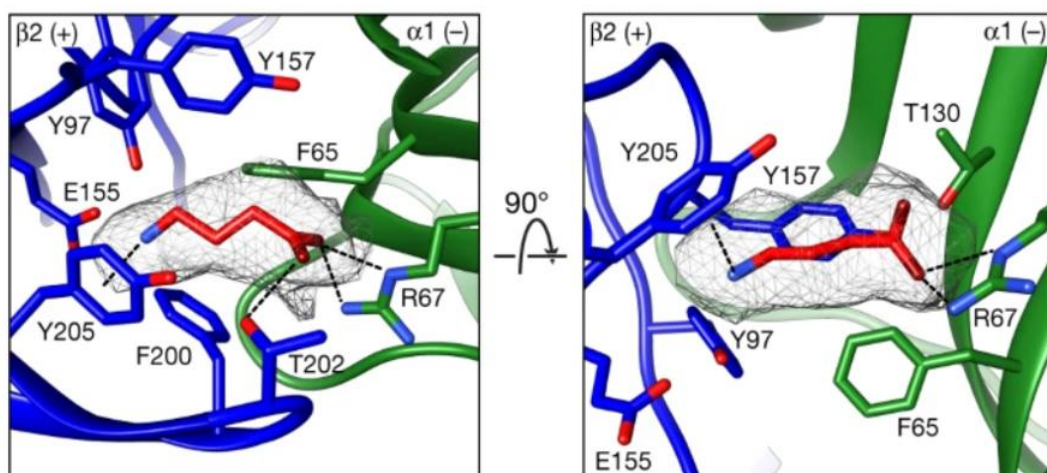


Figure 73. Structural depiction of the GABA pocket, with predicted hydrogen-bonding and cation- π contacts shown as dashed traces.⁶⁰⁹

On the other hand, benzodiazepines (BZD) bind at the $\alpha\gamma$ interface. As positive modulators of GABA_ARs, BZDs amplify GABA's effects by shifting the GABA concentration-response to lower agonist levels, leading to sedation, hypnosis, anxiolysis, anticonvulsant activity, and muscle relaxation.⁶¹⁰

Overall, GABA recognition emerges from a combination of aromatic stacking/cation- π contacts with its amino group and electrostatic/hydrogen-bond interactions with the carboxylate, with complementary-face variability exerting strong control over ligand selectivity. Electrophysiological evidence indicates that as the agonist concentration rises, the mean open time increases, consistent with preferential opening of doubly liganded receptors (which thus display long-lived open states).⁶¹¹ An interesting phenomenon was observed: in rat brain membrane preparations, the receptor's ion permeability shows a transient enhancement that decays despite the continued presence of agonists. This phenomenon, called desensitization, reverses rapidly upon agonist removal. Notably, it has been proposed that specialized GABA-binding sites initiate desensitization and are distinct from the sites that directly gate the Cl⁻ channel.⁶¹²

1.3.2. GABA_{A ρ} Rs

GABA_{A ρ} Rs, historically designated as GABA_CRs, are ionotropic members of the pentameric Cys-loop LGIC superfamily, which are assembled from ρ subunits ($\rho 1$, $\rho 2$, $\rho 3$) in homo- or pseudohomopentameric forms. This reclassification reflects sequence homology and shared LGIC topology with other GABA_ARs, while recognizing the distinctive pharmacology and kinetics of ρ assemblies (Figure 74, panel A). They were first described in spinal cord

interneurons.^{613–616} Shortly thereafter, expression in the retina was revealed.⁶¹⁷ Beyond the retina, GABA_A ρ Rs currents have been recorded in higher visual centers such as the superior colliculus, where they participate in GABA-dependent long-term potentiation.^{618,619} From an electrophysiologic point of view, these receptors are distinguished from canonical GABA_ARs by higher apparent sensitivity to GABA, smaller macroscopic currents, and minimal or absent desensitization; single-channel studies report longer mean open times and smaller unitary Cl[−] conductance.^{620–622} Their pharmacology is likewise distinctive: they are insensitive to classic GABA_ARs modulators (barbiturates, BDZs) and to the competitive antagonist bicuculline, and they are not activated by agonist baclofen.⁶²³

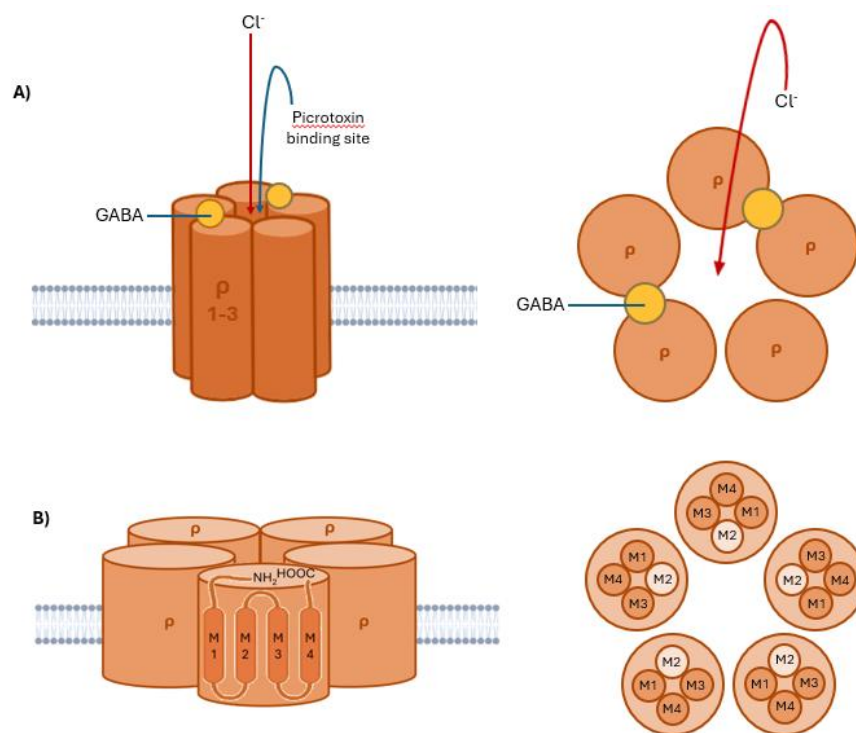


Figure 74. (A) Side and extracellular top view of GABA_A ρ Rs embedded in the membrane. The locations of the binding sites for GABA and antagonists picrotoxin are also shown. (B) Single-subunit topology: a large extracellular N-terminal domain contributing to agonist/antagonist binding, four transmembrane segments (M1–M4), and an extracellular C-terminus. M2 faces the pore and governs ion selectivity.

As already described for GABA_ARs, GABA_A ρ Rs subunits support the canonical Cys-loop/LGIC topology inferred from the nicotinic acetylcholine receptor: a long extracellular N-terminus, four transmembrane segments (M1–M4, with M2 lining the ion pore), and a short extracellular C-terminus (Figure 74, panel B).⁶²⁴ N-terminus hosts the orthosteric GABA-binding element, which is repeated at each subunit–subunit interface, yielding up to five

GABA-binding pockets per receptor; consequently, as many as five GABA molecules can bind simultaneously.⁶²⁵ Channel opening requires occupancy of multiple sites (typically ≥ 2). Full occupation of all five sites is observed at high agonist concentrations and modulates both efficacy and the cooperativity of gating.^{622,626} As GABA_ARs, also GABA_{A ρ} Rs are inhibited by picrotoxin, with a mechanism that includes non-competitive pore block and a competitive component.^{627–629} Cysteine-scanning of the GABA_ARs $\alpha 1$ subunit identified V257/C257, a position topologically analogous to $\rho 1$ P309, as pore-exposed and part of the picrotoxin footprint.⁶³⁰ Unlike GABA_ARs, GABA_{A ρ} Rs are markedly insensitive to barbiturates.⁶²⁰ A defining determinant is W328 in M3 of $\rho 1$: replacing W328 with hydrophobic residues (M, V, A, L, I) confers barbiturates sensitivity.⁶³¹ Notably, the reciprocal manipulation at the corresponding position in GABA_ARs subunits affects barbiturates sensitivity.⁶³²

Like GABA_ARs, GABA_{A ρ} Rs formed by $\rho 1$ subunits display selective modulation by several exogenous and endogenous factors, including ethanol, protons/pH, glycine and β -alanine, and halogenated fatty acids.^{629,633–635} A defined divalent-cation interaction site underlies modulation by Zn^{2+} , a transmitter co-localized with photoreceptor terminals and known to regulate GABA_{A ρ} Rs currents.^{629,636–639} In $\rho 1$, Zn^{2+} reduces GABA-evoked currents in a pH-dependent manner, implicating histidines in coordination: Zn^{2+} binds to one or more histidine residues on the receptor to exert its effect. Histidines are excellent “anchors” for transition-metal cations (such as Zn^{2+}), but their ability to coordinate the metal depends on their protonation state, which in turn depends on pH. At higher pH, the histidine imidazole is more often deprotonated, being able to coordinate Zn^{2+} more effectively. As a final result, Zn^{2+} inhibition tends to be stronger. Indeed, substitution H156Y in the N-terminal extracellular domain abolishes Zn^{2+} sensitivity without altering other pharmacological or electrical properties, and the same residue contributes to inhibition by Ni^{2+} , and Cd^{2+} .^{629,637}

Compared to GABA_ARs, GABA_{A ρ} Rs are characterized by higher GABA sensitivity ($EC_{50}=21 \mu M$ for GABA_ARs, compared to $EC_{50}=2.5 \mu M$ for GABA_{A ρ} Rs).^{640,641} Note that EC_{50} is influenced by recording configuration, expression level, chloride gradient, and receptor composition; it should not be equated strictly with microscopic affinity. Still, under comparable conditions, the potency gap between GABA_ARs and GABA_{A ρ} Rs remains robust. This pattern is consistent with classic observations that GABA_{A ρ} Rs are high-sensitivity, slowly gating anion channels, contrasting with the lower sensitivity, faster synaptic GABA_A complexes. Mutational analysis identified five N-terminal residues in human $\rho 1$ (Y198, Y200, Y241, T244, Y247)

between the Cys-loop and M1, as determinants of GABA affinity: substitutions at any of these sites decreased apparent agonist potency.⁶⁴²

In the context of GABA binding, GABA_ARs and GABA_{Aρ}Rs show a structural correspondence (Figure 75).⁶⁰⁹ Overall, the binding mode of GABA in the GABA_{Aρ}Rs pocket closely resembles GABA_ARs receptors. The lipophilic pocket defined by $\alpha 1\beta 2$ residues in GABA_ARs corresponds, in GABA_{Aρ}Rs, to Y123, Y219, Y262, and Y268. Likewise, the electrostatic contact to E155 in the $\alpha 1\beta 2$ site is mirrored by an interaction with E217 in the ρ site, and the arginine that stabilizes the ligand's carboxylate, R67 in the $\alpha 1\beta 2$ interface, corresponds to R125 in GABA_{Aρ}Rs.⁶⁴³ Site-directed mutagenesis further suggests that the GABA_{Aρ}Rs orthosteric pocket is narrower than its GABA_ARs counterpart because of an additional phenylalanine. This may help explain the lack of bicuculline binding at GABA_{Aρ}Rs; notably, the F240V substitution increases bicuculline affinity at GABA_{Aρ}Rs, consistent with a steric contribution to antagonist exclusion. In addition, D219 in GABA_{Aρ}Rs aligns with A181 in GABA_ARs (a small, lipophilic residue), implying that electrostatic repulsion from D219 (a charged residue) could further disfavour bicuculline binding.⁶⁴⁴

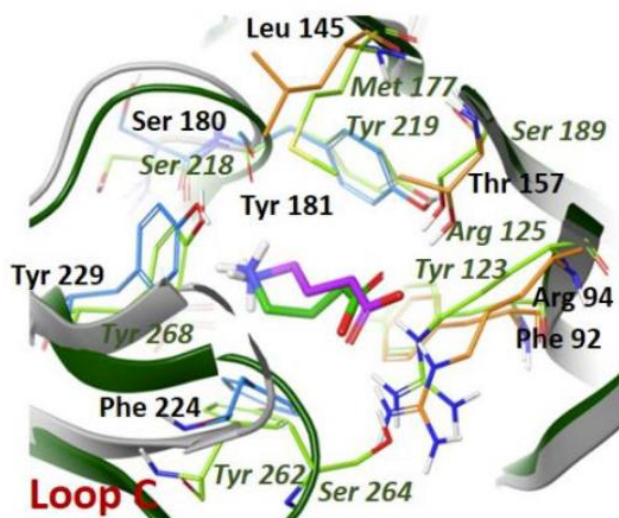


Figure 75. Comparison of the $\alpha 1\beta 2$ and $\rho 1$ orthosteric binding sites. Superposed pockets illustrate the conserved GABA binding mode across receptors. GABA at $\alpha 1\beta 2$ is shown in purple; GABA at $\rho 1$ in green. The $\rho 1$ site (residues in green/yellow) was generated by homology modelling from PDB: 4COF, 5TIN, and 3RIF. The $\alpha 1$ and $\beta 2$ contributions are depicted in orange and light blue, respectively. Key correspondences include the lipophilic cavity ($\rho 1$ Y123, Y219, Y262, Y268), the acidic contact (E217 in $\rho 1$ vs E155 in $\alpha 1\beta 2$), and the arginine stabilizing GABA's carboxylate (R125 in $\rho 1$ vs R67 in $\alpha 1\beta 2$). Mutagenesis indicates a narrower $\rho 1$ pocket,

with F240 contributing to bicuculline exclusion (affinity increases with F240V). Residue numbering follows the original articles and has not been renumbered here.

Moreover, functional studies of GABA_{Ap}Rs in the lateral nucleus of the amygdala, region critically involved in fear processing, have revealed intriguing differences from canonical GABA_ARs.⁶⁴⁵ Using electrophysiological recordings, the selective GABA_{Ap}Rs antagonist (1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) and the agonist muscimol were applied to probe receptor activity. Muscimol selectively activated GABA_{Ap}Rs at 1 μ M, whereas its EC₅₀ for GABA_ARs was higher (27 μ M vs. 2.3 μ M for GABA_{Ap}Rs).⁶⁴⁶ Typically, muscimol enhances inhibition through GABA_ARs and GABA_BRs activation; however, in the amygdala, muscimol paradoxically reduced inhibition and enhanced excitation via GABA_{Ap}Rs. In contrast, TPMPA increased inhibition and reduced excitatory drive. These data suggest that GABA_{Ap}Rs are presynaptically located, where they may modulate tonic GABA release and thereby indirectly control neuronal excitability in this fear-related circuit. Given the hyperactivity of the amygdala in anxiety disorders, GABA_{Ap}Rs represent an attractive therapeutic target for more selective anxiolytic agents. Current treatments acting on conventional GABA_ARs, such as BZDs, are effective but associated with tolerance, dependence, and cognitive side effects, making receptor-subtype-specific strategies particularly appealing. Emerging evidence suggests that GABA_{Ap}Rs may also represent novel therapeutic targets in memory-related disorders and ocular pathologies such as myopia.^{598,646} Their low abundance, restricted expression profile, and unique pharmacological properties relative to canonical GABA_ARs confer potential advantages for drug selectivity and side-effect minimization. Nevertheless, despite increasing interest, the physiological roles of GABA_{Ap}Rs remain incompletely characterized.⁵⁸ A significant obstacle to advancing GABA_{Ap}Rs research is the lack of selective pharmacological tools. Despite radio-labeled muscimol ([³H]-muscimol) serves as a reliable ligand for studying GABA_ARs, no radioligands currently exist for GABA_{Ap}Rs, largely due to the absence of sufficiently selective agonists or antagonists.⁶⁴⁵ Thus, the design and synthesis of subtype-selective GABA_{Ap}Rs ligands represents a critical step toward developing molecular probes that could elucidate the receptor's structure–function relationships and its contributions to both normal physiology and disease.

1.3.3. GABA_BRs

GABA_BRs are class C GPCR that inhibit the activity of adenylate cyclase and voltage-gated Ca²⁺ channels at axon terminals, resulting in modulation of intracellular activity and

neurotransmitter release, respectively. Class C GPCRs are structurally more complex than other GPCRs. GABA_BRs are heterodimers composed of two distinct subunits: GB1, which contains the orthosteric binding site for agonists, and GB2, which mediates G-protein activation.⁶⁴⁷ Each subunit comprises an extracellular Venus Flytrap (VFT) domain coupled to a transmembrane (TM) domain (Figure 76).^{648,649}

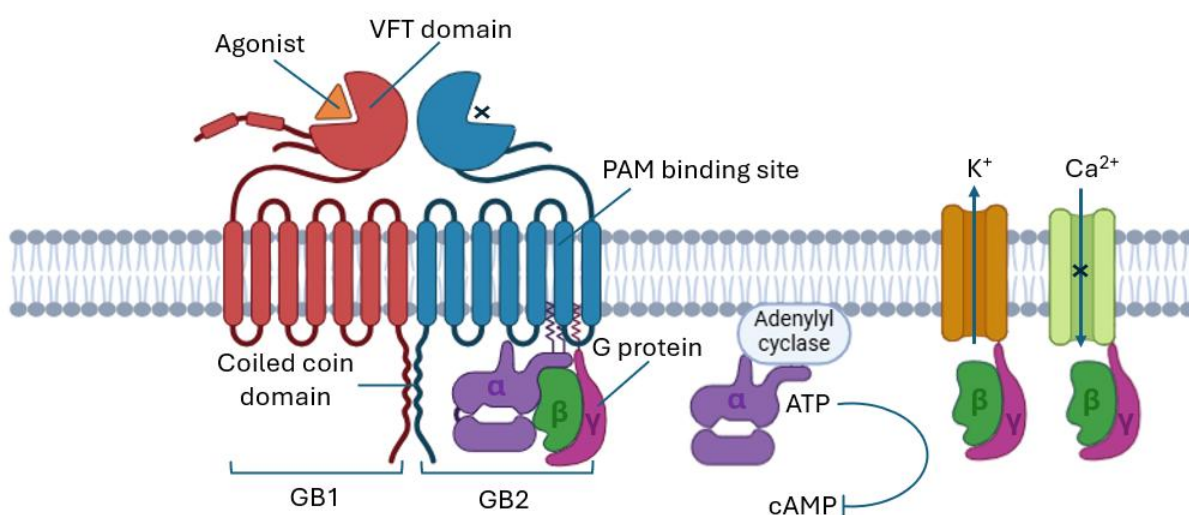


Figure 76. Structural organization and signalling of GABA_BRs. Functional GABA_BRs are heterodimers composed of the two subunits, GB1 (red) and GB2 (blue). Both subunits contain a large extracellular VFT domain, where agonist binding occurs exclusively at GB1, while GB2 provides a binding pocket for positive allosteric modulators (PAMs). The subunits heterodimerize through their VFT domains and coiled-coil intracellular regions. Upon agonist binding, conformational changes recruit and activate G proteins via GB2. The activated G protein α subunit inhibits adenylyl cyclase, reducing intracellular cAMP levels, whereas the G βγ dimer modulates ion channels by activating K⁺ channels and inhibiting Ca²⁺ channels, thereby producing neuronal inhibition.

Structural studies of GABA_BRs in different conformational states, including apo, antagonist-bound, agonist-bound, and agonist plus positive allosteric modulator (PAM)-bound, have provided valuable insights into conformational rearrangements associated with ligand binding.^{155,650} However, the precise atomic details of G-protein activation by this heterodimer remain incompletely understood.

GABA_BRs are present as autoreceptors, that inhibit GABA release, or heteroreceptors that reduce the release of glutamate, norepinephrine, serotonin, or dopamine. Binding of GABA to

presynaptic GABA_BRs also modulate three major effector systems: adenylyl cyclases, voltage-sensitive Ca²⁺ channels and G-protein inwardly-rectifying K⁺ (GIRK) channels to cause hyperpolarization, limiting glutamate release and general neuronal activity.^{597,651} Notably, the coupling of effectors is not strictly compartmentalized: accumulating evidence indicates that GABA_BRs can also activate K⁺ channels at presynaptic terminals, contributing to reduced transmitter release and, conversely, inhibit postsynaptic Ca²⁺ channels, thus regulating dendritic and spine excitability. Collectively, current data support a complex signalling topology in which GABA_BRs modulate multiple G protein–gated inwardly rectifying K⁺ channels and voltage-gated Ca²⁺ channels at both pre- and postsynaptic sites, culminating in neuronal inhibition.⁶⁴⁹

1.3.4. *Peripheral GABA Receptors*

Multiple pharmacological, biochemical, and autoradiographic studies demonstrate that GABA receptors are not confined to the CNS. Peripheral expression has been documented in gastric G cells, dorsal root ganglia, optic nerve, nodose and trigeminal ganglia, enteric nervous system, adrenal gland, salivary gland, small intestine, liver, kidney, male and female reproductive tissues, pancreas, pituitary, lung, and cardiac sinoatrial node.⁶⁵²

GABA_BRs are likewise reported across peripheral organs, including reproductive tissues, the nodose ganglion, stomach, adrenal, pituitary, spleen, kidney, and small intestine.^{653–658}

In the gut, GABA_BRs inhibit excitatory cholinergic output in several preparations, reducing electrically evoked twitch responses and dampening peristaltic drive in a tetrodotoxin- and antimuscarinic-sensitive manner. Species- and region-specific effects are evident, and the presynaptic inhibition of acetylcholine release (with limited effects on classic inhibitory cotransmitters in many contexts) is the common characteristic. By contrast, evidence for GABA_BRs participation in electrolyte transport is comparatively sparse and often overshadowed by GABA_ARs-mediated mechanisms, underlining the circuit selectivity of GABA_BRs control.^{657,659} Beyond motility, GABA_BRs modulate afferent pathways. Vagal and spinal afferents express functional GABA_BRs; agonism reduces responses to colorectal distension and can synergize with established analgesic pathways.⁶⁶⁰ At the foregut, GABA_BRs influence gastric tone and emptying via enteric and vagal mechanisms and critically suppress transient lower esophageal sphincter relaxation, the motor event driving gastroesophageal reflux.^{661,662}

Moreover, it is well established that GABA_BRs selective agonists reduce airway responsiveness to diverse bronchoconstrictors by inhibiting presynaptic acetylcholine release from parasympathetic nerves.⁶⁶³

Paradoxically, the GABA_BRs agonist baclofen has been reported to exacerbate methacholine-induced airway responses in patients with asthma.⁶⁶⁴ This unexpected facilitation suggests the presence of postganglionic GABA_BRs that couple to G proteins, starting a signalling pathway known to impair airway smooth muscle (ASM) relaxation.^{665,666}

In addition to this, studies in humans and rodents report GABA_BRs transcripts/proteins in β -cell lines and islets, with context-dependent effects on insulin secretion and pancreatic β -cell differentiation; some human islet datasets show limited GABA_BRs functionality compared with rodents, underscoring species and preparation differences.^{667,668}

On the other side, extrasynaptic GABA_ARs (commonly bearing $\alpha 4/\alpha 6\delta$ or $\alpha 5\gamma$ subunits) exhibit extrasynaptic-like features in the periphery, continuously sensing low, submicromolar GABA derived from the circulation, pancreatic islets, local paracrine sources, and even gut microbiota.^{669–672} The direction and magnitude of GABAergic currents in non-neuronal cells depend critically on intracellular chloride homeostasis, which is governed by sodium-potassium-chloride cotransporter (NKCC) and potassium-chloride cotransporter (KCC) transporters and can yield currents that resemble either extrasynaptic or synaptic profiles.⁶⁷²

Multiple GABA_ARs subunits are expressed in human islets at mRNA and protein levels, supporting the notion that GABA_ARs, more than GABA_BRs, dominates β -cell modulation. Further studies also indicated altered subunit expression in type 2 diabetes (reduced $\alpha 1$, $\alpha 2$, $\beta 2$, $\beta 3$), consistent with impaired GABAergic modulation).^{673,674}

Moreover, within the gastro-intestinal tract GABA_ARs activation can be excitatory in myenteric neurons due to Cl⁻ accumulation via NKCC1/2, resulting in depolarizing GABAergic responses. Pharmacological studies in rodent colon indicate that GABA contributes to both excitatory and inhibitory motor programs, including the peristaltic reflex, partly via GABA_ARs effects on cholinergic and VIP-ergic neurons.^{675,676}

In the liver, early binding studies on isolated hepatocytes established specific GABA_ARs sites, while expression analyses suggested atypical subunit profiles dominated by $\beta 3$ and ϵ in human tissue.^{677–679} The absence of $\gamma 2$ implies limited BZDs sensitivity at hepatic receptors. Intriguingly, GABA metabolism in liver deviates from the canonical brain/pancreas GABA

shunt; GAT may participate in GABA synthesis, and hepatocyte-derived GABA can act as a paracrine hepatokine linked to glucose homeostasis and steatosis.^{680,681}

Renal GAD is abundant (second only to brain), even though with cofactor/ligand sensitivities distinct from neural GAD; GAT is similar to the neural enzyme, and GAT2 supports active GABA uptake.^{682–684} These elements collectively indicate a complete renal GABA cycle, although human subunit stoichiometries and cell-type-specific functions remain to be defined.

The vasculature expresses GABA_ARs in endothelium and VSM. Some studies already demonstrated GABA-evoked relaxation in brain arteries.^{685,686} Clinical and *ex vivo* evidence shows BZDs directly vasodilate peripheral arteries independent of translocator protein (TSPO), implicating vascular GABA_ARs as drug targets.^{687–689} GABA_ARs agonists (muscimol, propofol) hyperpolarize VSM and attenuate myogenic tone.⁶⁹⁰ In addition, endothelial cells can synthesize and release GABA, supporting a local autacoid model in which endothelial GABA tonically engages GABA_ARs in VSM to modulate vascular tone.^{691,692} These observations elevate vascular GABA_ARs as potential antihypertensive targets.

In the lung, discrete GABA_ARs subtypes are present in ASM, epithelium, and immune cells. Laser capture microdissection and immunocytochemistry identified $\alpha 4$, $\alpha 5$, $\beta 3$, δ , $\gamma 1$ – 3 , π , and ρ transcripts/proteins in human ASM; $\alpha 4$ -selective modulators (gaboxadol, taurine; imidazobenzodiazepines) and an $\alpha 5$ -selective ligands further reduce ASM Ca^{2+} influx, store-operated Ca^{2+} entry, and oscillations, consistent with GABA_ARs -dependent Cl^- conductance affecting Ca^{2+} handling.^{693–696} These findings motivate lung-targeted GABA_ARs modulators for asthma, aiming to minimize CNS exposure while leveraging peripheral efficacy.^{696,697}

Reproductive organs display broad GABA_ARs subunit expression and GABAergic regulation of uterine contractility and male gamete function, with much evidence from animal studies.^{698,699} In humans, the uterus expresses an extensive subunit repertoire, with high levels of $\beta 3$, ϵ , θ , $\alpha 2$, $\beta 2$, π , and GABA/GAD enzymes increase with gestational context.^{700–703} Testis and sperm express GABAergic markers and selective $\alpha/\beta/\gamma$ subunits, though functional stoichiometries in human tissue require clarification.^{704,705}

Numerous lines of evidence suggest that human immune cells form functional, extrasynaptic-like GABA_ARs and that B cells, dendritic cells, macrophages, and T cells possess the machinery for GABA synthesis and catabolism.^{692,706,707} Components of the GABA signalling machinery have been identified in various human immune cells, such as peripheral blood mononuclear

cells (PBMC), CD4⁺ T cells and CD8⁺ T cells, macrophages, lymphocytes and neutrophils from psoriatic skin, peripheral T lymphocytes, myocardial macrophages and.^{708–713} Receptors composed of $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 1$, $\beta 2$, $\gamma 2$, δ , ϵ , $\rho 2$, and $\rho 3$ were highly expressed in peripheral blood mononuclear cells (PBMCs), with no differences between individuals, with the $\rho 2$ subunits having the most prominent expression level. Moreover, it has been found that GABA_ARs can influence the proliferation of immune cells, can cause the activation or suppression of cytokine secretion, and can even influence cell migration. The availability of GABA ensures GABA signalling in the immune system because GABA is released from the immune cell itself or when immune cells enter the brain.^{714,715} Myocardial macrophages express GABA_ARs as well and have key roles in cardiac remodelling and dysfunction.⁷¹³ Functional GABA_ARs are expressed in both human and rodent CD4⁺ and CD8⁺ T cells, but the subtypes of these receptors differ between species.⁷⁰⁶ The expression of GABA_ARs in PBMCs is determined by the physiological and pathological status of the individual, but is also sex-dependent, considering that different subunits are expressed in men and women, in pregnant women and people with mental disorders.⁷¹⁴ Similar changes could be found in the expression and function of some subtypes of GABA_ARs in the brain, due to the GABA modulation by different steroid hormones and also health/disease status.⁷¹⁶ Therefore, it is not unusual that a similar pattern of modulation is established for other subtypes of peripheral GABA_ARs. These differences highlight the importance that the physiological environment of cells has on receptor expression.⁷¹⁴ Although most GABA_ARs in the brain are composed of $\alpha\beta$ subunits which are most often combined with $\gamma 2$ or δ , in PBMCs it is possible for the $\rho 2$ subunit to combine with other subunits to form functional GABA_ARs. Given that ρ -containing GABA_ARs are involved in migration of newborn neurons, it is possible that these receptors may be involved in the migration of PBMCs also.^{603,717} T cells and macrophages express both GABA_ARs and GABA_BRs, and GABAergic signalling can dampen pro-inflammatory responses and bias toward regulatory phenotypes. In particular, GABA can also suppress immune activity. B cells, which can synthesize and release GABA, appear to dampen anti-tumour immunity by inhibiting nearby macrophages and cytotoxic CD8⁺ T cells, key effectors of tumour cell killing. In a murine model, B-cell-deficient mice exhibited smaller tumour volumes than WT controls, consistent with the absence of B-cell-derived GABA; exogenous GABA restored tumour growth toward WT levels. Conversely, antagonism of GABA_A receptors with picrotoxin in WT mice reduced tumour volume, consistent with enhanced CD8⁺ T-cell cytotoxicity. These findings suggest that GABA_ARs blockade could augment anti-tumour responses in the periphery.^{692,718}

1.4. Phasic and Tonic Inhibition

GABA receptors exert inhibitory control through two distinct mechanisms: phasic and tonic inhibition. Neuronal communication occurs via neurotransmitter release and propagation of action potentials, with GABA serving as the principal inhibitory neurotransmitter in the CNS. Upon arrival of an action potential at the presynaptic terminal, GABA stored in synaptic vesicles is released into the synaptic cleft. Because these vesicles contain high concentrations of GABA, the synaptic cleft experiences a transient but sharp increase in GABA levels, briefly activating postsynaptic GABA_ARs. This activation causes the receptor's chloride-permeable ion channels to open, increasing Cl⁻ influx into the postsynaptic neuron, thereby hyperpolarizing the membrane and reducing neuronal excitability (Figure 77, panel A). This transient inhibition defines phasic inhibition. The inhibitory event is short-lived due to the rapid reuptake of GABA by GATs and its diffusion away from the cleft, a process known as spillover. Spillover may result from synchronous release of multiple vesicles from the same terminal or from convergent activity of neighbouring GABAergic neurons (Figure 77, panel B).

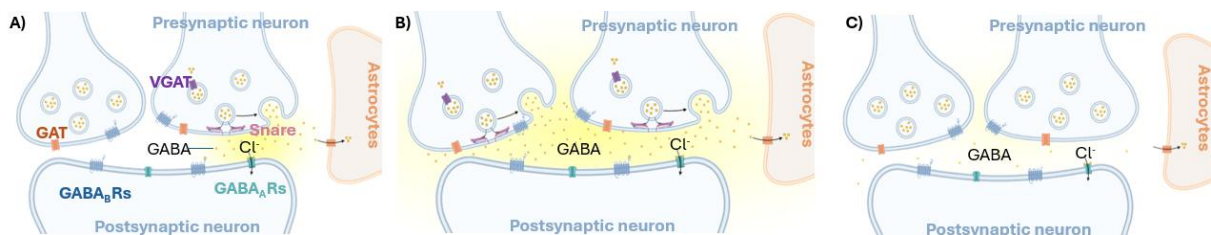


Figure 77. (A) Release of a single synaptic vesicle from the presynaptic terminal activates only the synaptic GABA_ARs clustered directly beneath the release site. The diffusion of GABA is indicated in yellow dots; the shaded area represents the total charge transfer. (B) Action potential-dependent release of multiple vesicles or from several terminals causes GABA spillover, activating both synaptic and extrasynaptic receptors. (C) Low concentrations of GABA, which persist despite the activity of neuronal and glial GATs, tonically activate high affinity extrasynaptic receptors. The conductance of tonically active GABA_ARs is several-fold greater than that of averaged phasic one.

Despite efficient reuptake, some GABA escapes the synaptic cleft and interacts with extrasynaptic or presynaptic receptors, which have higher affinity for GABA and exhibit lower rates of desensitization. These receptors mediate tonic inhibition, a sustained form of inhibitory control that modulates overall neuronal excitability rather than discrete synaptic events (Figure

77, panel C). Increased tonic inhibition via GABA_ARs has been implicated in several neurological disorders, most notably Alzheimer's disease, where elevated brain GABA levels correlate with enhanced tonic conductance.⁷¹⁹ Interestingly, selective reduction of α 5-containing GABA_ARs, a subtype strongly associated with tonic inhibition, has been shown to enhance memory and cognitive performance in animal models of Alzheimer's disease. Furthermore, negative allosteric modulators (NAMs) selective for α 5 improve cognitive outcomes without producing convulsant effects, which are commonly seen with non-selective GABA NAMs. Conversely, excessive reduction of α 5 has been observed in schizophrenia and autism spectrum disorders, suggesting that an optimal balance of tonic inhibition is necessary for normal cognitive and affective function.⁷²⁰ These findings highlight α 5 as a potential pharmacological target, where modulation rather than complete inhibition of tonic currents may offer therapeutic benefit.

Extrasynaptic GABA_ARs containing α 4, α 5, α 6, and/or δ subunits are the principal mediators of tonic inhibition.⁷²¹ However, GABA_{A ρ} Rs composed of ρ 1 or ρ 2 subunits also localize extrasynaptically, particularly within the hippocampus and retina.⁷²² The extrasynaptic positioning of these receptors corresponds to their exceptionally high sensitivity to GABA, with EC₅₀ values of approximately 1 μ M for GABA_{A ρ} Rs compared to ~16.5 μ M for typical GABA_ARs.⁶⁴⁶

If GABA_ARs mediate the fast component of synaptic inhibition (milliseconds timescale) and account for most of the extrasynaptic tonic inhibition in the brain, in contrast, GABA_BRs generate slow inhibitory responses lasting from hundreds of milliseconds to several seconds and exert powerful presynaptic control over neurotransmitter release through both auto- and hetero-inhibition. Although the effects mediated by GABA_BRs are less pronounced in terms of instantaneous current amplitude, they play crucial modulatory roles. After synaptic release, peri- and extrasynaptic GABA_BRs are engaged by GABA. On the postsynaptic membrane, GABA_BRs activation induces the opening of GIRK channels, producing membrane hyperpolarization and slow inhibitory postsynaptic potentials that typically last from hundreds of milliseconds to several seconds. At presynaptic terminals, the same signalling cascade inhibits voltage-gated Ca²⁺ channels, lowering intraterminal Ca²⁺ and thereby reducing transmitter release probability, both autoinhibition of GABAergic boutons and heteroinhibition of glutamatergic inputs. Together, these actions constitute the slow, phasic component of inhibition mediated by GABA_BRs.^{723,724} In parallel, when ambient GABA is present,

GABA_BRs can be persistently engaged, giving rise to tonic inhibition. Postsynaptically, this manifests as a baseline K⁺ conductance (again via GIRK) that shunts excitatory drive and stabilizes membrane potential. Presynaptically, sustained GABA_BRs signalling tonically suppresses vesicle release by keeping Ca²⁺ entry in check. The tonic GABA_BRs activity is enhanced when GATs are pharmacologically blocked and can be reinforced by glial GAT-3-dependent GABA release, underscoring the contribution of astrocytes and transport processes to slow inhibitory control at both pre- and postsynaptic sites.^{725,726}

1.5. GABAergic signalling in cancer

GABA signalling represents a complex and highly context-dependent network in cancer biology. Although primarily recognized for its inhibitory function in the CNS, accumulating evidence indicates that GABAergic signalling components, including GABA itself, its synthetic enzymes, transporters, and receptors, are aberrantly expressed across numerous malignancies and can profoundly influence tumour proliferation, migration, invasion, and metastasis. The functional output of this pathway depends largely on the specific receptor subtype engaged, the downstream signalling environment, and the histological origin of the tumour. In most cases, GABA_ARs regulate cellular proliferation, whereas GABA_BRs are more closely linked to invasive and metastatic behaviours. However, this dichotomy is not absolute, as both receptor classes can display oncogenic or tumour-suppressive properties depending on their subunit composition and cellular context.⁷²⁷ Several studies have reported that GABA and its receptors exhibit markedly altered expression patterns in malignant tissues compared with their normal counterparts, often correlating with clinical aggressiveness and patient outcomes. GABA can function as either a tumour promoter or suppressor, depending on the tissue type. Elevated GABA signalling promotes tumour development in prostate, gastric, ovarian, and oral squamous cell carcinoma, whereas in NSCLC, pulmonary adenocarcinoma (PAC), CRC, and cholangiocarcinoma it exerts inhibitory effects on cell proliferation and migration. Interestingly, certain malignancies such as HCC, pancreatic ductal adenocarcinoma (PDAC), and BC display bidirectional GABAergic influences.^{84,719,728–733} In HCC, GABA enhances proliferation through the GABA_AR θ and $\alpha 3$ subunits, yet restoration of the $\beta 3$ subunit attenuates tumour growth.^{679,733–735} Similar dualism occurs in pancreatic ductal adenocarcinoma (PDAC), where GABA promotes proliferation via GABA_AR π but inhibits cell growth and migration through suppression of β -adrenergic-dependent signalling. In BC, GABA_AR $\alpha 3$, GABA_AR π , and GABA_BR 1 subunits drive proliferation and invasiveness, although activation of GABA_BRs by

baclofen has been shown to inhibit norepinephrine-induced cell migration.^{736–741} Likewise, increased GABA_BRs 2 subunit expression in NSCLC correlates with a better prognosis, suggesting that receptor subtype balance may dictate whether signalling is pro- or anti-tumorigenic.⁸⁴ This complexity underscores the necessity of dissecting GABA receptor cross-talk in specific cellular contexts to determine its therapeutic potential.⁷²⁷

The contribution of GABA_ARs to tumorigenesis extends beyond classical neuronal inhibition. These pentameric ligand-gated chloride channels, composed of various subunit combinations (α , β , γ , δ , π , θ , ρ , etc.), can mediate oncogenic signalling through altered ion fluxes and secondary messenger cascades. Overexpression of GABA_AR θ and GABA_AR $\alpha 3$ in HCC tissues enhances malignant proliferation and invasiveness, whereas silencing these subunits suppresses tumour growth both *in vitro* and *in vivo*.^{734,735} Conversely, GABA_AR $\beta 3$ expression inversely correlates with HCC progression, functioning as a potential tumour suppressor.⁶⁷⁹ In PDAC and basal-like breast cancers, GABA_AR π promotes tumorigenesis, proliferation, and migration, while in oral squamous cell carcinoma (OSCC), its activation correlates with enhanced metastatic potential.^{730,736,740} GABA_ARs activity is also implicated in GC, where GABA stimulates tumour cell proliferation through the MAPK/ERK cascade and upregulation of cyclin D1.⁷²⁹ Furthermore, thyroid cancers show higher GABA_ARs expression than normal tissue, with subunit-specific distributions suggesting roles in angiogenesis and metastasis; GABA_AR $\beta 2$ localizes to tumour vasculature, while GABA_AR $\alpha 2$ appears enriched in metastatic derivatives.⁷⁴² Collectively, these findings indicate that GABA_ARs subunit composition determines whether the receptor functions as a promoter or inhibitor of tumour growth.

On the other hand, GABA_BRs stimulate invasion in prostate and breast cancers but suppress proliferation and migration in breast, colon, NSCLC, HCC, and cholangiocarcinoma.^{84,728,732,733,738} GABA_BR 1e, an alternatively spliced variant lacking a conventional transmembrane domain, enhances BC malignancy by facilitating proliferation and metastasis *in vitro* and *in vivo*.⁷³⁹ High GABA_BR 2 expression combined with low GABA_AR $\alpha 3$ expression predicts favourable clinical outcomes in NSCLC.⁸⁴ These observations imply that GABA_BR signalling can differentially regulate cancer cell behaviour depending on the subunit isoforms expressed and the intracellular signalling milieu.

The complexity of GABAergic signalling arises from its integration into multiple canonical oncogenic pathways. Among these, the cAMP, EGFR, AKT, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), and MMP cascades have been most

extensively characterized. These pathways not only control proliferation and survival but also orchestrate migration, invasion, and immune modulation. The cAMP pathway plays a pivotal role in determining the biological response to GABA.⁷²⁷ In several malignancies, including NSCLC, PAC, and cholangiocarcinoma, GABA suppresses proliferation and migration through downregulation of cAMP signalling.^{84,731,733} Similarly, in CRC and HCC, GABA_ARs and GABA_BRs activation inhibits migration by decreasing intracellular cAMP.^{704,732,743} The mechanistic connection likely involves inhibition of adenylyl cyclase through G protein coupling, leading to reduced protein kinase A (PKA) activity.

EGFR signalling is another critical axis influenced by GABA receptors. EGFR family members are frequently dysregulated in epithelial cancers, and GABAergic inputs can modulate their activity. In prostate cancer, GABA_ARs activation by 5 α -androstane-3 α ,17 β -diol converts androgen-dependent cells into an androgen-independent phenotype via EGFR activation.⁷⁴⁴ Increased GABA_AR α 1 expression enhances tumour growth by stimulating EGFR and its downstream kinase Src.⁷¹⁹ GABA_BR can transactivate EGFR in a ligand-dependent manner, promoting migration and invasion of prostate cancer cells: while GABA_BRs 1e displaces the phosphatase PTPN12 from EGFR, thereby sustaining receptor phosphorylation and amplifying downstream proliferative signalling in BC.^{738,745} These events integrate with the PI3K/AKT and MAPK pathways to propagate oncogenic outputs.

The AKT pathway serves as a central node downstream of GABA_BR. AKT, a serine/threonine kinase, regulates cell survival, migration, and EMT. Overexpression of GABA_AR α 3 enhances BC cell migration and metastasis by activating AKT independently of EGFR.⁷⁴¹

MAPK/ERK signalling provides another conduit for GABA-dependent modulation of tumour growth. The MAPK family transduces extracellular cues into transcriptional programs that govern proliferation and differentiation; dysregulation results in sustained mitogenic signalling. GABA_ARs activation elevates intracellular Ca²⁺ levels, leading to activation of Ras and ERK.⁷⁴⁶ In particular, GABA_AR π enhances tumour aggressiveness by stimulating the MAPK/ERK cascade in PDAC, basal-like breast, ovarian, and oral cancers.^{730,736,740} In gastric carcinoma, GABA promotes proliferation through MAPK/ERK activation and cyclin D1 upregulation.⁷²⁹

Recent research has expanded the role of GABA from a neurotransmitter to a key metabolite in immune regulation. Immune cells express functional GABA receptors and can both synthesize and respond to GABA, positioning this molecule as a link between metabolism and immunity. Activated B cells produce and secrete GABA, which in turn promotes IL-10 receptor

expression, facilitates the differentiation and survival of anti-inflammatory macrophages, and attenuates cytotoxic CD8⁺ T-cell responses via GABA_ARs activation. Pharmacologic modulation of this axis reveals potential therapeutic implications: the GABA_ARs agonist muscimol suppresses CD8⁺ T-cell cytotoxicity, affecting the antitumoral response, while the antagonist picrotoxin enhances T-cell activity sorting the opposite effect.⁷¹⁸ Hence, GABA metabolism within the tumour microenvironment can either dampen or amplify antitumour immunity depending on the dominant receptor pathway and metabolic context.

1.6. GABA_ARs antagonists

The way fast inhibitory neurotransmission in the mammalian brain is exerted by GABA_ARs was already described in this chapter. GABA_ARs subunit composition varies with synaptic localization and helps specify the mode of inhibition they mediate. At synapses, GABA_ARs are most often assembled from $\alpha 1$, $\alpha 2$, or $\alpha 3$ together with $\beta 2/\beta 3$ and $\gamma 2$ subunits, with $\alpha 1\beta 2\gamma 2$ emerging as the predominant synaptic subtype. Synaptic GABA_ARs convert brief, millisecond-scale GABA transients into short-lived phasic inhibitory chloride currents that can operate at high frequencies, supporting rapid information processing. By contrast, perisynaptic/extrasynaptic GABA_ARs are tuned to the lower, slowly fluctuating ambient GABA levels; they exhibit higher apparent GABA sensitivity and reduced desensitization relative to synaptic receptors, thereby mediating tonic inhibition. These extrasynaptic receptors are dominated by the $\alpha 4\beta \delta$, $\alpha 6\beta \delta$, and $\alpha 5\beta \gamma 2$ subtypes.⁷⁴⁷ Outside the brain, GABA_ApRs composed of $\rho 1$ – $\rho 3$ subunits are expressed mainly in the retina. In functional $\alpha 1\beta 2\gamma 2$ GABA_ARs, orthosteric GABA-binding pockets reside at each $\beta 2$ – $\alpha 1$ extracellular interface, while an additional allosteric BZD site is positioned at the $\alpha 1$ – $\gamma 2$ interface. Intensive drug discovery at the BZD site has yielded several marketed agents, like **zolpidem**, **clonazepam** (Figure 78).

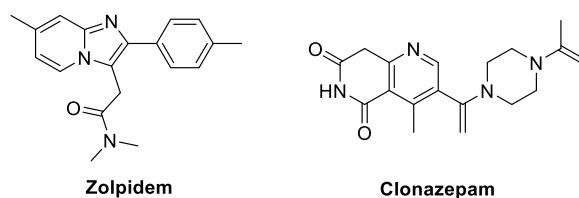


Figure 78. Structure of two allosteric binding BZD site marketed agents.

By contrast, comparatively little effort has focused on orthosteric ligands, likely because the high sequence/structural conservation of the orthosteric pocket across GABA_ARs subtypes hampers the attainment of subtype selectivity, increasing the risk of off-target effects.

Nonetheless, selective orthosteric modulation is achievable. An overview of several principal classes of GABA_ARs antagonists is outlined below.

1.6.1. Orthosteric competitive GABA antagonists

Selective receptor antagonists are indispensable probes for defining receptor functions in physiology and pharmacology. A canonical example is the plant alkaloid **bicuculline** (Figure 79), which rapidly became a reference ligand and substantially advanced the pharmacological characterization of GABA_ARs in the CNS.^{748,749} Subsequent medicinal-chemistry efforts yielded multiple bicuculline-related analogues that retain the same qualitative profile, albeit typically with reduced potency.⁷⁵⁰ A practical limitation of bicuculline is its modest chemical stability and aqueous solubility; accordingly, pharmacological studies frequently employ the more stable and water-soluble quaternary salts, like **bicuculline methochloride (BMC)** or **bicuculline methiodide** as functional surrogates.^{751–753} **Gabazine** (Figure 79) is, like bicuculline, an antagonist of GABA_ARs; however, the two compounds exhibit distinct pharmacological profiles. Notably, the determinants of insensitivity in $\rho 1$ – $\rho 3$ GABA_ARs differ for bicuculline versus gabazine, implying that these structurally unrelated antagonists engage the orthosteric site in mechanistically different ways.⁶⁴⁴ Moreover, gabazine competitively inhibits the binding of [³H]NCS382, a selective ligand for high-affinity γ -hydroxybutyrate (**GHB**) sites: a property unique among GABA_AR ligands suggesting that gabazine's actions are not confined to the classical $\alpha\beta$ orthosteric interfaces.^{747,754}

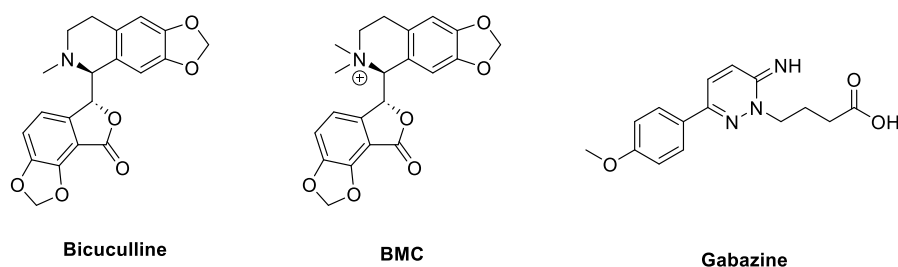


Figure 79. Structure of reference GABA_ARs antagonists **bicuculline**, **BMC** and **gabazine**.

A distinct chemotype of GABA_ARs antagonists, exhibiting mid–high nanomolar potency and built on a 3,9-diazaspiro[5.5]undecane core, has emerged from library screening.⁷⁵⁵ Notably, the primary hit **179** and the close analogue **180** (Figure 80) lack an acidic functionality, a departure from many classical orthosteric scaffolds. Functionally, these ligands preferentially engage receptors bearing $\alpha 3/\alpha 4/\alpha 5$ subunits over those containing $\alpha 1/\alpha 6$, while showing little discrimination among β or γ/δ partners. Madin-Darby canine kidney multidrug resistance 1

(MDCK-MDR1) permeability assays further indicate minimal passive permeability for both **179** and **180**, limiting their usefulness for probing central GABA_ARs mechanisms but, conversely, recommending them as probes of peripheral GABA_ARs-mediated phenomena.⁷⁵⁵ From a medicinal-chemistry standpoint, sp³-rich, three-dimensional scaffolds, and spirocycles have gained prominence as privileged platforms in contemporary drug design, often conferring improved target engagement, subtype selectivity, and pharmacokinetic profiles relative to flatter, sp²-dominated frameworks.^{756,757} Follow-up optimization identified a simpler spirocyclic scaffold, **181**, less potent than **180** but exhibiting substantially greater selectivity for $\alpha 4$ -containing extrasynaptic receptors over $\alpha 1/\alpha 2$ -containing synaptic subtypes. Consistent with prior evidence that the channel blocker **picROTOXIN** can reduce tumour in murine models by disinhibiting T cells via GABA_ARs blockade, the spirocyclic series was evaluated for immunomodulatory activity. In human and mouse CD8⁺ T cells, exposure to the antagonist **181** produced a more robust rescue of proliferative capacity than **bicuculline**, while maintaining minimal cytotoxicity despite the use of a lower concentration of **181** (50 μ M) compared with bicuculline (100 μ M).^{718,758,759}

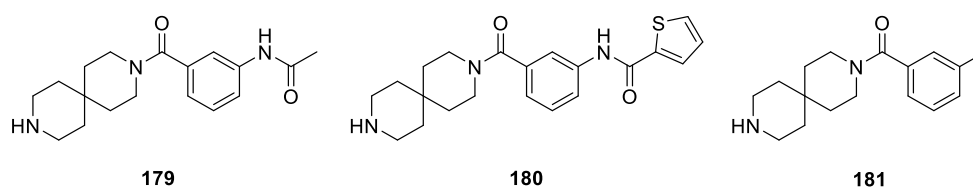


Figure 80. Structures of the GABA_AR antagonists **179–181**.

Muscimol, a natural constituent of *Amanita muscaria*, has served as a key scaffold for designing potent GABAergic ligands, including antagonists (Figure 80). Chemically, muscimol is a 3-hydroxyisoxazole bioisostere of the GABA carboxylate and acts as a high-potency GABA_ARs agonist. In addition to receptor activation, muscimol inhibits neuronal and glial GABA uptake and serves as a substrate of GABA transaminase.⁷⁶⁰ Systematic structural modifications, most notably the creation of conformationally restricted annulated and non-annulated bicyclic analogues, have yielded a suite of GABAergic compounds such as **4,5,6,7-tetrahydroisoxazol[5,4-*c*]pyridin-3-ol (THIP)**, **4,5,6,7-tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol (THPO)**, and **iso-THIP** (Figure 81).⁷⁶¹ Although these derivatives appear superficially similar, their pharmacological profiles diverge markedly: **THPO** functions as a selective GABA uptake inhibitor, with a degree of preference for glial transport systems, whereas **THIP** is a potent, selective GABA_ARs agonist.^{747,762,762–764}

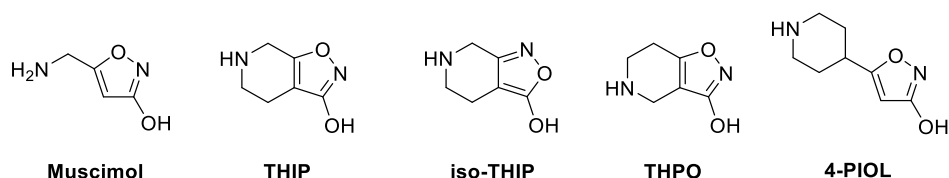


Figure 81. Chemical structures of muscimol and bicyclic muscimol analogues **THIP**, **iso-THIP**, **THPO** and **4-PIOL**.

A particularly instructive manipulation is the oxygen–nitrogen interchange within the five-membered ring of **THIP** to generate **iso-THIP**, which switches functional activity from agonism to antagonism. Despite close structural resemblance, electronic properties differ substantially between these scaffolds: in **THIP** and related 3-hydroxyisoxazoles, the aromatic bioisostere mimics the GABA carboxylate, whereas in the 5-hydroxyisoxazole system present in **iso-THIP** the negative charge is differently delocalized, a feature that likely underlies the antagonist behaviour at GABA_ARs.⁷⁶⁵

The non-annulated bicyclic muscimol analogue **5-(4-piperidyl)-3-hydroxyisoxazole (4-PIOL)** (Figure 82) also incorporates the 3-hydroxyisoxazole motif; however, **4-PIOL** acts as a low-efficacy partial agonist at GABA_ARs.⁷⁶⁶ This observation provided a rational starting point for further scaffold optimization of **4-PIOL** into series of compounds with robust GABA_ARs antagonism. **4-PIOL** has motivated the synthesis of multiple GABA_ARs antagonists that have been instrumental in defining both the dimensions and topology of the orthosteric site and the molecular features that distinguish agonism from antagonism. New antagonist chemotypes emerged by replacing the 3-hydroxyisoxazole of **4-PIOL** with 3-hydroxyisothiazole (**thio-4-PIOL**) and with 1- or 3-hydroxypyrazole rings, namely **4-(4-piperidyl)-1-hydroxypyrazole (4-PHP)** and **5-(4-piperidyl)-3-hydroxypyrazole (aza-4-PIOL)**, respectively (Figure 82).^{767–}

770

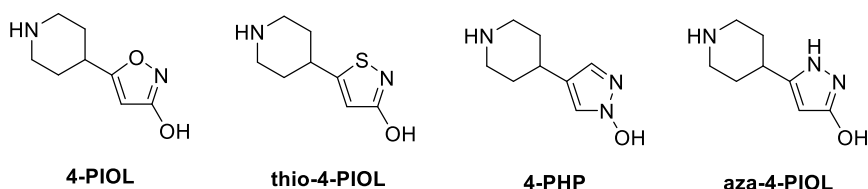


Figure 82. Chemical structures of bicyclic muscimol analogues **4-PIOL**, **thio-4-PIOL**, **4-PHP**, **aza-4-PIOL**.

The negative charge of the deprotonated hydroxy group in these analogues, a key determinant for orthosteric recognition, delocalizes into the heteroaromatic ring, producing an electrostatic

potential that mimics GABA carboxylate.⁷⁷¹ The mentioned examples provide evidence that orthosteric ligands for GABA_ARs are typically GABA mimetics, bearing a protonated ammonium and a carboxylate group. Nonetheless, bicuculline and related spirocyclic analogues lack a carboxylic acid, indicating that this moiety is not strictly required for occupation of the orthosteric site.⁷⁵⁶ From a drug-design perspective, replacing the carboxylate can be advantageous, because carboxylic acids are prone to glucuronidation, a metabolic pathway that may contribute to hepatotoxicity with potentially fatal outcomes.⁷⁷² An illustrative example is **2-guanidine-4-methylquinazoline (GMQ)**, originally identified as a H₂ and muscarinic receptors antagonist with antihistaminergic and antisecretory effects and as an acid-sensing ion channel 3 (ASIC3) agonist.^{773,774} More recently, **GMQ** was shown to act as a competitive GABA_ARs antagonist with potency comparable to bicuculline: at 10 μM, **GMQ** robustly inhibits GABA_ARs while exerting little or no effect on other Cys-loop family members, underscoring receptor selectivity. Structurally, **GMQ** is a small, rigid molecule (~200 g·mol⁻¹) comprising a hydrophobic quinazoline ring and a hydrophilic guanidinium group that is positively charged at physiological pH, without presenting a carboxylate group. This nonclassical scaffold thus broadens the chemical space available for orthosteric GABA_ARs antagonists.

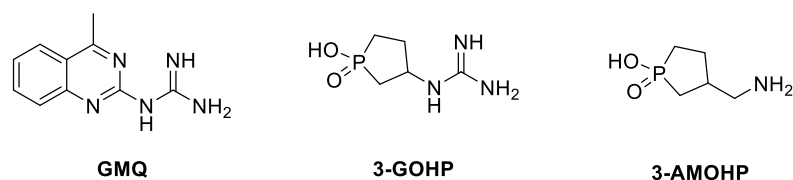


Figure 83. Structures of the GABA_ARs orthosteric antagonists, **GMQ**, **3-AMOHPP** and **3-GOHP**.

Mutagenesis at E155 of β2 and at F64 of α2 is critical for **GMQ** binding in the orthosteric pocket, since these residues govern GABA recognition, implying that **GMQ**'s guanidinium functionally mimics GABA's amine.⁷⁷⁵ Subsequent work revealed that **GMQ** also antagonizes GABA_ApRs (IC₅₀ ≈ 36 μM).⁷⁷⁶ Moreover, guanidinium substitution can improve selectivity for GABA_ApRs over canonical GABA_ARs: for example, **3-(guanidino)-1-oxo-1-hydroxy-phospholane (3-GOHP)** displays greater GABA_ApRs preference and reduced GABA_ARs inhibition compared to **3-(aminomethyl)-1-oxo-1-hydroxy-phospholane (3-AMOHPP)** (Figure 83). In the same study, replacing the basic headgroup (guanidine or amine) with a

carboxylic acid abolished activity across all GABA receptor classes, underscoring the essential role of a basic moiety for orthosteric engagement.⁷⁷⁷

Because the orthosteric pocket is highly conserved across most $\alpha\beta\gamma$ GABA_ARs assemblies, achieving genuine subtype selectivity with orthosteric ligands remains intrinsically difficult.⁷⁷⁸ Although subtle differences in the local microenvironment, such as those distinguishing α - from δ -containing receptors, can shift apparent potency, the pharmacological separation among common synaptic subtypes ($\alpha 1\beta 2\gamma 2$, $\alpha 2\beta 3\gamma 2$, $\alpha 3\beta 3\gamma 2$) is generally modest.⁶⁰³ Functionally, orthosteric antagonists preferentially suppress phasic synaptic inhibition, but at sufficiently high concentrations they also diminish tonic currents mediated by extrasynaptic receptors.⁷²¹ These compounds therefore serve as gold-standard probe ligands in systems neuroscience and receptor pharmacology, even if their global reduction of inhibitory tone is classically pro-convulsant and anxiogenic, confining clinical utility to short-term or diagnostic applications.⁷⁵⁵

1.6.2. Non-competitive channel blockers

Non-competitive GABA_ARs channel blockers, exemplified by picrotoxin and the convulsant **tert-butylbicyclophosphorothionate (TBPS)** (Figure 84), act within or adjacent to the transmembrane pore to curtail chloride flux independently of occupancy at the orthosteric GABA site.⁷⁷⁹

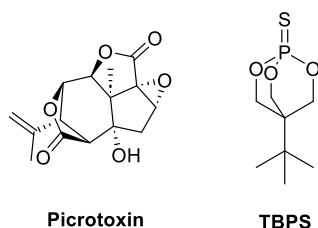


Figure 84. Chemical structures non-competitive GABA_ARs channel blockers *picrotoxin* and *TBPS*.

In particular, **picrotoxin** engages the M2 transmembrane helix of GABA_ARs and thus binds within the ion-conducting pore; indeed, picrotoxin can be trapped when the gate closes.⁷⁸⁰ Notwithstanding its intrapore interactions, pore residence does not necessarily imply simple steric occlusion as the mechanism of block. Although picrotoxin shows use dependence, kinetic analyses argue that its antagonism is allosteric in nature.⁷⁸¹ Consistent with an allosteric component, substituted-cysteine accessibility and site-directed fluorescence experiments reveal picrotoxin-induced conformational rearrangements, including changes around the central

vestibule that lie well above the membrane plane and distant from the putative binding locus.^{780,782} The picrotoxin site is preferentially accessible in the open state for GABA_ARs and computational modelling supports this intrapore mechanism.⁷⁸³

The bicyclophephosphorus ester **TBPS** is advantageous as a pore-site probe because it exhibits low nonspecific binding.⁷⁸⁴ Evidence that the convulsant binding site resides within the GABA_ARs receptor ion channel comes from the observation that anion-dependent enhancement of **TBPS** binding correlates with the permeability of those same anions through the channel.⁷⁸⁵ Moreover, **TBPS** binding is allosterically modulated by ligands acting not only at the orthosteric (GABA) site, but also at the benzodiazepine, barbiturate, loreclezole, anaesthetic, and neurosteroid recognition sites.⁷⁸⁶ Consequently, the modulation of **TBPS** binding has proved a useful *in vitro* indicator of efficacy and potency of compounds acting at the GABA_ARs.

Because the binding of non-competitive channel blockers perturbs the conductive pathway itself, the inhibition is typically non-surmountable by increasing GABA concentrations and often exhibits state dependence, with higher apparent affinity for open or desensitized channel states. Pharmacologically, these agents display broad activity across most $\alpha\beta\gamma$ receptor assemblies, although some degree of relative sparing of GABA_{A ρ} Rs has been reported for blockers.⁶²⁹ Functionally, they produce robust suppression of both phasic synaptic and tonic extrasynaptic inhibition. Crucially, their efficacy is maintained even under high-GABA conditions, such as within the synaptic cleft, because they do not compete with agonist binding. As a result, pore blockers serve as powerful tool compounds for dissecting network dependence on inhibitory tone and for mapping the contribution of GABAergic currents to circuit excitability.^{787,788} However, their lack of subtype selectivity and pronounced CNS liabilities, notably a high risk of seizures/convulsions and anxiogenic effects, preclude chronic therapeutic use, confining their value largely to experimental applications and tightly controlled settings.⁷⁷⁹

1.6.3. Benzodiazepine-site antagonists

Benzodiazepine-site ligands target an allosteric pocket located at the $\alpha\gamma$ interface of $\alpha\beta\gamma$ GABA_ARs, a site present when $\gamma 2$ assembles with $\alpha 1/2/3/5$ subunits.⁷⁷⁸ Within this pharmacological class, neutral antagonists, exemplified by flumazenil, exert little to no effect on basal channel gating but abolish the actions of PAMs such as diazepam or zolpidem.⁷⁸⁹ By contrast, inverse agonists and negative NAMs, classically represented by β -carboline derivatives like **methyl 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate (DMCM)** (Figure 85), reduce GABA efficacy and/or maximum response via the benzodiazepine site. Because

binding determinants differ among α subunits, these NAMs can display α -subtype bias, enabling a degree of functional selectivity across receptor populations.^{790–792}

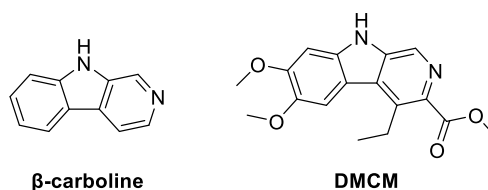


Figure 85. Chemical structures of the benzodiazepine-site antagonist **DMCM** and its parent compound β -carboline.

In detail, several β -carbolines interact with the BZD site of the GABA_ARs and can behave as full, partial, or mixed agonists, antagonists, or inverse agonists.^{793,794} Consistent with this pharmacological diversity, they elicit sedative, anxiolytic, anxiogenic, or even convulsant effects. High-affinity BZD-site inverse agonists such as **DMCM** are proconvulsant/anxiogenic *in vivo*, in line with their inhibitory modulation of GABA_ARs function in electrophysiological and binding assays.⁷⁹⁵ As already mentioned, the BZD site resides at the $\alpha\gamma$ subunit interface and many BZD ligands display subtype-dependent affinity determined by α ($\alpha 1$ – $\alpha 6$) and γ ($\gamma 1$ – $\gamma 3$) isoforms, with $\gamma 2$ -containing receptors forming the principal BZD-sensitive population.^{796,797} Mutagenesis has identified Phe77 of $\gamma 2$ subunit as a critical determinant for high-affinity binding of some BZD-site ligands: substituting Phe77 with an Ile reduces sensitivity to **DMCM** by 1000-fold, while sparing responsiveness to **diazepam** and **flunitrazepam**.⁷⁹⁸ Functionally, the BZD site mediates the GABA-antagonistic actions of **DMCM**, with a prominent contribution from $\alpha 1$ -containing receptors. Conversely, substituting the $\alpha 1$ subunit His100 with Arg confers relative agonist efficacy on **DMCM**, underscoring the fine structural determinants of efficacy at the BZD interface.^{792,795}

Beyond the BZD site, several β -carbolines bind with lower affinity to a second allosteric site on GABA_ARs, where they potentiate receptor function.⁷⁹⁹ This site overlaps functionally with the **loreclezole** site and requires $\beta 2$ or $\beta 3$ subunits.⁸⁰⁰ **Loreclezole** itself (Figure 86) displays anticonvulsant and anxiolytic-like properties through positive allosteric modulation of GABA_ARs.^{792,801,802}

The subtype landscape is critical for interpreting systems effects. Receptors containing $\alpha 1$ are enriched at synapses mediating fast inhibitory transmission and are often linked to sedation and pro-convulsant liability when negatively modulated; correspondingly, $\alpha 1$ -preferring inverse

agonists tend to produce anxiogenic and pro-excitatory outcomes. In contrast, $\alpha 5$ -containing receptors, which contribute disproportionately to tonic inhibition in hippocampal and cortical circuits, are attractive targets for cognition-oriented NAMs: selective reduction of $\alpha 5$ -mediated tonic tone can enhance certain mnemonic processes, although careful dosing is required to avoid network instability. More generally, because the benzodiazepine site can be present on both synaptic and extrasynaptic assemblies (depending on subunit composition and localization), benzodiazepine-site antagonists and NAMs may influence both phasic and tonic inhibition, with circuit-level consequences that reflect the regional distribution of α subtypes.^{31,720,721}

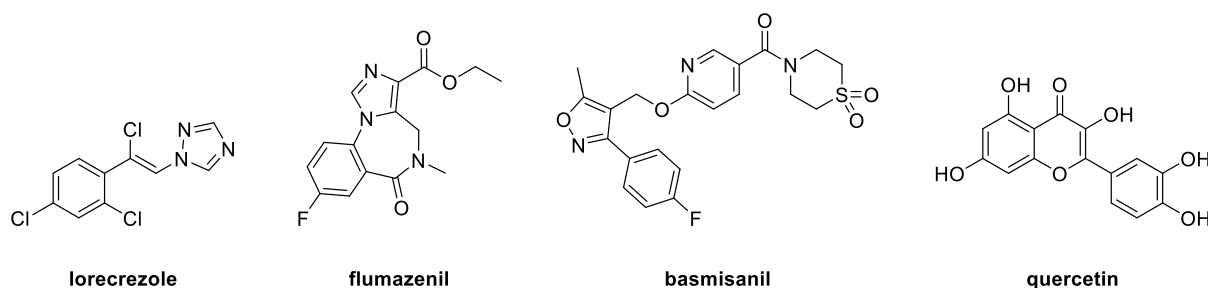


Figure 86. Chemical structures of GABA_ARs NAMs.

Clinically, **flumazenil** (Figure 86) underscores the therapeutic value of benzodiazepine-site antagonism as a reversal agent for benzodiazepine sedation or overdose. Conversely, the translational trajectory of inverse agonists/NAMs is shaped by a trade-off between mechanistic precision and safety: while α -selective designs (especially $\alpha 5$ -biased NAMs) promise more favourable profiles for cognitive indications, $\alpha 1$ -weighted activity remains constrained by anxiogenic and convulsant risks. As a result, current development emphasizes subtype-selective, titratable negative modulation to harness desired circuit effects while minimizing adverse outcomes.^{778,803}

NAMs can also bind outside the benzodiazepine site. Examples are provided by chemotypes such as $\alpha 5$ partial NAMs (e.g., **basmisanil**), phenolic NAMs, and newer series discovered through structure-guided screening (Figure 86).^{619,804} These ligands engage non-BZD allosteric pockets, often inter-subunit cavities in the extracellular domain or transmembrane domain, to dampen GABA_ARs responses without competing at the orthosteric site or physically occluding the pore.⁸⁰⁵ By design, many exhibit subunit selectivity (notably for $\alpha 5$ -containing assemblies), which confers regional bias toward hippocampal and cortical circuits and, in principle, reduces global CNS liabilities relative to nonselective channel blockers.^{806,807} Functionally, NAMs that target $\alpha 5/\delta$ -rich populations tend to exert a disproportionate effect on tonic inhibition, whereas

effects on phasic synaptic currents depend on the prevalence of the targeted subunit at synapses. Conceptually, this tunable, subtype-biased negative modulation offers a favourable therapeutic window compared with classical antagonists; however, clinical translation has been mixed, and rigorous monitoring for cognitive and affective side effects remains essential, particularly when modulating $\alpha 5$ -dependent inhibitory tone in mnemonic networks.

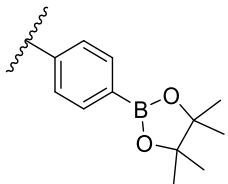
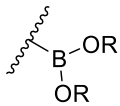
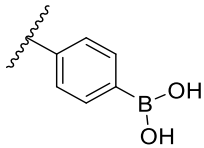
1.7. *ROS-Responsive Prodrugs*

Chemotherapy remains a cornerstone of cancer therapy, but it is hampered by severe systemic toxicities arising from nonspecific biodistribution of cytotoxic agents.^{36,808} To mitigate off-target effects, extensive effort has focused on stimuli-responsive drug delivery systems (DDSs), that release or activate active nucleus selectively within the tumour microenvironment in response to endogenous cues, most commonly pH, glutathione (GSH), enzymes, ROS, or combinations thereof.^{809–812} Because both malignant and healthy cells possess endo/lysosomal compartments with acidic pH and share high intracellular GSH concentrations (≈ 2 – 10 mM), platforms relying exclusively on pH or GSH often provide limited selectivity.^{169,813} By contrast, hypoxic tumour physiology elevates ROS, yielding levels in cancer cells (up to ~ 100 μ M) that far exceed those in normal tissues (≈ 20 nM).⁸¹⁴ This differential has motivated the development of ROS-responsive carriers based on poly(propylene sulfide), selenium-containing copolymers, and polythioether ketals.^{139,815–818} Nevertheless, clinical translation of such polymers has been constrained by complex syntheses, premature drug leakage, suboptimal biocompatibility and degradability, and notable cytotoxicity.^{819,820} In comparison, ROS-responsive prodrugs, small molecules bearing oxidation-labile motifs, offer streamlined synthesis, improved compatibility and degradability, and reduced off-target exposure.^{821,822}

ROS are highly reactive, oxygen-derived molecules, including hydrogen peroxide (H_2O_2), superoxide ($\text{O}_2^-/\text{O}_2^{\bullet-}$), and hydroxyl radical ($\bullet\text{OH}$), that function as second messengers across diverse signalling pathways. Strict regulation of ROS is essential for normal physiology, supporting metabolism, proliferation, and differentiation, whereas sustained elevation precipitates oxidative stress, cellular dysfunction, and tissue remodelling, contributing to cancer, chronic inflammation, and neurodegenerative diseases such as Alzheimer's and Parkinson's.^{823,824} Mitochondria are the principal ROS source through one-electron leakage from respiratory complexes I and III, supplemented by enzymatic generators including NADPH oxidases (NOXs), nitric-oxide synthases, and xanthine oxidase.^{825–827} In healthy cells, redox buffering maintains ROS at very low, quasi-steady concentrations; diseased tissues can sustain

markedly higher levels and adapt their signaling accordingly.^{828–830} These disease-associated gradients render ROS attractive both as biomarkers and as chemical triggers for selective drug release, imaging, and theragnostic intervention. Among ROS, H₂O₂ is particularly informative: less reactive and longer-lived than radical species, produced largely in mitochondria and tightly coupled to respiratory flux, it exerts dose- and cell-type-dependent effects on immune responses, signal transduction, and oxidative-stress programs. Selective mapping of H₂O₂ in living systems has therefore become central to understanding pathophysiology and pharmacodynamics.^{808,831}

Modern ROS-responsive prodrugs translate pathological oxidant tone into controlled active drug release by combining three modular elements: (i) a ROS-sensing trigger, (ii) a masked pharmacophore (the parent drug), and (iii) a self-immolative linker that converts an initial oxidative event into covalent bond scission. Arylboronate esters and arylboronic acids constitute the most widely exploited class.⁸³² Thiazolidinone caps mask carboxylate pharmacophores, while aryl oxalate esters undergo peroxide-promoted transformation of the oxalate, producing phenolic intermediates that hydrolyze to release parent drugs.⁸³³ In sulfur/selenium/tellurium-based systems, thioketals and thioethers, as well as Se/Te analogues, are oxidized to sulfoxides/sulfones or the corresponding selenoxides/telluroxides, which destabilizes adjacent bonds and facilitates unmasking the drug.^{834,835} Finally, proline oligomers serve as peptide triggers that are metal-catalytically oxidized, leading to backbone cleavage and drug release.^{836,837} Collectively, these chemotypes (Table 6) translate elevated oxidative tone in pathological tissues into covalent bond scission, enabling site-selective activation with tunable kinetics through the choice of trigger and linker architecture.

ROS-responsive moiety	Group	ROS-responsive site	Drug release process
	Arylboronate esters		Baeyer-Villiger oxidation-like rearrangement, hydrolysis, 1,6-elimination
	Arylboronic acids		

	Thiazolidinone		
	Aryl oxalate ester		Nucleophilic substitution, hydrolysis
	Thioketal		
	Thioether Selenium Tellurium		Oxidation, hydrolysis
	Y = S, Se, Te	Y = S, Se, Te	
	Proline oligomer		Metal-catalyzed oxidation

Table 6. Major ROS-responsive motifs used to engineer prodrugs and carriers, organized by chemical triggers and the corresponding drug-release mechanism.

Because many tumours maintain chronically elevated ROS relative to adjacent tissue, masking a cytotoxic pharmacophore with a ROS trigger can improve the therapeutic index by concentrating the active agent at the lesion while sparing healthy compartments.^{808,838}

Despite compelling preclinical results, relatively few ROS-responsive constructs have entered clinical trials, reflecting medicinal-chemistry headwinds. The dominant boronate/benzyl self-immolative motif increases lipophilicity and molecular size, particularly when combined with organelle or receptor-targeting elements, threatening compliance with conventional drug-likeness heuristics like Lipinski's rule of five.^{839,840} Moreover, many designs co-release *p*-benzoquinone methide (*p*-QM) during linker cleavage: this byproduct is undesirable for anti-inflammatory or neurotherapeutic indications where off-target injury must be minimized.^{841,842} Practical responses include direct arylboronate masks, that improve physicochemical profile at the cost of slower activation, and thiazolidinone capping of carboxylates (efficient unmasking with benign byproducts).^{843,844} Progress is likely to come from higher-efficiency triggers with lower lipophilic burden, more hydrophilic self-immolative linkers, and design principles that

harmonize activation kinetics with ADME constraints. At the end of the day, ROS-responsive strategies are well positioned to deliver clinically meaningful advances in precision pharmacology across oncology, inflammation, ischemia–reperfusion injury, and neurodegeneration.⁸²²

2. Rationale and aims of the project

Over the ensuing decades, GABAergic dysfunction has been implicated in a range of neurological and neuropsychiatric conditions, including anxiety disorders, alcohol use disorder, epilepsy, spastic syndromes, and idiopathic hypersomnia.⁸⁴⁵ Beyond its canonical inhibitory role in the CNS, GABA also exerts a wide range of functions in peripheral tissues. Many non-neuronal cells express the full molecular machinery for GABA synthesis, metabolism, transport, and signalling, including GADs, GABA-Ts, and GATs. Furthermore, both ionotropic GABA_ARs and metabotropic GABA_BRs are present in several peripheral organs, where GABA acts as an autocrine and paracrine signalling molecule rather than a neurotransmitter.⁶⁹² One of the most thoroughly investigated extra-neural roles of GABA is within the immune system.⁸⁴⁶ Immune cells such as B lymphocytes, macrophages, and CD4⁺/CD8⁺ T cells express functional GABA receptors. In particular, B cells are able to synthesize and secrete GABA, which acts locally to inhibit the activation of nearby macrophages and cytotoxic T cells. Through this mechanism, GABA exerts a potent immunosuppressive effect, dampening cytokine release and T-cell–mediated cytotoxicity.⁶⁷² This pathway contributes to the maintenance of immune homeostasis and the limitation of inflammatory damage, but it may also facilitate tumour immune evasion, as excessive GABAergic signalling can suppress the antitumor immune response.⁷¹⁸ Accordingly, pharmacological antagonism of GABA_AR has been proposed to enhance immune activation in the tumour microenvironment.⁸⁴⁷ In this context, pharmacological antagonism of GABA_ARs has the potential to relieve GABA-mediated immune suppression, thereby enhancing antitumor responses and serving as a mechanistic basis for anticancer therapy.

GMQ, originally characterized as a muscarinic receptor antagonist, functions as a competitive GABA_ARs antagonist.^{773,775,776}

GMQ is a small, rigid molecule comprising a hydrophobic quinazoline core and a hydrophilic, protonated guanidine group at physiological pH. This architecture distinguishes **GMQ** from classical GABA_ARs ligands such as bicuculline and diazaspiro[5.5]undecane derivatives and establishes a novel chemotype for antagonist discovery.^{755,759} Prior SAR work showed that

removing the 4-methyl substituent did not materially affect potency (Figure 87, compound **182**). On the other hand, replacement of the ring nitrogen at the 1-position with carbon (Figure 87, compound **183**) caused a marked loss of affinity (K_i 3.8 μ M $\rightarrow$ 65 μ M), indicating that nitrogen at the 1- and 4-positions is critical for activity. In contrast, isosteric ring editing from a quinazoline to a quinoxaline scaffold produced **GQX**, with markedly improved affinity at GABA_ARs ($K_i \approx$ 3.2 μ M $\rightarrow$ 0.50 μ M) and an even greater gain in apparent potency at GABA_{A ρ} Rs ($IC_{50} \approx$ 36 μ M $\rightarrow$ 0.21 μ M). Subsequent SAR exploration with hydrophobic substituents at the quinoxaline 6-position yielded promising inhibitors, including **184** and **185** (Figure 86).⁸⁴⁸

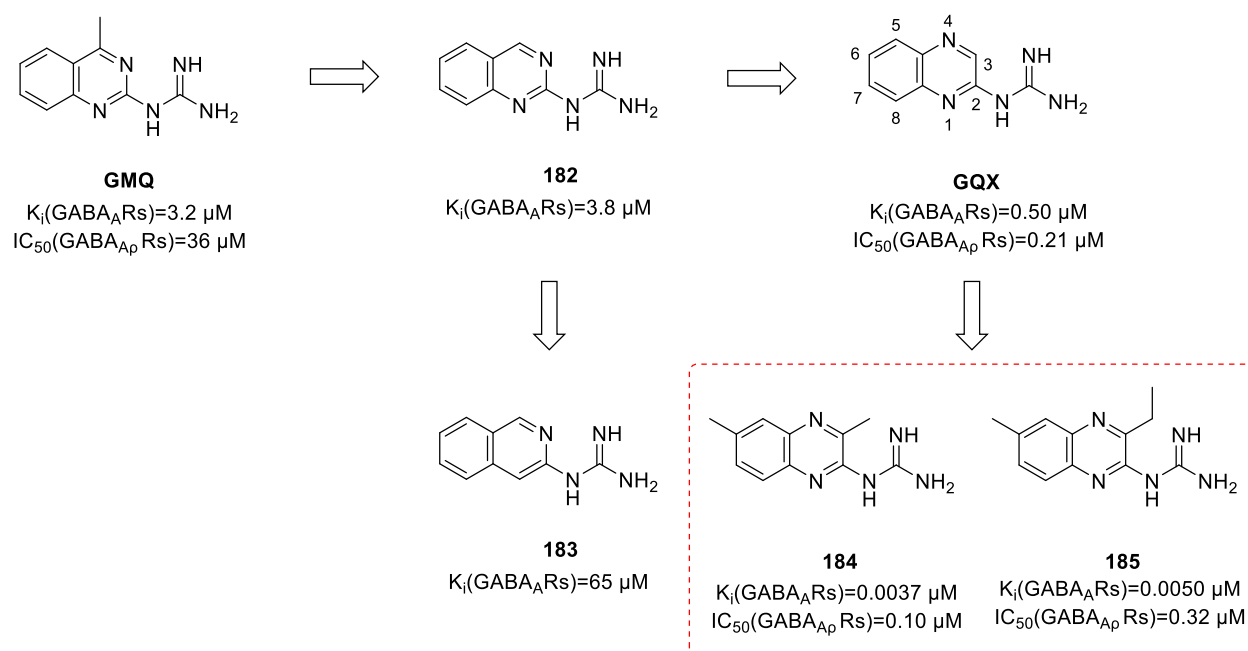


Figure 87. Modification of **GMQ** significantly improved the affinity of the GABA_ARs (K_i values) and GABA_{A ρ} Rs (IC_{50} values).

Docking studies utilizing $\alpha 1\beta 3$ GABA_ARs with **GQX** as the ligand were performed. With the available pharmacological data, the binding mode remains indeterminate. Nonetheless, the 6- and 7-positions appear to face a hydrophobic subpocket of the receptor, identifying them as attractive substitution points (Figure 87).

To further characterize the local environment, docking studies were conducted using an $\alpha 1\beta 3$ GABA_AR model with **184** as the ligand (Figure 88). In the resulting pose, the guanidinium group engages cation– π interactions in $\beta 3$ with Y205 and Y157, together with an electrostatic contact to E155; it also forms a hydrogen bond to the carbonyl of Y157. The quinazoline core is stabilized by π – π stacking with F46 and F64 of $\alpha 1$ and may experience an additional π –cation

interaction with R67. Consistent with a hydrophobic microenvironment, the 3-position projects toward $\alpha 1$ -L118, $\beta 3$ -Y157, and $\beta 3$ -Y97, rationalizing the affinity gains observed upon introducing moderate hydrophobic substituents at this site.

However, the selectivity towards GABA_ARs requires further optimization, as analogues **184** and **185** also display substantial potency at GABA_ARs. To address this, one strategy could be to exploit the pronounced steric hindrance imposed by F46 of $\alpha 1$ in GABA_ARs, in contrast to the smaller and more flexible Q34 of $\rho 1$ in GABA_ARs (Figure 88).

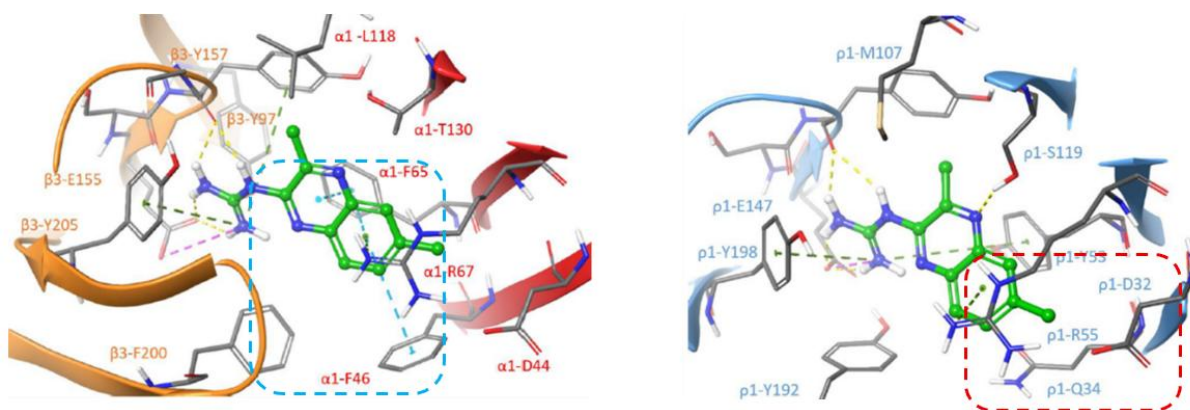


Figure 88. Predicted binding modes of ligand **184** in $\alpha 1\beta 3$ GABA_ARs (left) and $\rho 1$ GABA_ARs (right). In the $\alpha 1\beta 3$ model, the guanidinium engages cation- π contacts with $\beta 3$ -Y205 and $\beta 3$ -Y157 and an electrostatic interaction with $\beta 3$ -E155 (magenta dashed line), plus an H-bond to the $\beta 3$ Y157 carbonyl (yellow dashed line). The quinazoline core stacks π - π with $\alpha 1$ -F46 and $\alpha 1$ -F64 (blue dashed line) and may form a π -cation contact with $\alpha 1$ -R67. The 3-position projects into a hydrophobic pocket bounded by $\alpha 1$ -L118, $\beta 3$ -Y157, $\beta 3$ -Y97, consistent with affinity gains from moderate hydrophobic substituents. In GABA_ARs, the pocket is sterically constrained by $\alpha 1$ -F46 (blue), whereas in GABA_ARs the corresponding region features $\rho 1$ -Q34 adjacent to $\rho 1$ -D32 and $\rho 1$ -R55 (red dashed box), creating a more permissive cavity that could be exploited to bias potency toward $\rho 1$.⁸⁴⁸

This structural difference may allow the introduction of bulkier substituents at position 7 of the **GQX** core scaffold, which would be sterically disfavoured in GABA_ARs due to a narrower cavity. Accordingly, compound **186**, bearing a phenyl group at position 7, was synthesised as a first attempt to drive selectivity toward GABA_ARs (Figure 89).

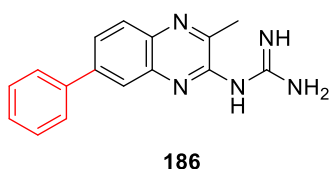


Figure 89. Structure of the newly synthesized **GQX** analogue **186**, bearing a bulky moiety at position 7.

Moreover, since many tumours exhibit rewired metabolism with enhanced mitochondrial activity and elevated ROS, a chemical gradient suitable for selective drug activation is then created.^{829,830} Boronate motifs undergo rapid, H₂O₂-triggered oxidation to phenols, which can be coupled to self-immolative linkers to unmask a caged pharmacophore.^{839,840} Embedding such ROS-responsive boronates into GABA_ARs antagonists enables tumour-selective release in oxidative microenvironments.

Accordingly, the aim of this project is appending a boron-containing trigger to the most potent **GQX** derivatives (compound **187**, **188**) as a strategy to: (i) improve delivery and lesion specificity; (ii) minimize systemic exposure of the active antagonist; and (iii) enhance on-target inhibition of GABA_ARs within GABA-high, ROS-rich tumours (Figure 90). This design integrates immune-disinhibition via GABA_ARs blockade with redox-gated drug activation, offering a coherent path toward cancer-targeted therapy.

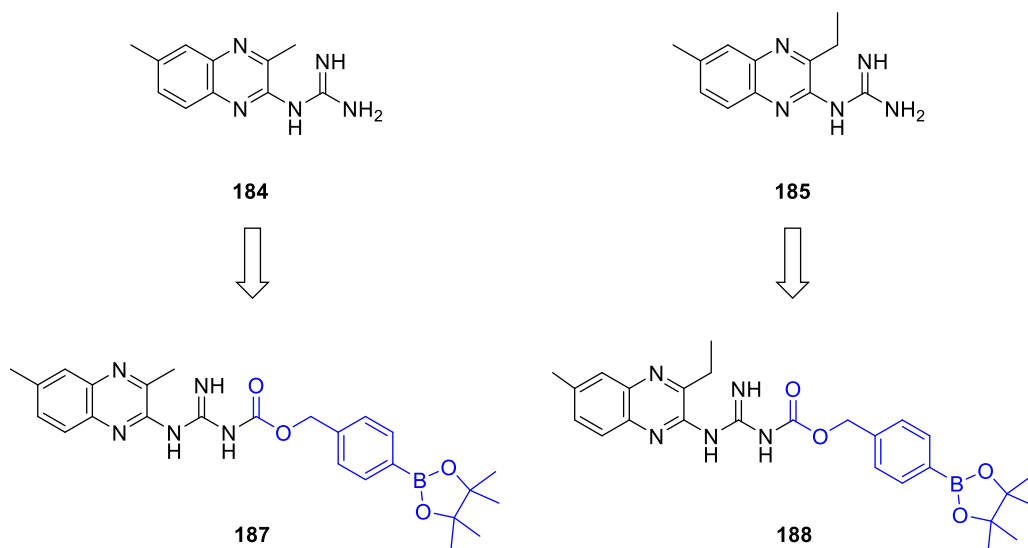


Figure 90. Structure of the newly synthesized **GQX** analogues coupled with a ROS-sensitive moiety (in blue).

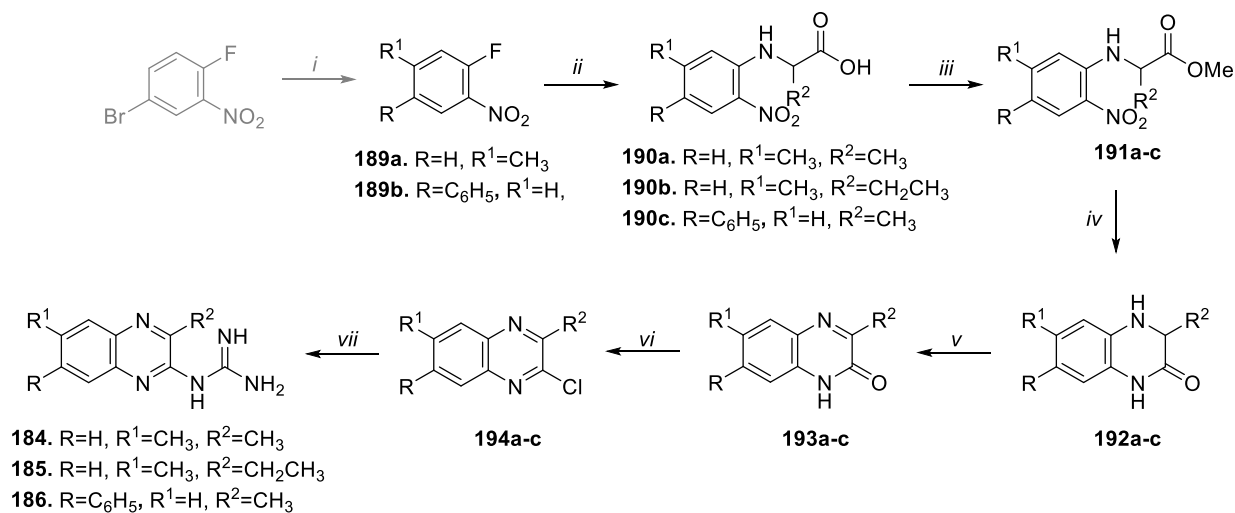
3. Results and Discussion

3.1. Chemistry

Prior works^{848,849} consolidated the SAR on the **GMQ/GQX** chemotype to establish how substituents at C3, C6, and C7 shape potency and indicated that ring editing from **GMQ** to **GQX** markedly increases affinity at GABA_ARs and even more so at GABA_ApRs, while preserving the essential N1 and N4 pharmacophores; removal of the 4-methyl group is largely neutral, whereas replacing N1 with carbon severely reduces affinity (K_i 3.8 μ M $\rightarrow$ 65 μ M) (Figure 86). Guided by these precedents, compound **186** was designed combining moderate lipophilic moieties at C3 and bulk introduction at C7 to probe potency and exploit GABA_ApRs subtype-specific steric constraints (Figures 87 and 88) to eventually start a new series of **GQX** analogues. On the other hand, a boron-containing moiety was appended to the most potent **GQX** derivatives **187** and **188** as a prodrug delivery system (Figure 90). The synthesis of compounds **184–188** is discussed below.

3.1.1. Synthesis of GQX analogues

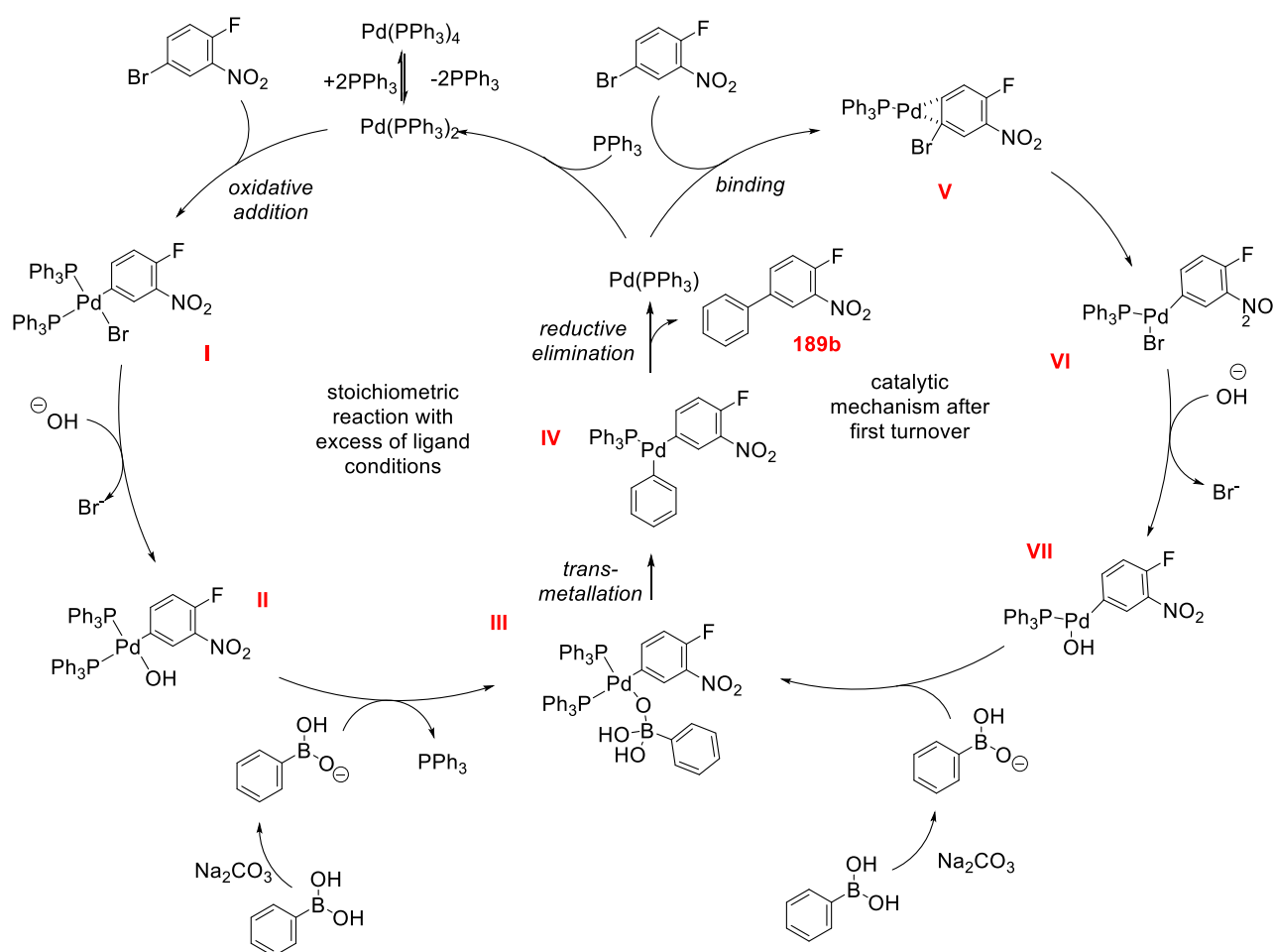
The synthesis of the designed compounds **186–188** was performed through a multi-step approach (Scheme 18).



Scheme 18. Synthesis of compounds **184–186**; Reaction conditions: (i) phenylboronic acid, tetrakis(triphenylphosphine)palladium(0), Na₂CO₃, Tol/EtOH (1:1), 85°C, 48h (to obtain **189b**); (ii) alanine (to obtain **190a,b**) or 2-aminobutanoic acid (to obtain **190c**), NaHCO₃, EtOH, 100°C, 24h; (iii) H₂SO₄, MeOH, 65°C, 5h; (iv) Fe, NH₄Cl/H₂O, 100°C, 16h; (v) 3% H₂O₂, 2 M NaOH, H₂O/MeOH, 100°, 1.5h; (vi) POCl₃, 100°C, 1h; (vii) guanidine hydrochloride, K₂CO₃, NMP, 140°C, 16h.

3.1.1.1. Suzuki-Miyaura coupling

The initial reaction for affording the final compound **186** was a Suzuki-Miyaura coupling. The reaction proceeds selectively at the C–Br bond, while the fluorine and nitro substituents remain intact. This chemoselectivity reflects the much faster oxidative addition of Pd(0) into C–Br compared to C–F, together with the excellent tolerance of the NO₂ group under palladium catalysis. The outcome is a biaryl in which F and NO₂ are preserved as orthogonal handles for the subsequent nucleophilic aromatic substitution (S_NAr) C–F and the derivatization of the nitro group (Scheme 19).



Scheme 19. Proposed mechanism for Suzuki-Miyaura cross-coupling step.⁸⁵⁰

The reaction proceeds through the canonical Pd(0)/Pd(II) cycle, with the oxidative addition of tetrakis(triphenylphosphine)palladium(0) (Pd(PPh₃)₄) into the aryl bromide to give an oxidative-addition Pd(II) aryl–bromide complex (intermediate **I**). The hydroxide exchange product, an aryl–palladium(II) hydroxo complex (intermediate **II**), reacts with base-activated

organoboron reagent to give a Pd(II) hydroxo–boronate adduct (intermediate **III**). The latter one goes through a transmetalation process (intermediate **IV**) and subsequently a reductive elimination, to release intermediate **189b** and regenerate Pd(0). After the first, stoichiometric-like pass, the catalyst enters its true catalytic regime as a more active, monoligated Pd(0)–PPh₃ complex (intermediate **VI**). Under the basic aqueous medium, bromide is exchanged for hydroxide, furnishing the crucial aryl–Pd(II)–OH intermediate **VII**. The latter engages the boronate (intermediate **III**) enabling rapid transmetalation to a Pd(II) hydroxo–boronate adduct (intermediate **IV**). Finally, reductive elimination forms the biaryl product and regenerates the Pd(0)–PPh₃ resting state, which re-enters the cycle. Functionally, this catalytic pathway is faster than the stoichiometric reaction, because the ligand-thinned Pd center promotes both oxidative addition and Pd–OH–mediated transmetalation. Throughout, the aryl–F bond remains intact, while the base continuously supplies OH[–] and activates the boron partner, sustaining efficient catalysis.⁸⁵¹

In 4-bromo-1-fluoro-2-nitrobenzene, chemoselectivity is dictated by the much faster oxidative addition into C–Br relative to C–F; the fluoro substituent therefore remains untouched. The nitro group strongly deactivates the ring, facilitating oxidative addition. Both the fluorine and the nitro group are therefore preserved as orthogonal handles for downstream transformations, like a S_NAr and a reduction. In the toluene/ethanol medium, Na₂CO₃ deprotonates the alcohol to give sodium ethoxide (NaOEt) and transient H₂CO₂, which collapses to CO₂ and H₂O. Exchange between NaOEt and the adventitious water generates a small but operative amount of NaOH that provides the hydroxyl ion which replaces bromide in the presence of the catalyst. The boronic acid is base-activated to a tetra-coordinated boronate species.⁸⁵²

Strictly oxygen free conditions are required due to the formation of triphenylphosphine oxide (OPPh₃), a ubiquitous by-product in Suzuki–Miyaura cross-couplings conducted with Pd(PPh₃)₄ or phosphine-ligated palladium precatalysts, that was detected by both NMR spectroscopy and liquid chromatography–mass spectrometry (LC–MS) analysis of the reaction mixture. Its formation reflects oxidation of P(III) of PPh₃ to P(V) and the associated kinetics can ultimately exhaust the free phosphine and deactivate the catalyst, leading to cessation of catalytic turnover.^{853,854} Practically, the reaction was driven to complete conversion prior to workup: the starting aryl bromide (4-bromo-1-fluoro-2-nitrobenzene) and compound **189b** exhibit near-identical silica retention and almost co-elute by LC, and under the used LC–MS conditions their masses can not be reliably discriminated (Figure 91).

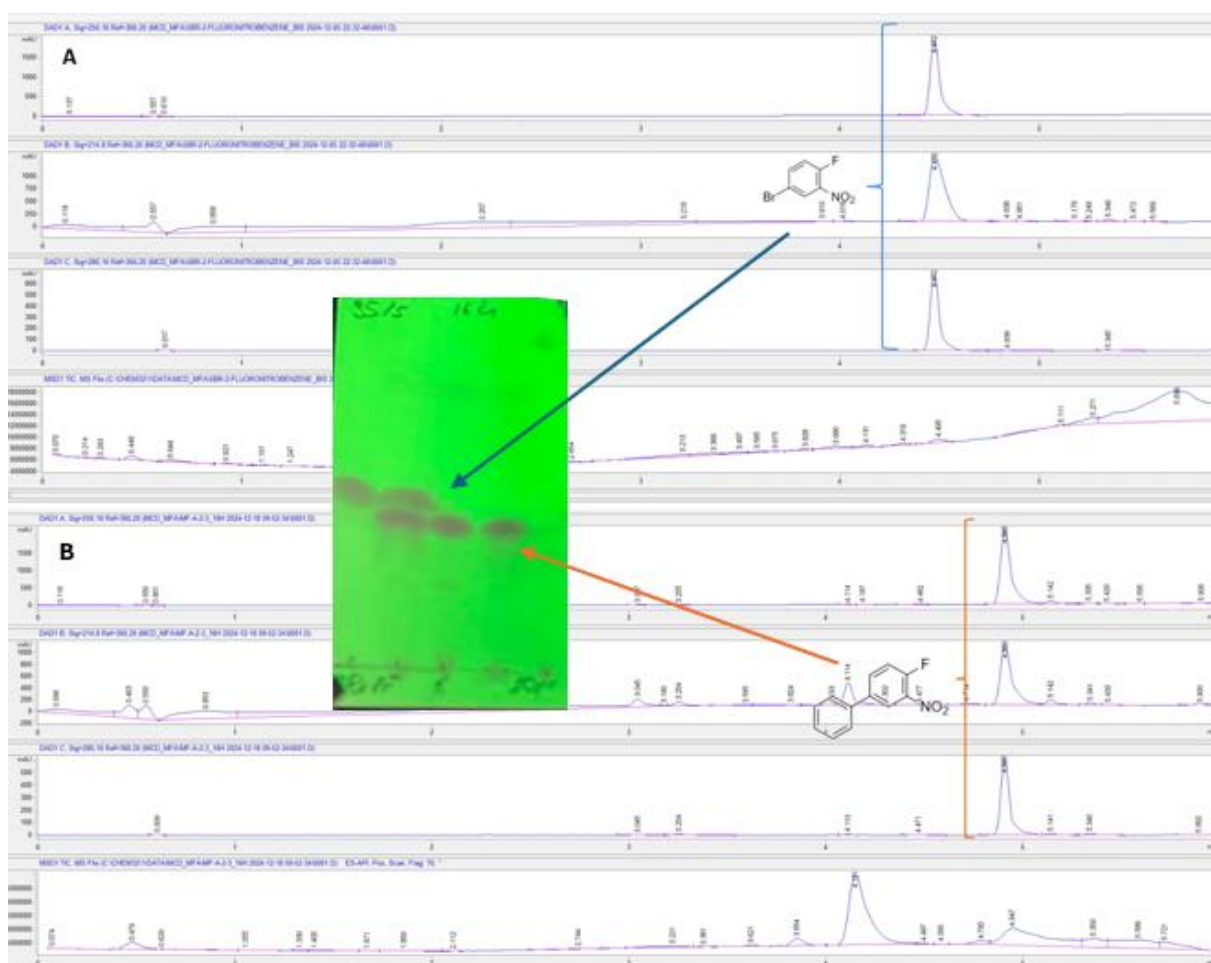
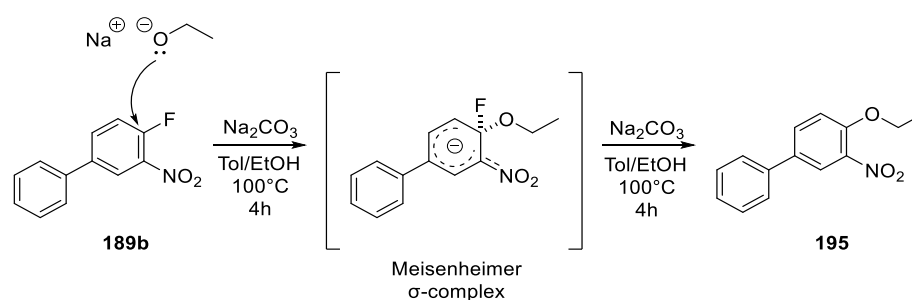


Figure 91. LC–MS traces comparing 4-bromo-1-fluoro-2-nitrobenzene (A, $t_R = 4.44$ min) with compound **189b** (B, $t_R = 5.04$ min). A representative TLC (silica gel, heptane/EtOAc 95/5) is shown alongside, where substrate and product present closely spaced R_f values. The chromatographic similarity highlights the challenge of separating partially converted mixtures.

The reaction conditions are extremely important considering that higher temperatures can lead to the substitution of fluorine (Scheme 20). Indeed, it was also observed that the aryl fluoride **189b** can be competitively converted to the corresponding aryl ethyl ether byproduct (compound **195**) via a base-promoted S_NAr pathway.



Scheme 20. Proposed mechanism for the substitution of compound **x**, affording byproduct **195**.

In this scenario, the ethoxide generated *in situ* by deprotonation of ethanol with carbonate and equilibrating traces of water acts as the nucleophile, attacking the carbon bearing fluorine on the nitro-activated arene to form a Meisenheimer σ -complex. Resonance delocalization of negative charge onto the nitro group lowers the barrier for the dearomatizing step; subsequent expulsion of fluoride restores the aromaticity and furnishes the corresponding aryl ethyl ether byproduct (Figure 92).⁸⁵⁵

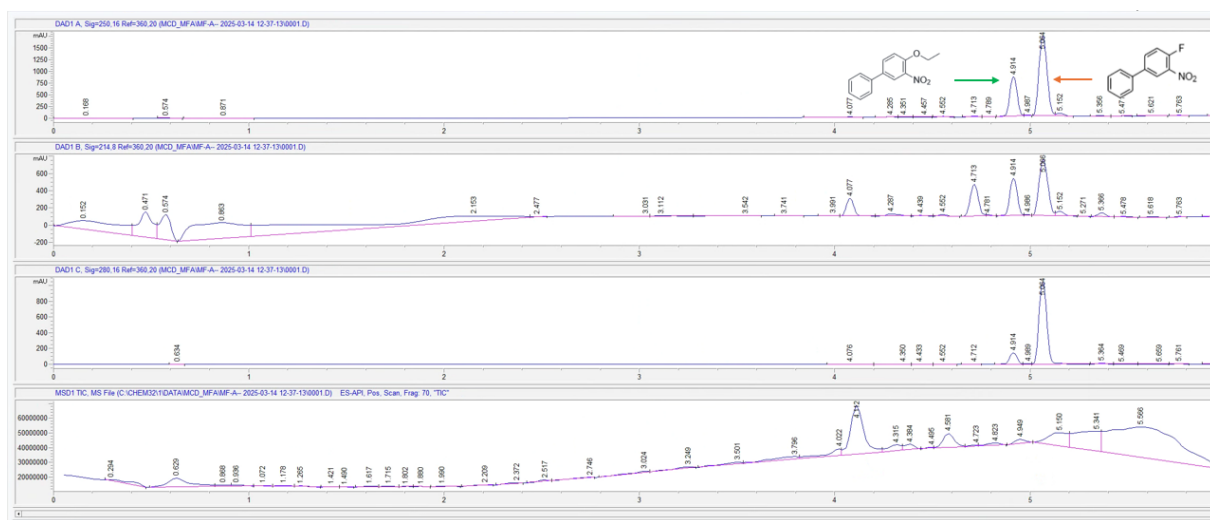
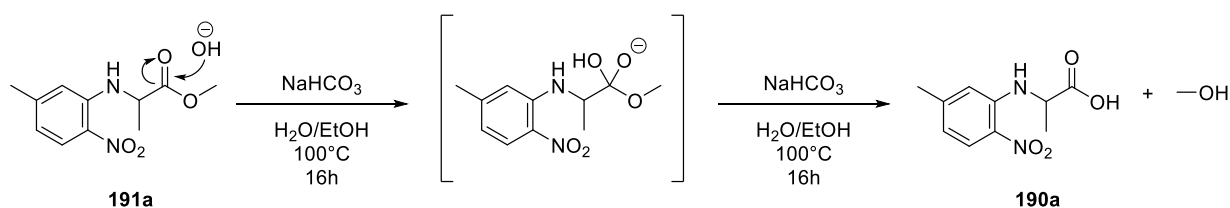


Figure 92. LC–MS traces comparing compound **189b** ($t_R = 5.06$ min) with byproduct **195** ($t_R = 4.91$ min). Also in this case, their masses can not be reliably discriminated under the used LC–MS conditions.

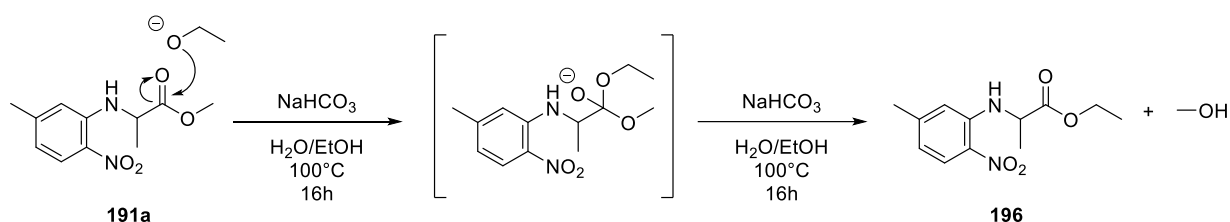
3.1.1.2. Nucleophilic aromatic substitution

The second step for obtaining compound **190c** and the first one for obtaining compound **190a** and **190b** (Scheme 18) was S_NAr , in which an appropriate aryl fluoride was displaced by the primary amine of alanine (to obtain **190a,c**) or 2-aminobutanoic acid (to obtain **190b**). Methyl-ester-protected amino acids were used at the beginning, anticipating that masking the carboxylate would simplify flash-chromatographic purification and to obtain directly intermediates **191a–c**. In practice, these reactions gave low isolated yields: LC–MS analysis revealed substantial *in situ* hydrolysis of the methyl ester under the substitution conditions, furnishing the corresponding carboxylic-acid products (Scheme 21).



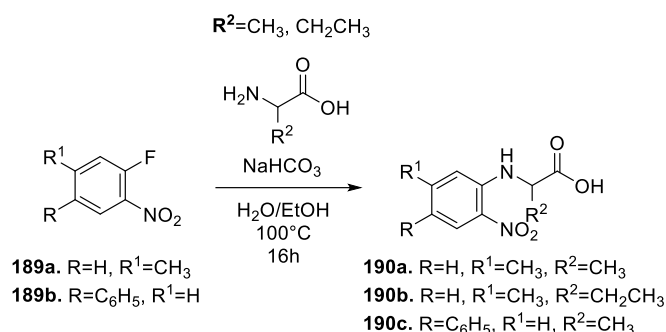
Scheme 21. Proposed mechanism for the hydrolysis of the methyl ester, affording **190a**. Under aqueous ethanolic, the bicarbonate buffer establishes small but sufficient concentrations of NaOH and, by transiently deprotonating ethanol, small concentrations of EtONa as well. The hydroxide attacks the carbonyl carbon of the carbamate, generating the tetrahedral intermediate in which the former carbonyl carbon is now sp^3 -hybridized. Collapse of this intermediate restores the carbonyl and expels the alkoxide as MeOH. Finally, buffer-mediated proton transfer forms the carboxylic acid. The bicarbonate medium is strong enough to cleave the ester but insufficiently basic to trigger rapid decarboxylation of the resulting carbamic acid, thus avoiding the release of the amine.⁷⁵¹

Despite hydrolysis predominates over transesterification in the presence of bulk water, common S_NAr conditions could also afford a small amount of **196** (Scheme 22).⁸⁵⁶



Scheme 22. Proposed mechanism for the transesterification of **191a**. Under the aqueous ethanolic, mildly basic conditions, the methyl carbamate is converted to the corresponding ethyl carbamate via a base-promoted transesterification. The bicarbonate buffer partially deprotonates ethanol to generate small, catalytic amounts of EtONa and simultaneously help maintaining a gentle basic environment that limits competing hydrolysis/decarboxylation. EtO^- attacks the carbonyl to form the tetrahedral intermediate. Collapse of the latter one re-forms the carbonyl and expels MeO^- as the leaving group, thereby furnishing the ethyl carbamate. In the protic medium, MeO^- is rapidly protonated to MeOH, whereas the EtO^- nucleophile is continuously regenerated by the bicarbonate/ethanol equilibrium, sustaining catalytic turnover. The reaction is thermodynamically driven by the large excess of and by the removal of the leaving group as neutral MeOH.^{857,858}

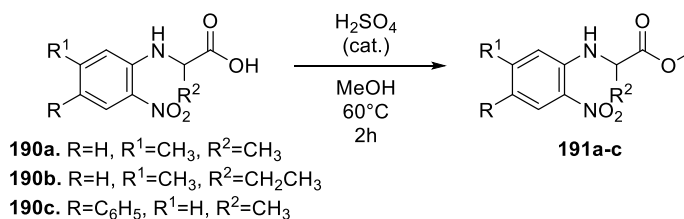
Therefore, the unprotected amino acids were used (Scheme 23). In these conditions the $\text{S}_{\text{N}}\text{Ar}$ step was high-yielding and chemoselective, typically reaching full conversion and providing the substituted intermediates **190a-c** with a yield >85%.



Scheme 23. Preparation of derivatives 190a-c.

3.1.1.3. Fischer esterification

Since the further reduction step triggers a spontaneous cyclization and water is a poor leaving group, a Fischer esterification step was introduced immediately after the $\text{S}_{\text{N}}\text{Ar}$ to generate the methyl ester (Scheme 24), thereby providing a more reactive intermediate for the reduction-induced cyclization, expelling MeOH (a superior leaving group) rather than water, thus providing a more labile moiety that accelerated the cyclization.



Scheme 24. Preparation of derivatives 191a-c.

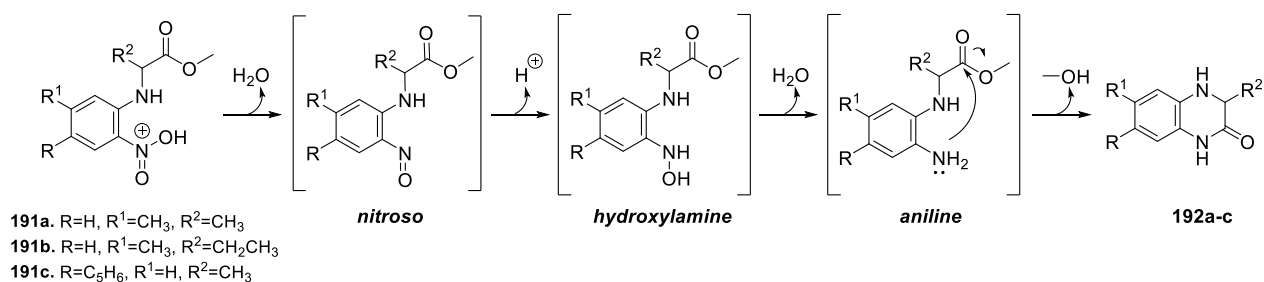
First attempts to obtain compounds **191a-c** were unsuccessful or afforded low yields, presumably due to insufficiently dry solvent. Because the process is equilibrium-limited ($\text{acid} + \text{MeOH} \rightleftharpoons \text{ester} + \text{H}_2\text{O}$), even residual water is sufficient to withdraw the reaction in the reverse direction and suppress product formation. With methanol, Dean–Stark removal is impractical, so the reaction can stall unless water is actively scavenged. For reasons of sustainability, using trimethyl orthoformate as a water scavenger was deliberately avoided, as it requires stoichiometric amounts. In practice, reliability is restored by making the medium strictly anhydrous, using absolute MeOH on molecular sieves.⁸⁵⁷

Operationally, the $\text{S}_{\text{N}}\text{Ar}$ products (**190a-c**) were dissolved in dry methanol containing concentrated H_2SO_4 ($\text{MeOH}/\text{H}_2\text{SO}_4 = 10:1$, v/v) and heated to reflux under argon atmosphere with stirring for 2 h. Reactions progress was monitored by LC-MS; upon complete consumption of the starting material, the mixture was concentrated *in vacuo*, diluted with EtOAc, and carefully neutralized with aqueous K_2CO_3 , slowly added to the ice-cooled reaction mixtures. The aqueous layer was extracted with EtOAc and the combined organic phases were dried over Na_2SO_4 , filtered, and evaporated to afford the methyl ester of the corresponding carboxylic acid in good yields. Despite the completion of the reactions, notably the yields were never quantitative. This outcome was attributed to partial hydrolysis during the carbonate quench: although K_2CO_3 is a mild base and generates only small amounts of OH^- , these are sufficient to hydrolyse the methyl carbamate to the carbamic acid with a mechanism similar to the one already discussed in Scheme 21, thereby eroding the isolated yield affording a small amount of starting material as well, suspended in the water phase.

Moreover, given the large volumes generated during acid quenching, K_2CO_3 was preferred over NaHCO_3 , used in prior work.⁸⁴⁸ As a stronger base/buffer (aqueous $\text{pH} \approx 10\text{--}11$ vs ≈ 8.3), K_2CO_3 neutralizes strong mineral and sulfonic acids more rapidly and more completely, enabling the use of smaller quench volumes and a more compact workup. From a practical point of view, a saturated K_2CO_3 solution also breaks emulsions more effectively than NaHCO_3 , particularly valuable with EtOAc.

3.1.1.4. *Bechamp reduction*

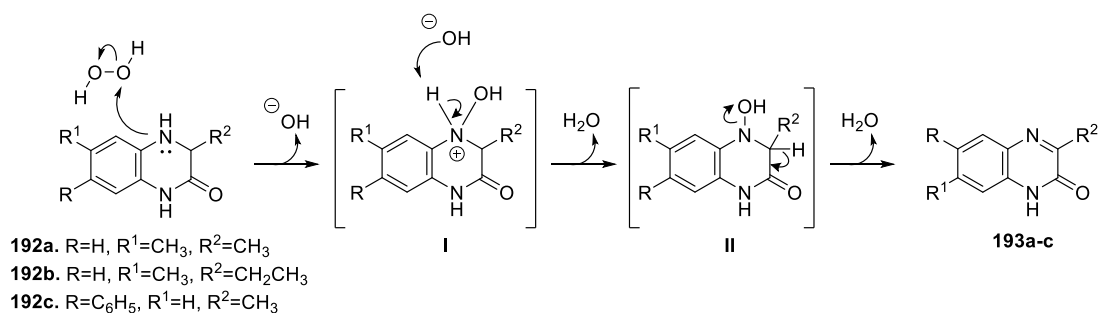
To obtain dihydroquinoxalinone derivatives **192a-c**, a reductive cyclization was executed in two consecutive events: reduction of the nitro group to the corresponding aniline, followed by a spontaneous intramolecular ring closure between the unmasked aniline and the ester, to furnish the bicyclic amide derivatives **192a-c** (Scheme 25). In an iron-mediated nitro reduction in mildly acidic medium, multiple single-electron transfers, protonation steps, and dehydrations are implicated. Under the slightly acidic conditions generated by NH_4Cl , the sequence can initiate via protonation of the nitro group and then proceed through three principal two-electron reductions started by iron: nitro $\rightarrow$ nitroso $\rightarrow$ hydroxylamine $\rightarrow$ aniline, each facilitated by Fe. Upon formation of the aniline, nucleophilic attack on the methyl-ester carbonyl triggers spontaneous cyclization, delivering the reduced bicyclic intermediates (Scheme 8).



Scheme 25. Proposed simplified mechanism for the Bechamp reduction of intermediates **191a-c**, followed by an intramolecular cyclization. In this transformation, iron serves as the sacrificial reductant in a Béchamp reduction ($\text{Fe}^0 \rightarrow \text{Fe}^{2+}$), delivering the electrons, and NH_4Cl the necessary protons are provided to convert the aryl nitro group to the aniline, sequentially through nitroso and hydroxylamine intermediates. The newly formed aniline then intramolecularly attacks the ester, effecting spontaneous cyclization to the bicyclic amide.

3.1.1.5. Oxidation

In this step, dihydroquinoxalinone derivatives **192a-c** were oxidized to corresponding quinoxalinone, furnishing intermediates **193a-c**. The oxidation was effected with 3% H_2O_2 in the presence of a NaOH . Nucleophilic attack on the peroxide generates the N-oxide, with hydroxyl displacement facilitated by the basic medium. The resulting N-oxide then undergoes two successive dehydration events, delivering the aromatized quinoxalinone (Scheme 26).

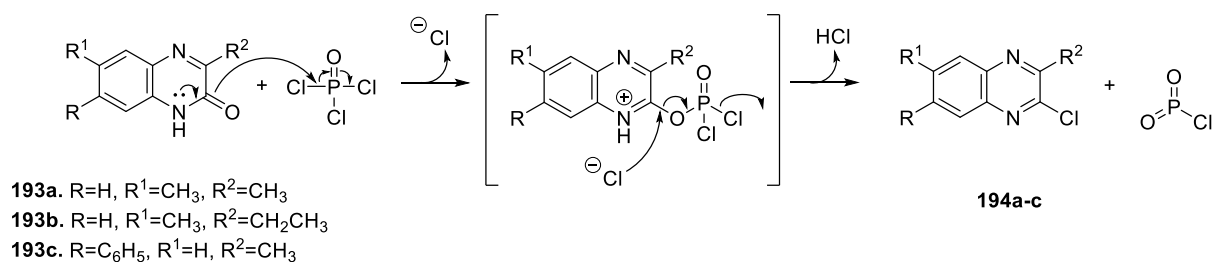


Scheme 26. Proposed mechanism for the oxidation of intermediates **192a-c**.

However, the quinoxalinone thus formed can over-oxidize to the N-oxide of the quinoxalinone under the same conditions. Mechanistically, this follows the same initial N-oxygenation pathway. Consequently, careful control of the oxidant stoichiometry, pH, and reaction time is required to favour the formation of the neutral quinoxalinone over its N-oxide.

3.1.1.6. Chlorination

To enable guanidine installation, the amide carbonyl of quinoxalinone intermediates **193a-c** was first activated by conversion to chlorine, a superior leaving group, using POCl₃ as a neat reagent (Scheme 27). The reagent's efficiency stems from the oxophilicity of phosphorus: formation of a strong P=O bond renders the transformation thermodynamically favourable and drives it to completion. Conversely, POCl₃ is readily hydrolysed by water to H₃PO₄, so all operations were performed in oven-dried glassware under argon.

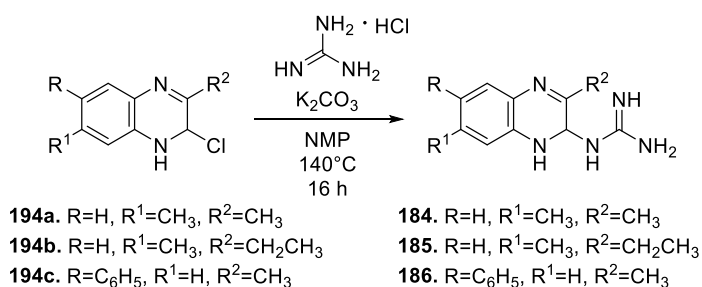


***Scheme 27.** Proposed mechanism for the chlorination of intermediates **193a-c**. Mechanistically, nucleophilic attack of the amide oxygen on phosphorus generates the P–O adduct; charge stabilization by the amide nitrogen produces a highly electrophilic acyl center bearing a good leaving group, which is then displaced by chloride.*

The reaction mixture was poured onto crushed ice to quench the excess POCl₃, and the product was subsequently extracted with dichloromethane (DCM) and washed with a saturated solution of NaHCO₃.

3.1.1.7. Guanylation

The last step for synthesizing compound **184–186** is the guanylation of intermediates **194a-c**. The reaction was accomplished by S_NAr substitution of the activated chloride with guanidine in dry *N*-methylpyrrolidone (NMP) at 140 °C (Scheme 28).



***Scheme 28.** Preparation of compounds **184–186**.*

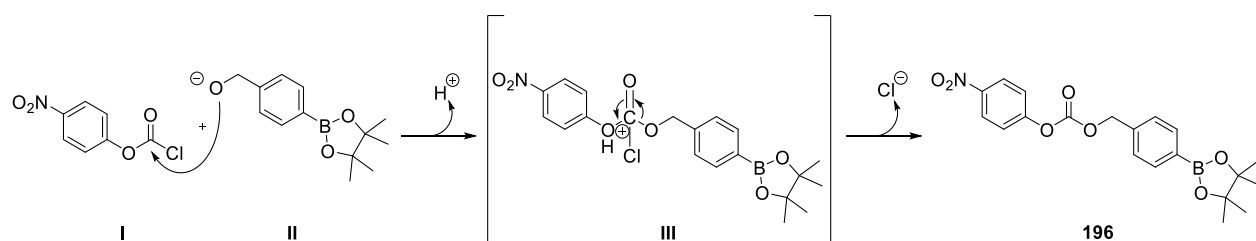
Elevated temperature, allowed high-boiling solvents like NMP and dimethylformamide (DMF), was required because aryl chlorides are less reactive than the corresponding fluorides in $\text{S}_{\text{N}}\text{Ar}$ processes. Moreover, NMP enhances the effective nucleophilicity of guanidine and efficiently solvates both guanidine hydrochloride and the chlorinated intermediates, thereby maintaining a high concentration of the free, nucleophilic nitrogen. Moreover, earlier attempts in DMF⁸⁴⁸ led to solvent decarbonylation and *in situ* formation of dimethylamine, which competed for substitution; switching to NMP eliminated this complication and delivered the desired products in moderate yields ($\approx 40\text{--}50\%$).

3.1.2. Synthesis of a ROS-responsive prodrug: exploration and optimization

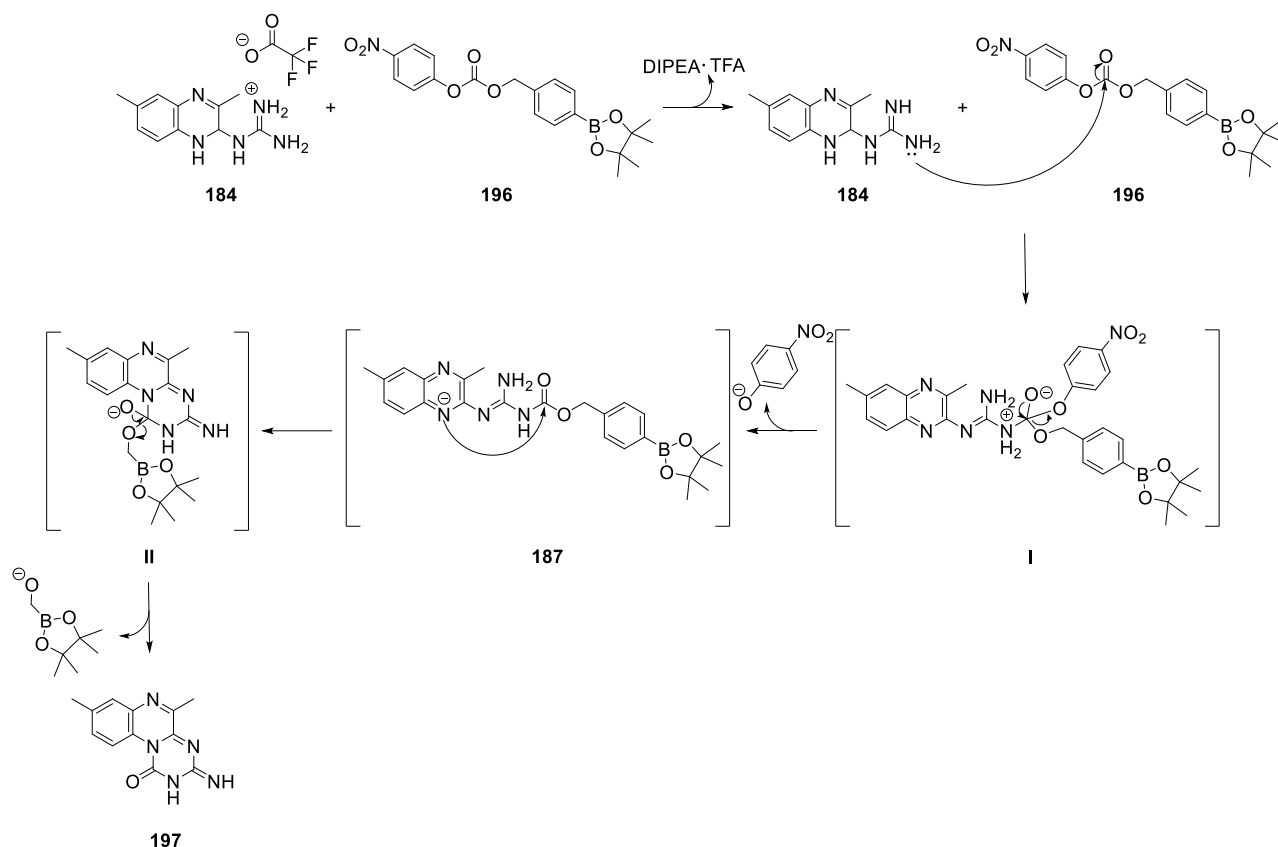
Cancer cells frequently exhibit increased mitochondrial activity and ROS levels, especially H_2O_2 , establishing an environment that can be exploited for site-selective prodrug activation. Thereby, the incorporation of a ROS-responsive moiety enables preferential drug release in oxidative tumour microenvironments, improving selectivity and potentially mitigating off-target effects. Several attempts for appending a boron-containing trigger to the most potent **GQX** derivatives were performed and are described below.

3.1.2.1. Assembly of a ROS-responsive boronate carbonate linker

The initial stage of this work involved the activation of phenylboronic acid pinacol ester (**II**), selected as the ROS-sensitive moiety intended for coupling to the **GQX** analogues. The reaction was performed following a literature procedure⁸⁵⁹ and affords the an aryl-alkyl carbonate via nucleophilic acyl substitution on *p*-nitrophenyl chloroformate (**I**). In dry THF at room temperature, TEA generates the phenoxide (**II**) from the substrate and scavenges the HCl produced during acyl transfer. Phenoxide attacks the carbonyl carbon **II**, affording a tetrahedral intermediate (**III**) that collapses with chloride expulsion to deliver the desired compound **16**.



Scheme 29. Proposed mechanism for the activation of phenylboronic acid pinacol ester (**II**) to desired compound **196**.



Scheme 31. Proposed mechanism for the obtainment of undesired byproduct **197**. The excess of DIPEA neutralizes the TFA salt of **184**, unleashing the corresponding free diamine and sequestering trifluoroacetic acid. One of the two amines of **184** then attacks the carbonyl carbon of the activated *p*-nitrophenyl carbonate **196**, generating a tetrahedral intermediate (**I**) that collapses with expulsion of *p*-nitrophenoxide. This process affords **187** as a transient carbamate intermediate, in which its **GQX** scaffold is linked through a carbamoyl group to the boronate-containing moiety. Under strongly basic and relatively high-temperature conditions (dry DMF, 65 °C), a neighbouring ring nitrogen of **187** is deprotonated and undergoes intramolecular nucleophilic attack on the carbamate carbonyl. The resulting tetrahedral intermediate collapses with displacement of the boron containing moiety as an oxide, leading to the formation of the undesired compound **197**.

The outcome of this intramolecular nucleophilic acyl substitution is therefore governed by the combined effect of the strongly basic environment, which promotes deprotonation of the heterocyclic nitrogen, the elevated temperature, which accelerates the intramolecular rearrangement, and the high reactivity of the *p*-nitrophenyl carbonate. Together, these factors favour cyclization over the desired coupling to the boronate linker. NMR data (notably a

strongly deshielded aromatic proton at 9.2 ppm) and LC-MS analysis supported this interpretation (Figure 93).

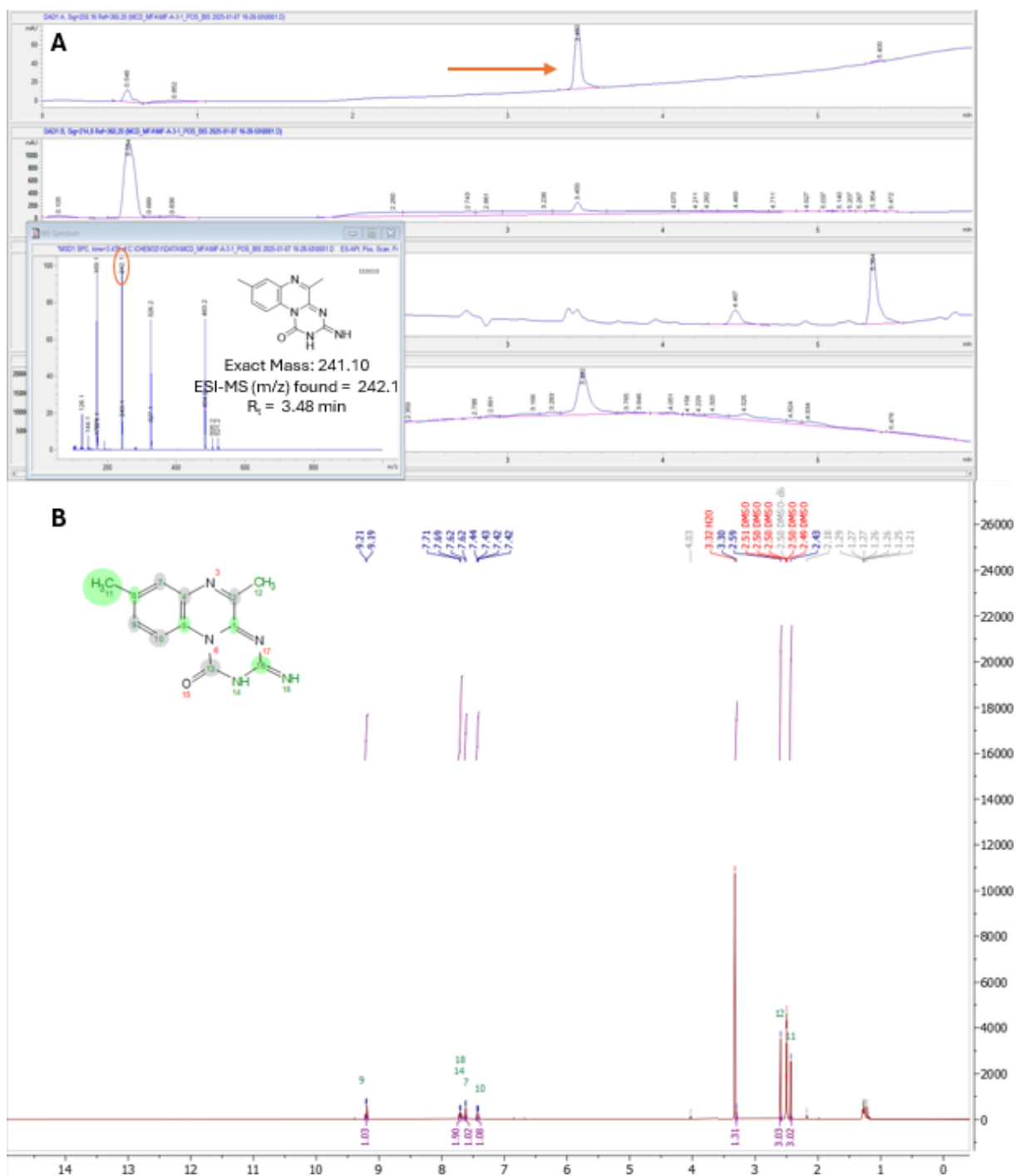
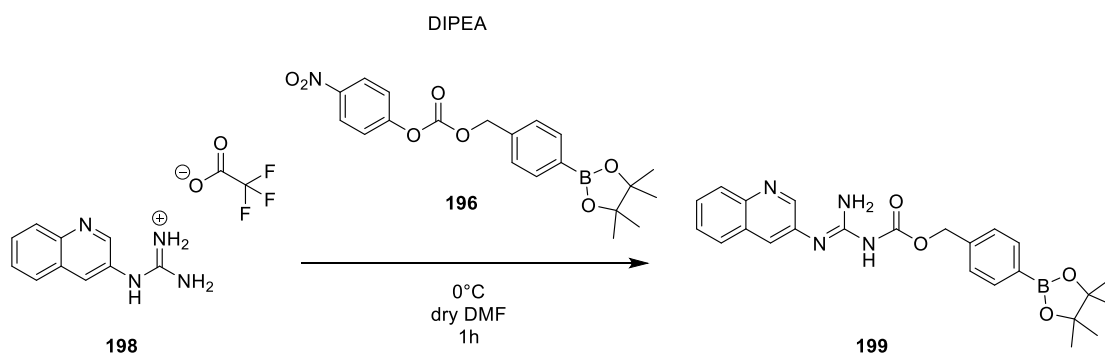


Figure 93. (A) LC–MS plot of 197 ($m/z = 242.1$). (B) ^1H NMR spectrum of 197, recorded in $\text{DMSO}-d_6$. A strongly deshielded singlet at $\delta \approx 9.2$ ppm (1H), is shown, attributable to an aromatic proton adjacent to a carbonyl or heteroatom within the newly formed heterocyclic framework. Additional aromatic resonances appear at $\delta \approx 8.1$ – 8.2 and 7.0 – 7.3 ppm, consistent

with the remaining protons of the heteroaromatic core. In the aliphatic region, two singlets at $\delta \approx 3.1\text{--}3.3$ ppm (6 H total) correspond to the two methyl groups attached to the aromatic core. Notably, no signals are observed around 1.3 ppm, where the protons of the boronate linker would normally resonate, indicating that this moiety is absent in the isolated product.

Altogether, the presence of the highly deshielded proton at 9.2 ppm, the aromatic pattern, and the disappearance of the boronate resonances, together with LC-MS data, support the occurrence of an intramolecular cyclization via a nucleophilic acyl substitution mechanism rather than the intended coupling to the boronate linker.

A second attempt employed *in-house* analogue **198** (Scheme 32), lacking the heterocyclic nitrogen implicated in the cyclization.



Scheme 32. Attempted coupling of *in-house* analogue **198** as TFA salt, selected to prevent intramolecular cyclization, with the boronate carbonate linker. The reaction was carried out at 0 °C in dry DMF with DIPEA (1 eq) as base, yielding a low amount of the expected product (**199**) along with several byproducts, likely originating from base-promoted degradation or partial loss of the pinacol moiety.

The reaction was conducted at 0 °C with one equivalent of DIPEA to neutralize the TFA salt. The low temperature was chosen to suppress fast side reactions. Multiple fractions were obtained after purification, yielding a small amount of impure product (~2 mg). LC-MS analysis indicated the presence of several byproducts, potentially arising from base-promoted side reactions or partial loss of the pinacol moiety, according to literature (Figure 94).^{861,862} Nevertheless, NMR confirmed the formation of the target compound.

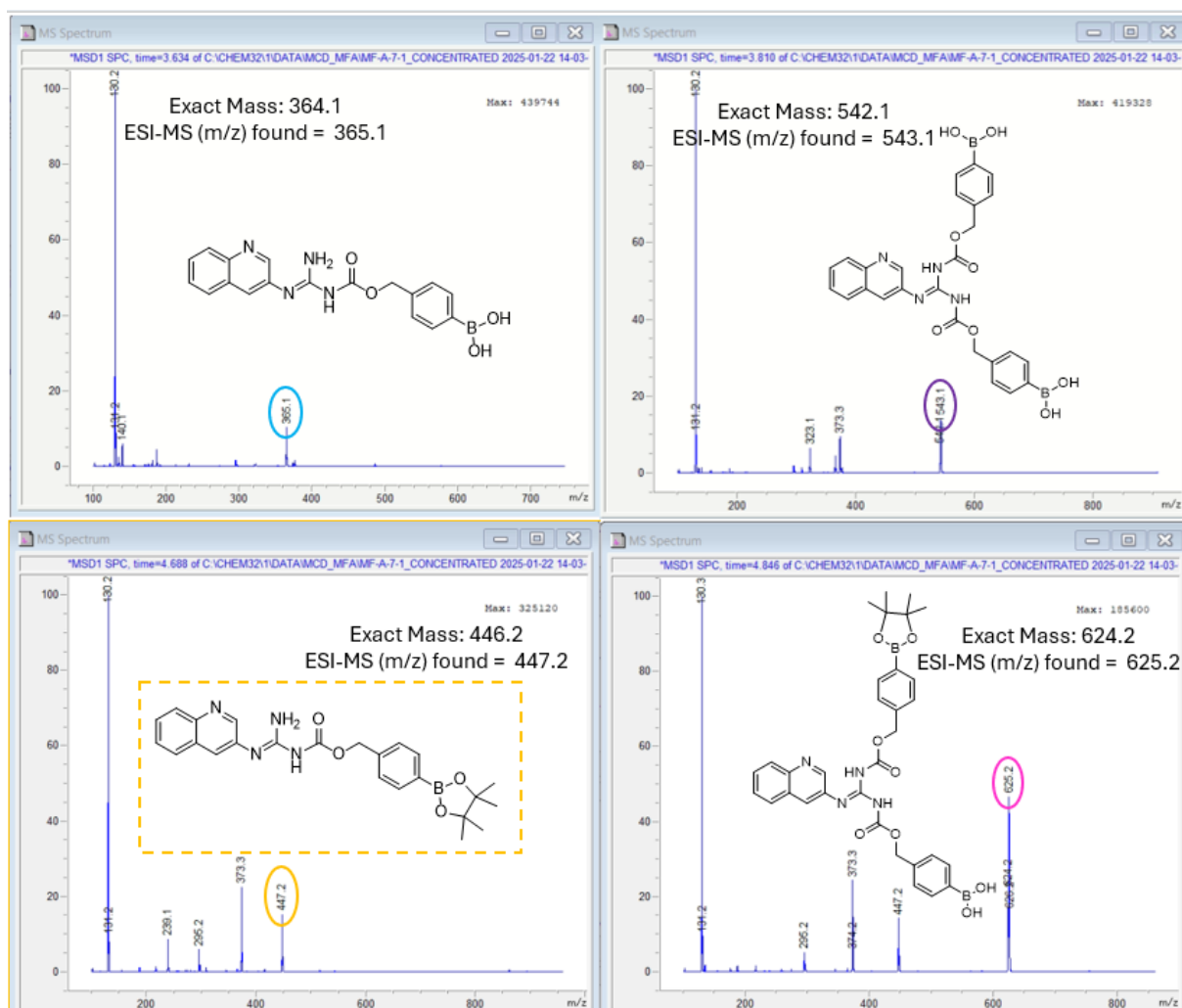
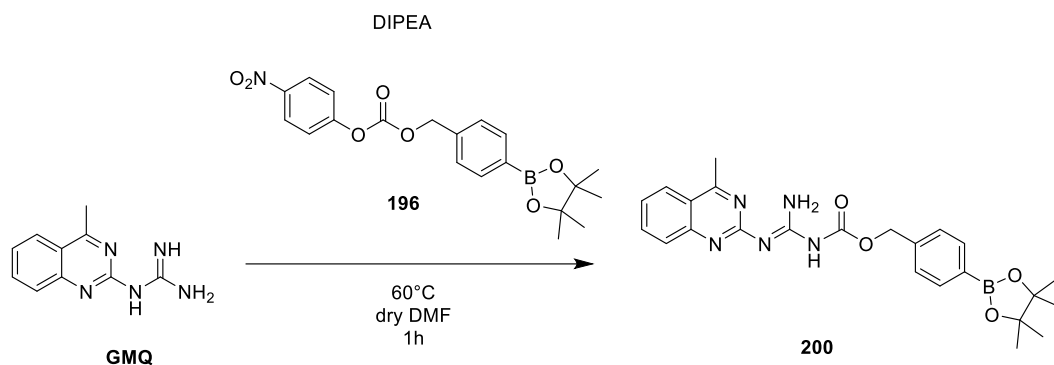


Figure 94. LC–MS analysis of the reaction crude revealed the coexistence of several species, including a compound resulting from the coupling of **198** with one ($m/z = 365.1$), or two hydrolysed boronic acid moieties ($m/z = 543.1$), a mono-pinacol disubstituted intermediate ($m/z = 625.2$), and the desired carbamate product ($m/z = 447.2$, yellow dashed box). These findings are consistent with partial hydrolysis of the pinacol ester and side reactions promoted under basic conditions.

A third attempt, using **GMQ** as a model substrate owing to the limited availability of the target **GQX** derivatives, and running the reaction with the free base with no external base added, successfully delivered the desired product (Scheme 33).



Scheme 33. Attempted coupling of **GMQ** as a free base with the boronate carbonate linker **196** in dry DMF at 60 °C with no external base to afford the desired compound **200**.

LC–MS analysis of the crude showed the expected species (**200**) together with a minor component consistent with partial loss of the dioxaborolane; the latter increased upon storage, confirming that the product is water-sensitive, according to previous results (Figure 95).

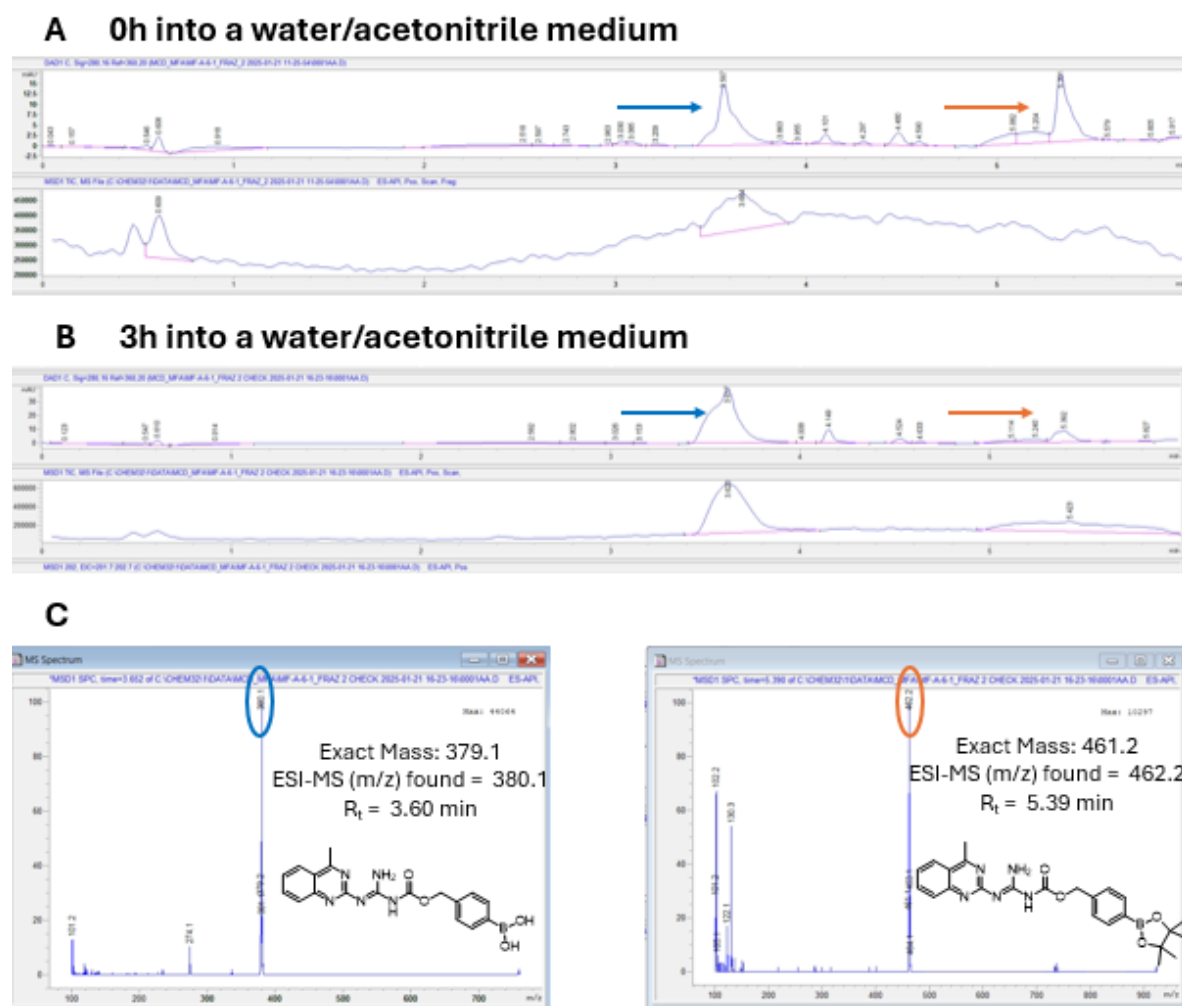


Figure 95. LC–MS analysis of compound **200** immediately after preparation and after exposure to water. (A) Chromatogram recorded immediately after dissolution ($t = 0$ h) showing the main product (orange) and a minor component, identified as the hydrolyzed species (blue). (B) After 3 h in aqueous medium, the relative intensity of the hydrolyzed species (blue) increased, indicating progressive degradation of the dioxaborolane moiety. (C) ESI–MS spectra corresponding to the hydrolysis product (left, $m/z = 380.1$, $R_t = 3.60$ min) and the parent compound (right, $m/z = 462.2$, $R_t = 5.39$ min), confirming partial conversion upon water exposure.

Notably, LC–MS analysis of the reaction mixture after 1 h revealed a cyclic species (Figure 96, compound **201**) analogous to **197** in Scheme 31, consistent with intramolecular nucleophilic acyl substitution, supporting the conclusion that even elevated temperature could promote cyclization.

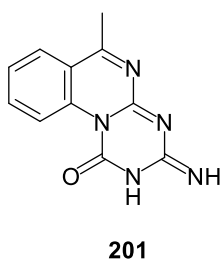
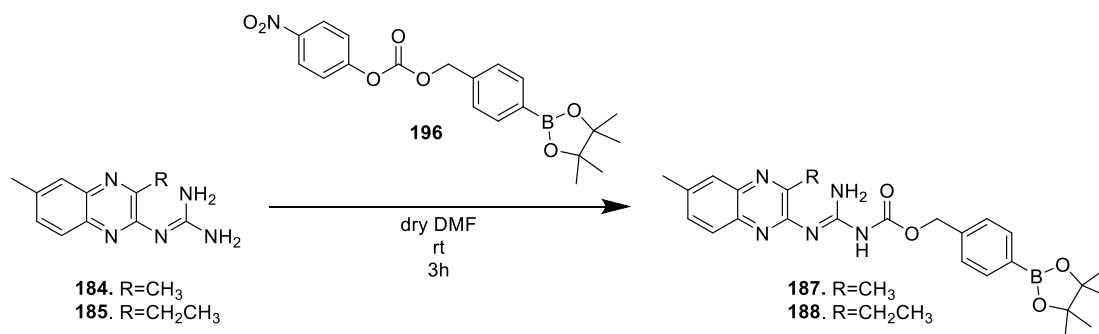


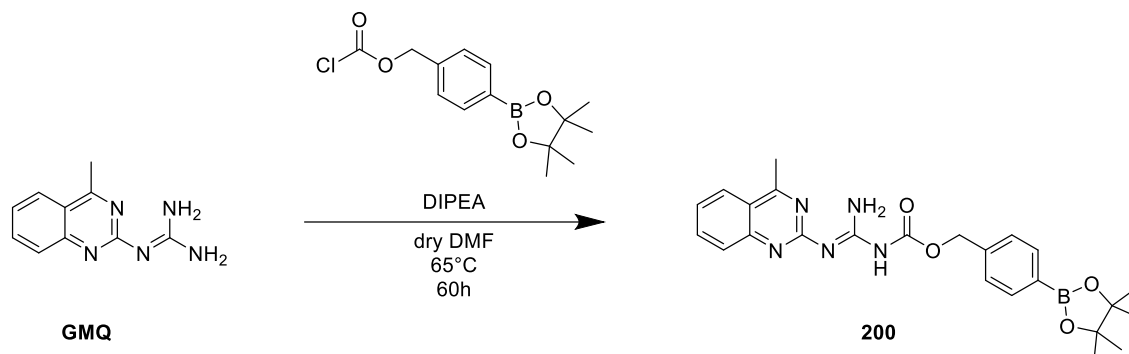
Figure 96. Proposed structure of **201**, detected in the reaction mixture after 1 h at elevated temperature.

The reaction was then repeated with **184**, after a basic wash to liberate the free base and before reacting it, again without any external base and at ambient temperature. Despite modest spectral quality, the NMR data were consistent with the target structure. On this basis, the optimized conditions were used to scale up the reaction and furnish the desired compounds **187** and **188** (Scheme 34).



Scheme 35. Preparation of final compounds **187** and **188**, following the optimized procedure.

To explore other conditions, the coupling was repeated using **GMQ** as the free base and an acyl-chloride derivative to engage a different and simpler mechanism (Scheme 35).



Scheme 35. Acyl-chloride route to obtain the boronate carbamate **200**. Coupling of **GMQ** (free base) with the acyl-chloride derivative of the *p*-(pinacol boronate)benzyl fragment affords the corresponding carbamate prodrug (**200**). This approach engages a simpler acylation mechanism than the carbonate route and is compatible with the free amine of **GMQ**, avoiding external bases.

Several sets of parameters were screened (Table 7). In an initial trial, the previous setup was reproduced; however, with this reagent a base is required to neutralize the HCl formed *in situ*. Accordingly, TEA and Na₂CO₃ were evaluated. Na₂CO₃ provided the best outcome, improving yield and facilitating purification, likely owing to the heterogeneous reaction medium.

Base (eq.)	Solvent	Temp (°C)	Observation / Outcome	Yield (%)
—	dry DMF	60	Incomplete conversion; HCl formation observed	20
TEA (1.0)	dry DMF	rt	Product detected; difficult purification	22
Na ₂ CO ₃ (1.0)	dry DMF	rt	Clean conversion; improved isolation	33

Table 7. Optimization of coupling conditions for **GMQ** with the acyl-chloride derivative (1 eq.). The use of Na₂CO₃ as base improved both yield and work-up efficiency, likely due to the heterogeneous nature of the reaction mixture.

The optimization study (Table 7) indicates that the presence and nature of the base are decisive for efficient coupling with the acyl-chloride reagent. In the absence of base, conversion

remained incomplete despite heating, consistent with *in situ* formation of HCl that protonates the amine nucleophile and simultaneously promotes competitive hydrolysis of the acyl chloride (and, potentially, degradation of the boronate).⁸⁶¹ Introducing TEA restored reactivity but provided only a modest improvement in isolated yield and complicated purification: as a homogeneous base, TEA and HCl form soluble salts that persist in the organic phase, favoring emulsions and leaving a broader impurity profile, while stronger basicity may also enable side reactions. In contrast, sodium carbonate afforded the cleanest conversion and the highest yield. Its mild, non-nucleophilic character efficiently scavenges HCl without competing with acylation, and the heterogeneous medium removes acid as insoluble inorganic salts, effectively shifting the equilibrium toward product and simplifying the workup. Performing the reaction at room temperature further limits hydrolysis of the acyl chloride and preserves the dioxaborolane unit, collectively explaining the superior outcome observed with Na₂CO₃. However, despite achieving reasonable success, this strategy and the use of this reagent were discontinued due to the reagent's high cost and the impracticality of *in-house* synthesis, which relies on hazardous inputs such as phosgene or triphosgene.

3.1.3. Release of the parent drug under oxidative conditions

As a proof of concept for the prodrug approach, the H₂O₂-triggered release of compound **188** under pathophysiological oxidant levels was evaluated. Physiological H₂O₂ levels are normally low ($\approx 0.5\text{--}7\ \mu\text{M}$ intracellular), whereas under pathophysiological oxidative stress they can rise substantially, locally reaching up to $\sim 1\ \text{mM}$, particularly in extracellular compartments. Activation was then initiated by adding 25 μL of a highly concentrated solution of H₂O₂ in PBS (50 mM) to 50 μL of a 1 mM DMSO solution of **188** combined with PBS (225 μL) and additional DMSO (150 μL), followed by a further addition of 50 μL of a 1 mM diclofenac solution in DMSO, used as the internal standard. The mixture was vortexed, and the reaction was incubated at 37 °C in an Eppendorf Thermomixer (1000 rpm). Aliquots (50 μL) were withdrawn at 0 min, 30 min, 1 h, 2 h, 4 h, and 6 h for analysis by analytic HPLC. Time-course chromatograms (Figure 97) were used to quantify depletion of prodrug **188** and release of active compound **185**, enabling direct comparison to negative controls without H₂O₂ and to reference compounds analysed in parallel.

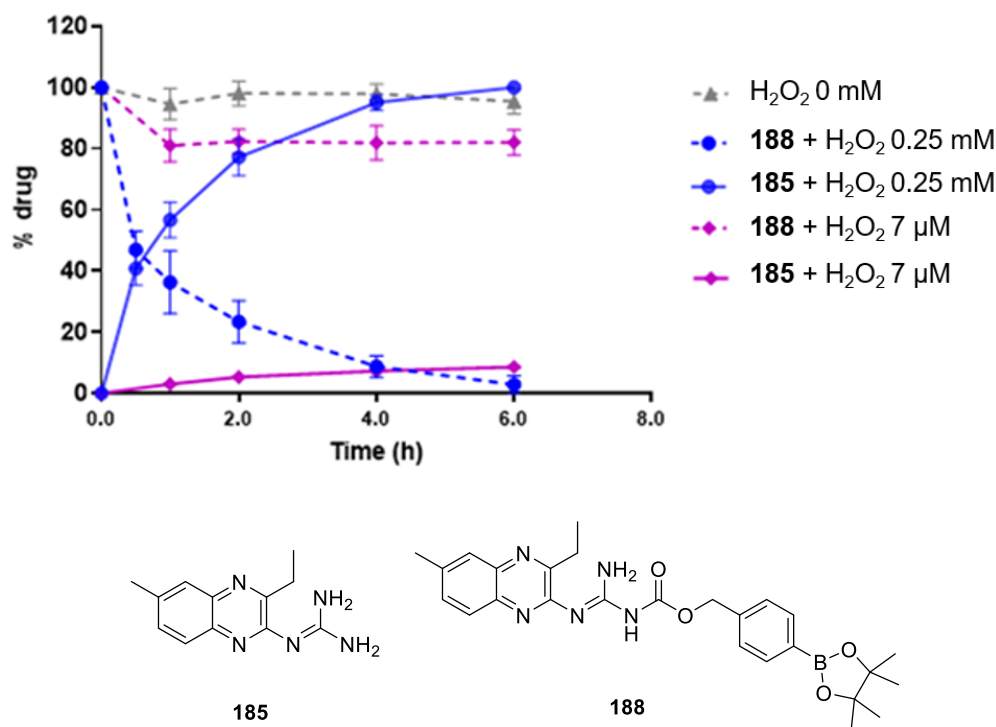


Figure 97. Time-dependent H_2O_2 release of **185** from prodrug **188**, tested as a mixture of the pinacol boronate ester and the free boronic acid derivatives.

In PBS at 37 °C, **188** is essentially unchanged over 6 h in the absence of H_2O_2 (≈ 95 –100% remaining; grey dashed line), indicating good chemical stability under assay conditions. At a near-physiological oxidant level (7 μM H_2O_2), only modest conversion is observed: **188** declines slowly toward $\sim 80\%$ (purple dashed line) with a corresponding, low-amplitude rise in **185** (~ 10 –15% at 6 h; purple plain line). By contrast, at a pathophysiological concentration (0.25 mM H_2O_2), **188** is rapidly consumed within the first hour and approaches near-complete depletion by 4–6 h (dashed blue line), with a reciprocal increase in **185** approaching quantitative formation (plain blue line). Error bars indicate variability consistent with a reproducible kinetic process.

Overall, the data demonstrate dose-dependent, selective release of **185** by H_2O_2 , minimal at basal ROS and efficient under elevated oxidative stress, supporting its utility as an oxidation-responsive prodrug.

On the other side, due to its rapid degradation under the activation conditions, compound **187** is not included in this discussion.

4. Conclusions

In this chapter a new medicinal chemistry platform was developed to exploit GABA_ARs antagonism and ROS-responsive prodrug design as a combined strategy for cancer therapy. Building on the GQX chemotype, previous SAR data were integrated with docking studies to define the key pharmacophoric elements and the role of substituents at C3, C6 and C7 in shaping potency and subtype selectivity at GABA_ARs and GABA_{A α} Rs. In this framework, compound **186** was conceived as a quinoxaline antagonist combining a moderately lipophilic C3 substituent with a bulkier C7 phenyl group to probe a sterically differentiated subpocket in GABA_{A α} Rs, while compounds **187** and **188** incorporated a boronate-based, ROS-sensitive linker to achieve H₂O₂-triggered release of potent GQX analogues **184** and **185**.

From a synthetic standpoint, this work established a robust multistep route to quinoxaline-based antagonists **184–186**, combining palladium-catalysed Suzuki–Miyaura coupling, S_N_{Ar} functionalisation with amino acids, Fischer esterification, Béchamp nitro reduction with concomitant ring closure, oxidative aromatisation, POCl₃-mediated chlorination and final guanylation. Particular attention was devoted to understanding and controlling the reactivity of the aryl fluoride and nitro groups during cross-coupling, as well as the stability of carbamate and ester functionalities under basic and oxidative conditions. Mechanistic analysis of side reactions (notably ester hydrolysis, transesterification and undesired intramolecular cyclisations) guided the optimisation of several steps, including the use of unprotected amino acids for S_N_{Ar}, strictly anhydrous conditions for Fischer esterification, and NMP instead of DMF for the guanylation stage.

A second major outcome of this chapter is the development and optimisation of a ROS-responsive boronate carbonate linker suitable for coupling to polybasic **GQX** derivatives. Systematic exploration of carbonate and acyl-chloride strategies revealed the competing tendency of highly basic conditions to promote intramolecular cyclisation and loss of the boronate moiety. These insights led to an optimised protocol in which the free-base form of the antagonist is coupled to an activated boronate carbonate under mild, base-limited conditions, enabling isolation of prodrugs **187** and **188** while largely preserving the sensitive dioxaborolane unit. Although yields remain moderate, the route is synthetically tractable and scalable.

Release studies on **188** confirmed that the boronate trigger responds selectively to oxidising conditions compatible with tumour microenvironments. In PBS at 37 °C, **188** is essentially stable in the absence of H₂O₂, whereas near-physiological ROS levels induce only slow, partial

conversion. By contrast, at pathophysiological H_2O_2 concentrations the prodrug is rapidly and almost quantitatively converted to the parent antagonist **185**, demonstrating that the linker can function as an effective chemical switch. The closely related prodrug **187**, on the other hand, proved too labile under the same activation conditions, underscoring the delicate balance between trigger reactivity and chemical stability and highlighting the need for careful tuning of the linker environment.

At the present stage, however, no binding or functional data are yet available for compounds **186–188**, and their affinity, efficacy and selectivity at GABA_A Rs and $\text{GABA}_\text{A}\rho$ Rs remain to be established.

Future work will therefore focus on completing the pharmacological characterisation of these molecules, including functional studies in immune and tumour cell models to assess their ability to relieve GABA-mediated immunosuppression. In parallel, the chemical space around the quinoxaline core and the boronate linker will be further expanded, with systematic variation of the C3/C7 substituents to optimise both receptor selectivity and prodrug stability. Taken together, the results presented here provide a synthetic and conceptual foundation for the development of ROS-activated GABA_A Rs antagonists as targeted anticancer agent

CHAPTER 4

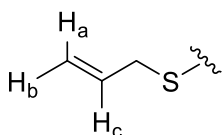
Experimental section

1. Materials and methods

Starting materials and reagents were purchased from Aldrich-Italia (Milan, Italy), Alfa Aesar (Lancashire, UK), Fluorochem Ltd. (Cork, Ireland) or other commercial suppliers and used without further purification unless otherwise stated. Commercial solvents were used as received. Air- and/or moisture-sensitive reactions were carried out under an argon atmosphere in oven- or flame-dried glassware. Anhydrous solvents were obtained either from an in-house purification system maintained under argon or by storage over 3 Å molecular sieves.

Melting points were determined on Büchi B-540 or Büchi 530 apparatus and are uncorrected. IR spectra were recorded in KBr pellets or CHCl₃ on a Perkin-Elmer 398 spectrophotometer.

¹H and ¹³C NMR spectra were recorded on Varian Gemini 200 (200 MHz for ¹H), Bruker Avance DPX400 (400 MHz for ¹H), JEOL JNM ECZ-400S/L1 FT (400 MHz for ¹H and 101 MHz for ¹³C), or Bruker Avance III 600 MHz spectrometers (600 MHz for ¹H and 151 MHz for ¹³C, where applicable). ¹⁹F NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer. Spectra were generally acquired in DMSO-d₆ or CDCl₃, as indicated for each compound. Chemical shifts (δ) are reported in parts per million (ppm) relative either to tetramethylsilane (TMS) as internal standard or to the residual solvent signal (DMSO-d₆: δ H = 2.50, δ C = 39.52; CDCl₃: δ H = 7.26, δ C = 77.16). Coupling constants (*J*) are given in hertz (Hz). Proton multiplicities are abbreviated as follows: s = singlet, br s = broad singlet, d = doublet, dd = doublet of doublets, dt = doublet of triplets, td = triplet of doublets, ddt = doublet of doublet of triplets, t = triplet, q = quartet, quint = quintet, sx (sex) = sextet, sept = septet, m = multiplet. For triazinic derivatives, ¹H NMR integrations were reported assuming a 1:1 mixture of the two tautomers. For allylic derivatives, ¹H NMR data were assigned consistently with the structural representations reported below.



Thin-layer chromatography (TLC) was carried out on Merck silica gel 60 F₂₅₄ aluminum plates, with visualization under UV light (254/366 nm). Column chromatography was performed on silica gel 60 Å (Merck or Merck Geduran[®], particle size 0.040–0.063 mm; 220–440 mesh, corresponding to approx. 35–75 µm), or using Biotage[®] SNAP Ultra cartridges packed with Biotage[®] HP-Sphere[™] spherical silica on an Isolera[™] One (Biotage) instrument. Automated flash column chromatography was also carried out on a CombiFlash NextGen 300+ system. For normal-phase separations, mixtures of ethyl acetate and heptane were used, with conditions guided by TLC. For reversed-phase separations on CombiFlash, solvent A was H₂O/acetonitrile (95:5, 0.1% TFA) and solvent B was acetonitrile/H₂O (90:10, 0.1% TFA); gradients or isocratic programs are specified in the individual compound procedures.

Reaction monitoring and LC–MS analyses were performed on an Agilent 1100 LC/MSD VL system (G1946C) or an Agilent 6130 single-quadrupole mass spectrometer equipped with an Agilent 1200 HPLC system (diode-array detector, autosampler) using a Zorbax Eclipse XDB-C18 column (4.6 × 50 mm). Typical analytical conditions employed a binary solvent system of methanol/water (95:5) at a flow rate of 0.4–1.0 mL min⁻¹. UV detection was generally monitored at 254 nm. Mass spectra were acquired in positive and negative electrospray ionization modes, scanning over the mass range *m/z* 50–1500. Typical ion source parameters were: drying gas flow 9 mL min⁻¹, nebulizer pressure 40 psig, and drying gas temperature 350°C.

Preparative HPLC purifications were carried out on a Thermo Scientific UltiMate system equipped with an LPG-3400BX pump (up to 20 mL min⁻¹), Rheodyne 9725i injector (10 mL loop), MWD-3000SD multi-wavelength detector (typically 200, 247, 254 and 280 nm), and AFC-3000SD fraction collector, using a Gemini-NX C18 column (21.2 × 250 mm, 5 µm, 110 Å; Phenomenex). Elution employed solvent A (H₂O/0.1% TFA) and solvent B (acetonitrile/H₂O/0.1% TFA, 90:10); gradients or isocratic conditions are reported with each compound. Data acquisition and processing for both preparative and analytical HPLC were performed using Chromeleon (versions 6.80 or 7.3).

Elemental analyses (C, H, N, and S when applicable) were carried out on a Thermo Scientific Flash 2000 analyzer. Experimental values were within ±0.4% of the theoretical values. Unless otherwise stated, all target compounds showed a purity ≥ 95% as determined by elemental analysis and/or HPLC.

Prodrug stability under oxidative conditions was evaluated on a Thermo Scientific UltiMate 3000 HPLC system equipped with an HPG-3400SD pump (1.0 mL min⁻¹), a WPS-3000SL autosampler, and a DAD-3000D diode-array detector, using a Gemini-NX C18 column (4.6 × 250 mm, 3 μm, 110 Å; Phenomenex). Elution employed solvent A (H₂O/0.1% TFA) and solvent B (acetonitrile/H₂O/0.1% TFA, 90:10), with a linear gradient from 0 to 100% B over 10 min; the injection volume was 2 μL. UV detection was carried out at 254 nm, and peak areas were integrated manually.

Synthetic procedures are reported in the order in which the compounds appear in the Schemes and in the Results and Discussion section.

2. General procedures

2.1. General procedure for the synthesis of compounds **95a–c**, **95e**, **95f**

The appropriate hydrazine derivatives **94a–e** (20 mmol) were added to a solution of ethyl(ethoxymethylene)cyanoacetate (3.38 g, 20 mmol) in anhydrous toluene (20 mL) and the mixture was heated at 80 °C for 8 h. The solution was concentrated under reduced pressure to half of the volume and allowed to cool to room temperature. The yellow pale solid was filtered and recrystallized from toluene to afford **95a–c**, **95e**, **95f** as white solids.

ethyl 5-amino-1-phenethyl-1H-pyrazole-4-carboxylate (**95a**)

Yield: 60% (white solid). Mp: 85–86°C. ¹H NMR (400 MHz DMSO-d₆): δ 7.59 (s, 1H, H-3), 7.28–6.90 (m, 5H Ar), 4.30 (br s, 2H, NH₂ disappears with D₂O), 4.13 (q, *J* = 7.2, 2H, CH₂O), 4.03 (t, *J* = 6.8, 2H, CH₂N), 3.02 (t, *J* = 6.8, 2H, CH₂Ar), 1.25 (t, *J* = 7.2, 3H, CH₃). IR (KBr): cm⁻¹ 3428, 3296 (NH₂), 1685 (CO). MS: *m/z* 259 [M+1]⁺. Elem. anal. calcd. for C₁₄H₁₇N₃O₂: C 64.85, H 6.61, N 16.21; found: C 64.92, H 6.80, N 16.02.

ethyl 5-amino-1-(2-phenylpropyl)-1H-pyrazole-4-carboxylate (**96b**)

Yield: 60% (white solid). Mp: 85–86°C. ¹H NMR (400 MHz DMSO-d₆): δ 7.55 (s, 1H, H-3), 7.00–7.16 (m, 5H Ar), 4.79 (br s, 2H, NH₂ disappears with D₂O), 4.10 (q, *J* = 7.0, 2H, CH₂O), 3.89–3.91 and 3.78–3.83 (2m, 2H, CH₂N), 3.24–3.26 (m, 1H, CHCH₃), 1.06–1.20 (m, 6H, 2CH₃). IR (KBr): cm⁻¹ 3398, 3291 (NH₂), 1681 (CO). MS: *m/z* 273 [M+1]⁺. Elem. anal. calcd. for C₁₅H₁₉N₃O₂: C 65.91; H 7.01; N 15.37; found: C 65.94; H 7.06; N 15.33.

ethyl 5-amino-1-(2-hydroxy-2-phenylethyl)-1H-pyrazole-4-carboxylate (95c)

Yield: 80% (white solid). Mp:136-137°C. ¹H NMR (400 MHz CHCl₃): δ 7.58 (s, 1H, H-3), 7.42-7.23 (m, 5H Ar), 5.30 (br s, 2H, NH₂, disappears with D₂O), 5.13-5.02 (m, 1H, CHOH), 4.25 (q, *J* = 7.0, 2H, CH₂O), 4.20-4.3.92 (m, 2H, CH₂N), 3.53 (m, 1H, OH, disappears with D₂O), 1.33 (t, *J* = 7.0, 3H, CH₃). IR (CHCl₃): cm⁻¹ 3470, 3330 (NH₂), 3300-3000 (OH), 1685 (CO). Elem. anal. calcd. for C₁₄H₁₇N₃O₃: C 61.08, H 6.22, N 15.26; found: C 61.12; H 6.18; N 15.25.

ethyl 5-amino-1-(2-(4-chlorophenyl)-2-hydroxyethyl)-1H-pyrazole-4-carboxylate (95e)

Yield: 75% (white solid). Mp:168-169°C. ¹H NMR (200 MHz DMSO-d₆): δ 8.59 (s, 1 H, H-3), 7.46-7.25 (m, 4 H, Ar), 5.33 (br s, 2 H, NH₂, disappears with D₂O), 5.18-5.05 (m, 1 H, CHO), 4.28 (q, *J*=7.0, 2 H, CH₂O), 4.19-3.91 (m, 2 H, CH₂N), 3.56 (br s, 1 H, OH, disappears with D₂O), 1.38 ppm (t, *J* = 7.0 Hz, 3 H, CH₃); IR (KBr): cm⁻¹ 3412, 3291 (NH₂), 3219-3100 (OH), 1685 (CO). Elem. anal. calcd. for C₁₄H₁₆ClN₃O₃: C 54.29, H 5.21, N 13.57; found: C 54.29, H 5.21, N 13.57.

ethyl 5-amino-1-(2-(4-bromophenyl)-2-hydroxyethyl)-1H-pyrazole-4-carboxylate (95f)

Yield: 65% (white solid). Mp:164-165°C. ¹H NMR (200 MHz DMSO-d₆): δ 7.55 (s, 1 H, H-3), 7.47-7.37 and 7.24-7.14 (2 m, 4 H, Ar), 5.12-5.03 (m, 1 H, CHO), 4.21 (q, *J* = 7.2 Hz, 2 H, CH₂O), 4.13-3.84 (m, 2 H); IR (KBr): cm⁻¹ 3411, 3291 (NH₂), 3157-2900 (OH), 1689 (CO). Elem. anal. calcd. for C₁₄H₁₆BrN₃O₃: C 47.47, H 4.55, N 11.86; found: C 47.51, H 4.54, N 11.83.

2.2. Synthesis of ethyl 5-amino-1-(2-oxo-2-phenylethyl)-1H-pyrazole-4-carboxylate (95d)

A solution of sulfur trioxide–pyridine complex (30 mmol, 4.77 g) in DMSO (20 mL) was added dropwise to a stirred solution of **95c** (2.75 g, 10 mmol) in DMSO (10 mL) and TEA (9 mL) at room temperature. The reaction mixture was stirred for 30 min, then carefully acidified to pH 5–6 with 1 M HCl and poured into water. The resulting precipitate was collected by filtration and purified by recrystallization from absolute ethanol, affording **95d**. Yield: 82% (white solid). Mp:138-139°C. ¹H NMR (200 MHz CDCl₃): δ 8.02 (m, 1H, H3), 7.75-7.45 (m, 4H, Ar), 5.48 (s, 2H, CH₂N), 5.20–5.10 (m, 2H, NH₂, disappears with D₂O), 4.27 (q, *J* = 7.1, 2H, CH₂O), 1.33 (t, *J* = 7.1, 3H, CH₃). IR (KBr): cm⁻¹ 3425, 3332 (NH₂), 1688 (CO), 1668 (C=COEt). Elem. anal. calcd. for C₁₄H₁₅N₃O₃: C 61.53; H 5.53; N 15.38; found: 61.55; H 5.55; N 15.39.

2.3. General procedure for the synthesis of compounds 96a–f

Benzoyl isothiocyanate (6.68 mmol, 2.0 g) was added dropwise to a solution of the intermediate **95a–f** (6.07 mmol, 0.4 mL) in dry THF (25 mL). The mixture was refluxed for 12 h and then the solvent was removed under reduced pressure to afford a yellow pale oil. The product was precipitated by addition of a mixture of diethyl ether and petroleum spirit to obtain a solid which was filtered affording the desired compounds **96a–f**.

ethyl 5-(3-benzoylthioureido)-1-phenethyl-1H-pyrazole-4-carboxylate (96a)

Yield: 60% (white solid). Mp: 168–169°C. ¹H NMR (200 MHz DMSO-*d*₆): δ 11.76 (s, 1H, NH, disappears with D₂O), 9.30 (s, 1H, NH, disappears with D₂O), 1.20 (t, *J* = 7.2, 3H, CH₃), 7.94 (s, 1H, H-3), 7.90–6.97 (m, 10H Ar), 4.30–4.10 (m, 4H, CH₂O + CH₂N), 3.18 (t, *J* = 7.0, 2H, CH₂Ar). IR (KBr) cm^{–1}: 3366, 3127 (NH), 1707 (COOEt), 1662 (CONH). MS: *m/z* 423 [M+1]⁺. Elem. anal. calcd. for C₂₂H₂₂N₄O₃S: C 62.54, H 5.25, N 13.26, S 7.59; found: C 62.56, H 5.26, N 13.29, S 7.62.

ethyl 5-(3-benzoylthioureido)-1-(2-phenylpropyl)-1H-pyrazole-4-carboxylate (96b)

Yield: 63% (white solid). Mp: 112–113°C. ¹H NMR (200 MHz DMSO-*d*₆): δ 11.60 (br s, 1H, NH disappears with D₂O), 9.19 (br s, 1H, NH disappears with D₂O), 7.93 (s, 1H, H-3), 7.89–7.07 (m 10H Ar), 4.22–4.17 (m, 4H, CH₂N + CH₂O), 3.49–3.43 (m, 1H, CHCH₃), 1.29–1.21 (m, 6H, 2CH₃). IR (KBr) cm^{–1}: 3384, 3129 (NH), 1696 (COOEt), 1666 (CONH). MS: *m/z* 437 [M+1]⁺. Elem. anal. calcd. for C₂₃H₂₄N₄O₃S: C 63.28, H 5.54, N 12.83, S 7.34; found: C 63.29, H 5.56, N 12.86, S 7.30.

ethyl 5-(3-benzoylthioureido)-1-(2-hydroxy-2-phenylethyl)-1H-pyrazole-4-carboxylate (96c)

Yield: 93% (white solid). Mp: 171–172°C. ¹H NMR (200 MHz DMSO-*d*₆): δ 12.05 (s, 1H, NH, disappears with D₂O), 8.70 (s, 1H, NH, disappears with D₂O), 8.02 (s, 1H, H-3), 7.98–7.05 (m, 10H Ar), 4.68–4.58 (m, 1H, CHO), 4.20–3.97 (m, 5H, 2CH₂ + OH, 1H disappears with D₂O), 1.29 (t, *J* = 7.0, 3H, CH₃). Elem. anal. calcd. for C₂₂H₂₂N₄O₄S: C 60.26, H 5.06, N 12.78, S 7.31; found C 60.22, H 5.20, N 12.95, S 7.50.

ethyl 5-(3-benzoylthioureido)-1-(2-oxo-2-phenylethyl)-1H-pyrazole-4-carboxylate (96d)

Yield: 71% (white solid). Mp: 143–144°C. ¹H NMR (400 MHz, DMSO-*d*₆): 12.18 (s, 1H, NH), 12.08 (s, 1H, NH), 8.04–7.99 (m, 2H Ar), 7.97 (s, 1H, H-3), 7.91 (d, *J* = 7.4 Hz, 2H Ar), 7.68–7.60 (m, 2H Ar), 7.54–7.46 (m, 3H Ar), 5.84 (s, 2H, NCH₂), 4.14–3.81 (q, *J* = 7.1 Hz, 2H, CH₂O), 1.16 (t, *J* = 7.1 Hz, 3H). ¹³C NMR (101 MHz, DMSO-*d*₆): δ 193.10, 192.92, 183.12,

169.20, 161.82, 140.98, 140.16, 134.76, 134.65, 133.99, 132.15, 129.45, 129.34, 129.03, 128.92, 125.84, 109.06, 60.35, 56.57, 14.52. Elem. anal. calcd. for C₂₂H₂₀N₄O₄S: C 60.54, H 4.62, N 12.84, S 7.35; found: C 60.62, H 4.62, N 12.62, S 7.20.

ethyl 5-(3-benzoylthioureido)-1-(2-(4-chlorophenyl)-2-hydroxyethyl)-1H-pyrazole-4-carboxylate (96e)

Yield: 85% (white solid). Mp: 129-130°C. ¹H NMR (200 MHz, DMSO-d₆): δ 12.16 (s, 1H, NH, disappears with D₂O), 9.36 (s, 1H, NH, disappears with D₂O), 8.05 (s, 1H, H-3), 7.90-7.00(m, 9HAr), 5.38-5.25 (m, 1H, CHO), 4.38-4.10 (m, 5H, 2CH₂+OH, 1H disappears with D₂O), 1.31 (t, *J* = 7.0, 3H, CH₃). IR (KBr): cm⁻¹: 3444, 3261 (NH), 3190-2940 (OH), 1683 (CO). Elem. anal. calcd. for C₂₂H₂₁N₄O₄S: C 55.87, H 4.48, Cl 7.50, N 11.85, S, 6.78; found: C 55.88, H 4.50, N 11.87, S 6.73.

ethyl 5-(3-benzoylthioureido)-1-(2-(4-bromophenyl)-2-hydroxyethyl)-1H-pyrazole-4-carboxylate (96f)

Yield: 91% (white solid). Mp: 173-174°C. ¹H NMR (400 MHz, DMSO-d₆): δ 12.13 (br s, 2H, NH + NH), 8.00-7.98 (m, 2H Ar), 7.86 (s, 1H, H-3), 7.68-7.64 (m, 1H Ar), 7.53 (t, *J* = 7.7 Hz, 2H Ar), 7.46 (d, *J* = 8.4 Hz, 2H Ar), 7.24 (d, *J* = 8.3 Hz, 2H Ar), 5.89 (br s., 1H, OH), 4.93 (t, *J* = 6.1 Hz, 1H, CHAr), 4.15-4.06 (m, 4H, OCH₂ + NCH₂), 1.14 (t, *J* = 7.1 Hz, 3, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 182.79, 168.86, 161.89, 142.10, 140.50, 133.96, 132.35, 131.58, 129.38, 129.09, 128.85, 121.17, 108.55, 71.20, 60.18, 56.42, 14.54. Elem. anal. calcd. for C₂₂H₂₁BrN₄O₄S: C 51.08, H 4.09, N 10.83, S 6.20; found: C 50.75, H 3.93, N 11.75, S 5.43.

2.4. General procedure for the synthesis of compounds 97a-f

A solution of intermediates **96a-f** (2.23 mmol, 1.0 g) in 2 M NaOH (10 mL) was refluxed for 10 min, then diluted with H₂O (10 mL) and acidified with glacial acetic acid. After 12 h at 4°C, the precipitated solid was filtered and recrystallized from absolute EtOH to give the desired compound **97a-f**.

1-phenethyl-6-thioxo-1,5,6,7-tetrahydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (97a)

Yield: 65% (white solid). Mp: 200-201°C. ¹H NMR (200 MHz, DMSO-d₆): δ 9.25 (s, 1H, NH, disappears with D₂O), 7.95 (s, 1H, H-3), 7.50-7.00 (m, 5H Ar), 4.20 (t, *J* = 7.0, 2H, CH₂N), 3.20 (t, *J* = 7.0, 2H, CH₂Ar). IR (KBr): cm⁻¹ 3400-3300 (NH + OH), 1660 (CO). Elem. anal. calcd. for C₁₃H₁₂N₄OS: C 57.34, H, 4.44, N 20.57, S, 11.77; found: C 57.37, H, 4.47, N 20.57, S, 11.75.

1-(2-phenylpropyl)-6-thioxo-1,5,6,7-tetrahydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (97b)

Yield: 60% (white solid). Mp: 192-193°C. ¹H NMR (200 MHz, DMSO-d₆): δ 10.49 (br s, 1H, disappears with D₂O), 7.82 (s, 1H, H-3), 7.21-7.12 (m, 5H Ar), 4.51-4.45 and 4.32-4.26 (2m, 2H, CH₂N), 3.42-3.36 (m, 1H, CHCH₃), 1.60-1.22 (m, 3H, CH₃). IR (KBr): cm⁻¹ 3455-2867 (NH + OH), 1677 (CO). MS: m/z 286 [M+1]⁺. Elem. anal. calcd. for C₁₄H₁₄N₄OS: C 58.72, H, 4.93, N 19.57, S, 11.20; found: C 58.75, H, 4.91, N 19.58, S, 11.25.

1-(2-hydroxy-2-phenylethyl)-6-thioxo-1,5,6,7-tetrahydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (97c)

Yield: 80% (white solid). Mp: 264-265°C. ¹H NMR (200 MHz, DMSO-d₆): δ 13.40 (s, 1H, NH, disappears with D₂O), 12.20 (s, 1H, NH, disappears with D₂O), 8.02 (s, 1H, H-3), 7.51-7.20 (m, 5H Ar), 5.66 (br s, 1H, OH, disappears with D₂O), 5.00-4.85 (m, 1H, CHO), 4.72-4.55 and 4.30-4.15 (2m, 2H, CH₂N). IR (KBr): cm⁻¹ 3362 (NH), 3242-2973 (OH), 1681 (CO). Elem. anal. calcd. for C₁₃H₁₂N₄O₂S: C 54.16, H 4.20, N 19.43, S 11.12; found: C 54.28, H 4.27, N 19.70, S 10.86.

1-(2-oxo-2-phenylethyl)-6-thioxo-1,5,6,7-tetrahydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (97d)

Yield: 64% (white solid). Mp: 268-269°C. ¹H NMR (400 MHz, DMSO-d₆): δ 13.41 (br s, 1H, NH) 12.24 (s, 1H, NH), 8.03-8.01 (m, 2H Ar), 7.97 (s, 1H, H-3), 7.73-7.69 (m, 1H Ar), 7.58 (t, *J* = 7.7 Hz, 2H Ar), 5.95 (s, 2H, NCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 192.63, 175.31, 156.66, 145.60, 136.52, 134.66, 134.59, 129.46, 128.72, 102.74, 56.71. Elem. anal. calcd. for C₁₃H₁₀N₄O₂S: C 54.54, H 3.52, N 19.57, S 11.20; found: C 54.50, H 3.62, N 19.90, S 10.61

1-(2-(4-chlorophenyl)-2-hydroxyethyl)-6-thioxo-1,5,6,7-tetrahydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (97e)

Yield: 70% (white solid). Mp: 249-250°C. ¹H NMR (200 MHz, DMSO-d₆): δ 13.38 (s, 1H, NH, disappears with D₂O), 12.19 (s, 1H, NH, disappears with D₂O), 7.97 (s, 1H, H-3), 7.51-7.30 (m, 4H Ar), 5.71 (br s, 1H, OH, disappears with D₂O), 5.00-4.84 (m, 1H, CHO), 4.65-4.13 (2m, 2H, CH₂N). IR (KBr): cm⁻¹ 3390, 3220 (NH), 3100-2700 (OH), 1675 (CO). Elem. anal. calcd. for C₁₃H₁₁N₄ClO₂S: C 48.38, H 3.44, N 17.36, S 9.93; found: C 48.38, H 3.44, N 17.36, S 9.93.

1-(2-(4-bromophenyl)-2-hydroxyethyl)-6-thioxo-1,5,6,7-tetrahydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (97f)

Yield: 90% (white solid). Mp: 281-283°C. ¹H NMR (400 MHz, DMSO-d₆): δ 13.33 (br s, 1H, NH), 12.13 (s, 1H, NH), 7.93 (s, 1H, H-3), 7.53-7.51 (m, 2H Ar), 7.37-7.35 (m, 2H Ar), 5.69 (br s, 1H, OH), 4.88-4.85 (m, 1H, CHAr), 4.57-4.51 and 4.21-4.16 (2m, 2H, NCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 175.26, 156.71, 145.33, 141.99, 136.45, 131.54, 129.23, 121.23, 102.24, 71.20, 55.06. Elem. anal. calcd. for C₁₃H₁₁BrN₄O₂: C 42.52, H 3.02, N 15.26, S 8.73; found: C 42.66, H 2.82, N 15.29, S 8.11.

2.5. General procedure for the synthesis of compounds 98a, 98b, 98d-f, 98h

A solution of **97a–d, 97f** (5.00 mmol, 1.5 g) and methyl iodide or ethyl iodide (37.50 mmol) in anhydrous THF (25 mL) was refluxed for 12 h. The solvent and the excess of alkyl iodide were removed by distillation under reduced pressure; the oily residue was crystallized by adding a mixture of diethyl ether and petroleum spirit (25 ml). The obtained solid was filtered and recrystallized from absolute EtOH to give **98a, 98b, 98d-f, 98h**.

6-(methylthio)-1-(1-phenylethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (98a)

Yield: 60% (white solid). Mp: 173-174°C. ¹H NMR (200 MHz, DMSO-d₆): δ 8.01 (s, 1H, H-3), 7.13-6.96 (m, 5H Ar), 4.50 (t, *J* = 7.0, 2H, CH₂N), 3.13 (t, *J* = 7.0, 2H, CH₂Ar), 2.41 (s, 3H, CH₃S). IR (KBr): cm⁻¹ 3200-2850 (NH + OH) 1678 (CO). Elem. anal. calcd. for C₁₄H₁₄N₄OS: C 58.72, H 4.93, N 19.57, S 11.20; found: C 58.88, H 5.05, N 19.73, S 11.03.

6-(ethylthio)-1-phenethyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (98b)

Yield: 69% (white solid). Mp: 182-183°C. ¹H NMR (400 MHz, DMSO-d₆): δ 12.25 (s, 1H, NH), 7.91 (s, 1H, H-3), 7.11-7.09 (m, 3H Ar), 7.05-7.03 (m, 2H Ar), 4.43 (t, *J* = 7.0 Hz, 2H, NCH₂), 3.10-3.04 (m, 4H, SCH₂ + CH₂Ar), 1.26 (t, *J* = 7.3 Hz, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 159.95, 158.13, 152.30, 138.64, 134.84, 129.12, 128.78, 126.89, 103.17, 48.46, 35.68, 24.98, 14.90. Elem. anal. calcd. for C₁₅H₁₆N₄OS: C 59.98, H 5.37, N 18.65, S 10.67; found: C 60.33, H 5.57, N 18.86, S 10.28.

6-(ethylthio)-1-(2-phenylpropyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (98d)

Yield: 61% (white solid). Mp: 155–156 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 12.10 (br s, 1H, NH, disappears with D₂O), 7.94 (s, 1H, H-3), 7.07-7.30 (m, 5H, Ar), 4.26–4.50 (m, 2H, CH₂N), 3.40-3.50 (m, 1H, CHCH₃), 3.17 (q, *J* = 7.2 Hz, 2H, SCH₂), 1.36 (t, *J* = 7.2 Hz, 3H, SCH₂CH₃),

1.22 (d, $J = 7.0$ Hz, 3H, CH₃CH). IR (KBr) cm⁻¹: 3110–2800 (NH), 1681 (C=O). MS: m/z 315 [M + H]⁺. Elem. anal. calcd. for C₁₆H₁₈N₄OS: C 59.98, H 5.37, N 18.65, S 10.67; found: C 60.33, H 5.57, N 18.86, S 10.28.

6-(methylthio)-1-(2-oxo-2-phenylethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (98e)

Yield: 74% (white solid). Mp: 261–261°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.04–7.97 (m, 3H, H-3 + 2 Ar), 7.68–7.64 (m, 1H Ar), 7.55–7.51 (m, 2H Ar), 5.87 (s, 2H, NCH₂), 2.39 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 193.16, 161.13, 157.94, 153.22, 135.27, 134.83, 134.49, 129.37, 128.58, 103.26, 53.86, 13.01. Elem. anal. calcd. for C₁₄H₁₂N₄O₂S: C 55.99, H 4.03, N 18.6CH₆, S 10.67; found: C 56.00, H 4.07, N 18.51, S 10.33.

1-(2-Hydroxy-2-phenylethyl)-6-(methylthio)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (98f)

Yield: 72% (white solid). Mp: 208–209°C. ¹H NMR: δ 7.97 (s, 1H, H-3), 7.42–7.20 (m, 5H Ar), 5.68 (d, 1H, OH, disappears with D₂O), 5.18–5.04 (m, 1H, CHO), 4.50–4.27 (m, 2H, CH₂N), 2.51 (s, 3H, CH₃S). IR (KBr) cm⁻¹: 3544 (NH), 3450–3100 (OH), 1678 (CO). Elem. anal. calcd. for C₁₄H₁₄N₄O₂S: C 55.62, H 4.67, N 18.53, S 10.60; found: C 55.64, H 4.64, N 18.57, S 10.60.

1-(2-(4-bromophenyl)-2-hydroxyethyl)-6-(methylthio)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (98h)

Yield: quantitative (white solid). Mp: 250–251°C. ¹H NMR (400 MHz, DMSO-d₆) δ 12.31 (s, 1H, NH), 7.91 (s, 1H, H-3), 7.43–7.40 (m, 2H Ar), 7.14–7.12 (m, 2H Ar), 5.74 (d, $J = 4.7$ Hz, 1H, OH), 5.01–4.99 (m, 1H, CHAr), 4.39–4.34 and 4.28–4.24 (2m, 2H, NCH₂), 2.45 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-d₆) δ 160.55, 158.06, 152.63, 142.42, 134.93, 131.41, 128.80, 120.97, 103.10, 71.06, 54.34, 13.34. Elem. anal. calcd. for C₁₄H₁₃BrN₄O₂: C 44.11, H 3.44, N 14.70, S 8.41; found: C 44.45, H 3.45, N 14.71, S 8.37.

2.6. Synthesis of 6-(allylthio)-1-phenethyl-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (98c)

Allyl bromide (2.86 mmol, 0.3 mL) was added to a solution of the intermediate **97a** (1.43 mmol, 390.1 mg) and NaOH (2.86 mmol, 114.1 mg) in absolute EtOH. The mixture was refluxed for 2 h and, after cooling, the solvent was removed under reduced pressure. The obtained slurry was suspended in 2M HCl and extracted with DCM. The organic layer was dried over Na₂SO₄ and concentrated under reduced pressure. The crude oil was purified by column

chromatography, using a mixture of EtOAc and PE (4:6) as eluent, to afford the pure product as a solid. Yield: 81% (white solid). Mp: 159°C. ¹H NMR (400 MHz, DMSO-d₆): δ 12.29 (s, 1H, NH), 7.92 (s, 1H, H-3), 7.19-7.09 (m, 3H Ar), 7.04-7.02 (m, 2H Ar), 5.85 (ddt, *J* = 16.9, 10.0, 6.9 Hz, 1H, H_c), 5.28 (dd, *J* = 16.9, 1.4 Hz, 1H, H_a), 5.10 (dd, *J* = 1.4, 10.00 Hz, 1H, H_b), 4.43 (t, *J* = 7.0 Hz, 2H, NCH₂), 3.75 (d, *J* = 6.9 Hz, 2H, SCH₂), 3.08 (t, *J* = 7.0 Hz, 2H, CH₂Ar). ¹³C NMR (101 MHz, DMSO-d₆): δ 159.33, 158.11, 152.19, 138.65, 134.89, 133.80, 129.15, 128.77, 126.91, 119.05, 103.20, 48.49, 35.68, 33.04. Elem. anal. calcd. for C₁₆H₁₆N₄OS: C 61.52, H 5.16, N 17.94, S 10.26; found: C 61.54, H 5.119, N 17.97, S 10.21.

2.7. General procedure for the synthesis of compounds 98g, 98i, 177

K₂CO₃ (8.5 mmol, 1.2 g) was added to a suspension of **97d-f** (3.4 mmol) in acetone. Pyridine (4.8 mmol, 0.40 mL) and allyl bromide (5.1 mmol, 0.60 g) were then added successively, and the reaction mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and the residue was suspended in water and dichloromethane. The resulting solid was collected by filtration, washed with water, and dried to give **98g**, **98i** or **177** as a white solids.

6-(allylthio)-1-(2-(4-chlorophenyl)-2-hydroxyethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (98g)

Yield: 84% (white solid). Mp: 203-204°C. ¹H NMR (400 MHz, DMSO-d₆): δ 12.10 (br s, 1H, NH), 7.90 (s, 1H, H-3), 7.45-7.20 (m, 4H, Ar-H), 5.98 (ddt, *J* = 16.7, 9.8, 1.3 Hz, 1H, H_a), 5.29 (dd, *J* = 9.8, 1.3 Hz, 1H, H_b), 5.23-4.81 (m, 1H, CHAr), 4.62 (br s, 1H, OH), 5.04 (dd, *J* = 16.7, 1.3 Hz, 1H, H_c), 4.50-4.30 (m, 2H, NCH₂), 3.95-3.70 (d, *J* = 6.9 Hz, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 161.00, 159.31, 150.05, 135.74, 133.23, 131.43, 131.11, 126.92, 126.04, 118.28, 105.81, 71.02, 62.72, 33.49. Elem. anal. calcd. for C₁₆H₁₅ClN₄O₂S: C 52.97, H 4.17, N 15.44, S 8.84; found: C 52.99, H 4.27, N 15.34, S 8.80.

6-(allylthio)-1-(2-(4-bromophenyl)-2-hydroxyethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (98i)

Yield: 81% (white solid). Mp: 204-205°C. ¹H NMR (400 MHz, DMSO-d₆): δ 12.32 (s, 1H, NH), 7.92 (s, 1H, H-3), 7.44-7.41 (m, 2H Ar), 7.13-7.10 (m, 2H Ar), 5.92-5.82 (m, 1H, H_c), 5.76 (d, *J* = 4.6 Hz, 1H, OH), 5.30 (dd, *J* = 17.0, 1.3 Hz, 1H, H_a), 5.12 (dd, *J* = 10.0, 1.3 Hz, 1H, H_b), 5.02-4.98 (m, 1H, CHAr), 4.39-4.34 and 4.27-4.22 (2m, 2H, NCH₂), 3.75 (d, *J* = 6.9 Hz, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 159.36, 158.19, 152.58, 142.46, 134.97,

133.75, 131.45, 128.70, 120.97, 119.18, 103.27, 71.02, 54.36, 33.02. Elem. anal. calcd. for C₁₆H₁₅BrN₄O₂S: C 47.18, H 3.71, N 13.76, S 7.87; found: C 47.32, H 3.48, N 13.93, S 8.19.

6-(allylthio)-1-(2-hydroxy-2-phenylethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (177)

Yield: 78% (white solid). Mp: 209-211°C. ¹H NMR (400 MHz, DMSO-d₆): δ 12.55 (s, 1H, NH), 8.30 (s, 1H, H-3), 7.35-7.29 (m, 2H Ar), 7.22-7.17 (m, 2H Ar), 5.02-5.96 (m, 1H, H_c), 5.17 (d, *J* = 5.2 Hz, 1H, OH), 5.04 (dd, *J* = 17.5, 1.4 Hz, 1H, H_a), 5.29 (dd, *J* = 9.8, 1.4 Hz, 1H, H_b), 4.93-4.87 (m, 1H, CHAr), 4.33-4.30 and 4.11-4.06 (2m, 2H, NCH₂), 3.54 (d, *J* = 7.0 Hz, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 159.36, 158.19, 152.58, 142.46, 134.97, 133.75, 131.45, 128.70, 120.97, 119.18, 103.27, 71.02, 54.36, 33.02. Elem. anal. calcd. for C₁₆H₁₆N₄O₂S: C, 58.52; H, 4.91; N, 17.06; S, 9.76; found: C, 58.61; H, 4.95; N, 17.10; S, 9.77.

2.8. General procedure for the synthesis of compounds 99a-i, 103a, 103b, 109, 178

The Vilsmeier complex, previously prepared from POCl₃ (20 mmol, 3.0 g) and anhydrous DMF (20 mmol, 2.92 g), was added to a suspension of the intermediates **98a-i**, **102a**, **102b**, or **177** (3.75 mmol) in CHCl₃ (10 mL), and the mixture was refluxed for 8 h. The solution was washed with H₂O, dried over Na₂SO₄, and concentrated under reduced pressure. The crude oil was purified by column chromatography, using diethyl ether or a mixture of PE and diethyl ether (95:5) as the eluant, to afford the pure products as a yellow oil (**99a**, **99c**, **99d**) or white solids (**99b**, **99f-i**, **103a**, **103b**, **109**, **178**). Compound **99e** was used as a crude in the subsequent step.

4-chloro-6-(methylthio)-1-(2-phenylethyl)-1H-pyrazolo[3,4-d]-pyrimidine (99a)

Yield: 83% (yellow oil). Mp: 190-194°C. ¹H NMR (200 MHz, DMSO-d₆): δ 8.02 (s, 1H, H-3), 7.15-7.00 (m, 5H Ar), 4.48 (t, *J* = 7.0, 2H, CH₂N), 3.15 (t, *J* = 7.0, 2H, CH₂Ar), 2.40 (s, 3H, SCH₃), MS: *m/z* 305 [M+1]⁺. Elem. anal. calcd. for C₁₄H₁₃N₄ClS: C 55.17, H 4.30, N 18.38, S 10.52; found: C 55.20, H 4.32, N 18.38; S 10.50.

6-(ethylthio)-4-chloro-1-phenethyl-4,5-dihydro-1H-pyrazolo[3,4-d]pyrimidine (99b)

Yield: 73% (white solid). Mp: 44-46°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.29 (s, 1H, H-3), 7.16-7.09 (m, 3H Ar), 7.04-7.02 (m, 2H Ar), 4.58 (t, *J* = 6.9 Hz, 2H, NCH₂), 3.15-3.06 (m, 4H, SCH₂ + CH₂Ar), 1.29 (t, *J* = 7.3 Hz, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.67, 154.07, 153.40, 138.40, 133.02, 129.11, 128.79, 126.97, 110.38, 48.83, 35.34, 25.52,

14.61. Elem. anal. calcd. for C₁₅H₁₅N₄ClS: C 56.51, H 4.74, N 17.57, S 10.06; found: C 56.39, H 5.08, N 16.99, S 10.35.

6-(allylthio)-4-chloro-1-phenethyl-1H-pyrazolo[3,4-d]pyrimidine (99c)

Yield: 98% (yellow oil). Mp: 199-201°C ¹H NMR (400 MHz, DMSO-d₆): δ 8.30 (s, 1H, H-3), 7.17-7.07 (m, 3H Ar), 7.04-7.02 (m, 2H Ar), 5.89 (ddt, *J* = 16.9, 10.0, 6.9 Hz, 1H, H_c), 5.32 (dd, *J* = 16.9, 1.3 Hz, 1H, H_a), 5.11 (dd, *J* = 10.0, 1.3 Hz, 1H, H_b), 4.59 (t, *J* = 6.9 Hz, 2H, NCH₂), 3.78 (d, *J* = 6.9 Hz, 2H, SCH₂), 3.13 (t, *J* = 6.9 Hz, 2H, CH₂Ar). ¹³C (101 MHz, DMSO-d₆): δ 167.99, 153.98, 153.47, 138.43, 133.75, 133.08, 129.14, 128.78, 126.98, 118.90, 110.48, 48.88, 35.36, 33.81. Elem. anal. calcd. for C₁₆H₁₅ClN₄S: C 58.09, H 4.57, N 16.94, S 9.69; found: C 58.11, H 4.61, N 16.94, S 9.64.

4-chloro-6-(ethylthio)-1-(2-phenylpropyl)-1H-pyrazolo[3,4-d]pyrimidine (99d)

Yield: 90% (yellow oil). Mp: 199-201°C ¹H NMR (400 MHz, CDCl₃): δ 8.01 (s, 1H, H-3), 7.31-7.12 (m, 5H Ar), 4.66-4.42 (m, 2H, CH₂N), 3.63-3.48 (m, 1H, CHCH₃), 3.21 (q, *J* = 7.4 Hz, 2H, SCH₂), 1.47 (t, *J* = 7.4 Hz, 3H, SCH₂CH₃), 1.31 (d, *J* = 7.2 Hz, 3H, CH₃CH). MS: *m/z* [M + 1]⁺ 334. Elem. anal. calcd. for C₁₆H₁₇N₄ClS: C 57.74, H 5.15, N 16.83, S 9.63; found: C 57.78, H 5.13, N 16.87, S 9.61.

4-chloro-1-(2-chloro-2-phenylethyl)-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (99f)

Yield: 76% (white solid). Mp: 95-96°C ¹H NMR (400 MHz, CDCl₃): δ 8.00 (s, 1H, H-3), 7.38-7.24 (m, 5H Ar), 5.53-5.45 (m, 1H CHCl), 4.97-4.92 and 4.84-4.78 (2m, 2H, CH₂N), 2.60 (s, 3H, CH₃). MS: 337.6 *m/z* [M-1]⁻. Elem. anal. calcd. for C₁₄H₁₂Cl₂N₄S: C 49.57, H 3.57, N 16.52, S 9.45; found: C 49.61, H 3.57, N 16.53, S 9.41.

6-(allylthio)-4-chloro-1-(2-chloro-2-(4-chlorophenyl)ethyl)-1H-pyrazolo[3,4-d]pyrimidine (99g)

Yield: 76% (white solid). Mp: 95-96°C ¹H NMR (400 MHz, DMSO-d₆): δ 7.97 (s, 1H, H-3), 7.17-7.07 (m, 3H Ar), 7.04-7.02 (m, 2H Ar), 6.00 (ddt, *J* = 16.7, 9.8, 7.3 Hz, 1H, H_c), 5.04 (dd, *J* = 16.7, 1.3 Hz, 1H, H_a), 5.29 (dd, *J* = 9.8, 1.3 Hz, 1H, H_b), 4.44-4.38 and 4.19-4.15 (2m, 2H, NCH₂), 4.8 (t, *J* = 6.9 Hz, 1H, CHCl), 3.97 (d, *J* = 6.9 Hz, 2H, SCH₂). ¹³C (101 MHz, DMSO-d₆): δ 172.51, 154.53, 150.46, 136.10, 133.14, 134.33, 132.01, 131.63, 129.14, 118.31, 106.04, 70.00, 58.84. Elem. anal. calcd. for C₁₆H₁₃Cl₃N₄S: C 48.08, H 3.28, N 14.02, S 8.02; found: C 48.12, H 3.26, N 14.03, S 8.04.

1-(2-(4-bromophenyl)-2-chloroethyl)-4-chloro-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidine (99h)

Yield: 86% (white solid). Mp: 125-126°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.36 (s, 1H, H-3), 7.53-7.50 (m, 2H Ar), 7.43-7.39 (m, 2H Ar), 5.68-5.64 (m, 1H, CHCl), 5.01-4.96 and 4.90-4.85 (2m, 2H, NCH₂), 2.58 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.84, 154.59, 153.58, 137.71, 133.84, 132.12, 130.37, 122.82, 110.35, 59.76, 53.55, 14.48. Elem. anal. calcd. for C₁₄H₁₁BrCl₂N₄S: C 40.22, H 2.65, N 13.40, S 7.67; found: C 39.81, H 2.50, N 13.61, S 7.75.

6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-4-chloro-1H-pyrazolo[3,4-d]pyrimidine (99i)

Yield: 87% (white solid). Mp: 70-72°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.37 (s, 1H, H-3), 7.54-7.51 (m, 2H Ar), 7.43-7.41 (m, 2H Ar), 5.96 (ddt, *J* = 16.9, 10.0, 6.8 Hz, 1H, H_c), 5.67-5.64 (m, 1H, CHCl), 5.37 (dd, *J* = 16.9, 1.6 Hz, 1H, H_a), 5.15 (dd, *J* = 10.0, 1.6 Hz, 1H, H_b), 5.03-4.97 and 4.89-4.84 (2m, 2H, NCH₂), 3.92-3.81 (m, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.62, 154.53, 153.76, 137.72, 133.88, 133.72, 132.16, 130.34, 122.84, 119.10, 110.56, 59.85, 53.56, 33.92. Elem. anal. calcd. for C₁₆H₁₃BrCl₂N₄S: C 43.27, H 2.95, N 12.61, S 7.22; found: C 43.07, H 2.79, N 12.94, 7.26.

4-chloro-1-(2-chloro-2-phenylethyl)-6-(pyrrolidin-1-yl)-1H-pyrazolo[3,4-d]pyrimidine (103a)

Yield: 47% (white solid). Mp: 134-135°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.00 (s, 1H, H-3), 7.46 (dd, *J* = 7.8, 1.6 Hz, 2H Ar), 7.35-7.29 (m, 3H Ar), 5.64-5.60 (m, 1H, CHCl), 4.83-4.77 and 4.72-4.67 (2 m, 2H, NCH₂), 3.52-3.50 (m, 4H, 2 CH₂N pyr), 1.92-1.89 (m, 4H, 2 CH₂CH₂N pyr). ¹³C NMR (101 MHz, DMSO-d₆): δ 158.23, 156.52, 153.49, 138.43, 133.31, 129.49, 129.19, 128.09, 105.90, 60.65, 53.15, 47.67, 25.50. Elem. anal. calcd. for C₁₇H₁₇Cl₂N₅: C 56.37, H 4.73, N 19.33; found: C 56.46, H 4.53, N 19.52.

4-chloro-1-(2-chloro-2-phenylethyl)-6-(piperidin-1-yl)-1H-pyrazolo[3,4-d]pyrimidine (103b)

Yield: 51% (white solid). Mp: 118.120°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.00 (s, 1H, H-3), 7.45 (dd, *J* = 7.6, 1.8 Hz, 2H Ar), 7.33- 7.28 (m, 3H Ar), 5.60-5.56 (m, 1H, CHCl), 4.83-4.77 and 4.72-4.67 (2m, 2H, NCH₂), 3.75 (br s, 4H, 2 CH₂N pip), 1.61-1.60 (m, 2H, CH₂ pip), 1.52-1.51 (m, 4H, 2 CH₂CH₂N pip). ¹³C NMR (101 MHz, DMSO-d₆): δ 159.37, 156.63, 153.80,

138.41, 133.37, 129.47, 129.14, 128.12, 105.90, 60.71, 53.01, 45.50, 25.81, 24.66. Elem. anal. calcd. for C₁₈H₁₉N₅Cl₂: C 57.45, H 5.09, N 18.61; found: C 57.23, H 5.26, N 18.43.

N-benzyl-1-(2-chloro-2-phenylethyl)-6-(trifluoromethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (109)

Yield: 21% (white solid). Mp: 105-106°C. ¹H NMR (200 MHz, DMSO-*d*₆): δ 10.88 (br s, 1H, NH disappears with D₂O), 8.06 (s, 1H, H-3), 7.38–7.26 (m, 10H Ar), 5.03-4.97 and 4.89-4.81 (2m, 5H, CH₂N + CH₂Ar + CHCl). IR (KBr): cm⁻¹ 3288 (NH). Elem. anal. calcd. for C₂₁H₁₇N₅ClF₃: C 58.41, H 3.97, N 16.22; found: C 58.25, H 4.16, N 15.93.

6-(allylthio)-4-chloro-1-(2-chloro-2-phenylethyl)-1*H*-pyrazolo[3,4-*d*]pyrimidine (178)

Yield: 77% (white solid). Mp: 86-88°C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.93 (s, 1H, H-3), 7.61-7.57 (m, 2H Ar), 7.35-7.31 (m, 2H Ar), 6.00 (ddt, *J* = 17.1, 10.0, 6.8 Hz, 1H, H_c), 4.89-4.81 (m, 1H, CHCl), 5.04 (dd, *J* = 17.1, 1.6 Hz, 1H, H_a), 5.29 (dd, *J* = 10.0, 1.6 Hz, 1H, H_b), 4.44-4.40 and 4.20-4.14 (2m, 2H, NCH₂), 3.90-3.85 (m, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-*d*₆): δ 172.53, 154.51, 150.46, 138.07, 133.12, 131.13, 128.91, 128.74, 128.62, 118.35, 106.11, 70.02, 53.8, 36.92. Elem. anal. calcd. for C₁₆H₁₄Cl₂N₄S: C 52.61, H 3.86, N 15.34, S, 8.78; found: C 52.63, H 3.86, N 15.36, S, 8.74.

2.9. General procedure for the synthesis of compounds 74, 79–81, 85–87, 100c–e, 100j–l, 104, 169, 175.

The appropriate aliphatic amine (4.60 mmol) was added to a solution of the 4-chloro derivative (1.15 mmol) in anhydrous toluene (10 mL), and the mixture was stirred at room temperature for 24-48 h. The organic phase was washed with water, dried over Na₂SO₄, and concentrated under reduced pressure. The crude oil was purified by column chromatography using a mixture of PE and EtOAc (8:2) as the eluent to afford compounds **74**, **79–81**, **84–87**, **100c–e**, **100j–l**, **104**, **169**, **175** as white solids.

N-(3-fluorobenzyl)-6-(methylthio)-1-phenethyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (74)

Yield: 28% (white solid). Mp: 141-143°C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.76 (t, *J* = 5.7 Hz, 1H, NH), 8.00 (s, 1H, H-3), 7.37-7.31 (m, 1H Ar), 7.20-7.02 (m, 8H Ar), 4.66 (d, *J* = 5.8 Hz, 2H, CHbenz), 4.42 (t, *J* = 7.2 Hz, 2H, NCH₂), 3.09 (t, *J* = 7.2 Hz, 2H, CH₂Ar), 2.39 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-*d*₆): δ 168.69, 162.74 (d, ¹*J*_{CF} = 244.5 Hz), 155.72, 153.90, 142.74 (d, ³*J*_{CF} = 7.0 Hz), 138.82, 132.21, 130.90 (d, ³*J*_{CF} = 8.4 Hz), 129.15, 128.80, 126.85,

124.00 (d, $^4J_{\text{CF}} = 2.7$ Hz), 114.71 (d, $^2J_{\text{CF}} = 21.72$ Hz), 114.28 (d, $^2J_{\text{CF}} = 21.0$ Hz), 98.61, 47.91, 43.33, 35.44, 14.05. Elem. anal. calcd. for $\text{C}_{21}\text{H}_{20}\text{FN}_5\text{S}$: C 64.10, H 5.12, N 17.80, S 8.15; found: C 64.17, H 4.93, N 17.94, S 8.64.

N-butyl-6-(ethylthio)-1-(2-phenylpropyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (79)

Yield: 47% (white solid). Mp: 174-175°C. ^1H NMR (400 MHz, DMSO-d_6): δ 8.15 (t, $J = 5.4$ Hz, 1H, NH), 7.93 (s, 1H, H-3), 7.23-7.18 (m, 4H Ar), 7.14-7.10 (m, 1H Ar), 4.36-4.31 and 4.28-4.22 (2m, 2H, NCH_2), 3.43-3.36 (m, 3H, $\text{CHCH}_3 + \text{CH}_2\text{NH}$), 3.04 (q, $J = 7.3$ Hz, 2H, SCH_2), 1.52 (p, $J = 7.3$ Hz, 2H, $\text{CH}_2\text{CH}_2\text{CH}_3$), 1.31 (m, 5H, $\text{CH}_2\text{CH}_2\text{CH}_3 + \text{SCH}_2\text{CH}_3$), 1.11 (d, $J = 7.0$ Hz, 3H, CHCH_3), 0.87 (t, $J = 7.3$ Hz, 3H, $\text{CH}_2\text{CH}_2\text{CH}_3$). ^{13}C NMR (101 MHz, DMSO-d_6): δ 168.22, 155.92, 154.01, 144.06, 132.14, 128.85, 127.57, 126.99, 98.49, 53.12, 31.46, 25.01, 20.19, 19.52, 15.56, 14.22. Elem. anal. Anal. calcd. for $\text{C}_{20}\text{H}_{27}\text{N}_5\text{S}$: C 65.01, H 7.37, N 18.95, S 8.68; found: C 65.32, H 7.11, N 19.22, S 8.01.

2-(4-((2-fluorobenzyl)amino)-6-(methylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-1-yl)-1-phenylethan-1-one (80)

Yield: 34% (white solid). Mp: 202-203°C. ^1H NMR (400 MHz, DMSO-d_6): δ 8.84 (t, $J = 5.6$ Hz, 1H, NH), 8.10 (s, 1H, H-3), 8.05-8.02 (m, 2H Ar), 7.69-7.65 (m, 1H Ar), 7.58-7.52 (m, 2H Ar), 7.43-7.39 (m, 1H Ar), 7.33-7.28 (m, 1H Ar), 7.20-7.13 (m, 2H Ar), 5.85 (s, 2H, NCH_2), 4.74 (d, $J = 5.6$ Hz, 2H, CH_2 Ar), 2.35 (s, 3H, SCH_3). ^{13}C NMR (101 MHz, DMSO-d_6): δ 193.64, 169.04, 160.84 (d, $^1J_{\text{CF}} = 244.7$ Hz), 155.78, 154.92, 134.92, 134.60, 133.08, 130.52, 129.71 (d, $^3J_{\text{CF}} = 8.1$ Hz), 129.49, 128.70, 126.22 (d, $^2J_{\text{CF}} = 14.7$ Hz), 124.96 (d, $^4J_{\text{CF}} = 3.5$ Hz), 115.77 (d, $^2J_{\text{CF}} = 21.2$ Hz), 98.76, 53.67, 37.82 (d, $^3J_{\text{CF}} = 4.3$ Hz), 13.99. Elem. anal. calcd. for $\text{C}_{21}\text{H}_{18}\text{FN}_5\text{OS}$: C 61.90, H 4.45, N 17.19, S 7.87; found: C 61.64, H 4.60, N 17.50, S 7.86.

2-(4-((4-fluorobenzyl)amino)-6-(methylthio)-1*H*-pyrazolo[3,4-*d*]pyrimidin-1-yl)-1-phenylethan-1-one (81)

Yield: 39% (white solid). Mp: 182-183°C. ^1H NMR (400 MHz, DMSO-d_6): δ 8.86 (t, $J = 5.8$ Hz, 1H, NH), 8.07 (s, 1H, H-3), 8.05-8.02 (m, 2H Ar), 7.67 (tt, $J = 7.0, 1.2$ Hz, 1H Ar), 7.56-7.53 (m, 2H Ar), 7.40-7.36 (m, 2H Ar), 7.17-7.11 (m, 2H Ar), 5.85 (s, 2H, NCH_2), 4.68 (d, $J = 5.8$ Hz, 2H, CH_2Ar), 2.36 (s, 3H, SCH_3). ^{13}C NMR (101 MHz, DMSO-d_6): δ 193.66, 169.09, 161.83 (d, $^1J_{\text{CF}} = 242.5$ Hz), 155.78, 154.92, 135.89 (d, $^4J_{\text{CF}} = 3.0$ Hz), 134.92, 134.60, 133.02, 130.05 (d, $^3J_{\text{CF}} = 8.2$ Hz), 129.49, 128.70, 115.68 (d, $^2J_{\text{CF}} = 21.3$ Hz), 98.73, 53.66, 43.18, 14.07.

Elem. anal. calcd. for C₂₁H₁₈FN₅OS: C 61.90, H 4.45, N 17.19, S 7.87; found: C 61.84, H 4.65, N 16.87, S 7.46.

1-(2-chloro-2-phenylethyl)-N-propyl-6-(pyrrolidin-1-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (85)

Yield: 34% (white solid). Mp :158-159°C. ¹H NMR (400 MHz, DMSO-d₆): δ 7.76 (s, 1H, H-3), 7.68 (t, *J* = 4.6 Hz 1H, NH), 7.47-7.45 (m, 2H Ar), 7.35-7.26 (m, 3H Ar), 5.65-5.61 (m, 1H, CHCl), 4.69-4.63 and 4.61-4.55 (2m, 2H, NCH₂), 3.47 (t, *J* = 6.5 Hz, 4H, 2 CH₂N pyr), 3.36-3.29 (m, 2H, NHCH₂), 1.87-1.84 (m, 4H, 2 CH₂CH₂N pyr), 1.56 (sx, *J* = 7.3 Hz, 2H, CH₂CH₃), 0.87 (t, *J* = 7.3 Hz, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 159.78, 156.66, 156.53, 138.84, 132.60, 129.33, 129.15, 128.10, 95.18, 60.85, 52.66, 46.79, 42.04, 25.50, 22.75, 12.17. Elem. anal. calcd. for C₂₁H₂₅ClN₆: C 62.41, H 6.55, N 21.83; found: C 62.62, H 6.61, N 22.09.

N-benzyl-1-(2-chloro-2-phenylethyl)-6-(piperidin-1-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (86)

Yield: 48% (white solid). Mp: 123-125°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.30 (t, *J* = 5.4 Hz, 1H, NH), 7.80 (s, 1H, H-3), 7.45 (dd, *J* = 7.8, 1.5 Hz, 2H Ar), 7.34-7.25 (m, 7H Ar), 7.21-7.17 (m, 1H Ar), 5.59 (t, *J* = 7.3 Hz, 1H, CHCl), 4.68-4.65 and 4.62-4.57 (2m, 4H, NHCH₂ + NCH₂), 3.71-3.69 (m, 4H, 2 CH₂N pip), 1.56-1.55 (m, 2H, CH₂ pip), 1.45-1.39 (m, 4H, 2 CH₂CH₂N pip). ¹³C NMR (101 MHz, DMSO-d₆): δ 160.78, 156.70, 156.55, 140.40, 138.79, 132.69, 129.35, 129.15, 128.81, 128.12, 128.07, 127.31, 95.15, 60.95, 52.52, 45.20, 43.82, 25.87, 25.06. Elem. anal. calcd. for C₂₅H₂₇N₆Cl: C 67.18, H 6.09, N 18.80; found: C 66.94, H 6.39, N 18.51.

N-benzyl-1-(2-chloro-2-phenylethyl)-6-(trifluoromethyl)-1H-pyrazolo[3,4-b]pyridin-4-amine (87)

Yield: 72% (white solid). Mp: 105-106°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.30 (t, 1H, NH, *J* = 6.0 Hz), 8.27 (s, 1H, H-3), 7.47-7.45 (m, 2H Ar), 7.36-7.28 (m, 7H Ar), 7.26-7.22 (m, 1H Ar), 5.63-5.59 (m, 1H, NCH), 4.97-4.92 and 4.82-4.77 (2m, 2H CH₂ Ar), 4.71 (d, *J* = 6.0 Hz, 2H, CH₂NH). ¹³C NMR (101 MHz, DMSO-d₆): δ 157.13, 153.36 (q, ⁴*J*_{CF} = 0.9 Hz), 153.09 (q, ²*J*_{CF} = 34.8 Hz), 138.80, 138.26, 133.47, 129.52, 129.18, 129.00, 128.43, 128.07, 127.81, 120.39 (q, ¹*J*_{CF} = 275.7 Hz), 101.06, 60.99, 53.40, 44.23. Elem. anal. calcd. for C₂₁H₁₇ClF₃N₅: C 58.41, H 3.97, N 16.22; found: C 58.25, H 4.16, N 15.93.

1-(2-(4-bromophenyl)-2-chloroethyl)-6-(methylthio)-N-(naphthalen-1-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (100c)

Yield: 73% (white solid). Mp: 76°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.33 (s, 1H, NH), 8.00-7.98 (m, 1H Ar), 7.92-7.89 (m, 2H, 1 Ar + H-3), 7.63-7.62 (m, 1H Ar), 7.58-7.48 (m, 6H Ar), 7.44-7.42 (m, 2H Ar), 5.61 (t, *J* = 7.2 Hz, 1H, CHCl), 4.85-4.80 and 4.75-4.69 (2m, 2H, NCH₂), 2.35 (s, 3H, SCH₃). ¹³C NMR (125 MHz, DMSO-d₆): δ 169.19, 156.21, 155.08, 137.99, 134.81, 134.46, 134.18, 133.25, 132.12, 130.39, 130.39, 130.04, 128.83, 126.94, 126.26, 124.98, 123.53, 122.71, 59.94, 54.23, 52.83, 13.97. Elem. anal. calcd. for C₂₄H₁₉BrN₅ClS: C 54.92, H 3.65, N 13.34, S 6.11; found: C 54.92, H 4.10, N 13.36, S 6.22.

5-((1-(2-(4-bromophenyl)-2-chloroethyl)-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)naphthalen-1-ol (100d)

Yield: 46% (white solid). Mp: 186-188°C. ¹H NMR (400 MHz, DMSO-d₆) δ 10.23 (s), 8.13 (d, *J* = 8.2 Hz, 1H Ar), 7.58-7.40 (m, 6H Ar + H-3), 7.33-7.25 (m, 2H Ar), 6.88 (d, *J* = 7.2 Hz, 1H Ar), 5.61-5.58 (m, 1H, CHCl), 4.84-4.78 and 4.73-4.69 (2 m, 2H, NCH₂), 2.37 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-d₆) δ 169.16, 156.42, 155.07, 154.12, 137.97, 133.85, 133.24, 132.11, 131.66, 130.37, 127.48, 126.06, 125.39, 124.69, 122.70, 121.77, 113.93, 109.03, 98.24, 59.93, 52.82, 13.98. Elem. anal. calcd. for C₂₄H₁₉BrClN₅OS: 53.30, H 3.54, N 12.95, S 5.93; found: C 53.59, H 3.41, N 12.95, S 5.93.

1-(2-(4-bromophenyl)-2-chloroethyl)-6-(methylthio)-N-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (100e)

Yield: 60% (white solid). Mp: 147-148°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.79 (t, *J* = 5.4 Hz, 1H, NH), 8.11-8.09 (m, 1H Ar), 8.03 (s, 1H, H-3), 7.95-7.92 (m, 1H Ar), 7.85 (d, *J* = 7.9 Hz, 1H Ar), 7.55-7.50 (m, 5H Ar), 7.47-7.42 (m, 3H Ar), 5.62 (t, *J* = 7.3 Hz, 1H, CHCl), 5.11 (d, *J* = 5.4 Hz, 2H, CH₂naph), 4.85-4.79 and 4.74-4.69 (2 m, 2H, NCH₂), 2.46-2.47 (m, 8H, SCH₃ + solvent). ¹³C NMR (125 MHz, DMSO-d₆): δ 169.29, 155.57, 154.51, 138.03, 134.53, 133.91, 133.25, 132.11, 131.64, 130.39, 129.12, 128.42, 126.94, 126.65, 126.44, 126.04, 124.09, 122.68, 98.48, 60.01, 52.83, 42.11, 14.14. Elem. anal. calcd. for C₂₅H₂₁BrClN₅S: C 55.72, H 3.93, N 13.00, S 5.95; found: C 55.43, H 3.48, N 13.45, S 6.00.

6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-N-(naphthalen-1-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (100j)

Yield: 82% (white solid). Mp: 136-138 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.37 (s, 1H, NH), 8.00-7.97 (m, 1H Ar), 7.92-7.89 (m, 2H, 1H Ar + H-3), 7.62-7.48 (m, 7H Ar), 7.42 (d, *J* = 8.4 Hz, 2H, Ar), 5.80-5.78 (m, 1H, H_c), 5.60 (t, *J* = 7.2 Hz, 1H, CHCl), 5.08 (d, *J* = 16.2 Hz, 1H, H_a), 4.95 (d, *J* = 9.8 Hz, 1H, H_b), 4.86-4.81 and 4.73-4.68 (2m, 2H, NCH₂), 3.60 (m, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.13, 155.05, 138.00, 134.98, 134.47, 134.15, 133.29, 133.25, 132.15, 130.36, 130.10, 128.84, 127.62, 126.98, 126.94, 126.26, 125.07, 125.04, 123.57, 123.52, 122.73, 117.79, 60.01, 52.84, 33.43. Elem. anal. calcd. for C₂₆H₂₁BrClN₅S: C 56.69, H 3.84, N 12.71, S 5.82; found: C 56.62, H 3.85, N 12.75, S 5.87.

5-((6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)naphthalen-1-ol (100k)

Yield: 30% (white solid). Mp: 197°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.28 (br s, 2H, OH + NH), 8.13 (d, *J* = 8.3 Hz, 1H Ar), 7.57-7.40 (m, 7H, 6 Ar + H-3), 7.32-7.25 (m, 2H Ar), 6.88 (d, *J* = 6.0 Hz, 1H Ar), 5.89-5.73 (m, 1H, H_c), 5.59 (t, *J* = 7.2 Hz, 1H, CHCl), 5.14-5.10 (m, 1H, H_a), 4.97 (d, *J* = 9.6 Hz, 1H, H_b), 4.84-4.79 and 4.71-4.68 (2m, 2H, NCH₂), 3.63 (s, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.09, 155.04, 154.13, 152.28, 138.00, 135.03, 133.84, 133.28, 132.14, 131.71, 130.34, 127.52, 126.07, 125.52, 124.68, 122.71, 122.00, 117.81, 113.92, 109.03, 106.57, 59.99, 52.83, 33.43. Elem. anal. calcd. for C₂₆H₂₁BrClN₅OS: C 55.09, H 3.73, N 12.35, S 5.66; found: C 55.11, H 3.77, N 12.39, S 5.71.

6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-N-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (100l)

Yield: 80% (white solid). Mp: 165-167°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.82 (t, *J* = 5.4 Hz, 1H, NH), 8.09-8.07 (m, 1H Ar), 8.04 (s, 1H, H-3), 7.94-7.91 (m, 1H Ar), 7.85 (d, *J* = 7.6 Hz, 1H Ar), 7.54-7.41 (m, 8H Ar), 5.95-5.85 (m, 1H, H_c), 5.63-5.59 (m, 1H, CHCl), 5.21 (dd, *J* = 17.0, 1.6 Hz, 1H, H_a), 5.11 (d, *J* = 5.4 Hz, 2H, CH₂naph), 5.00 (dd, *J* = 10.0, 1.6 Hz, 1H, H_b), 4.85-4.80 and 4.73-4.67 (2m, 2H, NCH₂), 3.79-3.71 (m, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.22, 155.71, 154.46, 138.04, 135.16, 134.44, 133.91, 133.28, 132.13, 131.60, 130.35, 129.12, 128.40, 126.93, 126.49, 126.44, 126.03, 124.04, 122.69, 117.75, 98.61, 60.05, 52.85, 42.16, 33.60. Elem. anal. calcd. for C₂₇H₂₃BrClN₅S: C 57.40, H 4.10, N 12.40, S 5.67; found: C 57.69, H 3.96, N 12.17, S 5.49.

1-(2-chloro-2-phenylethyl)-6-(methylthio)-N-phenethyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine
(104)

Yield: 76% (white solid). Mp: 73-74°C. ¹H NMR (400 MHz, DMSO-d₆): δ 7.73 (s, 1H, H-3), 7.48-7.19 (m, 10H Ar), 5.60-5.50 (m, 1H, CHCl), 5.30 (br s, 1H, NH, disappears with D₂O), 4.96-4.70 (m, 2H, CH₂N), 3.87 (q, *J* = 6.0, 2H, CH₂NH), 2.98 (t, *J* = 6.0, 2H, CH₂Ar), δ 2.59 (s, 3H, CH₃S). IR (KBr) cm⁻¹: 3445 (NH). Elem. anal. calcd. for C₂₂H₂₂N₅ClS: C 62.33, H 5.23, N 16.52, S 7.56; found: C 62.36, H 5.27, N 16.50, S 7.60.

6-(allylthio)-1-(2-chloro-2-phenylethyl)-N-phenethyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine
(169)

Yield: 66% (white solid). Mp: 190-192°C. ¹H NMR (400 MHz, DMSO-D₆) δ 8.96 (t, *J* = 5.8 Hz, 1H, NH), 7.97 (s, 1H, H-3), 7.59 (d, *J* = 8.4 Hz, 2H Ar), 7.33 (t, *J* = 8.1 Hz, 2H Ar), 7.29 (quint, 1H Ar), 7.27-7.23 (m, 3H Ar), 7.25 (d, *J* = 6.6 Hz, 2H Ar), 6.00 (m, 1H, H_c), 5.29 (d, *J* = 9.7 Hz, 1H, H_b), 5.04 (dd, *J* = 16.9, 9.7 Hz, 1H, H_a), 4.8 (t, *J* = 7.1 Hz, 1H, CHCl), 4.45-4.40 and 4.19-4.15 (2 m, 2H, NCH₂), 3.86 (t, *J* = 6.9 Hz, 2H, SCH₂), 3.43-3.39 (m, 2H, CH₂CH₂Ph), 2.94-2.92 (m, 2H, CH₂CH₂Ph). ¹³C NMR (101 MHz, DMSO-D₆) δ 171.04, 156.73, 154.52, 39.40, 138.00, 133.12, 132.89, 128.91, 128.86, 128.64, 127.75, 125.94, 118.33, 101.14, 71.11, 70.03, 53.84, 44.32, 36.94, 35.12. Elem. anal. calcd. for C₂₄H₂₄ClN₅S: C 64.06, H 5.38, N 15.56, S 7.12; found: C 64.09, H 5.41, N 15.52, S 7.16

6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-N-phenethyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine
(175)

Yield: 58% (white solid). Mp: 103-104°C. ¹H NMR (400 MHz, DMSO-d₆) δ 8.43 (t, *J* = 5.5 Hz, 1H, NH), 7.98 (s, 1H, H-3), 7.47-7.45 (m, 2H, Ar), 7.36-7.30 (m, 3H, Ar), 7.28-7.15 (m, 5H, Ar), 6.04-5.94 (1H, m), 5.59 (dd, *J* = 8.6, 6.0 Hz, 1H, CHO), 5.28 (dd, *J* = 16.9, 1.7 Hz, 1H), 5.09-5.06 (m, 1H), 4.82 (dd, *J* = 14.3, 8.6 Hz, 1H, CH₂N), 3.84-3.75 (m, 2H), 3.65-3.60 (m, 2H, CH₂NH). ¹³C NMR (101 MHz, DMSO-d₆) δ: 168.11, 155.86, 154.37, 139.85, 138.57, 135.33, 132.98, 129.50, 129.22, 128.93, 128.07, 126.75, 117.78, 98.66, 61.07, 53.05, 42.24, 35.34, 33.62. Elem. anal. calcd. for C₂₄H₂₃BrClN₅S: C 64.06, H 5.38, N 15.56, S 7.13; found C 63.81, H 5.07, N 15.71, S 6.90.

2.10. General procedure for the synthesis of compounds 75, 77, 78, 82, 83, 88, 89, 100a, 100f-i, 170-172, 176

To a solution of the appropriate 4-chloro derivative (1 mmol) in absolute ethanol (5 mL), the appropriate aniline (3.00 mmol) was added, and the mixture was held at reflux for 3-5 h. After cooling to room temperature, the solid precipitate was filtered, washed with H₂O, recrystallized from absolute ethanol or purified by column chromatography using a mixture of PE and EtOAc (8:2) or diethyl ether as the eluant. Intermediate **100b** was used without further purification in the further step.

4-methyl-3-((6-(methylthio)-1-phenethyl-6,7-dihydro-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)phenol (75)

Yield: 50% (white solid). Mp: 203-204°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.62 (s, 1H, OH), 9.39 (s, 1H, NH), 7.20-7.07 (m, 7H, 6 Ar + H-3), 6.70 (s, 1H Ar), 6.66 (d, *J* = 8.0 Hz, 1H Ar), 4.42 (t, *J* = 7.2 Hz, 2H, NCH₂), 3.08 (t, *J* = 7.2 Hz, 2H, CH₂Ar), 2.39 (s, 3H, SCH₃), 2.00 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.61, 156.38, 154.45, 138.77, 138.76, 137.43, 132.31, 131.75, 129.16, 129.14, 128.80, 126.87, 125.07, 114.68, 98.16, 47.90, 35.42, 17.41, 13.90. Elem. anal. calcd. for C₂₁H₂₃N₅OS: C 64.10, H 5.89, N 17.80, S 8.15; found: C 65.66, H 5.29, N 17.67, S 5.22.

3-((6-(allylthio)-1-phenethyl-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)-4-methylphenol (77)

Yield: 60% (white solid). Mp: 142-143°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.66 (s, 1H, OH), 9.39 (s, 1H, NH), 7.21-7.07 (m, 7H, 6 Ar + H-3), 6.69-6.65 (m, 2H Ar), 5.89-5.83 (m, 1H, H_c), 5.18 (d, *J* = 16.9 Hz, 1H, H_a), 5.01 (d, *J* = 9.9 Hz, 1H, H_b), 4.42 (t, *J* = 6.9 Hz, 2H, NCH₂), 3.67 (d, *J* = 6.5 Hz, 2H, SCH₂), 3.08 (t, *J* = 6.9 Hz, 2H, CH₂Ar), 1.99 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 167.54, 156.40, 154.37, 138.77, 137.39, 135.09, 132.38, 131.74, 129.13, 128.81, 126.88, 125.20, 117.80, 114.73, 100.00, 98.27, 47.93, 35.44, 33.39, 17.39, 15.37. Elem. anal. calcd. for C₂₃H₂₃N₅OS: C 66.16, H 5.55, N 16.77, S 7.68; found: C 66.18, H 5.59, N 16.77, S 7.63.

6-(allylthio)-N-(3-fluorophenyl)-1-phenethyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (78)

Yield: 60% (white solid). Mp: 133-134°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.21 (s, 1H, NH), 8.18 (s, 1H, H-3), 7.81 (dt, *J* = 11.9, 2.2 Hz, 1H Ar), 7.48-7.45 (m, 1H Ar), 7.40-7.34 (m, 1H Ar), 7.20-7.16 (m, 2H Ar), 7.13-7.08 (m, 3H Ar), 6.91-6.86 (m, 1H Ar), 5.92 (ddt, *J* =

16.8, 10.0, 6.8 Hz, 1H, H_c), 5.26 (dd, J = 16.8, 1.6 Hz, 1H, H_a), 5.06 (dd, J = 10.0, 1.6 Hz, 1H, H_b), 4.48 (t, J = 7.1 Hz, 2H, NCH₂), 3.76 (d, J = 6.8 Hz, 2H, SCH₂), 3.12 (t, J = 7.1 Hz, 2H, CH₂Ar). ¹³C NMR (101 MHz, DMSO-d₆): δ 167.54, 162.59 (d, $^1J_{\text{CF}}$ = 242.4 Hz), 154.02, 153.60, 141.26 (d, $^3J_{\text{CF}}$ = 11.2 Hz), 138.73, 134.77, 132.40, 130.78 (d, $^3J_{\text{CF}}$ = 9.7 Hz), 129.14, 128.81, 126.88, 118.00, 117.01, 110.32 (d, $^2J_{\text{CF}}$ = 21.3 Hz), 108.21 (d, $^2J_{\text{CF}}$ = 25.5 Hz), 99.41, 48.08, 35.46, 33.49. Elem. anal. calcd. for C₂₂H₂₀FN₅S: C 65.17, H 4.97, N 17.27, S 7.91; found: C 65.21, H 4.99, N 17.27, S 7.99

2-(6-(methylthio)-4-(phenylamino)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)-1-phenylethan-1-one (82)

Yield: 59% (white solid). Mp: 204-206°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.11 (s, 1H, NH), 8.17 (s, 1H, H-3), 8.07-8.04 (m, 2H Ar), 7.77 (d, J = 7.9 Hz, 2H Ar), 7.68 (tt, J = 7.5, 1.7 Hz, 1H Ar), 7.58-7.54 (m, 2H Ar), 7.39-7.35 (m, 2H Ar), 7.10 (t, J = 7.4 Hz, 1H Ar), 5.92 (s, 2H, NCH₂), 3.30 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 193.58, 169.20, 155.17, 153.91, 139.33, 134.90, 134.64, 133.18, 129.51, 129.28, 128.73, 124.31, 121.96, 99.26, 53.73, 14.21. Elem. anal. calcd. for C₂₀H₁₇N₅OS: C 63.98, H 4.56, N 18.65, S 8.54; found: C 63.78, H 4.75, N 18.60, S 8.42.

2-(4-((3-fluorophenyl)amino)-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)-1-phenylethan-1-one (83)

Yield: 59% (white solid). Mp: 222-223°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.27 (s, 1H, NH), 8.26 (s, 1H, H-3), 8.06-8.04 (m, 2H Ar), 7.88 (dt, J = 12.0, 2.1 Hz, 1H Ar), 7.70-7.67 (m, 1H Ar), 7.58-7.50 (m, 3H Ar), 7.42-7.37 (m, 1H Ar), 6.93-6.88 (m, 1H Ar), 5.93 (s, 2H, NCH₂), 2.45 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 193.52, 169.22, 162.61 (d, $^1J_{\text{CF}}$ = 241.3 Hz), 155.13, 153.62, 141.25 (d, $^3J_{\text{CF}}$ = 11.2 Hz), 134.87, 134.66, 133.11, 130.82 (d, $^3J_{\text{CF}}$ = 9.6 Hz), 129.51, 128.74, 117.06 (d, $^4J_{\text{CF}}$ = 2.1 Hz), 110.37 (d, $^2J_{\text{CF}}$ = 21.02 Hz), 108.32 (d, $^2J_{\text{CF}}$ = 26.5 Hz), 99.46, 53.77, 14.23. Elem. anal. calcd. for C₂₀H₁₆FN₅OS: C 61.06, H 4.10, N 17.80, S 8.15; found: C 60.78, H 4.40, N 17.77, S 8.50.

1-(2-(4-Bromophenyl)-2-chloroethyl)-N-(3-fluorophenyl)-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (88)

Yield: 53 % (white solid). Mp: 90-92°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.24 (s, 1H, NH), 8.20 (s, 1H, H-3), 7.84 (d, J = 11.9 Hz, 1H Ar), 7.54-7.34 (m, 6H Ar), 6.89 (J = 8.5, 2.4 Hz, 1H Ar), 5.64 (t, J = 7.3 Hz, 1H, CHCl), 4.90-4.84 and 4.79-4.74 (2m, 2H, NCH₂), 2.53 (s, 3H,

CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆): δ 169.30, 162.58 (d, ¹*J*_{CF} = 241.2 Hz), 154.69, 153.46, 141.20 (d, ³*J*_{CF} = 11.3 Hz), 137.95, 133.20, 132.11, 130.81 (d, ³*J*_{CF} = 9.6 Hz), 130.37, 122.71, 116.99 (d, ⁴*J*_{CF} = 2.3 Hz), 110.36 (d, ²*J*_{CF} = 21.2 Hz), 108.24 (d, ²*J*_{CF} = 26.8 Hz), 99.20, 59.94, 52.94, 14.27. Elem. anal. calcd. for C₂₀H₁₆BrClFN₅S: C 48.75, H 3.27, N 14.21, S 6.51; found: C 47.83, H 3.11, N 13.90, S 6.97.

6-(Allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-N-(3-fluorophenyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (89)

Yield: 59% (white solid). Mp: 138 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.28 (s, 1H, NH), 8.20 (s, 1H, H-3), 7.80 (dt, *J* = 11.8, 2.1 Hz, 1H Ar), 7.57-7.50 (m, 2H Ar), 7.49-7.33 (m, 4H Ar), 6.94-6.85 (m, 1H Ar), 6.02-5.92 (m, 1H, H_c), 5.65-5.61 (m, 1H, CHCl), 5.29 (dd, *J* = 17.0, 1.7 Hz, 1H, H_a), 5.09 (dd, *J* = 10.0, 1.7 Hz, 1H, H_b), 4.91-4.85 and 4.78-4.73 (2m, 2H, NCH₂), 3.88-3.76 (m, 2H, CH₂S). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 168.13, 162.59 (d, ¹*J*_{CF} = 241.5 Hz), 154.63, 153.61, 141.11 (d, ³*J*_{CF} = 11.0 Hz), 137.96, 134.73, 133.25, 132.14, 130.82 (d, ³*J*_{CF} = 9.5 Hz), 130.33, 122.72, 118.14, 117.15 (d, ⁴*J*_{CF} = 2.3 Hz), 110.50 (d, ²*J*_{CF} = 20.7 Hz), 108.37 (d, ²*J*_{CF} = 26.4 Hz), 99.34, 59.99, 52.97, 33.55. Elem. anal. calcd. for C₂₂H₁₈BrClFN₅S: C 50.93; H 3.50, N 13.50, S 6.18; found: C 50.80, H 3.43, N 13.44, S 6.24.

3-((6-(allylthio)-1-(2-chloro-2-(4-chlorophenyl)ethyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-yl)amino)-4-methylphenol (100a)

Yield: 68% (white solid). Mp: 202-204 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.60(br s, 1H, NH), 9.38 (br s, 1H, OH), 7.95 (s, 1H, H-3), 7.45 (d, *J* = 8.5 Hz, 2H Ar), 7.26 (d, *J* = 8.5 Hz, 2H Ar), 7.05 (d, *J* = 8.2 Hz, 1H Ar), 6.73 (m 1H Ar), 6.66 (d, *J* = 2.2 Hz, 1H), 5.94 (m, 1H, H_c), 5.20 (dd, *J* = 17.0, 1.6 Hz, 1H, H_a), 5.03 (dd, *J* = 10.0, 1.6 Hz, 1H, H_b), 4.90 (m, 1H, CHCl), 3.78 (d, *J* = 6.5 Hz, 2H, SCH₂), 3.30 (m, 2H, CH₂Ph), 2.10 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.02, 156.31, 153.36, 154.52, 153.11, 143.47, 136.13, 134.35, 133.10, 132.94, 132.00, 131.22, 129.01, 121.64, 118.33, 101.14, 100.91, 70.04, 53.84, 36.90, 17.62. Elem. anal. calcd. for C₂₃H₂₁Cl₂N₅OS: C 56.79, H 4.35, N 14.40, S 6.59; found: C 56.83, H 4.39, N 14.40, S 6.71.

6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-N-(3-methoxyphenyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (100f)

Yield: 52% (white solid). Mp: 97-98 °C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.07 (s, 1H, NH), 8.15 (s, 1H, H-3), 7.54-7.52 (m, 2H Ar), 7.45-7.41 (m, 3H Ar), 7.26-7.24 (m, 2H Ar), 6.68-6.66

(m, 1H Ar), 5.99-5.93 (m, 1H, H_c), 5.65-5.61 (m, 1H, CHCl), 5.28 (dd, *J* = 17.0, 1.7 Hz, 1H, H_a), 5.08 (dd, *J* = 10.0, 1.7 Hz, 1H, H_b), 4.90-4.84 and 4.77-4.72 (2m, 2H, NCH₂), 3.82-3.79 (m, 2H, CH₂S), 3.73 (s, 3H, OCH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.11, 160.02, 154.61, 153.81, 140.41, 137.98, 134.81, 133.26, 132.14, 130.34, 130.03, 122.71, 118.14, 113.93, 109.85, 99.21, 60.00, 55.57, 52.92, 33.55. Elem. anal. calcd. for C₂₃H₂₁BrClN₅OS: C 52.04, H 3.99, N 13.19, S 6.04; found: C 38.24, H 3.08, N 9.73, S 3.47.

4-((6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)-3-methylphenol (100g)

Yield: 54% (white solid). Mp: 173-174°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.74 (s, 1H, OH), 9.41 (s, 1H, NH), 7.53-7.51 (m, 2H Ar), 7.42-7.40 (m, 2H Ar), 7.08 (d, *J* = 8.3 Hz, 1H Ar), 6.68-6.65 (m, 2H Ar), 5.91-5.87 (m, 1H, H_c), 5.60 (t, *J* = 7.2 Hz, 1H, CHCl), 5.20 (d, *J* = 16.9 Hz, 1H, H_a), 5.04 (d, *J* = 9.9 Hz, 1H, H_b), 4.85-4.79 and 4.72-4.66 (2m, 2H, NCH₂), 3.72 (br s, 2H, SCH₂), 1.98 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.06, 156.42, 156.40, 154.98, 138.00, 137.26, 136.73, 135.03, 133.83, 133.23, 133.14, 132.12, 131.79, 130.33, 122.70, 117.96, 114.67, 60.00, 52.84, 33.45, 17.39. Elem. anal. calcd. for C₂₃H₂₁BrClN₅OS: C 52.04, H 3.99, N 13.19, S 6.04; found: C 52.01, H 3.88, N 13.45, S 6.51.

6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-N-(4-methoxy-2-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (100h)

Yield: 47% (white solid). Mp: 153-155°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.70 (s, 1H, NH), 7.53-7.51 (m, 2H Ar), 7.41-7.39 (m, 2H Ar), 7.17 (d, *J* = 8.6 Hz, 1H Ar), 6.90-6.80 (m, 2H Ar), 5.93-5.89 (m, 1H, H_c), 5.58 (br s, CHCl), 5.26-5.04 (m, 2H, H_a + H_b), 4.80-4.70 (m, 2H, NCH₂), 3.74 (br s, 5H, OCH₃ + SCH₂), 2.08 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.06, 155.01, 137.99, 135.08, 133.05, 133.02, 132.11, 130.32, 129.58, 122.69, 118.93, 118.60, 117.93, 116.22, 116.15, 112.26, 111.24, 98.35, 59.97, 55.78, 52.82, 33.44. Elem. anal. calcd. for C₂₄H₂₃BrClN₅OS: C 52.90, H 4.25, N 12.85, S 5.88; found: C 53.43, H 4.18, N 12.67, S 5.34.

3-((6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)-4-methylphenol (100i)

Yield: 50% (white solid). Mp: 171°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.75 (s, 1H, OH), 9.43 (br s, 1H, NH), 7.53-7.51 (m, 3H, 2H Ar + H-3), 7.41 (d, *J* = 8.5 Hz, 2H Ar), 7.08 (d, *J* = 8.2 Hz, 1H Ar), 6.68-6.65 (m, 2H Ar), 5.90 (dd, *J* = 16.8, 9.7 Hz, 1H, H_c), 5.59 (t, *J* = 7.2 Hz, 1H,

CHCl), 5.20 (d, $J = 16.8$ Hz, 1H, H_a), 5.04 (d, $J = 9.7$ Hz, 1H, H_b), 4.85-4.79 and 4.72-4.66 (2m, 2H, NCH_2) 3.72 (s, 2H, SCH_2), 1.98 (s, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO- d_6): δ 170.88, 168.06, 156.42, 154.98, 137.99, 137.26, 135.03, 133.14, 132.12, 131.78, 130.33, 129.38, 125.17, 122.70, 117.95, 114.73, 98.18, 60.00, 52.84, 33.45, 17.39. Elem. anal. calcd. for $C_{23}H_{21}BrClN_5OS$: C 52.04, H 3.99, N 13.19, S 6.04; found: C 52.50, H 3.84, N 13.00, S 6.04.

6-(allylthio)-N-(3-bromophenyl)-1-(2-(4-bromophenyl)-2-chloroethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (170)

Yield: 47% (white solid). Mp: 115-116°C. ^1H NMR (400 MHz, DMSO- d_6) ; δ 10.19 (bs, 1H, NH), 8.19 (s, 1H, H-3), 8.15 (t, $J = 2.0$ Hz, 1H, Ar), 7.67-7.64 (m, 1H, Ar), 7.48-7.46 (m, 2H, Ar), 7.48-7.24 (m, 5H, Ar), 6.04-5.94 (m, 1H), 5.63 (dd, $J = 8.6, 6.0$ Hz, 1H, CHO), 5.29 (dd, $J = 16.9, 1.6$ Hz, 1H), 5.11-5.08 (m, 1H), 4.89 (dd, $J = 14.3, 8.6$ Hz, 1H, CH_2N), 4.76 (dd, $J = 14.3, 6.0$ Hz, 1H, CH_2N), 3.88-3.78 (m, 2H). ^{13}C NMR (101 MHz, DMSO- d_6): 168.09, 154.67, 153.56, 140.96, 138.48, 134.70, 133.14, 131.21, 129.53, 129.24, 128.08, 126.60, 123.91, 121.97, 120.14, 118.18, 99.36, 61.02, 53.21, 33.63. Elem. anal. calcd. for $C_{22}H_{19}BrClN_5S$: C 52.76; H 3.82; N 13.98; S 6.40; found C 52.80; H 3.70; N 14.20; S 5.90.

3-((6-(allylthio)-1-(2-chloro-2-phenylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)-4-methylphenol (171)

Yield: 49% (white solid). Mp: 154-156°C. ^1H NMR (400 MHz, DMSO- d_6): δ 9.74 (s, 1H, OH), 9.38 (s, 1H, NH), 7.50-7.43 (m, 2H Ar), 7.38-7.24 (m, 4H, 3 H Ar + H-3), 7.08 (d, $J = 8.2$ Hz, 1H Ar), 6.71-6.63 (m, 2H Ar), 6.00-5.85 (m, 1H, H_c), 5.60 (t, $J = 8.7$ Hz, 1H, CHCl), 5.21 (d, $J = 17.0$ Hz, 1H, H_a), 5.04 (d, $J = 10.0$ Hz, 1H, H_b), 4.84 and 4.69 (2 dd, $J = 14.3, 8.8$ Hz, 2H, NCH_2), 3.81-3.63 (m, 2H, SCH_2), 1.99 (s, 3H, CH_3). ^{13}C NMR (101 MHz, DMSO- d_6): δ 168.02, 156.42, 154.97, 138.54, 137.29, 135.08, 133.06, 131.80, 129.53, 129.24, 128.07, 117.96, 114.74, 98.21, 61.03, 53.11, 33.46, 17.40. Elem. anal. calcd. for $C_{23}H_{22}ClN_5OS$: C 61.12, H, 4.91, N 15.50, S, 7.09; found: C 61.15, H, 4.95, N 15.48, S, 7.02.

3-((6-(allylthio)-1-(2-chloro-2-phenylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)-4-fluorophenol (172)

Yield: 69% (white solid). Mp: 193-194°C. ^1H NMR (400 MHz, DMSO- d_6) δ 9.95 (s, 1H, OH), 9.52 (s, 1H, H-3), 7.97 (br s, 1H, NH), 7.51-7.44 (m, 2H Ar), 7.38-7.28 (m, 3H Ar), 7.14-7.04 (m, 1H Ar), 6.99 (dd, $J = 6.8, 3.0$ Hz, 1H Ar), 6.63 (dd, $J = 7.9, 4.5$ Hz, 1H Ar), 5.90 (ddt, $J =$

16.9, 10.0, 6.9 Hz, 1H, H_c), 5.62 (dd, $J = 8.7, 5.9$ Hz, 1H, CHCl), 5.19 (dd, $J = 17.0, 1.9$ Hz, 1H, H_a), 5.03 (dd, $J = 10.0, 1.9$ Hz, 1H, H_b), 4.87 and 4.73 (2dd, $J = 14.3, 8.7$ Hz, 2H, NCH₂), 3.80-3.60 (m, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 168.01, 154.89 (d, $J = 4.3$ Hz), 154.03, 150.08 (d, $^1J_{\text{CF}} = 237.7$ Hz), 138.52, 134.97, 133.17, 129.55, 129.26, 128.10, 126.09 (d, $^3J_{\text{CF}} = 13.7$ Hz), 117.96, 116.80 (d, $^2J_{\text{CF}} = 23.0$ Hz), 114.03, 98.84, 61.06, 53.13, 40.70, 40.49, 33.54. Elem. anal. calcd. for C₂₂H₁₉ClFN₅OS: C 57.96, H 4.20, N 15.36, S 7.03; found: C 57.92, H 4.22, N 15.36, S 7.06.

6-(allylthio)-N-(3-bromophenyl)-1-(2-(4-bromophenyl)-2-chloroethyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (176)

Yield: 61% (white solid). Mp: 163-165°C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.22 (b s, 1H, NH), 8.18 (s, 1H, Ar), 8.15 (s, 1H, Ar), 7.65 (d, $J = 8.1$ Hz, 1H, Ar), 7.53 (d, $J = 8.4$ Hz, 2H, Ar), 7.42 (d, $J = 8.4$ Hz, 2H, Ar), 7.33-7.24 (m, 2H, Ar), 6.02-5.92 (m, 1H), 5.63 (dd, $J = 8.2, 6.3$ Hz, 1H), 5.28 (dd, $J = 16.9, 1.7$ Hz, 1H), 5.09 (dd, $J = 10.0, 1.7$ Hz, 1H), 4.88 (dd, $J = 14.3, 8.2$ Hz, 1H), 4.75 (dd, $J = 14.3, 6.3$ Hz, 1H), 3.87-3.77 (m, 2H). ¹³C NMR (101 MHz, DMSO-*D*₆) δ 168.14, 154.66, 153.53, 140.93, 137.97, 134.67, 133.21, 132.16, 131.23, 130.36, 126.61, 123.90, 122.74, 121.98, 120.14, 118.22, 99.34, 60.00, 52.95, 33.62. Elem. anal. calcd. for C₂₂H₁₈Br₂ClN₅S: C 45.58, H 3.13, N 12.08, S 5.53; found: C 45.62, H 3.16, N 12.11, S 5.48.

2.11. General procedure for the synthesis of compounds 60–73, 167, 168, 173, 174

A suspension of the proper intermediate in 4 M NaOH was added to absolute ethanol and the reaction mixture was refluxed for 4h. After cooling, the formed solid was filtered and recrystallized from ethanol to afford **60–73, 167, 168, 173, 174** as pure white solids.

(*E*)-3-((6-(allylthio)-1-(4-chlorostyryl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-yl)amino)-4-methylphenol (60)

Yield: 51% (white solid). Mp: 220-222 °C. ¹H NMR (400 MHz, DMSO-*d*₆) δ 9.91 (s, 1H, NH), 9.40 (s, 1H, OH), 7.96 (d, $J = 14.5$ Hz, 1H, NCH=), 7.59-7.57 (m, 3H, 1H Ar + H-3), 7.38-7.36 (m, 2H, 1H Ar), 7.26 (m, 1H Ar), 7.24 (d, $J = 14.5$ Hz, 1H, =CHAr), 7.10 (d, $J = 8.3$ Hz, 1H Ar), 6.73 (s, 1H Ar), 6.69-6.67 (m, 1H Ar), 5.94-5.86 (m, 1H, H_c), 5.20 (d, $J = 17.0$ Hz, 1H, H_a), 5.03 (d, $J = 10.0$ Hz, 1H, H_b), 3.78 (d, $J = 5.7$ Hz, 2H, SCH₂), 2.03 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 169.14, 156.46, 154.15, 137.10, 135.21, 134.83, 134.67, 132.19, 131.85, 131.47, 129.30, 128.43, 125.08, 123.23, 118.15, 115.40, 114.65, 99.25, 55.46, 33.58, 17.42. Elem. anal. calcd. for C₂₃H₂₀ClN₅OS C 61.40, H 4.48, N 15.56, S 7.13.

(E)-1-(4-Bromostyryl)-N-(3-fluorophenyl)-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (61)

Yield: 90 % (white solid). Mp: 218-219°C. ¹H NMR (400 MHz,) δ 8.36 (s, 1H, H-3), 7.95 (d, *J* = 14.5 Hz, 1H, NCH=), 7.83 (dt, *J* = 12.0, 2.1 Hz, 1H Ar), 7.52 – 7.45 (m, 5H Ar), 7.39-7.34 (m, 1H Ar), 7.24 (d, *J* = 14.5 Hz, 1H, =CHAr), 6.88 (dt, *J* = 8.3, 2.5 Hz, 1H Ar), 2.57 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 170.33, 162.58 (d, ¹*J*_{CF} = 241.2 Hz), 153.77, 153.48, 141.43 (d, ³*J*_{CF} = 11.2 Hz), 135.28, 134.98, 132.14, 130.73 (d, ³*J*_{CF} = 9.6 Hz), 128.74, 123.08, 120.70, 117.12 (d, ⁴*J*_{CF} = 1.7 Hz), 115.51, 110.33 (d, ²*J*_{CF} = 21.4 Hz), 108.35 (d, ²*J*_{CF} = 26.1 Hz), 100.34, 14.39. Elem. anal. calcd. for C₂₀H₁₅BrFN₅S: C 52.64, H 3.31, N 15.35, S 7.03; found: C 51.35, H 3.04, N 14.57, S 7.04.

(E)-1-(4-bromostyryl)-N-(3-methoxyphenyl)-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (62)

Yield: 31% (white solid). Mp: 199-200°C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.20 (s, 1H, NH), 8.37 (s, 1H, H-3), 8.00 (d, *J* = 14.5 Hz, 1H, NCH=), 7.56-7.50 (m, 5H, 4Ar + =CHAr), 7.32-7.25 (m, 3H Ar), 6.68 (d, *J* = 6.8 Hz, 1H, Ar), 3.74 (s, 3H, OCH₃), 2.57 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-*d*₆): δ 170.41, 160.04, 153.81, 153.66, 140.37, 135.34, 135.00, 132.18, 130.07, 128.80, 123.13, 120.73, 115.54, 113.82, 109.88, 107.44, 100.12, 55.60, 31.23. Elem. anal. calcd. for C₂₁H₁₈BrN₅OS: C 53.85, H 3.87, N 14.95, S 6.84; found: C 53.90, H 3.73, N 14.76, S 6.63.

(E)-1-(4-bromostyryl)-6-(methylthio)-N-(naphthalen-1-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (63)

Yield: 73% (white solid). Mp: 158-160°C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.99-7.96 (m, 3H, 1 Ar + H-3 + NCH=), 7.87 (d, *J* = 7.7 Hz, 1H Ar), 7.64 (d, *J* = 7.1 Hz, 1H Ar), 7.57-7.47 (m, 8H Ar), 7.23 (d, *J* = 14.5 Hz, 1H =CHAr), 2.38 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-*d*₆): δ 170.11, 167.01, 156.35, 154.35, 135.55, 135.32, 135.12, 134.50, 132.24, 132.17, 129.96, 128.74, 126.82, 126.69, 126.32, 124.22, 123.76, 123.30, 120.60, 115.13, 99.78, 14.06. Elem. anal. calcd. for C₂₄H₁₈BrN₅S: C 59.02, H 3.71, N 14.34, S 6.56; found: C 58.54, H 3.50, N 14.44, S 6.56.

(E)-5-((1-(4-bromostyryl)-6-(methylthio)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)naphthalen-1-ol (64)

Yield: 10% (white solid). Mp: 268-270°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.41 (br s, 2H, NH + OH), 8.14 (d, *J* = 7.9 Hz, 1H Ar), 7.98 (d, *J* = 14.6 Hz, 1H, NCH=), 7.62 (d, *J* = 7.0 Hz, 1H Ar), 7.55-7.46 (m, 5H Ar + H-3), 7.36 (d, *J* = 8.6 Hz, 1H Ar), 7.31-7.22 (m, 2H, 1 Ar + =CHAr), 6.89 (d, *J* = 7.3 Hz, 1H Ar), 2.42 (s, 3H SCH₃). DMSO-d₆. ¹³C NMR (101 MHz, DMSO-d₆): δ 170.29, 155.43, 154.27, 154.17, 135.36, 135.02, 133.68, 133.65, 132.86, 132.18, 128.79, 127.58, 126.09, 125.40, 124.70, 123.23, 120.80, 120.69, 115.41, 113.91, 109.06, 14.08. Elem. anal. calcd. for C₂₄H₁₈BrN₅OS: C 57.15, H 3.60, N 13.88, S 6.36; found: C 57.45, H 3.64, N 13.85, S 6.30.

(E)-1-(4-bromostyryl)-6-(methylthio)-N-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (65)

Yield: 60% (white solid). Mp: 189-191°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.98 (t, *J* = 5.1 Hz, 1H, NH), 8.24 (s, 1H, H-3), 8.13-8.10 (m, 1H Ar), 7.99-7.92 (m, 2H, 1H Ar + NCH=), 7.86 (d, *J* = 8.1 Hz, 1H Ar), 7.56-7.45 (m, 8H Ar), 7.25 (d, *J* = 14.5 Hz, 1H, =CHAr), 5.15 (d, *J* = 4.8 Hz, 2H, CH₂naph), 2.51 (s, 3H, SCH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 170.44, 155.53, 153.78, 135.44, 135.11, 134.37, 133.92, 132.17, 131.63, 129.14, 128.73, 128.48, 126.97, 126.71, 126.46, 126.05, 124.08, 123.34, 120.62, 115.18, 99.46, 42.20, 14.25. Elem. anal. calcd. for C₂₅H₂₀BrN₅S: C 59.76, H 4.01, N 13.94, S 6.38; found: C 59.63, H 3.88, N 14.21, S 6.25.

(E)-6-(allylthio)-1-(4-bromostyryl)-N-(3-fluorophenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (66)

Yield: 66% (white solid). Mp: 189-191 °C. ¹H NMR (400 MHz, DMSO-d₆) δ 8.35 (s, 1H, H-3), 7.97 (d, *J* = 14.5 Hz, 1H, NCH=), 7.77 (d, *J* = 11.8 Hz, 1H Ar), 7.55-7.47 (m, 4H Ar), 7.45-7.43 (m, 1H Ar), 7.40-7.34 (m, 1H Ar), 7.26 (d, *J* = 14.5 Hz, 1H, =CHAr), 6.89 (dt, *J* = 8.2, 2.1 Hz, 1H Ar), 6.03-5.92 (m, 1H, H_c), 5.29 (dd, *J* = 17.0, 1.5 Hz, 1H, H_a), 5.11-5.05 (m, 1H, H_b), 3.87 (d, *J* = 6.8 Hz, 2H, CH₂S). ¹³C NMR (101 MHz, DMSO-d₆) δ 169.10, 162.61 (d, ¹*J*_{CF} = 241.4 Hz), 153.75, 153.66, 141.58 (d, ³*J*_{CF} = 10.5 Hz), 135.33, 134.99, 134.61, 132.18, 130.74 (d, ³*J*_{CF} = 9.6 Hz), 128.74, 123.12, 120.73, 118.22, 117.38, 115.56, 110.42 (d, ²*J*_{CF} = 21.5 Hz), 108.55 (d, ²*J*_{CF} = 26.2 Hz), 100.57, 33.66. Elem. anal. calcd. for C₂₂H₁₇BrFN₅S: C 54.78; H, 3.55; N, 14.52; S: 6.65; found: C 54.44; H, 3.24; N, 14.99; S: 6.57.

(E)-6-(allylthio)-1-(4-bromostyryl)-N-(3-methoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (67)

Yield: 13% (white solid). Mp: 180-181°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.24 (s, 1H, NH), 8.36 (s, 1H, H-3), 8.01 (d, *J* = 14.5 Hz, 1H, NCH=), 7.56-7.51 (m, 4H, 3Ar + =CHAr), 7.46 (s, 1H Ar), 7.30-7.25 (m, 3H Ar), 6.71-6.89 (m, 1H Ar), 6.01-5.94 (m, 1H, H_c), 5.29 (dd, *J* = 17.0, 1.7 Hz, 1H, H_a), 5.08 (dd, *J* = 10.0, 1.7 Hz, 1H, H_b), 3.89 (d, *J* = 6.8 Hz, 2H, CH₂S), 3.75 (s, 3H, OCH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.20, 160.06, 153.75, 140.25, 135.36, 134.99, 134.61, 132.22, 130.08, 128.80, 123.16, 120.77, 118.33, 115.64, 114.13, 110.05, 107.64, 100.26, 55.61, 33.67. Elem. anal. calcd. for C₂₃H₂₀BrN₅OS: C 55.88, H 4.08, N 14.17, S 6.48; found: C 55.58, H 4.16, N 14.09, S 6.22.

(E)-4-((6-(allylthio)-1-(4-bromostyryl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)-3-methylphenol (68)

Yield: 45% (white solid). Mp: 287-288°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.91 (s, 1H, OH), 9.48 (s, 1H, NH), 7.96 (d, *J* = 14.6 Hz, 1H, NCH=), 7.57-7.45 (m, 5H, 4H Ar + H-3), 7.25-7.22 (m, 1H Ar), 7.10-7.05 (m, 1H Ar), 6.72-6.68 (m, 2H Ar), 5.93-5.87 (m, 1H, H_c), 5.24-5.17 (m, 1H, olephine), 5.05-5.01 (m, 1H, olephine), 3.79-3.77 (br, 2H, SCH₂), 2.02 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.13, 156.54, 154.15, 137.09, 135.24, 135.02, 134.82, 132.26, 132.19, 131.85, 128.74, 126.07, 123.25, 120.70, 118.15, 115.43, 114.74, 111.18, 99.80, 33.57, 17.41. Elem. anal. calcd. for C₂₃H₂₀BrN₅OS: C 55.88, H 4.08, N 14.17, S 6.48; found: C 55.88, H 4.12, N 14.17, S 6.69.

6-(allylthio)-1-(2-(4-bromophenyl)-2-chloroethyl)-N-(4-methoxy-2-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (69)

Yield: 47% (white solid). Mp: 153-155°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.70 (s, 1H, NH), 7.53-7.51 (m, 2H Ar), 7.41-7.39 (m, 2H Ar), 7.17 (d, *J* = 8.6 Hz, 1H Ar), 6.90-6.80 (m, 2H Ar), 5.93-5.89 (m, 1H, H_c), 5.58 (br s, CHCl), 5.26-5.04 (m, 2H, H_a + H_b), 4.80-4.70 (m, 2H, NCH₂), 3.74 (br s, 5H, OCH₃ + SCH₂), 2.08 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 168.06, 155.01, 137.99, 135.08, 133.05, 133.02, 132.11, 130.32, 129.58, 122.69, 118.93, 118.60, 117.93, 116.22, 116.15, 112.26, 111.24, 98.35, 59.97, 55.78, 52.82, 33.44. Elem. anal. calcd. for C₂₄H₂₃BrClN₅OS: C 52.90, H 4.25, N 12.85, S 5.88; found: C 53.43, H 4.18, N 12.67, S 4.34.

(E)-3-((6-(allylthio)-1-(4-bromostyryl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)-4-methylphenol (70)

Yield: 62% (white solid). Mp: 223-224°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.85 (br s, 1H, NH), 7.96 (d, *J* = 14.5 Hz, 1H, NCH=), 7.56-7.48 (m, 5H, 4 Ar + H-3), 7.24 (d, *J* = 14.5 Hz, 1H, =CHAr), 7.08 (d, *J* = 8.1 Hz, 1H Ar), 6.71-6.67 (m, 2H Ar), 5.95-5.85 (m, 1H, H_c), 5.21-5.18 (d, *J* = 13.8, 1H, H_a), 5.02 (d, *J* = 9.8 Hz, 1H, H_b), 3.77 (s, 2H, SCH₂), 2.02 (s, 3H, CH₃). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.09, 156.79, 154.48, 154.16, 137.21, 135.30, 135.17, 135.04, 134.84, 132.19, 131.77, 128.86, 128.73, 124.77, 123.26, 120.68, 118.13, 115.37, 114.85, 33.57, 17.40. Elem. anal. calcd. for C₂₃H₂₀BrN₅OS: C 55.88, H 4.08, N 14.17, S 6.48; found: C 55.58, H 3.64, N 14.72, S 6.93.

(E)-6-(allylthio)-1-(4-bromostyryl)-N-(naphthalen-1-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (71)

Yield: 68% (white solid). Mp: 202-204°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.56 (br s, 1H, NH), 7.99 (s, 1H, H-3), 7.97-7.90 (m, 3H Ar, 2H Ar + NCH=), 7.65-7.63 (m, 1H Ar), 7.58-7.49 (m, 8H Ar), 7.23 (d, *J* = 14.4 Hz, 1H, =CHAr), 5.78-5.76 (m, 1H, H_c), 5.13-4.98 (m, 1H, H_a), 4.92 (d, *J* = 8.4, 1H, H_b), 3.62 (br s, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.09, 156.36, 154.23, 139.92, 135.48, 135.04, 134.83, 134.49, 132.44, 132.19, 130.02, 128.99, 128.82, 128.74, 127.40, 126.91, 126.27, 124.70, 123.64, 123.21, 120.68, 117.88, 115.34, 33.52. Elem. anal. calcd. for C₂₆H₂₀BrN₅S: C 60.70, H 3.92, N 16.61, S 6.23; found: C 60.61, H 3.45, N 16.110, S 6.64.

(E)-5-((6-(allylthio)-1-(4-bromostyryl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)amino)naphthalen-1-ol (72)

Yield: 71% (white solid). Mp: 286-287°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.44 (s, 1H, OH), 10.29 (br s, 1H, NH), 8.15 (d, *J* = 7.4 Hz, 1H Ar), 7.97 (d, *J* = 14.6 Hz, 1H, NCH=), 7.60 (d, *J* = 7.1 Hz, 1H Ar), 7.51-7.46 (m, 6H, 5 Ar + H-3), 7.37-7.21 (m, 3H, 2 Ar + =CHAr), 6.89 (d, *J* = 7.3 Hz, 1H Ar), 5.87-5.73 (m, 1H, H_c), 5.18-5.01 (m, 1H, H_a), 5.00-4.90 (s, 1H, H_b), 3.66 (s, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.14, 154.20, 154.13, 147.20, 135.38, 135.01, 134.81, 133.64, 132.19, 131.61, 128.88, 128.75, 128.67, 127.57, 126.08, 125.42, 124.69, 123.20, 120.70, 117.97, 115.43, 113.95, 109.06, 33.54. Elem. anal. calcd. for C₂₆H₂₀BrN₅OS: C 58.87, H 3.80, N 13.20, S 6.04; found: C 58.84, H 3.65, N 13.33, S 6.50.

(E)-6-(allylthio)-1-(4-bromostyryl)-N-(naphthalen-1-ylmethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (73)

Yield: 84% (white solid). Mp: 169-170°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.00 (t, *J* = 5.3 Hz, 1H, NH), 8.25 (s, 1H, H-3), 8.10-8.08 (m, 1H Ar), 7.98-7.93 (m, 2H, NCH= + 1H Ar), 7.86 (d, *J* = 8.0 Hz, 1H Ar), 7.56-7.44 (m, 8H Ar), 7.24 (d, *J* = 14.5 Hz, 1H, =CHAr), 5.90 (ddt, *J* = 16.9, 10.0, 6.8 Hz, 1H, H_c), 5.21 (dd, *J* = 16.9, 1.5 Hz, 1H, H_a), 5.14 (d, *J* = 5.3 Hz, 2H, NHCH₂), 4.99 (dd, *J* = 10.0, 1.5 Hz, 1H, H_b), 3.80 (d, *J* = 6.8 Hz, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.30, 155.65, 153.70, 135.45, 135.09, 134.95, 134.28, 133.92, 132.19, 131.60, 129.14, 128.72, 128.46, 126.97, 126.52, 126.46, 126.04, 124.02, 123.30, 120.64, 117.92, 115.23, 99.60, 42.26, 33.71. Elem. anal. calcd. for C₂₇H₂₂BrN₅S: C 61.37, H 4.20, N 13.25, S 6.07; found: C 61.38, H 3.89, N 13.48, S 6.00.

(E)-6-(allylthio)-N-phenethyl-1-styryl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (167)

Yield: 52%. Mp: 125-126°C. ¹H NMR (400 MHz, DMSO-d₆): δ 8.62 (t, *J* = 5.6 Hz, 1H, NH), 8.18 (s, 1H, H-3), 7.90 (d, *J* = 14.5 Hz, 1H, CH), 7.55 (d, *J* = 8.7 Hz, 1H, Ar), 7.34 (t, *J* = 7.7 Hz, 2H, Ar), 7.29-7.16 (m, 7H, 6Ar + CH), 6.04-5.94 (m, 1H), 5.29 (dd, *J* = 17.0, 1.7 Hz, 1H), 5.09-5.06 (m, 1H), 3.86 (d, *J* = 6.9 Hz, 2H), 3.70-3.65 (m, 2H), 2.90 (t, *J* = 7.4 Hz, 2H). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.16, 155.81, 153.53, 139.78, 135.71, 135.13, 135.02, 129.65, 129.39, 129.24, 128.95, 128.35, 127.89, 126.78, 126.69, 122.63, 117.96, 116.47, 99.58, 42.29, 35.32, 33.73. Elem. anal. calcd. for C₂₄H₂₃N₅S: C 69.70, H 5.61, N 16.94, S 7.75; found: C 70.02, H 5.38, N 17.01, S 7.40.

(E)-6-(Allylthio)-N-(3-bromophenyl)-1-styryl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (168)

Yield: 43%. Mp: 173-174°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.36 (br s, 1H, NH), 8.37 (s, 1H, H-3), 8.18 (t, *J* = 1.9 Hz, 1H, Ar), 7.95 (d, *J* = 14.6 Hz, 1H, CH), 7.68-7.65 (m, 1H, Ar), 7.57-7.55 (m, 2H, Ar), 7.36-7.21 (m, 6H, 5Ar + CH), 6.03-5.94 (m, 1H), 5.30 (dd, *J* = 16.9, 1.7 Hz, 1H), 5.11-5.08 (m, 1H), 3.88 (d, *J* = 6.7 Hz, 2H). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.11, 153.62, 153.56, 140.81, 135.55, 135.04, 134.50, 131.22, 129.39, 128.02, 126.78, 124.01, 122.40, 122.00, 120.22, 118.34, 117.03, 100.37, 33.75. Elem. anal. calcd. for C₂₂H₁₈BrN₅S: C 56.90, H 3.91, N 15.08, S 6.90; found: C 56.77; H 3.84; N 15.20; S 6.56.

(E)-6-(allylthio)-1-(4-bromostyryl)-N-phenethyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (173)

Yield: 55%. Mp: 148–149°C. ¹H NMR (400 MHz, DMSO-d₆) δ 8.65 (s, 1H, NH), 8.20 (s, 1H, H-3), 8.00–7.89 (m, 1H Ar), 7.57–7.45 (m, 4H Ar), 7.31–7.13 (m, 6H, 4 H Ar + CH=CH), 6.05–5.92 (m, 1H, H_c), 5.28 (d, *J* = 17.0 Hz, 1H, H_a), 5.08 (t, *J* = 9.7 Hz, 1H, H_b), 3.90–3.81 (m, 2H, CH₂CH₂Ph), 3.67 (m, 2H, CH₂CH₂Ph), 2.95–2.84 (m, 2H, SCH₂). ¹³C NMR (101 MHz, DMSO-d₆): δ 169.24, 155.79, 153.64, 139.77, 135.28, 135.12, 132.20, 129.23, 128.94, 128.72, 126.78, 123.32, 120.63, 117.99, 115.17, 99.60, 42.30, 35.32, 33.74. Elem. anal. calcd. for C₂₄H₂₂BrN₅S: C 58.54, H 4.50, N 14.22, S, 6.51; found: C 58.58, H 4.53, N 14.224, S, 6.57.

(E)-6-(allylthio)-N-(3-bromophenyl)-1-(4-bromostyryl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine (174)

Yield: 43%. Mp: 173–174 °C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.34 (bs, 1H, NH), 8.36 (s, 1H, Ar), 8.17 (s, 1H, Ar), 7.52–7.51 (m, 4H, Ar), 7.34–7.24 (m, 3H, Ar), 7.98 (d, *J* = 14.5 Hz, 1H, Ar), 7.65 (d, *J* = 7.9 Hz, 1H, Ar), 6.02–5.95 (m, 1H), 5.29 (d, *J* = 16.9 Hz, 1H), 5.09 (d, *J* = 10.1 Hz, 1H), 3.88 (d, *J* = 6.8 Hz, 2H). ¹³C NMR (101 MHz, DMSO-d₆) δ 169.20, 153.71, 153.52, 140.75, 135.25, 134.93, 134.47, 132.20, 131.23, 128.81, 126.78, 124.01, 123.04, 122.00, 120.80, 120.21, 118.39, 115.72, 100.37, 33.75. Elem. anal. calcd. for C₂₄H₂₂BrN₅S: C 58.54, H 4.50, N 14.22, S, 6.51; found: C 58.58, H 4.53, N 14.224, S, 6.57

2.12. General procedure for the synthesis of compounds 101, 105

m-Chloroperoxybenzoic acid (5 mmol, 1.25 g of 77% suspension in mineral oil) was added portion wise to a solution of **98f** or **104** (2.5 mmol) in anhydrous CHCl₃ (25 mL) at 0°C. The mixture was stirred at room temperature for 12 h. The solvent was evaporated under reduced pressure, and diethyl ether (10 mL) was added. By standing in a refrigerator for 2 h, the pure products precipitated as white solids, which were filtered and recrystallized from absolute ethanol.

1-(2-hydroxy-2-phenylethyl)-6-(methylsulfonyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (101)

Yield: 57%. Mp: 170–171°C. ¹H NMR (400 MHz, DMSO-d₆): δ 3.69 (s, 3H, SO₂CH₃), 4.22–4.52 (m, 2H, CH₂N), 4.91–5.09 (m, 1H, CHO), 5.60 (br s, 1H, OH disappears with D₂O), 7.09–7.28 (m, 5H Ar), 8.15 (s, 1H, H-3), 13.20 (br s, 1H, NH disappears with D₂O). IR (CHCl₃): cm⁻¹ 3540 (NH), 3150–2900 (OH), 1693 (CO). MS: *m/z* 334 [M+1]⁺. Elem. anal. calcd. for C₁₄H₁₄N₄O₄S: C 50.29, H 4.22, N 16.76, S 9.59; found: C 50.33, H 4.22, N 16.80, S 9.64

1-(2-chloro-2-phenylethyl)-6-(methylsulfonyl)-N-phenethyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine (105)

Yield: 46 % (white solid). Mp:163°C. ¹H NMR (400 MHz, DMSO-d₆) δ 9.08 (t, *J* = 5.5 Hz, 1H, NH), 8.21 (s, 1H, H-3), 7.50-7.46 (m, 2H Ar), 7.36-7.29 (m, 3H Ar), 7.28-7.22 (m, 4H Ar), 7.17 (td, *J* = 6.3, 2.7 Hz, 1H Ar), 5.63 (dd, *J* = 8.6, 6.0 Hz, 1H, CHCl), 4.94 and 4.82 (2dd, *J* = 14.3, 8.7 Hz, 2H, NCH₂), 3.71 (q, *J* = 6.4 Hz, 2H, CH₂CH₂Ph), 3.33 (s, 3H, CH₃), 2.91 (t, *J* = 7.4 Hz, 2H, CH₂CH₂Ph). ¹³C NMR (101 MHz, DMSO-d₆) δ 163.48, 157.13, 152.90, 139.57, 138.27, 133.51, 129.57, 129.36, 129.27, 129.22, 128.94, 128.12, 126.81, 101.18, 60.94, 53.43, 42.63, 35.03. Elem. anal. calcd. for C₂₂H₂₂ClN₅O₂S: C 57.95, H 4.86, N 15.36, S 7.03; found: C 57.96, H 4.84, N 15.40, S 7.07

2.13. General procedure for the synthesis of compounds 84, 102a, 102b

To a solution of intermediate **101** or **105** (1.80 mmol, 600 mg) in DMSO (25 mL), the proper aliphatic amine (9 mmol) was added, and the mixture was heated at 100 °C for 12h. After cooling to room temperature, cold water was added; the pale-yellow solid was filtered, washed with water, and recrystallized from absolute EtOH, affording compounds **84**, **102a**, **102b**.

2-((1-(2-chloro-2-phenylethyl)-4-(phenethylamino)-1H-pyrazolo[3,4-d]pyrimidin-6-yl)amino)-ethan-1-ol (84)

Yield: 63% (white solid). Mp:181-182°C. ¹H NMR (400 MHz, DMSO-d₆): δ 7.83 (br s, 1H, NH), 7.75 (s, 1H, H-3), 7.47-7.45 (m, 2H Ar), 7.35-7.22 (m, 7H Ar), 7.19-7.15 (m, 1H Ar), 6.56 (br s, 1H, NH), 5.61 (t, *J* = 7.3 Hz, 1H, CHCl), 4.69-4.64 (m, 2H, NCH₂), 4.58 (br s, 1H, OH), 3.57-3.52 (m, 4H, CH₂OH + NHCH₂), 3.38-3.36 (m, 2H, NHCH₂), 2.82 (t, *J* = 7.4 Hz, 2H, CH₂Ar). ¹³C NMR (101 MHz, DMSO-d₆): δ 162.03, 156.90, 156.52, 140.14, 138.80, 132.70, 129.37, 129.23, 129.19, 128.89, 128.09, 126.63, 95.85, 61.00, 60.89, 52.59, 44.31, 42.11, 35.64. Elem. anal. calcd. for C₂₃H₂₅ClN₆O: C 63.22, H 5.77, N 19.23; found: C 63.26, H 5.76, N 19.25.

1-(2-Hydroxy-2-phenylethyl)-6-(pyrrolidin-1-yl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (102a)

Yield: 42% (white solid). Mp: 223-225°C. ¹H NMR (400 MHz, DMSO-d₆): δ 10.54 (br s, 1H, NH), 7.68 (s, 1H, H-3), 7.26-7.25 (m, 4H Ar), 7.19- 7.18 (m, 1H Ar), 5.61 (br s, 1H, OH), 5.05-5.03 (m, 1H, CHOH), 4.24-4.19 and 4.15-4.10 (2m, 2H, NCH₂), 3.42-3.40 (m, 4H, 2 CH₂N pyr), 1.86 (s, 4H, 2 CH₂CH₂N pyr). ¹³C NMR (101 MHz, DMSO-d₆): δ 155.17, 152.36,

143.38, 141.55, 134.43, 128.53, 127.75, 126.58, 99.33, 71.50, 54.33, 47.31, 25.30. Elem. anal. calcd. for C₁₇H₁₉N₅O₂: C 62.75, H 5.89, N 21.52; found: C 60.00, H 6.23, N 20.07

1-(2-Hydroxy-2-phenylethyl)-6-(piperidin-1-yl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (102b)

Yield: 60% (white solid). Mp: 178-180°C. ¹H NMR (400 MHz, DMSO-d₆): δ 7.71 (s, 1H, H-3), 7.23-7.17 (m, 5H Ar), 5.54 (br s, 1H, OH), 5.02 (t, *J* = 6.6 Hz, 1H, CHCl), 4.25-4.20 and 4.15-4.10 (2m, 2H, NCH₂), 3.53-3.51 (m, 4H, 2 CH₂N pip), 1.61-1.57 (m, 2H, CH₂ pip), 1.48-1.47 (m, 4H, 2 CH₂CH₂N pip). ¹³C NMR (101 MHz, DMSO-d₆): δ 159.23, 154.87, 153.46, 143.29, 134.51, 128.50, 127.76, 126.55, 99.47, 71.53, 54.05, 46.51, 25.53, 22.79. Elem. Anal. calcd. for C₁₈H₂₁N₅O₂: C 63.70, H 6.24, N 20.64; found: C 62.18, H 6.77, N 20.35.

2.14. Synthesis of 5-amino-1-(2-hydroxy-2-phenylethyl)-1H-pyrazole-4-carbo- nitrile (106)

Ethoxymethylenemalononitrile (2.44 g, 20 mmol) was added to a solution of the 2-hydrazino-1-phenylethanol **94d** (20 mmol) in absolute ethanol (40 ml) and the reaction mixture was refluxed for 6 h. The solvent was concentrated to 50% of the initial volume and cooled. The yellow solid obtained was filtered and recrystallized from absolute ethanol to afford **106** as a product. Yield: 63%. Mp: 164-166°C. ¹H NMR (200 MHz, DMSO-d₆): δ 7.46 (s, 1H, H-3), 7.45-7.26 (m, 5H Ar), 5.8-5.08 (m, 1H, CHOH), 4.80 (br s, 2H, NH₂, disappears with D₂O), 4.27-3.98 (m, 2H, CH₂N), 3.15 (br s, 1H, OH, disappears with D₂O). IR (KBr): cm⁻¹ 3438, 3339, 3199 (OH, NH₂), 2223 (CN). Elem. Anal. calcd. for C₁₂H₁₂N₄O: C 63.15, H 5.30, N 24.55; found: C 62.99, H 5.25, N 24.60.

2.15. Synthesis of 5-amino-1-(2-hydroxy-2-phenylethyl)-1H-pyrazole-4-carbo-xamide (107)

To a solution of compounds **106** (10 mmol) in absolute ethanol (10 mL) 2M NaOH (10 mL) was added, and the mixture was refluxed for 2 h. After cooling, the white solid obtained was filtered, washed with water and recrystallized from ethanol. Yield: 65%. Mp: 228-229°C. ¹H NMR (200 MHz, DMSO-d₆): δ 7.56 (s, 1H, H-3), 7.35-7.10 (m, 5H, Ar), 6.60 (br s, 2H, CONH₂, disappears with D₂O), 5.98 (s, 2H, NH₂, disappears with D₂O), 5.61 (d, *J* = 4, 1H, OH, disappears with D₂O), 4.90-4.80 (m, 1H, CHOH), 3.90-3.80 (m, 2H, CH₂N). IR (KBr): cm⁻¹ 3389 (OH), 3299, 3169 (NH₂), 1643 (CO). Elem. Anal. calcd. for C₁₂H₁₄N₄O₂: C 58.83, H 5.73, N 22.75; found: C 58.55, H 5.56, N 22.94.

2.16. *Synthesis of 1-(2-hydroxy-2-phenylethyl)-6-(trifluoromethyl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (108)*

To a solution of the 5-amino-1-(2-hydroxy-2-phenylethyl)-1*H*-pyrazole-4-carboxamide **107** (10 mmol) in absolute ethanol (90 mL), a solution of sodium ethoxide prepared from sodium (1.38 g, 60 mmol) and absolute ethanol (30 mL) and methyl trifluoroacetate (60 mmol) were added. The mixture was refluxed for 6 h; after cooling, ice water was added and the solution was acidified with 3% acetic acid. The precipitated solid was filtered, washed with water and recrystallized from absolute ethanol to afford **108** as a white solid. Yield: 58%. Mp: 212-214°C. ¹H NMR (200 MHz, DMSO-*d*₆): δ 13.60 (br s, 1H, NH disappears with D₂O), 8.23 (s, 1H, H-3), 7.38–7.25 (m, 5H Ar), 5.71 (br s, 1H, OH disappears with D₂O), 5.10-5.00 (m, 1H, CHOH), 4.50–4.41 (m, 2H, CH₂N). IR (KBr): cm⁻¹ 3450-2798 (OH + NH), 1704 (CO). Elem. Anal. calcd. for C₁₄H₁₁N₄O₂F₃: C 51.86, H 3.42, N 17.58; found: C 51.78, H 3.77, N 17.34.

2.17. *Synthesis of 4-chloro-N-(3-fluorophenyl)-1,3,5-triazin-2-amine (111)*

A suspension of 2,4-dichloro-1,3,5-triazine **110** (1.5 g, 10.0 mmol) in anhydrous THF (15 mL) was cooled to 0°C under a nitrogen atmosphere. DIPEA (1.8 mL, 10.0 mmol) and 3-fluoroaniline (0.9 mL, 3.1 mmol) were then added. The reaction mixture was stirred for 30 min at 0 °C and then for 2 h at room temperature. The solvent was removed under reduced pressure and the crude residue was washed with water and the product was further extracted with EtOAc, dried over Na₂SO₄, and concentrated under reduced pressure. The resulting solid was purified by column chromatography using a mixture of PE and EtOAc (85:15) as the eluent, affording **111** as a pure white solid. Yield: 82%. Mp: 142-144°C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 10.90 (s, 1H, NH), 8.66 (s, 1H, H-6), 7.61 (s, 1H Ar), 7.45-7.31 (m, 2H Ar), 6.92 (t, *J* = 8.2 Hz, 1H Ar). ¹³C NMR (101 MHz, DMSO-*d*₆): δ 163.73, 163.37, 161.33, 158.35, 140.76, 130.74, 116.99, 110.51, 108.16. Elem. Anal. calcd. for C₉H₆ClFN₄: C 48.12, H, 2.69, N 24.94; found: C 48.14, H, 2.71, N 24.93.

2.18. *General procedure for the synthesis of compounds 90, 91*

To a suspension of **111** (1.8 mmol, 400 mg) in *i*PrOH (10 mL), DIPEA (1.8 mmol, 230 mg) and 2-phenylethan-1-amine or 2-(4-bromophenyl)ethan-1-amine (1.8 mmol) were added sequentially. The reaction mixture was heated at reflux for 2 h. The resulting suspension was filtered and the solid was suspended in EtOAc and washed with 1 NaOH. The crude was dried over Na₂SO₄, concentrated and purified by column chromatography to afford **90** and **91** as a white solids.

N2-(3-fluorophenyl)-N4-phenethyl-1,3,5-triazine-2,4-diamine (90)

Yield: 25%. Mp: 206-208°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.78 (s, 1H, NH), 9.69 (s, 1H, NH), 8.23 (s, 1H, H-6), 8.12 (s, 1H, H-6), 7.88-7.67 (m, 4H, 2NHCH₂ + 2Ar), 7.46-7.41 (m, 2H Ar), 7.30-7.12 (m, 12H Ar), 6.79-6.71 (m, 2H Ar), 3.50-3.45 (m, 4H, 2CH₂NH), 2.84-2.79 (m, 4H, 2CH₂Ar). ¹³C NMR (101 MHz, DMSO-d₆): δ 166.55, 166.13, 165.34, 165.20, 164.04, 163.88, 163.71, 162.69 (d, ¹J_{CF} = 241.2 Hz), 161.74 (d, ¹J_{CF} = 241.3 Hz), 161.49, 142.18 (d, ³J_{CF} = 12.4 Hz), 142.13 (d, ³J_{CF} = 11.3 Hz), 139.95, 139.78, 130.46 (d, ³J_{CF} = 9.4 Hz), 130.39 (d, ³J_{CF} = 10.2 Hz), 129.18, 128.87, 126.68, 126.61, 115.95 (d, ⁴J_{CF} = 2.1 Hz), 115.79 (d, ⁴J_{CF} = 2 Hz), 109.91 (d, ²J_{CF} = 21.3 Hz), 108.72 (d, ²J_{CF} = 21.2 Hz), 106.93 (d, ²J_{CF} = 25.8 Hz), 106.83 (d, ²J_{CF} = 26.9 Hz), 42.38, 42.29, 35.58, 35.50. Elem. Anal. calcd. for C₁₇H₁₆FN₅: C 66.01, H 5.21, N 22.64; found: C 66.04, H 5.22, N 22.67.

N2-(4-bromophenethyl)-N4-(3-fluorophenyl)-1,3,5-triazine-2,4-diamine (91)

Yield: 20%. Mp: 210-212°C. ¹H NMR (400 MHz, DMSO-d₆): δ 9.77 (s, 1H, NH), 9.69 (s, 1H, NH), 8.22 (s, 1H, H-6), 8.11 (s, 1H, H-6), 7.84-7.64 (m, 4H, 2NHCH₂ + 2Ar), 7.44-7.42 (m, 6H Ar), 7.29-7.23 (m, 2H Ar), 7.18-7.16 (m, 4H Ar), 6.79-6.74 (m, 2H Ar), 3.51-3.43 (m, 4H, 2CH₂NH), 2.79 (m, 4H, 2CH₂Ar). ¹³C NMR (101 MHz, DMSO-d₆): δ 166.53, 166.15, 165.33, 165.20, 164.01, 163.92, 163.58, 162.85, 162.69 (d, ¹J_{CF} = 234.6 Hz), 162.58 (d, ¹J_{CF} = 225.4 Hz), 142.15 (d, ³J_{CF} = 12.3 Hz), 142.09 (d, ³J_{CF} = 12.1 Hz), 139.44, 139.28, 131.61 (d, ³J_{CF} = 13.7 Hz), 131.50, 130.50 (d, ³J_{CF} = 10.4 Hz), 130.36, 119.76, 119.71, 115.98 (d, ⁴J_{CF} = 2.3), 115.80 (d, ⁴J_{CF} = 2.3), 108.95 (d, ²J_{CF} = 21.5 Hz), 108.64, 106.95 (d, ²J_{CF} = 25.8), 106.84 (d, ²J_{CF} = 27), 42.03, 41.98, 34.84, 34.69. Elem. Anal. calcd. for C₁₇H₁₅BrFN₅: C 52.59, H 3.89, N 18.04; found: C 52.63, H 3.86, N 18.06.

2.19. General procedure for the synthesis of compounds 92, 93

To a mixture of 2,4-dichloro-6-(methylthio)-1,3,5-triazine **112** (1.3 mmol, 250 mg,) in water (3 mL) and acetone (3 mL) cooled to 0 °C, NaHCO₃ (1.3 mmol, 107 mg) and 3-fluoroaniline (1.2 mmol, 154 mg) were added. The reaction mixture was stirred for 1 h at 0 °C. After consumption of the aniline, additional NaHCO₃ (1.3 mmol, 107 mg) and the appropriate phenethylamine (1.3 mmol) were added dropwise. The reaction mixture was stirred at room temperature for 12 h. The solvents were removed *in vacuo* and the crude was washed 1 M NaOH and extracted with DCM, dried over anhydrous Na₂SO₄, filtered, concentrated and finally purification by column chromatography to afford **92** and **93** as white solids.

N2-(3-fluorophenyl)-6-(methylthio)-N4-phenethyl-1,3,5-triazine-2,4-diamine (92)

Yield: 30%. Mp: 135-136°C. ^1H NMR (400 MHz, DMSO- d_6): δ 9.75 (s, 1H, NH), 9.65 (s, 1H, NH), 7.90-7.71 (m, 4H, 2Ar + 2NHCH $_2$), 7.48-7.34 (m, 2H Ar), 7.30-7.11 (m, 12H Ar), 6.79-6.68 (m, 2H Ar), 3.47 (q, J = 7.6 Hz, 4H, 2NHCH $_2$), 2.81 (t, J = 7.6 Hz, 4H, 2CH $_2$ Ar), 2.43 (s, 3H, SCH $_3$), 2.39 (s, 3H, SCH $_3$). ^{13}C NMR (101 MHz, DMSO- d_6): δ 179.98, 179.43, 164.53, 164.21, 162.97, 162.73 (d, $^1J_{\text{CF}}$ = 241.3 Hz), 162.68 (d, $^1J_{\text{CF}}$ = 241.3 Hz), 162.67, 142.20 (d, $^3J_{\text{CF}}$ = 12.4 Hz), 142.12 (d, $^3J_{\text{CF}}$ = 11.3 Hz), 139.96, 139.75, 130.48 (d, $^3J_{\text{CF}}$ = 8.4 Hz), 130.38 (d, $^3J_{\text{CF}}$ = 8.3 Hz), 129.20, 129.17, 128.89, 128.88, 126.68, 126.64, 115.97 (d, $^4J_{\text{CF}}$ = 3.1 Hz), 115.76 (d, $^4J_{\text{CF}}$ = 3.0 Hz), 108.93 (d, $^2J_{\text{CF}}$ = 21.2 Hz), 108.69 (d, $^2J_{\text{CF}}$ = 21.3 Hz), 106.96 (d, $^2J_{\text{CF}}$ = 22.4 Hz), 106.82 (d, $^2J_{\text{CF}}$ = 27.2 Hz), 42.43, 35.64, 35.59, 12.93, 12.84. Elem. Anal. calcd. for C $_{18}$ H $_{18}$ FN $_5$ S: C 60.83, H 5.10, N 19.70, S 9.02; found: C 60.85, H 5.13, N 19.73.

N2-(4-bromophenethyl)-N4-(3-fluorophenyl)-6-(methylthio)-1,3,5-triazine-2,4-diamine (93)

Yield: 10%. Mp: 178-181°C. ^1H NMR (400 MHz, DMSO- d_6): δ 9.74 (s, 1H, NH), 9.63 (s, 1H, NH), 7.82-7.71 (m, 4H, 2Ar + 2NHCH $_2$), 7.45-7.38 (m, 6H Ar), 7.28-7.20 (m, 2H Ar), 7.18-7.16 (m, 4H Ar), 6.79-6.72 (m, 2H Ar), 3.49-3.44 (m, 4H, 2CH $_2$ Ar), 2.79 (t, J = 7.3 Hz, 4H, 2CH $_2$ Ar), 2.42 (s, 3H, SCH $_3$), 2.39 (s, 3H, SCH $_3$). ^{13}C NMR (101 MHz, DMSO- d_6): δ 180.01, 179.47, 164.55, 164.24, 162.96, 162.72 ($^1J_{\text{CF}}$ = 241.3 Hz), 162.67, 162.65 ($^1J_{\text{CF}}$ = 241.0 Hz), 142.23, 142.08 ($^3J_{\text{CF}}$ = 11.2 Hz), 139.45, 139.27, 131.69, 131.68, 131.51 ($^3J_{\text{CF}}$ = 4.9 Hz), 130.50 ($^3J_{\text{CF}}$ = 8.6 Hz), 130.35, 119.76, 119.73, 116.02 ($^4J_{\text{CF}}$ = 2.0 Hz), 115.79 ($^4J_{\text{CF}}$ = 2.0 Hz), 108.86 ($^2J_{\text{CF}}$ = 21.3 Hz), 108.72 ($^2J_{\text{CF}}$ = 21.2 Hz), 107.01 ($^2J_{\text{CF}}$ = 26.4 Hz), 106.86 ($^2J_{\text{CF}}$ = 26.3 Hz), 42.09, 34.90, 34.78, 12.94, 12.84. Elem. Anal. calcd. for C $_{18}$ C $_{17}$ BrFN $_5$ S: C 49.78, H 3.95, N 16.12, S 7.38; found: C 49.77, H 3.93, N 16.10, S 7.33.

2.20. Synthesis of 4-fluoro-3-nitro-1,1'-biphenyl (189b)

Phenylboronic acid (6.8 mmol, 1.5 g) was added to a solution of 4-bromo-1-fluoro-2-nitrobenzene (7.5 mmol, 0.91 g) in toluene/ethanol (1:1, 30 mL), followed by the addition of Na $_2$ CO $_3$ (13 mmol, 1.4 g) and tetrakis(triphenylphosphine)palladium(0) (0.17 mmol, 0.2 g). The mixture was heated at reflux (85°C) for 24 h under argon atmosphere, then the solvent was removed under reduced pressure to afford a crude oil. The residue was extracted with EtOAc, and the combined organics were washed with H $_2$ O and brine, dried over Na $_2$ SO $_4$, and concentrated. Purified by normal-phase flash chromatography (CombiFlash; Heptane/EtOAc 0 $\rightarrow$ 5%) afforded **189b** as a yellow oil that crystallises spontaneously. Yield: 95% (yellow solid).

LC-MS (ESI): m/z calculated for $C_{12}H_8FNO_2$ $[M+H]^+$: 217.5; found: -. ^1H NMR (600 MHz, DMSO- d_6) δ 8.36 (dd, J = 7.6, 2.5 Hz, 1H), 8.12 (ddd, J = 7.6, 4.3, 2.5 Hz, 1H), 7.78 – 7.73 (m, 2H), 7.69 (dd, J = 11.1, 8.7 Hz, 1H), 7.54 – 7.50 (m, 2H), 7.46 – 7.43 (m, 1H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 154.80 (d, $^1J_{CF}$ = 261.8 Hz), 137.29 (d, $^4J_{CF}$ = 4.0 Hz), 136.92, 134.22 (d, $^3J_{CF}$ = 8.7 Hz), 129.18, 128.48, 126.90, 123.76 (d, $^3J_{CF}$ = 2.7 Hz), 119.00 (d, $^2J_{CF}$ = 20.8 Hz). ^{19}F NMR (376 MHz, DMSO- d_6) δ -121.93.

2.21. General procedure for the synthesis of compounds 190a-c

Aniline or 2-aminobutanoic acid (50 mmol) and $NaHCO_3$ (60 mmol, 5 g) were added to the appropriate fluoro-containing substrate (**189a,b**) in a EtOH/ H_2O (1:1, 100 ml). The mixture was heated at reflux (100 °C) overnight, developing a red solution. Reaction progress was monitored by LC-MS. After completion, EtOH was removed by distillation and the remaining aqueous phase was acidified to pH 1-2 with HCl 6M, affording a suspended orange solid further extracted with DCM from the aqueous layer, dried over Na_2SO_4 and finally concentrated *in vacuo*. The obtained solid was recrystallised from a mixture of DCM and cyclohexane and filtered to obtain the desired compound as a fine orange/yellow solid.

(5-methyl-2-nitrophenyl)alanine (190a)

Yield: 95% (yellow solid). LC-MS (ESI): m/z calculated for $C_{10}H_{12}N_2O_4$ $[M+H]^+$: 224.08; found: 225.1. ^1H NMR (600 MHz, DMSO- d_6) δ 13.21 (s, 1H), 8.39 (d, J = 7.1 Hz, 1H), 7.99 (d, J = 8.7 Hz, 1H), 6.81 (s, 1H), 6.57 (dd, J = 8.7, 1.7 Hz, 1H), 4.48 (p, J = 6.9 Hz, 1H), 2.31 (s, 3H), 1.47 (d, J = 6.9 Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 173.94, 147.99, 143.68, 129.56, 126.24, 117.59, 114.18, 50.13, 21.57, 18.21.

2-((5-methyl-2-nitrophenyl)amino)butanoic acid (190b)

Yield: 90% (yellow solid). LC-MS (ESI): m/z calculated for $C_{11}H_{14}N_2O_4$ $[M+H]^+$: 239.1; found: 238.10. ^1H NMR (600 MHz, DMSO- d_6) δ 13.21 (s, 1H), 8.39 (d, J = 7.4 Hz, 1H), 7.99 (d, J = 8.7 Hz, 1H), 6.84 (s, 1H), 6.57 (dd, J = 8.7, 1.7 Hz, 1H), 4.48 (dt, J = 7.4, 5.4 Hz, 1H), 2.31 (s, 3H), 1.98-1.83 (m, 2H), 0.89 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 173.57, 148.50, 144.43, 130.10, 126.76, 118.11, 114.76, 55.78, 24.88, 22.05, 9.44.

(3-nitro-[1,1'-biphenyl]-4-yl)alanine (190c)

Yield: 95% (yellow solid). LC-MS (ESI): m/z calculated for $C_{15}H_{14}N_2O_4$ $[M+H]^+$: 286.10; found: 287.1. ^1H NMR (600 MHz, DMSO- d_6) δ 13.27 (s, 1H), 8.39 (d, J = 7.0 Hz, 1H), 8.34 (d, J = 2.3 Hz, 1H), 7.92 (dd, J = 9.0, 2.3 Hz, 1H), 7.66 (d, J = 7.6 Hz, 2H), 7.46 (t, J = 7.6 Hz,

2H), 7.34 (t, $J = 7.6$ Hz, 1H), 7.12 (d, $J = 9.0$ Hz, 1H), 4.56 (p, $J = 6.9$ Hz, 1H), 1.51 (d, $J = 6.9$ Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 173.88, 142.87, 137.91, 134.99, 131.77, 129.04, 127.83, 127.24, 125.87, 123.37, 115.70, 50.38, 18.15.

2.22. General procedure for the synthesis of compounds **191a-c**

A Fisher esterification is performed by the reaction of **190a**, **190b** or **190c** (4.9 mmol) refluxed at 65°C for 5h in abundant $\text{H}_2\text{SO}_4/\text{MeOH}$ (10:1, 20 mL), previously prepared. Upon completion monitored by LC-MS, the mixture was concentrated *in vacuo* and afterwards quenched with a saturated solution of K_2CO_3 . The aqueous layer was then extracted with DCM. The organic layer was dried over Na_2SO_4 , filtered and concentrated *in vacuo* to give the corresponding alkyl ester of the carboxylic acid as an orange solid (**191a-c**).

Methyl (5-methyl-2-nitrophenyl)alaninate (**191a**)

Yield: 73% (orange solid). LC-MS (ESI): m/z calculated for $\text{C}_{11}\text{H}_{14}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$: 239.1; found: 238.1. ^1H NMR (600 MHz, DMSO- d_6) δ 8.31 (d, $J = 7.5$ Hz, 1H), 7.99 (d, $J = 8.7$ Hz, 1H), 6.81 (s, 1H), 6.59 (dd, $J = 8.8, 1.4$ Hz, 1H), 4.63 (p, $J = 7.0$ Hz, 1H), 3.73 (s, 3H), 2.31 (s, 3H), 1.49 (d, $J = 6.9$ Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 172.94, 148.07, 143.62, 129.70, 126.24, 117.83, 114.15, 52.48, 50.21, 21.56, 18.17.

Methyl 2-((5-methyl-2-nitrophenyl)amino)butanoate (**191b**)

Yield: 77% (orange solid). LC-MS (ESI): m/z calculated for $\text{C}_{12}\text{H}_{16}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$: 252.11; found: 253.1. ^1H NMR (600 MHz, DMSO- d_6) δ 8.33 (d, $J = 7.7$ Hz, 1H), 8.00 (d, $J = 8.8$ Hz, 1H), 6.84 (s, 1H), 6.59 (dd, $J = 8.8, 1.3$ Hz, 1H), 4.61 (dt, $J = 7.7, 5.7$ Hz, 1H), 3.73 (s, 3H), 2.31 (s, 3H), 1.96 – 1.85 (m, 2H), 0.90 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 172.23, 148.12, 143.89, 129.76, 126.27, 117.90, 114.21, 55.45, 52.40, 39.94, 39.80, 39.66, 39.52, 39.38, 39.24, 39.10, 24.76, 21.56, 9.15.

Methyl (3-nitro-[1,1'-biphenyl]-4-yl)alaninate (**191c**)

Yield: 97% (orange solid). LC-MS (ESI): m/z calculated for $\text{C}_{16}\text{H}_{16}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$: 300.11; found: 301.1. ^1H NMR (600 MHz, DMSO- d_6) δ 8.34 (d, $J = 2.3$ Hz, 1H), 8.30 (d, $J = 7.4$ Hz, 1H), 7.92 (dd, $J = 8.9, 2.4$ Hz, 1H), 7.67 (dd, $J = 8.3, 1.2$ Hz, 2H), 7.47-7.44 (m, 2H), 7.36-7.33 (m, 1H), 7.12 (d, $J = 8.9$ Hz, 1H), 4.71 (p, $J = 7.0$ Hz, 1H), 3.74 (s, 3H), 1.53 (d, $J = 7.0$ Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 172.88, 142.80, 137.84, 134.98, 131.93, 129.04, 128.10, 127.28, 125.90, 123.38, 115.64, 52.52, 50.45, 18.08.

2.23. General procedure for the synthesis of compounds 192a-c

A Bechamp reduction is performed adding **191a**, **191b** or **191c** (5 mmol) and iron (60 mmol) to a mixture of EtOH/NH₄Cl (1:1, 50 mL), previously prepared. The mixture is refluxed at 100°C overnight and, upon completion monitored through LC-MS, filtered through a celite cartridge and extracted with EtOAc. The organic layer was dried on Na₂SO₄, filtered and concentrated *in vacuo* to give the corresponding intramolecular-cyclized product (**192a-c**). **192c** was used in the next step as a crude. The flasks and everything in contact with iron was washed with 6M HCl.

3,6-dimethyl-3,4-dihydroquinoxalin-2(1H)-one (**192a**)

Yield: quant. (white/yellow solid). LC-MS (ESI): *m/z* calculated for C₁₀H₁₂N₂O [M+H]⁺: 176.09; found: 177.2. ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.05 (s, 1H), 6.60 (d, *J* = 7.8 Hz, 1H), 6.48 (s, 1H), 6.40 (d, *J* = 7.8 Hz, 1H), 5.88 (s, 1H), 3.72 (q, *J* = 6.8 Hz, 1H), 2.14 (s, 3H), 1.23 (d, *J* = 6.8 Hz, 3H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 168.18, 134.37, 131.42, 123.93, 118.32, 114.49, 113.99, 50.87, 20.68, 17.42.

3-ethyl-6-methyl-3,4-dihydroquinoxalin-2(1H)-one (**192b**)

Yield: 80% (white/yellow solid). LC-MS (ESI): *m/z* calculated for C₁₁H₁₄N₂O [M+H]⁺: 190.11; found: 191.2. ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.05 (s, 1H), 6.57 (d, *J* = 7.8 Hz, 1H), 6.51 (s, 1H), 6.36 (ddd, *J* = 7.8, 1.9, 0.9 Hz, 1H), 5.89 (s, 1H), 3.61 (ddd, *J* = 6.8, 5.1, 1.9 Hz, 1H), 2.13 (s, 3H), 1.65 – 1.54 (m, 2H), 0.91 (t, *J* = 7.4 Hz, 3H). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 167.89, 134.50, 131.95, 123.90, 118.39, 114.84, 114.33, 56.97, 25.20, 21.18, 10.01.

2.24. General procedure for the synthesis of compounds 193a-c

To a solution of MeOH, 2 M NaOH and 3% H₂O₂ (2:1:1, 40 mL) previously prepared, **192a,b** or crude **192c** (7.5 mmol) were added. The mixture was stirred at 100°C under reflux for 1.5 h to obtain the oxidized form. Upon completion, monitored by LC-MS, the solution was cooled to 0°C, and acetic acid was added until precipitation. The precipitate solid was vacuum filtered, yielding the oxidized product.

3,6-dimethylquinoxalin-2(1H)-one (**193a**)

Yield: quant. (white/yellow solid). LC-MS (ESI): *m/z* calculated for C₁₀H₁₀N₂O [M+H]⁺: 174.08; found: 175.2. ¹H NMR (600 MHz, DMSO-*d*₆) δ 12.51 (s, 1H), 7.48 (s, 1H), 7.27 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.20 (d, *J* = 8.3 Hz, 1H), 2.38 (s, 3H), 2.36 (s, 3H). ¹³C NMR (151 MHz,

DMSO- d_6) δ 159.07, 155.16, 140.93, 139.15, 132.59, 131.14, 129.12, 128.33, 128.10, 126.85, 121.70, 112.99, 20.54.

3-ethyl-6-methylquinoxalin-2(1H)-one (193b)

Yield: 91% (white/yellow solid). LC-MS (ESI): m/z calculated for $C_{11}H_{12}N_2O$ $[M+H]^+$: 188.09; found: 189.2. ^1H NMR (600 MHz, DMSO- d_6) δ 12.21 (s, 1H), 7.52 (s, 1H), 7.28 (dd, $J = 8.3$, 1.9 Hz, 1H), 7.18 (d, $J = 8.3$ Hz, 1H), 2.79 (d, $J = 7.4$ Hz, 2H), 2.36 (s, 3H), 1.20 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 162.34, 154.60, 132.11, 131.60, 130.33, 129.61, 127.72, 115.03, 25.96, 20.36, 10.49.

3-methyl-7-phenylquinoxalin-2(1H)-one (193c)

Yield: 80% (yellow solid). LC-MS (ESI): m/z calculated for $C_{15}H_{12}N_2O$ $[M+H]^+$: 236.09; found: 237.1. ^1H NMR (600 MHz, DMSO- d_6) δ 12.45 (s, 1H), 7.76 (d, $J = 8.3$ Hz, 1H), 7.68 – 7.66 (m, 2H), 7.55 (dd, $J = 8.3$, 2.0 Hz, 1H), 7.52 – 7.49 (m, 3H), 7.44 – 7.41 (m, 1H), 2.42 (s, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 159.07, 155.16, 140.93, 139.15, 132.59, 131.14, 129.12, 128.33, 128.10, 126.85, 121.70, 112.99, 20.54.

2.25. General procedure for the synthesis of compounds 194a-c

Intermediates **193a-c** (2.5 mmol) are chlorinated by $POCl_3$ (4 mL) in a solvent-free reaction heated at 100°C under argon atmosphere. After cooling, the reaction mixture is slowly poured into ice-cooled water and extracted with DCM. The organic layer was washed with 1M NaOH, dried over Na_2SO_4 and concentrated under reduced pressure.

2-chloro-3,6-dimethylquinoxaline (194a)

Yield: 86% (brown solid). LC-MS (ESI): m/z calculated for $C_{10}H_9ClN_2$ $[M+H]^+$: 192.05; found: 193.1. ^1H NMR (600 MHz, DMSO- d_6) δ 7.80 (d, $J = 8.5$ Hz, 1H), 7.74 (s, 1H), 7.59 (dd, $J = 8.5$, 1.9 Hz, 1H), 2.66 (s, 3H), 2.46 (s, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 153.02, 146.84, 141.27, 140.87, 139.10, 132.87, 127.75, 127.45, 23.39, 21.73.

2-chloro-3-ethyl-6-methylquinoxaline (194b)

Yield: 98% (brown solid). LC-MS (ESI): m/z calculated for $C_{11}H_{11}ClN_2$ $[M+H]^+$: 206.6; found: 207.1. ^1H NMR (600 MHz, DMSO- d_6) δ 7.88 (d, $J = 8.5$ Hz, 1H), 7.86 (s, 1H), 7.67 (dd, $J = 8.5$, 1.9 Hz, 1H), 3.07 (q, $J = 7.4$ Hz, 2H), 2.55 (s, 3H), 1.34 (t, $J = 7.4$ Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 155.93, 146.12, 140.78, 140.38, 138.50, 132.39, 127.24, 127.18, 8.27, 21.20, 11.16.

2-chloro-3-methyl-7-phenylquinoxaline (194c)

Yield: 70% (brown solid). LC-MS (ESI): m/z calculated for $C_{15}H_{11}ClN_2$ $[M+H]^+$: 254.06; found: 255.1. ^1H NMR (600 MHz, DMSO- d_6) δ 8.25 (d, J = 2.0 Hz, 1H), 8.19 (dd, J = 8.7, 2.0 Hz, 1H), 8.11 (d, J = 8.7 Hz, 1H), 7.89-7.86 (m, 2H), 7.56-7.53 (m, 2H), 7.48-7.44 (m, 1H), 2.77 (s, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 152.63, 147.78, 141.77, 140.57, 139.66, 138.33, 129.54, 129.18, 128.62, 128.46, 127.28, 124.67, 22.91.

2.26. General procedure for the synthesis of compounds 184–186

Intermediates **194a-c** (0.6 mmol) were added to a solution of K_2CO_3 (3 mmol) and guanidine HCl (1.8 mmol) in NMP, previously prepared, and stirred overnight at 140°C. After completion monitored by LCMS, the solution was diluted with EtOAc and washed with water to remove the solvent. The organic layer was dried with Na_2SO_4 and evaporated *in vacuo*. Compounds **184** and **185** were purified by CombiFlash with a gradient 0-100B (ACN/ H_2O 90:10) over 15 column volumes (CV) to afford the desired compounds as free bases. Final compound **186** was purified by preparative HPLC in 0-100B (ACN/ H_2O 90:10, 0.1 % TFA) for 20 minutes to give a 95.8% pure product (analytical HPLC, λ = 210 nm).

1-(3,6-dimethylquinoxalin-2-yl)guanidine (184)

Yield: 37% (yellow solid). LC-MS (ESI): m/z calculated for $C_{11}H_{13}ClN_5$ $[M+H]^+$: 215.12; found: 216.2. ^1H NMR (600 MHz, DMSO- d_6) δ 7.48 (s, 1H), 7.47 (d, J = 8.4 Hz, 1H), 7.30 (d, J = 8.4 Hz, 1H), 7.20 (s, 4H), 2.52 (s, 3H), 2.42 (s, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 159.43, 156.23, 154.35, 137.89, 136.03, 133.24, 129.56, 126.62, 124.97, 22.39, 20.85.

1-(3-ethyl-6-methylquinoxalin-2-yl)guanidine (185)

Yield: 53% (orange solid). LC-MS (ESI): m/z calculated for $C_{12}H_{15}ClN_5$ $[M+H]^+$: 229.13; found: 230.2. ^1H NMR (600 MHz, DMSO- d_6) δ 7.51 (s, 1H), 7.47 (d, J = 8.5 Hz, 1H), 7.31 (s, 4H), 7.30 (dd, J = 8.5, 1.8 Hz, 1H), 2.97 (q, J = 7.4 Hz, 2H), 2.42 (s, 3H), 1.24 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 159.40, 157.89, 155.68, 137.65, 136.08, 133.27, 129.56, 126.88, 124.95, 27.20, 20.82, 11.82.

1-(3-methyl-7-phenylquinoxalin-2-yl)guanidine (186)

Yield: 58% (white solid). LC-MS (ESI): m/z calculated for $C_{16}H_{15}ClN_5$ $[M+H]^+$: 277.13; found: 278.3. ^1H NMR (600 MHz, DMSO- d_6) δ 9.90 (s, 1H, TFA), 8.92 (s, 3H), 8.44 (d, J = 2.0 Hz, 1H), 8.06 (dd, J = 8.6, 2.1 Hz, 1H), 8.03 (d, J = 8.6 Hz, 1H), 7.88-7.85 (m, 2H), 7.55 (t, J = 7.7 Hz, 2H), 7.48-7.44 (m, 1H), 2.74 (s, 3H). ^{13}C NMR (151 MHz, DMSO- d_6) δ 158.80 (q, J = 35.9

Hz, TFA, CO), 156.05, 147.31, 146.55, 141.82, 139.20, 138.05, 137.94, 129.64, 128.83, 128.76, 127.75, 127.57, 124.81, 116.28 (q, $J = 310.3$ Hz, TFA, CF₃), 21.88.

2.27. General procedure for the synthesis of compounds **187**, **188**.

To a solution of **184** or **185** (0.46 mmol) in dry DMF, 4-nitrophenyl (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) carbonate (0.70 mmol) is added under argon. The reaction mixture is stirred at room temperature for 3 h. Upon completion, the reaction mixture is diluted with heptane (10 ml) to azeotrope DMF. The combined solvents are removed under reduced pressure after several washes. The crude is directly purified by CombiFlash using a gradient elution (EtOAc/Heptane, from 0% to 50%) to obtain **187** and **188** as white solids.

4-[(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl]methyl[(3,6-dimethylquinoxalin-2-yl)carbamimidoyl]carbamate (**187**)

Yield: 66% (white solid). LC-MS (ESI): m/z calculated for C₂₅H₃₀BN₅O₄ [M+H]⁺: 475.24 found: 476.3. ¹H NMR (600 MHz, CDCl₃) δ 10.39 (s, 1H), 7.82 (d, $J = 7.6$ Hz, 2H), 7.70 (s, 1H), 7.62 (d, $J = 8.4$ Hz, 1H), 7.46 (d, $J = 8.4$ Hz, 1H), 7.42 (d, $J = 7.6$ Hz, 2H), 5.22 (s, 2H), 2.73 (s, 3H), 2.54 (s, 3H), 1.35 (s, 12H). ¹³C NMR (151 MHz, CDCl₃) δ 139.30, 138.53, 137.81, 136.75, 135.15, 131.63, 127.63, 127.19, 126.33, 126.22, 121.92, 83.99, 67.19, 25.02, 21.70.

4-[(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl]methyl [(3-ethyl-6-methylquinoxalin-2-yl)carbamimidoyl]carbamate (**188**)

Yield: 73% (white solid). LC-MS (ESI): m/z calculated for C₂₅H₃₀BN₅O₄ [M+H]⁺: 489.25 found: 490.3. ¹H NMR (600 MHz, CDCl₃) δ 10.43 (s, 3H), 7.82 (d, $J = 7.6$ Hz, 2H), 7.75 (s, 1H), 7.63 (d, $J = 8.5$ Hz, 1H), 7.46 (d, $J = 8.5$ Hz, 1H), 7.43 (d, $J = 7.6$ Hz, 2H), 5.23 (s, 2H), 3.04 (d, $J = 7.8$ Hz, 2H), 2.54 (s, 3H), 1.42 (t, $J = 7.8$ Hz, 3H), 1.35 (s, 12H). ¹³C NMR (151 MHz, CDCl₃) δ 139.40, 138.63, 137.72, 136.49, 135.14, 131.56, 127.87, 127.77, 127.20, 126.18, 125.45, 121.92, 83.98, 67.15, 27.44, 25.02, 21.66, 11.52.

2.28. Synthesis of 4-nitrophenyl (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) carbonate (**196**)

To a solution of 4-(hydroxymethyl)phenylboronic acid pinacol ester (4.06 mmol, 950 mg) in dry THF, triethylamine (8.12 mmol, 821 mg) and 4-nitrophenyl chloroformate (9.4 mmol, 1.89 g) were added. The reaction mixture is stirred at room temperature for 24h. The solvent is removed under reduced pressure and the obtained solid is suspended in DCM and washed with 1 M HCl, saturated sodium bicarbonate, and brine. The organic layer is dried over Na₂SO₄ and

concentrated under reduced pressure. The obtained crude is purified by CombiFlash using gradient elution (EtOAc/Heptane, from 0% to 5%). Yield: 86% (white solid). LC-MS (ESI): m/z calculated for $C_{20}H_{22}BNO_7$ $[M+H]^+$: 399.15 found: -. ^1H NMR (600 MHz, $CDCl_3$) δ 8.27 (d, $J = 9.2$ Hz, 2H), 7.85 (d, $J = 7.9$ Hz, 2H), 7.44 (d, $J = 7.9$ Hz, 2H), 7.38 (d, $J = 9.2$ Hz, 2H), 5.31 (s, 2H), 1.35 (s, 12H). δ 155.67, 152.57, 145.58, 137.20, 135.35, 127.77, 125.45, 121.92, 84.14, 77.16, 76.95, 70.96, 25.02, 1.16.

List of abbreviations

3-AMOHP	3-(aminomethyl)-1-oxo-1-hydroxy-phospholane
3-GOHP	3-(guanidino)-1-oxo-1-hydroxy-phospholane
4-PHP	4-(4-piperidyl)-1-hydroxypyrazole
4-PIOL	5-(4-piperidyl)-3-hydroxyisoxazole
5-FU	5-fluorouracil
ACLY	ATP citrate lyase
ACN	acetonitrile
ADPR	ADP-ribose
AIBN	azo(bis)isobutyronitrile
AKT	protein kinase B
AKR1C1	aldo-keto reductase family 1 member C1
AMPK	AMP-activated protein kinase
APTES	(3-aminopropyl)triethoxysilane
AR	androgen receptor
ARE	antioxidant response element
AS	astrocyte
ASS	argininosuccinate-synthetase
ASIC3	acid-sensing ion channel 3
ASM	airway smooth muscle
aza-4-PIOL	5-(piperidin-4-yl)-1 <i>H</i> -pyrazol-3-ol
BBB	blood brain barrier
BC	breast cancer
BCa	bladder cancer
BMC	bicuculline methochloride
BARD1	BRCA1-associated RING domain protein 1
BRCA1	breast cancer type-1 susceptibility protein
BZD	benzodiazepine
CAT	catalase

CC	cervical cancer
CDK	cyclin-dependent kinase
CML	chronic myeloid leukaemia
CNS	central nervous system
CPP	cell-penetrating peptides
CRC	colorectal cancer
CRM1	chromosomal maintenance 1
CRPC	castration-resistant prostate cancer
Csh	Csk-homologous kinase
Csk	C-terminal Src kinase
CUR	curcumin
CV	column volumes
DCM	dichloromethane
DDS	drug delivery systems
DIPEA	<i>N,N</i> -diisopropylethylamine
DMCM	methyl 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate
DMF	dimethylformamide
DSPE	distearoylphosphoethanolamine
DXR	doxorubicin
E2F1	E2F1 transcription factor 1
EC	endometrial cancer
EGFR	epidermal growth factor receptor
ELT	encoded library technology
EMT	epithelial-mesenchymal transition
FAK	focal adhesion kinase
FC	flash column chromatography
FDA	Food and Drugs Administration
FEP	free energy perturbation
FGF	fibroblast growth factor
FoxO	forkhead box O

HGF	hepatocyte growth factor
GABA	γ -aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase
GAT	GABA-transporter
GB	glioblastoma
GC	gastric cancer
GDH	glycerol-3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GHB	γ -hydroxybutyrate
GIRK	G-protein–gated inwardly rectifying K ⁺
GLS	glutaminase
Glu	glutamate
GLUT	glucose transporter
GMQ	2-guanidine-4-methylquinazoline
GPCR	G-protein-coupled receptors
GQX	guanidinium–quinoxaline
GSH	glutathione
HCC	hepatocellular carcinoma
HER2	human epidermal growth factor receptor 2
HIF	hypoxia-inducible factor
HNT	halloysite nanotubes
IDH	isocitrate dehydrogenase
IGF-1R	insulin-like growth factor 1
IL	interleukin
iNOS	inducible nitric oxide synthase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KCC	potassium-chloride cotransporter
KDR	kinase insert domain receptor

KGN	kartogenin
LC	lung cancer
LC-3	microtubule-associated proteins 1A/1B light chain 3B
LCAD	acyl-CoA dehydrogenase
LC-MS	liquid chromatography-mass spectrometry
LGIC	ligand gating chloride channels
LKB1	liver kinase B1
lncRNA	long non-coding RNA
LPS	lipopolysaccharide
MAPK/ERK	mitogen-activated protein kinase/extracellular signal-regulated kinase
MB	medulloblastoma
<i>m</i> CPBA	<i>m</i> -chloroperbenzoic acid
MCPIP1	monocyte chemoattractant protein-induced protein 1
MDCK-MDR1	Madin-Darby canine kidney multidrug resistance 1
MEK	mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
MnSOD	manganese superoxide dismutase
NAD	nicotinamide adenine dinucleotide
NAM	negative allosteric modulator
NB	neuroblastoma
ncRNA	non coding RNA
NES	nuclear export signal
NF	nuclear factor
NKCC	sodium-potassium-chloride cotransporter
NLRP	nucleotide-binding oligomerization domain-like receptors containing pyrin
NMP	<i>N</i> -methylpyrrolidone
Nrf2	nuclear factor erythroid 2
NRTK	non-receptor tyrosine kinase
NSCLC	non-small-cell lung cancer
NSL	nuclear localization signal

OC	ovarian cancer
OSCC	oral squamous cell carcinoma
PAC	pulmonary adenocarcinoma
PAM	positive allosteric modulator
PAP	phenylaminopyrimidine
PPAP	pyridylpyrimidinylaminophenyl
PC	pancreatic cancer
PDGEFR	platelet-derived growth factor receptor
PDH	pyruvate dehydrogenase
PDI	polydispersion index
PD-L1	programmed death-ligand 1
PDX1	pancreatic and duodenal homeobox 1
PEPCK	phosphoenolpyruvate carboxykinase
PGAM1	phosphoglycerate mutase 1
PGC-1 α	receptor gamma coactivator-1 alpha
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PLA	polylactic acid
PPII	polyproline type II
PROTAC	proteolysis targeting chimera
PTX	paclitaxel
PSA	prostate-specific antigen
<i>p</i> -QM	<i>para</i> -benzoquinone methide
PTP1B	protein tyrosine phosphatase 1B
PTM	post-translational modification
PTP1B	protein tyrosine phosphatase 1B
Rb	retinoblastoma
RhoGAP	RHO GTPase-activating protein
ROS	reactive oxygen species
RTK	receptor tyrosine kinase

SAR	structure activity relationships
SCC	squamous-cell carcinoma
SFK	Src family of protein tyrosine kinases
SH	Src homology
Shc	Src homology 2 domain-containing
SHMT2	serine hydroxymethyltransferase 2
Shp	Src homology region 2 domain-containing phosphatase-1
SIRT	sirtuin
SMKI	small molecule kinase inhibitor
S _N	nucleophilic substitution
S _N Ar	nucleophilic aromatic substitution
SNARE	<i>N</i> -ethylmaleimide-sensitive factor attachment protein receptor
SREBP1	sterol regulatory element-binding protein 1
STAT3	signal transducer and activator of transcription 3
TEA	triethylamine
TCA	tricarboxylic acids
TFEB	transcription factor EB
TGF	transforming growth factor
THF	tetrahydrofuran
thio-4-PIOL	5-(piperidin-4-yl)isothiazol-3-ol
THIP	tetrahydroisoxazol[5,4- <i>c</i>]pyridin-3-ol
TK	tyrosine kinase
TLC	thin layer chromatography
TM	transmembrane
TME	tumour microenvironment
TNBT	triple-negative breast cancer
TNF	tumour necrosis factor
TPMPA	(1,2,5,6-tetrahydropyridin-4-yl)methylphosphinic acid
TRANCE	TNF-related activation-induced cytokine
TSPO	translocator protein

UV	ultraviolet
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VGAT	vesicular GABA Transporter
VIAAT	vesicular Inhibitory Amino Acid Transporter
VSM	vascular smooth muscle
WT	wild-type
ZEB1	zinc finger E-Box binding homeobox 1

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