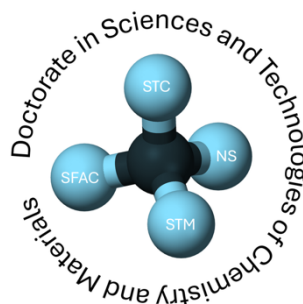
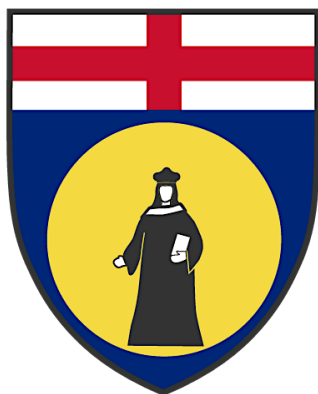




**DOCTORATE SCHOOL IN SCIENCES AND TECHNOLOGIES OF
CHEMISTRY AND MATERIALS**

Curriculum in pharmaceutical, food and cosmetic sciences
XXXVIII cycle

**Synthesis and preformulation studies of
natural-inspired antioxidant hybrids
with neuroprotective or antiproliferative activity**

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Abstract

This PhD thesis focuses on the design and synthesis of novel hybrid compounds inspired by natural antioxidants with potential antiproliferative or neuroprotective activities. The research is based on a fragment-based molecular hybridization strategy that combines pharmacophoric motifs relevant in medicinal chemistry, in which at least one fragment is a natural antioxidant product (e.g., coumarin, vanillin, isovanillin, cinnamic acids or cytosine) or one of their synthetic derivatives. This approach aims to generate compounds with polypharmacological profiles capable of modulating cancer-related pathways as well as biological targets involved in the neurodegenerative processes associated with Alzheimer's disease (AD).

A total of 150 final compounds were synthesized, including 64 derivatives (compounds 1–64) investigated for antiproliferative activity and 86 compounds (65–150) designed as potential anti-AD agents. The chemical radical scavenging activity of each compound was assessed by DPPH assay, revealing structural features-dependent variations.

In the anticancer section, two main series were developed: vanillin/isovanillin-based hydrazone hybrids and 3-cinnamoyl coumarins. Several hydrazone derivatives showed strong growth-inhibitory effects in a neuroblastoma cellular model, including in chemotherapy-resistant cells. One compound in particular reduced tumor cell proliferation while sparing non-malignant cells; mechanistic studies indicated that its activity was associated with increased ROS production, induction of cellular senescence, and reduced clonogenic potential. Moreover, micellar encapsulation using non-ionic surfactants improved the aqueous solubility of one of the most active but poorly soluble candidates. The 3-cinnamoyl coumarin series also displayed a favorable antiproliferative profile against breast and lung cancer cell lines, with several derivatives showing activity in the low micromolar range.

The anti-AD section explored a library of sixty coumarin-based multitarget ligands incorporating alicyclic amines. Several derivatives showed potent cholinesterase inhibition, with submicromolar activity and tunable selectivity depending on linker length and amine fragment. All compounds of this series

were also evaluated for their ability to inhibit A β ₄₂ self-aggregation during a six-month research internship at the University of Cambridge (UK). Additional scaffold combinations (phenothiazine–cinnamic acids, cytosine–cinnamic acids, and coumarin–indole hybrids) were investigated to expand the chemical diversity of the anti-AD library. Among these, the coumarin–alicyclic amine and phenothiazine–cinnamic acid series yielded compounds with promising polypharmacological profiles, selected for further evaluation of their neuroprotective potential in cellular models.

Overall, these results support molecular hybridization of natural-inspired scaffolds as an effective strategy to obtain bioactive compounds and provide useful structure–activity relationship insights for the future optimization of multitarget agents for cancer and AD.

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PhD project overview

The overarching aim of my PhD project was to design, synthesize, and conduct preformulation studies on new series of compounds with potential polypharmacological activity, focusing on two major therapeutic areas: neurodegenerative diseases, particularly Alzheimer's disease (AD), and cancer. Both AD and cancer are chronic multifactorial disorders involving numerous interconnected biochemical pathways. This biological complexity challenges the identification of a single dominant molecular target and complicates the development of effective therapeutic strategies.

In AD, despite extensive research over several decades, no curative or disease-modifying therapy is currently available. FDA-approved treatments, primarily cholinesterase inhibitors and NMDA receptor antagonists, provide only transient symptomatic improvement and do not prevent the progressive neurodegeneration. A similar limitation is observed across many neoplastic conditions, where treatment still largely relies on surgery and classical chemotherapeutic agents, with outcomes often compromised by toxicity, relapse, or resistance.

These considerations highlight a fundamental weakness of the traditional "one drug-one target" paradigm. In complex and heterogeneous disorders, interventions directed toward a single pathway rarely produce durable clinical benefits. Consequently, growing attention has turned toward polypharmacology, which aims to modulate multiple disease-relevant mechanisms simultaneously. Clinically, this concept is reflected in combination therapies, whereas in medicinal chemistry it has inspired the design of single molecules capable of interacting with more than one biological target. However, creating efficient multitarget ligands remains scientifically challenging, as it requires assembling pharmacophoric fragments that retain appropriate affinity, efficacy, and selectivity while maintaining favorable ADMET properties.

Within this framework, my research employed a fragment-based hybridization strategy, combining naturally inspired antioxidant scaffolds into new molecular architectures. The structural fragments selected coumarin, vanillin, isovanillin,

cinnamic acids and indole, share several advantageous features are present in many compounds of natural origin, exhibit intrinsic antioxidant properties, synthetically versatility, widely used as pharmacophores in drug-design, and possess documented biological activities.

The focus on antioxidant scaffolds stems from the well-established role of oxidative stress in the onset and progression of both AD and cancer. The imbalance in the cellular levels of reactive oxygen species (ROS) induce neuronal dysfunction and death in AD and contribute to genomic instability, tumor proliferation, and therapy resistance in cancer. Therefore, incorporating antioxidant moieties into hybrid molecules represents a rational strategy to modulate redox homeostasis while simultaneously targeting additional biological pathways.

Over the course of the project, I designed and synthesized hybrid compounds intended to exert either neuroprotective effects in AD or antiproliferative effects in cancer. Their antioxidant properties were evaluated through radical-scavenging assay (DPPH assay), and broader biological studies were carried out through dedicated collaborations. This work also included a six-month research period at the Centre for Misfolding Diseases (University of Cambridge), where I performed advanced assays to investigate the potential anti-amyloid activity of selected compounds from the neurodegeneration-focused series. Moreover, preformulation studies were conducted to address solubility constraints that could affect the drug-likeness and pharmacokinetic profiles of the most promising anticancer molecules.

Overall, the integration of fragment-based design, molecular hybridization strategies and the use of antioxidant scaffolds enabled the exploration of new chemical spaces and supported the development of compounds with polypharmacological potential which, with further optimization, may contribute to therapeutic strategies better suited to the multifactorial nature of AD and cancer.

Antiproliferative activity

Introduction

Cancer and anticancer medicinal chemistry

Cancer remains one of the leading causes of morbidity and mortality worldwide and represents a major public-health and socioeconomic burden. Global estimates for 2020 indicated ~19.3 million new cancer cases and ~10.0 million cancer deaths ^[1], underscoring both the scale of the disease and the urgent need for improved prevention, diagnosis, and treatment strategies.

At the biological level, cancer is a heterogeneous family of diseases driven by the progressive accumulation of genetic and epigenetic alterations, together with profound rewiring of cellular signaling and tissue homeostasis. A useful conceptual framework to describe malignant progression is the “hallmarks of cancer,” which organize the complex traits acquired during tumor evolution into a set of functional capabilities ^[2]. This conceptual model of tumor heterogeneity assumes that the transition from normal to neoplastic cells occurs through a gradual accumulation of functional capabilities. Within this view, human tumor development is understood as a multistep process in which emerging cancer cells progressively acquire the biological traits required to initiate tumor formation and, eventually, to sustain malignant growth. In detail, capabilities acquired by tumor cells include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, later expanded to incorporate additional enabling features such as genome instability and tumor-promoting inflammation, as well as emerging hallmarks related to deregulated cellular energetics and immune evasion ^[3].

These premises highlight that, from a therapeutic perspective, and consequently from a drug discovery standpoint, tumors are difficult to control with a single therapeutic intervention. Despite major advances in molecular oncology, the treatment of the most common cancers still relies on a limited number of well-established therapeutic modalities. Surgery, radiotherapy, and chemotherapy remain the backbone of cancer treatment for solid tumors such as breast, lung, colorectal, and prostate cancers, particularly in early or localized disease stages ^[4]. Cytotoxic chemotherapy, based on non-specific agents such as platinum compounds, antimetabolites, taxanes, and anthracyclines, continues to play a central role in both curative and palliative settings, either as monotherapy or in

combination regimens. In parallel, hormone therapy represents a cornerstone for hormone-dependent malignancies, including estrogen receptor–positive breast cancer where endocrine manipulation significantly improves disease control and survival [5].

Over the past two decades, targeted therapies, such as tyrosine kinase inhibitors and monoclonal antibodies directed against specific oncogenic targets (e.g., EGFR, HER2, BRAF), have become standard of care in selected patient populations, improving clinical outcomes while reducing nonspecific toxicity compared to conventional chemotherapy [6]. More recently, cancer immunotherapy, particularly immune checkpoint inhibitors targeting PD-1/PD-L1 and CTLA-4, has transformed the treatment landscape of several highly prevalent cancers, including melanoma, non-small cell lung cancer, and renal cell carcinoma, although primary and acquired resistance remain significantly limiting clinical challenges [7].

Indeed, therapeutic resistance, whether intrinsic or acquired, remains a dominant limitation of anticancer treatment. Resistance can arise through diverse mechanisms, including target mutation, pathway reactivation, bypass signaling, altered drug transport, enhanced DNA damage repair, and adaptive phenotypic switching, frequently in the context of substantial intratumoral heterogeneity [3,8]. Moreover, the selective pressure imposed by therapy often promotes outgrowth of resistant clones. These features complicate durable tumor control and help explain why initial responses to targeted therapies are often followed by relapses, particularly in advanced solid tumors. As a result, modern oncology increasingly relies on rational combination regimens and treatment sequencing strategies designed to suppress compensatory pathways and delay resistance [3,8].

In this context, redox biology is particularly relevant and particularly considered in the rationale of the work presented in this thesis.

The role of reactive oxygen species (ROS) in cancer biology, together with the availability of molecular scaffolds endowed with intrinsic redox-modulating properties, supports the development of alternative therapeutic strategies, particularly in light of the limited long-term efficacy and frequent emergence of resistance associated with conventional chemotherapeutic agents.

Importantly, ROS play a paradoxical role in cancer biology, as moderate increases in ROS levels promote tumor initiation, proliferation, and survival signaling, whereas excessive oxidative stress can overwhelm cellular defenses and trigger irreversible damage leading to cell death. Many tumors exhibit an altered redox environment characterized by elevated ROS production and adaptive increases in antioxidant defenses.

Experimental observations demonstrated that certain human tumor cells can constitutively generate substantial amounts of hydrogen peroxide (H_2O_2), suggesting a potential contribution of ROS to mutation, tissue injury, and malignant progression [9–18]. At the same time, this redox imbalance can be therapeutically exploitable: because cancer cells often operate near the upper limit of tolerable oxidative stress, further ROS insult or disruption of antioxidant defense mechanisms may selectively push malignant cells toward cell death, while sparing normal tissues with greater redox reserve [19]. These principles motivate medicinal-chemistry efforts aimed at designing compounds that can modulate oxidative stress either elevate ROS beyond a cytotoxic threshold or interfere with redox-adaptation pathways that support tumor survival.

This redox dysregulation and liability have been successfully exploited in clinical oncology, particularly through natural products and their derivatives. Indeed, a still highly productive strategy in anticancer drug discovery is the investigation of natural products and natural-product-inspired scaffolds, which have historically contributed in a significant and sustained manner to oncology therapeutics. Their persistent relevance in medicinal chemistry arises from their evolved structural complexity, intrinsic bioactivity, and ability to engage cancer-relevant molecular targets with high specificity.

For instance, anthracyclines such as doxorubicin, widely used in breast cancer therapy, induce cytotoxicity through a combination of DNA intercalation, topoisomerase II inhibition, and ROS generation [20]. Similarly, etoposide, a semisynthetic derivative of podophyllotoxin, is used in combination regimens for neuroblastoma treatment, where oxidative stress contributes to its antitumor activity [21].

Natural products targeting the microtubule cytoskeleton further exemplify this paradigm. Vinca alkaloids (e.g., vincristine) are essential agents in pediatric oncology, including neuroblastoma, while taxanes such as paclitaxel are

standard treatments for non-small cell lung cancer and breast cancer [22,23]. Beyond their antimitotic effects, these compounds have also been shown to influence ROS levels and mitochondrial function, thereby amplifying tumor cell death [24,25].

Collectively, these examples highlight how natural-product scaffolds have enabled the effective exploitation of cancer-specific vulnerabilities, including redox imbalance, and continue to provide a strong foundation for medicinal chemistry efforts aimed at developing novel redox-modulating agents with improved selectivity for malignant cells and reduced toxicity toward normal tissues.

Overview of the design of antiproliferative compounds

Given the premises earlier discussed, the work presented in this section was directed toward the design and biological evaluation of novel compounds with potential antiproliferative activity, obtained by incorporating structural fragments of natural origin into synthetically accessible molecular frameworks. In particular, the design strategy focused on the use of coumarin, vanillin and isovanillin, and cinnamic acid derivatives. These scaffolds have been widely reported to interfere with key cellular processes involved in tumor cell proliferation, including cell-cycle progression, redox homeostasis, and stress-response pathways. Moreover, their suitability for structural modification makes them attractive starting points for the generation of compound libraries aimed at exploring structure–activity relationships in antiproliferative assays.

In this context, the combination of phenolic and aromatic fragments within a single molecular framework was pursued as a strategy to obtain hybrid compounds capable of exerting multiple biological activities, potentially enhancing growth inhibition in tumor cells.

Accordingly, the synthesized compounds were evaluated for their antiproliferative effects against selected human cancer cell lines, chosen as representative models of highly prevalent malignancies. This experimental approach was intended to provide an initial assessment of the ability of the designed molecules, and therefore of the chosen scaffolds and their combination, to impair cancer cell proliferation, while laying the groundwork for subsequent functional and structure-based investigations.

The compounds developed in this section of thesis were conceived as synthetic derivatives inspired by natural scaffolds, rather than direct mimics of existing entire natural molecules, with the objective of combining favorable structural features through the fragment-based strategy design into new chemical entities suitable for biological evaluation. A detailed discussion of the individual scaffolds, their known biological activities, and the specific design rationale underlying their combination is provided in the following sections.

Coumarin- vanillin/ isovanillin hybrid hydrazides

Coumarin (*2H-chromen-2-one* or *1-benzopyran-2-one*) is a bicyclic heterocycle made of a benzene ring fused to an α -pyrone moiety, a structural arrangement that imparts planarity, aromatic stabilization, and extensive electron delocalization. These properties make coumarins highly attractive scaffolds in medicinal chemistry, where they are widely regarded as privileged structures capable of engaging a broad range of biological targets. Naturally occurring as plant secondary metabolites within the flavonoid family, coumarins and their derivatives, whether isolated from natural sources or synthetically modified, exhibit a wide spectrum of pharmacological activities, including anti-inflammatory, antioxidant, antimicrobial, antiviral, anticoagulant, and anticancer [18,13,15].

In oncology-oriented medicinal chemistry, coumarin derivatives have gained particular importance due to their ability to interact with key molecular targets involved in tumor growth and survival [26,27]. Various coumarins have been shown to modulate topoisomerases [12], kinases [28], histone deacetylase [11], tubulin polymerization [12], estrogen receptors [29], and enzymes regulating oxidative-stress homeostasis [30,31].

Their synthetic accessibility and the possibility of functionalization at different positions, especially C-3, C-4, C-7 and C-8, has also encouraged their incorporation into hybrid molecules designed to achieve multitarget activity, a strategy increasingly relevant for complex and multifactorial diseases such as cancer.

Complementing the coumarin scaffold, naturally occurring phenolic aldehydes, including vanillin (*4-hydroxy-3-methoxybenzaldehyde*) and isovanillin (*3-hydroxy-4-methoxybenzaldehyde*), represent another class of simple yet biologically versatile building blocks. These aromatic aldehydes have been extensively investigated for their anticancer and anti-inflammatory properties [32,33], as well as for their capacity to modulate reactive oxygen species (ROS) levels [34–37]. Moreover, the possibility of their chemical derivatization has enabled the design of numerous analogues with enhanced bioactivity [7,8].

A further structural motif relevant to the present work is the acyl-hydrazide/hydrazone moiety ($-\text{CO}-\text{NH}-\text{N}=\text{CH}-$), which serves as a versatile

linker in medicinal chemistry. Hydrazones are readily obtained through the condensation of hydrazides with carbonyl compounds ^[38], and they offer multiple sites for chemical modulation at either the carbon or nitrogen center. Their favorable physicochemical properties, together with the capacity of the hydrazone function to establish stabilizing hydrogen bonds or engage in metal coordination, contribute directly to their pharmacological profiles. Notably, several studies have emphasized the relevance of hydrazide and hydrazone groups at the C-3 position of the coumarin ring as key features of compounds exhibiting notable antitumor activity ^[39,40].

Moreover, as discussed in the previous sections, since many tumors exhibit profound alterations in redox homeostasis, often characterized by elevated ROS production ^[34–37], molecules capable of modulating oxidative stress (either as suppressors or enhancers) are of particular interest.

In this context and considering the aforementioned characteristics of the chemical fragments employed, the strategy was to design and synthesize a new series of antiproliferative agents through a molecular-hybridization approach, combining natural scaffolds endowed with intrinsic antioxidant activity (coumarin, vanillin and isovanillin) and joined with a hydrazide–hydrazone linkers. The overarching goal was to generate hybrid entities with dual function: antiproliferative effects, and the ability to influence oxidative-stress pathways implicated in tumor progression.

On these bases, 46 new compounds (Figure 1) consisting of the coumarin fragment (3-carboxycoumarin either unsubstituted or substituted at C-7 or C-8 with OH or OCH₃ group) linked to vanillin or isovanillin or their phenoxy or benzyloxy derivatives via an acylhydrazone linker were synthesized.

To assess the biological potential of these new compounds, the molecules were grouped into three sets (Set I-III, Figure 1), consistent with the chemical structure, each of which was subjected to different biological assays as detailed later in the Biological activity section.

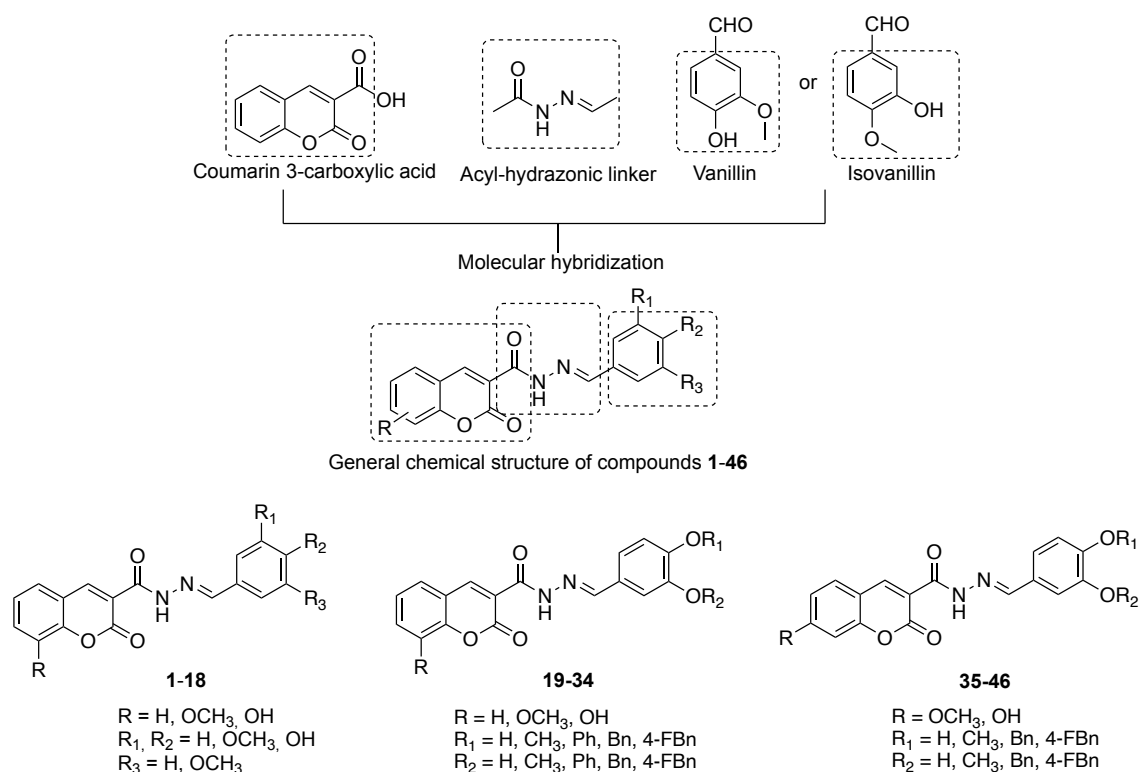


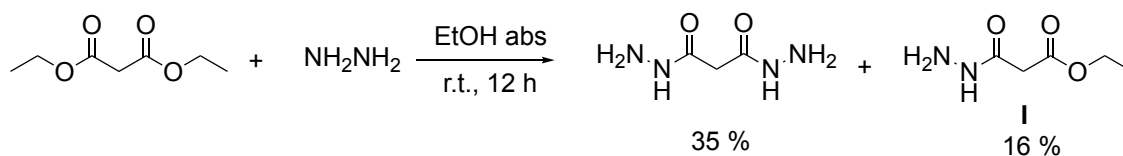
Figure 1. Design of compounds **1-46** and their general chemical structure.

Synthesis

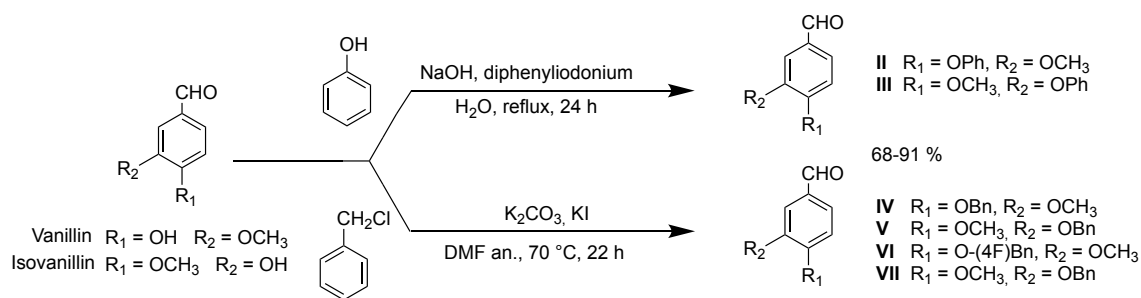
For the synthesis of the new compounds, it was first necessary to prepare intermediate **I** (hydrazonic fragment) and, when not commercially available, aldehydes **II-VII** (fragment derived from vanillin or isovanillin), which served as starting reagents for the synthesis of hydrazide intermediates. The synthetic procedures for obtaining intermediate **I** and aldehydes **II-VII** are reported in Scheme 1 and Scheme 2, respectively. In detail, the malonic ester was reacted with hydrazine monohydrate in ethanol, leading to the formation of both malonohydrazide and malonomonohydrazide, as described by Abdel-Aziz et al.^[41] and separated through fractional crystallization. Malonohydrazide was discarded, while malonomonohydrazide was subsequently reacted with the desired aldehyde **II-VII** or other commercially available aldehydes.

Phenoxy, benzyloxy, and 4-fluoro-benzyloxy aldehydes (**II-VII**) were prepared from vanillin or isovanillin following the procedures outlined in Scheme 2 and as previously described in literature^[42,43].

Scheme 1. Synthesis of intermediate **I**.



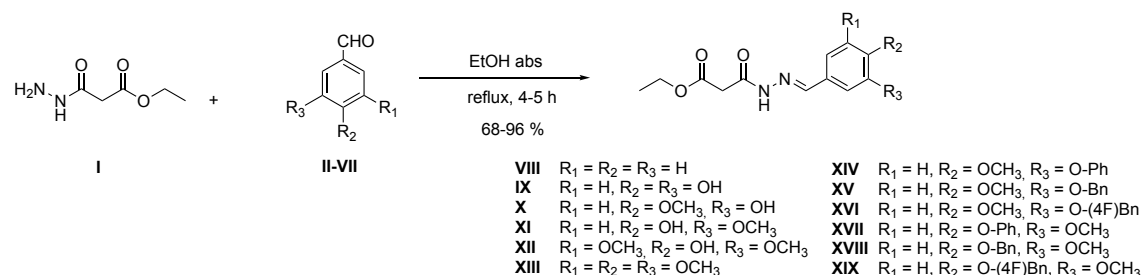
Scheme 2. Synthesis of aldehydes **II-VII**.



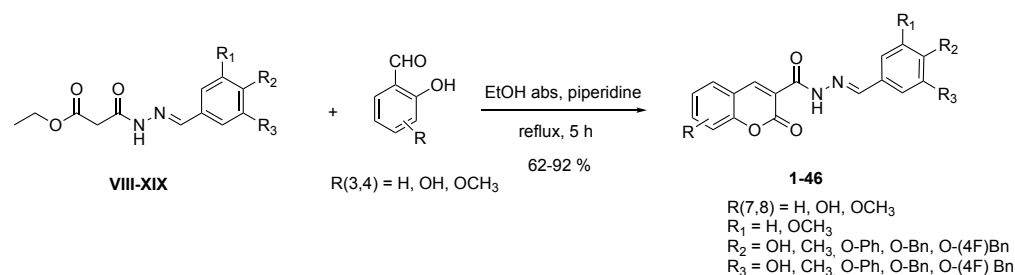
Intermediate **I** was then reacted with aldehydes **II-VII** in absolute ethanol in the presence of piperidine to obtain the linear acylhydrazones **VIII-XIX** (Scheme 3), which crystallized directly from the reaction mixture. The final compounds **1-46** were synthesized by reacting intermediates **VIII-XIX** with the appropriate 2-hydroxybenzaldehyde (Scheme 3) in ethanol with the addition of piperidine to afford the cyclization and creation of the coumarin scaffold and the desired R substituent. The final products crystallized directly from the reaction mixture and were obtained as yellow crystals in good yields (> 70 %). Compounds in which R = OH were also obtained as yellow crystals, but under modified conditions: a lower amount of piperidine was used, followed by acidic workup. This additional step justifies a reduction in the yields (30–50 %). The intermediates **VIII-XIX** and all final compounds **1-46** were characterized by TLC, melting point and NMR spectra (^1H and ^{13}C).

Scheme 3. General synthetic pathway for the synthesis of final compound **1-46**.

Synthesis of linear hydrazides VIII-XIX



Synthesis of final compounds 1-46



In the ^1H -NMR spectra of all target compounds **1-46** as well of all linear intermediates **VIII-XIX**, the N-H bond signal was described at a chemical shift that ranges approximatively from δ 12-11.50, characteristic of the *E* isomer. The absence of a signal above δ 14.00 demonstrates that the *Z* isomer is not formed. The N=CH signal was also described at a range of chemical shift of approximatively 8.7-8.2, confirming that the *E* isomer is the only single product formed by the reaction.

Moreover, it is interesting to underline the presence of duplicate signals caused by the possible geometric stereoisomerism in the N-acylhydrazone linker. For example, next to the signals of the prevalent rotamer (**27 ap**, Figure 2 panel A) for N-H bond (11.62 and 11.75), N=CH (8.43 and 8.22), aromatic H-4 of coumarin nucleus (8.89 and 8.77). The total of the integrals of each pair of signals corresponds to one hydrogen. This chemical behavior has already been studied by several authors and described in literature [44-46].

To confirm that compounds **1-46** exist as a mixture of rotamers at room temperature, sequential ^1H NMR spectra of compound **27**, a compound taken as an example, were obtained at different increasing temperatures (25, 35, 50, 60, and 70 °C). As Figure 2 panel A shows, the signals tend progressively approach and coalesce as the temperature increases, as expected in the presence of the equilibrium between rotamers thereby confirming the hypothesis of the presence

of a less stable syn-periplanar conformer (sp, minor rotamer) that is gradually converted into the thermodynamically favored anti-periplanar conformer (ap, major rotamer) as the temperature rises, consistently with what already described in the literature [44–46].

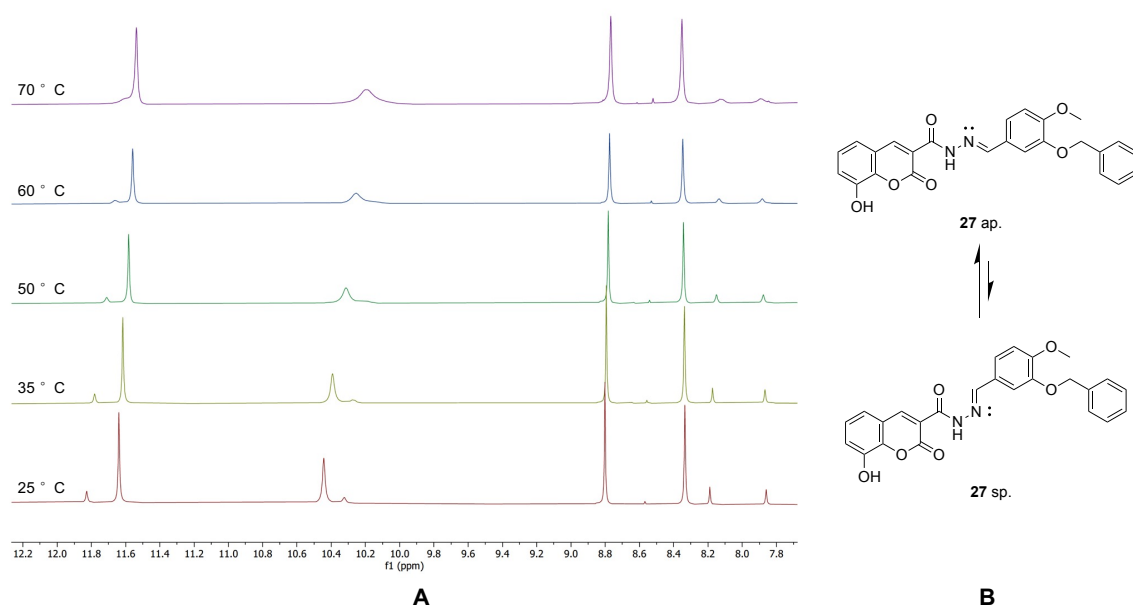
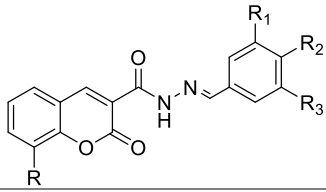


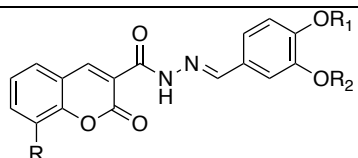
Figure 2. (A) ^1H NMR spectrum of (*E*)-*N'*-(3-(benzyloxy)-4-methoxybenzylidene)-8-hydroxy-2-oxo-2*H*-chromene-3-carbohydrazide (**27**) at different temperatures (400 MHz, DMSO- d_6); **(B)** chemical structures of its anti-periplanar (ap) and syn-periplanar (sp) conformers.

Given these observations and considering previous literature reports describing rotameric equilibria in solution for derivatives containing a hydrazone moiety, the same rationale was applied to the other hydrazone-based compounds discussed later in this thesis.

Details of the chemical structure of all target compounds (Series I-III) are listed in Table 1, 2, 3, respectively.

Table 1. Details of the chemical structure of compounds **1-18** (Set I).


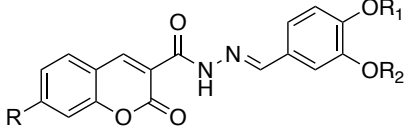
Compound	R	R ₁	R ₂	R ₃
1	H	H	H	H
2	H	OH	OH	H
3	H	OH	OCH ₃	H
4	H	OCH ₃	OH	H
5	H	OCH ₃	OH	OCH ₃
6	H	OCH ₃	OCH ₃	OCH ₃
7	OH	H	H	H
8	OH	OH	OH	H
9	OH	OH	OCH ₃	H
10	OH	OCH ₃	OH	H
11	OH	OCH ₃	OH	OCH ₃
12	OH	OCH ₃	OCH ₃	OCH ₃
13	OCH ₃	H	H	H
14	OCH ₃	OH	OH	H
15	OCH ₃	OH	OCH ₃	H
16	OCH ₃	OCH ₃	OH	H
17	OCH ₃	OCH ₃	OH	OCH ₃
18	OCH ₃	OCH ₃	OCH ₃	OCH ₃

Table 2. Details of the chemical structure of compounds **19-34** (Set II).


Compound	R	R ₁	R ₂
19	H	Ph	CH ₃
20	H	Bn	CH ₃
21	H	4F-Bn	CH ₃
22	H	CH ₃	Ph
23	H	CH ₃	Bn
24	H	CH ₃	4F-Bn
25	OH	Ph	CH ₃
26	OH	CH ₃	Ph
27	OH	CH ₃	Bn
28	OH	CH ₃	4F-Bn
29	OCH ₃	CH ₃	Ph
30	OCH ₃	CH ₃	Bn
31	OCH ₃	CH ₃	4F-Bn
32	OCH ₃	Ph	CH ₃

33	OCH ₃	Bn	CH ₃
34	OCH ₃	4-F-Bn	CH ₃

Table 3. Details of the chemical structure of compounds **35-46** (Set III).

			
Compound	R	R ₁	R ₂
35	OH	CH ₃	H
36	OH	CH ₃	Bn
37	OH	CH ₃	4F-Bn
38	OH	H	CH ₃
39	OH	Bn	CH ₃
40	OH	4F-Bn	CH ₃
41	OCH ₃	CH ₃	H
42	OCH ₃	CH ₃	Bn
43	OCH ₃	CH ₃	4F-Bn
44	OCH ₃	H	CH ₃
45	OCH ₃	Bn	CH ₃
46	OCH ₃	4F-Bn	CH ₃

Chemical radical scavenging activity

In order to evaluate the variation of the antioxidant activity depending on chemical modifications, all the compounds **1-46** were tested for their chemical radical scavenging activity throughout the colorimetric assay DPPH. The assay uses the stable organic radical *2,2-diphenyl-1-picrylhydrazide* (DPPH) which appears as a dark purple crystalline powder. When the reagent is dissolved in methanol, the solution shows a deep purple color with a pronounced absorption band at the wavelength of 515 nm when analyzed with a UV/Vis spectrophotometer. When the DPPH radical is neutralized, the solution takes on a pale-yellow coloration, hence a decrease in absorption intensity is recorded. This method is relatively quick and simple and it is therefore widely used for the determination of the antioxidant activity of various substances, particularly matrices of natural origin ^[47]. Details about the execution of the assay are reported in the experimental section.

The results showing antioxidant radical-scavenging activity above 10 % are reported in Table 4. For comparison purposes, vanillin and 3-coumarinic acid

were included as reference compounds, allowing the activity of the newly synthesized derivatives to be evaluated relatively to their parent scaffolds. Moreover, although the antioxidant activity does not exceed 10 %, three compounds from Set II which were found to be more active in the biological assays, are reported as representative examples to highlight the differences in activity among the three sets.

Table 4. Radical scavenging activity determined using DPPH assay.

Compound	A (517 nm)	DPPH %	AA %
Set I			
2	0.606	64.90 ± 1.05	35.10 ± 1.21
4	0.698	74.78 ± 1.85	25.22 ± 2.10
5	0.545	58.44 ± 2.40	41.56 ± 2.99
8	0.633	74.85 ± 1.21	25.15 ± 1.21
10	0.644	69.02 ± 0.55	30.98 ± 0.68
11	0.436	48.53 ± 0.28	51.48 ± 0.35
14	0.770	85.75 ± 0.80	14.25 ± 0.95
16	0.645	69.13 ± 1.22	30.87 ± 1.44
17	0.454	50.50 ± 1.10	50.99 ± 1.35
Set II			
23	0.812	96.01 ± 0.04	3.99 ± 0.04
31	0.810	96.04 ± 0.08	3.96 ± 0.08
34	0.807	95.39 ± 0.17	4.61 ± 0.17
Set III			
35	0.735	85.61 ± 0.52	14.39 ± 0.41
38	0.518	60.37 ± 0.44	39.39 ± 0.33
44	0.531	61.83 ± 0.58	38.69 ± 0.49
Vanillin	0.700	87.70 ± 0.95	12.30 ± 0.95
3-Cumarinic acid	0.725	89.40 ± 0.90	10.60 ± 0.90

Confirming the expected chemical structure-related trends, antioxidant activity remains moderate within Set I (**1-18**) where the presence of one or more free hydroxyl groups supports a measurable radical-scavenging response. In contrast, Set II and III (**19-46**), characterized by the absence (Set II, <10 %) or reduced (Set III) availability of such functionalities due to functionalization, shows a markedly diminished antioxidant profile. As a confirmation only compounds belonging to Series III which have unsubstituted R₁ and R₂ exhibit radical scavenging activity, as compounds belonging to set I. In particular, these results suggest that the presence of peripheral free hydroxyl groups at positions

R₁, R₂, or R₃ exerts a stronger influence on radical-scavenging activity than those embedded within the coumarin core (R), either at C-7 or C-8.

However, it is important to emphasize that this assay provides a qualitative rather than a quantitative measure of antioxidant activity. Moreover, the observed radical-scavenging capacity reflects the intrinsic chemical reactivity of the compounds and may not directly translate to their behavior in the cellular environment, where the redox state is tightly regulated by complex biochemical pathways.

Biological evaluation

The hydrazone hybrids derived from coumarin and vanillin or isovanillin were grouped into three sets (Figure 1) and subjected to different biological evaluations.

As detailed below, the compounds in Set I and two compounds of Set II were submitted to the National Cancer Institute (NCI, USA) for anticancer screening. The derivatives belonging to Set II and III were instead tested against neuroblastoma cell lines. Since Set III was designed as a further exploration of the SAR of Set II, whose members had already shown promising anti-neuroblastoma activity, the assays performed on the third series are intended as preliminary evaluations.

Set I

The compounds belonging to the first set (compounds **1-18**, Table 1) were submitted to the NCI for biological evaluation. However, unfortunately, the results are not yet available at the time of writing.

Set II and III

Two compounds of set II were submitted to NCI for a 60-cell-line screening panel (Developmental Therapeutics Project, DTP^[48]) at the concentration of 10 μ M and results are reported in Figure 3 and Figure 4, respectively.

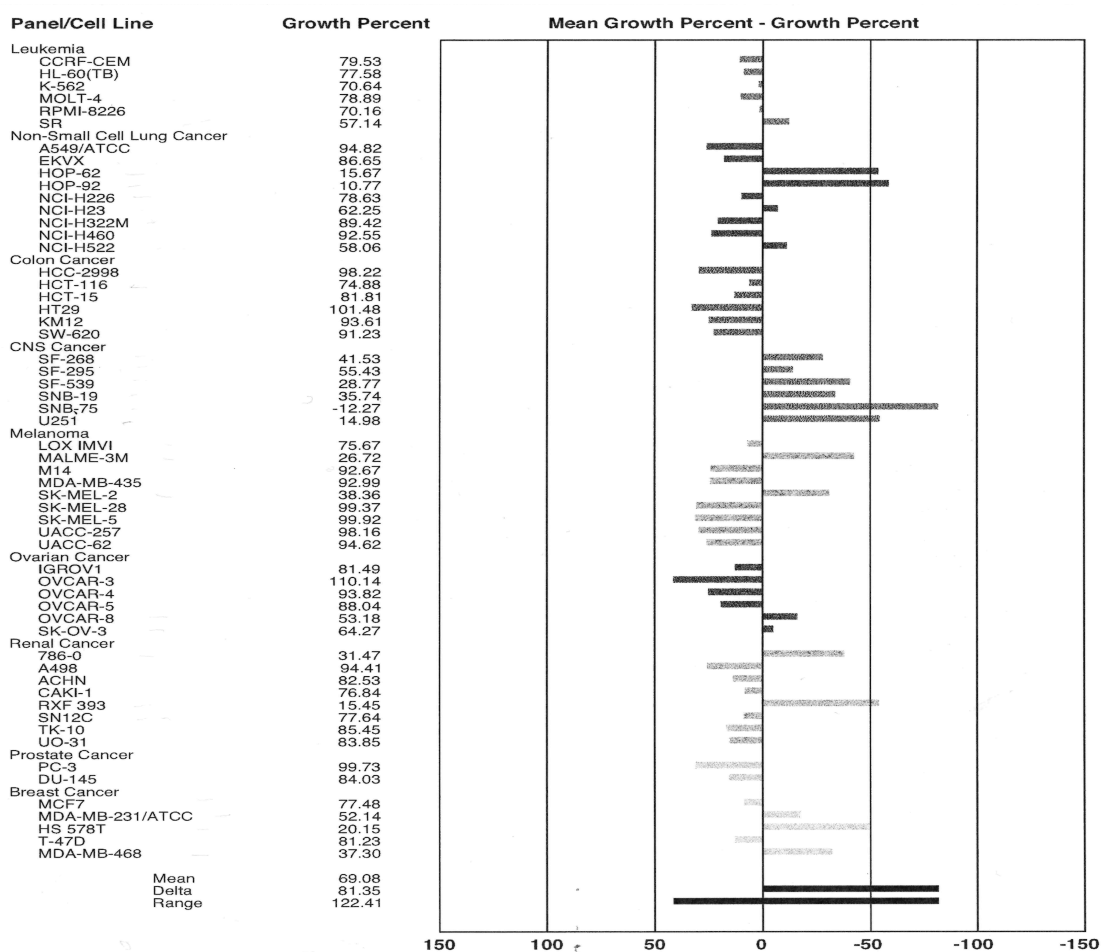


Figure 3. Results from NCI of the 60-cell-line screening panel of compound **33** at the concentration of 10 μ M.

Results are reported as growth percent values. Values lower than 100 indicate growth inhibition compared with untreated controls, while negative values reflect cytotoxicity. Values below 50, which correspond to growth inhibition and cytotoxicity, are reported on the right side of the graph while values above 50 are reported on the left side.

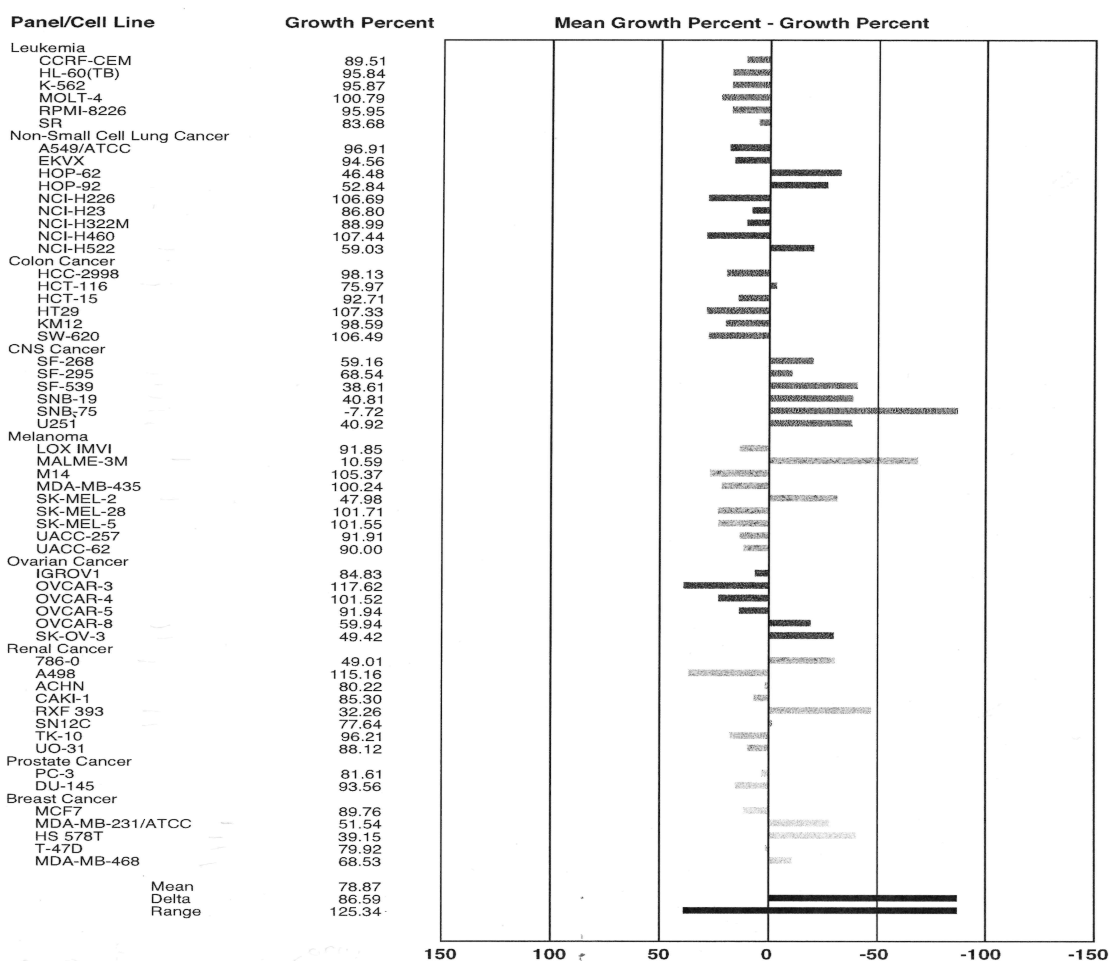


Figure 4. Results from NCI of the 60-cell-line screening panel of compound **34** at the concentration of 10 μ M.

Results are reported as growth percent values. Values lower than 100 indicate growth inhibition compared with untreated controls, while negative values reflect cytotoxicity. Values below 50, which correspond to growth inhibition and cytotoxicity, are reported on the right side of the graph while values above 50 are reported on the left side.

Compound **33** and **34** showed a comparable profile with moderate but consistent growth-inhibitory effect across different tumor types at the concentration of 10 μ M. The growth-percentage (GP) values were mostly in the ~50–95% range indicating partial growth suppression rather than marked cytotoxicity.

The strongest responses were reported in central nervous system (CNS) cancer cell lines where cytotoxicity (GP < 0, -12.27 and -7.72, respectively) was highlighted in a glioblastoma cell line (SNB-75). Minor growth-inhibitory activity was also found in some melanoma, ovarian and renal cancer lines indicating that the structural differences between the two derivatives mainly affected potency rather than target selectivity.

Thanks to a pre-existing collaboration, compounds belonging to Set II (to which compounds **33** and **34** belong), as well as compounds from Set III, were investigated in neuroblastoma cell lines, although these cells do not originate from the central nervous system, unlike glioblastoma cell lines such as SNB-75, in which the NCI screening had revealed cytotoxic effects.

The work presented in this section hereafter was done in collaboration with Professor Cinzia Domenicotti, Professor Barbara Marengo (Department of experimental medicine- DIMES, University of Genoa, Italy; Policlinic research hospital IRCCS San Martino, Genoa, Italy), Dr. Giulia Elda Valenti (Department of experimental medicine- DIMES, University of Genoa, Italy) and Dr. Sara Tirendi (Department of experimental medicine- DIMES, University of Genoa, Italy; Inter-University Center for the Promotion of the 3Rs Principles in Teaching & Research (Centro 3R), Pisa, Italy).

Introduction to Neuroblastoma

Neuroblastoma (NB) is an embryonal tumor arising from neural crest-derived precursors ^[49] and is diagnosed almost exclusively during early childhood. Although relatively uncommon, affecting approximately 1 in 8000 live births and accounting for only 6–10 % of all pediatric cancers, it represents 12–15 % of cancer-related mortality in children and remains the most frequent and lethal extracranial solid tumor in this age group ^[50].

One of the hallmark features of NB is its remarkable clinical heterogeneity. The disease may present as a localized tumor curable by surgery alone, or with limited chemotherapy, while other cases exhibit widespread metastases and poor therapeutic response. A unique subset of metastatic NB, however, can undergo spontaneous regression without any medical intervention ^[51,52].

This clinical diversity is mirrored by distinct molecular alterations. High-risk disease is often associated with amplification of the *MYCN* oncogene, observed in roughly 20–25 % of tumors and strongly predictive of unfavorable outcome. Additional genetic abnormalities, including recurrent segmental chromosomal alterations, similarly correlate with aggressive tumor behavior, even in *MYCN*-non-amplified cases ^[53].

Because of this biological and clinical heterogeneity, therapeutic approaches are tailored according to established risk categories ^[54]. Children with biologically favorable tumors benefit from de-escalated treatment strategies, whereas high-risk patients require markedly intensified multimodal regimens. Standard therapy for high-risk NB typically involves an intensive induction chemotherapy protocol (COJEC), which administers Cisplatin, Vincristine, Etoposide, Cyclophosphamide, and Carboplatin in rapid succession. Induction is followed by consolidation, consisting of surgical removal of the primary mass, myeloablative chemotherapy with autologous stem cell rescue, and local radiotherapy. Maintenance treatment includes retinoic acid to induce differentiation and immunotherapy aimed at eliminating minimal residual disease.

Despite this aggressive approach, survival outcomes vary drastically: low-risk patients achieve disease-free survival rates of approximately 95 %, whereas high-risk cases face relapse rates approaching 50 %, largely due to the emergence of chemoresistant tumor cell populations ^[55]. Ultimately, most high-risk patients develop progressive, treatment-refractory disease despite maximal therapy ^[56].

Drug resistance in NB can be either intrinsic or acquired after repeated exposure to chemotherapeutic agents. Both forms arise from genetic and epigenetic alterations affecting pathways that regulate cell death, proliferation, and genome stability. Molecular features such as aberrant telomere maintenance, *MYCN* amplification, and mutations affecting the RAS–MAPK or p53 pathways contribute to the aggressive, therapy-resistant phenotype observed in high-risk NB ^[57].

Given the limited effectiveness of current treatments for relapsed or refractory NB, the development of innovative therapeutic strategies remains an urgent priority.

Results of Set II

To assess the potential efficacy of the synthesized compounds against neuroblastoma, their biological activity, initially for Set II and subsequently for Set III, was evaluated in vitro using a malignant *MYCN*-amplified NB cell line (HTLA-230) obtained from the IRCCS Giannina Gaslini Pediatric Institute, (Genoa, Italy) and derived from a stage IV patient. In view of the negative prognostic impact of resistance to standard chemotherapeutic agents, the

compounds were also evaluated against a multidrug-resistant (ER) counterpart, generated through chronic exposure to etoposide and exhibiting cross-resistance to commonly used drugs, including etoposide and doxorubicin, as previously described by Colla et al. and Marengo et al.^[58,59].

During the biological evaluation of Set II, compound **8**, originally belonging to Set I, was also included in the assays. This decision was made to enable a direct comparison of its behavior with the newly synthesized derivatives and to determine whether the structural modifications introduced in Set II conferred any improvement in antiproliferative or mechanistic profiles relative to the earlier scaffold.

In fact, compound **8** differs from the others since it lacks phenyl, benzyl, or 4-fluorobenzyl substituents and is instead characterized by the presence of three hydroxyl groups ($R = R_1 = R_2 = OH$, Table 1). This compound was selected as a reference to highlight the importance of an additional aromatic ring. Moreover, since free radical scavenging activity (as assessed by the DPPH assay) is strongly influenced by the presence of free hydroxyl groups, and this compound bears such groups in all positions, it serves as a structural analogue reference with higher antioxidant activity.

Compounds 23, 31 and 34 are able to reduce the growth of cancer cells but not that of healthy keratinocytes (HaCat).

Both NB cell lines (HTLA-230 and ER) and healthy keratinocytes (HaCat) were exposed to all tested compounds (20 μ M) for 72 hours. As shown in Figure 5, nine compounds demonstrated a reduction in cancer cell growth, and in particular were more effective on ER cells rather than on HTLA-230. In fact, among the compounds tested, **28** was the most effective in reducing the growth of ER and HTLA cells by 30 % and 20 % respectively, but it also reduced the growth of HaCat cells by 20 %. A similar effect was observed in cells treated with **19**, while **27** was cytotoxic for both ER and HaCat cells (Figure 5). Compounds **32** and **20** were ineffective on HaCat and exerted a limited effect on both NB cell lines. Interestingly, **23**, **31** and **34** were able to reduce the growth of HTLA and ER cells by 10-15 % and 20-25 % without affecting the viability of HaCat cells.

Furthermore, **8** was found on one hand to reduce the growth of ER cells by 18 % and on the other, to stimulate the cell proliferation of HTLA and HaCat (Figure 5).

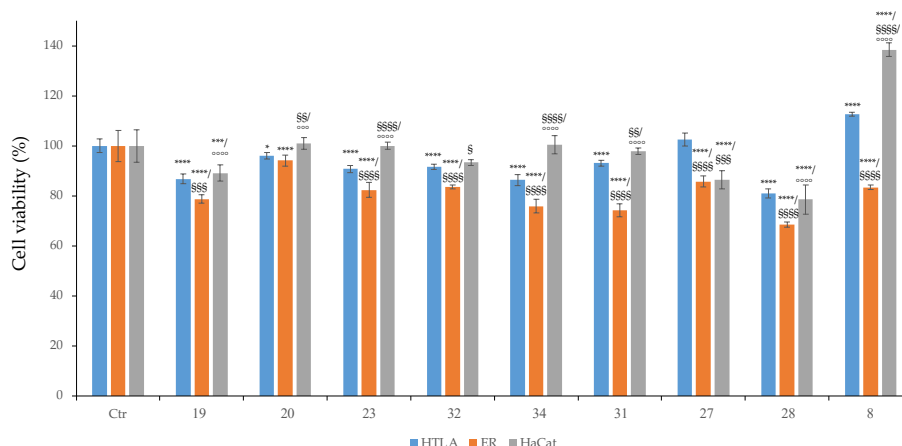


Figure 5. Compounds **23**, **31** and **34** reduce the growth of HTLA-230 and ER cells. Cell viability was evaluated using MTS assay in HTLA-230 (blue lines), in HTLA-ER cells (orange lines) and HaCat cells (gray lines) treated with 20 μ M in DMSO of all tested compounds for 72 h. DMSO was used as control (Ctr). Results are expressed as percentage variations in cell viability of treated cells with respect to that of untreated ones (100 %). Histograms summarize quantitative data of means $\pm$ SEM of at least four independent experiments. * $p<0.05$ vs Ctr; *** $p<0.001$ vs Ctr; **** $p<0.0001$ vs Ctr; § $p<0.05$ vs HTLA; §§ $p<0.01$ vs HTLA; §§§ $p<0.001$ vs HTLA; §§§§ $p<0.0001$ vs HTLA; °°° $p<0.001$ vs ER; °°°° $p<0.0001$ vs ER.

Compounds **23**, **31** and **34** stimulate ROS overproduction in NB cells but not in healthy cells.

Since the resistance to etoposide and other chemotherapeutics of ER cells was found to be dependent on the increase in their antioxidant response to the prooxidant effect of the drugs^[58], the evaluation of the redox state was considered useful to investigate the mechanisms of action of compounds **8** and **19-34** (Set I and II, respectively). As shown in Figure 6, compound **31** was the most efficient in stimulating ROS production in all three tested cell lines and in particular in ER cells in which it induced a 50 % increase in ROS levels in respect to untreated cells. Compound **20** stimulated ROS production only in HaCat cells with a 30 % increase in respect to untreated cells, while **19** and **32** only partially increased ROS in both NB cells and **31** only in HTLA (Figure 4). Instead, **23**, **31**, and **34** were able to induce ROS over-production in HTLA and ER cells without changes in HaCat cells. In particular, especially **34** and **31** were observed to stimulate ROS

production by 30 % in HTLA and by 35-40 % in ER cells. Furthermore, **8** was not able to increase ROS production in NB cells, leading to a reduction in HaCat (Figure 6).

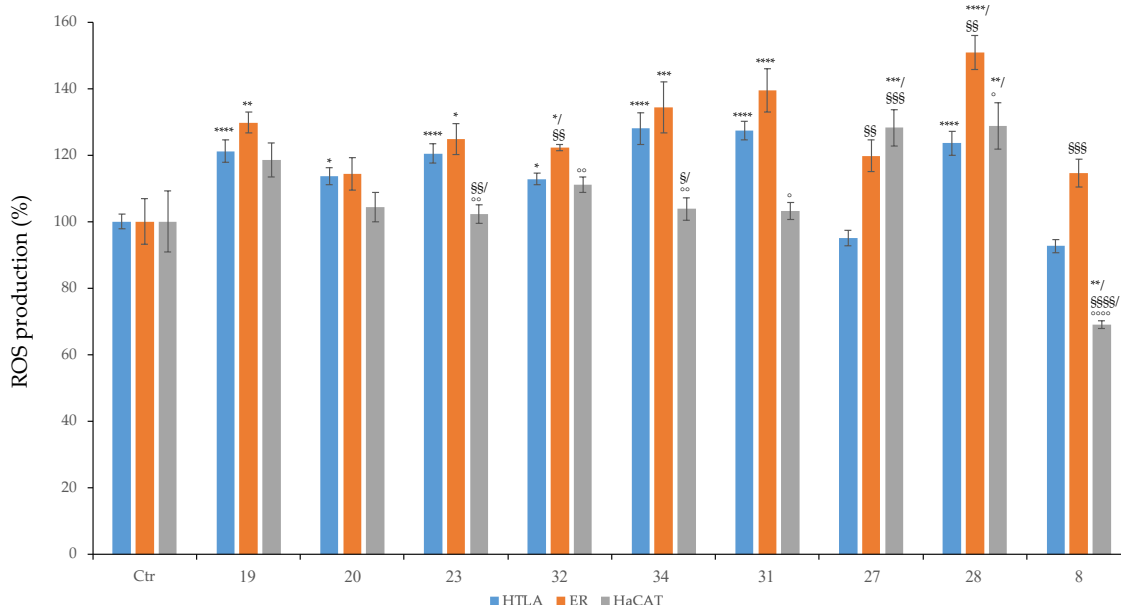


Figure 6. Compounds **23**, **31**, and **34** enhance ROS production in HTLA-230 and ER cells.

ROS production was analyzed in HTLA-230 (blue), HTLA-ER cells (orange), and HaCat cells (gray) treated with 20 μ M in DMSO of all tested compounds for 72 h. DMSO was used as control (Ctr). Results are expressed as percentage variations in DCFH-positive cells, under treatment conditions in comparison with untreated ones (100 %). Histograms summarize quantitative data of means $\pm$ SEM of four independent experiments. * p <0.05 vs Ctr; ** p <0.01 vs Ctr; *** p <0.001 vs Ctr; **** p <0.0001 vs Ctr; § p <0.05 vs HTLA; §§ p <0.01 vs HTLA; §§§ p <0.001 vs HTLA; §§§§ p <0.0001 vs HTLA; ° p <0.05 vs ER; °° p <0.01 vs ER; °°°° p <0.0001 vs ER.

Based on the results obtained, further analyses were performed on both NB cell lines exposed to **23**, **31**, **34** and **8**.

Compounds **23**, **31**, **34** and **8** do not induce apoptotic death.

To shed light on the mechanisms underlying the effect of **23**, **31**, **34** and **8** on the survival of cancer cells, the levels of full length (116 kDa) and cleaved (89 kDa) PARP, a known marker of apoptosis [60], were evaluated. As shown in Figure 7, all compounds did not induce PARP cleavage in either HTLA or ER cells.

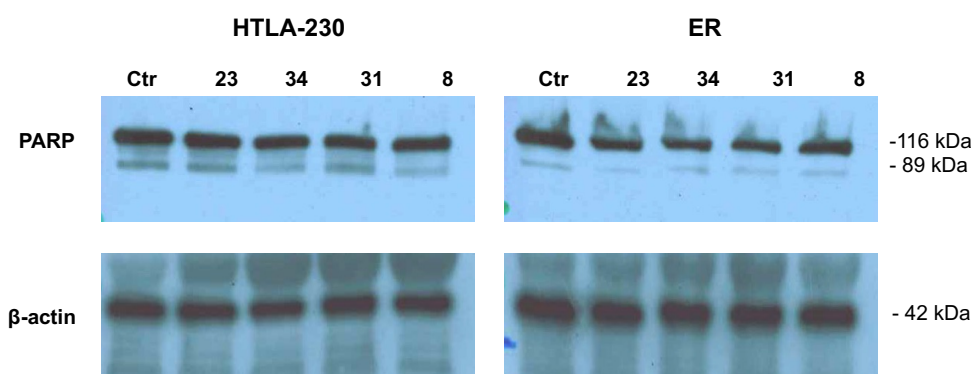


Figure 7. Compounds **23**, **31** and **34** do not induce apoptotic death of NB cells. The expression levels of full length (116 kDa) and cleaved (89 kDa) PARP were evaluated in HTLA (left panels) and ER (right panels) cells exposed to 20 μ M in DMSO of all tested compounds for 72h. DMSO was used as control (Ctr). Immunoblots shown are representative of three independent experiments. β -actin is the internal loading control.

Compounds **23**, **31**, **34** and **8** do not induce lipoperoxidation and compound **8** slightly stimulates it only in ER cells.

To better characterize cell damage, the involvement of membrane lipid oxidative degradation was evaluated by analyzing lipid peroxidation (LPO) [61]. As shown in Figure 8, only **8** was able to increase LPO (+10 %) in ER cells, while **23**, **31**, **34** and **8** reduced LPO by 15-20 % in HTLA cells, in respect to untreated cells (Ctr).

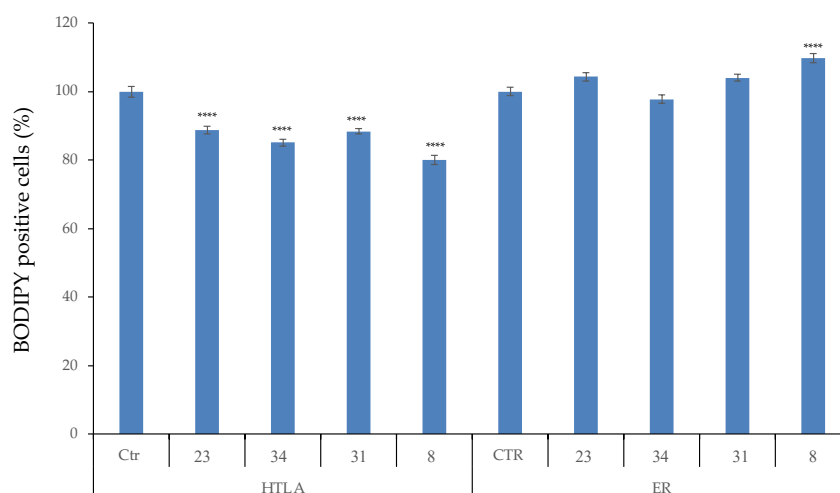


Figure 8. Compound **8** induces lipoperoxidation in ER cells. Lipid peroxidation (BODIPY-positive cells) was analyzed in HTLA-230 and HTLA-ER cells untreated or treated with 20 μ M in DMSO of all tested compounds for 72 h. DMSO was used as control (Ctr). Results are expressed as percentage variations in BODIPY-positive cells under

treatment conditions in comparison with untreated ones (100 %). Histograms summarize quantitative data of means $\pm$ SEM of four independent experiments. ****p<0.0001 vs Ctr.

Compounds 23, 31, 34 and 8 do not induce ferroptotic death.

Based on the evidence that **23**, **31** and **34** induced ROS overproduction and non-apoptotic death, the involvement of ferroptosis, a newly described type of programmed cell death, has been considered. For this purpose, both cell populations were exposed to ferroptosis-inhibitory agents (ferrostatin or liprostatin) and to ferroptosis-inducing compounds (RSL3 or erastin) for 72 h, either given alone or co-administered with **23**, **31**, **34** and **8**. As reported in Figure 9 panel A, ferrostatin or liprostatin, given alone, did not alter the viability of both cell populations, and the co-administration with **23**, **31**, **34** or **8** was not able to prevent the cytotoxic effects of the compounds.

On the other hand, erastin and RSL3, given alone reduced the viability of HTLA cells by 52 % and 45 %, respectively and of ER cells by 20 % and 25 %, respectively (Figure 9 panel B), and the co-treatment with the compounds did not further increase the cytotoxic action of compounds **23**, **31**, **34** and **8**. In fact, although a reduction in viability was observed in co-treated HTLA cells, the effect is due to the presence of erastin or RSL3, which directly reduced cell viability, and not to a synergistic action with the tested compounds.

Taken together these results demonstrate that **23**, **31**, **34** and **8** limited NB growth without inducing ferroptotic death.

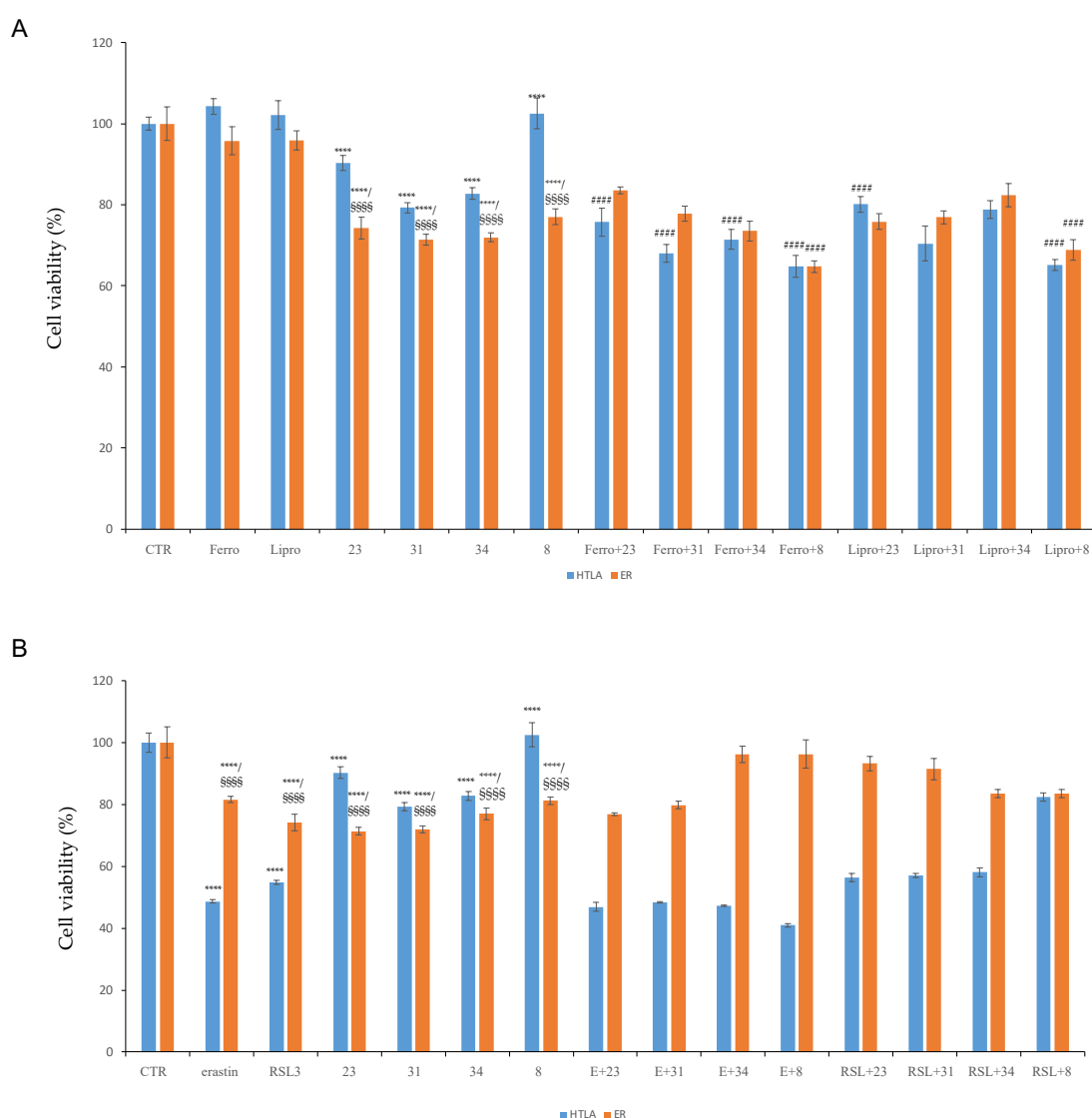


Figure 9. Compounds **23**, **31**, **34** and **8** do not induce ferroptosis in HTLA and in ER cells.

Cell viability was evaluated using MTS assay in HTLA and ER cells treated for 72 h with 20 μ M in DMSO of all tested compounds and ferroptosis-inhibitory drugs (**A**) 5 μ M of ferrostatin and 1 μ M of liprostatin) or ferroptosis-inducing compounds; (**B**) 250 nM of RSL3 and 2.5 μ M of erastin), given alone or in combination. DMSO was used as control (Ctr). Results are expressed as percentage variations in the viability of treated cells with respect to that of untreated ones (100 %). Histograms summarize quantitative data of means $\pm$ SEM of four independent experiments. *** p <0.001 vs Ctr; **** p <0.0001 vs Ctr; §§§§ p <0.0001 vs HTLA; #### p <0.0001 vs relative treatment without inhibitors (**A**) or activators (**B**).

Compound 23, 31, and 34 decrease NB cell growth by inducing senescence.

Mounting evidence suggests that cellular senescence can act as a tumor suppressor mechanism able to prevent the growth of NB cells [62–66]. For this reason, the presence of senescent cells was evaluated in HTLA and ER cells treated with **23**, **31**, **34** and **8** for 72 h. As shown in Figure 10, compound **23**, **31**, and **34** induced a 50 % increase in the senescent positive cells in HTLA, a 40 % (**23** and **34**) and 25 % (**31**) increase in ER cells, while exposure to compound **8** did not induce the appearance of senescence in both NB cell populations.

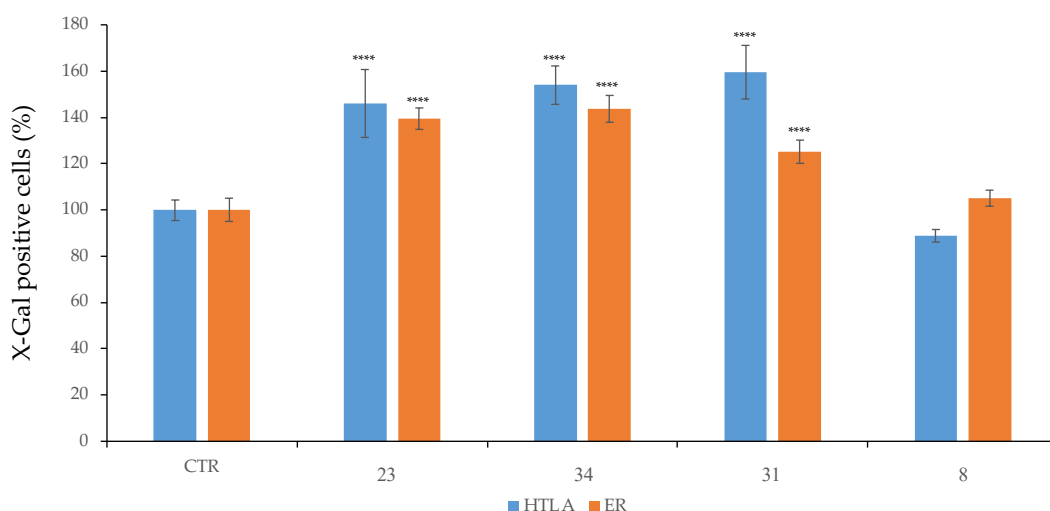


Figure 10. Compounds **23**, **31** and **34** induce senescence in HTLA and in ER cells. Cell senescence was evaluated using CellEvent Senescence Green detection kit in HTLA and ER cells treated for 72 h with 20 μ M in DMSO of all tested compounds. DMSO was used as control (Ctr). Results are expressed as percentage variations in CellEvent-positive cells under treatment conditions in comparison with untreated ones (100 %). Histograms summarize quantitative data of means $\pm$ SEM of four independent experiments. ****p<0.0001 vs Ctr.

Compound 23 decreases cell growth by reducing the cellular clonogenic potential.

To further investigate the cytotoxic effects of the most active compounds tested, the reproductive activity of both treated cell populations was assessed by clonogenic assay. As shown in Figure 11, only compound **23** was able to markedly reduce colonies formation in both cell populations, while compound **8** exerted a similar effect only in HTLA parental cells.

Noteworthy, the other compounds were unable to counteract the clonogenic potential of HTLA and ER cells.

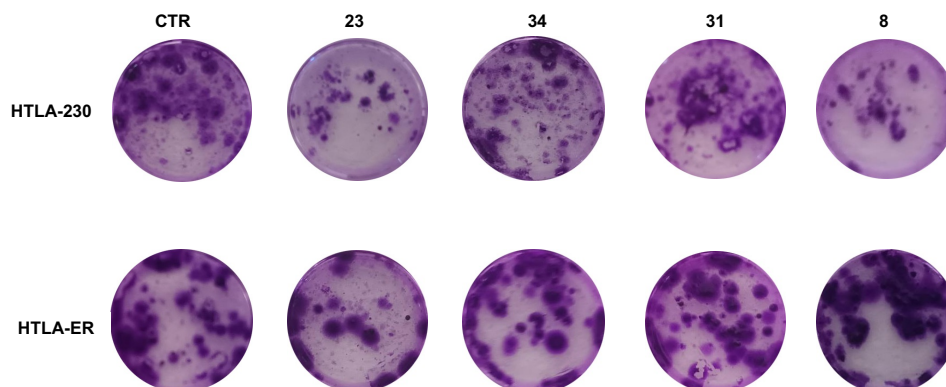


Figure 11. Compound **23** reduces the clonogenic potential of HTLA and ER cells. HTLA-230 and ER cells were seeded in six-well plates and then incubated for 72 h with 20 μ M in DMSO of all tested compounds. DMSO was used as control (Ctr). Subsequently, cells were incubated in fresh medium without drugs for additional 20 days before staining. The images are representative of three independent experiments.

Results of Set III

Set III, as earlier described for Set II, was evaluated against neuroblastoma. Following the promising antiproliferative activity observed for some of the compounds belonging to Set II, Set III was designed as a structural refinement aimed at further exploring and optimizing the SARs of the most active compounds (benzyloxy derivatives). As highlighted in Figure 12, the compounds in Set III differ from those in Set II only in the position of the R substituent on the coumarin ring, which is shifted from C-8 in Set II to C-7 in Set III.

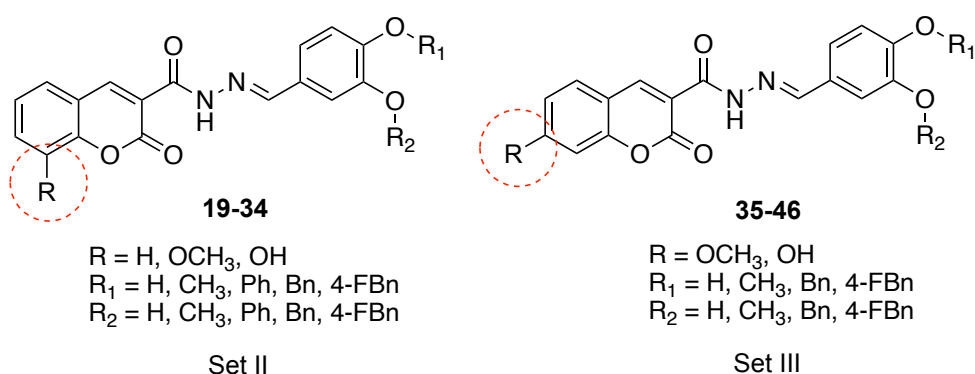


Figure 12. Structural differences between Set II and Set III.

After assessing the importance of the presence of another aromatic ring in the vanillin- or isovanillin- derived part of the structure with the biological evaluation results of Set I and II, this modification characteristic of Set III allowed us to directly assess the impact of substituent positioning on the coumarin ring on biological activity and potentially identify more favorable interactions with the molecular targets involved in the considered NB cells proliferation.

The details of the structure of the compounds were previously reported in Table 2 and 3, respectively.

Results of the cell viability assay on HTLA-230 and HTLA-ER cells at the concentration of 20 μ M are reported in Figure 13, panel A and B, respectively.

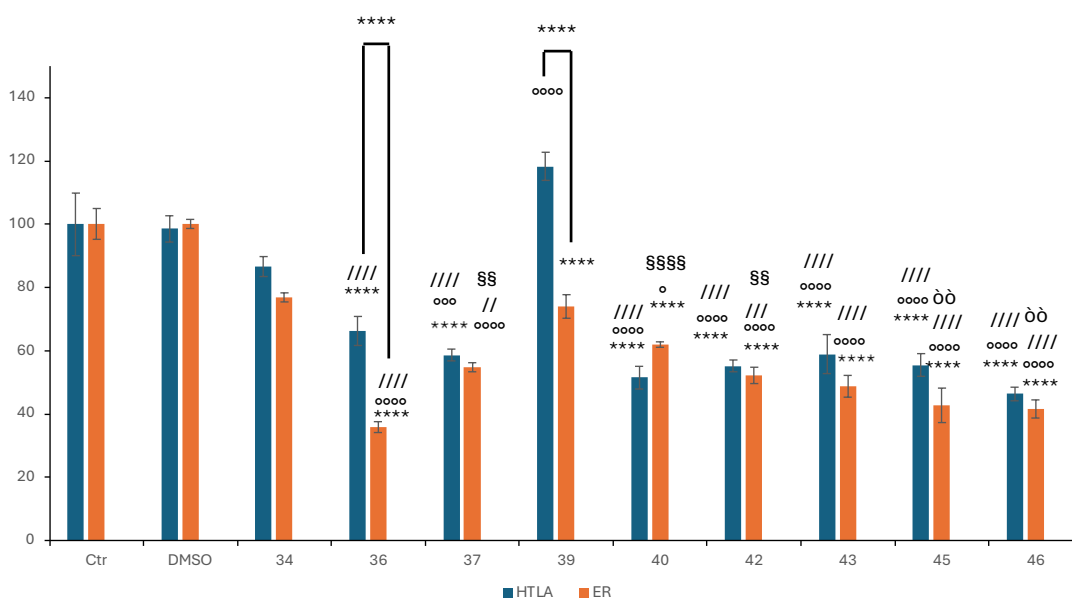


Figure 13. Compounds 36, 37, 40, 42, 43, 45, 46 reduce the growth of HTLA-230 and ER cells.

Cell viability was evaluated using MTS assay in HTLA-230 (blue) and in HTLA-ER cells (orange,) treated with 20 μ M of all tested compounds for 72 h. Results are expressed as percentage variations in cell viability of treated cells with respect to that of untreated/treated with DMSO ones (Ctr/DMSO, 100 %). Histograms summarize quantitative data of means $\pm$ SEM of at least four independent experiments. *p<0.05 vs Ctr; ***p<0.001 vs Ctr; ****p<0.0001 vs Ctr ****p<0.00001 vs Ctr/DMSO, °p<0.01 vs 31; °°°°p<0.00001 vs 31, // p<0.001 vs 39; ///p<0.00001 vs 39; §§p<0.001 vs 36; §§§§p<0.00001 vs 36. Compound 31 is reported as the most active compound of the previously synthesized series.

Preliminary results on eight tested compounds (36-37, 39-40, 42-43, 45-46) suggest that this structural modification improves antiproliferative activity at 20

μM . All the tested compounds, except compound **39** ($R = \text{OH}$, $R_1 = \text{Bn}$, $R_2 = \text{CH}_3$), caused a reduction in cell viability in both sensitive and multidrug-resistant NB cell lines. Interestingly, compound **39** increased viability in the sensitive line while moderately reducing it in the multidrug-resistant one. Also of note is the activity of compound **36**, which shows 35 % viability on the multi-resistant line, a 65 % reduction compared with compound **34** which obtained the highest antiproliferative activity among the compounds from set II.

Most of the newly tested derivatives exhibited higher antiproliferative activity than the most active compounds from the previous series and maintained or slightly enhanced their effect on the resistant cell line, indicating that substitution at position C-7 on the coumarin ring is more favorable than substitution at position C-8. Indeed, considering, for example, two of the most active compounds from Set II, **31** and **34**, these showed cell viability of approximately 95 and 90 %, respectively, in HTLA cells and about 75 % for both in ER cells. In contrast, their structural analogues substituted at position C-7, compounds **43** and **46**, displayed cell viability of approximately 58 and 46 %, respectively, in HTLA cells and about 50 and 40 %, respectively, in ER cells, clearly demonstrating that substitution at C-7 is more advantageous than at C-8.

Similarly, compound **28** (Set II) showed viability of about 80 % in HTLA cells and about 70 % in ER cells, whereas its analogue **37** (Set III) exhibited viability of about 60 % in HTLA cells and about 55 % in ER cells, identifying **37** as the most active compound across the two series.

Moreover, the presence of fluorine at position 4 on the benzaldehyde ring appears to exert only a minor and not fully clear influence on biological activity. For instance, fluorine slightly improves the activity of compound **46** compared to its non-fluorinated analogue **45**, with cell viability decreasing from approximately 55 and 40 % in HTLA and ER cells, respectively, to about 45 and 40 %. Conversely, in compound **37**, the presence of fluorine slightly enhances activity compared to the non-fluorinated analogue **36** in HTLA cells (from about 65 % to about 60 %) but reduces activity in ER cells (from about 35 % to about 54 %).

Furthermore, it is important to emphasize that the trend observed for Set II where the compounds displayed higher activity against the multidrug-resistant cell line compared to the drug-sensitive line (Figure 5), was also confirmed for Set III. This result is particularly significant, as it indicates a selective mode of action of these molecules. The observation of a similar trend suggests that they may share the same mechanism of action, which in turn could point to the identification of a distinct and previously unexplored class of compounds. The recognition of a new class of molecules may ultimately contribute to addressing current therapeutic gaps.

Overall, the results justify further biological investigation to clarify the mechanism of action of this class of compounds and the observed differences between sensitive and resistant neuroblastoma lines. Additional assays on HaCat are planned to confirm the absence of cytotoxicity in healthy cells, consistent with previous findings

Preformulation studies on compounds 8 and 34

According to estimates suggest that as many as 90% of drug candidates under investigation lack adequate water solubility ^[67,68], a limitation that significantly compromises their therapeutic potential. Poorly soluble drugs often display unpredictable absorption profiles, reduced bioavailability, and may necessitate higher doses to achieve therapeutic effects, conditions that increase the risk of gastrointestinal, cardiovascular, neurological, and respiratory side effects ^[67–69]. Consequently, enhancing drug solubility and encapsulation efficiency has become a central strategy for improving drug absorption, increasing bioavailability, and minimizing dose-related toxicities ^[70,71].

In addition to these challenges, low-solubility drug molecules frequently exhibit high lipophilicity, extensive first-pass metabolism, and strong affinity for plasma proteins, all of which further limit their systemic availability. These issues not only complicate formulation development but also contribute to significant inter-patient variability, making it difficult to achieve consistent therapeutic outcomes.

Over the past decade, substantial research efforts have been devoted to developing advanced formulation technologies such as amorphous solid dispersions ^[72], lipid-based delivery systems ^[73], polymeric micelles, and nanocarriers, to overcome solubility-related barriers and enhance clinical performance. Moreover, innovations in nanotechnology have enabled the design of targeted delivery platforms capable of improving tissue penetration, controlling drug release kinetics, and reducing off-target toxicity. Collectively, these advancements highlight the critical role of solubility enhancement strategies in modern pharmaceutical development and underscore the need for continued exploration of multifunctional delivery systems that can address the complex physicochemical limitations of emerging drug candidates.

Modern drug-delivery systems aim to transport active pharmaceutical ingredients efficiently to their target tissues, while protecting them from premature degradation and maintaining therapeutically relevant concentrations at the site of action. Among the various carriers explored, surfactants have garnered significant attention due to their amphiphilic nature, which enables them to lower interfacial tension between immiscible phases and facilitate solubilization, emulsification, and molecular transport ^[74–76]. When present

above their critical micelle concentration (CMC), surfactants self-assemble into micelles, nanoscale structures capable of markedly enhancing the solubility of hydrophobic drugs ^[76]. Both single-surfactant micellar systems and mixed micelles composed of multiple surfactants have demonstrated considerable potential in drug solubilization, often outperforming traditional delivery vehicles ^[77].

Within this context, nonionic surfactants have emerged as particularly advantageous for pharmaceutical applications. Their low protein binding, high solubility, and low CMC values make them compatible with physiological environments and suitable for drug-delivery formulations. Nonionic micelles typically form thermodynamically stable nanoscale assemblies (10–100 nm) with hydrophobic cores and hydrophilic coronas, creating an ideal environment for the dissolution and transport of poorly water-soluble molecules.

The process of micellar solubilization involves encapsulating a hydrophobic drug molecule within the micelle's core, shielding it from the aqueous medium and thereby enhancing its chemical stability and bioavailability. Interactions between drug molecules and surfactants, both hydrophobic and electrostatic, dictate the drug's position within the micelle and determine the efficiency of solubilization ^[78–80].

Based on the biological activity results obtained in the initial phase of the study (Set II), compounds **8** and **34** were selected for preformulation studies because they previously demonstrated excellent biological performance, with **34** representing one of the most active yet least water-soluble derivatives in the series, and **8** serving as structural analogue reference compound in the biological assays.

Hydrazone-based compounds are well known for their very low aqueous solubility, with experimental values often reported in the range of 10^{-5} – 10^{-4} mol/L under standard conditions ^[81,82]. Their strong preference for organic phases reflects a pronounced hydrophobic character that compromises dissolution in water and creates significant challenges in drug formulation. As a result, hydrazone derivatives frequently require co-solvents, solubilizing excipients, or encapsulation strategies to achieve pharmaceutically relevant bioavailability ^[83].

In this context, the present chapter investigates the micellar solubilization of compounds **8** and **34** using nonionic surfactants, specifically Tween-80 (TW-80), Ricinoleth-40 and Triton X-100 (TX-100) in a single micellar system. Using UV–Visible spectroscopy, the study explores how these surfactants influence CMC values of poorly water-soluble compounds.

To evaluate the behavior of **8** and **34** in solution, a pre-formulative study was carried out ^[84–86]. Initially, the UV-visible spectra of **8** and **34** in water (0.05 mM) was recorded to identify the maximum absorbance value (λ max **8** = 370 nm; λ max **34** = 415 nm). Then, the absorption profile of **8** and **34** in the presence of different amount of the different surfactants (Tween-80, Ricinoleth-40, Triton X-100) was measured and the CMC was determined.

The CMC of compounds **8** and **34** with Tween-80 are reported in Figure 14 A-D and in Figure 15 A-D, respectively. In the presence of different surfactant concentrations, the compound absorbance raised sharply in the pre-micellar region until reaching the CMC, after which no further increase was detected, and the system reached a plateau corresponding to complete drug incorporation into micelles.

The results indicate that all tested surfactants can encapsulate the new molecules, significantly enhancing their solubilization and, consequently, their potential bioavailability.

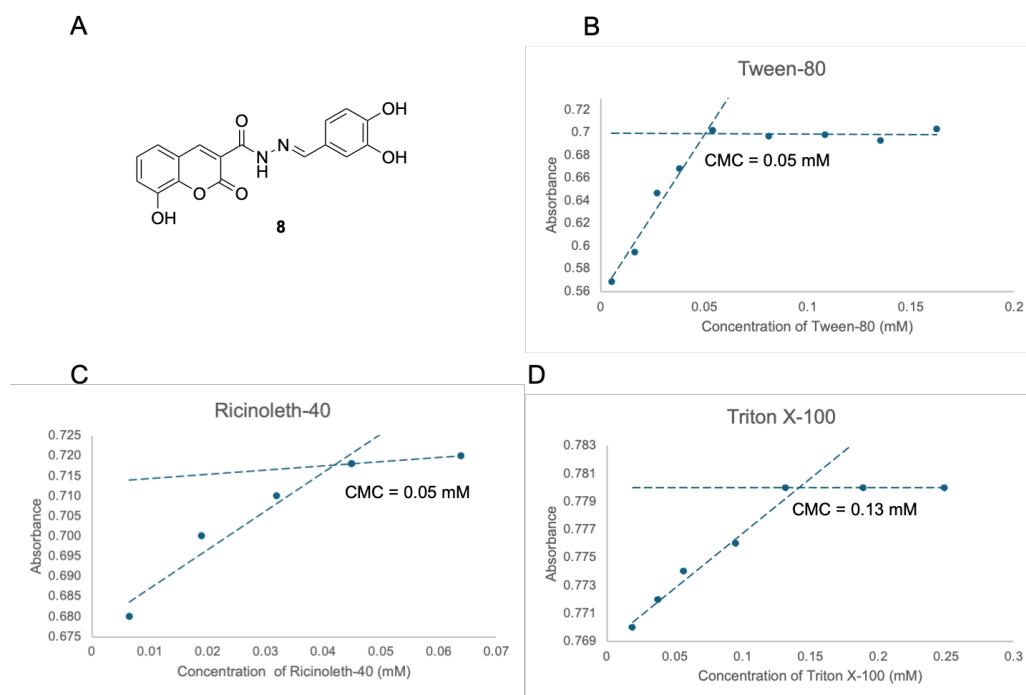


Figure 14. Molecular structure of compound **8** and plots of its absorbance in the presence of different surfactants at different concentrations and determination of the CMC. (A-D) Compound **8** (A) in the presence of TW-80 (B), Ricinoleth-40 (C), TX-100 (D).

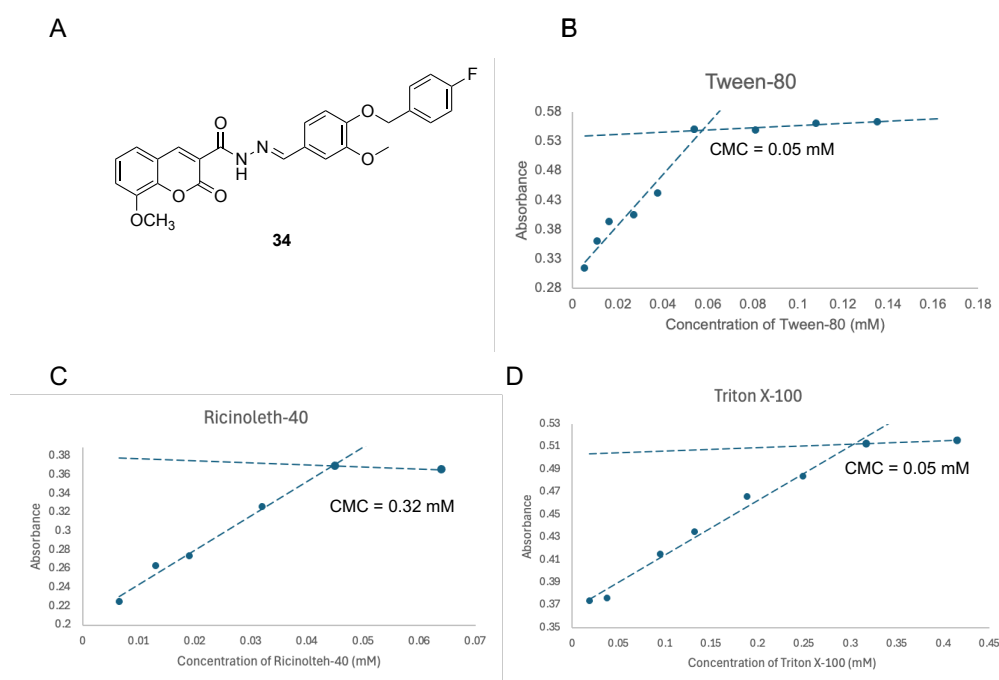


Figure 15. Molecular structure of compound **34** and plots of its absorbance in the presence of different surfactants at different concentrations and determination of the CMC. (A-D) Compound **34** (A) in the presence of TW-80 (B), Ricinoleth-40 (C), TX-100 (D).

3-Cinnamoyl coumarins

Given the solubility limitations frequently associated with hydrazone-containing scaffolds, an issue widely reported for acylhydrazone derivatives ^[81] due to their tendency to form extensive hydrogen-bond networks, alternative structural strategy was explored to improve the physicochemical profile of the previously synthesized coumarin hybrids. To address these drawbacks, new analogues were designed by replacing the hydrazone linker characteristic of the coumarin-vanillin and isovanillin- hydrazone hybrids (compounds **1-46**, Set I-III) with an α,β -unsaturated carbonyl system, thereby preserving the key structural motif of a carbonyl group conjugated to a double bond while simplifying the overall molecular architecture (Figure 16).

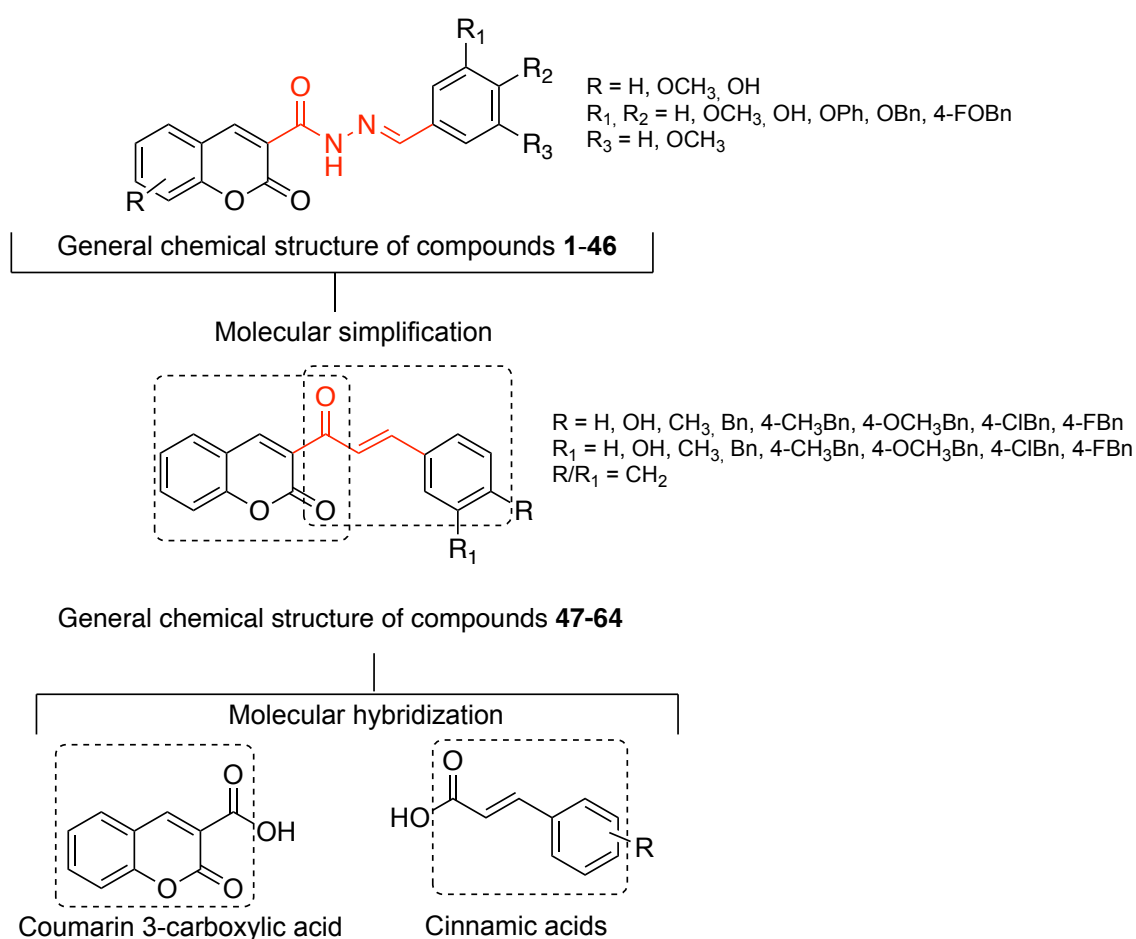


Figure 16. Design and general chemical structure of compounds **47-64**.

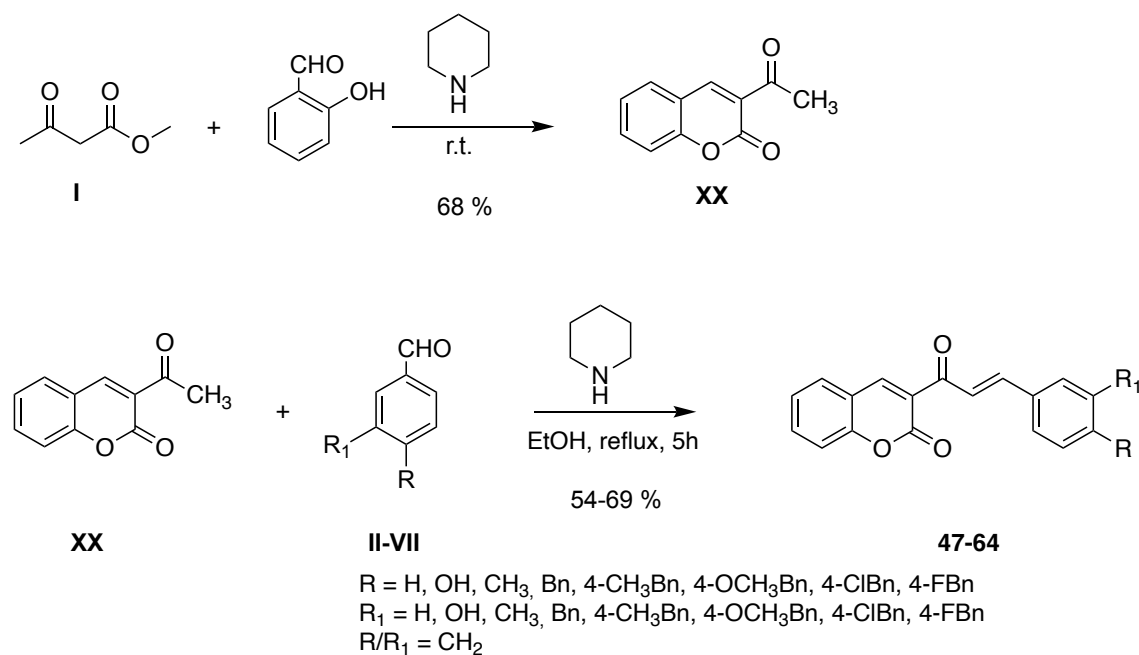
This modification not only enhances the likelihood of improved aqueous solubility but also aligns the scaffold with the structural features of cinnamic acid

derivatives, enabling a new cinnamic–coumarin hybrid framework through a molecular hybridization approach. The resulting compounds can therefore be formally regarded as 3-cynnaomyl–coumarin hybrids, specifically coumarin-3-carboxylic acid derivatives, designed to retain the pharmacophoric essence of the original series while overcoming their solubility limitations.

Synthesis

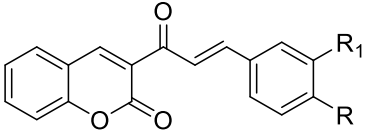
For the synthesis of the coumarin derivatives (Scheme 4), the first step involved the preparation of the coumarin scaffold (**XX**) through the condensation of salicylaldehyde with methyl acetoacetate under piperidine catalysis. This reaction proceeded rapidly, and the intermediate coumarin crystallized directly after the addition of cold ethanol. This scaffold (**XX**) was then used for the second condensation step, in which it was reacted with the aldehydes **II–VII** (the synthesis of which has already been described in Scheme 2) in ethanol in the presence of piperidine, leading to the formation of the final derivatives **47–64** that precipitated upon cooling, giving yellow or pale-grey solids. Overall, the two-step sequence allowed the efficient preparation of the desired compounds in good purity and reproducible yields.

Scheme 4. Synthesis of compounds **47–64**.



Details of the chemical structure of targets compounds **47–64** are listed in Table 5.

Table 5. Details of the chemical structure of 3-cinnamoyl coumarins **47-64**.



Compound	R	R ₁
47	OH	H
48	OCH ₃	H
49	O-Bn	H
50	H	OH
51	H	OCH ₃
52	H	O-Bn
53	OCH ₃	OCH ₃
54		OCH ₂ O
55	OCH ₃	O-Bn
56	OCH ₃	O-(4F)Bn
57	OCH ₃	O-(4Cl)Bn
58	OCH ₃	O-(4OCH ₃)Bn
59	OCH ₃	O-(4CH ₃)Bn
60	O-Bn	OCH ₃
61	O-(4F)Bn	OCH ₃
62	O-(4Cl)Bn	OCH ₃
63	O-(4OCH ₃)Bn	OCH ₃
64	O-(4CH ₃)Bn	OCH ₃

Chemical radical-scavenging activity

The radical-scavenging capacity of these new cinnamate–coumarin hybrids was evaluated using the DPPH assay. However, none of the compounds exhibited antioxidant activity above 10 %, and therefore the corresponding data were not included.

Biological evaluation

The work presented in this section was done in collaboration with Professor Francesco Piacente (Department of Experimental Medicine – DIMES, University of Genoa, Italy).

The cytotoxicity assays were performed on the human breast cancer cell line MCF-7 and the lung adenocarcinoma cell line A549, two widely used *in vitro* models of these two types of cancer ^[87,88]. These complementary profiles allow a broader evaluation of SARs.

Estrogen receptor–positive (ER⁺) breast cancer represents the most prevalent molecular subtype of breast cancer, accounting for approximately 70% of all diagnosed cases. This subtype is defined by the expression of estrogen receptor α (ER α), which drives tumor growth through estrogen-dependent signaling pathways ^[5]. ER⁺ breast cancers generally display a more favorable prognosis compared to other subtypes; however, long-term disease control is often limited by the development of resistance to endocrine therapies such as tamoxifen and aromatase inhibitors ^[5,89]. Tumor heterogeneity, adaptive signaling, and metabolic rewiring contribute to therapy resistance and disease recurrence, underscoring the need for novel therapeutic strategies capable of complementing or enhancing current treatments ^[89].

Lung adenocarcinoma is the most common histological subtype of non-small cell lung cancer (NSCLC), accounting for approximately 40–50 % of lung cancer cases worldwide. It originates from glandular epithelial cells of the distal lung and is characterized by marked molecular heterogeneity, frequently involving oncogenic alterations in genes such as EGFR, KRAS, ALK, and BRAF ^[90,91]. Lung adenocarcinoma often exhibits aggressive behavior and a strong propensity for early metastasis, contributing to its poor prognosis. Despite the introduction of targeted therapies and immunotherapy, resistance and disease progression remain major clinical challenges, particularly in advanced-stage disease ^[91,92].

In this context, compound **47**, a literature-known compound active against A549 cell line ($IC_{50} = 32.34 \pm 0.58 \mu M$ ^[31]) structurally analogue to the new derivatives either present in literature active against MCF-7 and A549 cell line and synthesized in this work ^[11,31,93], was resynthesized and tested as reference

standard. Its inclusion in biological assays enables direct comparison with previously published data and enables to assess the impact of the structural modifications introduced.

Table 6. Cytotoxicity of compounds **47-64** expressed as IC₅₀ (μM) ± SEM on MCF-7 and A549 cell lines. All dose–response curves showed good quality fits, with R² values ranging from 0.8626 to 0.9827 (data not shown).

Compound	IC ₅₀ MCF-7 (μM)	IC ₅₀ A549 (μM)
47	31.39 ± 7.65	28.44 ± 8.11
48	24.67 ± 4.14	20.05 ± 2.22
49	22.99 ± 6.79	49.01 ± 15.02
50	10.12 ± 2.75	6.84 ± 0.79
51	3.74 ± 0.94	13.81 ± 3.46
52	11.07 ± 3.26	9.27 ± 1.20
53	11.21 ± 3.74	1.72 ± 0.63
54	30.05 ± 11.08	3.50 ± 2.02
55	15.13 ± 2.55	11.12 ± 1.55
56	9.21 ± 3.7	1.45 ± 0.73
57	2.84 ± 1.17	3.41 ± 1.44
58	33.24 ± 12.07	20.16 ± 11.19
59	ND	ND
60	18.33 ± 5.30	2.02 ± 0.87
61	10.01 ± 3.50	1.79 ± 0.58
62	26.12 ± 7.24	6.86 ± 5.42
63	47.91 ± 13.39	5.33 ± 6.81
64	4.22 ± 1.77	22.82 ± 12.07

ND = not determined

The IC₅₀ values revealed a good antiproliferative profile across the series, with several compounds displaying noteworthy potency, particularly against the lung cancer cell line (A549). Overall, the lung cancer cell line (A549) therefore appears more sensitive to these compounds than breast cancer cells (MCF-7).

Among the most active derivatives against A549, compounds **53**, **54**, **56**, **60** and **61**, showed sub-5 μM IC₅₀ values, with compound **56**, bearing a 4F-benzyloxy substituent in the meta position, emerging as one of the strongest inhibitors (IC₅₀

= $1.45 \pm 0.73 \mu\text{M}$). The same substituent is present in compound **61**, another compound that achieved an IC_{50} of approximately $1 \mu\text{M}$; however, in this case the 4F-benzyloxy substituent is in the para position, suggesting that the effect is primarily due to the nature of the substituent itself rather than its positional orientation.

The same substituent is present in compound **61**, which also exhibited an IC_{50} of approximately $1 \mu\text{M}$; however, in this case, the substituent is in the para position, suggesting that the observed effect is mainly attributable to the intrinsic nature of the substituent rather than its positional orientation.

The same reasoning, however, cannot be extended to other structurally related compounds bearing either benzyloxy or substituted-benzyloxy groups and the other = OCH_3 . Indeed, the presence of para-substituted benzyloxy (**60**) or OCH_3 -benzyloxy (**58**) groups enhances the activity of the compounds compared to structurally analogous derivatives with meta substitution (**55** and **58**, respectively). Conversely, for 4-chlorobenzyl-oxy compounds, it is the meta-substituted derivative (**57**) that shows higher activity relative to the para-substituted analogue (**62**). These observations indicate that the influence of substituent position on biological activity is highly context-dependent, likely reflecting the interplay between steric effects, electronic distribution, and specific interactions within the binding site.

It is also noteworthy that two compounds, in which the substituents are OCH_3 (**53**) or where the structure is modified to resemble a piperonal moiety (**54**), exhibit low micromolar activity. This suggests that both the presence of electron-donating methoxy groups and the incorporation of a piperonal scaffold contribute positively to the interaction with the target, potentially enhancing binding affinity through favorable electronic and steric effects, without the need to introduce an additional aromatic fragment,

In contrast, activity on MCF-7 is generally weaker, although compounds **51**, **57** and **64** show sub- $5 \mu\text{M}$ IC_{50} values indicating good potency and several compounds show IC_{50} values below $15 \mu\text{M}$, indicating moderate potency.

On the MCF-7 cell line, the most active compounds ($\text{IC}_{50} < 5 \mu\text{M}$) are characterized by the presence of a single methoxy substituent in the meta position (**51**), a meta-substituted 4-chlorobenzyl-oxy group (**57**), or 4-

methylbenzyl-oxy group in the para position (**64**). When compounds with sub-15 μM activity are also considered, most active molecules are clustered within the monosubstituted derivatives (**50–53**) bearing substituents in the meta position. This trend suggests that, in this cellular model, limited aromatic functionalization and meta substitution are favorable for biological activity.

The 4-fluorobenzyl-oxy group again proves to be active, both in the meta and para positions (**56** and **61**, respectively), displaying comparable IC_{50} values of approximately 10 μM . This observation highlights that, for this substituent, its strong inductive electron-withdrawing effect plays a more decisive role in influencing biological activity than its positional orientation.

However, it is worth to underline that the IC_{50} value of compound **47** on A549 cells ($28.44 \pm 8.11 \mu\text{M}$), used as already literature-known reference compound was consistent with what already found on the same cell line ($32.34 \pm 0.58 \mu\text{M}$ [31]). Moreover, all the compounds except compound **49** exhibited lower IC_{50} values on this cell line, indicating the positivity of the structural features explored.

Notably, compound **57** (bearing a 4Cl-benzyloxy group at R_1) has good IC_{50} on both cell lines ($2.84 \pm 1.17 \mu\text{M}$ on MCF7 and $3.41 \pm 1.44 \mu\text{M}$ on A549), while some derivatives (compounds **53**, **54**, **56**, **60** and **63**) show strong selectivity toward A549, with much higher IC_{50} values in MCF-7. On the other hand, compounds **54** and **61** show selectivity for MCF7 cells highlighting divergent SARs between the two cancer models providing useful guidance for target identification and consequent optimization of the selectivity.

In silico predictions of ADME properties

Predictions of pharmacokinetic properties of the most active compounds of each series (Coumarin- vanillin/isovanillin hybrid hydrazones Set II: compounds **19-34**, Set III: compounds **35-46** and 3-cinnamoyl coumarin hybrids **47-64**) were made using SwissADME web tool ^[94,95]. The values of the predicted physicochemical properties and water solubility are reported in Table 7, while predicted values of pharmacokinetics and druglikeness are reported in Table 8. Predictions of ADME properties of compound **8** are reported as this compound was included in the biological assays of set II and, moreover, it constitutes a structural representative of set I.

Table 7. Predicted physico-chemical properties and water solubility of selected compounds.

Physico-chemical Properties					
Cmpd	MW	#H-bond acceptors	#H-bond donors	TPSA	Consensus LogP o/w
Coumarin- vanillin/isovanillin hybrid hydrazones					
SET I					
8	340.29	7	4	132.36	1.75
SET II					
23	430.41	7	2	110.36	3.73
31	428.44	6	1	90.13	4.09
34	446.43	7	1	90.13	4.4
SET III					
36	476.45	8	1	99.36	4.41
39	458.46	7	1	99.36	4.07
3-Cinnamoyl coumarins					
51	306.31	4	0	56.51	3.38
52	382.41	4	0	56.51	4.7
56	420.43	6	0	65.74	4.81

MW: molecular weight; #H-bond acceptors: number of H bond acceptors; H-bond donors: number of H bond donors, TPSA: topological polar surface area; Consensus LogP o/w: average of five predictions (iLOGP, XLOGP3, WLOGP, MLOGP, SILICOS-IT).

Table 8. Predicted pharmacokinetics and druglikeness of selected compounds.

Pharmacokinetics								Druglikeness	
Cmpd	GI	Pgp	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4	#Lipinski	Bioav.
Coumarin- vanillin/isovanillin hybrid hydrazones									
SET I									
8	High	No	No	No	No	No	No	0	0.55
SET II									
23	High	No	No	Yes	Yes	No	No	0	0.55
31	High	No	No	Yes	Yes	No	Yes	0	0.55
34	High	No	No	Yes	Yes	No	Yes	0	0.55
SET III									
36	High	No	No	Yes	Yes	No	Yes	0	0.55
39	High	No	No	Yes	Yes	No	Yes	0	0.55
3-Cinnamoyl coumarins									
51	High	No	Yes	Yes	Yes	No	No	0	0.55
52	High	No	Yes	Yes	Yes	No	Yes	0	0.55
56	High	No	Yes	Yes	Yes	No	Yes	0	0.55

GI: gastrointestinal absorption; Pgp: P-glycoprotein substrate; CYP1A2: cytochrome P450 1A2; CYP2C19 cytochrome P450; CYP2C9: cytochrome P450 2C9, CYP2D6: cytochrome P450 2D6; CYP3A4: cytochrome P450 3A4; Lipinsky (Pfizer) filter number (#) of violation: MW $\leq$ 500, MLOGP $\leq$ 4.15, N or O $\leq$ 10, NH or OH $\leq$ 5; Bioav.: Abbott bioavailability score.

All the compounds do not violate Lipinski's rules as they have a molecular weight lower than 500 g/mol, LogP below 5, N or O number less than 10, and NH or OH number less than 5. From the point of view of physicochemical properties, all compounds have a number of hydrogen bond acceptors between 6 and 8, and a number of hydrogen bond donors equal to 1 or 2, except for compound **8**, where it is equal to 4 (R = OH and the ex-aldehyde nucleus is catechol). In addition, the topological polar surface area (TPSA) is lower than 140 Å² in all compounds.

All compounds have a predicted high gastrointestinal absorption and are predicted not to be substrates of P-glycoprotein. None of the compounds is predicted to simultaneously inhibit all the cytochromes considered in the pharmacokinetic parameters and compound **8** does not inhibit any of the considered cytochrome. The bioavailability score is 0.55 for all compounds, which combined with 0 Lipinski rule violations, indicates a good probability of oral bioavailability.

The predicted ADME properties are, as expected from the molecular structure, similar between set II and III, while compound **8**, belonging to set I, mainly differs

for the higher number of H bond donors, lower LogP value and none predicted interaction with the cytochromes.

Overall, these data would seem to indicate that these compounds have the potential to lead to the development of molecules characterized by adequate physicochemical properties and good drug-likeness.

Conclusions

In this part of the PhD project, a natural-inspired medicinal chemistry strategy was applied to the design and synthesis of new hybrid molecules with potential antiproliferative activity. The antiproliferative section focused on the combination of bioactive fragments of natural origin within synthetically accessible frameworks, with the aim of modulating cancer-related processes involved in tumor growth and resistance.

Two main series were developed: (i) vanillin- and isovanillin-based hydrazone hybrids and (ii) 3-cinnamoyl coumarins. The two series mainly differ in the nature of the linker connecting the coumarin-derived portion to the vanillin/isovanillin-derived fragment (hydrazonic and an α,β -unsaturated carbonyl).

In particular, the hydrazone series was further organized into three sets based on structural features, including the functionalization pattern of the vanillin/isovanillin moiety and the substitution position on the coumarin ring, in order to enable a systematic exploration of structure–activity relationships. Overall, all series and sub-series (with the exception of Set I, for which results are not yet accessible) yielded biologically active compounds.

Antiproliferative activity was evaluated separately for the hydrazone derivatives and the 3-cinnamoyl coumarins. Hydrazone hybrids were tested on neuroblastoma models, including a chemotherapy-resistant cell line, and several derivatives showed strong growth-inhibitory effects. Comparative testing against a non-functionalized analogue highlighted the relevance of vanillin/isovanillin functionalization and supported specific SAR trends. Within Set II, one compound emerged as particularly promising, as it reduced proliferation in resistant cell lines while sparing non-malignant cells. Mechanistic investigations indicated that its growth-inhibitory effect was associated with the induction of ROS production and senescence, and notably, the compound also reduced the clonogenic potential of tumor cells, supporting a sustained antiproliferative phenotype.

In addition, given the limited aqueous solubility of hydrazone-containing compounds, preformulation studies were carried out on one of the most active yet poorly soluble candidates. Screening of three non-ionic surfactants demonstrated the feasibility of micellar encapsulation, significantly improving

aqueous solubilization and supporting the potential to proceed toward formulation-oriented development.

Preliminary results also suggest that structural optimization within Set III may lead to a notable increase in antiproliferative activity. However, these findings require confirmation and a broader biological characterization comparable to the previous sets.

The 3-cinnamoyl coumarin series was evaluated on human breast cancer and adenocarcinoma cell lines (MCF-7 and A549, respectively). This library was specifically designed to investigate whether replacing the hydrazone linker, often associated with solubility limitations, with an α,β -unsaturated carbonyl system could maintain or improve biological activity while introducing a cinnamic-acid-like fragment into the hybrid architecture. The assays revealed an overall favorable antiproliferative profile, with most compounds outperforming a structurally related analogue reported in the literature. In this series, derivatives bearing a non-functionalized aldehydic fragment still exhibited good activity on both cell lines; nevertheless, consistently with what was observed in neuroblastoma models, functionalization generally produced superior outcomes, further reinforcing the role of substitution pattern as a key determinant of potency in this series of compounds.

Overall, the antiproliferative results demonstrate that molecular hybridization of selected natural-inspired scaffolds can generate compounds with detectable anticancer activity and defined structure–activity trends. Further optimization will be required to increase potency and selectivity, alongside mechanistic studies aimed at identifying molecular targets and guiding rational structural refinement. Nonetheless, the present findings validate the adopted design strategy and highlight key structural features that may support the development of improved antitumoral candidates.

Experimental section

Chemistry

All chemicals (reagent grade) were purchased from Merck (formerly Aldrich Chemical). Solvents were reagent-grade. All commercial reagents were used without further purification. Reaction progress was monitored using thin layer chromatography (TLC) on precoated silica gel plates (Merck DC-Alufolien Kieselgel 60; F254, Darmstadt, Germany) and the spots were detected under UV light (254 nm). Column chromatography was performed on silica gel (60; 0.063-0.200 mm). Melting point was measured on a Stuart SMP3 micromelting point apparatus and uncorrected. Elemental analyses were performed on a Flash 2000 CHNS (Thermo Scientific). ^1H NMR spectra (400 MHz) and ^{13}C spectra (101 MHz) were measured on a JEOL JNM ECZ-400S/L1 spectrometer (Tokyo, Japan) at 25 °C or at variable temperature using DMSO- d_6 or CDCl_3 as solvents. Chemical shifts are reported in ppm (δ), and the splitting patterns are described as follows: s(singlet), d(doublet), dd(doublet of doublets), t(triplet), q(quartet) and m(multiplet). The purity of all final compounds was confirmed through ^1H NMR spectra.

Coumarin- vanillin/isovanillin hybrid hydrazones

Synthesis of ethyl 3-hydrazineyl-3-oxopropanoate (I).

A solution of diethyl malonate (50 mmol) and hydrazine monohydrate (20 mmol) in absolute ethanol (10 mL) was stirred at room temperature for 12 hours. The reaction lead to the formation of both malonohydrazide and malonomonohydrazide as described by Abdel-Aziz et al. ^[41]. The white precipitate, formed at room temperature, (malonohydrazide, melting point: 148-150 °C) was removed by filtration and the ethanol was evaporated under vacuum. The solution was cooled at -20 °C overnight to afford the crystallization of a white solid (malonomonohydrazide, I) which is separated by filtration and washed with diethyl ether. The remaining solution is reacted again with hydrazine monohydrate (10 mmol) in absolute ethanol (10 mL). The entire process is repeated up to three times. The monohydrazide fractions obtained are reunited and recrystallized from ethanol.

White solid. Yield: 16 % (calculated on diethyl malonate). Melting point: 69 – 71 °C. Molecular formula: $C_5H_{10}N_2O_3$. Molecular weight: 146.15 g/mol. Elemental analysis calculated for $C_5H_{10}N_2O_3$ C% 41.09, H% 6.90, N% 19.17; found C% 40.85, H% 6.94, N% 19.35.

General procedure for the synthesis of phenoxy aldehydes II-III.

Phenoxy aldehydes were synthesized following the method described by Crowder et al. [42]. In detail, an equimolar amount of solid NaOH (5 mmol) and of diphenyliodonium chloride (1.58 g, 5 mmol) are added to vanillin or isovanillin (5 mmol) in H_2O (35 mL). The reaction mixture is heated to 100 °C for 24 hours. After extraction with diethyl ether (3x30 mL), the organic phase is washed with 1N NaOH (3x20 mL), dried on Na_2SO_4 , filtered and evaporated under vacuum. Finally, the aldehydes are purified by chromatography 1:15 SiO_2 /n-hexane:ethyl Acetate (80:20).

3-Methoxy-4-phenoxybenzaldehyde (II)

White solid. Melting point: 38 – 40 °C. (40-42 °C [96]). Yield: 68 %. Molecular formula: $C_{14}H_{12}O_3$. Molecular weight: 228.25 g/mol. Elemental analysis calculated for $C_{14}H_{12}O_3$. C% 73.67, H% 5.30; found C% 73.45, H% 5.12.

4-Methoxy-3-phenoxybenzaldehyde (III)

White solid. Melting point: 43 – 45 °C. (48 °C [97]). Yield: 78 %. Molecular formula: $C_{14}H_{12}O_3$. Molecular weight: 228.25 g/mol. Elemental analysis calculated for $C_{14}H_{12}O_3$. C% 73.67, H% 5.30; found C% 73.82, H% 5.47.

General procedure for the synthesis of benzyloxy and 4-fluorobenzyloxy aldehydes IV-VII.

Benzyloxy and 4-fluorobenzyloxy aldehydes were synthesized following the method described by Ashton et al. [43]. Briefly, to a solution of vanillin or isovanillin (5 mmol) in an. DMF (5 mL) were added an. K_2CO_3 (5 mmol) and a catalytic amount of KI (50 mg). After stirring at 60 °C for 20 min, a solution of the appropriate benzyl chloride (5 mmol) in an. DMF (2 mL) was added dropwise. The reaction mixture was heated at 70 °C for 22 hours. Then, water (30 mL) was added and extractions with ethyl acetate (2x20 mL, 1x15 mL) were performed. The organic phases were reunited and washed with 1N NaOH (10 mL) and water (15 mL). The aqueous phases were reunited and re-extracted with ethyl acetate

(10 mL). Then, the organic phases were reunited and washed with water (10 mL) and Brine (15 mL), dried on Na₂SO₄, filtered and evaporated under vacuum to obtain colorless oils that crystallize at room temperature possibly by adding a few drops of anhydrous diethyl ether.

4-(Benzyloxy)-3-methoxybenzaldehyde (IV)

White solid. Yield: 91 %. Melting point: 61–62 °C. (55–57 °C ^[98]). Molecular formula: C₁₅H₁₄O₃. Molecular weight: 242.27 g/mol. Elemental analysis calculated for C₁₅H₁₄O₃ C% 74.36, H% 5.82; found C% 74.54, H% 5.86.

4-((4-Fluorobenzyl)oxy)-3-methoxybenzaldehyde (V)

White solid. Yield: 85 %. Melting point: 62–63 °C. (60–62 °C ^[98]). Molecular formula: C₁₅H₁₃FO₃. Molecular weight: 260.26 g/mol. Elemental analysis calculated for C₁₅H₁₃FO₃: C% 69.22; H% 5.03; found C% 69.04, H% 5.01.

3-(Benzyloxy)-4-methoxybenzaldehyde (VI)

White solid. Yield: 90%. Melting point: 61–63 °C. (61–63 °C ^[99]). Molecular formula: C₁₅H₁₄O₃. Molecular weight: 242.27 g/mol. Elemental analysis calculated for C₁₅H₁₄O₃: C% 74.36, H% 5.82; found C% 74.12, H% 5.78.

3-((4-Fluorobenzyl)oxy)-4-methoxybenzaldehyde (VII)

White solid. Yield: 91 %. Melting point: 80–81°C. Molecular formula: C₁₅H₁₃FO₃. Molecular weight: 260.26 g/mol. Elemental analysis calculated for C₁₅H₁₃FO₃: C% 69.23; H% 5.03; found C% 69.38, H% 5.07.

General procedure for the synthesis of intermediate hydrazides VIII-XIX.

A solution of I and of the desired aldehyde (**II-VII** or commercially available) in EtOH (8 mL) was refluxed for 5 hours. All intermediates **VIII-XIX** crystallize in the reaction mixture. The volume was reduced under vacuum and the solution cooled overnight at +4 °C to allow complete crystallization. Intermediates **VIII-XIX** were then filtered out and washed with cold ethanol.

As discussed in detail in the results section, linear hydrazides **VIII-XIX**, as well as their corresponding final compounds **1-46**, exist in solution as an equilibrium of two possible rotamers. In the ¹H NMR spectra, signals for which the corresponding resonance of the alternative rotamer is detectable are annotated as “and.” The first value reported represents the signal of the major rotamer.

Ethyl 3-(2-benzylidenehydrazineyl)-3-oxopropanoate (VIII)

White solid. Yield: 76 %. Melting point: 111-112 °C. Molecular formula: C₁₂H₁₄N₂O₃. Molecular weight: 234.10 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.54 and 11.58 (s, 1H, NH), 7.92 and 8.12 (s, 1H, CH=N), 7.70 – 7.54 (m, 2H, Ar), 7.45 – 7.33 (m, 3H, Ar), 4.04 and 4.08 (q, J = 7.0 Hz, 2H, CH₂), 3.60 and 3.31 (s, 2H, CH₂), 1.12 and 1.17 (t, J = 7.0 Hz, 3H, CH₃).
¹³C NMR (101 MHz, DMSO-D₆) δ 168.47, 168.25, 143.63, 134.59, 130.45, 129.36, 129.32, 127.67, 127.26, 60.99, 41.68, 14.56.

Ethyl 3-(2-(3,4-dihydroxybenzylidene)hydrazineyl)-3-oxopropanoate (IX)

White solid. Yield: 95 %. Melting point: 168-169 °C. Molecular formula: C₁₃H₁₆N₂O₅. Molecular weight: 280.28 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.32 and 11.34 (s, 1H, NH), 9.11 and 9.38 (s, 1H, OH), 7.78 and 3.94 (s, 1H, CH=N), 7.06 (d, J = 1.9 Hz, 1H, Ar), 7.03 – 6.87 (m, 2H, Ar), 4.05 and 4.07 (q, J = 7.1 Hz, 2H, CH₂), 3.76 and 3.79 (s, 3H, OCH₃), 3.57 and 3.30 (s, 2H, CH₂), 1.13 and 1.15 (t, J = 7.1 Hz, 3H, CH₃).
¹³C NMR (101 MHz, DMSO-d₆) δ: 168.24, 168.07, 147.28, 144.00, 127.48, 120.31, 112.86, 112.32, 60.97, 56.15, 41.47, 14.55.

Ethyl 3-(2-(3-hydroxy-4-methoxybenzylidene)hydrazineyl)-3-oxopropanoate (X)

White solid. Yield: 55 %. Melting point: 156-157 °C. Molecular formula: C₁₂H₁₄N₂O₅. Molecular weight: 266.26 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.25 and 11.30 (s, 1H, NH), 9.38 (s, 1H, OH), 9.06 and 9.21 (s, 1H, OH), 7.74 and 7.92 (s, 1H, CH=N), 7.04 – 6.68 (m, 3H, Ar), 4.04 and 4.07 (q, J = 7.1 Hz, 2H, OCH₂), 3.56 and 3.27 (s, 2H, CH₂), 1.13 and 1.16 (t, J = 7.1 Hz, 3H, CH₃).
¹³C NMR (101 MHz, DMSO-d₆) δ: 168.25, 148.27, 146.23, 146.16, 144.39, 120.42, 116.03, 113.39, 60.96, 41.41, 14.57.

Ethyl 3-(2-(4-hydroxy-3-methoxybenzylidene)hydrazineyl)-3-oxopropanoate (XI)

White solid. Yield: 81 %. Melting point: 136-140 °C. Molecular formula: C₁₃H₁₆N₂O₅. Molecular weight: 280.28 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.36 and 11.37 (s, 1H, NH), 9.46 and 9.52 (s, 1H, OH), 7.79 and 7.99 (s, 1H, CH=N), 7.20 (dd, J = 16.9, 1.9 Hz, 1H, Ar), 7.07 –

6.93 (m, 1H, Ar), 6.74 and 6.78 (dd, J = 12.7, 8.1 Hz, 1H, Ar), 4.04 and 4.07 (q, J = 7.1 Hz, 2H, CH₂), 3.76 and 3.77 (s, 3H, OCH₃), 3.57 and 3.29 (s, 2H, CH₂), 1.10 and 1.16 (t, J = 7.1 Hz, 3H, CH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 168.30, 168.13, 149.25, 148.49, 143.95, 126.09, 121.85, 115.94, 109.79, 60.90, 56.02, 41.84, 14.56.

Ethyl 3-(2-(4-hydroxy-3,5-dimethoxybenzylidene)hydrazineyl)-3-oxopropanoate (XII)

White solid. Yield: 71 %. Melting point: 110-111 °C. Molecular formula: C₁₄H₁₈N₂O₆. Molecular weight: 310.31 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.43 and 11.42 (d, J = 4.3 Hz, 1H, NH), 8.83 and 8.89 (s, 1H, OH), 7.78 and 7.99 (s, 1H, CH=N), 6.87 and 6.92 (s, 2H, Ar), 4.04 and 4.07 (q, J = 6.8 Hz, 2H, OCH₂), 3.75 and 3.76 (s, 6H, 2 OCH₃), 3.58 and 3.29 (s, 2H, C(O)CH₂), 1.09 and 1.15 (t, J = 7.1 Hz, 3H, CH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 168.32, 168.23, 148.59, 143.97, 138.13, 124.90, 104.84, 61.22, 60.90, 56.52, 56.45, 41.97, 14.57.

Ethyl 3-oxo-3-(2-(3,4,5-trimethoxybenzylidene)hydrazineyl)propanoate (XIII)

White solid. Yield: 88 %. Melting point: 114-116 °C. Molecular formula: C₁₅H₂₀N₂O₆. Molecular weight: 324.33 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.57 and 11.55 (s, 1H, NH), 7.82 and 8.04 (s, 1H, CH=N), 6.92 and 6.97 (s, 2H, Ar), 4.04 and 4.07 (q, J = 7.1 Hz, 2H, CH₂), 3.77 and 3.78 (s, 6H, 2OCH₃), 3.64 and 3.65 (s, 3H, OCH₃), 3.59 and 3.32 (s, 2H, CH₂), 1.09 and 1.16 (t, J = 7.1 Hz, 3H, CH₃).

¹³C NMR (101 MHz, DMSO-D₆) δ 168.27, 153.69, 153.65, 143.31, 139.43, 130.17, 104.83, 104.54, 61.26, 60.94, 60.63, 56.46, 56.38, 42.02, 14.57.

Ethyl 3-(2-(4-methoxy-3-phenoxybenzylidene)hydrazineyl)-3-oxopropanoate (XIV)

White solid. Yield: 68 %. Melting point: 129-131 °C. Molecular formula: C₁₉H₂₀N₂O₅. Molecular weight: 356.38 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.42 and 11.46 (s, 1H, NH), 7.84 and 8.04 (s, 1H, CH=N), 7.44 – 6.76 (m, 8H, Ar), 3.93 and 4.06 (q, J = 7.1 Hz, 2H, OCH₂), 3.74 and 3.77 (s, 3H, OCH₃), 3.52 and 3.30 (s, 2H, COCH₂CO), 1.03 and 1.15 (t, J = 7.1 Hz, 3H, CH₂CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 168.25, 158.25, 144.16, 142.83, 130.41, 130.27, 128.02, 126.04, 122.75, 119.46, 117.11, 116.34, 113.90, 60.88, 56.41, 41.70, 14.44.

Ethyl 3-(2-(3-(benzyloxy)-4-methoxybenzylidene)hydrazineyl)-3-oxopropanoate (XV)

White solid. Yield: 94 %. Melting point: 110-112 °C. Molecular formula: C₂₀H₂₂N₂O₅. Molecular weight: 370.41 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 9.98 and 10.28 (s, 1H, NH), 7.69 and 8.01 (s, 1H, CH=N), 7.50 – 7.44 (m, 2H, Ar), 7.41 – 7.25 (m, 4H, Ar), 7.08 and 7.16 (dd, J = 8.3, 1.9 Hz, 1H, Ar), 6.86 (d, J = 8.3 Hz, 1H, Ar), 5.18 and 5.21 (s, 2H, CH₂Ar), 4.18 and 4.23 (q, J = 7.2 Hz, 2H, OCH₂), 3.90 and 3.95 (s, 3H, OCH₃), 3.72 and 3.84 (s, 2H, CH₂), 1.22 and 1.30 (t, J = 7.1 Hz, 3H, CH₂CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 169.07, 167.58, 151.87, 148.57, 144.45, 136.88, 128.67, 128.64, 128.08, 127.67, 127.57, 126.51, 122.39, 111.28, 110.95, 71.02, 61.41, 56.11, 41.39, 14.28.

Ethyl 3-(2-(3-((4-fluorobenzyl)oxy)-4-methoxybenzylidene)hydrazineyl)-3-oxopropanoate (XVI)

White solid. Yield: 76 %. Melting point: 110-111 °C. Molecular formula: C₂₀H₂₁FN₂O₅. Molecular weight: 388.40 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 9.96 and 10.30 (s, 1H, NH), 7.69 and 8.02 (t, J = 2.0 Hz, 1H, CH=N), 7.51 – 7.38 (m, 2H, Ar), 7.30 (d, J = 1.9 Hz, 1H, Ar), 7.18 – 6.92 (m, 3H, Ar), 6.86 and 6.74 (d, J = 8.3 Hz, 1H, Ar), 5.13 and 5.09 (s, 2H, CH₂Ar), 4.18 and 4.23 (q, J = 7.1 Hz, 2H, OCH₂), 3.89 and 3.95 (s, 3H, OCH₃), 3.72 and 3.83 (s, 2H, CH₂), 1.22 and 1.30 (t, J = 7.1 Hz, 3H, CH₂CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 167.55, 163.86, 161.41, 151.90, 148.40, 132.60, 129.53, 129.45, 126.49, 122.63, 115.69, 115.64, 115.47, 111.28, 110.90, 70.37, 61.42, 56.10, 41.47, 14.26.

Ethyl 3-(2-(3-methoxy-4-phenoxybenzylidene)hydrazineyl)-3-oxopropanoate (XVII)

White solid. Yield: 79 %. Melting point: 105-108 °C. Molecular formula: C₁₉H₂₀N₂O₅. Molecular weight: 356.38 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.55 and 11.44 (s, 1H, NH), 7.91 and 8.12 (s, 1H, CH=N), 7.64 – 6.74 (m, 8H, Ar), 4.05 and 4.09 (q, J = 7.1 Hz, 2H, OCH₂), 3.76 and 3.77 (s, 3H, OCH₃), 3.61 and 3.33 (s, 2H, COCH₂CO), 1.11 and 1.17 (t, J = 7.1 Hz, 3H, CH₂CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 168.45, 168.26, 157.77, 151.88, 145.95, 143.01, 131.82, 130.37, 123.21, 121.57, 121.06, 117.19, 111.03, 60.97, 56.20, 41.84, 14.57.

Ethyl 3-(2-(4-(benzyloxy)-3-methoxybenzylidene)hydrazineyl)-3-oxopropanoate (XVIII)

White solid. Yield: 94 %. Melting point: 139-41 °C. Molecular formula: C₂₀H₂₂N₂O₅. Molecular weight: 370.41 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 9.97 and 10.28 (s, 1H, NH), 7.72 and 8.02 (s, 1H, CH=N), 7.46 – 7.24 (m, 6H, Ar), 7.01 (dt, J = 8.3, 2.5 Hz, 1H, Ar), 6.85 (dd, J = 8.3, 3.2 Hz, 1H, Ar), 5.17 and 5.24 (s, 2H, CH₂Ar), 4.18 and 4.23 (q, J = 7.1 Hz, 2H, OCH₂), 3.92 and 3.94 (s, 3H, OCH₃), 3.74 and 3.81 (s, 2H, CH₂), 1.22 and 1.30 (t, J = 7.1 Hz, 3H, CH₂CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 169.07, 167.59, 150.39, 150.05, 144.50, 136.66, 128.73, 128.72, 128.12, 128.10, 127.33, 126.96, 121.89, 113.23, 108.85, 70.96, 61.40, 56.11, 41.59, 14.26.

Ethyl 3-(2-(4-((4-fluorobenzyl)oxy)-3-methoxybenzylidene)hydrazineyl)-3-oxopropanoate (XIX)

White solid. Yield: 96 %. Melting point: 148-149 °C. Molecular formula: C₂₀H₂₁FN₂O₅. Molecular weight: 388.40 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 9.95 and 10.29 (s, 1H, NH), 7.72 and 8.03 (s, 1H, CH=N), 7.48 – 7.39 (m, 2H, Ar), 7.28 (d, J = 1.9 Hz, 1H, Ar), 7.11 – 6.99 (m, 3H, Ar), 6.84 (dd, J = 8.3, 3.0 Hz, 1H, Ar), 5.12 and 5.18 (s, 2H, CH₂Ar), 4.19 and 4.23 (q, J = 7.1 Hz, 2H, OCH₂), 3.92 and 3.93 (s, 3H, OCH₃), 3.74 and 3.80 (s, 2H, CH₂), 1.22 and 1.30 (t, J = 7.1 Hz, 3H, CH₂CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 169.06, 167.57, 150.18, 150.10, 144.39, 132.41, 132.38, 129.33, 129.25, 127.16, 121.82, 115.74, 115.55, 113.32, 108.88, 70.36, 61.40, 56.09, 41.59, 14.26.

General procedure for the synthesis of final hydrazones 1-46.

To a solution of proper intermediate **VIII-XIX** (0.8 mmol) and of the appropriate 2-hydroxybenzaldehyde (0.8 mmol) in abs. ethanol (8 mL), piperidine (3 mmol) was added. The reaction mixture was stirred for 5 h under reflux. Afterwards, it was concentrated under vacuum and crystallized at -20 °C. The precipitate was filtered and washed with anhydrous diethyl ether to obtain the desired compounds as yellow solids.

For compounds in which R = OH, either at position C-8 or C-7 of the coumarin ring, the amount of piperidine was reduced (0.1 mL, 1 mmol) and the purification

required and additional step. In detail, the reaction mixture was allowed to cool down, and a solution of iced water and hydrochloric acid (10 mL + 5 mL of 1N HCl) was added to afford the complete precipitation of the compounds. After filtration and washing with water, the compounds were purified by crystallization from ethanol.

As will be discussed in detail later, compounds **1-46** and their corresponding linear hydrazides **VIII-XIX** exist in solution as an equilibrium of two possible rotamers. In the ^1H NMR spectra, signals for which the corresponding resonance of the alternative rotamer is detectable are annotated as “and.” The first value reported represents the signal of the major rotamer.

(E)-N'-Benzylidene-2-oxo-2H-chromene-3-carbohydrazide (1)

Compound was obtained according to the general procedure reported above with intermediate **VIII** and 2-hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 82 %. Melting point: 258-262 °C. Molecular formula: $\text{C}_{17}\text{H}_{12}\text{N}_2\text{O}_3$. Molecular weight: 292.29 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ : 11.73 and 11.97 (s, 1H, NH), 8.89 and 8.67 (s, 1H, Ar, CH=N), 8.44 and 8.28 (s, 1H, Ar), 7.99 (dd, $J = 7.9, 1.7$ Hz, 1H, Ar), 7.82 – 7.67 (m, 3H, Ar), 7.56 – 7.41 (m, 5H, Ar).

^{13}C NMR (101 MHz, DMSO- d_6) δ : 160.43, 158.66, 154.45, 149.96, 148.43, 134.89, 134.50, 130.99, 130.85, 129.42, 129.37, 127.93, 127.22, 125.82, 119.84, 118.97, 116.80.

(E)-N'-(3,4-Dihydroxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (2)

Compound was obtained according to the general procedure reported above with intermediate **IX** and hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 52 %. Melting point: > 260 °C. Molecular formula: $\text{C}_{17}\text{H}_{12}\text{N}_2\text{O}_5$. Molecular weight: 324.29 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ : 11.55 and 11.71 (s, 1H, NH), 9.43 and 9.28 (s, 1H, OH), 9.24 and 9.11 (s, 1H, OH), 8.88 and 8.67 (s, 1H, Ar), 8.21 (s, 1H, CH=N), 8.01 - 7.94 (m, 2H, Ar), 7.83-7.22 (m, 3H, Ar), 6.98 – 6.73 (m, 2H, Ar).

^{13}C NMR (101 MHz, DMSO- d_6) δ : 160.14, 158.11, 154.40, 150.37, 149.02, 148.49, 148.15, 146.30, 130.86, 130.09, 125.79, 121.59, 119.09, 117.01, 116.82, 116.10, 113.43.

(E)-N'-(3-Hydroxy-4-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (3)

Compound was obtained according to the general procedure reported above with intermediate **X** and hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 74 %. Melting point: > 270 °C. Molecular formula: C₁₈H₁₄N₂O₆. Molecular weight: 354.32 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: ¹H NMR (400 MHz, DMSO-d₆): δ 11.57 and 11.76 (s, 1H, NH); 9.29 and 9.14 (s, 1H, OH); 8.85 and 8.67 (s, 1H, CH=C); 8.26 and 8.24 (s, 1H, N=CH); 8.04-6.87 (m, 7H, Ar); 3.78 and 3.71 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-D₆) δ 160.46, 158.34, 150.38, 149.87, 148.58, 148.55, 145.05, 143.05, 125.84, 125.78, 123.02, 120.83, 120.61, 119.95, 119.69, 116.02, 109.93, 56.11.

(E)-N'-(4-Hydroxy-3-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (4)

Compound was obtained according to the general procedure reported above with intermediate **XI** and hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 88 %. Melting point: 227-228 °C. Molecular formula: C₁₈H₁₄N₂O₅. Molecular weight: 388.40 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.58 and 11.81 (s, 1H, NH); 9.60 and 9.44 (s, 1H, OH); 8.86 and 8.67 (s, 1H, CH=C); 8.29 and 8.24 (s, 1H, N=CH); 8.03-6.77 (m, 7H, Ar); 3.80 and 3.56 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-D₆) δ 160.51, 158.26, 154.40, 150.43, 149.89, 148.53, 148.11, 134.79, 130.79, 125.82, 125.80, 123.05, 119.96, 119.00, 116.78, 116.02, 109.92, 56.11.

(E)-N'-(4-Hydroxy-3,5-dimethoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (5)

Compound was obtained according to the general procedure reported above with intermediate **XII** and hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 70 %. Melting point: 246-247 °C. Molecular formula: C₁₉H₁₆N₂O₆. Molecular weight: 368.35 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.62 and 11.89 (s, 1H, NH), 9.18 and 8.91 (s, 1H, OH), 8.83 and 8.25 (s, 1H, Ar), 8.28 and 7.98 (s, 1H, CH=N), 7.84 – 7.70 (m, 2H, Ar), 7.54-7.39 (m, 2H, Ar), 7.00 and 6.70 (s, 2H, Ar), 3.79 and 3.58 (s, 6H, 2 OCH₃).

¹³C NMR (101 MHz, DMSO-D₆) δ 160.55, 158.27, 154.40, 150.57, 148.65, 148.10, 138.83, 134.81, 130.80, 125.81, 124.59, 119.96, 118.99, 116.78, 105.49, 56.55.

(E)-2-Oxo-N'-(3,4,5-trimethoxybenzylidene)-2H-chromene-3-carbohydrazide (6)

Compound was obtained according to the general procedure reported above with intermediate **XIII** and hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 87 %. Melting point: 192-194 °C. Molecular formula: C₂₀H₁₈N₂O₆. Molecular weight: 382.37 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.71 and 12.03 (s, 1H, NH), 8.87 and 8.62 (d, J = 0.7 Hz, 1H, Ar), 8.34 and 8.27 (s, 1H, CH=N), 7.99 (dd, J = 7.8, 1.6 Hz, 1H, Ar), 7.77 – 7.71 and 7.68 – 7.61 (m, 1H, Ar), 7.51 (d, J = 8.2 Hz, 1H, Ar), 7.43 and 7.40 – 7.33 (td, J = 7.6, 1.1 Hz, 1H, Ar), 7.04 (s, 2H, Ar), 3.80 and 3.70 (s, 6H, 2 OCH₃), 3.68 and 3.59 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.48, 158.54, 154.43, 153.73, 149.99, 148.23, 140.00, 134.87, 130.83, 129.91, 125.83, 119.94, 118.96, 116.80, 105.15, 60.68, 56.48, 56.47.

(E)-N'-Benzylidene-8-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (7)

Compound was obtained according to the general procedure reported above with intermediate **VIII** and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 48 %. Melting point: > 280 °C. Molecular formula: C₁₇H₁₂N₂O₄. Molecular weight: 308.29 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.72 and 11.95 (s, 1H, NH), 10.16 (s, 1H, OH), 8.87 and 8.66 (s, 1H, Ar, CH=N), 8.44 and 8.27 (s, 1H, Ar), 8.01 (dd, J = 7.8, 1.6 Hz, 1H, Ar), 7.95 – 7.65 (m, 3H, Ar), 7.55 – 7.42 (m, 4H, Ar).

¹³C NMR (101 MHz, DMSO-d₆) δ: 163.21, 160.17, 154.51, 149.96, 148.43, 134.89, 134.58, 131.13, 130.85, 129.44, 129.38, 127.93, 127.22, 125.82, 119.84, 118.97, 116.80.

(E)-N'-(3,4-Dihydroxybenzylidene)-8-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (8)

Compound was obtained according to the general procedure reported above with intermediate **IX** and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 57 %. Melting point: > 280 °C. C₁₇H₁₂N₂O₆. Molecular weight: 340.29 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.66 and 11.54 (s, 1H, NH), 10.44 and 10.31 (s, 1H, OH), 9.40 and 9.30 (s, 1H, OH), 9.26 and 9.10 (s, 1H, OH), 8.80 and 8.16 (s, 1H, Ar), 8.21 and 7.81 (s, 1H, CH=N), 7.38 (dd, J = 6.8, 2.5 Hz, 1H, Ar), 7.26-7.10 (m, 3H, Ar), 6.95 (dd, J = 8.2, 2.0 Hz, 1H, Ar), 6.75 (d, J = 8.1 Hz, 1H, Ar).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.43, 158.31, 150.30, 148.88, 148.60, 146.25, 145.05, 143.04, 125.92, 125.77, 121.55, 120.81, 120.61, 119.96, 119.66, 116.12, 113.51.

(E)-8-Hydroxy-N'-(3-hydroxy-4-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (9)

Compound was obtained according to the general procedure reported above with intermediate **X** and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 91 %. Melting point: 253-254 °C. Molecular formula: $C_{18}H_{14}N_2O_5$.

^1H NMR (400 MHz, DMSO- d_6): δ 11.59 and 11.79 (s, 1H, NH); 10.14 and 10.28 (s, 1H, OH), 9.57 and 9.42 (s, 1H, OH); 8.80 and 8.29 (s, 1H, CH=C); 8.17 and 7.85 (s, 1H, N=CH); 7.42 – 7.06 (m, 6H, Ar); 6.81 (d, J = 8.1 Hz, 1H, Ar), 3.80 and 3.58 (s, 3H, OCH₃).

^{13}C NMR (101 MHz, DMSO- D_6) δ 160.46, 158.34, 150.38, 149.87, 148.58, 148.55, 145.05, 143.05, 125.84, 125.78, 123.02, 120.83, 120.61, 119.95, 119.69, 116.02, 109.93, 56.11.

(E)-8-Hydroxy-N'-(4-hydroxy-3-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (10)

Compound was obtained according to the general procedure reported above with intermediate **XI** and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 46 %. Melting point: > 300 °C. Molecular formula: $C_{18}H_{14}N_2O_6$. Molecular weight: 354.32 g/mol.

^1H NMR (400 MHz, DMSO- D_6) δ 11.60 and 11.75 (s, 1H, NH), 10.44 and 10.31 (s, 1H, OH), 9.30 and 9.15 (s, 1H, OH), 8.81 (s, 1H, CH=C), 8.26 and 8.17 (s, 1H, N=CH), 7.42 – 6.83 (m, 6H, Ar), 3.77 and 3.72 (s, 3H, OCH₃).

^{13}C NMR (101 MHz, DMSO- D_6) δ 160.42, 158.42, 150.64, 149.99, 148.68, 147.40, 145.05, 143.05, 127.31, 125.77, 121.30, 120.62, 119.96, 119.63, 113.04, 112.40, 56.12.

(E)-8-Hydroxy-N'-(4-hydroxy-3,5-dimethoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (11)

Compound was obtained according to the general procedure reported above with intermediate **XII** and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 28 %. Melting point: > 250 °C. Molecular formula: $C_{19}H_{16}N_2O_7$. Molecular weight: 384.34 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ : 11.64 and 11.88 (s, 1H, NH), 10.46 and 10.28 (s, 1H, OH), 8.97 (s, 1H, OH), 8.83 and 8.17 (s, 1H, Ar), 8.28 and 7.82 (s, 1H, CH=N), 7.44-7.22 (m, 3H, Ar), 7.01 and 6.71 (s, 2H, Ar), 3.78 and 3.59 (s, 6H, 2 OCH₃).

^{13}C NMR (101 MHz, DMSO- d_6) δ : 160.50, 158.35, 150.53, 148.64, 148.57, 145.05, 143.03, 138.81, 125.79, 124.61, 120.62, 119.95, 119.69, 105.48, 56.54.

(E)-8-Hydroxy-2-oxo-N'-(3,4,5-trimethoxybenzylidene)-2H-chromene-3-carbohydrazide (12)

Compound was obtained according to the general procedure reported above with intermediate **XIII** and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 33 %. Melting point: > 260 °C. Molecular formula: C₂₀H₁₈N₂O₇. Molecular weight: 398.37 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.73 and 12.01 (s, 1H, NH), 10.47 and 10.29 (s, 1H, OH), 8.80 and 8.19 (d, J = 0.7 Hz, 1H, Ar), 8.34 and 7.86 (s, 1H, CH=N), 7.40 (dd, J = 7.8, 1.6 Hz, 2H, Ar), 7.25 – 7.14 (m, 2H, Ar), 7.04 and 6.75 (s, 2H, Ar), 3.80 and 3.61 (s, 6H, 2 OCH₃), 3.67 and 3.59 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.44, 158.60, 153.73, 149.95, 148.71, 143.06, 140.00, 129.92, 125.80, 120.89, 120.64, 119.92, 119.65, 105.15, 56.48, 56.07.

(E)-N'-Benzylidene-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (13)

Compound was obtained according to the general procedure reported above with intermediate **VIII** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 80 %. Melting point: > 260 °C. Molecular formula: C₁₈H₁₄N₂O₄. Molecular weight: 322.32 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.73 and 11.98 (s, 1H, NH), 8.87 and 8.25 (s, 1H, Ar, CH=N), 8.44 and 8.00 (s, 1H, Ar), 7.80 – 7.67 (m, 2H, Ar), 7.52 (dd, J = 7.7, 1.5 Hz, 2H, Ar), 7.47 – 7.40 (m, 4H, Ar), 7.37 (d, J = 7.8 Hz, 1H, Ar), 7.35 – 7.30 (m, 1H, Ar), 3.91 and 3.78 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 169.62, 160.16, 158.62, 150.01, 148.67, 146.86, 143.73, 134.50, 131.00, 129.43, 127.94, 125.77, 121.73, 119.94, 119.53, 116.80, 56.76.

(E)-N'-(3,4-Dihydroxybenzylidene)-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (14)

Compound was obtained according to the general procedure reported above with intermediate **IX** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 66 %. Melting point: 195-196 °C. Molecular formula: C₁₈H₁₄N₂O₆. Molecular weight: 354.32 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.61 and 11.78 (s, 1H, NH), 9.41 and 9.29 (s, 1H, OH), 9.19 and 9.08 (s, 1H, OH), 8.84 and 8.17 (s, 1H, Ar), 8.21 and 7.80 (s, 1H, CH=N), 7.59 – 6.68 (m, 7H, Ar), 3.91 and 3.77 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.24, 158.15, 150.49, 149.41, 148.32, 146.85, 146.46, 143.67, 125.73, 125.55, 121.67, 121.59, 120.02, 119.58, 116.07, 113.35, 56.75.

(E)-N'-(3-Hydroxy-4-methoxybenzylidene)-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (15)

Compound was obtained according to the general procedure reported above with intermediate **X** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 89 %. Melting point: 259-260 °C. Molecular formula: C₁₈H₁₆N₂O₅. Molecular weight: 340.34 g/mol.

¹H NMR (400 MHz, DMSO-d₆): δ 11.59 AND 11.76 (s, 1H, NH); 9.30 and 9.15 (s, 1H, OH); 8.85 and 8.22 (s, 1H, CH=); 8.26 and 7.85 (s, 1H, N=CH); 7.55-6.99 (m, 6H, Ar); 3.93 and 3.91 (s, 3H, OCH₃); 3.81 and 3.71 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-D₆) δ 160.21, 158.29, 150.65, 150.09, 148.45, 147.40, 146.86, 143.70, 127.28, 125.74, 121.69, 121.32, 119.98, 119.56, 113.05, 112.39, 56.77, 56.12.

(E)-N'-(4-Hydroxy-3-methoxybenzylidene)-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (16)

Compound was obtained according to the general procedure reported above with intermediate **XI** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 87 %. Melting point: 274-275 °C. Molecular formula: C₁₈H₁₆N₂O₅.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.58 and 11.82 (s, 1H, NH); 9.60 (s, 1H, OH); 8.83 and 8.21 (s, 1H, CH=); 8.29 and 7.84 (s, 1H, N=CH); 7.54-6.78 (m, 6H, Ar); 3.91 and 3.90 (s, 3H, OCH₃); 3.80 and 3.57 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-D₆) δ 160.25, 158.21, 150.47, 149.89, 148.55, 148.35, 146.85, 143.69, 125.81, 125.74, 123.06, 121.68, 119.56, 116.73, 116.01, 109.90, 56.76, 56.10.

(E)-N'-(4-Hydroxy-3,5-dimethoxybenzylidene)-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (17)

Compound was obtained according to the general procedure reported above with intermediate **XII** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 65 %. Melting point: 256-257 °C. Molecular formula: C₂₀H₁₈N₂O₇. Molecular weight: 398.37 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.62 and 11.90 (s, 1H, NH), 8.95 (s, 1H, OH), 8.83 and 8.22 (s, 1H, Ar), 8.28 and 7.81 (s, 1H, CH=N), 7.52 (dd, J = 7.8, 1.5 Hz, 1H, Ar), 7.42-7.29 (m, 3H, Ar), 7.00 and 6.70 (s, 2H, Ar), 3.91 and 3.91 (s, 3H, OCH₃), 3.78 and 3.59 (s, 6H, 2 OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.29, 158.22, 150.62, 148.64, 148.34, 146.85, 143.68, 138.82, 125.76, 124.58, 121.69, 120.05, 119.55, 116.72, 105.48, 56.76, 56.54, 56.07.

(E)-8-Methoxy-2-oxo-N'-(3,4,5-trimethoxybenzylidene)-2H-chromene-3-carbohydrazide (18)

Compound was obtained according to the general procedure reported above with intermediate XIII and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 78 %. Melting point: 183-188°C. Molecular formula: C₂₁H₂₀N₂O₇. Molecular weight: 412.40 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.71 and 12.04 (s, 1H, NH), 8.84 and 8.62 (s, 1H, Ar), 8.34 and 7.86 (s, 1H, CH=N), 7.52 (dd, J = 7.8, 1.5 Hz, 1H, Ar), 7.42 (dd, J = 8.3, 1.5 Hz, 1H, Ar), 7.37 (d, J = 7.8 Hz, 1H, Ar), 7.04 and 6.74 (s, 2H, Ar), 3.91 and 3.90 (s, 3H, OCH₃), 3.80 and 3.60 (s, 6H, 2 OCH₃), 3.67 and 3.59 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.23, 158.48, 153.72, 150.04, 148.48, 146.85, 143.70, 140.00, 129.90, 125.77, 121.71, 120.01, 119.52, 116.79, 105.14, 60.67, 56.76, 56.47, 55.97.

(E)-N'-(3-Methoxy-4-phenoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (19)

Compound was obtained according to the general procedure reported above with intermediate XVII and 2-hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 85 %. Melting point: 256-261 °C. Molecular formula: C₂₄H₁₈N₂O₅. Molecular weight: 414.42 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.74 and 12.03 (s, 1H, NH), 8.89 and 8.29 (s, 1H, Ar), 8.43 and 7.97 (s, 1H, CH=N), 8.07 – 6.71 (m, 12H, Ar), 3.80 and 3.57 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.48, 157.59, 154.45, 149.57, 148.36, 134.89, 131.45, 130.86, 130.44, 125.83, 123.40, 122.10, 121.37, 119.89, 118.98, 117.47, 116.81, 111.15, 56.27.

(E)-N'-(4-(Benzyloxy)-3-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (20)

Compound was obtained according to the general procedure reported above with intermediate **XVIII** and 2-hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 86 %. Melting point: 213-214 °C. Molecular formula: C₂₅H₂₀N₂O₅. Molecular weight: 428.44 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.64 and 11.91 (s, 1H, NH), 8.87 and 8.26 (s, 1H, Ar), 8.34 and 7.88 (s, 1H, CH=N), 8.05 – 6.92 (m, 12H, Ar), 5.11 and 5.03 (s, 2H, CH₂Ph), 3.80 and 3.55 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.50, 158.38, 154.41, 150.55, 150.07, 149.84, 148.20, 137.27, 134.83, 130.82, 129.01, 128.53, 128.49, 127.40, 125.81, 122.72, 119.92, 118.98, 116.78, 113.53, 109.28, 70.38, 56.06, 55.52.

(E)-N'-(4-((4-Fluorobenzyl)oxy)-3-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (21)

Compound was obtained according to the general procedure reported above with intermediate **XIX** and 2-hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 91 %. Melting point: 235-256 °C. Molecular formula: C₂₅H₁₉FN₂O₅. Molecular weight: 428,44 g/mol.

¹H NMR (400 MHz, DMSO-d₆): δ 11.63 and 11.89 (s, 1H, NH), 8.87 and 8.25 (s, 1H, CH=N), 8.34 and 7.89 (s, 1H, Ar), 8.04 – 6.95 (m, 11 H, Ar), 5.10 and 5.02 (s, 2H, CH₂Ph), 3.80 and 3.55 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.48, 158.39, 154.42, 150.45, 150.06, 149.89, 148.18, 134.82, 133.55, 130.81, 130.75, 130.67, 127.54, 125.81, 122.65, 119.95, 118.99, 116.78, 115.93, 115.72, 113.70, 109.43, 69.70, 56.10.

(E)-N'-(4-Methoxy-3-phenoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (22)

Compound was obtained according to the general procedure reported above with intermediate **XIV** and 2-hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 92 %. Melting point: 243–244 °C. Molecular formula: C₂₄H₁₈N₂O₅. Molecular weight: 414.42 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.64 and 11.87 (s, 1H, NH), 8.85 and 8.19 (s, 1H, Ar), 8.34 and 7.89 (s, 1H, CH=N), 8.03 – 6.70 (m, 12H, Ar), 3.78 and 3.71 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.46, 158.43, 157.88, 154.42, 153.67, 149.22, 148.29, 144.81, 134.85, 130.82, 130.45, 130.19, 127.69, 126.14, 125.81, 123.21, 119.82, 119.29, 118.97, 117.56, 117.17, 116.78, 113.95, 56.45.

(E)-N'-(3-(Benzyloxy)-4-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (23)

Compound was obtained according to the general procedure reported above with intermediate **XV** and 2-hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 92 %. Melting point: 214-215 °C. Molecular formula: C₂₅H₂₀N₂O₅. Molecular weight: 428.44 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.63 and 11.87 (s, 1H, NH), 8.88 and 8.27 (s, 1H, Ar), 8.33 and 7.86 (s, 1H, CH=N), 8.07 – 6.87 (m, 12H, Ar), 5.10 and 4.86 (s, 2H, CH₂Ph), 3.79 and 3.72 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.49, 158.36, 154.41, 151.87, 150.08, 148.61, 148.20, 137.37, 134.83, 130.81, 128.96, 128.89, 128.51, 128.02, 127.09, 125.81, 123.03, 119.92, 118.98, 116.79, 112.35, 110.97, 70.45, 56.21.

(E)-N'-(3-((4-Fluorobenzyl)oxy)-4-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (24)

Compound was obtained according to the general procedure reported above with intermediate **XVI** and 2-hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 93 %. Melting point: 235-236 °C. Molecular formula: C₂₅H₁₉FN₂O₅. Molecular weight: 446.43 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.62 and 11.86 (s, 1H, NH), 8.87 and 8.24 (s, 1H, Ar), 8.34 and 7.87 (s, 1H, CH=N), 8.02 – 6.91 (m, 11H, Ar), 5.09 and 4.84 (s, 2H, CH₂Ph), 3.79 and 3.72 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 161.18, 160.48, 158.37, 154.42, 151.91, 150.05, 148.54, 148.18, 134.82, 133.67, 130.81, 130.74, 130.66, 127.13, 125.81, 123.12, 119.95, 118.99, 116.78, 115.88, 115.67, 112.43, 111.18, 69.81, 56.24.

(E)-8-Hydroxy-N'-(3-methoxy-4-phenoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (25)

Compound was obtained according to the general procedure reported above with intermediate **XVII** and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 93 %. Melting point: 243-246 °C. Molecular formula: C₂₄H₁₈N₂O₆. Molecular weight: 430.42 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.75 and 12.01 (s, 1H, NH), 10.47 and 10.30 (s, 1H, OH), 8.83 and 8.21 (s, 1H, Ar), 8.43 and 7.96 (s, 1H, CH=N), 7.56 – 6.76 (m, 11H, Ar), 3.80 and 3.58 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.42, 158.65, 157.59, 151.81, 149.52, 148.83, 146.63, 145.07, 143.08, 131.47, 130.43, 130.33, 125.81, 123.38, 122.07, 121.37, 120.90, 120.66, 119.93, 119.59, 117.46, 117.03, 111.16, 56.27.

(E)-8-Hydroxy-N'-(4-methoxy-3-phenoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (26)

Compound was obtained according to the general procedure reported above with intermediate XIV and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 62 %. Melting point: 270-271 °C. Molecular formula: C₂₄H₁₈N₂O₆. Molecular weight: 430.42 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.66 and 11.85 (s, 1H, NH), 10.46 and 10.34 (s, 1H, OH), 8.78 and 8.12 (s, 1H, Ar), 8.35 and 7.89 (s, 1H, CH=N), 7.60 – 6.73 (m, 11H, Ar), 3.78 and 3.71 (s, 3H, CH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.40, 158.51, 157.87, 153.65, 149.16, 148.76, 145.05, 144.80, 143.04, 130.45, 127.70, 126.13, 125.78, 123.21, 120.87, 120.63, 119.92, 119.55, 119.27, 117.17, 113.94, 56.45.

(E)-N'-(3-(Benzyloxy)-4-methoxybenzylidene)-8-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (27)

Compound was obtained according to the general procedure reported above with intermediate XV and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 75 %. Melting point: 258-260 °C. Molecular formula: C₂₅H₂₀N₂O₆. Molecular weight: 444.44 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.65 and 11.85 (s, 1H, NH), 10.46 and 10.35 (s, 1H, OH), 8.80 and 8.19 (s, 1H, Ar), 8.33 and 7.86 (s, 1H, CH=N), 7.51 – 6.88 (m, 11H, Ar), 5.10 and 4.88 (s, 2H, CH₂Ph), 3.79 and 3.73 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.43, 158.44, 151.86, 150.03, 148.66, 148.61, 145.06, 143.05, 137.38, 128.96, 128.87, 128.51, 127.89, 127.11, 125.79, 123.01, 120.85, 120.62, 119.94, 119.66, 112.36, 110.98, 70.45, 56.21.

(E)-N'-(3-((4-Fluorobenzyl)oxy)-4-methoxybenzylidene)-8-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (28)

Compound was obtained according to the general procedure reported above with intermediate XVI and 2,3-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 74 %. Melting point: 282-284 °C. Molecular formula: C₂₅H₁₉FN₂O₆. Molecular weight: 462.43 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.64 and 11.84 (s, 1H, NH); 10.44 (s, 1H, OH), 8.80 and 8.19 (s, 1H, Ar), 8.34 and 7.87 (s, 1H, CH=N), 7.64 – 6.84 (m, 9H, Ar), 5.09 and 4.86 (s, 3H, OCH₃), 3.79 and 3.72 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.42, 158.45, 151.90, 150.00, 148.66, 148.53, 145.02, 143.05, 133.66, 130.75, 130.67, 127.14, 125.79, 123.11, 120.83, 120.65, 119.94, 119.64, 115.88, 115.67, 112.42, 111.17, 69.80, 56.23.

(E)-8-Methoxy-N'-(4-methoxy-3-phenoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (29)

Compound was obtained according to the general procedure reported above with intermediate **XIV** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 74 %. Melting point: 228-229 °C. Molecular formula: C₂₅H₂₀N₂O₆. Molecular weight: 444.44 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.64 and 11.87 (s, 1H, NH), 8.82 and 8.17 (s, 1H, Ar), 8.35 and 7.89 (s, 1H, CH=N), 7.59 – 6.67 (m, 11H, Ar), 3.91 and 3.92 (s, 3H, OCH₃), 3.78 and 3.71 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.19, 158.37, 157.88, 153.67, 149.26, 148.54, 146.84, 144.79, 143.69, 130.44, 130.11, 127.68, 126.15, 125.75, 123.21, 121.70, 119.88, 119.52, 119.29, 117.47, 117.16, 116.77, 113.93, 56.75, 56.44.

(E)-N'-(3-(Benzyloxy)-4-methoxybenzylidene)-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (30)

Compound was obtained according to the general procedure reported above with intermediate **XV** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 93 %. Melting point: 228-229 °C. Molecular formula: C₂₆H₂₂N₂O₆. Molecular weight: 458.47 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.63 and 11.88 (s, 1H, NH), 8.84 and 8.24 (s, 1H, Ar), 8.34 and 7.86 (s, 1H, CH=N), 7.60 – 6.86 (m, 11H, Ar), 5.10 and 4.83 (s, 2H, CH₂Ph), 3.91 and 3.75 (s, 3H, OCH₃), 3.79 and 3.72 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.22, 158.31, 151.87, 150.12, 148.61, 148.43, 146.85, 143.69, 137.37, 128.96, 128.88, 128.51, 128.17, 127.08, 125.75, 123.03, 121.69, 120.01, 119.55, 116.74, 115.38, 112.35, 110.97, 70.45, 56.76, 56.21.

(E)-N'-(3-((4-Fluorobenzyl)oxy)-4-methoxybenzylidene)-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (31)

Compound was obtained according to the general procedure reported above with intermediate **XVI** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 93 %. Melting point: 219-220 °C. Molecular formula: C₂₆H₂₁FN₂O₆. Molecular weight: 476.46 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.62 and 11.86 (s, 1H, NH), 8.84 and 8.23 (s, 1H, CH=N), 8.34 and 7.86 (s, 1H, Ar), 7.90 – 6.88 (m, 10H, Ar), 5.09 and 4.82 (s, 2H, CH₂Ph), 3.92 and 3.76 (s, 3H, OCH₃), 3.79 and 3.71 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.21, 158.33, 151.92, 150.10, 148.53, 148.42, 146.87, 143.72, 130.75, 130.66, 127.12, 125.76, 123.13, 121.71, 120.04, 119.56, 116.79, 115.88, 115.66, 112.42, 111.18, 69.81, 56.79, 56.24.

(E)-8-Methoxy-N'-(3-methoxy-4-phenoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (32)

Compound was obtained according to the general procedure reported above with intermediate **XVII** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 75 %. Melting point: 239-240 °C. Molecular formula: C₂₅H₂₀N₂O₆. Molecular weight: 444.44 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.73 and 12.03 (s, 1H, NH), 8.86 and 8.26 (s, 1H, Ar), 8.43 and 7.96 (s, 1H, CH=N), 7.59 – 6.76 (m, 11H, Ar), 3.92 and 3.88 (s, 3H, OCH₃), 3.79 and 3.57 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.21, 158.53, 157.59, 151.81, 149.61, 148.59, 146.86, 146.65, 143.73, 131.45, 130.43, 130.33, 125.77, 123.39, 122.09, 121.73, 121.37, 119.96, 119.53, 117.47, 117.03, 116.80, 111.15, 56.77, 56.27.

(E)-N'-(4-(Benzyloxy)-3-methoxybenzylidene)-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (33)

Compound was obtained according to the general procedure reported above with intermediate **XVIII** and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 91 %. Melting point: 233-234 °C. Molecular formula: C₂₆H₂₂N₂O₆. Molecular weight: 458.47 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.64 and 11.91 (s, 1H, NH), 8.84 and 8.23 (s, 1H, Ar), 8.34 and 7.87 (s, 1H, CH=N), 7.60 – 6.86 (m, 11H, Ar), 5.11 and 5.03 (s, 2H, CH₂Ph), 3.91 and 3.90 (s, 3H, OCH₃), 3.80 and 3.56 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 160.23, 158.34, 150.56, 150.12, 149.85, 148.43, 146.85, 143.70, 137.27, 129.01, 128.53, 128.49, 127.40, 125.76, 122.73, 121.70, 120.02, 119.55, 116.76, 113.55, 109.30, 70.38, 56.77, 56.07.

(E)-N'-(4-((4-Fluorobenzyl)oxy)-3-methoxybenzylidene)-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (34)

Compound was obtained according to the general procedure reported above with intermediate XIX and 2-hydroxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 92 %. Melting point: 254-255 °C. Molecular formula: C₂₆H₂₁FN₂O₆. Molecular weight: 476.46 g/mol.

¹H NMR (400 MHz, DMSO-d₆): ¹H NMR (400 MHz, DMSO-d₆) δ: 11.63 and 11.89 (s, 1H, NH), 8.84 and 8.22 (s, 1H, CH=N), 8.34 and 7.88 (s, 1H, Ar), 7.89 – 6.95 (m, 10 H, Ar) 5.09 and 5.02 (s, 2H, CH₂Ph), 3.92 and 3.90 (s, 3H, OCH₃), 3.80 and 3.56 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 159.79, 158.39, 150.19, 148.32, 147.03, 146.57, 142.60, 133.66, 130.46, 128.01, 127.43, 125.73, 122.50, 121.79, 120.16, 119.65, 117.11, 115.85, 115.63, 114.40, 110.35, 70.05, 56.96, 56.38.

(E)-7-Hydroxy-N'-(3-hydroxy-4-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (35)

Compound was obtained according to the general procedure reported above with intermediate X and 2,4-dihydroxybenzaldehyde as starting material.

Yellow-orange solid. Yield: 88 %. Melting point: >290 °C. Molecular formula: C₁₈H₁₄N₂O₆. Molecular weight: 354.32 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.61 and 11.85 (s, 1H, NH), 11.09 and 10.74 (s, 1H, OH), 9.29 and 9.15 (s, 1H, OH), 8.80 and 8.52 (s, 1H, CH=N), 8.25 and 8.18 (s, 1H, Ar), 7.82 and 7.59 (d, *J* = 8.7 Hz, 1H, Ar), 7.26 (d, *J* = 2.1 Hz, 1H, Ar), 7.07 (dd, *J* = 8.3, 2.0 Hz, 1H, Ar), 6.93 (d, *J* = 8.4 Hz, 1H, Ar), 6.86 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar), 6.80 (d, *J* = 2.3 Hz, 1H, Ar), 3.77 and 3.71 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 164.43, 161.25, 158.73, 156.88, 150.55, 149.60, 149.07, 147.38, 132.64, 127.41, 121.19, 115.01, 114.16, 113.05, 112.40, 111.75, 102.43, 56.12.

(E)-N'-(3-(Benzyloxy)-4-methoxybenzylidene)-7-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (36)

Compound was obtained according to the general procedure reported above with intermediate **XV** and 2,4-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 95 %. Melting point: 264-265 °C. Molecular formula: C₂₅H₂₀N₂O₆. Molecular weight: 444.44 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.60 and 11.75 (s, 1H, NH), 11.09 and 10.75 (s, 1H, OH), 8.76 and 8.52 (s, 1H, CH=N), 8.44 and 8.19 (d, *J* = 1.8 Hz, 1H, Ar), 7.81 and 7.59 (dd, *J* = 8.7, 1.9 Hz, 1H, Ar), 7.47 – 7.25 (m, 6H, Ar), 7.17 – 7.05 (m, 2H, Ar), 6.87 (dt, *J* = 8.6, 2.2 Hz, 1H, Ar), 6.80 (t, *J* = 2.2 Hz, 1H, Ar), 5.09 and 5.00 (d, *J* = 1.9 Hz, 2H, CH₂Ar), 3.85 and 3.81 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 164.41, 160.83, 159.13, 156.91, 153.32, 148.94, 147.10, 144.83, 137.55, 132.59, 129.00, 128.85, 128.68, 128.46, 125.02, 117.80, 114.97, 114.51, 111.66, 102.45, 75.43, 56.43.

(E)-N'-(3-((4-Fluorobenzyl)oxy)-4-methoxybenzylidene)-7-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (37)

Compound was obtained according to the general procedure reported above with intermediate **XVI** and 2,4-dihydroxybenzaldehyde as starting material.

Pale yellow solid. Yield: 89 %. Melting point: 272-273°C. Molecular formula: C₂₅H₁₉FN₂O₆. Molecular weight: 462.43 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.59 and 11.82 (s, 1H, NH), 11.10 and 10.78 (s, 1H, OH), 8.77 and 8.51 (s, 1H, CH=N), 8.44 and 8.18 (s, 1H, Ar), 7.81 and 7.58 (d, *J* = 8.6 Hz, 1H, Ar), 7.47 (ddd, *J* = 9.0, 5.6, 2.7 Hz, 2H, Ar), 7.40 (dd, *J* = 6.3, 3.2 Hz, 1H, Ar), 7.21 – 7.09 (m, 4H, Ar), 6.87 (dd, *J* = 8.6, 2.2 Hz, 1H, Ar), 6.80 (d, *J* = 2.3 Hz, 1H, Ar), 5.09 and 4.98 (s, 2H, CH₂Ar), 3.84 and 3.79 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-D₆) δ 164.41, 160.83, 159.13, 156.91, 153.32, 148.94, 147.10, 144.83, 137.55, 132.59, 129.00, 128.85, 128.68, 128.46, 125.02, 117.79, 114.97, 114.51, 111.66, 102.45, 75.43, 56.43.

(E)-7-Hydroxy-N'-(4-hydroxy-3-methoxybenzylidene)-2-oxo-2H-chromene-3-carbohydrazide (38)

Compound was obtained according to the general procedure reported above with intermediate **XI** and 2,4-dihydroxybenzaldehyde as starting material.

Yellow-orange solid. Yield: 85 %. Melting point: >290 °C. Molecular formula: C₁₈H₁₄N₂O₆. Molecular weight: 354.32 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.52 and 11.75 (s, 1H, NH), 11.09 and 10.74 (s, 1H, OH), 9.28 (s, 1H, OH), 8.82 and 8.73 (s, 1H, CH=N), 8.25 and 8.12 (s, 1H, Ar), 7.82 (d, *J* = 8.7 Hz, 1H, Ar), 7.24 (d, *J* = 2.1 Hz, 1H, Ar), 7.06 (dd, *J* = 8.3, 2.0 Hz, 1H, Ar), 6.95 (d, *J* = 8.4 Hz, 1H, Ar), 6.87 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar), 6.80 (d, *J* = 2.3 Hz, 1H, Ar), 3.80 and 3.77 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 164.47, 161.28, 158.76, 156.89, 150.48, 149.64, 149.06, 132.65, 129.00, 128.51, 122.59, 115.03, 114.15, 111.73, 109.36, 102.43, 70.41, 56.08.

(E)-N'-(4-(Benzyloxy)-3-methoxybenzylidene)-7-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (39)

Compound was obtained according to the general procedure reported above with intermediate XVIII and 2,4-dihydroxybenzaldehyde as starting material.

Yellow solid. Yield: 91 %. Melting point: 255-256 °C. Molecular formula: C₂₅H₂₀N₂O₆. Molecular weight: 444.44 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.58 and 11.75 (s, 1H, NH), 11.11 and 10.74 (s, 1H, OH), 8.84 and 8.61 (s, 1H, CH=N), 8.32 and 8.12 (s, 1H, Ar), 7.83 (d, *J* = 8.7 Hz, 1H, Ar), 7.46 – 7.28 (m, 6H, Ar), 7.20 (dd, *J* = 8.4, 1.9 Hz, 1H, Ar), 7.10 (d, *J* = 8.4 Hz, 1H, Ar), 6.87 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar), 6.81 (d, *J* = 2.2 Hz, 1H, Ar), 5.11 and 5.02 (s, 2H, CH₂Ar), 3.80 and 3.76 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 164.47, 161.28, 158.76, 156.89, 150.48, 149.86, 149.64, 149.06, 132.65, 129.00, 128.51, 128.46, 127.55, 122.59, 115.03, 114.15, 113.60, 111.73, 109.36, 102.43, 70.41, 56.08, 44.24.

(E)-N'-(4-((4-Fluorobenzyl)oxy)-3-methoxybenzylidene)-7-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (40)

Compound was obtained according to the general procedure reported above with intermediate XIX and 2,4-dihydroxybenzaldehyde as starting material.

Pale yellow solid. Yield: 91 %. Melting point: 265-266 °C. Molecular formula: C₂₅H₁₉FN₂O₆. Molecular weight: 462.43 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.58 and 11.84 (s, 1H, NH), 11.11 and 10.76 (s, 1H, OH), 8.81 and 8.25 (s, 1H, CH=N), 8.33 and 8.11 (s, 1H, Ar), 7.83 and 7.59 (d, *J* = 8.7 Hz, 1H, Ar), 7.52 – 7.44 (m, 2H, Ar), 7.34 (d, *J* = 1.9 Hz, 1H, Ar), 7.25 – 7.14 (m, 3H, Ar), 7.10 (d, *J* = 8.4 Hz, 1H, Ar), 6.87 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar), 6.80 (d, *J* = 2.3 Hz, 1H, Ar), 5.09 and 4.90 (s, 2H, CH₂Ar), 3.84 and 3.80 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 164.47, 161.29, 158.76, 156.90, 150.35, 149.86, 149.62, 149.07, 133.53, 132.66, 130.76, 130.68, 127.64, 122.55, 115.93, 115.72, 115.03, 114.14, 113.67, 111.73, 109.38, 102.43, 69.69, 56.08.

(E)-N'-(3-hydroxy-4-methoxybenzylidene)-7-methoxy-2-oxo-2H-chromene-3-carbohydrazide (41)

Compound was obtained according to the general procedure reported above with intermediate **X** and 2-hydroxy-4-methoxybenzaldehyde as starting material.

Pale yellow solid. Yield: 90 %. Melting point: 285-287 °C. Molecular formula: C₁₉H₁₆N₂O₆. Molecular weight: 368.35 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.53 and 11.73 (s, 1H, NH), 9.28 and 9.15 (s, 1H, OH), 8.86 and 8.53 (d, *J* = 0.6 Hz, 1H, CH=N), 8.26 and 8.11 (s, 1H, Ar), 7.91 and 7.60 (d, *J* = 8.8 Hz, 1H, Ar), 7.25 (d, *J* = 2.0 Hz, 1H, Ar), 7.12 (d, *J* = 2.5 Hz, 1H, Ar), 7.05 (ddd, *J* = 12.4, 8.5, 2.2 Hz, 2H, Ar), 6.95 (d, *J* = 8.4 Hz, 1H, Ar), 3.88 and 3.85 (s, 3H, OCH₃), 3.81 and 3.77 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 165.16, 161.06, 158.59, 156.77, 150.58, 149.74, 148.78, 147.40, 132.16, 127.38, 121.21, 115.46, 114.32, 113.07, 112.74, 112.41, 100.95, 56.86, 56.12.

(E)-N'-(3-(Benzyloxy)-4-methoxybenzylidene)-7-methoxy-2-oxo-2H-chromene-3-carbohydrazide (42)

Compound was obtained according to the general procedure reported above with intermediate **XV** and 2-hydroxy-4-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 88 %. Melting point: 233-234 °C. Molecular formula: C₂₆H₂₂N₂O₆. Molecular weight: 458.47 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.62 and 11.89 (s, 1H, NH), 8.80 and 8.51 (s, 1H, CH=N), 8.46 and 8.19 (s, 1H, Ar), 7.89 and 7.58 (d, *J* = 8.7 Hz, 1H, Ar), 7.44 (s, 1H, Ar), 7.41 (dd, *J* = 9.6, 2.2 Hz, 3H, Ar), 7.38 – 7.33 (m, 2H, Ar), 7.33 – 7.27 (m, 1H, Ar), 7.16 – 7.09 (m, 2H, Ar), 7.03 (dd, *J* = 8.7, 2.4 Hz, 1H, Ar), 5.07 and 5.00 (s, 2H, CH₂Ar), 3.86 and 3.83 (s, 3H, OCH₃), 3.81 and 3.77 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 165.13, 160.60, 159.02, 156.80, 153.32, 148.61, 147.11, 144.96, 137.56, 132.10, 128.98, 128.85, 128.67, 128.44, 125.03, 117.81, 115.85, 115.23, 114.29, 112.64, 100.96, 75.41, 56.86, 56.43.

(E)-N'-(3-((4-Fluorobenzyl)oxy)-4-methoxybenzylidene)-7-methoxy-2-oxo-2H-chromene-3-carbohydrazide (43)

Compound was obtained according to the general procedure reported above with intermediate **XVI** and 2-hydroxy-4-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 86 %. Melting point: 248-249 °C. Molecular formula: C₂₆H₂₁FN₂O₆. Molecular weight: 476.46 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.62 and 11.76 (s, 1H, NH), 8.81 and 8.52 (s, 1H, CH=N), 8.46 and 8.20 (s, 1H, Ar), 7.90 and 7.58 (d, *J* = 8.7 Hz, 1H, Ar), 7.52 – 7.44 (m, 2H, Ar), 7.40 (dd, *J* = 6.3, 3.1 Hz, 1H, Ar), 7.22 – 7.09 (m, 5H, Ar), 7.04 (dd, *J* = 8.7, 2.4 Hz, 1H, Ar), 5.09 and 4.98 (s, 2H, CH₂Ar), 3.86 and 3.83 (s, 3H, OCH₃), 3.82 and 3.79 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 165.15, 163.70, 160.61, 159.02, 156.81, 153.29, 148.66, 146.96, 144.97, 133.88, 132.11, 131.29, 131.21, 128.47, 125.09, 117.84, 115.82, 115.75, 115.54, 115.01, 114.30, 112.64, 100.97, 74.60, 56.86, 56.43.

(E)-N'-(4-Hydroxy-3-methoxybenzylidene)-7-methoxy-2-oxo-2H-chromene-3-carbohydrazide (44)

Compound was obtained according to the general procedure reported above with intermediate **XI** and 2-hydroxy-4-methoxybenzaldehyde as starting material.

Yellow-orange solid. Yield: 93 %. Melting point: 260-261 °C. Molecular formula: C₁₉H₁₆N₂O₆. Molecular weight: 368.35 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 11.53 and 11.74 (s, 1H, NH), 9.56 and 9.39 (s, 1H, OH), 8.85 and 8.52 (s, 1H, CH=N), 8.28 and 8.12 (s, 1H, Ar), 7.92 and 7.59 (d, *J* = 8.9 Hz, 1H, Ar), 7.29 (d, *J* = 1.9 Hz, 1H, Ar), 7.14 – 7.07 (m, 2H, Ar), 7.03 (dd, *J* = 8.7, 2.4 Hz, 1H, Ar), 6.81 (d, *J* = 8.1 Hz, 1H, Ar), 3.87 and 3.83 (s, 3H, OCH₃), 3.80 and 3.77 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ: 165.14, 161.10, 158.50, 156.76, 150.13, 149.82, 148.69, 148.55, 132.15, 125.90, 122.99, 116.01, 115.50, 114.32, 112.73, 109.88, 100.94, 56.86, 56.11.

(E)-N'-(4-(Benzyloxy)-3-methoxybenzylidene)-7-methoxy-2-oxo-2H-chromene-3-carbohydrazide (45)

Compound was obtained according to the general procedure reported above with intermediate **XVIII** and 2-hydroxy-4-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 89 %. Melting point: 238-239 °C. Molecular formula: $C_{26}H_{22}N_2O_6$. Molecular weight: 458.47 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ : 11.59 and 11.73 (s, 1H, NH), 8.86 and 8.50 (s, 1H, CH=N), 8.33 and 8.18 (s, 1H, Ar), 7.92 and 7.60 (d, J = 8.8 Hz, 1H, Ar), 7.46 – 7.28 (m, 6H, Ar), 7.20 (dd, J = 8.4, 1.9 Hz, 1H, Ar), 7.15 – 7.07 (m, 2H, Ar), 7.04 (dd, J = 8.7, 2.4 Hz, 1H, Ar), 5.11 and 5.02 (s, 2H, CH_2 Ar), 3.88 and 3.85 (s, 3H, OCH_3), 3.80 and 3.76 (s, 3H, OCH_3).

^{13}C NMR (101 MHz, DMSO- d_6) δ : 161.08, 158.63, 156.78, 150.51, 149.78, 148.77, 137.29, 132.18, 129.00, 128.52, 128.46, 122.63, 115.48, 113.61, 112.73, 110.37, 109.37, 109.06, 100.95, 70.41, 56.87, 56.10.

(E)-N'-(4-((4-Fluorobenzyl)oxy)-3-methoxybenzylidene)-7-methoxy-2-oxo-2H-chromene-3-carbohydrazide (46)

Compound was obtained according to the general procedure reported above with intermediate XIX and 2-hydroxy-4-methoxybenzaldehyde as starting material.

Pale yellow solid. Yield: 88 %. Melting point: 245-246 °C. Molecular formula: $C_{26}H_{21}FN_2O_6$. Molecular weight: 476.46 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ : 11.59 and 11.78 (s, 1H, NH), 8.86 and 8.52 (s, 1H, CH=N), 8.34 and 8.11 (s, 1H, Ar), 7.92 and 7.60 (d, J = 8.7 Hz, 1H, Ar), 7.48 (dd, J = 8.4, 5.5 Hz, 2H, Ar), 7.35 (d, J = 1.9 Hz, 1H, Ar), 7.20 (t, J = 8.8 Hz, 3H, Ar), 7.15 – 7.07 (m, 2H, Ar), 7.04 (dd, J = 8.7, 2.4 Hz, 1H, Ar), 5.09 and 5.01 (s, 2H, CH_2 Ar), 3.88 and 3.84 (s, 3H, OCH_3), 3.80 and 3.77 (s, 3H, OCH_3).

^{13}C NMR (101 MHz, DMSO- d_6) δ : 162.07, 161.28, 158.74, 155.87, 150.36, 149.86, 149.62, 149.08, 133.53, 132.66, 130.84, 130.68, 127.64, 122.54, 115.93, 115.64, 115.03, 114.19, 113.67, 111.73, 109.38, 101.23, 70.02, 56.08.

3-Cinnamoyl coumarins

Synthesis of 3-acetyl-2H-chromen-2-one (XX).

A mixture of salicylaldehyde (10 mmol) and methyl acetoacetate (10 mmol) was placed in a round-bottom flask and stirred for 1 minute at room temperature. Piperidine (0.3 mL) was then added, and the reaction mixture was stirred vigorously for 10 minutes, during which the formation of a thick paste was observed. Cold ethanol (4 mL) was added, and the resulting solid was collected

by filtration. The crude product were recrystallized from ethanol to afford the purified compound.

White solid. Melting point: 120-121 °C (119 °C ^[100]). Yield: 68 %. Molecular formula: C₁₁H₈O₃. Molecular weight: 118.18 g/mol. Elemental analysis calculated for C₁₁H₈O₃: C% 70.21, H% 4.29; found C% 70.07, H% 4.12.

General procedure for the synthesis of 3-Cinnamoyl coumarins 47-64.

To a solution of **XX** (1.2 mmol) and of the appropriate aldehyde (1.2 mmol) in abs. EtOH (4 mL), piperidine (0.3 mL, 3 mmol) was added. The synthesis of the aldehydes used has already been described in the section on the synthesis of hydrazone compounds derived from vanillin and isovanillin (aldehydes **II-VII**). The reaction mixture was stirred for 5 h at 90 °C in a sealed tube. Then, was allowed to cool down and crystallized at -20 °C. The precipitate was filtered and washed with cold ethanol to obtain the desired compounds as yellow or grey/light yellow solids.

(E)-3-(3-(4-Hydroxyphenyl)acryloyl)-2H-chromen-2-one (47)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 4-hydroxybenzaldehyde as starting material.

Yellow solid. Yield: 66 %. Melting point: 247-249 °C. Molecular formula: C₁₈H₁₂O₄. Molecular weight: 292.29 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 10.12 (s, 1H, OH), 8.58 (s, 1H, Ar), 7.89 (d, J = 15.6 Hz, 1H, CH=), 7.75 – 7.55 (m, 4H, 1H CH= + 3H Ar), 7.51 – 7.34 (m, 3H, Ar), 6.86 – 6.63 (m, 2H, Ar).

¹³C NMR (101 MHz, DMSO-D₆) δ 187.63, 161.01, 159.00, 154.90, 146.91, 145.53, 134.53, 131.49, 130.84, 129.71, 125.88, 125.45, 125.40, 121.86, 118.99, 116.72, 116.59, 116.55.

(E)-3-(3-(4-Methoxyphenyl)acryloyl)-2H-chromen-2-one (48)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 4-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 62 %. Melting point: 159-161 °C. Molecular formula: C₁₉H₁₄O₄. Molecular weight: 306.32 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.56 (d, J = 0.7 Hz, 1H, Ar), 7.86 (d, J = 15.7 Hz, 1H, CH=), 7.79 (d, J = 15.7 Hz, 1H, CH=), 7.73 – 7.57 (m, 4H, Ar), 7.45 – 7.21 (m, 2H, Ar), 6.97 – 6.83 (m, 2H, Ar), 3.84 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 186.42, 162.08, 155.29, 147.92, 145.21, 134.18, 130.92, 130.06, 128.54, 127.70, 125.64, 125.03, 124.97, 121.74, 117.88, 116.78, 116.12, 114.51, 55.52.

(E)-3-(3-(4-(Benzyloxy)phenyl)acryloyl)-2H-chromen-2-one (49)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 4-(benzyloxy)benzaldehyde as starting material.

Yellow solid. Yield: 63 %. Melting point: 168-169 °C. Molecular formula: C₂₅H₁₈O₄. Molecular weight: 382.42 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.56 (d, J = 0.8 Hz, 1H, Ar), 7.86 (d, J = 15.6 Hz, 1H, CH=), 7.80 (d, J = 15.6 Hz, 1H, CH=), 7.72 – 7.59 (m, 4H, Ar), 7.47 – 7.27 (m, 7H, Ar), 7.14 – 6.92 (m, 2H, Ar), 5.11 (s, 2H, CH₂Ar).

¹³C NMR (101 MHz, CDCl₃) δ: 186.41, 161.24, 159.47, 155.30, 147.94, 145.12, 136.47, 134.19, 130.92, 130.07, 128.77, 128.28, 127.59, 125.62, 125.03, 121.86, 118.72, 116.79, 115.38, 70.21.

(E)-3-(3-(3-Hydroxyphenyl)acryloyl)-2H-chromen-2-one (50)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 3-hydroxybenzaldehyde as starting material.

Grey solid. Yield: 54 %. Melting point: 233-235 °C. Molecular formula: C₁₈H₁₂O₄. Molecular weight: 292.29 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ: 9.65 (s, 1H, OH), 8.64 (s, 1H, Ar), 7.91 (dd, J = 7.8, 1.6 Hz, 1H, Ar), 7.77 – 7.68 (m, 1H, Ar), 7.64 (d, J = 15.9 Hz, 1H, CH=), 7.59 (d, J = 15.9 Hz, 1H, CH=), 7.52 – 7.36 (m, 2H, Ar), 7.28 – 7.02 (m, 3H, Ar), 6.84 (m, 2H, Ar).

¹³C NMR (101 MHz, CDCl₃) δ: 192.41, 166.14, 163.82, 163.08, 159.79, 152.37, 149.55, 139.53, 135.78, 135.40, 130.76, 130.27, 129.82, 125.41, 123.52, 123.19, 121.51, 119.79.

(E)-3-(3-(3-Methoxyphenyl)acryloyl)-2H-chromen-2-one (51)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 3-methoxybenzaldehyde as starting material.

Grey solid. Yield: 57 %. Melting point: 122-124 °C. Molecular formula: C₁₉H₁₄O₄. Molecular weight: 306.32 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.57 (d, J = 0.7 Hz, 1H, Ar), 7.91 (d, J = 15.7 Hz, 1H, CH=), 7.82 (d, J = 15.7 Hz, 1H, CH=), 7.70 – 7.49 (m, 3H, Ar), 7.44 – 7.24 (m, 2H, Ar), 7.17 (dd, J = 2.6, 1.5 Hz, 1H, Ar), 6.96 (ddd, J = 8.0, 2.6, 1.2 Hz, 2H, Ar), 3.84 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 186.62, 160.00, 159.42, 155.36, 148.21, 145.13, 136.25, 134.36, 130.14, 130.00, 125.39, 125.10, 124.29, 121.79, 118.65, 116.99, 116.83, 113.63, 55.45.

(E)-3-(3-(3-(Benzyloxy)phenyl)acryloyl)-2H-chromen-2-one (52)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 3,4-(benzyloxy)benzaldehyde as starting material.

Grey solid. Yield: 59 %. Melting point: 139-141 °C. Molecular formula: C₂₅H₁₈O₄. Molecular weight: 382.42 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.52 (s, 1H, Ar), 8.07 (d, J = 15.9 Hz, 1H, CH=), 7.86 (d, J = 15.9, 1H, CH=), 7.71 – 7.59 (m, 2H, Ar), 7.47 – 7.41 (m, 3H, Ar), 7.41 – 7.22 (m, 6H, Ar), 7.09-6.98 (m, 2H, Ar), 5.09 (s, 2H, CH₂Ar).

¹³C NMR (101 MHz, CDCl₃) δ: 186.91, 159.26, 154.26, 153.29, 147.85, 147.63, 140.78, 137.11, 134.14, 133.01, 129.64, 128.75, 128.49, 128.21, 125.72, 125.11, 125.02, 124.43, 119.56, 118.65, 116.80, 114.59, 75.94.

(E)-3-(3-(3,4-Dimethoxyphenyl)acryloyl)-2H-chromen-2-one (53)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 3,4-dimethoxybenzaldehyde as starting material.

Yellow solid. Yield: 65 %. Melting point: 179-181 °C. Molecular formula: C₂₀H₁₆O₅. Molecular weight: 336.34 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.57 (d, J = 0.7 Hz, 1H, Ar), 7.84 (d, J = 15.4 Hz, 1H, CH=), 7.78 (d, J = 15.4 Hz, 1H, CH=), 7.68 – 7.58 (m, 2H, Ar), 7.45 – 7.31 (m, 2H, Ar), 7.27 – 7.23 (m, 1H, Ar), 7.19 (d, J = 2.0 Hz, 1H, Ar), 6.88 (d, J = 8.3 Hz, 1H, Ar), 3.94 (s, 3H, OCH₃), 3.92 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 186.38, 159.54, 155.28, 151.87, 149.34, 147.95, 145.46, 134.22, 130.07, 127.97, 125.64, 125.07, 124.11, 121.91, 118.72, 116.79, 111.13, 110.34, 56.10, 56.05.

(E)-3-(3-(Benzo[d][1,3]dioxol-5-yl)acryloyl)-2H-chromen-2-one (54)

Compound was obtained according to the general procedure reported above with intermediate **XX** and benzo[d]dioxole-5-carbaldehyde as starting material.

Yellow solid. Yield: 69 %. Melting point: 172-173 °C. Molecular formula: C₁₉H₁₂O₅. Molecular weight: 320.30 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.56 (s, 1H, Ar), 7.82 (d, J = 15.3 Hz, 1H, CH=), 7.77 (d, J = 15.3 Hz, 1H, CH=), 7.70 – 7.57 (m, 2H, Ar), 7.45 – 7.27 (m, 2H, Ar), 7.24 – 7.15 (m, 2H, Ar), 6.88 (d, J = 8.3 Hz, 1H, Ar), 5.20 (s, 2H, OCH₂O).

¹³C NMR (101 MHz, CDCl₃) δ 186.37, 159.51, 155.28, 151.00, 147.94, 145.40, 136.58, 134.21, 130.07, 128.75, 128.15, 125.06, 123.85, 122.03, 118.71, 116.79, 113.44, 110.95, 70.93.

(E)-3-(3-(3-(Benzyloxy)-4-methoxyphenyl)acryloyl)-2H-chromen-2-one (55)

Compound was obtained according to the general procedure earlier reported with intermediate **XX** and 3-(benzyloxy)-4-methoxybenzaldehyde as starting material.

Grey solid. Yield: 65 %. Melting point: 148-149 °C. Molecular formula: C₂₆H₂₀O₅. Molecular weight: 412.44 g/mol.

¹H NMR (400 MHz, CDCl₃) 8.47 (s, 1H, Ar), 8.15 (d, J = 15.9 Hz, 1H, CH=), 7.77 (d, J = 15.9, 1H, CH=), 7.67 – 7.61 (m, 2H, Ar), 7.47 – 7.41 (m, 2H, Ar), 7.41 – 7.22 (m, 6H, Ar), 7.09 (t, J = 8.0 Hz, 1H, Ar), 6.98 (dt, 1H, Ar), 5.04 (s, 2H, CH₂Ar), 3.89 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ 186.91, 159.26, 155.26, 153.29, 147.85, 147.63, 140.23, 137.11, 134.14, 130.01, 129.64, 128.75, 128.49, 128.21, 125.72, 125.11, 125.02, 124.43, 119.56, 118.65, 116.80, 114.59, 75.94, 56.03.

(E)-3-(3-(3-((4-Fluorobenzyl)oxy)-4-methoxyphenyl)acryloyl)-2H-chromen-2-one (56)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 3-(4-fluorobenzyloxy)-4-methoxybenzaldehyde as starting material.

Grey solid. Yield: 58 %. Melting point: 172-173 °C. Molecular formula: C₂₆H₁₉FO₅. Molecular weight: 430.43 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.49 (s, 1H, arom), 8.10 (d, J = 15.9 Hz, 1H, CH=), 7.78 (d, J = 15.9 Hz, 1H, CH=), 7.72 – 7.58 (m, 2H, Ar), 7.50 – 7.30 (m, 5H, Ar), 7.09 (t, J = 8.0 Hz, 1H, Ar), 7.05 – 6.95 (m, 3H, Ar), 5.00 (s, 2H, CH₂Ar), 3.89 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ 186.88, 163.99, 161.54, 159.31, 155.27, 153.22, 147.79, 147.54, 140.05, 134.22, 132.96, 132.93, 130.74, 130.65, 130.05, 125.64, 125.16, 125.06, 124.55, 119.63, 118.63, 116.81, 115.48, 115.27, 114.52, 75.10, 56.01.

(E)-3-(3-(3-((4-Chlorobenzyl)oxy)-4-methoxyphenyl)acryloyl)-2H-chromen-2-one (57)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 3-(4-chlorobenzyl)oxy-4-methoxybenzaldehyde as starting material.

Grey solid. Yield: 65 %. Melting point: 172-173 °C. Molecular formula: C₂₆H₁₉ClO₅. Molecular weight: 446.88 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.57 (s, 1H, Ar), 7.82 (d, J = 15.6 Hz, 1H, CH=), 7.73 (d, J = 15.6 Hz, 1H, CH=), 7.72 – 7.59 (m, 2H, Ar), 7.47 – 7.18 (m, 9H, Ar), 6.90 (d, J = 8.4 Hz, 1H, Ar), 5.15 (s, 2H, CH₂Ar), 3.92 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ 186.22, 159.53, 155.31, 152.47, 148.15, 148.07, 145.20, 135.26, 134.26, 133.96, 130.10, 129.01, 128.96, 125.50, 125.07, 124.63, 121.98, 118.72, 116.79, 113.13, 111.63, 70.43, 56.14.

(E)-3-(3-(4-Methoxy-3-((4-methoxybenzyl)oxy)phenyl)acryloyl)-2H-chromen-2-one (58)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 3-(4-methoxybenzyl)oxy-4-methoxybenzaldehyde as starting material.

Grey solid. Yield: 64 %. Melting point: 181-182 °C. Molecular formula: C₂₇H₂₂O₆. Molecular weight: 442.47 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.56 (s, 1H, Ar), 7.79 (d, J = 15.3 Hz, 1H, CH=), 7.74 (d, J = 15.3 Hz, 1H, CH=), 7.71 – 7.55 (m, 2H, Ar), 7.50 – 7.34 (m, 4H, Ar), 7.27 – 7.19 (m, 3H, Ar), 6.96 – 6.82 (m, 3H, Ar), 5.12 (s, 2H, CH₂Ar), 3.90 (s, 3H, OCH₃), 3.80 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ 186.29, 159.57, 159.49, 155.29, 152.56, 148.45, 147.94, 145.45, 134.20, 130.08, 129.41, 128.79, 127.83, 125.60, 125.04, 124.31, 121.85, 118.73, 116.77, 114.18, 113.27, 111.52, 70.98, 56.11, 55.38.

(E)-3-(3-(4-Methoxy-3-((4-methylbenzyl)oxy)phenyl)acryloyl)-2H-chromen-2-one (59)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 3-(4-methylbenzyl)oxy-4-methoxybenzaldehyde as starting material.

Grey solid. Yield: 65 %. Melting point: 163-165 °C. Molecular formula: C₂₇H₂₂O₅. Molecular weight: 426.47 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.56 (s, 1H, Ar), 7.79 (d, J = 15.7 Hz, 1H, CH=), 7.73 (d, J = 15.7 Hz, 1H, CH=), 7.69 – 7.59 (m, 2H, Ar), 7.41 – 7.29 (m, 4H, Ar), 7.21 – 7.13 (m, 4H, Ar), 6.88 (d, J = 8.1 Hz, 1H, Ar), 5.16 (s, 2H, CH₂Ar), 3.93 (s, 3H, OCH₃), 2.34 (s, 3H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 186.32, 159.48, 155.30, 152.52, 148.46, 147.93, 145.48, 137.88, 134.20, 133.68, 130.07, 129.44, 127.83, 127.75, 125.61, 125.04, 124.18, 121.86, 118.73, 116.78, 113.28, 111.55, 71.08, 56.13, 21.31.

(E)-3-(3-(4-(Benzyloxy)-3-methoxyphenyl)acryloyl)-2H-chromen-2-one (60)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 4-(benzyloxy)-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 59 %. Melting point: 199-200 °C. Molecular formula: C₂₆H₂₀O₅. Molecular weight: 412.44 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.56 (s, 1H, Ar), 7.80 (d, J = 15.3 Hz, 1H, CH=), 7.74 (d, J = 15.3 Hz, 1H, CH=), 7.73 – 7.22 (m, 7H, Ar), 7.14 – 6.98 (m, 4H, Ar), 6.87 (d, J = 8.8 Hz, 1H, Ar), 5.09 (s, 2H, CH₂Ar), 3.91 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 186.32, 158.92, 155.31, 154.22, 148.04, 147.83, 145.13, 134.25, 130.09, 129.47, 127.67, 125.97, 125.50, 125.05, 124.11, 122.13, 118.70, 116.79, 112.39, 108.73, 107.25, 101.77, 71.47, 55.74.

(E)-3-(3-(4-((4-Fluorobenzyl)oxy)-3-methoxyphenyl)acryloyl)-2H-chromen-2-one (61)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 4-(4-fluorobenzyloxy)-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 65 %. Melting point: 146-147 °C. Molecular formula: C₂₆H₁₉FO₅. Molecular weight: 430.43 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.56 (s, 1H, Ar), 7.81 (d, J = 15.3 Hz, 1H, CH=), 7.75 (d, J = 15.3 Hz, 1H, CH=), 7.71 – 7.57 (m, 2H, Ar), 7.50 – 7.28 (m, 4H, Ar), 7.21 – 7.14 (m, 2H, Ar), 7.12 – 7.01 (m, 2H, Ar), 6.88 (d, J = 8.8 Hz, 1H, Ar), 5.14 (s, 2H, CH₂Ar), 3.93 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ: 186.36, 163.88, 161.43, 159.53, 155.28, 150.75, 149.87, 148.01, 145.29, 134.25, 132.33, 132.30, 130.09, 129.33, 129.25, 128.48, 125.57, 125.08, 123.76, 122.15, 118.70, 116.79, 116.78, 115.79, 115.58, 113.46, 110.95, 70.32, 56.13.

(E)-3-(3-(4-((4-Chlorobenzyl)oxy)-3-methoxyphenyl)acryloyl)-2H-chromen-2-one (62)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 4-(4-chlorobenzyl)oxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 63 %. Melting point: 171-172 °C. Molecular formula: C₂₆H₁₉ClO₅. Molecular weight: 446.88 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.57 (d, J = 0.7 Hz, 1H, Ar), 7.84 (d, J = 15.7 Hz, 1H, CH=), 7.76 (d, J = 15.7 Hz, 1H, CH=), 7.69 – 7.58 (m, 2H, Ar), 7.41 – 7.31 (m, 6H, Ar), 7.23 – 7.16 (m, 2H, Ar), 6.86 (d, J = 8.1 Hz, 1H, Ar), 5.15 (s, 2H, CH₂Ar), 3.94 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ 186.36, 159.52, 155.29, 150.65, 149.87, 148.01, 145.24, 135.09, 134.25, 133.98, 130.09, 128.70, 128.58, 125.57, 125.08, 123.73, 122.21, 118.71, 116.80, 113.50, 110.99, 70.21, 56.14.

(E)-3-(3-(3-Methoxy-4-((4-methoxybenzyl)oxy)phenyl)acryloyl)-2H-chromen-2-one (63)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 4-(4-methoxybenzyl)oxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 66 %. Melting point: 183-184 °C. Molecular formula: C₂₇H₂₂O₆. Molecular weight: 442.47 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.56 (s, 1H, Ar), 7.81 (d, J = 15.7 Hz, 1H, CH=), 7.76 (d, J = 15.7 Hz, 1H, CH=), 7.68 – 7.61 (m, 2H, Ar), 7.44 – 7.29 (m, 4H, Ar), 7.18 (dd, J = 6.0, 2.2 Hz, 2H, Ar), 6.95 – 6.83 (m, 3H, Ar), 5.12 (s, 2H, CH₂Ar), 3.92 (s, 3H, OCH₃), 3.79 (s, 3H, OCH₃).

¹³C NMR (101 MHz, CDCl₃) δ 186.37, 159.60, 159.51, 155.28, 151.09, 149.89, 147.91, 145.45, 134.20, 130.07, 129.12, 128.59, 128.19, 125.65, 125.05, 123.86, 121.96, 118.72, 116.78, 114.14, 113.49, 110.93, 70.76, 56.13, 55.38.

(E)-3-(3-(3-Methoxy-4-((4-methylbenzyl)oxy)phenyl)acryloyl)-2H-chromen-2-one (64)

Compound was obtained according to the general procedure reported above with intermediate **XX** and 4-(4-methylbenzyl)oxy-3-methoxybenzaldehyde as starting material.

Yellow solid. Yield: 63 %. Melting point: 164-166 °C. Molecular formula: C₂₇H₂₄O₅. Molecular weight: 426.47 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 8.56 (s, 1H, Ar), 7.79 (d, J = 15.4 Hz, 1H, CH=), 7.73 (d, J = 15.4 Hz, 1H, CH=), 7.73 – 7.57 (m, 3H, Ar), 7.44 – 7.28 (m, 5H, Ar), 7.23 – 7.12 (m, 2H, Ar), 6.88 (d, J = 8.1 Hz, 1H, Ar), 5.16 (s, 2H, CH₂Ar), 3.93 (s, 3H, OCH₃), 2.34 (s, 3H, CH₃).

¹³C NMR (101 MHz, CDCl₃) δ 186.38, 159.51, 155.28, 151.08, 149.85, 147.92, 145.47, 137.91, 134.20, 133.53, 130.07, 129.42, 128.17, 127.41, 125.65, 125.05, 123.88, 121.95, 118.72, 116.79, 113.43, 110.93, 70.87, 56.15, 21.29.

Chemical radical scavenging activity

The antioxidant activity of compounds **1-64** was measured using DPPH assay [101,102]. The compounds (3 mg) were first dissolved in 1 mL DMSO then methanol was added to take up the volume to 50 mL. A total of 0.1 mL of the compound solution was mixed with 3.9 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanol solution (65 mM). Absorbance was measured at 517 nm after reacting for 30 min in the dark. The linear calibration curve was obtained using Trolox standards (ranging between 20 and 200 mg/L, R² = 0.9955). After confirming linear behavior within the concentration interval used, the antioxidant activity (AA%) of the samples was determined by the ratio of the decrease in absorbance of each sample (A_s) with that of the DPPH blank solution (A₀) as reported in Equation 1 [103].

$$AA\% = \frac{A_0 - A_s}{A_0} \times 100$$

All analyses were performed in triplicate (n = 3), and values are given ± standard deviation (SD).

Preparation of micellar solutions

The surfactants Tween-80 (TW-80), Ricinoleth-40, Triton X-100 (TX-100) were purchased from Sigma-Aldrich. The stock solutions of **8** and **34**, at the concentration of 0.05 mM, were prepared for spectroscopic measurement in deionized water. The surfactant solutions were prepared in deionized water and then combined with compound's solution. The concentrations were set to range: from 0.014 to 0.14 mM for TW-80, from 0.0065 to 0.064 mM for Ricinoleth-40 and from 0.019 to 0.415 mM for TX-100. The UV absorption spectra were carried out using a Thermo Scientific UV/VIS spectrophotometer (Evolution 300, Fischer Scientific, GmbH, Schwerte, Germany) at λ = 370 nm for compound **8** and at λ = 415 nm for compound **34**.

Biological evaluation

Biological evaluation methods on NB

Biological evaluations were carried out using patient-derived malignant neuroblastoma cell lines (HTLA-230) at stage IV obtained from Giannina Gaslini Pediatric Institute (Genoa, Italy), and the multidrug-resistant derivative cell line (ER)^[58,59], together with the human keratinocyte line (HaCat), all maintained under standard culture conditions. Stock solutions of compounds **1–46** were prepared using dimethyl sulfoxide (DMSO) as solvent. Cells were exposed to compounds **1–46** and, when required, to ferroptosis modulators including ferrostatin-1, liprostatin-1, RSL3 and erastin. In order to exclude the interference of DMSO, employed to solubilize these compounds, cells were exposed to the highest concentration used as control (Ctr). Cell viability was assessed by the MTS colorimetric assay, while total protein extracts were prepared for biochemical analysis and Western blot experiments performed according to established procedures^[104]. Intracellular oxidative stress and lipid peroxidation were evaluated through fluorometric assays employing DCFH-DA and BODIPY probes, respectively, following previously described protocols^[58,105] and kit's manufacturer instructions. Cellular senescence was measured using a fluorescence-based commercial kit following manufacturer's instructions, and long-term proliferative capacity was evaluated through clonogenic assays. Results were expressed as mean $\pm$ SEM of at least four independent experiments. The statistical significance of the parametric differences between the experimental data sets was assessed by one-way ANOVA and Dunnett's test for multiple comparisons; $p < 0.05$ was considered statistically significant.

Biological evaluation methods on MCF-7 and A549 cell lines

The work presented in this section was done in collaboration with Professor Francesco Piacente (Department of Experimental Medicine – DIMES, University of Genoa, Italy).

Cytotoxicity of compounds **47–59** was investigated against human breast adenocarcinoma (MCF7) and lung adenocarcinoma epithelial (A549). The results are expressed as the IC₅₀, the concentration of the compound to cause 50%

inhibition of cell viability. Cells were treated in triplicate with increasing concentrations of each compound between 0.2 and 50 μM for 72 h. Cells were cultivated in 37 °C and 5 % CO_2 and at the end of treatment were blocked with TCA, and viability was evaluated following the colorimetric Sulforhodamine B assay ^[106]. The IC_{50} was evaluated on the obtained viability data by the [Inhibitor] vs. normalized response -- Variable slope function of GraphPad Prims v. 8.0.2.

***In silico* predictions of ADME properties**

In silico prediction of ADMET-related parameters was performed using the freely available SwissADME web tool ^[94,95], which provides physicochemical profiling, lipophilicity estimation, water solubility prediction, permeability assessment and drug-likeness analysis based on validated computational models.

Anti-neurodegeneration activity

Introduction

Alzheimer's Disease: Clinical and Epidemiological Background

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder and is characterized by a progressive and irreversible decline in cognitive functions, predominantly affecting older individuals.

Aging is the primary risk factor for dementia, and according to the World Health Organization (WHO), AD accounts for 60–70% of all dementia cases in the elderly people ^[107]. In the context of global population aging and increased life expectancy, the number of people living with AD is expected to rise dramatically. The World Alzheimer Report 2024 estimated that approximately 55 million people were affected in 2019, with projections suggesting that this number may exceed 100 million by 2050 ^[108]. Altogether, these data firmly establish AD as one of the major health challenges worldwide.

Its clinical presentation includes memory impairment, deficits in learning and language, impaired executive functions, and progressive loss of autonomy. This occurs together with a wide spectrum of non-cognitive manifestations, collectively known as Behavioral and Psychological Symptoms of Dementia (BPSDs) which include affective and psychological disturbances, particularly apathy and depression, as well as mood changes, alterations in personality and behavior, and insomnia ^[109,110].

These non-cognitive symptoms substantially worsen patients' quality of life and significantly increase the social and emotional burden of the disease.

Biochemical Background

From a biochemical perspective, neurodegenerative diseases, including AD, share several converging pathological mechanisms. These include protein misfolding and aggregation ^[111], dysregulation of key neurotransmitters such as acetylcholine and dopamine ^[112–114], metal ion dyshomeostasis ^[115,116], mitochondrial dysfunction, oxidative stress and chronic neuroinflammation ^[117–119]. The complex interplay of these processes contributes to synaptic dysfunction, neuronal damage, and ultimately neuronal loss.

Models of AD Pathophysiology

Two major conceptual frameworks have guided the understanding of AD pathogenesis: the cholinergic hypothesis and the amyloid hypothesis. Although they emphasize different mechanisms, both contribute to the current view of AD as a multifactorial disorder.

The Cholinergic Hypothesis

The cholinergic hypothesis was formulated in the 1970s based on postmortem studies showing profound cholinergic deficits in the brains of AD patients. The development of AD symptoms was linked to structural alterations in cholinergic synapses, loss of acetylcholine (ACh) receptors, degeneration of ACh-producing neurons, and a consequent impairment of cholinergic neurotransmission. Because ACh plays a pivotal role in cognitive processes, including learning, memory, and attention, these alterations were proposed to underlie the cognitive decline observed in AD ^[112,120].

However, reductions in the activity of cholinergic enzymes such as choline acetyltransferase (ChAT), which is responsible for ACh synthesis, have also been reported in senile dementia ^[113,114]. Since cholinergic dysfunction is therefore not exclusive to Alzheimer's disease, this observation strongly suggests that additional pathological mechanisms must necessarily contribute to the onset and progression of AD, supporting its multifactorial nature.

The role of Butyryl Cholinesterase

Butyrylcholinesterase (BChE), a serine hydrolase belonging to the cholinesterase family alongside AChE, has gained increasing attention as a relevant modulator in the pathophysiology of AD and therefore as a potential therapeutic target. While AChE is predominantly synaptic in the brain, accounting for about 80% of total ChEs activity and highly selective for ACh hydrolysis, BChE is more abundant peripherally and in glial cells, functioning efficiently at higher ACh concentrations and metabolizing various neuroactive peptides ^[121,122]. In the healthy brain, AChE predominates in hydrolyzing ACh, while BChE contributes to a lesser extent. However, during the progression of AD, especially during

advanced stages of the disease, AChE activity declines, whereas BChE activity is preserved or even elevated, particularly in regions exhibiting amyloid pathology [123–126]. This shift results in an increased BChE:AChE ratio that may accentuate cholinergic deficits as the disease advances and suggests that BChE plays a compensatory role in ACh metabolism [124]. Beyond its enzymatic function, as AChE, BChE has been localized to A β plaques and has been implicated in plaque maturation and neuroinflammatory processes, further supporting its involvement in AD progression [127–129]. From a medicinal chemistry perspective, these biological insights have motivated the design of BChE-selective and dual cholinesterase inhibitors. Although no BChE-selective drug is to date in clinical use (see the “FDA-Approved Treatments for Alzheimer’s Disease” section below), dual cholinesterase inhibitors such as rivastigmine, have proven symptomatic efficacy in Alzheimer’s disease. Consequently, current medicinal chemistry efforts are increasingly focused on improving enzyme selectivity and on the rational design of optimized multitarget profiles aimed at addressing not only cholinergic dysfunction but also the complex interplay among multiple pathological hallmarks of Alzheimer’s disease.

The Amyloid Hypothesis

A central feature of many neurodegenerative disorders is the aberrant aggregation of normally soluble proteins into amyloid structures [130] which, despite the obvious differences in amino acid sequences and native structure, all appear to share a common β -sheet conformation of their polypeptide backbone and it is likely that this characteristic confers thermodynamic stability to the higher order aggregates, proteolytic resistance and insoluble characteristics [131,132].

In AD, alongside cholinergic disfunction, the typical histopathological picture is dominated by extracellular β -amyloid (A β) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau (pTau) [111]. These aggregated species accumulate in vulnerable brain regions, disrupt synaptic connectivity, induce neurotoxicity, and eventually lead to neuronal death.

In 1984, George Glenner and co-workers identified A β as the major amyloidogenic protein in senile plaques [133], leading Hardy and Higgins to formulate the amyloid cascade hypothesis in 1992 [134]. Initially, this hypothesis proposed that accumulation of A β peptides in the brain is an early and causative event in AD, leading to plaque formation and downstream neurodegeneration.

Later studies refined this view by emphasizing the role of an imbalance between A β production and clearance, which promotes gradual accumulation and aggregation of the peptide. This imbalance is thought to initiate a neurodegenerative cascade that culminates in synaptic dysfunction, tau pathology, neuronal loss, and clinical symptoms [135,136].

However, it is worth noting that A β can also be generated through normal protein processing pathways [137] and A β deposits have been found in non-demented elderly individuals [138]. This suggests that amyloid deposition becomes pathologic only when a critical threshold is exceeded, leading to tau hyperphosphorylation, neurofibrillary tangles, and progressive neurodegeneration. Dense A β aggregates are major constituents of neocortical neuritic plaques, which, together with tau neurofibrillary tangles, represent core pathological hallmarks of AD [139]. These features, in combination with metal-ion involvement, oxidative stress, and neuroinflammation, delineate a complex and non-linear pathological network typical of a multifactorial disease.

FDA-Approved Treatments for Alzheimer's Disease

To date, the U.S. Food and Drug Administration (FDA) has approved five small-molecule drugs (Figure 17), tacrine, rivastigmine, galantamine, donepezil, and memantine, and three monoclonal, antibodies aducanumab, lecanemab, and donanemab, for the treatment of AD. Despite these therapeutic options, none of these agents is curative; rather, they provide symptomatic relief or modestly slow disease progression.

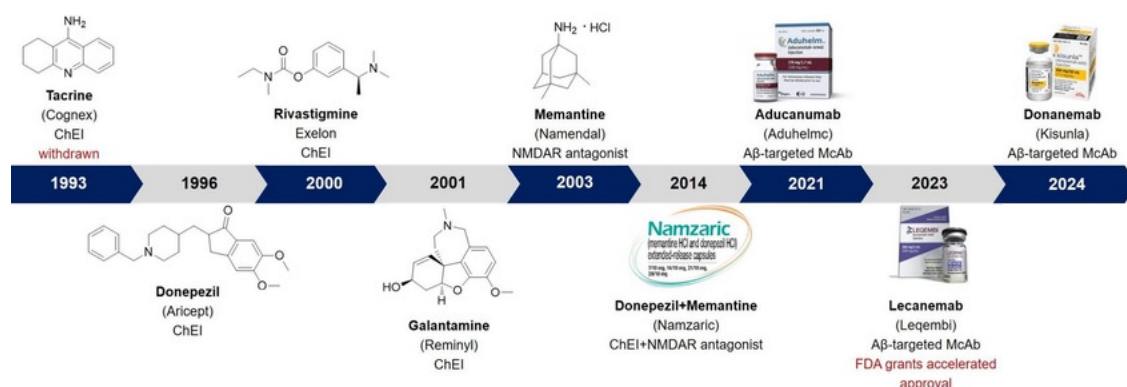


Figure 17. FDA-approved drug for the treatment of Alzheimer disease. Figure modified from Mao et al [140].

Cholinesterase Inhibitors and NMDA Receptor Antagonist

Early therapeutic efforts for AD were guided by the cholinergic hypothesis, leading to the development of cholinesterase inhibitors (ChIs) aimed at counteracting ACh deficits. The first approved drug, tacrine, showed potent and BChE-selective inhibitory activity but suffered from poor bioavailability and hepatotoxicity, resulting in its withdrawal ^[141,142]. Later, second-generation AChE inhibitors donepezil ^[143–145], strongly selective for AChE, rivastigmine ^[146,147], selective for AChE, and galantamine ^[148–150], dual AChE-BChE inhibitor, became the standard symptomatic treatments, with multiple randomized clinical trials demonstrating their cognitive and functional benefits.

A different pharmacological strategy is represented by memantine, a noncompetitive NMDA receptor antagonist that modulates glutamatergic signaling and reduces toxicity ^[151,152]. Memantine is often administered alongside donepezil, and the fixed-dose combination (Namzaric®) has been approved for moderate-to-severe AD ^[153]. Notably, dual inhibition of cholinesterase and NMDA pathways has been shown to improve survival compared to monotherapy ^[154], highlighting the therapeutic potential of multi-pathway targeting.

Although these drugs constitute the first line of pharmacological intervention in AD, they provide only modest and temporary symptomatic relief. As neurodegeneration progresses and cholinergic neurons are progressively lost, the efficacy of cholinesterase inhibition inevitably diminishes, underscoring the need for alternative or complementary therapeutic strategies.

Immunotherapies Targeting Amyloid- β

Growing support for the amyloid hypothesis has driven the development of monoclonal antibodies designed to selectively target aggregated A β . Three antibodies, aducanumab, lecanemab and donanemab, have been approved for early symptomatic AD. Aducanumab binds aggregated A β species and interferes with secondary nucleation processes, though despite its accelerated FDA approval in 2021, its development was discontinued in 2024. Lecanemab, approved in 2023, targets soluble and insoluble A β aggregates, reducing amyloid burden and modestly slowing cognitive decline ^[155]. Donanemab, approved in

2024, binds pyroglutamate-modified A β present in mature plaques and promotes their clearance via microglial activation ^[156].

While these immunotherapies can reduce amyloid load and slightly delay clinical progression, they fall short of offering a cure and remain limited by significant challenges. The production of biologics is complex, costly, and time-intensive, raising concerns regarding large-scale accessibility. Moreover, cost-effectiveness analyses indicate that these treatments may only become economically viable under specific dosing paradigms, such as early discontinuation once substantial amyloid reduction is achieved ^[157], leaving open important questions regarding the feasibility and applicability of antibody-based therapies within large-scale treatment strategies.

From Combination Therapies to Multi-Target Directed Ligands (MTDLs)

Given the multifactorial nature of AD, single-target drugs often show limited clinical efficacy and fail to adequately modulate the complex pathophysiological network underlying the disease. Combination therapies, also referred to as multiple-medication therapy (MMT) or “cocktail” approaches, have therefore emerged as an attractive strategy. By combining two or more agents with different mechanisms of action, MMT can enhance therapeutic efficacy, overcome resistance, reduce toxicity through dose sparing, and better address disease heterogeneity ^[158]. However, polypharmacy can compromise patient adherence, particularly in elderly individuals with multiple comorbidities and cognitive impairment. Clinical studies indicate that adherence improves as the number of daily doses decreases, with once-daily or twice-daily regimens being preferred over more frequent dosing ^[159,160].

An alternative strategy is the use of multiple-compound medications (MCMs), or “single-pill combinations”, in which different drugs are formulated together to simplify dosing and improve compliance. Nevertheless, both MMT and MCM approaches still face challenges related to the differing pharmacokinetic and ADMET profiles of each component and the risk of drug–drug interactions.

In this context, increasing attention has been devoted to the rational design of multi-target directed ligands (MTDLs), single molecular entities optimized to interact with two or more relevant targets. In principle, a MTDL could provide synergistic effects comparable to combination therapies while avoiding many of

their drawbacks. A single compound with a suitably balanced polypharmacological profile can be optimized pharmacokinetically as any other lead molecule, and the risk of drug–drug interactions is therefore eliminated.

While target promiscuity has traditionally been viewed negatively, as a source of off-target toxicity, it can be therapeutically advantageous when carefully directed toward multiple beneficial targets, the so-called “selective promiscuity”. Indeed, in other therapeutic areas, such as antipsychotic drug development, dual antagonism has been shown to be necessary for full clinical efficacy^[161].

Overall, these considerations have led to a growing interest in MTDLs as promising candidates for the treatment of complex, multifactorial disorders such as AD, where simultaneous modulation of cholinesterases, monoaminergic systems, and amyloid-related pathways may offer superior therapeutic outcomes compared with classical single-target approaches.

Other important targets: monoamine oxidase

Monoamine oxidases (MAOs) are flavin-containing enzymes localized on the outer mitochondrial membrane, where they catalyze the oxidative deamination of endogenous and exogenous amines, thereby regulating neurotransmitter homeostasis. Two isoforms, MAO-A and MAO-B, have been identified; although they share approximately 73% sequence similarity, they differ in substrate specificity, inhibitor selectivity, and tissue distribution^[162].

MAO-A preferentially metabolizes larger endogenous monoamines such as serotonin, epinephrine, and norepinephrine and is predominantly expressed in peripheral tissues, whereas MAO-B is mainly localized in platelets and glial cells and accounts for nearly 80% of total MAO activity in the human brain. MAO-B shows higher affinity for smaller amines such as benzylamine and β -phenylethylamine, while dopamine and tyramine are substrates for both isoforms^[163].

The relevance of MAOs in neurodegenerative disorders has attracted increasing attention, as their catalytic activity produces hydrogen peroxide as a byproduct, contributing to oxidative stress, mitochondrial dysfunction, and neuronal damage. This aspect is particularly significant for MAO-B, which has been reported to be overexpressed in the brains of Alzheimer’s disease patients, especially in the hippocampus and cortex^[164], and has been correlated with

cognitive decline and disease progression ^[165] These findings support MAO inhibition as a valuable complementary strategy to cholinesterase modulation within a multi-target therapeutic framework.

Other targets

Beyond ChEs, MAOs and A β , several additional enzymatic and molecular targets have emerged as relevant contributors to AD pathology. Among these, carbonic anhydrases (CAs), β -secretase (BACE-1), serotonergic receptors (5-HT₄ and 5-HT₆), tau neurofibrillary tangles (NFTs), tau-related kinases (GSK-3 and Cdk5), neuroinflammation, oxidative stress and metal ion homeostasis.

Carbonic anhydrases (CAs), mitochondrial enzymes highly expressed in the central nervous system and involved in acid–base homeostasis, have attracted increasing attention. Alterations in CA expression and activity have been reported in AD and are associated with amyloid- β –induced mitochondrial dysfunction, ultimately contributing to oxidative stress and neuronal damage ^[166–169].

Other key targets include β -secretase (BACE-1) and serotonergic receptors, particularly 5-HT₄ and 5-HT₆, which are involved in the regulation of amyloidogenic and non-amyloidogenic processing of amyloid precursor protein (APP) ^[170–172]. In parallel, pathological aggregation of tau protein into neurofibrillary tangles (NFTs) represents a central hallmark of AD ^[173]. Tau hyperphosphorylation, driven by dysregulated activity of tau-related kinases such as glycogen synthase kinase-3 (GSK-3) and cyclin-dependent kinase 5 (Cdk5), promotes tau misfolding and aggregation, leading to cytoskeletal disruption and synaptic dysfunction ^[174].

In addition, multiple signaling pathways involved in neuroinflammation have been identified as critical contributors to disease progression, reflecting the prominent role of chronic glial activation in AD ^[118,175–177]. Dysregulation of metal ion homeostasis, particularly involving copper, zinc, and iron, further exacerbates oxidative stress, protein aggregation, and neuronal toxicity, reinforcing its relevance as a therapeutic target ^[116].

At the same time, an increasing number of studies in AD drug discovery are being directed toward non-conventional targets, including for example, α -glycosidase ^[178,179] and ryanodine receptors ^[180], which are implicated in cellular metabolism and calcium signaling dysregulation, respectively. Collectively, these approaches reflect a paradigm shift toward integrated target networks rather than isolated biochemical pathways, in line with the multifactorial and interconnected nature of AD pathology.

Overview of the design of anti-neurodegeneration compounds

Given the multifactorial and degenerative nature of Alzheimer's disease, the work presented in this section was directed toward the rational design and biological evaluation of novel compounds with potential anti-neurodegenerative activity. In light of the limited efficacy of current single-target therapies and the complex network of biochemical pathways involved in AD pathophysiology, a multitarget-oriented medicinal chemistry approach was adopted. This strategy aimed to generate small molecules capable of simultaneously modulating multiple disease-relevant mechanisms, including cholinergic dysfunction, amyloid aggregation and oxidative stress.

In this context, the design strategy focused on the incorporation of privileged scaffolds of natural or natural-inspired origin, such as coumarins, cinnamic acid derivatives, cytosine and indole nuclei, into synthetically accessible hybrid molecular frameworks. These scaffolds have been extensively reported in the literature to interact with key targets implicated in AD, including cholinesterases, monoamine oxidases, amyloid- β aggregation pathways, and redox-related processes. Their structural versatility and favorable drug-like features make them particularly suitable for molecular hybridization approaches aimed at exploring structure–activity relationships across multiple biological activities. Accordingly, different classes of hybrid compounds were designed by combining aromatic and heterocyclic fragments modulating molecular flexibility (e.g.: linker length), and functional group distribution, to identify the best activity profiles with AD-relevant molecular targets.

A detailed discussion of the individual scaffolds, their known biological relevance in the context of AD, the specific rationale underlying their selection and combination, together with their biological evaluation, is presented in the following sections.

Coumarin-alicyclic amine hybrids

Coumarin (*2H-chromen-2-one*) has become a widely used scaffold in anti-Alzheimer medicinal chemistry. Beyond its natural occurrence, favorable drug-like properties and intrinsic antioxidant activity, coumarin can be easily functionalized at the C-3, C-4, C-5, C-7 and C-8 positions, allowing tuning of physicochemical and pharmacological properties. Numerous synthetic coumarin derivatives have shown activity on AD-relevant targets, including mostly AChE and BChE ^[181–185], MAO-A and MAO-B ^[182,186], but other targets such as BACE-1 ^[185], and A β aggregation ^[183] are also being investigated. Moreover, it is interesting to note that many examples of the extensive studies directed through different AD-relevant targets aim at the combination of different activities in a single coumarin-based molecule ^[187–189].

In parallel, the donepezil scaffold is one of the most exploited pharmacophores in AD medicinal chemistry. As earlier discussed, donepezil itself is an FDA-approved reversible AChE inhibitor that binds both the catalytic active site (CAS) and peripheral anionic site (PAS) of the enzyme ^[143,190], making it an ideal starting point for MTDL design. Structural elements of donepezil, such as its benzylpiperidine moiety and indanone core, have been hybridized with other pharmacophores (e.g., chalcones, rivastigmine, tacrine, salicylic acid, metal-chelating units, or antioxidant fragments) to generate hybrids able to inhibit AChE/BChE, modulate MAO isoforms, reduce A β aggregation, or provide antioxidant/neuroprotective effects ^[191].

Given these premises, the fragment-based and molecular-hybridization strategy were used to synthesize a library of sixty compounds featuring the alicyclic amine moiety of donepezil linked to a coumarin nucleus via an alkyloxy linker at either the C-4 or C-7 position (Figure 18). This design allowed the systematic exploration of how spacer length, coumarin substitution pattern and the nature of the alicyclic amine influence the multifunctional profile of the hybrids, particularly with respect to cholinesterase inhibition and additional AD-relevant targets.

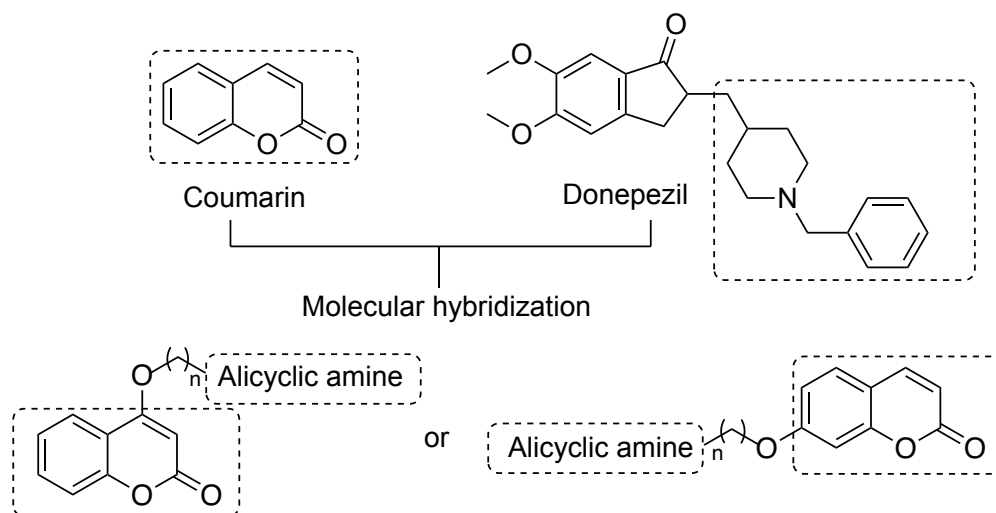
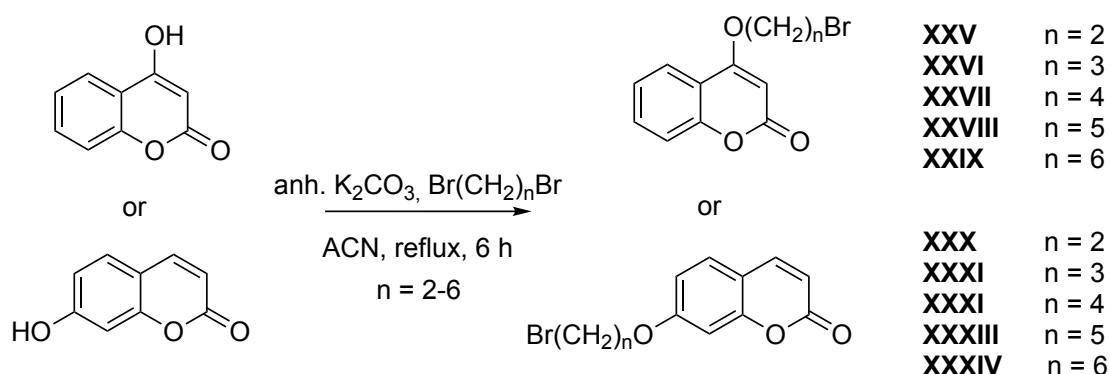


Figure 18. Schematic representation of the design of compounds **64-124**.

Synthesis

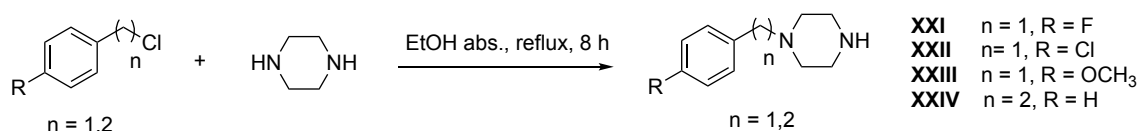
For the synthesis of the final coumarin-alicyclic amine hybrids, it was first necessary to obtain intermediate **XXV-XXIX** or **XXX-XXXIV** (Scheme 5) starting from 4- or 7-hydroxy coumarin, respectively, with the appropriate α,ω -dibromoalkane in acetonitrile under reflux in the presence of K_2CO_3 . After aqueous work-up and solvent removal, the crude products were purified by SiO_2 column chromatography.

Scheme 5. Synthesis of intermediates **XXV-XXXIV**.



In some cases, the alicyclic amine was also synthesized. In detail, the *p*-substituted benzyl piperazines and phenylethyl piperazine intermediates **XXI–XXIV** were synthesized through a straightforward nucleophilic substitution reaction between piperazine and the appropriate *p*-substituted benzyl or phenethyl chloride (Scheme 6). In general, the reaction was carried out between the selected precursor and an excess of anhydrous piperazine in absolute ethanol under reflux conditions. After completion of the reaction, removal of the solvent and basic aqueous work-up allowed efficient separation of the mono-substituted crude products which were then purified by column chromatography on SiO₂ for *p*-substituted piperazines and on Al₂O₃ for phenylethyl piperazine.

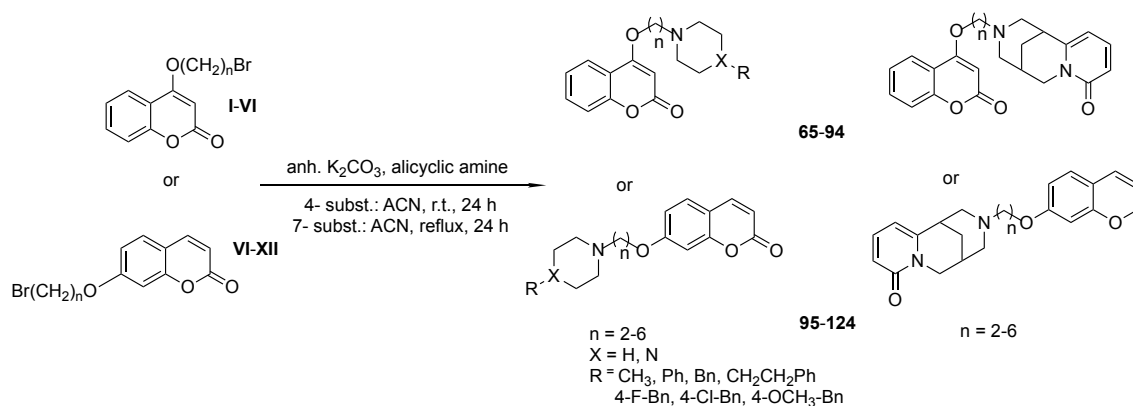
Scheme 6. Synthesis of intermediates **XXI–XXIV**.



After obtaining all the necessary intermediates, the appropriate ω -bromoalkoxy coumarin **XXV–XXXIV** is reacted in a closed tube with the desired alicyclic amine in the presence of K₂CO₃ in ACN, heating for 24 hours (Scheme 7).

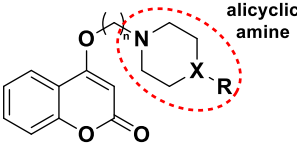
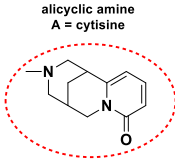
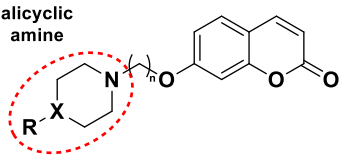
It is interesting to note that for compounds **95–124** (4-subst.) prolonged heating leads to decomposition with the formation of complex mixtures. Only by leaving the reaction at room temperature for 24 hours in a closed tube the desired compounds were obtained in good yields and then purified by chromatography (SiO₂; dichloromethane).

Scheme 7. Synthesis and reaction conditions of final compounds **65–124**.



All final compounds **65–124** were obtained in good yields, generally above 60 %. Overall, slightly lower yields were observed for compounds bearing shorter alkyloxy linker chains, with no significant differences between the 4- and 7-substituted series. A plausible explanation is that steric effect arising from the proximity of the coumarin core and the alicyclic amine may reduce the efficiency of the coupling step. Most derivatives appeared as white solids, with the exception of the 4-substituted benzyl-piperazine analogues, which were isolated as light-yellow oils that subsequently solidified, and the cytosine-based hybrids, which formed orange oils that in some cases solidified. Melting points fell within the approximate range of 50–100 °C. All final compounds were fully characterized by TLC, melting point determination, and ¹H and ¹³C NMR spectroscopy. Table 9 lists the structural details of the target compounds.

Table 9. Structural details of target compounds **65-124**.

 alicyclic amine 4-substituted  alicyclic amine A = cytosine  alicyclic amine 7-substituted				
Compound	n	X	R	Compound
65	2	N	Bn	95
66	2	CH	Bn	96
67	3	N	CH ₃	97
68	3	N	Ph	98
69	3	N	Bn	99
70	3	CH	Ph	100
71	3	CH	Bn	101
72	3		A	102
73	3	N	4F-Bn	103
74	3	N	4Cl-Bn	104
75	3	N	4OCH ₃ -Bn	105
76	3	N	CH ₂ CH ₂ Ph	106
77	4	N	CH ₃	107
78	4	N	Ph	108
79	4	N	Bn	109
80	4	CH	Ph	110
81	4	CH	Bn	111
82	4		A	112
83	5	N	CH ₃	113
84	5	N	Ph	114
85	5	N	Bn	115
86	5	CH	Ph	116
87	5	CH	Bn	117
88	5		A	118
89	6	N	CH ₃	119
90	6	N	Ph	120
91	6	N	Bn	121
92	6	CH	Ph	122
93	6	CH	Bn	123
94	6		A	124

Chemical radical-scavenging activity

The radical-scavenging capacity of these new coumarin-alicyclic amine hybrids was evaluated using the DPPH assay. However, none of the compounds exhibited antioxidant activity above 10 % and therefore the corresponding data were not included.

These findings are consistent with the structure–activity considerations discussed earlier. As observed for other series of compounds, the DPPH radical-scavenging response is primarily driven by the presence of free hydroxyl groups. Compounds retaining accessible peripheral OH substituents show measurable activity, whereas derivatives in which these groups are absent or functionalized, as in compounds **65-124** discussed in this section, display a markedly reduced antioxidant effect.

Nonetheless, it should be noted that the DPPH assay offers a qualitative indication of radical-scavenging capacity and reflects only the intrinsic chemical reactivity of the compounds. Such activity may not directly correlate with their performance in a cellular context, where redox homeostasis is governed by complex biochemical pathways.

Biological evaluation

ChEs and MAOs inhibition

The work presented in this section was done in collaboration with Professor Marco Catto and Dr. Gabriella La Spada (University of Bari, Italy).

The library of sixty coumarin-donepezil like hybrids derivatives (compounds **65-124**) was tested to investigate their potential polypharmacological activity against cholinesterases (ChEs; AChE and BChE) and monoamine oxidases (MAOs, MAO-A and MAO-B) using standardized spectrophotometric and fluorometric methods ^[181] ^[192].

Results are reported in Table 10 and Table 11 for 4- and 7- substituted compound, respectively. Results are reported as inhibition percentage at the concentration of

10 μ M. When the value exceeded 60 %, IC_{50} was determined and is reported $\pm$ SEM of five experiments at different concentration executed in triplicate.

The selectivity index (SI) is calculated as the ratio of the IC_{50} for AChE to the IC_{50} for BChE ($IC_{50}(AChE) / IC_{50}(BChE)$). An SI value greater than 1.0 indicates a preference for BChE while an SI value lower than 1.0 indicates a preference for AChE and a value of ~ 1 indicates no selectivity towards the two isoforms.

Table 10. Results of ChEs and MAOs inhibition assays on 4- substituted coumarin-alicyclic amine donepezil-like hybrids **65-94**.

	<i>h</i> AChE		<i>es</i> BChE		SI AChE/BChE	<i>h</i> MAO-A	<i>h</i> MAO-B
Compound	Inh. % (10 μM)	IC ₅₀ (μM)	Inh. % (10 μM)	IC ₅₀ (μM)		Inh. % (10 μM)	Inh. % (10 μM)
Ethoxy (n = 2)							
65	43 ± 4		8 ± 4				
66	30 ± 2		12 ± 2				
Propoxy (n = 3)							
67	47 ± 2		20 ± 4		1.73	NA	20 ± 5
68	56 ± 3		17 ± 1				
69		1.79 ± 0.34	39 ± 3				
70	47 ± 3		46 ± 2				
71	57 ± 4			15.0 ± 0.6			
72	54 ± 2		20 ± 2			17±5	28 ± 4
73	51 ± 4		14 ± 3				
74	19 ± 2		NA				
75	22 ± 4		NA				
76	35 ± 4		14 ± 6				
Butoxy (n = 4)							
77	50 ± 4		25 ± 4		1.73	NA	46 ± 6
78	42 ± 4		NA				
79		2.23 ± 0.30	35 ± 4				
80	53 ± 1			19.6 ± 2.0			
81	57 ± 4		39 ± 1				
82	48 ± 2		20 ± 1				
Pentyloxy (n = 5)							
83	NA		NA		1.73		
84	11 ± 3		46 ± 7				
85		4.96 ± 0.32		2.87 ± 0.11			
86	20 ± 4			7.93 ± 1.01			
87	31 ± 2			4.03 ± 0.17			
88	NA		38 ± 3				
Hexyloxy (n = 6)							
89		2.22 ± 0.37	35 ± 4		0.08	12 ± 4	14 ± 3
90	47 ± 5		46 ± 2				
91		0.36 ± 0.08	45 ± 2			19 ± 5	7 ± 4
92		11.10 ± 1.60	38 ± 3				
93		0.67 ± 0.11		7.86 ± 1.34			
94	48 ± 1		31 ± 3				
Donepezil		0.017 ± 0.002		2.30 ± 0.20	0.01		

*h*AChE = human Acetyl Cholinesterase; *es*BChE = equine Butyryl Cholinesterase; NA = not active

Within the 4-substituted series, a clear structure–activity relationship emerged, strongly influenced by both the length of the O-alkyl linker and the identity of the amine fragment. Shorter chains ($n = 2\text{--}4$) generally displayed weaker ChEs inhibition, whereas the hexyloxy derivatives ($n = 6$) produced the largest number of active compounds and the lowest IC_{50} values across the panel. Interestingly, when considering AChE inhibition specifically, the propoxy derivatives ($n = 3$) showed superior potency compared with the butoxy ($n = 4$) and pentyloxy ($n = 5$) analogues, following the trend $3 > 4 > 5$.

Hexyloxy derivatives were overall the most effective: compounds **91** and **93** reached submicromolar activity, with IC_{50} values of $0.36 \pm 0.08 \mu\text{M}$ and $0.67 \pm 0.11 \mu\text{M}$, respectively, representing the best AChE inhibitors in the 4-substituted library. From a SAR perspective, all compounds achieving $>60\%$ inhibition at $10 \mu\text{M}$ on AChE, and for which IC_{50} values were determined, shared a common feature: the presence of a benzyl-piperazinyl fragment, with the exception of compound **93** which carries a benzyl piperidine fragment. Although the methylpiperazine derivatives in the hexyloxy subgroup also displayed low-micromolar potency, the most active compounds of the entire 4-substituted set were those bearing benzyl-piperazine or benzyl-piperidine moieties.

Regarding BChE inhibition, the benzyl-piperidine fragment emerged as the most effective. Notably, the pentyloxy series ($n = 5$) contained the largest number of compounds active below $10 \mu\text{M}$ against BChE. In this subgroup, both benzyl-piperidine and benzyl-piperazine derivatives performed well. Among them, the pentyloxy benzyl-piperazine analogue (**85**) emerged as the compound with the most balanced dual AChE/BChE inhibitory profile within the 4-substituted family.

With regard to MAO activity, it is noteworthy that the butoxy benzyl-piperazine derivative (**79**) is selective for the MAO-B isoform, showing approximately 50% inhibition at $10 \mu\text{M}$. Equally interesting is the hexyloxy cytosine derivative (**94**), which displays a balanced inhibition of both isoforms, with $54 \pm 4\%$ for MAO-A and $54 \pm 6\%$ for MAO-B.

Table 11. Results of ChEs and MAOs inhibition assays on 7-substituted coumarin-alicyclic amine donepezil-like hybrids **65-124**.

	<i>h</i> AChE		<i>es</i> BChE		SI AChE/BChE	<i>h</i> MAO-A	<i>h</i> MAO-B
Cmpd	Inh. % (10 μM)	IC ₅₀ (μM)	Inh. % (10 μM)	IC ₅₀ (μM)		Inh. % (10 μM)	Inh. % (10 μM)
Ethoxy (n = 2)							
95		1.04 ± 0.08	NA				
96	38 ± 4		11 ± 2				
Propoxy (n = 3)							
97	54 ± 3		21 ± 1		0.71	58 ± 5	
98		6.05 ± 0.59	17 ± 5				
99		0.31 ± 0.06		4.58 ± 1.24			
100	32 ± 1		58 ± 1				
101		4.05 ± 1.02		5.69 ± 0.76			
102	51 ± 3		19 ± 1				
103	73 ± 3		49 ± 5				
104	34 ± 5		NA				
105	39 ± 1		NA				
106	32 ± 5		26 ± 4				
Butoxy (n = 4)							
107		3.04 ± 0.62	18 ± 2		1.03	36 ± 2	23 ± 2
108	56 ± 3		28 ± 4				
109		1.30 ± 0.32	43 ± 5				
110	14 ± 4			4.82 ± 0.87			
111		2.15 ± 0.07		2.08 ± 0.38			
112	52 ± 5		12 ± 3				
Pentyloxy (n= 5)							
113	26 ± 3		10 ± 1		0.63		
114	18 ± 1		48 ± 2				
115		5.54 ± 0.15		8.82 ± 1.00			
116	36 ± 2			4.70 ± 0.58			
117	47 ± 2			3.50 ± 0.06			
118	8 ± 1		18±5				
Hexyloxy (n = 6)							
119		1.02 ± 0.15	24 ± 4		0.41		
120	51 ± 3		37 ± 2				
121		2.18 ± 0.23	44 ± 2				
122		3.07 ± 0.26		7.43 ± 0.56			
123		1.64 ± 0.58		7.38 ± 1.16			
124	54 ± 2		30 ± 2				
Donepezil		0.017 ± 0.002		2.30 ± 0.20	0.01		

*h*AChE = human Acetyl Cholinesterase; *es*BChE = equine Butyryl Cholinesterase; NA = not active

A comparable yet distinct SAR pattern emerged for the 7-substituted derivatives. As observed in the 4-substituted library, the O-alkyl chain length again played a decisive role. However, the 7-substituted analogues generally displayed greater potency, particularly toward AChE, with several compounds showing clear selectivity for this isoform (**95**, **99**, **107**, **109**, **119** and **121**). Notably, short chains ($n = 2-3$) were already sufficient to confer strong activity. Among them, the propoxy derivative **99**, bearing a benzyl-piperidinyl moiety, demonstrated a submicromolar IC_{50} of $0.305 \pm 0.059 \mu M$, making it one of the most potent inhibitors not only within the 7-substituted series but across both substitution patterns.

As the chain length increased, potency generally remained high, with the exception of the pentyloxy subseries, which showed higher IC_{50} values than the adjacent homologues. Conversely, the hexyloxy derivatives formed the subgroup with the highest number of compounds exhibiting IC_{50} values below $5 \mu M$. From a structural perspective, benzyl-piperidine and benzyl-amine fragments again emerged as key contributors to activity against both AChE and BChE, underscoring the importance of an extended hydrophobic–aromatic motif for productive interactions with ChEs. It is worth noting that the 7-substituted compounds included a greater number of BChE inhibitors, predominantly those featuring benzyl-piperazinyl, benzyl-piperidinyl, or phenyl-piperidinyl units, with several low-micromolar and selective derivatives (**110**, **116**, **117**). Nevertheless, it is noteworthy—and certainly deserving of further investigation—that compound **107** and **119**, bearing only a methyl group as a substituent ($n = 4$ and $n = 6$, respectively), still exhibited selective AChE inhibitory activity, with IC_{50} values in the sub-5 micromolar range.

Several derivatives stand out in this series. Compound **99**, for instance, not only exhibits submicromolar AChE inhibition, significantly superior to its 4-substituted analogue **69**, but also displays low-micromolar activity on BChE. Likewise, compound **101** shows a balanced low-micromolar profile toward both AChE and BChE as well as moderate selectivity for MAO-A ($58 \pm 5\%$ vs $30 \pm 6\%$ on MAO-B).

When comparing the two substitution patterns, the overall SAR conclusions regarding chain length are essentially superimposable, and the same amine fragments, benzyl-piperazine and benzyl-piperidine, consistently emerge as key

pharmacophoric drivers for tuning both potency and selectivity. An interesting observation arises when directly comparing some of the most active analogues: the propoxy benzyl-piperazine derivative loses potency when shifting from 7- to 4-substitution (compounds **99** and **69**, with IC_{50} values of $0.31 \pm 0.06 \mu M$ vs $1.79 \pm 0.34 \mu M$), whereas the opposite trend is seen for the hexyloxy benzyl-piperazine pair (**121** and **91**, with IC_{50} values of $2.18 \pm 0.23 \mu M$ vs $0.36 \pm 0.08 \mu M$).

Moreover, compounds bearing *para*-substituted benzyl piperazines, whether carrying electron-withdrawing (halo) or electron-donating (methoxy) substituents, as well as phenethyl piperazine derivatives, showed only modest inhibitory activity compared to the other compounds in both series, underscoring that both the further extension of this part of the molecule and the interaction mediated by the unsubstituted benzyl aromatic ring are critical determinants of inhibitory potency.

Taken together, these findings highlight how the substitution position on the coumarin core, together with the choice of the appropriate alicyclic amine, can significantly modulate both potency and selectivity, providing a valuable handle for fine-tuning multitarget ligand profiles of these hybrid molecules.

The inhibitory activities of the synthesized compounds toward AChE and BChE were evaluated and compared with donepezil as the reference drug. Overall, donepezil remained the most potent AChE inhibitor, exhibiting an IC_{50} value of $0.017 \pm 0.002 \mu M$, which was not matched by any compound in the present series. Nevertheless, as discussed above, several derivatives displayed noteworthy AChE inhibition, particularly among the 7-substituted series.

Among them, the hexyloxy compounds **91** and **93** ($IC_{50} = 0.36 \pm 0.08 \mu M$ and $0.67 \pm 0.11 \mu M$, respectively) and the propoxy derivative **99** ($IC_{50} = 0.31 \mu M$) emerged as the most active AChE inhibitors, although they were approximately one order of magnitude less potent than donepezil.

In contrast, a number of compounds exhibited inhibitory activity toward BChE comparable to that of donepezil ($IC_{50} = 2.30 \pm 0.20 \mu M$), with IC_{50} values that were similar than that of donepezil (e.g., sub- $5 \mu M$ values were obtained for **85** and **87** in the 4-substituted series and **99**, **110**, **111**, **116** and **117** in the 7-substituted series). Notably, **85**, a 7-substituted compound bearing a benzyl piperazine moiety, and **111**, 4-substituted compound bearing a phenyl piperidine fragment, showed IC_{50} values on BChE of 2.87 and 2.08 μM , respectively, and, interestingly, with SI

values suggesting a more balanced dual inhibition of AChE and BChE compared to the pronounced AChE selectivity of donepezil.

Such a dual inhibitory profile may be advantageous in later stages of AD, where AChE activity decreases while BChE activity increases. From a drug discovery perspective, the objective of this work was not to maximize potency against a single enzyme nor to achieve uniformly high inhibition across all investigated targets, but rather to identify compounds with a balanced and complementary activity profile, while also exploring new scaffold combinations to generate novel chemical entities with potential therapeutic relevance. This polypharmacological approach aims to capture an optimal trade-off between multiple mechanisms relevant to disease progression, potentially translating into a more robust therapeutic benefit. However, the overall efficacy of such multitarget candidates should be further validated in cellular models of AD able to better reflect its complex and multifactorial pathophysiology.

A β ₁₋₄₂ aggregation assays in the presence of the compounds 65-124

The work presented in this section was carried out at the Centre for Misfolding Diseases at the University of Cambridge (UK) during a six-month internship under the supervision of Professor Michele Vendruscolo and with the kind help of Dr. Vaidehi Roy Chowdhury.

The library of sixty coumarin-donepezil-like hybrids derivatives (compounds **65-124**) revealed several promising multitarget results in the low micromolar range (Table 10 and Table 11) and allowed to establish a clear SAR.

To test whether these compounds could also exert additional polypharmacological effect, they were evaluated for their ability to inhibit A β peptide aggregation, an ability that could be conferred by the basic part of the molecule ^[193]. Because our targets of interest act in the cerebral context in AD, we selected A β ₁₋₄₂ peptide for these assays, as amyloid plaques in AD brains predominantly comprise A β ₄₂ aggregates ^[194].

To conduct A β ₁₋₄₂ aggregation assays in the presence of compounds **65-124**, recombinant A β ₁₋₄₂ peptide was produced in-house. Since expressing A β in *Escherichia coli* (*E. coli*) has proved difficult due to its toxicity and low solubility, the peptide was expressed as fusion protein (Figure 19) ^[195,196]. The fusion protein contains an N-terminal His-tag, necessary for purification, an NT*FISp solubilizing sequence necessary to enhance the solubility of the peptide while simultaneously reducing its intrinsic tendency to aggregate, and a TEV protease cleavage site, inserted between the NT*FISp domain and A β sequence, necessary to separate A β from the rest of the fusion protein after the first step of purification.

There are various ways of cleaving the fusion protein in order to obtain the protein of interest, and certain viral proteases, such as those encoded by the tobacco etch virus (TEV), have high cleavage accuracy, making them very useful for this purpose ^[197].

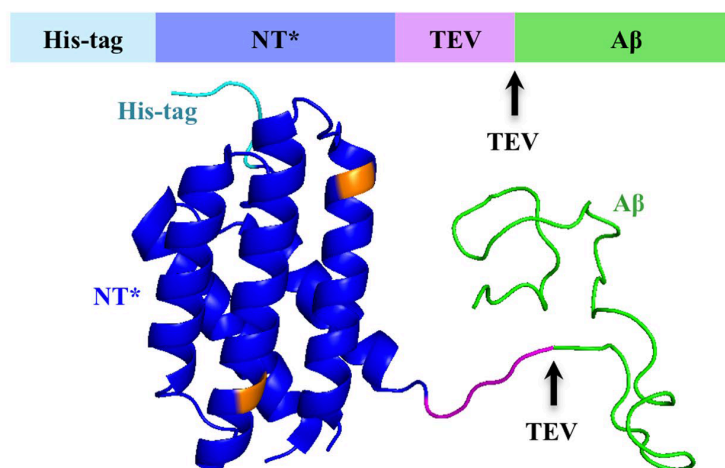


Figure 19. Schematic representation of sequence and structure of the NT*-A β fusion protein.

The arrows indicate the TEV protease cleavage site. Image from ^[198].

The TEV protease was also produced in-house. After expression and purification of the A β fusion protein, the protease was used to cleave and release the A β_{1-42} peptide. The liberated peptide was subsequently purified and obtained in monomeric form, which required kinetic validation to ensure that it behaved as expected. Specifically, it was necessary to confirm that its aggregation followed a secondary-nucleation-dominated mechanism which involve the formation and proliferation of larger aggregates such as fibrils ^[199]. Once experimental kinetic data were collected, they were analyzed using the free online platform AmyloFit ^[200,201] to determine the dominant molecular mechanisms of the reaction.

After the kinetic validation, aggregation assays were performed in presence of compounds **65-124** at a concentration of 20 μ M (20:1 compound-to-peptide molar ratio).

Kinetic validation and aggregation in the presence of the compounds were monitored by steady-state fluorescence using well-established fluorescent dye Thioflavin T (ThT) that emits at 480 nm upon binding to β -sheet-rich structures, such as amyloid fibrils ^[202].

Production of TEV protease

To overcome the limited solubility of TEV protease, an issue that occurs when the enzyme is overexpressed in *E. coli*, the protein can be produced as a fusion protein with maltose-binding protein (MBP), generating the MBP-TEV construct.

Fusion to MBP maintains the protease in a soluble, properly folded conformation and prevents accumulation into insoluble aggregates ^[203].

Following induction of protein expression in *E. coli* and subsequent reduction of the incubation temperature, the MBP-TEV fusion protein undergoes *in vivo* autocleavage, yielding His₆-TEV(S219V)-Arg₅, a highly soluble and active form of the protease that contains an N-terminal polyhistidine tag essential for purification ^[204].

After collection of the cell lysate, TEV protease was purified from the remaining cellular components by immobilized metal affinity chromatography (IMAC), exploiting the N-terminal His₆ tag and using nickel-containing affinity columns. Figure 20 shows the identification of His₆-TEV(S219V)-Arg₅ protease (~28 kDa) using SDS-PAGE after IMAC. The production and purification protocol for His₆-TEV(S219V)-Arg₅ protease (hereafter referred to as TEV) originally included an additional purification step by size-exclusion chromatography (SEC). However, the protein was deemed sufficiently pure to proceed without this further chromatographic step.

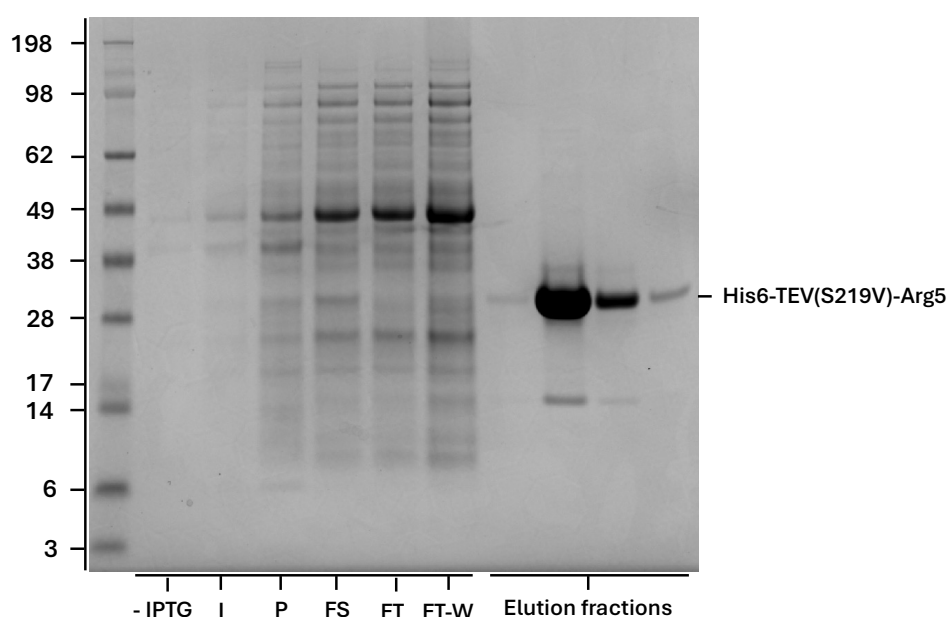


Figure 20. SDS-PAGE gel of TEV purification through IMAC. -IPTG: supernatant of the main culture before induction of protein production with IPTG. I: supernatant of the main culture after induction of protein production with IPTG. FS: supernatant from sonication and centrifugation, filtered through a 0.22 mm pore size filter. FT: flow through from the loading of the protein onto the IMAC column. FT-W: flow through when washing the IMAC column with equilibration buffer.

Expression and purification of A β ₁₋₄₂

The β -amyloid peptide, like the TEV protease, was expressed as a fusion protein containing an N-terminal polyhistidine tag together with an N-terminal domain derived from spider silk (NT*(FISp)). This domain protects the β -amyloid peptide throughout the production process and maintains it in solution at high concentrations preventing aggregation ^[198].

Similarly to the purification of TEV protease, once the *E. coli* lysate was obtained, the NTFISp-A β fusion protein was isolated from the remaining cellular components by nickel-based affinity chromatography (IMAC). Figure 21 (A) shows the chromatogram corresponding to the elution of the NTFISp-A β protein, while Figure 21 (B) reports its identification by SDS-PAGE.

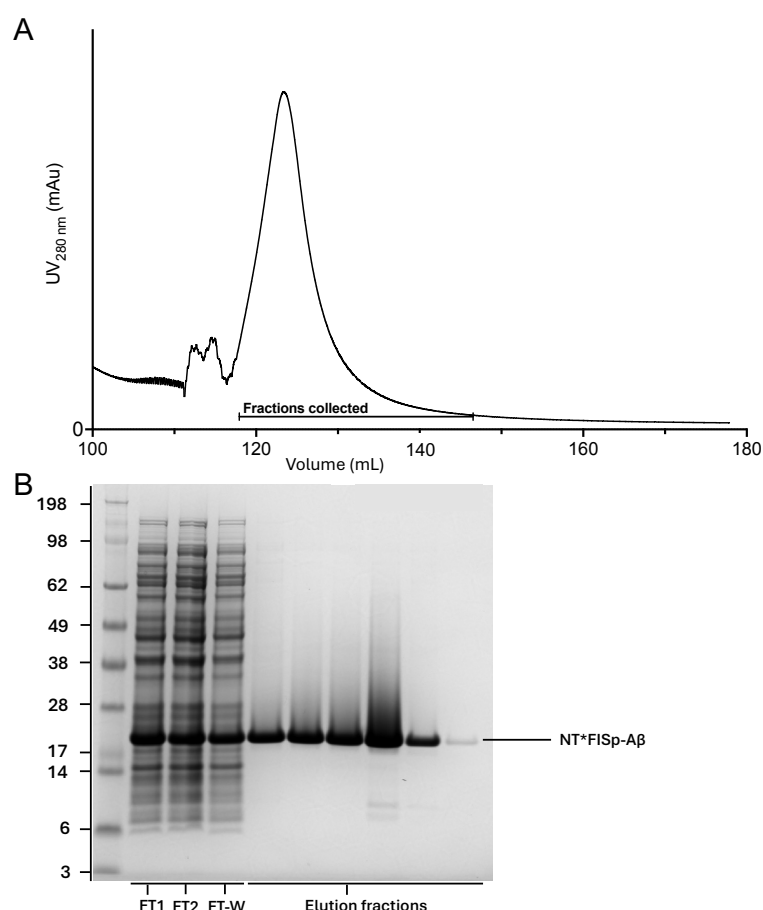


Figure 21 (A) Chromatogram of IMAC purification of NT*FISp-A β fusion protein. (B) SDS-PAGE gel of NT*FISp-A β purification through IMAC. FT1 and FT2: flow through from the loading of the protein onto the IMAC column.

FT-W: flow through when washing the IMAC column with equilibration buffer.

After purification of the NT*FISp-A β fusion protein, the A β peptide was released from the fusion construct by cleavage with TEV protease. Figure 22 shows the SDS-PAGE analysis confirming successful cleavage and separation of the A β peptide from the remaining fusion domains.

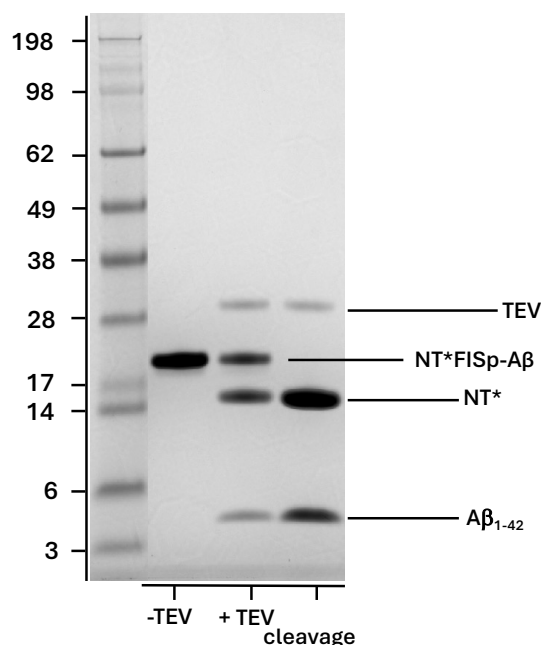


Figure 22. Cleavage of NT*FISp-A β fusion protein to obtain A β ₁₋₄₂. -TEV: before adding TEV to the solution containing NT*FISp-A β (~20 kDa). +TEV: TEV was added to the solution containing NT*FISp-A β .

The second purification step consists in isolating the A β ₁₋₄₂ peptide, which is released by TEV protease cleavage, from the remaining fusion tags and the TEV protease itself via size-exclusion chromatography (SEC). The separation of these components is illustrated in the chromatogram (Figure 23, panel **A**) and confirmed by SDS-PAGE analysis (Figure 23, panel **B**). The molecular mass of the peptide was further validated using mass spectrometry, as shown in Figure 23, panel **C**.

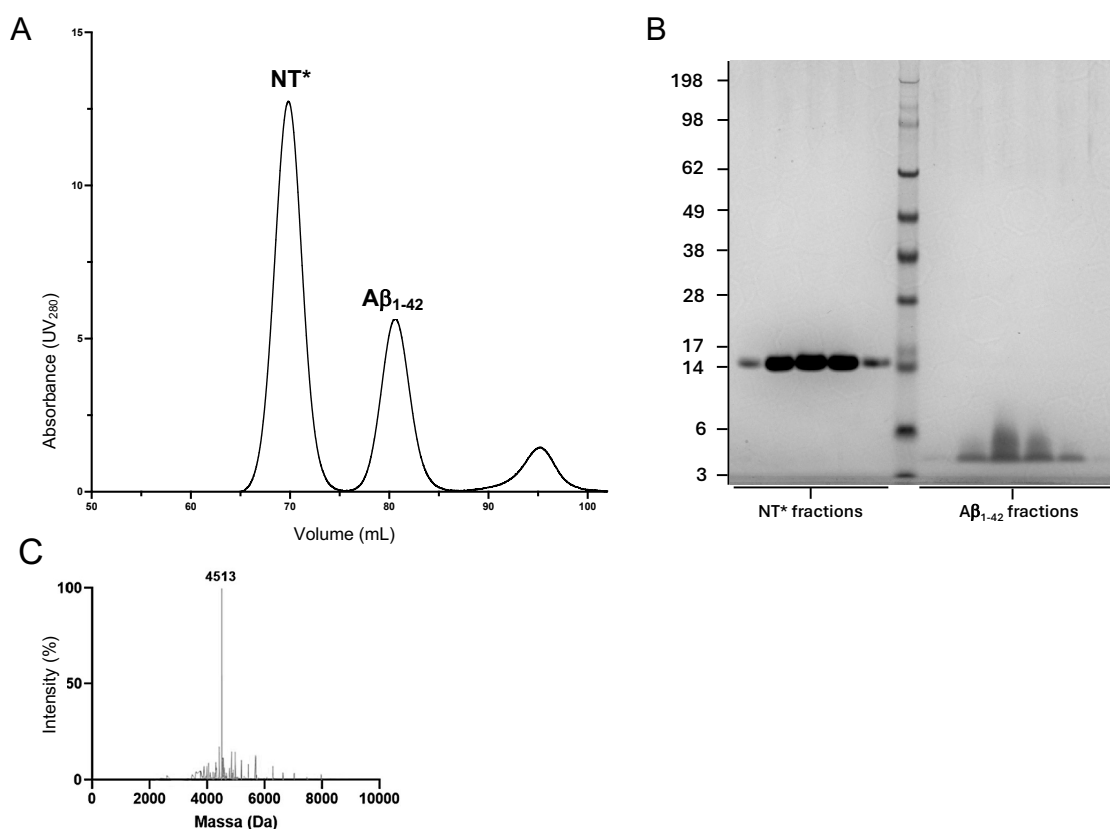


Figure 23. (A) Chromatogram of the SEC of Aβ₁₋₄₂. (B) SDS-PAGE gel of Aβ₁₋₄₂ purification through SEC. (C) Mass spectrometry of Aβ₁₋₄₂.

Kinetic validation

Before using the purified peptides to assess the effects of molecules on their aggregation, the quality of Aβ₄₀ and Aβ₄₂ must be kinetically validated through a series of aggregation assays. Aβ aggregation is primarily driven by secondary nucleation processes [199]. To ensure accurate assessment, it is essential that the peptide is in its monomeric form. Therefore, analytical size-exclusion chromatography (SEC) is performed immediately prior to the aggregation assay to separate monomeric species from any pre-formed aggregates.

The kinetics of Aβ aggregation at various concentrations (Figure 24, panel A) were monitored using steady-state fluorescence with ThT as a probe [199,202]. The resulting data were analyzed using AmyloFit [200,201].

The dependence of the half-life ($t_{1/2}$) on the initial monomer concentration (m_0) can be expressed as $t_{1/2} \sim m_0^\gamma$, where γ is the scalar exponent. By applying rate laws describing the increase in aggregate mass over time, γ can be compared to

the reaction order predicted by different aggregation models. Practically, it is convenient to plot the half-life versus monomer concentration on a logarithmic scale, as $\log(t_{1/2}) = \gamma \log(m_0) + \text{constant}$. The slope of this line thus provides the scalar exponent (Figure 24, panel B).

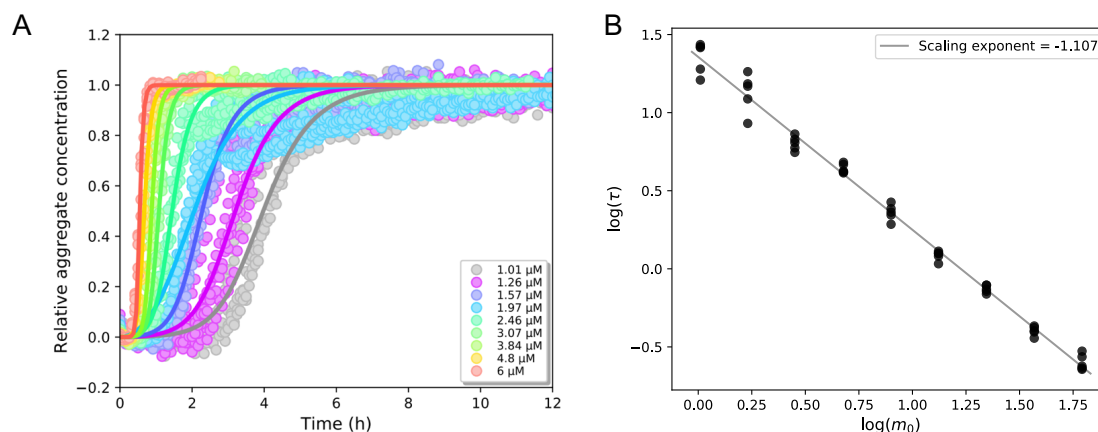


Figure 24. Aggregation kinetics of Ab₁₋₄₂. (A) Aggregation curves of Ab₁₋₄₂ at different concentrations. (C) Log of $\log(t_{1/2})$ versus $\log(m_0)$ (scalar exponent $\gamma = -1.107$, $R^2 = 0.9874$).

Each experimental sigmoidal curve was uploaded to the AmyloFit platform, where independent replicates at each concentration were combined and normalized. The platform was then used to elucidate the dominant molecular mechanisms governing A β aggregation through global fitting. A specific molecular aggregation model (secondary nucleation-dominated, unseeded) was selected in AmyloFit, which applies the integrated rate equations of the chosen model to the experimental kinetic data.

The scaling exponent was compared with that reported by Cohen et al., 2013 (-1.33 ± 0.03)^[199] and found to be consistent with an aggregation mechanism dominated by secondary nucleation. This indicates that the rate of fibril formation is dependent on the initial monomer concentration.

A β_{1-42} aggregation kinetics in the presence of compounds 65–124

The aim of this internship project was to investigate the effect of compounds 65–124 on the aggregation kinetics of A β_{1-42} . Since 7-substituted coumarin-based compounds are known to exhibit fluorescence ^[205], using a representative subset of 7-substituted derivatives from the 95–124 series, was verified that they did not display significant fluorescence at the wavelength used in the assay. The measurement was performed in triplicate for each sample, and no statistically significant emission was detected for the compounds tested.

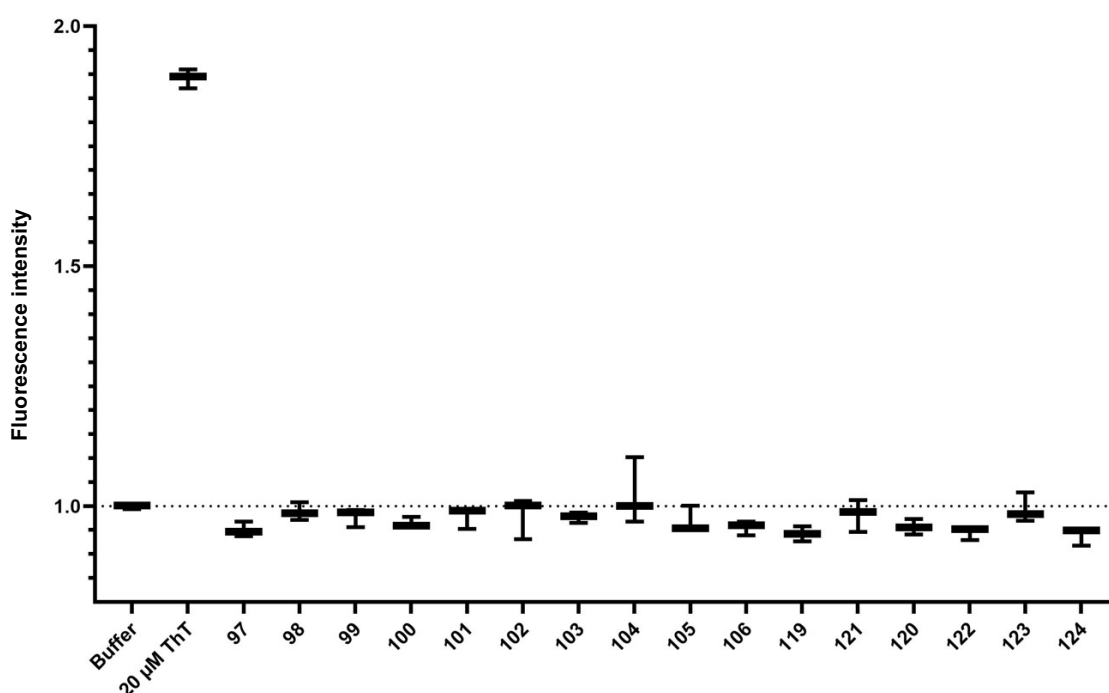


Figure 25. Fluorescence check of the compounds (20 μ M) compared to ThT (20 μ M) under the kinetic assay conditions (excitation: 440 nm, emission: 480).

Once it was confirmed that the compounds did not exhibit fluorescence that could interfere with the experiment, the assays were carried out using monomeric A β_{1-42} , obtained following the same purification procedure described above. The aggregation assay was conducted in the same conditions described for kinetic validation, and aggregation was monitored using steady-state fluorescence with ThT (20 μ M) as probe ^[199,202]. The data were analyzed using GraphPad Prism 10 (GraphPad Software). After manual removal of outlier replicates, curves were normalized from 0 to 1 by taking the average of the

baseline and plateau values. The half-times ($t^{1/2}$) of A β_{1-42} aggregation curves (1 μ M) were evaluated in the presence of each compound at 20 μ M, corresponding to a 20:1 compound-to-peptide molar ratio.

Unfortunately, none of the tested compounds produced a significant and reproducible reduction in the aggregation half-time of A β_{1-42} . Figure 26 presents data from some of the compounds as representative examples.

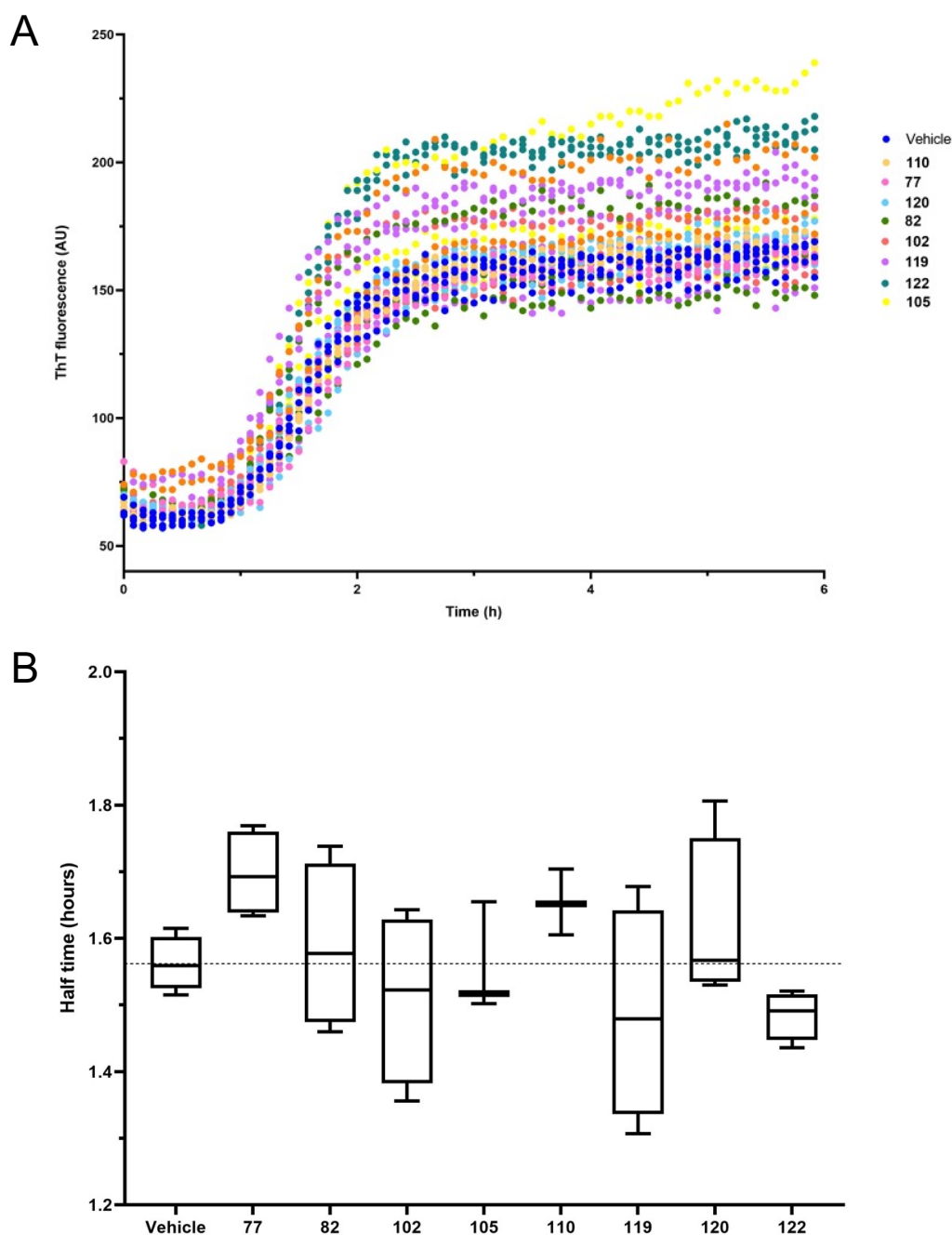


Figure 26. (A) Non-normalized aggregation curves of A β_{1-42} in the presence of compounds x-y. (B) Half-times ($t^{1/2}$) data.

Other scaffolds

Phenothiazine- or Cytisine- Cinnamic Acid hybrids

Cinnamic acids and their derivatives represent another class of naturally inspired scaffolds widely employed in medicinal chemistry. Cinnamic acid is an α,β -unsaturated aromatic carboxylic acid produced in plants through the phenylpropanoid pathway and serves as a precursor for numerous bioactive natural products. Cinnamic acid derivatives exhibit a wide range of pharmacological properties, including antioxidant, anti-inflammatory, antimicrobial, anticancer, and neuroprotective activities [44,206].

From a medicinal chemistry perspective, cinnamic acids are particularly valuable as modular building blocks for molecular hybridization strategies. Their conjugated α,β -unsaturated carbonyl system can act as a Michael acceptor, enabling interaction with nucleophilic residues of biological targets, while the aromatic ring allows extensive functionalization to tune lipophilicity, electronic properties, and target selectivity. In the context of neurodegenerative diseases, several cinnamic acid derivatives have been shown to inhibit A β aggregation, reduce oxidative stress, modulate neuroinflammation, and interact with key enzymes such as ChEs and MAOs, all of which are involved in AD pathophysiology [44,207,208].

Among cinnamic acid derivatives, ferulic acid (*4-hydroxy-3-methoxycinnamic acid*) is one of the most extensively studied scaffolds in anti-Alzheimer's medicinal chemistry [208–211]. As a naturally occurring hydroxycinnamate, Ferulic acid is regarded as a privileged fragment for the development of multifunctional hybrid molecules aimed at addressing the multifactorial nature of AD, owing to the combination of a redox-active phenolic moiety and a conjugated side chain well suited to chemical modification. Ferulic acid derivatives have been reported to modulate multiple pathological features of AD. In particular, many studies investigate the interaction between ferulic acid A β and amyloid-related pathways [212–214]. However, activity on other targets including ChEs, oxidative-stress and neuroinflammatory processes such as the ones involving MAOs have also been reported [215,215–219] making this scaffold particularly attractive in the development of new anti-AD agents.

In parallel, phenothiazine represents one of the most historically significant heterocyclic scaffolds in CNS-related medicinal chemistry. Structurally, it consists of a tricyclic system formed by two benzene rings fused to a central thiazine ring containing both sulfur and nitrogen atoms. This rigid and highly conjugated framework confers distinctive electronic and physicochemical properties that have been extensively exploited in drug discovery. Phenothiazine derivatives were among the earliest synthetic drugs introduced into clinical practice and have found broad application in psychiatry, neurology, and internal medicine.

Several phenothiazine-based drugs have been approved for clinical use, particularly as antipsychotic and antiemetic agents. Chlorpromazine, introduced in the early 1950s, was the first antipsychotic drug and revolutionized the treatment of schizophrenia and other psychotic disorders, marking the beginning of modern psychopharmacology ^[220]. Other clinically relevant phenothiazines include thioridazine, fluphenazine, and prochlorperazine, which have been employed for their antipsychotic, sedative, or antiemetic properties. Beyond central nervous system effects, phenothiazine derivatives have also demonstrated antihistaminic, anti-inflammatory, antimicrobial, and antiparasitic activities, highlighting the broad pharmacological versatility of this scaffold.

In recent years, the phenothiazine nucleus has regained attention in medicinal chemistry as a privileged scaffold for the design of new chemical entities. Numerous studies have explored phenothiazine derivatives such as anticancer, antimicrobial, antiviral, and antioxidant agents, as well as modulators of mitochondrial function and redox homeostasis. The presence of heteroatoms within the tricyclic core enables phenothiazines to participate in redox processes, π - π stacking interactions, and hydrogen bonding, making them particularly attractive for the multitarget-oriented drug design.

Importantly, phenothiazine-based compounds have also emerged as promising candidates in the field of neurodegenerative diseases, including AD. Phenothiazine derivatives have been extensively reported as ChEs inhibitors, exploiting their planar aromatic framework to engage π - π interactions within the active and peripheral anionic sites (PAS) of AChE and BChE. Structural modulation of the phenothiazine core has been shown to influence both potency and selectivity, with several derivatives displaying preferential inhibition of BChE, a feature considered particularly relevant in advanced stages of AD.

Moreover, several derivatives have been reported to interfere with A β aggregation, inhibit tau fibrillization, and modulate enzymes implicated in AD pathogenesis [221,222,220].

A paradigmatic example is methylene blue, a phenothiazine-derived compound extensively investigated for its ability to inhibit tau aggregation, modulate mitochondrial activity, and reduce oxidative stress. Methylene blue and its derivatives have progressed to advanced clinical evaluation for AD and other tauopathies, underscoring the relevance of the phenothiazine scaffold for the drug design of neuroprotective agents [223].

Alongside phenothiazines, cytisine, a quinolizidine alkaloid, represents another naturally occurring compound of significant interest in medicinal chemistry, particularly in the context of CNS disorders. Cytisine is a rigid, bicyclic quinolizidine alkaloid characterized by a nitrogen-rich framework that closely resembles the pharmacophoric features of nicotine. Owing to this structural similarity, cytisine acts as a partial agonist at nicotinic acetylcholine receptors (nAChRs) and has been extensively studied for its effects on cholinergic neurotransmission. Its ability to modulate nicotinic receptors, which are critically involved in cognition, memory, and neuroprotection, makes cytisine an attractive scaffold for the development of CNS-active compounds [224–226]. Moreover, the presence of a tertiary amine within a conformationally constrained system confers favorable interactions with cholinergic targets. On the other hand, the secondary amine and the pyrone ring provide sites for chemical functionalization aimed at fine-tuning receptor selectivity and pharmacokinetic properties. These features have prompted increasing interest in cytisine-based derivatives as potential ligands for neurodegenerative diseases, including AD, where cholinergic dysfunction represents a key pathological hallmark.

The combined incorporation of phenothiazine and cinnamic acid scaffolds into new chemical entities aims to overcome the limitations of single-target therapies and to address the multifactorial nature of complex disorders such as AD more effectively. Given the properties of the scaffold discussed above, phenothiazine- and cytisine- cinnamic acids hybrids (compounds **125-134** and **135-140**, respectively, Figure 27) were designed and synthesized. The present series were conceived to retain aromatic domains capable of engaging π - π interactions, while modulating the nature of the basic nitrogen-containing fragment through the introduction of cytisine- or the use of an alkanoyl-piperazinyl linker.

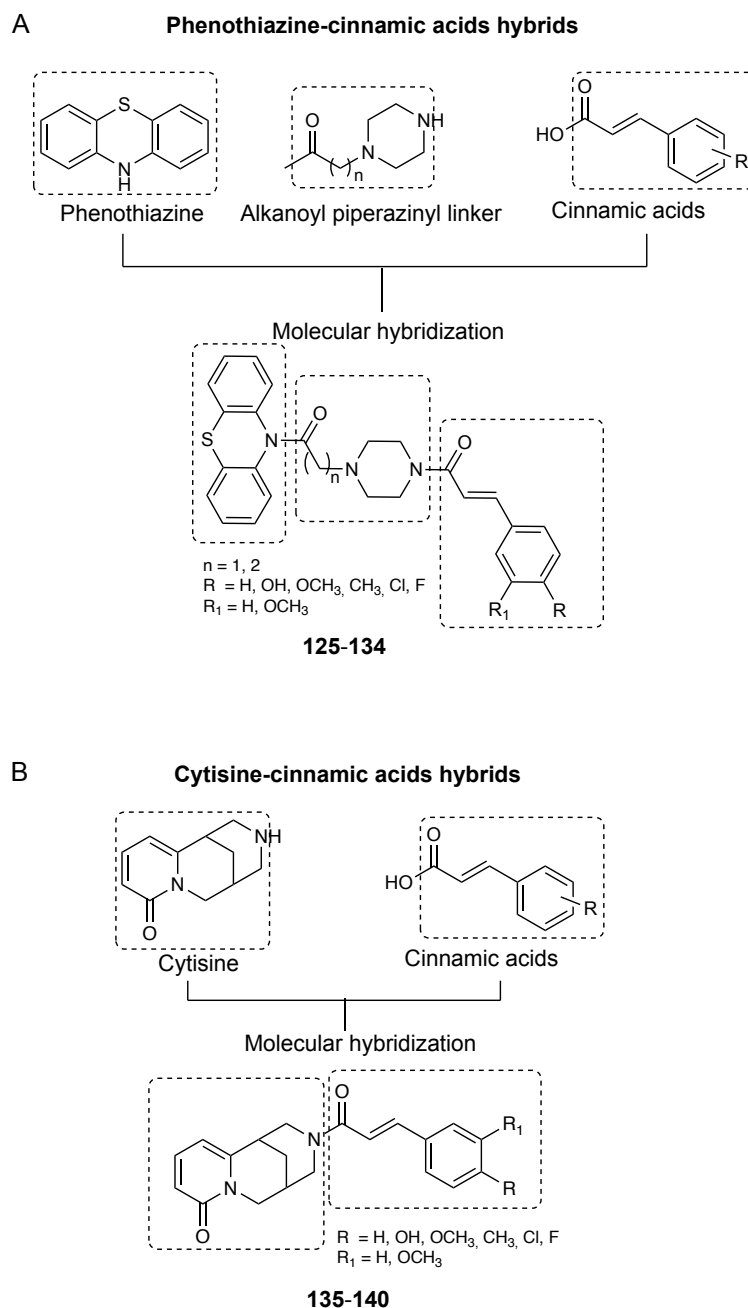


Figure 27. Design and general chemical structure of **(A)** Phenothiazine-cinnamic acids hybrids **125-134**; **(B)** Cytisine-cinnamic acids hybrids **135-140**.

The series of the phenothiazine-cinnamic acids hybrids discussed in the present section, as Figure 27 shows, are characterized by the phenothiazine ring joined to the desired cinnamic acid through an alkanoyl piperazinyl linker of different lengths to modulate flexibility and the dimensions of the molecule.

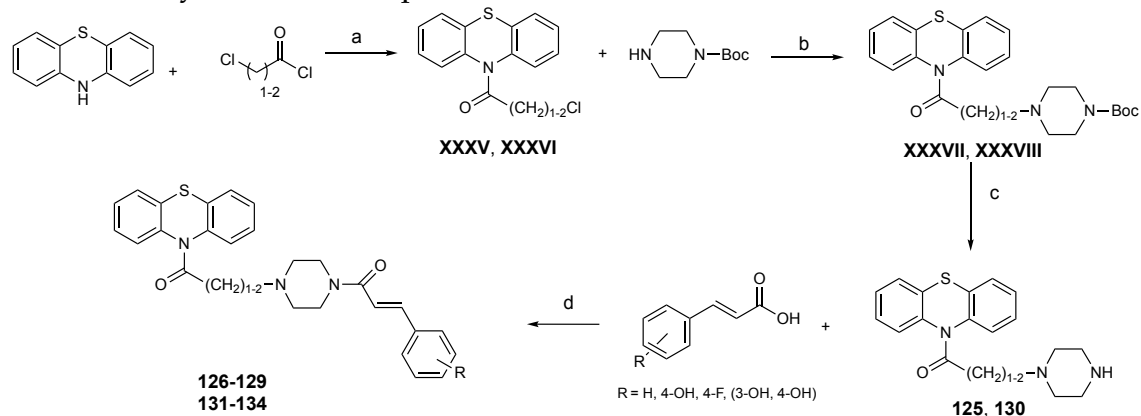
On the other hand, the cytosine-cinnamic acid hybrids (**125-134**) are characterized by the cytosine fragment directly linked to the cinnamic acid fragment and their general chemical structure is reported in Figure 27.

Phenothiazine- Cinnamic Acid hybrids

Synthesis

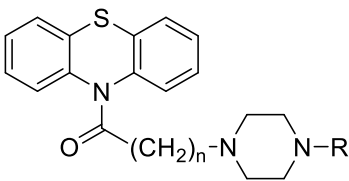
To synthesize the desired compounds, phenothiazine is first reacted with the appropriate ω -chloroacyl chloride in anhydrous toluene (Scheme 8). The N-(chloroalkoyl) derivatives of phenothiazine obtained (**XXXV** or **XXXVI**) is reacted with monoprotected N-Boc-piperazine to obtain the corresponding Boc-protected phenothiazine–piperazine derivatives (**XXXVIII** and **XXXVIII**, respectively). The reaction is carried out in acetonitrile in the presence of anhydrous K_2CO_3 , heating to reflux for 5 hours. Subsequently, the piperazine is deprotected with trifluoroacetic acid to obtain compounds **125** or **130**. The final reaction involves the direct reaction between compounds **125** or **130** and the cinnamic acids in anhydrous dimethylformamide in presence of 1-hydroxybenzotriazole (HOBT) and 3-dimethylamino-propyl)-ethyl-carbodiimide hydrochloride (EDC·HCl) as described by Cui et al. ^[227]. All final compounds (**125-134**) are purified by column chromatography on SiO_2 eluting with dichloromethane + Methanol 2 % (dichloromethane + Methanol 10 % for compounds **125** and **130**) and then characterized by TLC, melting point, ^1H and ^{13}C NMR spectra.

Scheme 8. Synthesis of compounds **125-134**.



Details of the chemical structure of the target compounds are listed in Table 12.

Table 12. Details of the chemical structure of phenothiazine-cinnamic acids hybrids **125-134**.

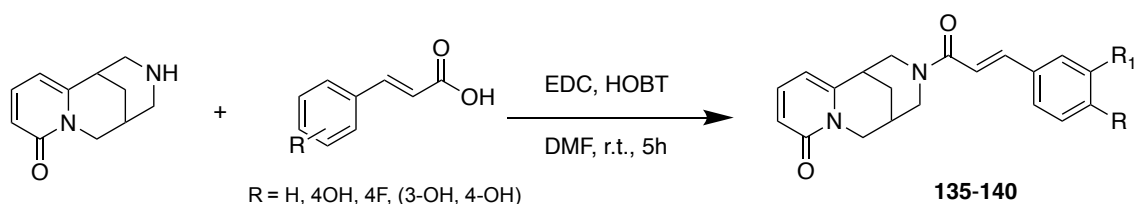
		
Compound	n	R
125	1	H
126	1	cinnamoyl
127	1	4OH-cinnamoyl
128	1	4F-cinnamoyl
129	1	feruloyl
130	2	H
131	2	cinnamoyl
132	2	4OH-cinnamoyl
133	2	4F-cinnamoyl
134	2	feruloyl

Cytisine - Cinnamic Acid hybrids

Synthesis

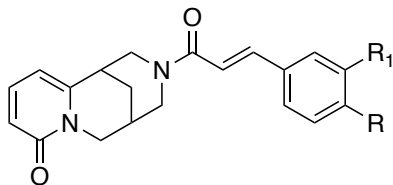
The synthesis of these compounds was accomplished in a single step involving the direct coupling between the cytisine core and the desired cinnamic acid, following the same procedure previously described for phenothiazine–cinnamic acid hybrids (Scheme 8, reaction d). The reaction was carried out in anhydrous dimethylformamide in the presence of *1-hydroxybenzotriazole* (HOBT) and *3-dimethylamino-propyl)-ethyl-carbodiimide hydrochloride* (EDC·HCl) as the coupling reagents, according to the protocol reported by Cui et al.^[227].

Scheme 9. Synthesis of compounds **135-140**.



Details of the chemical structure of the target compounds are listed in Table 13.

Table 13. Details of the chemical structure of cytisine-cinnamic acids hybrids **135-140**.



Compound	R ₁	R
135	H	H
136	OH	H
137	F	H
138	Cl	H
139	CH ₃	H
140	OH	OCH ₃

Chemical radical scavenging activity

In order to evaluate the variation of the antioxidant activity depending on chemical modifications, compounds **125-140** were tested for their chemical radical scavenging activity throughout the colorimetric assay DPPH.

The results showing antioxidant radical-scavenging activity above 10% are reported in Table 14 and Table 15 for phenothiazine-cinnamic acids hybrids **125-134** and cytosine-cinnamic acid hybrids **135-140**, respectively.

Table 14. Results of DPPH assay on phenothiazine-cinnamic acids hybrids **125-134**.

Compound	A (517 nm)	DPPH %	AA %
129	0.597	69.61 ± 2.76	30.39 ± 2.76
134	0.606	70.66 ± 1.28	29.34 ± 1.28

Table 15. Results of DPPH assay on cytosine-cinnamic acids hybrids **134-140**.

Compound	A (517 nm)	DPPH %	AA %
140	0.697	74.92 ± 3.7	25.08 ± 3.74

The DPPH assay results confirm, in agreement with previous observations, that the absence of peripheral free hydroxyl groups strongly affects radical-scavenging activity, which remains moderate or negligible (<10 %) when such functionalities are lacking. For both cinnamic acid-based hybrid series reported here, it is noteworthy that radical-scavenging activity above 10 % was observed exclusively for ferulic acid-derived compounds (**129**, **134** and **140**), whereas no comparable activity was detected for the 4-hydroxycinnamic acid derivatives, despite the presence of a free hydroxyl group. Furthermore, the antioxidant response appears to be largely independent of the nature of the heterocyclic core and of the length of the alkyl linker connecting the phenothiazine moiety to the cinnamic fragment.

Biological evaluation

ChEs inhibition

The work presented in this section was done in collaboration with Professor Marco Catto and Dr. Gabriella La Spada (University of Bari, Italy).

Phenothiazine- Cinnamic Acid hybrids

The library of phenothiazine-cinnamic acids hybrids and cytisine-cinnamic acids hybrids (compounds **125-134** and compounds **134-140**, respectively, Figure 27) was tested to investigate their potential polypharmacological activity against AChE and BChE using standardized spectrophotometric methods [181,228,229].

Results are reported in Table 16 and Table 17 for phenothiazine-cinnamic acids hybrids and cytisine-cinnamic acids hybrids, respectively. Results are reported as inhibition percentage at the concentration of 10 μ M. When the value exceeded 60 %, IC₅₀ was determined and is reported $\pm$ SEM of five experiments at different concentration executed in triplicate. The activity of donepezil was reported as reference.

Table 16. Details of the chemical structure and results of ChEs inhibition assays on phenothiazine-cinnamic acids hybrids **125-134**.

Compound	<i>h</i> AChE		<i>es</i> BChE	
	Inh. % (10 μ M)	IC ₅₀ (μ M)	Inh. % (10 μ M)	IC ₅₀ (μ M)
125	43 $\pm$ 1			3.42 $\pm$ 0.03
126	50 $\pm$ 2			6.89 $\pm$ 0.64
127	< 15		52 $\pm$ 4	
128	< 15		59 $\pm$ 3	
129	< 15			3.00 $\pm$ 0.29
130	39 $\pm$ 5		50 $\pm$ 4	
131	41 $\pm$ 3		52 $\pm$ 2	
132	45 $\pm$ 3		30 $\pm$ 5	
133	< 15		18 $\pm$ 3	
134	41 $\pm$ 3		24 $\pm$ 5	
Donepezil		0.017 $\pm$ 0.002		2.30 $\pm$ 0.20

hAChE = human Acetyl Cholinesterase; *esBChE* = equine Butyryl Cholinesterase; NA = not active

The results confirm a higher inhibitory activity toward BChE compared with AChE, in agreement with what was previously observed for phenothiazine derivatives synthesized earlier in our research group ^[193,230] and for several related compounds reported in the literature ^[231–233]. The most favorable linker length was identified as the shortest one (*n* = 1, acetyl derivatives). Indeed, the three compounds (**125**, **126**, and **129**) exhibiting IC₅₀ values in the low micromolar range (3.00–6.89 μM), belong to this subclass.

It is noteworthy that compound **125**, which does not contain a cinnamic moiety, also displayed strong inhibitory activity and the introduction of a cinnamic fragment leads to a reduction in activity (compound **126**). A plausible explanation for the observed decrease in potency is likely the loss of basicity of the terminal nitrogen atom. Several recent studies have underscored the structural importance of a basic nitrogen and its positioning relative to the pharmacophore for effective cholinesterase inhibition. For example, La Spada et al. ^[234] demonstrated that homologation at a basic nitrogen atom can switch selectivity between AChE and BChE by altering key interactions within the enzyme active sites, highlighting the role of protonable amine fragments in modulating both potency and selectivity.

The presence of an unsubstituted aromatic cinnamic nucleus results only in a modest reduction in activity. In contrast, the introduction of a single substituent at the para position (hydroxyl group and fluorine atom, **127** and **128**, respectively) caused a marked loss of inhibitory effect. Interestingly, the additional presence of a second substituent at the meta position (methoxy group), characteristic of ferulic acid, led to the most pronounced activity, yielding an IC₅₀ value of approximately 3 μM, closer to that of donepezil (compound **129**). Moreover, this compound is interesting also thanks to its antioxidant properties, which were confirmed by the DPPH radical scavenging assay (Table 14).

Since oxidative stress is widely recognized as a mechanism involved in neurodegenerative processes ^[235,236], additional cellular assays have already been planned to evaluate its neuroprotective effects against oxidative stress (H₂O₂-induced damage) and Aβ-mediated toxicity in a cellular environment in addition to the assessment of its possible effect on Aβ aggregation.

Cytisine - Cinnamic Acid hybrids

Results of Results of ChEs inhibition assays on cytisine-cinnamic acids hybrids **135-140** are reported in Table 17.

Table 17. Results of ChEs inhibition assays on cytisine-cinnamic acids hybrids **135-140**.

Compound	<i>ee</i> AChE	<i>es</i> BChE
	Inh. % (10 μ M)	Inh. % (10 μ M)
135	NA	NA
136	NA	NA
137	NA	NA
138	NA	17 $\pm$ 5
139	NA	NA
140	NA	NA

*ee*AChE = *electric eel* Acetyl Cholinesterase;
*es*BChE = *equine* Butyryl Cholinesterase;
 NA = not active

As reported in Table 17, only a marginal inhibitory activity was observed for compound **138**, which showed weak inhibition exclusively toward BChE (17 $\pm$ 5% at 10 μ M).

In this context, comparison with the previously reported series of cinnamic hybrids bearing a phenothiazine core (compounds **125–134**) clearly indicates that replacement of the phenothiazine scaffold with the cytisine nucleus resulted in a complete loss of inhibitory activity, even for those derivatives that were highly active in the phenothiazine series, such as the ferulic acid derivative (compound **129**, compared with compound **140**).

A plausible explanation for this behavior may be attributed to the increased steric hindrance arising from the direct linkage between the cinnamic moiety and the cytisine core, which could impair favorable interactions within the enzyme binding pockets.

Moreover, in agreement with our previous findings on phenothiazine-based hybrids lacking the cinnamic fragment and therefore possessing a free basic NH group (compound **125**), and consistently with literature reports ^[234], the loss of nitrogen basicity observed in this series of cytosine-based derivatives **135-140** appears to be a critical factor for ChEs inhibition. This observation further supports the notion that basicity is essential for ChE inhibitory activity, regardless of the presence of other fragments that may be active in alternative molecular combinations.

Coumarin-indole hybrids

Continuing the design of molecular hybrids by combining bioactive scaffolds, we combined two privileged structures such as coumarin and indole nuclei, in the development of potential anti-Alzheimer agents. These two fragments have independently emerged as biologically active scaffolds in the development of CNS-active compounds. Notably, in our previous work, coumarin–alicyclic amine donepezil-like hybrids (Figure 18) demonstrated promising inhibitory activity against both ChEs and MAOs, thereby supporting the effectiveness of coumarin-based hybridization strategies. Based on these findings, the combination of coumarin with an indole scaffold was considered as an attractive approach for the development of new hybrid molecules (Figure 28). Furthermore, the indole nucleus is already present in molecules such as melatonin and serotonin with important activity on the CNS.

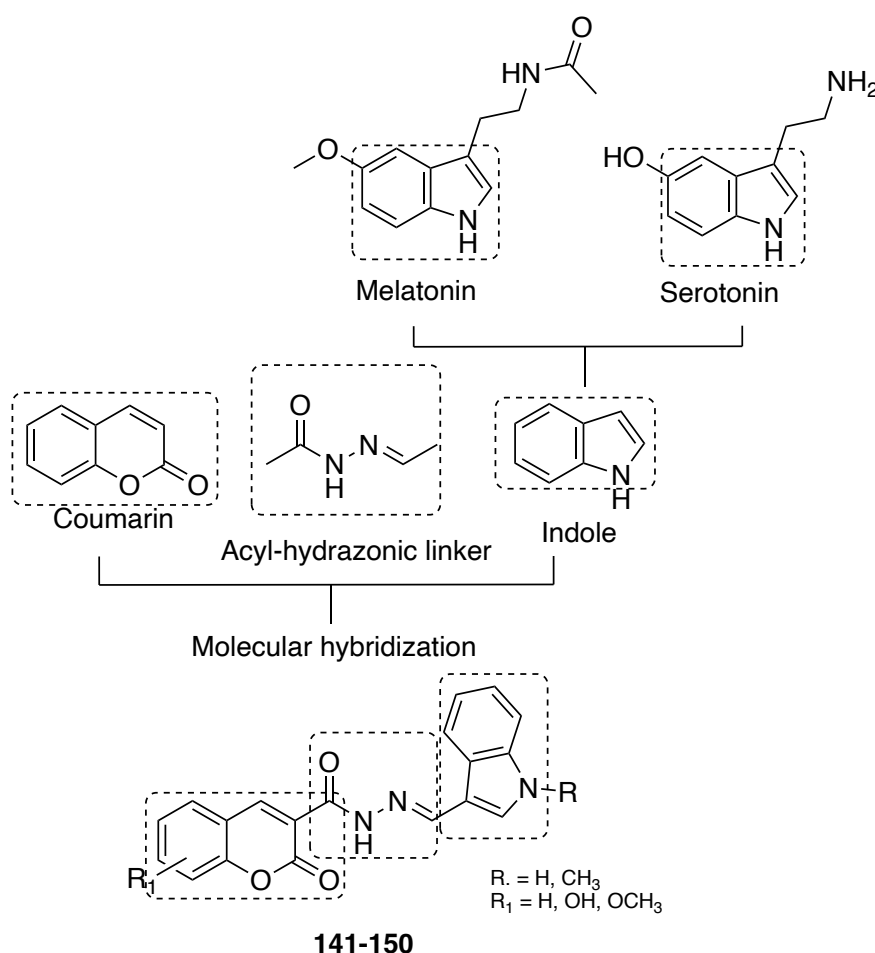


Figure 28. Design of coumarin-indole hybrids **141-150** and their general chemical structure.

Indole is a bicyclic structure of benzene fused to a five-membered pyrrole ring. The indole nucleus is a widely occurring heterocycle in biologically active compounds and plays a prominent role in central nervous system (CNS) physiology. Notably, indole is the core structural motif of serotonin (5-hydroxytryptamine) and melatonin (Figure 28), two endogenous molecules critically involved in neurochemical signaling, circadian rhythm regulation, cognition, and neuroprotection. Dysregulation of the serotonergic system and alterations in melatonin levels have both been implicated in the pathogenesis of AD [237–240].

Beyond its presence in endogenous neurotransmitters and neurohormones, the indole scaffold has been extensively explored in medicinal chemistry for the development of compounds with neuroprotective, antioxidant, anti-amyloid, and cholinesterase inhibitory activities [238,241]. Its planar aromatic structure enables favorable π – π stacking interactions, while the NH (when unsubstituted) can act as a hydrogen bond donor, contributing to binding interactions with enzyme active sites or protein aggregates relevant to AD pathology.

These features make indole-based derivatives particularly suitable for the design of CNS-active molecules with polypharmacological activity aimed at modulating multiple targets involved in neurodegeneration.

Based on the considerations discussed above, a series of coumarin–indole hybrid compounds were designed and synthesized. These molecules are characterized by a coumarin core substituted at the C-7 or C-8 position, which is linked to an indole nucleus through an acylhydrazone linker connecting the C-3 position of the coumarin to the C-3 position of the indole moiety (Figure 28).

Synthesis

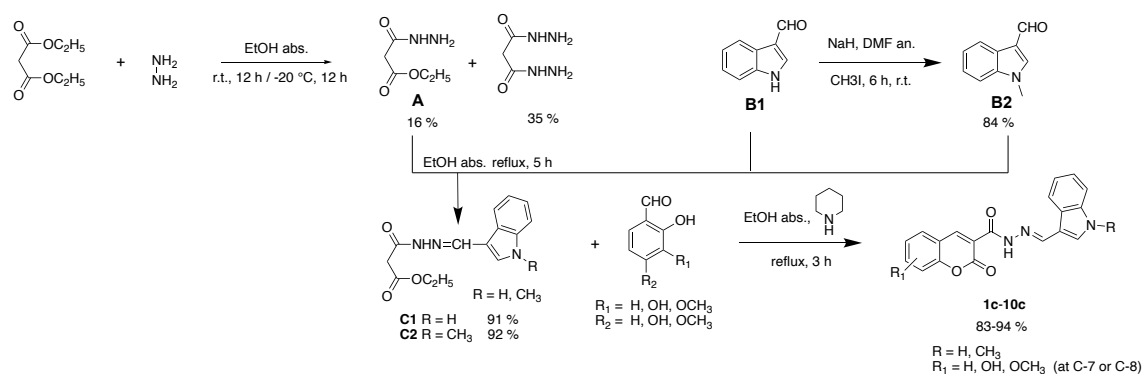
For the synthesis of the new compounds, the same synthetic rationale already reported in the antiproliferative compounds section for the coumarin- vanillin/ isovanillin hybrid hydrazones (Scheme 1 and 3) was employed.

In detail, it was first necessary to obtain intermediate **I** (hydrazonic fragment, Scheme 1). The indole fragment is represented by indole-3-carbaldehyde (commercially available) and the N-methylated analogue (intermediate **XXXIX**). These three reagents served as starting materials for the synthesis of hydrazides **XL** and **XLI**. The synthetic procedures are reported in Scheme 9.

Briefly, the malonic ester was reacted with hydrazine monohydrate in ethanol, leading to the formation of both malonohydrazide and malonomonohydrazide, as described by Abdel-Aziz et al. [41] and separated through fractional crystallization. The malonohydrazide was discarded, while malonomonohydrazide was subsequently reacted with the appropriate indole fragment. Intermediate **XXXIX** was obtained through methylation with iodomethane.

The final coumarin-indole carbohydrazides **141–150** were obtained by condensing the appropriate hydrazone intermediate (**XL** or **XLI**) with the desired 2-hydroxybenzaldehydes. As shown in Scheme 9, the hydrazones served as key precursors for the construction of the coumarin core through a piperidine-catalyzed condensation. The reaction proceeded smoothly in ethanol under reflux, gradually yielding yellow solutions from which the products crystallized either directly upon cooling or after standing at low temperature. Overall, the synthetic route afforded the final compounds in good yields and as highly crystalline solids.

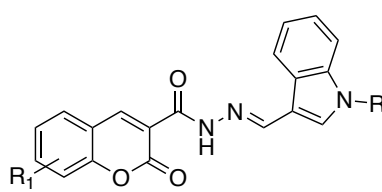
Scheme 9. Synthesis of compounds **141-150**.



In the ¹H-NMR spectra of all target compounds **141-150** and of their corresponding linear hydrazides **XL** and **XLI**, the presence of signals from two rotamers was detected as already observed for other hydrazone-containing compounds (**VII-XIX** and **1-46**). Therefore, the rotameric behavior described for those compounds can also be extended to the hydrazone-containing derivatives discussed in the present section.

Details of the chemical structure of target compounds are listed in Table 18.

Table 18. Details of the chemical structure of coumarin-indole hybrids **141-150**.



Compound	R	R ₁
141	H	H
142	H	7-OH
143	H	7-OCH ₃
144	H	8-OH
145	H	8-OCH ₃
146	CH ₃	H
147	CH ₃	7-OH
148	CH ₃	7-OCH ₃
149	CH ₃	8-OH
150	CH ₃	8-OCH ₃

Chemical radical scavenging activity

In order to evaluate the variation of the antioxidant activity depending on chemical modifications, compounds **141-150** were tested for their chemical radical scavenging activity through the colorimetric assay DPPH.

The results showing antioxidant radical-scavenging activity above 10 % are reported in Table 19.

Table 19. Results of DPPH assay of coumarin-indole hybrids **141-150**.

Compound	A (517 nm)	DPPH %	AA %
142	0.800	86.02 ± 0.30	13.98 ± 0.30
144	0.717	77.07 ± 1.63	22.93 ± 1.63
147	0.806	86.69 ± 0.72	13.31 ± 0.72
149	0.776	83.49 ± 1.29	16.51 ± 1.29

In line with previous observations made for other compound series, also within the hybrid series described in this section the presence of free hydroxyl groups emerges as a consistent structural feature among compounds exhibiting radical

scavenging activity as assessed by the DPPH assay (activity >10 %; compounds **142**, **144**, **147** and **149**).

Interestingly, despite the presence of a methylated indole nucleus (**147** and **149**), this structural element does not appear to significantly influence the overall activity. Indeed, in all the remaining compounds lacking this moiety, radical scavenging activity remains below the 10 % threshold.

A further noteworthy observation among the active compounds of this series concerns the position of the hydroxyl group on the coumarin ring. In particular, higher radical scavenging activity appears to be associated with derivatives bearing a hydroxyl group at the C-8 position (**144** and **149**), whereas lower activity is observed for analogues substituted at C-7 (**142** and **147**). However, given the qualitative nature of the DPPH assay employed in this study, this trend requires confirmation through a quantitative antioxidant assay.

Biological evaluation

ChEs inhibition

The work presented in this section was done in collaboration with Professor Marco Catto and Dr. Gabriella La Spada (University of Bari, Italy).

The library of coumarin-indole hybrids **141-150** was tested to investigate their activity against AChE and BChE using standardized spectrophotometric methods [228,181,229].

Results are reported in Table 20 along with the details about the chemical structure. Results are reported as inhibition percentage at the concentration of 10 μ M. When the value exceeded 60 % IC₅₀ was determined and is reported as $\pm$ SEM of five experiments at different concentration executed in triplicate.

Table 20. Results of ChEs inhibition assays on phenothiazine-cinnamic acids hybrids **141-150**.

Compound	<i>ee</i> AChE	<i>es</i> BChE
	Inh. % (10 μ M)	Inh. % (10 μ M)
141	NA	NA
142	NA	NA
143	20 $\pm$ 5	NA
144	NA	9 $\pm$ 1
145	25 $\pm$ 3	NA
146	NA	28 $\pm$ 1
147	NA	NA
148	NA	24 $\pm$ 5
149	NA	NA
150	NA	16 $\pm$ 2

*ee*AChE = electric eel Acetyl Cholinesterase;

*es*BChE = equine Butyryl Cholinesterase;

NA = not active

At a concentration of 10 μ M, all tested compounds exhibited weak or negligible inhibitory activity toward both ChEs isoforms. Nevertheless, some preliminary structure–activity relationships can be discerned from the obtained data.

Substitution at either the C-7 or C-8 position of the coumarin ring with a methoxy group resulted in a modest inhibitory effect (compounds **143**, **145** and **150**) when compared with the corresponding hydroxyl-substituted analogues (compounds **142**, **144**, **147**, and **149**), which were inactive.

For compounds bearing an unsubstituted coumarin scaffold (compounds **141** and **146**), the presence of a methyl group on the indole nitrogen (**146**) was associated with weak inhibition of BChE. In this context, the influence of indole N-methylation is particularly noteworthy. While indole derivatives with a free NH group showed activity, although moderate, exclusively toward AChE, N-methylation completely shifted the inhibitory profile toward BChE.

This observation suggests that alkylation at the indole nitrogen represents a structural feature capable of modulating isoform selectivity within this series of compounds and provides an interesting starting point for the rational design and

synthesis of new derivatives bearing the indole scaffold with improved selectivity profiles.

***In silico* predictions of ADME properties**

Predictions of pharmacokinetic properties of the most active compounds of each series (coumarin-alicyclic amine hybrids **65-124**, phenothiazine-cinnamic acid hybrids **125-134**, cytosine cinnamic acid hybrids **135-140** and coumarin-indole hybrids **141-150**) were made using SwissADME web tool ^[94,95]. The values of the predicted physicochemical properties and water solubility are reported in Table 21, while predicted values of pharmacokinetics and druglikeness are reported in Table 22. In the case of the non-particularly active series of cytosine cinnamic acid hybrids and coumarin-indole hybrids, some compounds were chosen as representative of the series.

Table 21. Predicted physicochemical properties and water solubility of selected compounds.

Cmpd	Physicochemical Properties				Consensus LogP o/w
	MW	#H-bond acceptors	#H-bond donors	TPSA	
Coumarin-alcyclic amine hybrids					
69	378.46	5	0	45.92	3.29
71	377.48	4	0	42.68	4.37
79	392.49	5	0	45.92	3.6
80	377.48	4	0	42.68	4.36
85	406.52	5	0	45.92	3.92
86	406.52	5	0	45.92	3.92
87	391.5	4	0	42.68	4.73
89	344.45	5	0	45.92	3
91	344.45	5	0	45.92	3
92	405.53	4	0	42.68	5.03
93	419.56	4	0	42.68	5.33
95	363.45	4	0	42.68	4.12
98	364.44	4	0	45.92	3.32
99	378.46	5	0	45.92	3.37
101	377.48	4	0	42.68	4.42
107	316.39	5	0	45.92	2.48
109	377.48	4	0	42.68	4.52
110	392.49	5	0	45.92	3.7
111	391.5	4	0	42.68	4.78
115	406.52	5	0	45.92	4
116	391.5	4	0	42.68	4.82
117	405.53	4	0	42.68	5.12
119	344.45	5	0	45.92	3.13
121	420.54	5	0	45.92	4.33
122	405.53	4	0	42.68	5.13
123	405.53	4	0	42.68	5.03
Phenothiazine-cinnamic acids hybrids					
125	325.43	3	1	60.88	2.41
126	455.57	3	0	69.16	3.93
129	501.6	5	1	98.62	3.58
Cytisine-cinnamic acids hybrids					
136	354.83	2	0	42.31	3.03
Coumarin-indole hybrids					
145	361.35	5	2	96.69	3.01
148	375.38	5	1	85.83	2.89

MW: molecular weight; #H-bond acceptors: number of H bond acceptors; #H-bond donors: number of H bond donors, TPSA: topological polar surface area; Consensus LogP o/w: average of five predictions (iLOGP, XLOGP3, WLOGP, MLOGP, SILICOS-IT).

Table 22. Predicted pharmacokinetics and druglikeness of selected compounds.

Cmpd	Pharmacokinetics								Druglikeness	
	GI	BBB	Pgp	CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4	#Lip.	Bioav.
Coumarin-alicyclic amine hybrids										
69	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
71	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
79	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
80	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
85	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
86	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
87	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
89	High	Yes	No	No	Yes	No	Yes	Yes	0	0.55
91	High	Yes	No	No	Yes	No	Yes	Yes	0	0.55
92	High	Yes	Yes	Yes	Yes	No	Yes	Yes	0	0.55
93	High	Yes	Yes	Yes	No	No	Yes	Yes	1	0.55
95	High	Yes	No	Yes	Yes	Yes	Yes	Yes	0	0.55
98	High	Yes	No	Yes	Yes	Yes	Yes	Yes	0	0.55
99	High	Yes	No	Yes	Yes	Yes	Yes	Yes	0	0.55
101	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
107	High	Yes	No	Yes	Yes	No	Yes	No	0	0.55
109	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
110	High	Yes	No	Yes	Yes	Yes	Yes	Yes	0	0.55
111	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
115	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
116	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
117	High	Yes	Yes	Yes	Yes	No	Yes	Yes	0	0.55
119	High	Yes	No	Yes	Yes	No	Yes	Yes	0	0.55
121	High	Yes	Yes	Yes	Yes	Yes	Yes	Yes	0	0.55
122	High	Yes	Yes	Yes	Yes	No	Yes	Yes	0	0.55
123	High	Yes	Yes	Yes	Yes	No	Yes	Yes	0	0.55
Phenothiazine-cinnamic acids hybrids										
125	High	Yes	Yes	Yes	No	No	Yes	Yes	0	0.55
126	High	Yes	Yes	No	Yes	Yes	Yes	Yes	0	0.55
129	High	No	Yes	No	Yes	Yes	Yes	Yes	1	0.55
Cytisine-cinnamic acids hybrids										
136	High	Yes	No	No	Yes	Yes	No	Yes	0	0.55
Coumarin-indole hybrids										
145	High	No	No	Yes	Yes	Yes	No	No	0	0.55
148	High	No	No	No	Yes	Yes	No	No	0	0.55

GI: gastrointestinal absorption; BBB: blood brain barrier permeability Pgp: P-glycoprotein substrate; CYP1A2: cytochrome P450 1A2; CYP2C19 cytochrome P450; CYP2C9: cytochrome P450 2C9, CYP2D6: cytochrome P450 2D6; CYP3A4: cytochrome P450 3A4; Lipinsky (Pfizer) filter number (#) of violation: MW ≤ 500, MLOGP ≤ 4.15, N or O ≤ 10, NH or OH ≤ 5; Bioav.: Abbott bioavailability score.

Coumarin–alicyclic amine hybrids

All coumarin–alicyclic amine hybrids exhibit favorable physicochemical properties and good drug-likeness. The molecular weight of the compounds is consistently below 500 g/mol, and almost all compounds show no violations of Lipinski's rules. Only compound **93** presents a single violation, which can be attributed to a slightly elevated LogP value.

Regarding polarity-related parameters, the number of hydrogen bond acceptors ranges between 4 and 5, while no hydrogen bond donors are present, in agreement with the tertiary amine nature of the alicyclic moiety. The topological polar surface area (TPSA) is low and narrowly distributed (42.68–45.92 Å²), well below the threshold commonly associated with poor membrane permeability. The consensus LogP values fall mostly between 2.48 and 5.33, indicating moderate lipophilicity compatible with oral drug candidates.

Moreover, all compounds in this series are predicted to have high gastrointestinal absorption and display a uniform bioavailability score of 0.55, suggesting a good probability of oral bioavailability. From a pharmacokinetic standpoint, many compounds are predicted to be P-glycoprotein substrates, which may influence cellular efflux but does not necessarily compromise absorption.

A key feature of this series is the predicted blood–brain barrier (BBB) permeability: all coumarin–alicyclic amine hybrids are predicted to be BBB-permeable. This behavior is consistent with their predicted low TPSA, absence of hydrogen bond donors, and balanced lipophilicity, making this series particularly attractive for the development of compounds with potential CNS activity.

With respect to cytochrome P450 isoforms, although none showed simultaneous inhibition of all major cytochromes, indicating a manageable pharmacokinetic profile at this stage, interactions were observed for several compounds, suggesting a potential metabolic liability that should be further investigated experimentally. Nevertheless, some derivatives (e.g., compounds **89**, **91**, **107**, and **119**) exhibit a more selective inhibition pattern, highlighting intraclass variability in metabolic liability.

Phenothiazine–cinnamic acid hybrids

The phenothiazine–cinnamic acid hybrids display a more heterogeneous physicochemical profile compared to the coumarin-based series. Molecular weights range from approximately 325 to 502 g/mol, with only compound **129** exceeding 500 g/mol and presenting one Lipinski rule violation. The LogP values (2.41–3.93) indicate moderate lipophilicity, which is generally favorable for oral bioavailability.

The number of hydrogen bond acceptors varies between 3 and 5, while hydrogen bond donors are limited to 0 or 1. The TPSA values are higher than those observed for the coumarin–alicyclic amine hybrids (60.88–98.62 Å²), reflecting the presence of the cinnamic acid moiety and contributing to increased molecular polarity.

All compounds in this series are predicted to have high gastrointestinal absorption and a bioavailability score of 0.55. However, in contrast to the coumarin–alicyclic amine hybrids, BBB permeability is variable within this series. Compounds **125** and **126** are predicted to be BBB-permeable, whereas compound **129**, the most interesting compound from biological activity perspective, is predicted not to be BBB-permeable, likely due to its higher molecular weight and increased TPSA. These findings suggest that BBB penetration in this series is strongly influenced by overall polarity and molecular size.

From a metabolic perspective, none of the compounds inhibit all the considered cytochrome P450 isoforms simultaneously.

Cytisine–cinnamic acid hybrids

The cytisine–cinnamic acid hybrid **136** shows good drug-likeness properties, with a molecular weight below 500 g/mol, no Lipinski rule violations, and a LogP value of 3.03. The compound possesses two hydrogen bond acceptors and no hydrogen bond donors, while the TPSA is relatively low (42.31 Å²), supporting membrane permeability.

The compound is predicted to have high gastrointestinal absorption, but it is predicted to be not BBB-permeable, despite favorable TPSA values. The

bioavailability score remains 0.55, and the cytochrome inhibition profile appears selective, without broad-spectrum CYP inhibition.

Coumarin–indole hybrids

The coumarin–indole hybrids **145** and **148** have molecular weights ranging from approximately 361 to 375 g/mol and show no violations of Lipinski's rules. Compared to the coumarin–alicyclic amine hybrids, these compounds exhibit higher polarity, as evidenced by elevated TPSA values (85.83–96.69 Å²) and the presence of hydrogen bond donors (1–2).

All compounds in this series are predicted to have high gastrointestinal absorption, but none are predicted to be BBB-permeable. This lack of BBB penetration is consistent with the increased polar surface area and hydrogen bonding capacity, which are known to limit passive diffusion across the BBB. In terms of pharmacokinetics, these compounds show limited cytochrome P450 inhibition, particularly lacking inhibition of CYP2D6 and CYP3A4, suggesting a potentially more favorable metabolic profile.

Overall, all analyzed compounds display adequate physicochemical properties, high predicted gastrointestinal absorption, and a uniform bioavailability score of 0.55, supporting their overall drug-likeness. However, clear differences emerge among the different series with respect, in particular, to BBB permeability. The coumarin–alicyclic amine hybrids emerge for their consistently positive BBB permeability, making them particularly promising candidates for CNS-oriented drug development. In contrast, the other series show limited or no BBB permeability, suggesting the need for appropriate formulation to overcome this limitation, particularly in the case of compound **129** phenothiazine-ferulic acid hybrid which showed an interesting profile in terms of biological activity.

Conclusions

The coumarin–alicyclic amine hybrids (**65–124**) were designed following a multitarget-oriented approach and enabled a systematic investigation of structure–activity relationships as a function of coumarin substitution position (C-4 vs C-7), O-alkyl linker length ($n = 2–6$), and nature of the alicyclic amine. The synthetic strategy proved modular and efficient, affording most final compounds in good yields ($>60\%$). Slightly reduced yields for shorter linkers are likely due to steric constraints during the coupling step, while the 4-substituted derivatives required milder reaction conditions to prevent decomposition.

None of the compounds exhibited significant radical-scavenging activity in the DPPH assay, in agreement with the absence of free phenolic OH groups. This result indicates that antioxidant properties are not a defining feature of this series in a chemical assay; however, antioxidant effects at the cellular level cannot be excluded and should be further investigated, as the compounds may interact with biochemical pathways regulating oxidative stress within the cellular system.

Cholinesterase inhibition emerged as the dominant biological activity. In the 4-substituted series, longer linkers favored potency, with hexyloxy derivatives ($n = 6$) yielding the most active AChE inhibitors; compounds **91** and **93** reached submicromolar IC_{50} values. High AChE inhibition was associated mainly with benzyl-piperazinyl fragments, whereas BChE inhibition was favored by benzyl-piperidine moieties, with compound **85** displaying the most balanced dual AChE/BChE profile. Limited but noteworthy MAO activity was observed, including selective MAO-B inhibition for compound **79** and balanced MAO-A/MAO-B inhibition for the cytosine derivative **94**.

The 7-substituted series generally showed higher AChE potency and selectivity, with effective inhibition already at shorter linker lengths. The propoxy benzyl-piperidinyl derivative **99** was among the most potent compounds across the entire library, combining submicromolar AChE inhibition with low-micromolar BChE activity. Across both substitution patterns, benzyl-piperidine and benzyl-piperazine fragments consistently emerged as key pharmacophoric elements, whereas para-substituted benzyl or extended phenethyl derivatives were less effective.

Overall, these results demonstrate that careful modulation of linker length, substitution position, and amine fragment enables fine-tuning of cholinesterase potency and selectivity, providing a solid basis for further optimization of coumarin-based multitarget ligands for Alzheimer's disease.

The "Other scaffolds" section highlighted how changes to the heterocyclic core and the connectivity between molecular fragments influence both antioxidant activity and ChEs inhibition. In the phenothiazine–cinnamic acid hybrids (**125–134**), activity shifted toward preferential BChE inhibition, with the shortest linker ($n = 1$) providing the most active derivatives (low-micromolar IC_{50} values for **125**, **126**, and **129**). Data indicate that preserving a suitably positioned basic nitrogen is critical for potency. Indeed, introducing the cinnamic fragment generally reduced inhibition (**125–126**), and moreover, mono para-substitution on the cinnamoyl ring (**127–128**) caused a marked activity loss. Notably, the feruloyl derivative **129** combined the best BChE inhibition ($\approx 3 \mu\text{M}$) with the only measurable antioxidant behavior within this scaffold class. Consistently, DPPH activity above 10 % was observed exclusively for ferulic acid–derived hybrids (**129**, **134**, and cytosine analogue **140**), suggesting that the 3-methoxy, 4-hydroxy substitution pattern is a key determinant of chemical radical-scavenging in these systems, largely independent of the heterocyclic core or linker length. Conversely, cytosine–cinnamic acid hybrids (**135–140**) were essentially inactive on ChEs (only marginal BChE inhibition was found for one compound), supporting the conclusion that loss of nitrogen basicity and/or steric constraints associated with direct cytosine–cinnamate linkage can abolish productive binding. Finally, coumarin–indole hybrids (**141–150**) showed weak or negligible ChE inhibition at $10 \mu\text{M}$, with only minor trends linked to methoxy substitution and indole N-methylation.

In silico ADMET predictions supported overall drug-likeness and high GI absorption across series, but highlighted BBB permeability as a key differentiator: coumarin–alicyclic amine hybrids were consistently BBB-permeable, whereas phenothiazine–cinnamate (notably **129**) and coumarin–indole representatives were predicted to have limited BBB penetration, indicating that future optimization should balance potency with polarity (TPSA) to improve CNS exposure.

Experimental section

Chemistry

All chemicals (reagent grade) were purchased from Merck (formerly Aldrich Chemical). Solvents were reagent-grade. All commercial reagents were used without further purification. Reaction progress was monitored using thin layer chromatography (TLC) on precoated silica gel plates (Merck DC-Alufolien Kieselgel 60; F254, Darmstadt, Germany) and the spots were detected under UV light (254 nm). Column chromatography was performed on silica gel (60; 0.063-0.200 mm). Melting point was measured on a Stuart SMP3 micromelting point apparatus and uncorrected. ^1H NMR spectra (400 MHz) and ^{13}C spectra (101 MHz) were measured on a JEOL JNM ECZ-400S/L1 spectrometer (Tokyo, Japan) at 25 °C or at variable temperature using DMSO- d_6 or CDCl_3 as solvents. Chemical shifts are reported in ppm (δ), and the splitting patterns are described as follows: s(singlet), d(doublet), dd(doublet of doublets), t(triplet), q(quartet) and m(multiplet). The purity of all final compounds was confirmed through ^1H NMR spectra.

Coumarin-alicyclic amine hybrids

General procedure for the synthesis of p-substituted piperazines XXI-XXIII.

To a solution of the appropriate 4-substituted-benzylchloride in ethanol abs., 3 eq. of an. piperazine were added. The solution is heated to reflux for 8 h. After cooling, the solvent is removed under vacuum and the residue taken up with a 10 % Na_2CO_3 solution. The aqueous phase is extracted with ethyl acetate and then, the combined organic phases were washed with brine solution, dried on Na_2SO_4 , filtered and evaporated under vacuum. A pasty residue is obtained, which after SiO_2 column chromatography (1:20; dichloromethane and successively dichloromethane + methanol 2 %) appears as a colorless oil that very slowly solidifies at room temperature.

1-(4-fluorobenzyl)piperazine (XXI)

White solid. Melting point: 87-89 °C (64-65 °C ^[242]). Yield: 76 %. Molecular formula: C₁₁H₁₅FN₂. Molecular weight: 194.25 g/mol.

1-(4-chlorobenzyl)piperazine (XXII)

White solid. Melting point: 99-102 °C (93-97 °C ^[243]). Yield: 74 %. Molecular formula: C₁₁H₁₅ClN₂. Molecular weight: 210.71 g/mol.

1-(4-methoxybenzyl)piperazine (XXIII)

White solid. Melting point: 99-102 °C (105-106 °C ^[244]). Yield: 70 %. Molecular formula: C₁₂H₁₈N₂O. Molecular weight: 206.29 g/mol.

Synthesis of N-(2-Phenylethyl)piperazine (XXIV)

To a solution of *2-phenethylbromide* in EtOH abs. 3 eq. of an. piperazine is added and heated to reflux for 4h. After cooling, the solvent is removed under vacuum and the residue taken up with a 10 % Na₂CO₃ solution. The aqueous phase is extracted with ethyl acetate and then, the combined organic phases washed with brine solution and dried with Na₂SO₄, filtered and evaporated under vacuum. A pasty residue is obtained, which after Al₂O₃ column chromatography (1:20; dichloromethane + methanol 2 %) appears as a colorless oil (^[245]).

Colorless oil. Yield: 72 %. Molecular formula: C₁₂H₁₈N₂. Molecular weight: 190.29 g/mol.

General procedure for the synthesis of the intermediates XXV-XXXIV.

To obtain the intermediates **XXV-XXXIV** 4- or 7-hydroxy coumarin (10 mmol) was reacted with the suitable α,ω -dibromoalkane in the presence of K₂CO₃ in a stoichiometric ratio of 1:3:2 ^[184] in ACN at reflux for 6 hours with a KOH trap obtaining a semi-solid reaction mixture pink- or yellow-colored respectively for 4- or 7-substituted derivatives. After evaporation of the solvent under vacuum, the residue was dissolved in dichloromethane (25 mL) and washed with water (2 x 15 mL). The organic phase was dried with Na₂SO₄ and the solvent was evaporated under vacuum. A colorless oily residue which tends to solidify at room temperature is obtained and then purified by column chromatography (SiO₂; dichloromethane; 1:17). The intermediates **XXV-XXXIV** are obtained as colorless oils which rapidly crystallize into white solids. The intermediate,

purified by chromatography (SiO₂; dichloromethane; 1:17) were characterized by TLC and melting point and compared to literature.

4-(2-bromoethoxy)-2H-chromen-2-one (XXV)

Intermediate **XXV** was obtained according to the general procedure reported above with 4-hydroxy coumarin and 1,2 dibromoethane as starting materials. White solid. Melting point: 164-166 °C (160-162 °C [246]). Yield: 30 %. Molecular formula: C₁₁H₉BrO₃. Molecular weight: 237.10 g/mol. Elemental Analysis calculated for C₁₁H₉BrO₃: C% 49.10, H% 3.37; found: C% 48.73, H% 3.35.

4-(3-bromopropoxy)-2H-chromen-2-one (XXVI)

Intermediate **XXVI** was obtained according to the general procedure reported above with 4-hydroxy coumarin and 1,3-dibromopropane as starting materials. White solid. Melting point: 110-111 °C (112-113 °C [246]). Yield: 64 %. Molecular formula: C₁₂H₁₁BrO₃. Molecular weight: 283.12 g/mol. Elemental Analysis calculated for C₁₂H₁₁BrO₃: C% 50.91, H% 3.92; found: C% 50.75, H% 3.89.

4-(4-bromobutoxy)-2H-chromen-2-one (XXVII)

Intermediate **XXVII** was obtained according to the general procedure reported above with 7-hydroxy flavone and 1,4-dibromobutane as starting materials. White solid. Melting point: 164-166 °C (163-165 °C [183]). Yield: 30 %. Molecular formula: C₁₃H₁₃BrO₃. Molecular weight: 297.15 g/mol. Elemental Analysis calculated for C₁₃H₁₃BrO₃: C% 52.55, H% 4.41; found: C% 52.37, H% 4.38.

4-((5-bromopentyl)oxy)-2H-chromen-2-one (XXVIII)

Intermediate **XXVIII** was obtained according to the general procedure reported above with 4-hydroxy flavone and 1,5-dibromopentane as starting materials. White solid. Melting point: 110-111 °C (98 °C [247]). Yield: 64 %. Molecular formula: C₁₄H₁₅BrO₃. Molecular weight: 311.18 g/mol. Elemental Analysis calculated for C₁₄H₁₅BrO₃: C% 54.04, H% 4.86; found: C% 53.85, H% 4.80.

4-((6-bromohexyl)oxy)-2H-chromen-2-one (XXIX)

Intermediate **XXIX** was obtained according to the general procedure reported above with 4-hydroxy coumarin and 1,6-dibromohexane as starting materials. White solid. Melting point: 105-106 °C (116-118 °C [17]). Yield: 44 %. Solid, white. Molecular formula: C₁₅H₁₇BrO₃. Molecular weight: 325.20 g/mol. Elemental Analysis calculated for C₁₅H₁₇BrO₃: C% 55.40, H% 5.27; found: C% 55.18, H% 5.24.

7-(2-bromoethoxy)-2H-chromen-2-one (XXX)

Intermediate **XXX** was obtained according to the general procedure reported above with 7-hydroxy coumarin and 1,2 dibromoethane as starting materials.

White solid. Melting point: 136-138 °C (134-136 °C ^[248]). Yield: 43 %. Molecular formula: C₁₁H₉BrO₃. Molecular weight: 237.10 g/mol. Elemental Analysis calculated for C₁₁H₉BrO₃: C% 49.10, H% 3.37; found: C% 49.50, H% 3.41.

7-(3-bromopropoxy)-2H-chromen-2-one (XXXI)

Intermediate **XXXI** was obtained according to the general procedure reported above with 7-hydroxy coumarin and 1,3-dibromopropane as starting materials.

White solid. Melting point: 104-105 °C (106-107 °C ^[249]). Yield: 71. Molecular formula: C₁₂H₁₁BrO₃. Molecular weight: 283.12 g/mol. Elemental Analysis calculated for C₁₂H₁₁BrO₃: C% 50.91, H% 3.92; found: C% 51.02, H% 3.95.

7-(4-bromobutoxy)-2H-chromen-2-one (XXXII)

Intermediate **XXXII** was obtained according to the general procedure reported above with 7-hydroxy coumarin and 1,4-dibromobutane as starting materials.

White solid. Melting point: 66-68 °C (64-65 °C ^[249]). Yield: 75 %. Molecular formula: C₁₃H₁₃BrO₃. Molecular weight: 297.15 g/mol. Elemental Analysis calculated for C₁₃H₁₃BrO₃: C% 52.55, H% 4.41; found: C% 52.72, H% 4.44.

7-((5-bromopentyl)oxy)-2H-chromen-2-one (XXXIII)

Intermediate **XXXIII** was obtained according to the general procedure reported above with 7-hydroxy coumarin and 1,5-dibromopentane as starting materials.

White/light grey solid. Melting point: 55-56 °C (61-63 °C ^[249]). Yield: 64 %. Molecular formula: C₁₄H₁₅BrO₃. Molecular weight: 311.18 g/mol. Elemental Analysis calculated for C₁₄H₁₅BrO₃: C% 54.04, H% 4.86; found: C% 54.22, H% 4.59.

7-((6-bromohexyl)oxy)-2H-chromen-2-one (XXXIV)

Intermediate **XXXIV** was obtained according to the general procedure reported above with 7-hydroxy coumarin and 1,6-dibromohexane as starting materials.

White solid. Melting point: 63-64 °C (58-60 °C ^[249]). Yield: 53 %. Molecular formula: C₁₅H₁₇BrO₃. Molecular weight: 325.20 g/mol. Elemental Analysis calculated for C₁₅H₁₇BrO₃: C% 55.40, H% 5.27; found: C% 55.62, H% 5.30.

General procedures for the preparation of final compounds 65-124.

The appropriate intermediate is reacted (1 mmol) in a closed tube with the desired alicyclic amine or cytosine in the presence of K_2CO_3 in a stoichiometric ratio of 1:1:1 in ACN, heating under stirring for 20-24 hours with a KOH trap. For the synthesis of the 4-substituted compounds (**65-94**), the reaction was conducted at room temperature for 24 hours.

After the evaporation of the solvent under vacuum, the residue was dissolved in 10 mL water and extracted with dichloromethane (3 x 15 mL). The organic phase was dried with Na_2SO_4 and the solvent was evaporated under vacuum.

Pure final compounds (**65-124**) were obtained by column chromatography (SiO_2 /dichloromethane + methanol 2 % MeOH or + methanol 5 % in the case of derivatives containing methyl piperazine and benzyl piperazine; 1:15), characterized by TLC, melting point, elemental analysis, ^1H and ^{13}C NMR spectra. In the ^1H NMR spectra H atoms relative to the pyrrolidine ring are reported as "pyr" while H atoms belonging to the non-aromatic part of cytosine nuclei are reported as "cyt".

4-(2-(4-Benzylpiperazin-1-yl)ethoxy)-2H-chromen-2-one (65)

Compound was obtained according to the general procedure reported above with intermediate **XXV** and N-benzylpiperazine as starting material.

Solid, white. Yield: 59 %; Melting point: 117-118°C. Molecular formula: $C_{22}H_{24}N_2O_3$. Molecular weight: 364,45 g/mol.

^1H NMR (400 MHz, $CDCl_3$) δ : 7.75 (dt, J = 8.0, 1.3 Hz, 1H, Ar), 7.62 – 7.04 (m, 8H, Ar), 5.65 (s, 1H, Ar), 4.25 (t, J = 5.4 Hz, 2H, OCH_2), 3.71 (s, 2H, NCH_2Ar), 3.08 – 2.27 (m, 10H, CH_2N + piperazine).

^{13}C NMR (101 MHz, $CDCl_3$) δ : 165.44, 162.85, 153.41, 132.61, 130.05, 128.72, 128.24, 124.10, 123.04, 116.93, 115.63, 90.82, 67.79, 62.38, 56.12, 52.51

4-(2-(4-Benzylpiperidin-1-yl)ethoxy)-2H-chromen-2-one (66)

Compound was obtained according to the general procedure reported above with intermediate **XXV** and 4-benzylpiperidine as starting material.

Solid, white/pale yellow. Yield: 62 %; Melting point: 117-118 °C. Molecular formula: $C_{23}H_{25}NO_3$. Molecular weight: 363,46 g/mol.

^1H NMR (400 MHz, $CDCl_3$) δ : 7.75 (dd, J = 8.0, 1.6 Hz, 1H, Ar), 7.55 - 7.51 (m, 1H, Ar), 7.33 – 7.05 (m, 7H, Ar), 5.68 (s, 1H, Ar), 4.39 (t, J = 5.8 Hz, 2H, OCH_2), 3.47 –

2.79 (m, 4H, piperidine), 2.55 (d, $J = 6.4$ Hz, 2H, CH_2Ar), 2.32 (t, $J = 5.8$ Hz, 2H, CH_2N), 1.80 – 1.32 (m, 5H, piperidine).

^{13}C NMR (101 MHz, CDCl_3) δ : 165.18, 162.72, 153.41, 140.15, 132.61, 129.17, 129.07, 128.40, 126.13, 124.08, 122.98, 116.94, 115.56, 91.05, 66.74, 56.30, 54.29, 42.83, 37.26, 31.29.

4-(3-(4-Methylpiperazin-1-yl)propoxy)-2H-chromen-2-one (67)

Compound was obtained according to the general procedure reported above with intermediate **XXVI** and N-methylpiperazine as starting material.

Solid, white/pale yellow. Yield: 53 %; Melting point: 76-78 °C. Molecular formula: $\text{C}_{17}\text{H}_{22}\text{N}_2\text{O}_3$. Molecular weight: 302.37 g/mol.

^1H NMR (400 MHz, CDCl_3): δ 7.79 (dd, $J = 8.0, 1.6$, 1 H, Ar); 7.61-7.51 (m, 1H, Ar); 7.34-7.22 (m, 2H, Ar); 5.69 (s, 1H, Ar); 4.19 (t, $J = 6.2$, 2H, OCH_2); 2.83-2.48 (m, 10H, CH_2N + piperazine); 2.39 (s, 3H, N- CH_3); 2.08 (q, $J = 6.6$, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$).

^{13}C NMR (101 MHz, CDCl_3): δ 165.74; 163.08; 153.44; 132.46; 123.95; 123.04; 116.89; 115.84; 90.62; 67.66; 55.10; 53.13; 45.98; 26.12.

4-(3-(4Phenylpiperazin-1-yl)propoxy)-2H-chromen-2-one (68)

Compound was obtained according to the general procedure reported above with intermediate **XXVI** and N-phenylpiperazine as starting material.

Solid, white. Yield: 81 %; Melting point: 118-119°C. Molecular formula: $\text{C}_{22}\text{H}_{24}\text{N}_2\text{O}_3$. Molecular weight: 364.45 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.79 (dd, 1H, 8.0, 1.6, Ar); 7.59-7.49 (m, 1H, Ar); 7.37-7.19 (m, 4H, Ar); 6.97-6.80 (m, 3H, Ar); 5.70 (s, 1H, Ar); 4.22 (t, $J = 6.2$, 2H, OCH_2); 3.31-3.18 (m, 6H, CH_2N + piperazine); 2.78-2.59 (m, 4H, piperazine); 2.14 (q, $J = 6.6$, 2H, $\text{CH}_2\text{CH}_2\text{CH}_2$).

^{13}C NMR (101 MHz, CDCl_3): δ 165.71; 163.05, 153.46; 151.27; 132.74; 132.49; 129.23; 123.97; 123.04; 122.84; 119.97; 117.01; 116.93; 116.25; 115.84; 91.00; 90.68; 67.64; 54.83; 53.38; 49.19; 26.08.

4-(3-(4-Benzylpiperazin-1-yl)propoxy)-2H-chromen-2-one (69)

Compound was obtained according to the general procedure reported above with intermediate **XXVI** and N-benzylpiperazine as starting material.

Solid, pale yellow. Yield: 74 %; Melting point: 89-92 °C. Molecular formula: $\text{C}_{23}\text{H}_{26}\text{N}_2\text{O}_3$. Molecular weight: 378.47 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.78 (dd, $J = 7.9, 1.6$ Hz, 1H, Ar), 7.52 (dd, $J = 8.4, 1.6$, 1H, Ar), 7.38 – 7.14 (m, 7H, Ar), 5.68 (s, 1H, Ar), 4.18 (t, $J = 6.3$ Hz, 2H, OCH_2),

3.52 (s, 2H, CH₂Ar), 2.84-2.23 (m, 10H, CH₂N + piperazine), 2.07 (q, J = 6.6 Hz, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 165.74, 163.07, 153.44, 132.45, 129.36, 128.34, 127.23, 123.95, 123.05, 116.89, 115.85, 90.62, 67.70, 63.07, 54.76, 53.26, 53.05, 26.13.

4-(3-(4-Phenylpiperidin-1-yl)propoxy)-2H-chromen-2-one (70)

Compound was obtained according to the general procedure reported above with intermediate XXVI and 4-phenylpiperidine as starting material.

Solid, light pink. Yield: 78 %; Melting point: 103-104 °C. Molecular formula: C₂₃H₂₆N₂O₃. Molecular weight: 363.46 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.81 (dd, 8.0, 1.8, 1H, Ar); 7.54 (dd, J = 8.8, 1.8, 1H, Ar); 7.39-7.11 (m, 7H, Ar); 5.71 (s, 1H, Ar); 4.22 (t, J = 6.4, 2H, OCH₂); 3.21-2.98 (m, 2H, CH₂N); 2.74-2.56 (m, 4H, piperidine); 2.56-2.42 (m, 1H, CH-Ar); 2.29-2.04 (m, 4H, piperidine); 1.95 (q, J = 6.6, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃): δ 165.75; 163.08; 153.47; 146.17; 132.47; 128.55; 126.95; 126.33; 123.96; 123.07; 116.91; 115.87; 90.67; 67.84; 55.21; 54.56; 42.70; 33.40; 26.30.

4-(3-(4-Benzylpiperidin-1-yl)propoxy)-2H-chromen-2-one (71)

Compound was obtained according to the general procedure reported above with intermediate XXVI and 4-benzylpiperidine as starting material.

Solid, pale pink. Yield: 58 %; Melting point: 80-81. Molecular formula: C₂₄H₂₇NO₃. Molecular weight: 377.48 g/mol.

¹H NMR (400 MHz, CDCl₃): δ 7.79 (dd, J = 8.0, 1.6, 1H, Ar); 7.59 (dd, J = 8.6, 1.6, 1H, Ar); 7.36-7.06 (m, 7H, Ar); 5.68 (s, 1H, Ar); 4.17 (t, J = 6.4, 2H, OCH₂); 2.93 (d, J = 11.0, 2H, CH₂Ar); 2.62-2.44 (m, 4H, piperidine); 2.07 (t, J = 7.0, 2H, CH₂N); 2.02-1.84 (m, 2H, CH₂CH₂CH₂); 1.71-1.60 (m, 2H, piperidine); 1.59-1.47 (m, 1H, CHCH₂); 1.41-1.26 (m, 2H, piperidine).

¹³C NMR (101 MHz, CDCl₃): δ 163.08; 153.45; 140.68; 132.44; 129.20; 129.13; 128.28; 125.92; 123.94; 123.07; 116.88; 115.86; 90.62; 67.85; 65.94; 55.16; 54.11; 43.21; 37.97; 32.13; 26.29; 15.36.

3-(3-((2-Oxo-2H-chromen-4-yl)oxy)propyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (72)

Compound was obtained according to the general procedure reported above with intermediate XXVI and cytosine as starting material.

Solid, white/pale-yellow. Yield: 69 %. Melting point: 159-160 °C. Molecular formula: C₂₃H₂₄N₂O₄. Molecular weight: 392.46 g/mol.

¹H NMR (400 MHz, CDCl₃) δ 7.70 (dd, J = 7.9, 1.6, 1H, Ar); 7.54-7.46 (m, 1H, Ar); 7.31-7.18 (m, 3H, 1H pyr + 2H Ar); 6.45 (dd, J = 9.0, 1.4, 1H, pyr); 5.91 (dd, J = 6.9, 1.4, 1H, pyr); 5.41 (s, 1H, Ar); 4.03 (d, J = 15.4, 1H, cyt); 3.93-3.72 (m, 3H, 1H cyt + 2H OCH₂); 3.02-2.79 (m, 3H, 1H cyt + 2H CH₂N); 2.54-2.28 (m, 4H, cyt); 2.09-1.70 (m, 5H, 3H cyt + 2H CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 165.34, 163.55, 162.87, 153.40, 151.29, 138.82, 132.33, 123.84, 122.94, 116.90, 116.84, 115.84, 104.54, 90.62, 66.84, 60.76, 60.36, 53.51, 50.14, 35.56, 28.11, 25.95, 25.87.

4-(3-(4-(4-Fluorobenzyl)piperazin-1-yl)propoxy)-2H-chromen-2-one (73)

Compound was obtained according to the general procedure reported above with intermediate **XXVI** and N-(4-fluorobenzyl)piperazine as starting material.

Solid, white. Yield: 68 %. Melting point: 125-126 °C. Molecular formula: C₂₃H₂₅FN₂O₃. Molecular weight: 396.46 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.77 (dd, J = 8.0, 1.9 Hz, 1H, Ar), 7.63 – 7.49 (m, 1H, Ar), 7.39 – 7.22 (m, 4H, Ar), 7.12 – 6.92 (m, 2H, Ar), 5.68 (s, 1H, Ar), 4.20 (t, J = 6.2, 2H, OCH₂), 3.63 (s, 2H, CH₂-Ar), 3.07 – 2.37 (m, 10H, CH₂N + piperazine), 2.18 (p, J = 6.2, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 165.32 163.34 153.42 136.59 132.64 132.86 130.49 128.59 124.08 122.95 116.96 115.67 90.79 67.39 62.73 54.33 53.08 53.38 26.09.

4-(3-(4-(4-Chlorobenzyl)piperazin-1-yl)propoxy)-2H-chromen-2-one (74)

Compound was obtained according to the general procedure reported above with intermediate **XXVI** and N-(4-chlorobenzyl)piperazine as starting material.

Solid, white. Yield: 73 %. Melting point: 115-116 °C. Molecular formula: C₂₃H₂₅ClN₂O₃. Molecular weight: 412.91 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.78 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.58 – 7.48 (m, 1H, Ar), 7.34 – 7.20 (m, 6H, Ar), 5.68 (s, 1H, Ar), 4.18 (t, J = 6.3 Hz, 2H, OCH₂), 3.46 (s, 2H, CH₂Ar), 2.77 – 2.26 (m, 10H, CH₂N + piperazine), 2.07 (p, J = 6.6 Hz, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 165.74, 163.09, 153.45, 136.69, 132.88, 132.46, 130.54, 128.47, 123.04, 116.90, 115.85, 90.63, 67.69, 62.28, 54.73, 53.28, 53.05, 26.13.

4-(3-(4-(4-Methoxybenzyl)piperazin-1-yl)propoxy)-2H-chromen-2-one (75)

Compound was obtained according to the general procedure reported above with intermediate **XXVI** and N-(4-methoxybenzyl)piperazine as starting material.

Solid, pale-yellow. Yield: 74 %. Melting point: 90-91 °C. Molecular formula: $C_{24}H_{28}N_2O_4$. Molecular weight: 408.50 g/mol.

^1H NMR (400 MHz, $CDCl_3$) δ : 7.78 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.57 – 7.48 (m, 1H, Ar), 7.34 – 7.15 (m, 5H, Ar), 6.88 – 6.78 (m, 1H, Ar), 5.68 (s, 1H, Ar), 4.18 (t, J = 6.3 Hz, 2H, OCH_2), 3.77 (s, 3H, OCH_3), 3.44 (s, 2H, CH_2Ar), 2.74 – 2.27 (m, 10H, CH_2N + piperazine), 2.06 (p, J = 7.0 Hz, 2H, $CH_2CH_2CH_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ : 165.75, 163.10, 158.84, 158.79, 153.45, 132.45, 130.54, 130.51, 130.08, 123.95, 123.05, 116.89, 115.86, 113.67, 113.64, 90.61, 67.74, 62.49, 55.34, 54.78, 53.33, 53.01, 52.96, 26.17.

4-(3-(4-Phenethylpiperazin-1-yl)propoxy)-2H-chromen-2-one (76)

Compound was obtained according to the general procedure reported above with intermediate **XXVI** and N-(phenylethyl)piperazine as starting material.

Solid, white. Yield: 72 %. Melting point: 128-130 °C. Molecular formula: $C_{24}H_{28}N_2O_3$. Molecular weight: 392.50 g/mol.

^1H NMR (400 MHz, $CDCl_3$) δ : 7.80 (dd, J = 8.0, 1.6 Hz, 1H, Ar), 7.58 – 7.49 (m, 1H, Ar), 7.34 – 7.14 (m, 7H, Ar), 5.69 (s, 1H, Ar), 4.19 (t, J = 6.3 Hz, 2H, OCH_2), (m, 14H, CH_2N + CH_2CH_2Ar + piperazine), 2.09 (p, J = 6.6 Hz, 2H, $CH_2CH_2CH_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ : 165.75, 163.08, 153.46, 140.30, 132.46, 128.78, 128.50, 126.18, 123.95, 123.05, 116.91, 115.86, 90.64, 67.70, 60.54, 54.77, 53.31, 53.23, 33.66, 26.15.

4-(4-(4-Methylpiperazin-1-yl)butoxy)-2H-chromen-2-one (77)

Compound was obtained according to the general procedure reported above with intermediate **XXVII** and N-methylpiperazine as starting material.

Solid, light yellow. Yield: 73 %. Melting point: 82-83 °C. Molecular formula: $C_{18}H_{24}N_2O_3$. Molecular weight: 316.40 g/mol.

^1H NMR (400 MHz, $CDCl_3$) δ : 7.79 (dd, J = 8.0, 1.4 Hz, 1H, Ar), 7.57 – 7.48 (m, 1H, Ar), 7.33 – 7.20 (m, 2H, Ar), 5.66 (s, 1H, Ar), 4.14 (t, J = 6.3 Hz, 2H, OCH_2), 2.82 – 2.35 (m, 10H, CH_2N + piperazine), 2.31 (s, 3H, CH_3), 1.92 (p, J = 7.0 Hz, 2H $CH_2(CH_2)_2CH_2$), 1.71 (p, J = 6.8 Hz, 2H, $CH_2(CH_2)_2CH_2$).

^{13}C NMR (101 MHz, $CDCl_3$) δ : 165.76, 163.16, 153.42, 132.49, 123.97, 123.09, 116.89, 115.83, 90.53, 77.34, 69.23, 57.88, 54.96, 52.91, 45.86, 26.55, 23.31.

4-(4-(4-Phenylpiperazin-1-yl)butoxy)-2H-chromen-2-one (78)

Compound was obtained according to the general procedure reported above with intermediate **XXVII** and N-phenylpiperazine as starting material.

Solid, white. Yield: 76 %. Melting point: 120-121 °C. Molecular formula: C₂₃H₂₆N₂O₃. Molecular weight: 378.47 g/mol.

¹H NMR (400 MHz, CDCl₃): δ 7.81 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.58 – 7.49 (m, 1H, Ar), 7.34 – 7.20 (m, 4H, Ar), 6.99 – 6.79 (m, 3H, Ar), 5.67 (s, 1H, Ar), 4.16 (t, J = 6.3 Hz, 2H, OCH₂), 3.24-3.17 (m, 4H, piperazine), 2.66 – 2.59 (m, 4H, piperazine), 2.53-2.45 (m, 2H, CH₂N), 1.95 (p, J = 6.8 Hz, 2H, CH₂(CH₂)₂CH₂), 1.77 (p, J = 6.8 Hz, 2H, CH₂(CH₂)₂CH₂).

¹³C NMR (101 MHz, CDCl₃): δ 165.76, 163.12, 153.45, 151.34, 132.50, 129.22, 123.97, 123.10, 119.88, 116.91, 116.18, 115.85, 90.56, 69.27, 58.08, 53.37, 49.22, 26.64, 23.44.

4-(4-(4-Benzylpiperazin-1-yl)butoxy)-2H-chromen-2-one (79)

Compound was obtained according to the general procedure reported above with intermediate XXVII and N-benzylpiperazine as starting material.

Solid, pale-yellow. Yield: 72 %. Melting point: 68-70 °C. Molecular formula: C₂₄H₂₈N₂O₃. Molecular weight: 392.50 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.79 (dd, J = 8.0, 1.6 Hz, 1H, Ar), 7.57 – 7.48 (m, 1H, Ar), 7.35 – 7.16 (m, 7H, Ar), 5.65 (s, 1H, Ar), 4.14 (t, J = 6.3 Hz, 2H, OCH₂), 3.50 (s, 2H, CH₂Ar), 2.88 – 2.17 (m, 10H, CH₂N + piperazine), 1.91 (p, J = 6.6 Hz, 2H, CH₂(CH₂)₂CH₂), 1.71 (p, J = 6.4 Hz, 2H, CH₂(CH₂)₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ 165.75, 163.12, 153.43, 138.04, 132.46, 129.34, 128.32, 127.18, 123.95, 123.10, 116.88, 115.85, 90.53, 69.27, 63.12, 58.07, 53.28, 53.07, 26.66, 23.38.

4-(4-(4-Phenylpiperidin-1-yl)butoxy)-2H-chromen-2-one (80)

Compound was obtained according to the general procedure reported above with intermediate XXVII and 4-phenylpiperidine as starting material.

Solid, white/pale-yellow. Yield: 71 %. Melting point: 110-111 °C. Molecular formula: C₂₄H₂₇NO₃. Molecular weight: 377.48 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.81 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.58 – 7.49 (m, 1H, Ar), 7.34 – 7.20 (m, 4H, Ar), 6.97 – 6.79 (m, 3H, Ar), 5.67 (s, 1H, Ar), 4.16 (t, J = 6.3 Hz, 2H, OCH₂), 3.25 – 3.17 (m, 4H, piperidine), 2.56 – 2.42 (m, 7H, CH₂N + piperidine + CH-Ar), 1.96 (p, J = 6.4 Hz, 2H, CH₂(CH₂)₂CH₂), 1.77 (p, J = 6.4 Hz, 2H, CH₂(CH₂)₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 165.76, 163.12, 153.45, 151.34, 132.50, 129.22, 123.97, 123.10, 119.88, 116.91, 116.18, 115.85, 90.56, 69.27, 58.08, 53.37, 49.22, 26.64, 23.44.

4-(4-(4-benzylpiperidin-1-yl)butoxy)-2H-chromen-2-one (81)

Compound was obtained according to the general procedure reported above with intermediate **XXVII** and 4-benzylpiperidine as starting material.

Solid, pale-yellow. Yield: 78 %. Melting point: 78 °C. Molecular formula: C₂₅H₂₉NO₃. Molecular weight: 391.51 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.79 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.58 – 7.42 (m, 1H, Ar), 7.37 – 7.03 (m, 7H, Ar), 5.65 (s, 1H, Ar), 4.13 (t, J = 6.3 Hz, 2H, OCH₂), 3.00 – 2.87 (m, 2H, CH₂N), 2.52 (d, J = 7.0 Hz, 2H, CH₂-Ar), 2.44 – 2.35 (m, 2H, piperidine), 1.96 – 1.83 (m, 3H, piperidine), 1.78 – 1.59 (m, 3H, piperidine), 1.57 – 1.44 (m, 1H, CH), 1.42 – 1.24 (m, 4H, CH₂(CH₂)₂CH₂).

¹³C NMR (101 MHz, CDCl₃) 165.77, 163.14, 153.43, 140.69, 132.46, 129.21, 128.27, 125.91, 123.95, 123.13, 116.87, 115.85, 90.52, 69.27, 58.41, 54.05, 43.22, 37.99, 32.09, 26.73, 23.51.

3-(4-((2-Oxo-2H-chromen-4-yl)oxy)butyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (82)

Compound was obtained according to the general procedure reported above with intermediate **XXVII** and cytosine as starting material.

Vitreous oil, orange. Yield: 60 %; Melting point: N/A. Molecular formula: C₂₄H₂₆N₂O₄. Molecular weight: 406.48 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.75 (dd, J = 7.9, 1.6, 1H, Ar); 7.54-7.47 (m, 1H, Ar); 7.32-7.18 (m, 3H, 1H pyr + 2H, Ar); 6.36 (dd, J = 9.0, 1.4, 1H, pyr); 5.96 (dd, J = 6.9, 1.4, 1H, pyr); 5.53 (s, 1H, Ar); 4.05 (d, J = 15.4, 1H, cyt); 3.98-3.79 (m, 3H, 1H cyt + 2H OCH₂); 2.99-2.81 (m, 3H, 1H cyt + 2H CH₂N); 2.47-2.23 (m, 4H, cyt); 1.94 -1.73 (m, 3H, 3H cyt), 1.71 -1.41 (m, 4H, CH₂(CH₂)₂CH₂).

¹³C NMR (101 MHz, CDCl₃) 165.73, 163.64, 163.14, 153.39, 151.65, 138.75, 132.43, 123.96, 123.15, 116.82, 116.61, 115.86, 104.56, 90.41, 68.96, 60.51, 60.31, 56.54, 50.18, 35.59, 28.14, 26.06, 25.72, 22.83.

4-((5-(4-Methylpiperazin-1-yl)pentyl)oxy)-2H-chromen-2-one (83)

Compound was obtained according to the general procedure reported above with intermediate **XXVIII** and N-methylpiperazine as starting material.

Solid, white. Yield: 70 %. Melting point: 74-75 °C. Molecular formula: C₁₉H₂₆N₂O₃. Molecular weight: 330.43 g/mol.

¹H NMR (400 MHz, CDCl₃) 7.89 – 7.71 (m, 1H, Ar), 7.59 – 7.43 (m, 1H, Ar), 7.34 – 7.20 (m, 2H, Ar), 5.64 (d, J = 2.4 Hz, 1H, Ar), 4.11 (t, J = 6.4 Hz, 2H, OCH₂), 2.46 –

2.22 (m, 10H, CH₂N + piperazine), 2.27 (s, 3H, CH₃), 1.99 – 1.83 (m, 2H, CH₂(CH₂)₃CH₂), 1.67 – 1.46 (m, 4H, CH₂(CH₂)₃CH₂).

¹³C NMR (101 MHz, CDCl₃) 165.78, 163.14, 153.44, 132.45, 123.93, 123.09, 116.90, 115.88, 90.49, 69.30, 58.45, 55.21, 53.34, 46.15, 28.52, 26.66, 24.06.

4-((5-(4-Phenylpiperazin-1-yl)pentyl)oxy)-2H-chromen-2-one (84)

Compound was obtained according to the general procedure reported above with intermediate **XXVIII** and N-phenylpiperazine as starting material.

Solid, white. Yield: 82 %. Melting point: 109-110 °C. Molecular formula: C₂₄H₂₈N₂O₃. Molecular weight: 392.50 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.81 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.63 – 7.47 (m, 1H, Ar), 7.37 – 7.17 (m, 4H, Ar), 7.00 – 6.79 (m, 3H, Ar), 5.66 (s, 1H, Ar), 4.13 (t, J = 6.3 Hz, 2H, OCH₂), 3.32 – 3.07 (m, 4H, piperazine), 2.71 – 2.52 (m, 4H, piperazine), 2.51 – 2.30 (m, 2H, CH₂N), 1.94 (p, J = 6.6 Hz, 2H, CH₂(CH₂)₃CH₂), 1.72 – 1.41 (m, 4H, CH₂(CH₂)₃CH₂).

¹³C NMR (101 MHz, CDCl₃) 165.79, 163.14, 153.45, 151.39, 132.48, 129.21, 123.96, 123.09, 119.82, 116.91, 116.13, 115.88, 90.52, 69.30, 58.49, 53.42, 49.24, 28.53, 26.66, 24.07.

4-((5-(4-Benzylpiperazin-1-yl)pentyl)oxy)-2H-chromen-2-one (85)

Compound was obtained according to the general procedure reported above with intermediate **XXVIII** and N-(benzyl)piperazine as starting material.

Solid, orange. Yield: 74 %. Melting point: 74-75 °C. Molecular formula: C₂₅H₃₀N₂O₃. Molecular weight: 406.53 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.80 (dd, J = 8.0, 1.6 Hz, 1H, Ar), 7.66 – 7.39 (m, 1H, Ar), 7.38 – 7.12 (m, 7H, Ar), 5.64 (s, 1H, Ar), 4.11 (t, J = 6.3 Hz, 2H, OCH₂), 3.51 (s, 2H, CH₂Ar), 3.00 – 2.12 (m, 10H, piperazine + CH₂N), 1.99 – 1.83 (m, 2H, CH₂(CH₂)₃CH₂), 1.68 – 1.47 (m, 4H, CH₂(CH₂)₃CH₂).

¹³C NMR (101 MHz, CDCl₃) 165.78, 163.14, 137.88, 132.46, 129.40, 129.37, 128.35, 128.30, 127.24, 127.18, 123.96, 123.10, 116.89, 115.86, 90.50, 69.26, 63.09, 63.06, 58.36, 53.20, 53.02, 52.81, 28.48, 26.40, 24.04.

4-((5-(4-Phenylpiperidin-1-yl)pentyl)oxy)-2H-chromen-2-one (86)

Compound was obtained according to the general procedure reported above intermediate **XXVIII** and 4-phenylpiperidine as starting material.

Solid, white. Yield: 74 %. Melting point: 83-84 °C. Molecular formula: C₂₅H₂₉NO₃. Molecular weight: 391.51 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.82 (dd, J = 7.9, 1.5 Hz, 1H, Ar), 7.63 – 7.47 (m, 1H, Ar), 7.43 – 7.08 (m, 7H, Ar), 5.66 (s, 1H, Ar), 4.13 (t, J = 6.3 Hz, 2H, OCH₂), 3.06 (d, J = 3.8 Hz, 2H, CH₂N), 2.66 – 2.37 (m, 3H, CH + piperidine), 2.10 – 1.99 (m, 2H, piperidine), 1.94 (p, J = 6.6 Hz, 2H, CH₂(CH₂)₃CH₂), 1.88 – 1.72 (m, 4H, piperidine), 1.73 – 1.59 (m, 2H, CH₂(CH₂)₃CH₂), 1.55 (p, J = 7.5 Hz, 2H, CH₂(CH₂)₃CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 163.16, 153.45, 146.42, 132.46, 128.52, 126.96, 126.26, 123.95, 123.11, 116.91, 115.89, 90.51, 69.33, 58.93, 54.57, 42.84, 33.54, 28.56, 26.87, 24.20.

4-((5-(4-Benzylpiperidin-1-yl)pentyl)oxy)-2H-chromen-2-one (87)

Compound was obtained according to the general procedure reported above with intermediate **XXVIII** and 4-benzylpiperidine as starting material.

Solid, pale yellow. Yield: 74 %. Melting point: 78-79 °C. Molecular formula: C₂₆H₃₁NO₃. Molecular weight: 405.54 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.80 (d, J = 1.5 Hz, 1H, Ar), 7.63 – 7.42 (m, 1H, Ar), 7.42 – 6.99 (m, 7H, Ar), 5.64 (d, J = 1.2 Hz, 1H, Ar), 4.11 (t, J = 6.3 Hz, 2H, OCH₂), 2.90 (d, J = 5.6 Hz, 2H, CH₂Ar), 2.51 (d, J = 7.0 Hz, 2H, CH₂N), 2.32 (t, J = 7.5 Hz, 2H, CH₂(CH₂)₃CH₂), 2.09 – 1.75 (m, 4H, piperidine), 1.75 – 1.44 (m, 7H, 2H, CH₂(CH₂)₃CH₂ + 1H CH + 4H piperidine), 1.44 – 1.10 (m, 2H, CH₂(CH₂)₃CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 165.79, 163.16, 153.44, 140.78, 132.45, 129.22, 129.15, 128.26, 125.88, 123.94, 123.11, 116.89, 115.89, 90.48, 69.31, 58.88, 54.11, 43.30, 38.05, 32.23, 28.53, 26.81, 24.17.

3-(5-((2-Oxo-2H-chromen-4-yl)oxy)pentyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (88)

Compound was obtained according to the general procedure reported above with intermediate **XXVIII** and cytosine as starting material.

Solid, white. Yield: 79%. Melting point: 148-150°C. Molecular formula: C₂₅H₂₈N₂O₄. Molecular weight: 420.51 g/mol.

¹H NMR (400 MHz, CDCl₃) δ 7.77 (dd, J = 7.9, 1.4, 1H, Ar); 7.56-7.47 (m, 1H, Ar); 7.33-7.19 (m, 3H, 1H pyr + 2H Ar); 6.39 (dd, J = 9.0, 1.4, 1H, pyr); 5.95 (dd, J = 6.8, 1.4, 1H, pyr); 5.60 (s, 1H, Ar); 4.04 (d, J = 15.4, 1H, cyt); 3.98-3.80 (m, 3H, 1H cyt + 2H OCH₂); 3.00 -2.79 (m, 3H, 1H cyt + 2H CH₂N); 2.47-2.18 (m, 4H, cyt); 1.83 -1.66 (m, 5H, 2H, CH₂(CH₂)₃CH₂ + 3H cyt), 1.43 -1.22 (m, 4H, CH₂(CH₂)₃CH₂).

¹³C NMR (101 MHz, CDCl₃) δ 163.65, 153.43, 151.82, 138.73, 132.40, 123.93, 123.16, 116.84, 116.55, 104.51, 90.46, 69.38, 60.57, 60.40, 57.16, 50.19, 35.65, 28.16, 28.06, 26.08, 23.35.

7-((6-(4-Methylpiperazin-1-yl)hexyl)oxy)-2H-chromen-2-one (89)

Compound was obtained according to the general procedure reported above with intermediate **XXVIII** and N-methylpiperazine as starting material.

Solid, white/pale-yellow. Yield: 68 %. Melting point: 50-51 °C. Molecular formula: C₂₀H₂₈N₂O₃. Molecular weight: 344.46 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.33 (d, J = 8.5 Hz, 1H, Ar), 6.84 – 6.74 (m, 2H, Ar), 6.22 (d, J = 9.4 Hz, 1H, Ar), 3.98 (t, J = 6.4 Hz, 2H, OCH₂), 2.86 – 2.33 (m, 10H, CH₂N + piperazine), 2.30 (s, 3H, CH₃), 1.79 (d, J = 6.9 Hz, 2H, CH₂(CH₂)₄CH₂), 1.61 – 1.40 (m, 4H, CH₂(CH₂)₄CH₂), 1.41 – 1.28 (m, 2H, CH₂(CH₂)₄CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 162.45, 161.43, 155.99, 143.59, 128.81, 113.09, 113.01, 112.47, 101.38, 68.57, 58.54, 54.86, 52.98, 45.91, 28.97, 27.32, 26.67, 25.97.

7-((6-(4-Phenylpiperazin-1-yl)hexyl)oxy)-2H-chromen-2-one (90)

Compound was obtained according to the general procedure reported above with intermediate **XXIX** and N-phenylpiperazine as starting material.

Solid, white/pale-yellow. Yield: 87 %. Melting point: 120-123 °C. Molecular formula: C₂₅H₃₀N₂O₃. Molecular weight: 406.53 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.62 (d, J = 9.5 Hz, 1H, Ar), 7.35 (d, J = 8.5 Hz, 1H, Ar), 7.30 – 7.20 (m, 2H, Ar), 6.96 – 6.88 (m, 2H, Ar), 6.88 – 6.76 (m, 3H, Ar), 6.23 (d, J = 9.5 Hz, 1H, Ar), 4.00 (t, J = 6.4 Hz, 2H, OCH₂), 3.25 – 3.11 (m, 4H, piperazine), 2.65 – 2.58 (m, 4H, piperazine), 2.46 – 2.37 (m, 2H, CH₂N), 1.88 – 1.76 (m, 2H, CH₂(CH₂)₄CH₂), 1.64 – 1.34 (m, 6H, CH₂(CH₂)₄CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 162.47, 161.42, 156.02, 151.37, 143.57, 129.40, 129.21, 128.82, 119.83, 113.10, 113.05, 112.49, 101.40, 68.60, 58.72, 53.38, 49.16, 29.01, 27.38, 26.82, 26.03.

7-((6-(4-Benzylpiperazin-1-yl)hexyl)oxy)-2H-chromen-2-one (91)

Compound was obtained according to the general procedure reported above with intermediate **XXIX** and N-benzylpiperazine as starting material.

Solid, white. Yield: 67 %; Melting point: 76-78 °C. Molecular formula: C₂₆H₃₂N₂O₃. Molecular weight: 420.55 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.37 – 7.18 (m, 6H, Ar), 6.84 – 6.74 (m, 2H, Ar), 6.22 (d, J = 9.5 Hz, 1H, Ar), 3.98 (t, J = 6.5 Hz, 2H, OCH₂), 3.49 (s, 2H, CH₂Ar), 2.80 – 2.16 (m, 10H, CH₂N + piperazine), 1.79 (p, J = 7.1 Hz, 2H, CH₂(CH₂)₄CH₂), 1.58 – 1.28 (m, 6H, CH₂(CH₂)₄CH₂).

^{13}C NMR (100 MHz, CDCl_3) δ : 162.46, 161.45, 155.99, 143.61, 137.99, 129.38, 128.81, 128.32, 127.18, 113.10, 112.99, 112.47, 101.38, 68.59, 63.13, 58.68, 53.25, 52.97, 28.98, 27.38, 26.75, 25.99.

4-((6-(4-Phenylpiperidin-1-yl)hexyl)oxy)-2H-chromen-2-one (92)

Compound was obtained according to the general procedure reported above with intermediate **XXIX** and 4-phenylpiperidine as starting material.

Solid, white/pale-yellow. Yield: 76 %; Melting point: 79-80 °C. Molecular formula: $\text{C}_{26}\text{H}_{31}\text{NO}_3$. Molecular weight: 405.54 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.81 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.58 – 7.49 (m, 1H, Ar), 7.34 – 7.14 (m, 7H, Ar), 5.66 (s, 1H, Ar), 4.12 (t, J = 6.4 Hz, 2H, OCH_2), 3.14 – 3.05 (m, 2H, piperidine), 2.57 – 2.37 (m, 3H, CH + CH_2N), 2.14 – 2.02 (m, 2H, piperidine), 1.97 – 1.79 (m, 6H, $\text{CH}_2(\underline{\text{CH}_2})_4\text{CH}_2$ + piperidine), 1.68 – 1.49 (m, 4H, $\text{CH}_2(\underline{\text{CH}_2})_4\text{CH}_2$), 1.48 – 1.36 (m, 2H, $\text{CH}_2(\underline{\text{CH}_2})_4\text{CH}_2$).

^{13}C NMR (100 MHz, CDCl_3) δ : 165.83, 163.18, 153.45, 146.24, 132.46, 128.55, 126.97, 126.31, 123.97, 123.12, 116.89, 115.91, 90.49, 69.39, 59.02, 54.50, 42.72, 33.32, 28.52, 27.39, 26.90, 26.03.

4-((6-(4-Benzylpiperidin-1-yl)hexyl)oxy)-2H-chromen-2-one (93)

Compound was obtained according to the general procedure reported above with intermediate **XXIX** and 4-benzylpiperidine as starting material.

Oil, orange. Yield: 67 %. Melting point: N/A. Molecular formula: $\text{C}_{27}\text{H}_{33}\text{NO}_3$. Molecular weight: 419.57 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.80 (dd, J = 8.0, 1.6 Hz, 1H, Ar), 7.57 – 7.48 (m, 1H, Ar), 7.37 – 7.04 (m, 7H, Ar), 5.64 (s, 1H, Ar), 4.10 (t, J = 6.4 Hz, 2H, OCH_2), 2.96 – 2.87 (m, 4H, piperidine), 2.52 (d, J = 7.0 Hz, 2H, CH_2Ar), 2.33 (t, J = 7.6 Hz, 2H, CH_2N), 1.95 – 1.83 (m, 3H, CH + $\text{CH}_2(\underline{\text{CH}_2})_4\text{CH}_2$), 1.74 – 1.20 (m, 10H, piperidine + $\text{CH}_2(\underline{\text{CH}_2})_4\text{CH}_2$).

^{13}C NMR (101 MHz, CDCl_3) δ : 165.82, 163.18, 153.43, 140.69, 132.45, 129.22, 129.15, 128.28, 125.92, 123.97, 123.12, 116.88, 115.89, 90.47, 69.37, 59.15, 58.94, 54.04, 43.21, 37.96, 31.97, 28.50, 27.36, 26.82, 26.71, 25.98.

3-(6-((2-Oxo-2H-chromen-4-yl)oxy)hexyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (94)

Compound was obtained according to the general procedure reported above with intermediate **XXIX** and cytosine as starting material.

Solid, pale-yellow. Yield: 33 %. Melting point: 108-110 °C. Molecular formula: C₂₆H₃₀N₂O₄. Molecular weight: 434.54 g/mol.

¹H NMR (400 MHz, CDCl₃) δ 7.79 (dd, J = 7.9, 1.3, 1H, Ar); 7.56-7.48 (m, 1H, Ar); 7.33-7.20 (m, 3H, 1H pyr + 2H, Ar); 6.39 (dd, J = 9.0, 1.3, 1H, pyr); 5.95 (dd, J = 6.9, 1.4, 1H, pyr); 5.63 (s, 1H, Ar); 4.07 – 3.96 (m, 3H, CH₂O + 1H, cyt); 3.85 (dd, J = 15.3, 6.7, 1H cyt); 2.97 -2.79 (m, 3H, 1H cyt + 2H CH₂N); 2.47-2.18 (m, 5H, 2H, CH₂(CH₂)₄CH₂ + cyt); 1.92 -1.67 (m, 4H, 2H, CH₂(CH₂)₄CH₂ + 2H cyt), 1.41 -1.07 (m, 6H, CH₂(CH₂)₄CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 165.83, 163.68, 163.20, 153.43, 151.90, 138.68, 132.40, 123.94, 123.17, 116.84, 116.51, 115.93, 104.50, 90.43, 69.42, 60.57, 60.33, 57.15, 50.21, 35.69, 28.52, 28.18, 26.51, 26.39, 26.11, 25.59.

7-(2-(4-Benzylpiperazin-1-yl)ethoxy)-2H-chromen-2-one (95)

Compound was obtained according to the general procedure reported above with intermediate XXX and N-benzylpiperazine as starting material.

Solid, pale yellow. Yield: 76 %. Melting point: 64-66°C. Molecular formula: C₂₂H₂₄N₂O₃. Molecular weight: 364,45 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.36 – 7.21 (m, 7H, Ar), 6.87 – 6.75 (m, 1H, Ar), 6.23 (d, J = 9.5 Hz, 1H, Ar), 4.15 (t, J = 5.6, 2H, OCH₂), 3.70 (s, 2H, NCH₂Ar), 3.12 – 2.23 (m, 10H CH₂N + piperazine).

¹³C NMR (101 MHz, CDCl₃) δ: 161.81, 161.28, 155.89, 143.49, 129.82, 128.91, 128.71, 128.60, 127.93, 113.37, 112.98, 112.83, 101.66, 66.35, 62.54, 56.68, 52.88, 52.38.

7-(2-(4-Benzylpiperidin-1-yl)ethoxy)-2H-chromen-2-one (96)

Compound was obtained according to the general procedure reported above with intermediate XXX and 4-benzylpiperidine as starting material.

Solid, pale yellow. Yield: 74 %; Melting point: 69-71 °C. Molecular formula: C₂₃H₂₅NO₃. Molecular weight: 363,46 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.35 (s, 1H, Ar), 7.31 – 7.08 (m, 5H, Ar), 6.86 – 6.75 (m, 2H, Ar), 6.24 (d, J = 9.5 Hz, 1H, Ar), 4.32 (d, J = 5.0 Hz, 2H, OCH₂), 3.30 – 2.88 (m, 4H, piperidine), 2.56 (d, J = 5.4 Hz, 2H, CH₂Ar), 2.33 (t, J = 5.4 Hz, 2H, CH₂N), 1.85-1.37 (m, 5H, piperidine).

¹³C NMR (101 MHz, CDCl₃) δ: 161.37, 161.17, 155.83, 143.41, 140.05, 129.02, 128.42, 126.17, 113.57, 113.06, 112.56, 102.04, 65.51, 56.79, 54.27, 42.70, 37.16, 30.90.

7-(3-(4-Methylpiperazin-1-yl)propoxy)-2H-chromen-2-one (97)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and N-methylpiperazine as starting material.

Solid, white/pale yellow. Yield: 67 %. Melting point: 105-106 °C. Molecular formula: C₁₇H₂₂N₂O₃. Molecular weight: 302.37 g/mol.

¹H NMR (400 MHz, CDCl₃): δ 7.61 (dd, J = 9.6, 2.8, 1H, Ar); 7.34 (dd, J = 8.8, 3.2, 1H, Ar); 6.85-8.76 (m, 2H, Ar); 6.22 (dd, J = 8.4, 3.2, 1H, Ar); 4.05 (t, J = 6.4, 2H, OCH₂); 2.71-2.34 (m, 10H, CH₂N + piperazine); 2.29 (s, 3H, N-CH₃); 1.98 (q, J = 6.4, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃): δ 162.37; 161.34; 155.99; 143.51; 128.80; 113.11; 113.06; 112.55; 101.47; 66.91; 55.10; 54.88; 53.10; 53.08; 45.98; 26.55.

7-(3-(4-Phenylpiperazin-1-yl)propoxy)-2H-chromen-2-one (98)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and N-phenylpiperazine as starting material.

Solid, white. Yield: 75 %; Melting point: 108-109 °C. Molecular formula: C₂₂H₂₄N₂O₃. Molecular weight: 364.45 g/mol.

¹H NMR (400 MHz, CDCl₃): δ 7.62 (d, J = 9.6, 1H, Ar); 7.35 (dd, J = 8.4, 1H, Ar); 7.30-7.19 (m, 2H, Ar); 6.97-6.79 (m, 5H, Ar); 6.23 (d, J = 9.6, 1H, Ar); 4.10 (t, J = 6.2, 2H, OCH₂); 3.30-3.17 (m, 6H, CH₂N + piperazine); 2.78-2.53 (m, 4H, piperazine); 2.06 (q, J = 5.6, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃): δ 162.34; 161.33; 156.00; 151.32; 143.50; 129.39; 129.22; 128.84; 119.90; 117.23; 116.21; 113.17; 112.98; 112.61; 101.56; 66.88; 55.02; 53.35; 49.18; 26.51.

7-(3-(4-Benzylpiperazin-1-yl)propoxy)-2H-chromen-2-one (99)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and N-benzylpiperazine as starting material.

Solid, white/pale yellow. Yield: 69 %. Melting point: 75-77 °C. Molecular formula: C₂₃H₂₆N₂O₃. Molecular weight: 378.47 g/mol.

¹H NMR (400 MHz, CDCl₃): δ 7.61 (d, J = 9.4, 1H, Ar); 7.37-7.20 (m, 6H, Ar); 6.85-6.76 (m, 2H, Ar); 6.23 (d, J = 9.4, 1H, Ar); 4.06 (t, J = 6.2, 2H, OCH₂); 3.52 (s, 2H, CH₂Ar); 2.75-2.36 (m, 10H, CH₂N + piperazine); 1.99 (q, J = 6.6, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃): δ 162.36; 161.35; 155.99; 143.51; 137.94; 129.36; 128.80; 128.33; 127.21; 113.12; 113.03; 112.56; 101.50; 63.07; 54.97; 53.24; 52.99; 26.50.

7-(3-(4-Phenylpiperidin-1-yl)propoxy)-2H-chromen-2-one (100)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and 4-phenylpiperidine as starting material.

Solid, light pink. Yield: 72 %. Melting point: 88-89 °C. Molecular formula: C₂₃H₂₆N₂O₃. Molecular weight: 363.46 g/mol.

¹H NMR (400 MHz, CDCl₃): δ 7.62 (d, J = 9.4, 1H, Ar); 7.35 (d, J = 8.4, 1H, Ar); 7.32-7.13 (m, 7H, Ar); 6.23 (d, J = 9.4, 1H, Ar); 4.09 (t, J = 6.2, 2H, OCH₂); 3.18-3.02 (m, 2H, piperidine); 2.65-2.54 (m, 2H, piperidine); 2.54-2.44 (m, 1H, CH₂-Ar); 2.22-1.75 (m, 8H, CH₂CH₂CH₂ + piperidine).

¹³C NMR (101 MHz, CDCl₃): δ 162.39; 161.36; 156.01; 146.26; 143.52; 128.82; 128.54; 126.96; 126.29; 113.13; 113.02; 112.58; 101.57; 67.10; 55.41; 54.52; 42.72; 33.42; 26.71.

7-(3-(4-Benzylpiperidin-1-yl)propoxy)-2H-chromen-2-one (101)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and 4-benzylpiperidine as starting material.

Solid, white/pale yellow. Yield: 84 %. Melting point: 80-81 °C. Molecular formula: C₂₄H₂₇NO₃. Molecular weight: 377.48 g/mol.

¹H NMR (400 MHz, CDCl₃): δ 7.61 (d, J = 9.4, 1H, Ar); 7.34 (d, J = 8.4, 1H, Ar); 7.32-7.06 (m, 6H, Ar); 6.86-6.74 (m, 2H, Ar); 4.05 (t, J = 6.2, 2H, OCH₂); 2.94 (d, J = 11.5, 2H, CH₂Ar); 2.63-2.40 (m, 4H, piperidine); 2.10-1.82 (m, 4H, CH₂N + CH₂CH₂CH₂); 1.72-1.46 (m, 2H, piperidine); 1.60-1.46 (m, 1H, CHCH₂); 1.43-1.24 (m, 2H, piperidine).

¹³C NMR (101 MHz, CDCl₃): δ 162.36; 161.35; 155.99; 143.51; 140.67; 129.20; 128.80; 128.28; 125.92; 113.12; 112.98; 112.57; 101.55; 77.31; 67.07; 55.36; 54.05; 43.18; 37.93; 32.04; 26.61.

3-(3-((2-Oxo-2H-chromen-7-yl)oxy)propyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (102)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and cytosine as starting material.

Vitreous oil, orange. Yield: 69 %. Melting point: N/A. Molecular formula: C₂₃H₂₄N₂O₄. Molecular weight: 392.46 g/mol.

¹H NMR (400 MHz, CDCl₃): δ 7.60 (d, J = 9.4, 1H, Ar); 7.32 (dd, J = 8.7, 1.1, 1H, pyr); 7.24-7.15 (m, 1H, Ar); 6.71-6.57 (m, 2H, Ar); 6.37 (dt, J = 9.0, 1.4, 1H, pyr); 6.21 (dd, J = 9.5, 1.4, 1H, Ar); 5.93 (dd, J = 6.9, 1.4, 1H, pyr); 3.99 (d, J = 15.3, 1H, cyt); 3.88-3.64 (m, 3H, 1H cyt + 2H OCH₂); 3.05-2.78 (m, 3H, 1H cyt + 2H CH₂N); 2.55-2.23 (m, 5H, cyt); 1.92-1.67 (m, 4H, 2H cyt + 2H CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃) 163.53, 162.23, 161.44, 155.81, 151.53, 143.61, 138.77, 128.92, 116.68, 113.02, 112.59, 112.29, 104.56, 101.94, 66.10, 60.42, 53.61, 50.15, 35.58, 28.13, 26.24, 25.98.

7-(3-(4-(4-Fluorobenzyl)piperazin-1-yl)propoxy)-2H-chromen-2-one (103)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and N-(4-fluorobenzyl)piperazine as starting material. Solid, white/pale-yellow. Yield: 80 %. Melting point: 75-76 °C. Molecular formula: C₂₃H₂₅FN₂O₃. Molecular weight: 396.46 g/mol.

¹H NMR (400 MHz, CDCl₃): δ 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.44 – 7.19 (m, 3H, Ar), 7.08 – 6.92 (m, 2H, Ar), 6.86 – 6.71 (m, 2H, Ar), 6.23 (dd, J = 9.5, 1.9 Hz, 1H, Ar), 4.07 (t, J = 6.4, 2H, OCH₂), 3.54 (s, 2H, CH₂-Ar), 3.10 – 2.18 (m, 12H, CH₂N + piperazine + CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃): 162.07, 161.25, 155.95, 143.46, 131.01, 128.89, 115.46, 115.25, 113.28, 112.83, 101.58, 77.43, 77.12, 76.80, 66.50, 61.77, 54.85, 52.67, 51.87, 25.78.

7-(3-(4-(4-Chlorobenzyl)piperazin-1-yl)propoxy)-2H-chromen-2-one (104)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and N-(4-chlorobenzyl)piperazine as starting material. Solid, white/pale-yellow. Yield: 85 %. Melting point: 70-71 °C. Molecular formula: C₂₃H₂₅ClN₂O₃. Molecular weight: 412.91 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.41 – 7.18 (m, 5H, Ar), 6.81 (d, J = 8.1 Hz, 2H, Ar), 6.23 (d, J = 9.5 Hz, 1H, Ar), 4.06 (t, J = 6.3 Hz, 2H, Ar), 3.46 (s, 2H, CH₂Ar), 2.59-2.46 (m, 10H, CH₂N + piperazine), 1.98 (p, J = 6.4 Hz, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 162.37, 161.35, 156.00, 143.51, 136.76, 132.84, 130.53, 128.79, 128.45, 113.11, 113.06, 112.55, 101.48, 66.95, 62.31, 54.95, 53.28, 53.07, 26.56.

7-(3-(4-(4-Methoxybenzyl)piperazin-1-yl)propoxy)-2H-chromen-2-one (105)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and N-(4-methoxybenzyl)piperazine as starting material.

Solid, pale-yellow. Yield: 76 %. Melting point: 87-88°C. Molecular formula: C₂₄H₂₈N₂O₄. Molecular weight: 408.50 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.33 (d, J = 8.3 Hz, 1H, Ar), 7.27 – 7.15 (m, 3H, Ar), 6.88 – 6.77 (m, 3H, Ar), 6.22 (d, J = 9.5 Hz, 1H, Ar),

4.05 (t, J = 6.3 Hz, 2H, OCH₂), 3.78 (s, 3H, OCH₃), 3.44 (s, 2H, CH₂Ar), 2.72 – 2.23 (m, 10H, CH₂N + piperazine), 1.98 (p, J = 6.4 Hz, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 162.40, 161.36, 158.82, 158.78, 156.00, 143.52, 130.51, 130.12, 128.79, 113.66, 113.63, 113.09, 113.06, 112.54, 101.49, 67.00, 65.94, 62.50, 55.34, 54.98, 53.31, 53.03, 52.99, 26.60.

7-(3-(4-Phenethylpiperazin-1-yl)propoxy)-2H-chromen-2-one (106)

Compound was obtained according to the general procedure reported above with intermediate **XXXI** and N-(phenylethyl)piperazine as starting material.

Solid, white. Yield: 73 %. Melting point: 102-104 °C. Molecular formula: C₂₄H₂₈N₂O₃. Molecular weight: 392.50 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.40 – 7.12 (m, 7H, Ar), 6.86 – 6.78 (m, 1H, Ar), 6.23 (d, J = 9.4 Hz, 1H, Ar), 4.07 (t, J = 6.3 Hz, 2H, OCH₂), 2.92 – 2.37 (m, 14H, CH₂N + CH₂CH₂-Ar + piperazine), 2.06 – 1.94 (p, J = 7.3 Hz, 2H, CH₂CH₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 162.39, 161.35, 156.01, 143.52, 140.35, 128.79, 128.49, 126.16, 113.11, 113.07, 112.56, 101.49, 66.97, 60.59, 55.00, 53.31, 53.25, 33.68, 26.58.

7-(4-(4-Methylpiperazin-1-yl)butoxy)-2H-chromen-2-one (107)

Compound was obtained according to the general procedure earlier reported with intermediate **XXXII** and N-methylpiperazine as starting material.

Solid, pale yellow. Yield: 72 %. Melting point: 58-62 °C. Molecular formula: C₁₈H₂₄N₂O₃. Molecular weight: 316.40 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (dd, J = 9.5, 1.4 Hz, 1H, Ar), 7.33 (dd, J = 8.4, 1.6 Hz, 1H, Ar), 6.84 – 6.75 (m, 2H, Ar), 6.22 (dd, J = 9.5, 2.1 Hz, 1H, Ar), 4.01 (t, J = 6.3 Hz, 2H, OCH₂), 3.02 – 2.31 (m, 10H, CH₂N + piperazine), 2.19 (s, 3H, CH₃), 1.79 (p, J = 6.8 Hz, 2H, CH₂(CH₂)₂CH₂), 1.67 (p, J = 6.8 Hz, 2H, CH₂(CH₂)₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 162.38, 161.40, 155.99, 143.56, 128.81, 113.08, 113.05, 112.52, 101.39, 68.42, 58.09, 55.06, 53.08, 45.98, 27.04, 23.36.

7-(4-(4-Phenylpiperazin-1-yl)butoxy)-2H-chromen-2-one (108)

Compound was obtained according to the general procedure earlier reported with intermediate **XXXII** and N-phenylpiperazine as starting material.

Solid, white. Yield: 76 %. Melting point: 154-155 °C. Molecular formula: C₂₃H₂₆N₂O₃. Molecular weight: 378.47 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.62 (d, J = 9.5 Hz, 1H, Ar), 7.35 (d, J = 8.5 Hz, 1H, Ar), 7.30 – 7.20 (m, 2H, Ar), 6.96 – 6.77 (m, 5H, Ar), 6.23 (d, J = 9.5 Hz, 1H, Ar),

4.05 (t, J = 6.3 Hz, 2H, OCH₂), 3.24 – 3.17 (m, 4H, piperazine), 2.66 – 2.57 (m, 4H, piperazine), 2.48 (t, J = 7.4 Hz, 2H, CH₂N), 1.86 (p, J = 6.3 Hz, 2H, CH₂(CH₂)₂CH₂), 1.74 (p, J = 6.4 Hz, 2H, CH₂(CH₂)₂CH₂).

¹³C NMR (101 MHz, CDCl₃): δ 162.38, 161.35, 156.02, 151.36, 143.53, 129.21, 128.83, 119.84, 116.17, 113.10, 113.04, 112.55, 101.44, 68.42, 58.20, 53.32, 49.18, 27.09, 23.38.

7-(4-(4-Benzylpiperazin-1-yl)butoxy)-2H-chromen-2-one (109)

Compound was obtained according to the general procedure reported above with intermediate XXXII and N-benzylpiperazine as starting material.

Solid, white. Yield: 77 %. Melting point: 91-93°C. Molecular formula: C₂₄H₂₈N₂O₃. Molecular weight: 392.50 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.40 – 7.17 (m, 6H, Ar), 6.84 – 6.75 (m, 2H, Ar), 6.23 (dd, J = 9.5, 1.1 Hz, 1H, Ar), 4.02 (t, J = 6.3 Hz, 2H, OCH₂), 3.51 (s, 2H, CH₂Ar), 2.72 – 2.28 (m, 10H, CH₂N + piperazine), 1.82 (p, J = 6.5 Hz, 2H, CH₂(CH₂)₂CH₂), 1.68 (p, J = 6.6 Hz, 2H, CH₂(CH₂)₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 162.38, 161.36, 156.01, 143.52, 138.05, 129.34, 128.80, 128.31, 127.17, 113.07, 112.52, 101.42, 68.41, 63.10, 58.14, 53.23, 53.01, 27.06, 23.35.

7-(4-(4-Phenylpiperidin-1-yl)butoxy)-2H-chromen-2-one (110)

Compound was obtained according to the general procedure reported above with intermediate XXXII and 4-phenylpiperidine as starting material.

. Solid, light yellow. Yield: 78 %. Melting point: 77-79 °C. Molecular formula: C₂₄H₂₇NO₃. Molecular weight: 377.48 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.62 (d, J = 9.5 Hz, 1H, Ar), 7.38 – 7.14 (m, 7H, Ar), 6.86 – 6.77 (m, 1H, Ar), 6.23 (dd, J = 9.5, 1.0 Hz, 1H, Ar), 4.05 (t, J = 6.2 Hz, 2H, OCH₂), 3.13 – 3.04 (m, 3H, CH₂N + CH-Ar), 2.56 – 2.42 (m, 4H, piperidine), 2.13 – 2.02 (m, 4H, piperidine), 1.91 – 1.68 (m, 4H, CH₂(CH₂)₂CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 162.41, 161.37, 156.02, 146.29, 143.53, 128.82, 128.53, 126.95, 126.27, 113.07, 112.53, 101.45, 68.46, 58.59, 54.46, 42.74, 33.40, 27.22, 23.54.

7-(4-(4-Benzylpiperidin-1-yl)butoxy)-2H-chromen-2-one (111)

Compound was obtained according to the general procedure reported above with intermediate XXXII and 4-benzylpiperidine as starting material.

Solid, light yellow. Yield: 69 %. Melting point: 69-70 °C. Molecular formula: C₂₅H₂₉NO₃. Molecular weight: 391.51 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.46 – 7.04 (m, 7H, Ar), 6.87 – 6.72 (m, 1H, Ar), 6.23 (d, J = 9.5 Hz, 1H, Ar), 4.01 (t, J = 6.2 Hz, 2H, OCH₂),

2.95 (d, $J = 10.9$ Hz, 2H, CH_2N), 2.52 (d, $J = 7.1$ Hz, 2H, $\text{CH}_2\text{-Ar}$), 2.48 – 2.30 (m, 2H, piperidine), 1.91 (t, $J = 11.7$ Hz, 2H, piperidine), 1.81 (p, $J = 6.6$ Hz, 2H, $\text{CH}_2(\underline{\text{CH}_2})_2\text{CH}_2$), 1.76 – 1.59 (m, 4H, piperidine), 1.52 (m, 1H, CH), 1.44 – 1.23 (m, 2H, $\text{CH}_2(\underline{\text{CH}_2})_2\text{CH}_2$).

^{13}C NMR (101 MHz, CDCl_3) δ : 162.35, 161.37, 155.99, 143.54, 140.59, 129.19, 128.82, 128.29, 125.94, 113.08, 113.01, 112.54, 101.45, 68.35, 58.40, 53.91, 43.11, 37.86, 31.80, 27.11, 23.26.

3-(4-((2-Oxo-2H-chromen-7-yl)oxy)butyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (112)

Compound was obtained according to the general procedure reported above with intermediate XXXII and cytosine as starting material.

Vitreous oil, orange. Yield: 78 %. Melting point: N/A. Molecular formula: $\text{C}_{24}\text{H}_{26}\text{N}_2\text{O}_4$. Molecular weight: 406.48 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.61 (d, $J = 9.5$ Hz, 1H, Ar), 7.27 – 7.19 (m, 1H, pyr), 7.27 – 7.19 (m, 1H, Ar), 6.76 (dd, $J = 8.6, 2.4$ Hz, 1H, Ar), 6.70 (d, $J = 2.4$ Hz, 1H, Ar), 6.39 (dd, $J = 9.0, 1.4$ Hz, 1H, pyr), 6.21 (d, $J = 9.5$ Hz, 1H, Ar), 5.96 (dd, $J = 6.9, 1.4$, 1H, pyr), 4.03 (d, $J = 15.3$ Hz, 1H, cyt), 3.90 – 3.74 (m, 3H, 1H cyt + 2H OCH_2), 2.97 – 2.83 (m, 3H, 1H cyt + 2H CH_2N), 2.45 – 2.22 (m, 5H, cyt), 1.93 – 1.71 (m, 2H, cyt), 1.63 – 1.42 (m, 4H, 2H, $\text{CH}_2(\underline{\text{CH}_2})_2\text{CH}_2$),

^{13}C NMR (101 MHz, CDCl_3) δ : 163.67, 162.41, 161.44, 155.94, 151.71, 143.61, 138.72, 128.82, 116.61, 112.97, 112.45, 104.58, 101.46, 68.15, 60.54, 60.25, 56.85, 50.17, 35.62, 31.05, 28.14, 26.25, 26.08, 22.96.

7-((5-(4-Methylpiperazin-1-yl)pentyl)oxy)-2H-chromen-2-one (113)

Compound was obtained according to the general procedure reported above with intermediate XXXIII and N-methylpiperazine as starting material.

Solid, white. Yield: 70 %. Melting point: 92-93 °C. Molecular formula: $\text{C}_{19}\text{H}_{26}\text{N}_2\text{O}_3$. Molecular weight: 330.43 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.61 (d, $J = 9.5$ Hz, 1H, Ar), 7.33 (d, $J = 8.5$ Hz, 1H, Ar), 6.83 – 6.74 (m, 2H, Ar), 6.22 (d, $J = 9.5$ Hz, 1H, Ar), 3.99 (t, $J = 6.4$ Hz, 2H, OCH_2), 2.87 – 2.33 (m, 10H, CH_2N + piperazine), 2.28 (s, 3H, CH_3), 1.87 – 1.73 (m, 2H, $\text{CH}_2(\underline{\text{CH}_2})_3\text{CH}_2$), 1.62 – 1.40 (m, 4H, $\text{CH}_2(\underline{\text{CH}_2})_3\text{CH}_2$).

^{13}C NMR (101 MHz, CDCl_3) δ : 162.42, 161.42, 155.99, 143.58, 128.81, 113.04, 112.49, 101.38, 68.49, 58.51, 55.05, 53.16, 46.03, 28.96, 26.60, 24.06.

7-((5-(4-Phenylpiperazin-1-yl)pentyl)oxy)-2H-chromen-2-one (114)

Compound was obtained according to the general procedure reported above with intermediate **XXXIII** and N-phenylpiperazine as starting material.

Solid, white. Yield: 79 %. Melting point: 115-117 °C. Molecular formula: C₂₄H₂₈N₂O₃. Molecular weight: 392.50 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.62 (d, J = 9.5 Hz, 1H, Ar), 7.35 (d, J = 8.5 Hz, 1H, Ar), 7.30 – 7.20 (m, 2H, Ar), 6.98 – 6.89 (m, 2H, Ar), 6.88 – 6.72 (m, 3H, Ar), 6.23 (d, J = 9.4 Hz, 1H, Ar), 4.01 (t, J = 6.4 Hz, 2H, OCH₂), 3.24 – 3.17 (m, 4H, piperazine), 2.60 (d, J = 5.0 Hz, 4H, piperazine), 2.46 – 2.35 (m, 2H, CH₂N), 1.85 (p, J = 7.3 Hz, 2H, CH₂(CH₂)₃CH₂), 1.67 – 1.46 (m, 4H, CH₂(CH₂)₃CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 162.43, 161.40, 156.01, 151.41, 143.56, 129.20, 128.82, 119.79, 116.13, 113.07, 112.51, 101.40, 68.52, 58.62, 53.42, 49.22, 29.00, 26.70, 24.10.

7-((5-(4-Benzylpiperazin-1-yl)pentyl)oxy)-2H-chromen-2-one (115)

Compound was obtained according to the general procedure reported above with intermediate **XXXIII** and N-(benzyl)piperazine as starting material.

White solid. Yield: 59 %. Melting point: 132-134 °C. Molecular formula: C₂₅H₃₀N₂O₃. Molecular weight: 406.53 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.62 (t, J = 1.9 Hz, 1H, Ar), 7.38 – 7.19 (m, 6H, Ar), 6.85 – 6.75 (m, 2H, Ar), 6.23 (dd, J = 9.4, 2.0 Hz, 1H, Ar), 3.99 (t, J = 6.5, 2H, OCH₂), 3.50 (s, 2H, CH₂Ar), 2.85 – 2.21 (m, 10H, piperazine + CH₂N), 1.98 – 1.69 (m, 2H, CH₂(CH₂)₃CH₂), 1.62 – 1.41 (m, 4H, CH₂(CH₂)₃CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 161.42, 156.00, 143.56, 138.15, 129.35, 128.80, 128.30, 128.28, 127.13, 127.10, 113.05, 112.49, 101.40, 77.33, 68.52, 63.19, 58.62, 53.35, 53.15, 53.14, 28.99, 26.70, 24.11.

7-((5-(4-Phenylpiperidin-1-yl)pentyl)oxy)-2H-chromen-2-one (116)

Compound was obtained according to the general procedure reported above with intermediate **XXXIII** and 4-phenylpiperidine as starting material.

Solid, white. Yield: 64 %. Melting point: 112-113 °C. Molecular formula: C₂₅H₂₉NO₃. Molecular weight: 391.51 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.62 (d, J = 9.5 Hz, 1H, Ar), 7.44 – 7.10 (m, 7H, Ar), 6.95 – 6.71 (m, 1H, Ar), 6.23 (d, J = 9.5 Hz, 1H, Ar), 4.01 (t, J = 6.4 Hz, 2H, OCH₂), 3.05 (d, J = 3.3 Hz, 2H, CH₂N), 2.49 (p, J = 5.0 Hz, 1H, CH), 2.03 (td, J = 11.2, 3.7 Hz, 2H, piperidine), 1.94 – 1.70 (m, 8H, 2H, CH₂(CH₂)₃CH₂ + 6H piperidine), 1.67 – 1.55 (m, 2H, CH₂(CH₂)₃CH₂), 1.55 – 1.44 (m, 2H, CH₂(CH₂)₃CH₂).

^{13}C NMR (101 MHz, CDCl_3) δ : 162.46, 161.42, 156.01, 146.50, 143.57, 128.81, 128.51, 126.97, 126.22, 113.05, 101.42, 68.55, 59.07, 54.58, 42.87, 33.57, 29.03, 26.93, 24.23.

7-((5-(4-Benzylpiperidin-1-yl)pentyl)oxy)-2H-chromen-2-one (117)

Compound was obtained according to the general procedure reported above with intermediate **XXXIII** and 4-benzylpiperidine as starting material.

Solid, pale-yellow. Yield: 69 %. Melting point: 85-86 °C. Molecular formula: $\text{C}_{26}\text{H}_{31}\text{NO}_3$. Molecular weight: 405.54 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.62 (d, J = 9.5 Hz, 1H, Ar), 7.39 – 7.06 (m, 7H, Ar), 6.84 – 6.75 (m, 1H, Ar), 6.23 (d, J = 9.4 Hz, 1H, Ar), 3.99 (t, J = 6.5 Hz, 2H, OCH_2), 2.89 (d, J = 11.2 Hz, 2H, CH_2Ar), 2.52 (d, J = 7.0 Hz, 2H, CH_2N), 2.31 (t, J = 7.6 Hz, 2H, $\text{CH}_2(\underline{\text{CH}_2})_3\text{CH}_2$), 1.89 – 1.76 (m, 5H, 1H CH + 4H piperidine), 1.62 – 1.19 (m, 8H, 4H $\text{CH}_2(\underline{\text{CH}_2})_3\text{CH}_2$ + 4H piperidine).

^{13}C NMR (101 MHz, CDCl_3) δ : 162.46, 156.01, 143.56, 140.83, 129.23, 128.79, 128.25, 125.86, 113.05, 112.48, 101.41, 77.32, 68.54, 59.03, 54.13, 43.32, 38.08, 32.27, 29.00, 26.89, 24.20.

3-(5-((2-Oxo-2H-chromen-7-yl)oxy)pentyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (118)

Compound was obtained according to the general procedure reported above with intermediate **XXXIII** as starting material.

Solid, white. Yield: 76 %. Melting point: 87-88 °C. Molecular formula: $\text{C}_{25}\text{H}_{28}\text{N}_2\text{O}_4$. Molecular weight: 420.51 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.61 (d, J = 9.5, 1.4 Hz, 1H, Ar), 7.34 (dd, 8.6, 1.6, 1H, Ar), 7.28 – 7.19 (m, 1H, pyr), 6.83 – 6.70 (m, 2H, Ar), 6.38 (m, 1H, pyr), 6.22 (dd, J = 9.4, 2.5 Hz, 1H, Ar), 5.96 (dd, J = 6.8, 1.3, 1H, pyr), 4.03 (d, J = 15.3 Hz, 1H, cyt), 3.92 – 3.78 (m, 3H, 1H cyt + 2H OCH_2), 2.96 – 2.80 (m, 3H, 1H cyt + 2H CH_2N), 2.45 – 2.17 (m, 5H, cyt), 1.92 – 1.71 (m, 2H, cyt), 1.68 – 1.56 (m, 2H, $\text{CH}_2(\underline{\text{CH}_2})_3\text{CH}_2$), 1.42 – 1.15 (m, 4H, $\text{CH}_2(\underline{\text{CH}_2})_3\text{CH}_2$).

^{13}C NMR (101 MHz, CDCl_3) δ : 163.67, 162.46, 161.47, 155.96, 151.85, 143.62, 138.73, 128.83, 116.51, 112.97, 112.93, 112.45, 104.57, 101.54, 68.63, 60.53, 60.35, 57.14, 50.20, 35.66, 28.54, 28.16, 26.10, 23.31.

4-((6-(4-Methylpiperazin-1-yl)hexyl)oxy)-2H-chromen-2-one (119)

Compound was obtained according to the general procedure reported above with intermediate **XXXIV** and N-methylpiperazine as starting material.

Solid, white. Yield: 68 %. Melting point: 91-92 °C. Molecular formula: C₂₀H₂₈N₂O₃. Molecular weight: 344.46 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.79 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.57 – 7.48 (m, 1H, Ar), 7.33 – 7.21 (m, 2H, Ar), 5.64 (s, 1H, Ar), 4.10 (t, J = 6.4 Hz, 2H, OCH₂), 2.80 – 2.32 (m, 8H, piperazine), 2.29 (s, 3H, CH₃), 1.89 (t, J = 6.6 Hz, 2H, CH₂(CH₂)₄CH₂), 1.61 – 1.46 (m, 4H, CH₂(CH₂)₄CH₂), 1.45 – 1.32 (m, 4H, CH₂(CH₂)₄CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 165.81, 163.16, 153.43, 132.45, 123.96, 123.09, 116.89, 115.89, 90.48, 69.36, 58.52, 54.97, 53.08, 45.98, 28.51, 27.25, 26.74, 25.99.

4-((6-(4-Phenylpiperazin-1-yl)hexyl)oxy)-2H-chromen-2-one (120)

Compound was obtained according to the general procedure reported above with intermediate **XXXIV** and N-phenylpiperazine as starting material.

Solid, white/pale-yellow. Yield: 78 %. Melting point: 98-100 °C. Molecular formula: C₂₅H₃₀N₂O₃. Molecular weight: 406.53 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.81 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.61 – 7.40 (m, 1H, Ar), 7.35 – 7.11 (m, 4H, Ar), 6.96 – 6.88 (m, 2H, Ar), 6.84 (t, J = 1.1 Hz, 1H, Ar), 5.66 (s, 1H, Ar), 4.12 (t, J = 6.3 Hz, 2H, OCH₂), 3.27 – 3.08 (m, 4H, piperazine), 2.70 – 2.54 (m, 4H, piperazine), 2.45 – 2.30 (m, 2H, CH₂N), 1.97 – 1.85 (m, 2H, CH₂(CH₂)₄CH₂), 1.70 – 1.32 (m, 6H, CH₂(CH₂)₄CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 165.82, 163.17, 153.45, 151.37, 132.46, 129.39, 129.21, 123.96, 123.09, 121.03, 119.84, 117.23, 116.91, 116.16, 115.91, 90.50, 69.38, 58.67, 53.39, 49.19, 28.54, 27.31, 26.84, 26.04.

4-((6-(4-Benzylpiperazin-1-yl)hexyl)oxy)-2H-chromen-2-one (121)

Compound was obtained according to the general procedure reported above with intermediate **XXXIV** and N-benzylpiperazine as starting material.

Oil, orange. Yield: 64 %. Melting point: N/A. Molecular formula: C₂₆H₃₂N₂O₃. Molecular weight: 420.55 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.79 (dd, J = 7.9, 1.6 Hz, 1H, Ar), 7.57 – 7.48 (m, 1H, Ar), 7.35 – 7.18 (m, 7H, Ar), 5.64 (s, 1H, Ar), 4.10 (t, J = 6.3 Hz, 2H, OCH₂), 3.52 (s, 2H, CH₂Ar), 2.97 – 2.20 (m, 10 H, CH₂N + piperazine), 1.89 (p, J = 7.2 Hz, 2H, CH₂(CH₂)₄CH₂), 1.72 – 1.33 (m, 6H, CH₂(CH₂)₄CH₂).

^{13}C NMR (100 MHz, CDCl_3) δ : 165.80, 163.17, 153.42, 137.64, 132.47, 129.38, 128.40, 127.35, 127.24, 123.98, 123.11, 116.88, 115.87, 90.49, 69.31, 62.90, 58.37, 53.04, 52.95, 52.32, 28.48, 27.16, 26.22, 25.93.

7-((6-(4-Phenylpiperidin-1-yl)hexyl)oxy)-2H-chromen-2-one (122)

Compound was obtained according to the general procedure reported above with intermediate XXXIV 4-phenylpiperidine as starting material.

Solid, pale-yellow. Yield: 74 %. Melting point: 79-81 °C. Molecular formula: $\text{C}_{26}\text{H}_{31}\text{NO}_3$. Molecular weight: 405.54 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.62 (d, J = 9.5 Hz, 1H, Ar), 7.39 – 7.14 (m, 6 H, Ar), 6.86 – 6.77 (m, 2H, Ar), 6.23 (d, J = 9.5 Hz, 1H, Ar), 4.01 (t, J = 6.5 Hz, 2H, OCH_2), 3.11 – 3.01 (m, 2H, piperidine), 2.55 – 2.42 (m, 1H, CH-Ar) 2.42 – 2.33 (m, 2H, CH_2N), 2.08 – 1.95 (m, 2H, piperidine), 1.88 – 1.72 (m, 6H, piperidine + 4H, $\text{CH}_2(\underline{\text{CH}_2})_4\text{CH}_2$), 1.65 – 1.31 (m, 6H, $\text{CH}_2(\underline{\text{CH}_2})_4\text{CH}_2$).

^{13}C NMR (100 MHz, CDCl_3) δ : 162.49, 161.42, 156.02, 146.47, 143.58, 128.81, 128.51, 126.97, 126.23, 113.10, 113.03, 112.48, 101.41, 68.63, 59.17, 54.56, 42.86, 33.52, 29.01, 27.53, 27.08, 26.05.

7-((6-(4-Benzylpiperidin-1-yl)hexyl)oxy)-2H-chromen-2-one (123)

Compound was obtained according to the general procedure reported above with intermediate XXXIV and 4-benzylpiperidine as starting material.

Solid, light yellow. Yield: 81 %. Melting point: 53-55 °C. Molecular formula: $\text{C}_{27}\text{H}_{33}\text{NO}_3$. Molecular weight: 419.57 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.61 (d, J = 9.5 Hz, 1H, Ar), 7.40 – 7.06 (m, 7H, Ar), 6.87 – 6.72 (m, 1H, Ar), 6.22 (d, J = 9.4 Hz, 1H, Ar), 3.98 (t, J = 6.4 Hz, 2H, OCH_2), 2.95 – 2.85 (m, 4H, piperidine), 2.52 (d, J = 7.0 Hz, 2H, CH_2Ar), 2.31 (t, J = 7.7 Hz, 2H, CH_2N), 1.94 – 1.83 (m, 3H, piperidine + $\text{CH}_2(\underline{\text{CH}_2})_4\text{CH}_2$), 1.70 – 1.29 (m, 10H, piperidine + $\text{CH}_2(\underline{\text{CH}_2})_4\text{CH}_2$).

^{13}C NMR (101 MHz, CDCl_3) δ : 162.48, 161.43, 156.01, 143.58, 140.75, 129.22, 128.80, 128.26, 125.89, 113.09, 113.02, 112.47, 101.40, 77.33, 68.61, 59.06, 54.25, 54.05, 43.25, 38.00, 32.07, 30.86, 28.98, 27.47, 26.90, 26.00.

3-(6-((2-Oxo-2H-chromen-7-yl)oxy)hexyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (124)

Compound was obtained according to the general procedure reported above with intermediate XXXIV and cytosine as starting material.

Solid, pale-yellow. Yield: 65 %. Melting point: 71-79 °C. Molecular formula: C₂₆H₃₀N₂O₄. Molecular weight: 434.54 g/mol.

¹H NMR (400 MHz, CDCl₃) 7.60 (d, J = 9.5 Hz, 1H, Ar), 7.33 (dd, J = 8.6, 1.9 Hz, 1H, pyr), 7.29 – 7.15 (m, 1H, Ar), 6.89 – 6.71 (m, 2H, Ar), 6.38 (dq, J = 9.0, 1.7 Hz, 1H, pyr), 6.20 (dd, J = 9.4, 2.0 Hz, 1H, Ar), 5.94 (dd, J = 6.9, 1.5 Hz, 1H, pyr), 4.00 (d, J = 15.1, 1H, cyt), 3.93 – 3.78 (m, 3H, OCH₂ + cytosine), 3.09 – 2.72 (m, 3H cytosine), 2.42 – 2.06 (m, 5H, 2H CH₂(CH₂)₄CH₂ + cyt), 1.90 – 1.52 (m, 4H, 2H CH₂(CH₂)₄CH₂ + 2H cyt), 1.34 – 1.07 (m, 6H, CH₂(CH₂)₄CH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 163.70, 162.51, 161.47, 155.96, 151.91, 143.63, 138.71, 128.82, 116.47, 113.01, 112.92, 112.43, 104.58, 101.48, 68.61, 60.49, 60.35, 57.24, 50.21, 35.66, 28.98, 28.16, 26.60, 26.38, 26.10, 25.61.

Phenothiazine-Cinnamic Acid hybrids

General procedure for the synthesis of the intermediates XXXV-XXXVI.

Phenothiazine (8 mmol) was reacted with 2-chloroacetyl chloride or 3-chloropropionyl chloride (8.8 mmol), respectively, in anh. toluene at reflux for 4 hours under a CaCl₂ trap. After evaporation of the solvent under vacuum, the residue was washed with 5% K₂CO₃ and water, dried over Na₂SO₄ and the solvent was removed under vacuum. A yellow oily residue which solidifies upon addition of Et₂O is obtained. Then, the solid was finely triturated and washed with n-pentane, affording the intermediates as white solids. The intermediates were characterized by TLC, melting point and ¹H NMR spectra.

2-chloro-1-(10H-phenothiazin-10-yl)ethan-1-one (XXXV)

White solid. Melting point: 114–115 °C. Yield: 95 %. Molecular formula C₁₄H₁₀ClNOS. Molecular weight: 275.79 g/mol. Elemental analysis calculated for C₁₄H₁₀ClNOS: C% 60.98, H% 3.66, N% 5.08; found: C% 61.02, H% 3.60, N% 5.01.

¹H-NMR (CDCl₃) δ: 7.69-7.12 (m, 4H, Ar); 7.02-6.75 (m, 4H, Ar); 3.43 (s, 2H, C(O)CH₂Cl).

3-chloro-1-(10H-phenothiazin-10-yl)propan-1-one (XXXVI)

White solid. Melting point: 131–133 °C. Yield: 88 %. Molecular formula C₁₅H₁₂ClNOS. Molecular weight: 289.78 g/mol. Elemental analysis calculated for C₁₅H₁₂ClNOS: C% 62.15, H% 4.17, N% 4.83; found: C% 62.05, H% 4.12, N% 4.79.

¹H-NMR (CDCl₃) δ: 7.61-7.08 (m, 4H, Ar); 6.98-6.73 (m, 4H, Ar); 3.39 (t, J = 6.2, 2H, CH₂Cl); 2.75 (t, J = 6.2, 2H, CH₂N).

General procedure for the synthesis of the intermediates XXXVII-XXXVIII.

Phenothiazine derivative XXXVII-XXXVIII (5 mmol), respectively, was reacted with N-BOC-piperazine (5 mmol) in the presence of anh. K₂CO₃ (5 mmol) in ACN at reflux for 8 hours with a KOH trap. After evaporation of the solvent under vacuum, the residue was partitioned between water and dichloromethane and the organic phase was washed with brine, dried on Na₂SO₄ and evaporated under vacuum. A foamy residue is obtained and purified by column chromatography (SiO₂/ dichloromethane; 1:15, then dichloromethane + methanol 2 %), yielding a white amorphous solid. The intermediates were characterized by TLC, melting point and ¹H NMR spectra.

tert-butyl 4-(2-oxo-2-(10H-phenothiazin-10-yl)ethyl)piperazine-1-carboxylate (XXXVII)

White amorphous solid. Melting point: 133–135 °C. Yield: 90 %. Molecular formula C₂₃H₂₇N₃OS. Molecular weight: 425.55 g/mol. Elemental analysis calculated for C₂₃H₂₇N₃OS: C% 70.22, H% 6.92, N% 10.68; found: C% 70.10, H% 6.85, N% 10.55. ¹H NMR (400 MHz, CDCl₃) δ: 7.58-7.40 (m, 4H, Ar), 7.34-7.16 (m, 4H, Ar), 3.30 (s, 2H, CH₂), 2.89 – 2.75 (m, 4H, piperazine), 2.51-2.38 (m, 4H, piperazine), 1.46 (s, 9H, CH₃).

tert-butyl 4-(3-oxo-3-(10H-phenothiazin-10-yl)propyl)piperazine-1-carboxylate (XXXVIII)

White solid. Melting point: 137–139 °C. Yield: 86 %. Molecular formula C₂₄H₂₉N₃OS. Molecular weight: 439.57 g/mol. Elemental analysis calculated for C₂₄H₂₉N₃OS: C% 65.59, H% 6.65, N% 9.56; found: C% 65.40, H% 6.72, N% 9.48. ¹H NMR (400 MHz, CDCl₃) δ: 7.58 – 7.41 (m, 4H, Ar), 7.31-7.15 (m, 4H, Ar), 2.95 (t, J = 4.9 Hz, 2H), 2.66 (dtd, J = 11.4, 6.4, 2.8 Hz, 6H), 2.40 (d, J = 4.9 Hz, 4H), 1.45 (s, 9H, CH₃).

General procedure for the synthesis of the final compounds 125 and 130.

To a solution of intermediates XXXVII or XXXVIII (4 mmol) in anhydrous dichloromethane (20 mL) was added dropwise 4 mL of trifluoroacetic acid. The

solution was left under vigorous stirring at rt for 6 hours. Finally, the solution was washed with 10% K₂CO₃, water, and brine. The organic solution, dried over Na₂SO₄, filtered, and evaporated to dryness, leaves a residue that was purified by chromatography on SiO₂ (1:10) by eluting with dichloromethane + 10% methanol.

1-(10H-Phenothiazin-10-yl)-2-(piperazin-1-yl)ethan-1-one (125)

White amorphous solid. Melting point: 162–165 °C. Yield: 96 %. Molecular formula C₁₈H₁₉N₃OS. Molecular weight: 325.43 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.58-7.40 (m, 4H, Ar), 7.34-7.16 (m, 4H, Ar), 3.30 (s, 2H, CH₂), 3.24 (s, 1H, NH), 2.89 – 2.75 (m, 4H, piperazine), 2.51-2.38 (m, 4H, piperazine).

¹³C NMR (101 MHz, CDCl₃) δ: 168.59, 138.70, 128.06, 127.98, 127.08, 127.01, 126.87, 76.80, 60.66, 52.91, 52.70, 51.34, 45.19.

1-(10H-Phenothiazin-10-yl)-3-(piperazin-1-yl)propan-1-one (130)

White amorphous solid. Melting point: 180–183 °C. Yield: 95 %. Molecular formula C₁₉H₂₁N₃OS. Molecular weight: 339.46 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.53 – 7.39 (m, 4H, Ar), 7.31-7.15 (m, 4H, Ar), 3.76 (s, 1H, NH), 2.92 (t, *J* = 5.0 Hz, 2H), 2.66 (dtd, *J* = 11.6, 6.5, 2.8 Hz, 6H), 2.41 (d, *J* = 5.0 Hz, 4H).

¹³C NMR (101 MHz, CDCl₃) δ: 170.89, 138.73, 138.70, 128.14, 127.34, 127.15, 127.11, 127.02, 126.96, 122.64, 54.41, 54.05, 52.84, 52.75, 51.36, 45.17.

General procedure for the synthesis of the final compounds 126-129 and 131-134.

The desired cinnamic acid (1.2 mmol) was activated with 1-hydroxybenzotriazole (HOBt, 1.2 mmol) and 3-dimethylamino-propyl-ethylcarbodiimide hydrochloride (EDC.HCl, 1.2 mmol) in DMF at room temperature for 30 minutes, then reacted with intermediate **125** or **130** (1 mmol) under stirring for 24 hours. After evaporation of the solvent under vacuum, the residue was dissolved in dichloromethane washed with an aqueous solution of K₂CO₃ (5 %) and brine. The organic phase was dried with Na₂SO₄ and evaporated under vacuum. A solid residue is obtained and purified by column chromatography (SiO₂; dichloromethane; 1:15) eluting with different quantity of methanol, in detail: compounds **125** and **130** (free NH group) with methanol 10 % ,

compounds **126-129** and **131-134** with 2 % MeOH. The final compounds were characterized by TLC, melting point, ^1H and ^{13}C NMR spectra.

(E)-1-(4-(2-Oxo-2-(10H-phenothiazin-10-yl)ethyl)piperazin-1-yl)-3-phenylprop-2-en-1-one (126)

White solid. Melting point: 193–196 °C. Yield: 78 %. Molecular formula $\text{C}_{27}\text{H}_{25}\text{N}_3\text{O}_2\text{S}$. Molecular weight: 455.58 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.65 (d, J = 15.4 Hz, 1H, CH=CH), 7.58 – 7.30 (m, 10H, Ar), 7.28 – 7.20 (m, 3H, Ar), 6.78 (d, J = 15.4 Hz, 1H, CH=CH), 3.78 (s, 2H, C(O)CH₂N), 3.14–2.83 (m, 4H, NCH₂), 2.79–2.31 (m, 4H, NCH₂).

^{13}C NMR (101 MHz, CDCl_3) δ : 165.47, 143.67, 138.35, 135.07, 129.96, 129.33, 129.19, 128.95, 128.27, 127.93, 127.33, 116.41, 53.67, 53.11, 52.50, 31.03, 29.80.

(E)-3-(4-Hydroxyphenyl)-1-(4-(2-oxo-2-(10H-phenothiazin-10-yl)ethyl)piperazin-1-yl)prop-2-en-1-one (127)

White amorphous solid. Melting point: 197–200 °C. Yield: 53 %. Molecular formula $\text{C}_{27}\text{H}_{25}\text{N}_3\text{O}_3\text{S}$. Molecular weight: 471.58 g/mol.

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ : 9.79 (s, 1H, OH), 7.67 – 7.60 (m, 2H, CH=CH e Ar), 7.56 – 7.43 (m, 4H, Ar), 7.41 – 7.22 (m, 5H, Ar), 6.90 (d, J = 15.3 Hz, 1H, CH=CH), 6.76 – 6.67 (m, 2H, Ar), 3.37 (s, 2H, C(O)CH₂N), 3.32–3.12 (m, 4H, NCH₂), 2.32–2.01 (m, 4H, NCH₂).

^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ : 168.70, 165.25, 159.47, 142.38, 139.22, 132.64, 130.35, 130.27, 128.32, 127.73, 127.38, 126.72, 116.05, 114.74, 60.39, 55.47, 52.89, 52.25, 45.41, 42.03.

(E)-3-(4-Fluorophenyl)-1-(4-(2-oxo-2-(10H-phenothiazin-10-yl)ethyl)piperazin-1-yl)prop-2-en-1-one (128)

White solid. Melting point: 196–198 °C. Yield: 65 %. Molecular formula $\text{C}_{27}\text{H}_{24}\text{FN}_3\text{O}_2\text{S}$. Molecular weight: 473.57 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.63 – 7.43 (m, 8H, CH=CH + Ar), 7.38 – 7.25 (m, 3H, Ar), 7.04 (t, J = 8.7 Hz, 2H, Ar), 6.67 (d, J = 15.4 Hz, 1H, CH=CH), 3.86 (s, 2H, C(O)CH₂N), 3.51–3.32- (m, 4H, NCH₂), 3.32–3.12 (m, 4H, NCH₂).

^{13}C NMR (101 MHz, CDCl_3) δ : 165.30, 165.00, 162.50, 142.75, 137.49, 131.21, 129.84, 129.76, 128.44, 127.85, 127.62, 126.81, 116.21, 116.18, 116.00, 115.96, 115.82, 57.38, 51.87, 44.04, 40.41.

(E)-3-(4-Hydroxy-3-methoxyphenyl)-1-(4-(2-oxo-2-(10H-phenothiazin-10-yl)ethyl)piperazin-1-yl)prop-2-en-1-one (129)

White amorphous solid. Melting point: 169–172 °C. Yield: 56 %. Molecular formula C₂₈H₂₇N₃O₄S. Molecular weight: 501.60 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.59 – 7.49 (m, 2H, CH=CH + Ar), 7.45 (dd, *J* = 7.7, 1.5 Hz, 2H), 7.37 – 7.20 (m, 5H), 7.04 (dd, *J* = 8.2, 1.9 Hz, 1H), 6.95 (d, *J* = 1.9 Hz, 1H), 6.88 (d, *J* = 8.1 Hz, 1H), 6.61 (d, *J* = 15.3 Hz, 1H, CH=CH), 6.00 (s, 1H, OH), 3.89 (s, 3H, OCH₃), 3.69–3.46 (m, 6H, NCH₂), 2.93–2.59 (m, 4H, NCH₂).

¹³C NMR (101 MHz, CDCl₃) δ: 166.89, 165.76, 147.59, 146.82, 143.64, 138.11, 133.22, 128.25, 127.68, 127.45, 127.38, 126.77, 122.18, 114.85, 113.92, 109.92, 65.96, 58.86, 56.10, 52.39, 44.90, 41.29.

(E)-1-(4-(3-Oxo-3-(10H-phenothiazin-10-yl)propyl)piperazin-1-yl)-3-phenylprop-2-en-1-one (131)

White solid. Melting point: 182–185 °C. Yield: 68 %. Molecular formula C₂₈H₂₇N₃O₂S. Molecular weight: 469.60 g/mol.

¹H NMR (400 MHz, CDCl₃) δ: 7.65 (d, *J* = 15.4 Hz, 1H, CH=CH), 7.58 – 7.29 (m, 10H, Ar), 7.28 – 7.20 (m, 3H, Ar), 6.79 (d, *J* = 15.4 Hz, 1H, CH=CH), 4.09–3.54 (m, 6H, CH₂N e C(O)CH₂), 2.96–2.30 (m, 6H, CH₂N).

¹³C NMR (101 MHz, CDCl₃) δ: 165.47, 143.67, 138.32, 135.05, 133.12, 129.99, 128.95, 128.28, 127.94, 127.35, 127.31, 116.46, 77.47, 53.65, 52.46, 44.47, 36.60, 31.06, 31.06.

(E)-3-(4-Hydroxyphenyl)-1-(4-(3-oxo-3-(10H-phenothiazin-10-yl)propyl)piperazin-1-yl)prop-2-en-1-one (132)

White solid. Melting point: 202–205 °C. Yield: 47 %. Molecular formula C₂₈H₂₇N₃O₃S. Molecular weight: 485.60 g/mol.

¹H NMR (400 MHz, DMSO-*D*₆) δ: 8.01 (s, 1H, OH), 7.62 – 7.39 (m, 5H, Ar), 7.37 – 7.17 (m, 6H, Ar), 6.87 – 6.72 (m, 2H, Ar), 6.58 (d, *J* = 15.4 Hz, 1H, CH=CH), 4.17–3.40 (m, 6H, CH₂N e C(O)CH₂), 2.89–2.41 (m, 6H, CH₂N).

¹³C NMR (101 MHz, DMSO-*D*₆) δ: 166.10, 162.76, 158.68, 138.22, 129.79, 128.28, 127.37, 116.14, 53.58, 36.67, 31.62.

(E)-3-(4-fluorophenyl)-1-(4-(3-oxo-3-(10H-phenothiazin-10-yl)propyl)piperazin-1-yl)prop-2-en-1-one (133)

White amorphous foam. Melting point: 178–181 °C. Yield: 74 %. Molecular formula C₂₈H₂₆FN₃O₂S. Molecular weight: 487.59 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.61 (d, J = 15.4 Hz, 1H, CH=CH), 7.58 – 7.41 (m, 6H, Ar), 7.34 (td, J = 7.7, 1.5 Hz, 2H, Ar), 7.24 (td, J = 7.6, 1.3 Hz, 2H, Ar), 7.04 (t, J = 8.6 Hz, 2H, Ar), 6.70 (d, J = 15.4 Hz, 1H, CH=CH), 4.14-3.41 (m, 6H, CH_2N e $\text{C}(\text{O})\text{CH}_2$), 2.97-2.34 (m, 6H, CH_2N).

^{13}C NMR (101 MHz, CDCl_3) δ : 165.30, 164.97, 162.64, 162.48, 142.60, 138.29, 131.27, 129.82, 129.74, 128.28, 127.36, 116.18, 115.96, 53.60, 53.05, 52.47, 36.59, 31.52, 31.05.

(E)-3-(4-Hydroxy-3-methoxyphenyl)-1-(4-(3-oxo-3-(10H-phenothiazin-10-yl)propyl)piperazin-1-yl)prop-2-en-1-one (134)

White amorphous solid. Melting point: 180–183 °C. Yield: 52 %. Molecular formula $\text{C}_{29}\text{H}_{29}\text{N}_3\text{O}_4\text{S}$. Molecular weight: 515.63 g/mol.

^1H NMR (400 MHz, CDCl_3) δ : 7.62 – 7.49 (m, 3H, CH=CH + Ar), 7.48 – 7.38 (m, 2H, Ar), 7.34 (td, J = 7.6, 1.5 Hz, 2H, Ar), 7.24 (t, J = 7.7 Hz, 2H, Ar), 7.11 – 7.02 (m, 1H, Ar), 6.96 (d, J = 1.9 Hz, 1H, Ar), 6.92 – 6.86 (m, 1H, Ar), 6.62 (d, J = 15.3 Hz, 1H, CH=CH), 3.90 (s, 3H, OCH_3), 2.91 (m, 6H, CH_2N e $\text{C}(\text{O})\text{CH}_2$), 2.62 (m, 6H, CH_2N).

^{13}C NMR (101 MHz, CDCl_3) δ : 165.74, 147.69, 146.86, 138.34, 128.26, 127.60, 127.33, 122.24, 114.91, 109.95, 65.96, 56.10, 53.66, 52.52, 31.05.

Cytisine-Cinnamic Acid hybrids

General procedure for the synthesis of the final compounds 135-140.

To obtain the final compounds **135-140**, the desired cinnamic acid (1.2 mmol) was activated with 1-hydroxybenzotriazole (HOBt, 1.2 mmol) and 3-dimethylamino-propyl)-ethyl-carbodiimide hydrochloride (EDCHCl, 1.2 mmol) in DMF at room temperature for 30 minutes, then reacted with cytosine (1 mmol) under stirring for 24 hours. After evaporation of the solvent under vacuum, the residue was dissolved in dichloromethane washed with an aqueous solution of K_2CO_3 (5 %) and brine. The organic phase was dried with Na_2SO_4 and evaporated under vacuum. A solid residue is obtained and purified by column chromatography (SiO_2 ; dichloromethane; 1:15) eluting with dichloromethane + methanol 5 %. The final compounds were characterized by TLC, melting point, ^1H and ^{13}C NMR spectra. In the ^1H NMR spectra H atoms relative to the pyrrolidine ring are

reported as “pyr” while H atoms belonging to the non-aromatic part of cytosine nuclei are reported as “cyt”.

3-cinnamoyl-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (135)

White solid. Melting point: 125-126 °C. Yield: 58 %. Molecular formula: C₂₀H₂₀N₂O₂. Molecular weight: 320.39 g/mol.

¹H NMR (400 MHz, CDCl₃) δ 7.46 – 7.11 (m, 6H, 2H CH=, + 3H arom, Ph + 1H pyr), 6.95 – 6.45 (m, 2H, arom), 6.41 (dd, J = 9.1, 1.4 Hz, 1H, pyr), 6.07 (d, J = 6.8 Hz, 1H, pyr), 4.15 (d, J = 15.8 Hz, 1H, cyt), 3.87 (dd, J = 16.3, 6.6 Hz, 1H, cyt), 3.27 – 2.74 (m, 4H, cyt), 2.66 – 2.44 (m, 2H, cyt), 2.04 – 1.93 (m, 2H, cyt).

¹³C NMR (101 MHz, CDCl₃) δ 166.75, 163.48, 148.52, 138.76, 135.12, 129.75, 128.84, 127.83, 117.77, 116.59, 105.54, 48.97, 34.92, 27.83, 26.38.

(E)-3-(3-(4-hydroxyphenyl)acryloyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (136)

White solid. Melting point: >260 °C. Yield: 64 %. Molecular formula: C₂₀H₂₀N₂O₃. Molecular weight: 336.39 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ 9.95 (s, 1H, OH), 7.56 – 7.03 (m, 7H, 2H CH=, + 4H arom, Ph + 1H pyr), 6.51 (dd, J = 9.1, 1.4 Hz, 1H, pyr), 6.12 (dd, J = 16.6, 7.9 Hz, 1H, pyr), 4.18 (d, J = 15.8 Hz, 1H), 3.88 (dd, J = 16.0, 6.5 Hz, 1H), 3.64 – 2.97 (m, 4H, cyt), 2.59 – 2.36 (m, 2H, cyt), 2.03 – 1.82 (m, 2H, cyt).

¹³C NMR (101 MHz, DMSO-D₆) δ 165.98, 162.63, 159.54, 155.77, 141.59, 139.16, 130.16, 126.56, 122.76, 119.40, 116.34, 116.07, 114.14, 110.59, 105.25, 51.33, 48.98, 34.83, 27.81, 25.95.

(E)-3-(3-(4-fluorophenyl)acryloyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (137)

White solid. Melting point: 204-205 °C. Yield: 61 %. Molecular formula: C₂₀H₁₉FN₂O₂. Molecular weight: 338.38 g/mol.

¹H NMR (400 MHz, CDCl₃) δ 7.39 – 6.94 (m, 7H, 2H CH=, + 4H arom, Ph + 1H pyr), 6.40 (dd, J = 9.1, 1.4 Hz, 1H, pyr), 6.06 (d, J = 6.8 Hz, 1H, pyr), 4.15 (d, J = 15.8 Hz, 1H), 3.86 (dd, J = 16.0, 6.5 Hz, 1H), 3.58 – 3.02 (m, 4H, cyt), 2.64 – 2.49 (m, 2H, cyt), 2.04 – 1.87 (m, 2H, cyt).

¹³C NMR (101 MHz, CDCl₃) δ 166.54, 164.82, 163.46, 162.33, 148.51, 141.79, 138.85, 131.36, 131.33, 129.68, 129.60, 117.78, 116.30, 116.04, 115.82, 105.72, 48.94, 34.92, 27.85, 26.38.

(E)-3-(3-(4-chlorophenyl)acryloyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (138)

White solid. Melting point: 182-184 °C. Yield: 74 %. Molecular formula: C₂₀H₁₉ClN₂O₂. Molecular weight: 354.83 g/mol.

¹H NMR (400 MHz, CDCl₃) δ 7.47 – 7.13 (m, 7H, 2H CH=, + 4H arom, Ph + 1H pyr), 6.42 (dd, J = 9.1, 1.4 Hz, 1H, pyr), 6.07 (d, J = 6.9 Hz, 1H, pyr), 4.15 (d, J = 15.7 Hz, 1H, cyt), 3.87 (dd, J = 16.3, 6.6 Hz, 1H, cyt), 3.52 – 2.87 (m, 4H, cyt), 2.63 – 2.49 (m, 2H, cyt), 2.10 – 1.98 (m, 2H, cyt).

¹³C NMR (101 MHz, CDCl₃) δ 166.40, 163.43, 148.47, 135.56, 133.62, 129.10, 129.01, 117.81, 117.15, 55.85, 48.95, 34.78, 27.85, 26.39.

(E)-3-(3-(p-tolyl)acryloyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (139)

White solid. Melting point: 179-181 °C. Yield: 71 %. Molecular formula: C₂₁H₂₂N₂O₂. Molecular weight: 334.42 g/mol.

¹H NMR (400 MHz, CDCl₃) δ 7.46 – 7.06 (m, 7H, 2H CH=, + 4H arom, Ph + 1H pyr), 6.41 (dd, J = 9.1, 1.6 Hz, 1H, pyr), 6.07 (d, J = 6.8 Hz, 1H, pyr), 4.15 (d, J = 15.7 Hz, 1H, cyt), 3.87 (dd, J = 16.3, 6.6 Hz, 1H, cyt), 3.46 – 2.98 (m, 4H, cyt), 2.62 – 2.50 (m, 2H, cyt), 2.33 (s, 3H, CH₃), 2.09 – 1.97 (m, 2H, cyt).

¹³C NMR (101 MHz, CDCl₃) δ 166.94, 163.47, 148.56, 140.05, 132.37, 129.56, 127.83, 115.44, 57.71, 48.99, 35.01, 27.83, 26.39.

(E)-3-(3-(4-hydroxy-3-methoxyphenyl)acryloyl)-1,2,3,4,5,6-hexahydro-8H-1,5-methanopyrido[1,2-a][1,5]diazocin-8-one (140)

White solid. Melting point: 156-160 °C. Yield: 59 %. Molecular formula: C₂₁H₂₂N₂O₄. Molecular weight: 366.42 g/mol.

¹H NMR (400 MHz, CDCl₃) δ 7.99 (s, 1H, OH), 7.42 – 7.17 (m, 6H, 2H CH=, + 4H arom, Ph + 1H pyr), 6.41 (dd, J = 9.1, 1.4 Hz, 1H, pyr), 6.08 (dd, J = 6.9 Hz, 1H, pyr), 4.16 (d, J = 15.7 Hz, 1H), 3.86 (dd, J = 16.0, 6.5 Hz, 1H), 3.52 – 3.04 (m, 4H, cyt), 2.61 – 2.38 (m, 2H, cyt), 2.09 – 1.94 (m, 2H, cyt).

¹³C NMR (101 MHz, CDCl₃) δ 167.11, 163.59, 162.70, 148.67, 147.67, 146.89, 143.46, 139.01, 127.52, 122.05, 117.66, 114.89, 113.71, 110.18, 56.07, 49.02, 36.62, 34.93, 31.55, 27.83, 26.35.

Coumarin-indole hybrids

Synthesis of 1-methyl-1H-indole-3-carbaldehyde (XXXIX).

To obtain the intermediate **XXXIX**, *1H-indole-3-carbaldehyde* (6 mmol) was dissolved in anhydrous DMF (2 mL) and the solution was cooled to 0 °C in an ice bath. Sodium hydride (6 mmol) was added in two portions and the mixture was stirred for 15 minutes under a KOH trap. Methyl iodide (7.2 mmol) dissolved in anhydrous DMF (1 mL) was then added dropwise, and the reaction mixture was stirred at room temperature for 6 hours. After being dissolved into an ice–water mixture (25 mL), the reaction mixture was extracted with EtOAc (2 × 10 mL). The combined organic phases were washed with water (10 mL) and then with brine (2 × 10 mL), dried over Na₂SO₄ and filtered. The solid collected was washed twice with anhydrous ether affording the desired compound.

Grey solid. Melting point: 68–69 °C. Yield: 84 %. Molecular formula: C₁₀H₉NO. Molecular weight: 159.19 g/mol. Elemental analysis calculated for: C₁₀H₉NO: C% 75.45, H% 5.70, N% 8.80; found: C% 75.32, H% 5.68, N% 8.72. ¹H NMR (400 MHz, CDCl₃) δ 9.97 (s, 1H, CHO), 8.33–8.26 (m, 1H, ArH), 7.66 (s, 1H, ArH), 7.35 (m, 3H, ArH), 3.86 (s, 3H, NCH₃). ¹³C NMR (101 MHz, CDCl₃) δ 184.53, 139.38, 137.97, 125.36, 124.15, 123.07, 122.16, 118.14, 109.96, 33.82.

General procedure for the synthesis of the intermediates XL-XLI.

A mixture of malonic monohydrazide (**I**) (6 mmol) and *1H-indole-3-carbaldehyde* or *1-methyl-1H-indole-3-carbaldehyde* (**XXXIX**) (6 mmol) in absolute ethanol (15 mL) was heated at reflux for 5 h under a KOH trap. During the reaction, a clear solution was formed. Upon completion, the mixture was concentrated under reduced pressure and the resulting solution was stored at –20 °C overnight. The following day, the formation of crystalline material was observed. The solid was collected by vacuum filtration, washed with a small amount of cold ethanol, and dried to afford intermediate **XL** and **XLI** as light-gray crystals.

Ethyl (E)-3-(2-((1H-indol-3-yl)methylene)hydrazineyl)-3-oxopropanoate (XL)

Light gray crystals. Yield: 91 %. Melting point: 173–174 °C. Molecular formula: C₁₄H₁₅N₃O₃. Molecular weight: 273.29 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ 11.52 and 11.73 (s, 1H, NH), 11.15 and 11.20 (s, 1H, CH=N), 8.12 and 8.16 (s, 1H, N=NH), 8.02 (d, J = 7.9 Hz, 1H, ArH), 7.73 (d, J

= 2.7 Hz, 1H, ArH), 7.39 (dd, J = 8.1, 3.1 Hz, 1H, ArH), 7.19–7.03 (m, 2H, ArH), 4.04 and 4.07 (q, J = 7.0 Hz, 2H, OCH₂), 3.65 and 3.61 (s, 2H, C(O)CH₂), 1.14 and 1.18 (t, J = 7.1 Hz, 3H, CH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ 168.40, 167.39, 141.47, 137.60, 131.15, 124.48, 123.13, 122.09, 120.91, 112.36, 111.84, 60.96, 41.47, 14.58.

Ethyl (E)-3-(2-((1-methyl-1H-indol-3-yl)methylene)hydrazineyl)-3-oxopropanoate (XLI)

White crystals. Yield: 92 %. Melting point: 172–173 °C. Molecular formula: C₁₅H₁₇N₃O₃. Molecular weight: 287.13 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ 11.15 and 11.21 (s, 1H, CH=N), 8.08 and 8.15 (s, 1H, N=NH), 8.02 (d, J = 7.8 Hz, 1H, ArH), 7.72 (s, 1H, ArH), 7.44 (d, J = 8.2 Hz, 1H, ArH), 7.26–7.07 (m, 2H, ArH), 4.03 and 4.07 (q, J = 7.1 Hz, 2H, OCH₂), 3.76 and 3.78 (s, 3H, NCH₃), 3.64 and 3.61 (s, 2H, C(O)CH₂C(O)), 1.12 and 1.17 (t, J = 7.1 Hz, 3H, CH₃CH₂O).

¹³C NMR (101 MHz, DMSO-d₆) δ 168.39, 167.38, 140.97, 138.10, 134.81, 124.83, 123.22, 122.24, 121.17, 110.75, 60.97, 41.48, 33.30, 14.58.

General procedure for the synthesis of the final compounds 141–150.

Hydrazone intermediate **XL** or **XLI** (1.0 mmol) was dissolved in absolute ethanol (8–10 mL). The desired 2-hydroxybenzaldehyde derivative (1 mmol) was then added, followed by piperidine (0.1–0.2 mL). The reaction mixture was heated at reflux for 3 h under a KOH trap, during which a clear, intensely yellow to orange solution gradually formed and a pale-yellow precipitate began to appear. At the end of the reflux period, the mixture was allowed to cool to room temperature. The suspension was stored in the freezer overnight to complete crystallization, and the resulting solid was collected by vacuum filtration, washed with small volume of cold ethanol, and dried to give yellow crystalline final compounds. All final compounds were characterized by TLC, melting point and NMR spectroscopy (¹H and ¹³C).

(E)-N'-((1H-indol-3-yl)methylene)-2-oxo-2H-chromene-3-carbohydrazide (141)

Yellow solid. Yield: 94 %. Melting point: 284–285 °C. Molecular formula: C₁₉H₁₃N₃O₃. Molecular weight: 331.33 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ 11.61 and 11.84 (s, 1H, NH), 11.47 and 11.07 (s, 1H, CH=N), 8.86 and 8.67 (s, 1H, N=NH), 8.54 and 8.13 (s, 1H, ArH), 8.23 (d, J =

7.6 Hz, 1H, ArH), 8.03–7.95 (m, 1H, ArH), 7.83–7.66 (m, 3H, ArH), 7.57–7.35 (m, 2H, ArH), 7.23–7.09 (m, 2H, ArH).

¹³C NMR (101 MHz, DMSO-d₆) δ 160.59, 157.80, 154.35, 147.64, 146.85, 137.58, 131.66, 130.72, 125.77, 124.86, 123.28, 122.42, 121.12, 116.76, 112.47, 111.90.

(E)-N'-((1H-indol-3-yl)methylene)-7-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (142)

Yellow-orange solid. Yield: 86 %. Melting point: 280–281 °C. Molecular formula: C₁₉H₁₃N₃O₄. Molecular weight: 347.33 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ 11.62 and 11.83 (s, 1H, NH), 11.50 and 11.16 (s, 1H, CH=N), 10.45 (s, 1H, OH), 8.79 and 8.63 (s, 1H, N=NH), 8.54 and 8.32 (s, 1H, ArH), 8.26–8.11 (m, 1H, ArH), 7.80 (d, J = 2.8 Hz, 1H, ArH), 7.45–7.34 (m, 2H, ArH), 7.24–7.09 (m, 4H, ArH).

¹³C NMR (101 MHz, DMSO-d₆) δ 160.55, 157.87, 148.12, 146.80, 145.22, 145.06, 142.97, 137.57, 131.67, 125.75, 124.85, 123.28, 122.43, 121.11, 120.66, 120.53, 120.02, 112.48, 111.90.

(E)-N'-((1H-indol-3-yl)methylene)-7-methoxy-2-oxo-2H-chromene-3-carbohydrazide (143)

Yellow solid. Yield: 86 %. Melting point: 288–289 °C. Molecular formula: C₂₀H₁₅N₃O₄. Molecular weight: 361.36 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ 11.61 and 11.78 (s, 1H, NH), 11.47 and 11.12 (s, 1H, CH=N), 8.82 and 8.66 (s, 1H, N=NH), 8.54 and 8.32 (d, J = 2.1 Hz, 1H, ArH), 8.26–8.19 (m, 1H, ArH), 7.80 (d, J = 2.6 Hz, 1H, ArH), 7.52 (d, J = 7.6 Hz, 1H, ArH), 7.45–7.29 (m, 3H, ArH), 7.22–7.09 (m, 2H, ArH), 3.91 and 3.93 (s, 3H, OCH₃).

¹³C NMR (101 MHz, DMSO-d₆) δ 160.33, 157.75, 147.88, 146.88, 146.86, 143.62, 137.57, 131.70, 125.71, 124.85, 123.28, 122.42, 121.62, 121.12, 120.38, 119.62, 116.57, 112.48, 111.89, 56.76.

(E)-N'-((1H-indol-3-yl)methylene)-8-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (144)

Yellow-orange solid. Yield: 83 %. Melting point: 257–258 °C. Molecular formula: C₁₉H₁₃N₃O₄. Molecular weight: 347.33 g/mol.

¹H NMR (400 MHz, DMSO-d₆) δ 11.61 and 11.78 (s, 1H, NH), 11.43 and 11.08 (s, 1H, CH=N), 11.11 (s, 1H, OH), 8.80 and 8.65 (s, 1H, N=NH), 8.52 and 8.14 (s, 1H, ArH), 8.22 (d, J = 7.7 Hz, 1H, ArH), 7.86–7.75 (m, 2H, ArH), 7.41 (d, J = 8.0 Hz, 1H, ArH), 7.21–7.08 (m, 4H, ArH).

^{13}C NMR (101 MHz, DMSO- d_6) δ 164.27, 161.37, 158.19, 156.76, 148.52, 146.42, 137.55, 132.51, 131.48, 124.85, 123.24, 121.06, 114.97, 114.57, 112.46, 111.94, 111.76, 102.41.

(E)-N'-((1H-indol-3-yl)methylene)-8-methoxy-2-oxo-2H-chromene-3-carbohydrazide (145)

Yellow solid. Yield: 89 %. Melting point: 285–286 °C. Molecular formula: $\text{C}_{20}\text{H}_{15}\text{N}_3\text{O}_4$. Molecular weight: 361.36 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ 11.57 and 11.77 (m, 1H, NH), 11.43 and 11.19 (s, 1H, CH=N), 8.84 and 8.68 (s, 1H, N=NH), 8.53 and 8.30 (s, 1H, ArH), 8.25–8.11 (m, 1H, ArH), 7.92 (d, J = 8.7 Hz, 1H, ArH), 7.79 (d, J = 2.8 Hz, 1H, ArH), 7.49–7.34 (m, 1H, ArH), 7.19 (dd, J = 7.1, 1.5 Hz, 1H, ArH), 7.17–7.09 (m, 2H, ArH), 7.03 (dd, J = 8.7, 2.4 Hz, 1H, ArH), 3.87 and 3.91 (s, 3H, OCH_3).

^{13}C NMR (101 MHz, DMSO- d_6) δ 164.98, 161.18, 158.05, 156.65, 148.23, 146.53, 137.56, 132.06, 131.52, 123.25, 122.42, 121.08, 115.87, 114.26, 112.77, 112.46, 111.94, 100.92, 56.83.

(E)-N'-((1-methyl-1H-indol-3-yl)methylene)-2-oxo-2H-chromene-3-carbohydrazide (146)

Yellow solid. Yield: 94 %. Melting point: 276–277 °C. Molecular formula: $\text{C}_{20}\text{H}_{15}\text{N}_3\text{O}_3$. Molecular weight: 345.36 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ 11.47 and 11.09 (s, 1H, CH=N), 8.86 and 8.67 (s, 1H, N=NH), 8.53 and 8.15 (s, 1H, ArH), 8.28–8.20 (m, 1H, ArH), 7.99 (dd, J = 7.8, 1.6 Hz, 1H, ArH), 7.82–7.66 (m, 3H, ArH), 7.57–7.34 (m, 2H, ArH), 7.26 (ddd, J = 8.3, 7.1, 1.4 Hz, 1H, ArH), 7.19 (ddd, J = 8.0, 7.1, 1.1 Hz, 1H, ArH), 3.81 and 3.72 (s, 3H, NCH_3).

^{13}C NMR (101 MHz, DMSO- d_6) δ 169.92, 160.54, 157.80, 154.37, 147.66, 146.34, 138.12, 135.10, 134.62, 130.72, 125.77, 123.34, 121.38, 119.06, 116.77, 110.92, 33.41.

(E)-7-hydroxy-N'-((1-methyl-1H-indol-3-yl)methylene)-2-oxo-2H-chromene-3-carbohydrazide (147)

Yellow-orange solid. Yield: 87 %. Melting point: 252–253 °C. Molecular formula: $\text{C}_{20}\text{H}_{15}\text{N}_3\text{O}_4$. Molecular weight: 361.36 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ 11.42 and 1.60 (s, 1H, CH=N), 11.07 and 11.26 (s, 1H, OH), 8.80 and 8.62 (s, 1H, N=NH), 8.52 and 8.41 (s, 1H, ArH), 8.23 (d, J = 7.2 Hz, 1H, ArH), 7.87–7.69 (m, 1H, ArH), 7.48 (d, J = 8.2 Hz, 2H, ArH), 7.31–7.11 (m, 2H, ArH), 6.91–6.74 (m, 2H, ArH), 3.81 and 3.75 (s, 3H, NCH_3).

^{13}C NMR (101 MHz, DMSO- d_6) δ 161.33, 158.20, 156.75, 148.52, 145.87, 143.53, 143.15, 138.09, 134.89, 132.52, 125.29, 121.31, 114.94, 114.60, 111.79, 110.99, 102.42, 33.39.

***(E)*-7-methoxy-*N'*-((1-methyl-1*H*-indol-3-yl)methylene)-2-oxo-2*H*-chromene-3-carbohydrazide (148)**

Yellow solid. Yield: 87 %. Melting point: 292–293 °C. Molecular formula: $\text{C}_{21}\text{H}_{17}\text{N}_3\text{O}_4$. Molecular weight: 375.38 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ 11.43 and 11.09 (s, 1H, CH=N), 8.85 and 8.52 (s, 1H, N=NH), 8.58 and 8.33 (s, 1H, ArH), 8.09 (d, J = 8.0 Hz, 2H, ArH), 7.92 (dd, J = 8.8, 5.0 Hz, 1H, ArH), 7.77 (d, J = 3.3 Hz, 1H, ArH), 7.40 (d, J = 3.0 Hz, 1H, ArH), 7.17–6.99 (m, 3H, ArH), 3.88 and 3.80 (s, 3H, OCH_3), 3.76 and 3.73 (s, 3H, NCH_3). ^{13}C NMR (101 MHz, DMSO- d_6) δ 161.29, 158.03, 156.66, 148.34, 145.91, 143.53, 143.22, 138.14, 134.75, 132.51, 125.28, 121.19, 114.92, 114.62, 111.78, 110.46, 102.44, 56.82, 33.39.

***(E)*-8-hydroxy-*N'*-((1-methyl-1*H*-indol-3-yl)methylene)-2-oxo-2*H*-chromene-3-carbohydrazide (149)**

Yellow-orange solid. Yield: 83 %. Melting point: 238–239 °C. Molecular formula: $\text{C}_{20}\text{H}_{15}\text{N}_3\text{O}_4$. Molecular weight: 361.36 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ 11.50 (s, 1H, CH=N), 11.07 (s, 1H, OH), 8.79 and 8.84 (s, 1H, CH=N), 8.53 and 8.33 (s, 1H, N=NH), 8.24 (dt, J = 7.8, 1.1 Hz, 1H, ArH), 8.19–8.09 (m, 1H, ArH), 7.79 (s, 1H, ArH), 7.48 (dt, J = 8.2, 1.0 Hz, 1H, ArH), 7.30–7.14 (m, 4H, ArH), 3.81 and 3.75 (s, 3H, NCH_3). ^{13}C NMR (101 MHz, DMSO- d_6) δ 160.60, 157.91, 148.25, 146.28, 143.16, 138.11, 135.07, 125.77, 125.29, 123.33, 122.53, 121.37, 119.87, 110.86, 110.94, 33.40.

***(E)*-8-methoxy-*N'*-((1-methyl-1*H*-indol-3-yl)methylene)-2-oxo-2*H*-chromene-3-carbohydrazide (150)**

Yellow solid. Yield: 90 %. Melting point: 283–284 °C. Molecular formula: $\text{C}_{21}\text{H}_{17}\text{N}_3\text{O}_4$. Molecular weight: 375.38 g/mol.

^1H NMR (400 MHz, DMSO- d_6) δ 11.47 and 11.26 (s, 1H, CH=N), 8.83 (s, 1H, N=NH), 8.54 and 8.33 (s, 1H, ArH), 8.23 (d, J = 8.2 Hz, 1H, ArH), 7.74 (s, 1H, ArH), 7.50 (dd, J = 14.8, 8.1 Hz, 2H, ArH), 7.45–7.32 (m, 2H, ArH), 7.14–7.05 (m, 2H, ArH), 3.92 and 3.96 (s, 3H, OCH_3), 3.76 and 3.72 (s, 3H, NCH_3).

¹³C NMR (101 MHz, DMSO-d₆) δ 160.47, 157.79, 148.21, 146.65, 143.62, 138.09, 134.68, 132.49, 125.73, 124.98, 123.27, 122.48, 121.50, 120.38, 119.65, 116.55, 112.39, 110.94, 56.74, 33.38.

Chemical radical scavenging activity

The antioxidant activity of compounds **65-105** was measured using DPPH assay [101,102]. The compounds (3 mg) were first dissolved in 1 mL DMSO then methanol was added to take up the volume to 50 mL. A total of 0.1 mL of the compound solution was mixed with 3.9 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanol solution (65 mM). Absorbance was measured at 517 nm after reacting for 30 min in the dark. The linear calibration curve was obtained using Trolox standards (ranging between 20 and 200 mg/L, R² = 0.9955). After confirming linear behavior within the concentration interval used, the antioxidant activity (AA%) of the samples was determined by the ratio of the decrease in absorbance of each sample (A_s) with that of the DPPH blank solution (A₀) as reported in Equation 1 [103].

$$AA\% = \frac{A_0 - A_s}{A_0} \times 100$$

All analyses were performed in triplicate (n = 3), and values are given ± standard deviation (SD).

Biological evaluation

ChEs and MAO inhibition

The work presented in this section was done in collaboration with Professor Marco Catto and Dr. Gabriella La Spada (University of Bari, Italy).

The inhibitory activity of the compounds toward ChEs (*hAChE* and *eqChE*) and monoamine oxidases (*hMAO-A* and *hMAO-B*) was assessed using standardized spectrophotometric and fluorometric methods. AChE and BChE inhibition were determined through the Ellman colorimetric assay adapted to 96-well plate [228,181,229] using acetylthiocholine or butyrylthiocholine iodide as a substrate. Inhibition of MAO-A and MAO-B was evaluated using recombinant human

enzymes and the kynuramine-based fluorescence method ^[250]. IC₅₀ values were calculated using the nonlinear regression method 'log[inhibitor] vs. response' of three independent experiments executed in duplicate and are expressed as $\pm$ SEM.

Proteins expression, purification, kinetic validation of Ab1-42 peptide and aggregation assays in presence of compounds 65-124

The work presented in this section was carried out at the Centre for Misfolding Diseases at the University of Cambridge during a six-month internship under the supervision of Professor Michele Vendruscolo and with the kind help of Vaidehi Roy Chowdhury.

General

All chemicals were purchased from Sigma-Aldrich. All solutions were prepared in MilliQ water and filtered through 0.22 μ m pore size filters (Whatman). Stock solutions of compounds **65-124** were prepared in DMSO and filtered through 0.02 μ m pore size aluminum filters (Anotop 10, Whatman). Agar plates were from MP Biomedicals, USA. The flasks containing the cell cultures were incubated in Multitron Standard incubators (Infors HT). The growth of the bacteria was monitored through optical dispersion measurements over time using an OD600 spectrophotometer (Mettler Toledo). Cells were isolated by centrifugation of the cell culture with an Avanti JXN-26, centrifuge (Beckman Coulter Life Sciences). Cell pellets resuspended in lysis buffer were sonicated with a Q700 sonicator (Qsonica). HiPrep excel nickel columns for affinity chromatography, Superdex 30 26/600 pg and Superdex 200 Increase 10/300 GL columns for size exclusion chromatography, ÄKTA Prime and ÄKTA Pure purification systems were from Cytiva (formerly GE Healthcare Life Sciences). All operations involving A β were carried out using low-binding tubes.

Production of TEV protease (His6-TEV(S219V)-Arg5)

Pre-culture

E. coli BL21(DE3) pLysS bacteria (New England BioLabs Inc.) containing pRK793 plasmid (addgene) were used for the expression of TEV protease. From a glycerol stock of transformed cells, bacterial colonies were grown overnight on agar plates containing 100 µg/mL ampicillin and 30 µg/mL chloramphenicol at 37 °C. A single colony was selected and used to inoculate the pre-culture LB medium (120 mL), ampicillin (100 µg/mL) and chloramphenicol (30 µg/mL). The pre-culture was incubated overnight at 37 °C, 180 rpm.

Main culture

For the main culture, 12.5 mL of pre-culture were incubated independently in eight sterile flasks each containing LB culture medium (500 mL), ampicillin (100 µg/mL), chloramphenicol (30 µg/mL) and 0.2 % D-Glucose. The cultures were incubated at 37 °C, 250 rpm until OD₆₀₀ ~ 0.5 was obtained. The production of the MBP-TEV fusion protein was induced by adding IPTG (final concentration of 1 mM) individually to each flask and incubated at 30 °C, 250 rpm until OD₆₀₀ ~ 0.8 was reached. After induction and reduction of the incubation temperature, the MBP-TEV fusion protein undergoes *in vivo* self-cleavage, generating His6-TEV(S219V)-Arg5.

Harvest and cell lysis

Cells were isolated by centrifugation (Avanti JXN-26, Beckman Coulter Life Sciences) at 5000xg for 10 minutes at 4 °C. The supernatant was discarded and the cell pellet were frozen in liquid nitrogen and kept at -80°C until use.

Cells were thawed on ice and resuspended in cold lysis buffer (50 mM NaPi, pH 8.0, 200 mM NaCl, 10% glycerol, 25 mM imidazole, 10 mL/g per cell pellet). The solution was then sonicated 3 times (amplitude: 70, pulse: 15 seconds, pause: 45 seconds). After sonication, polyethyleneimine was added (final concentration of 0.1%) to remove DNA and RNA, and the solutions were centrifuged at 15000xg for 30 minutes at 4 °C. The supernatants were separated from cells lysate pellet and filtered through 0.22 µm vacuum filter.

IMAC purification

Affinity chromatography was performed using HiPrep Excel nickel columns (5 mL) on an ÄKTA Prime purification system. Two 5 mL columns serially connected were used to increase the efficiency of the process. After being loaded onto the column (3 mL/min flow), the protein was eluted (3 mL/min flow, linear gradient to 100%), using a buffer containing high imidazole concentration (50 mM NaPi pH 8, 200 mM NaCl, 10% glycerol, 250 mM imidazole).

SDS-PAGE

SDS- PAGE was performed to identify the fractions containing the His6-TEV(S219V)-Arg5 protein (~28 kDa). For SDS-PAGE, 10 μ L of the sample was added to 10 μ L of LDS NuPAGE sample buffer (Invitrogen) and applied to a pre-prepared NuPAGE 4 12% Bis-Tris gel (Invitrogen). The SeeBlue reference (Invitrogen) was used as a standard. NuPAGE MES SDS buffer (Invitrogen) was used for measurement. A voltage of 200 V was applied for 35 minutes to separate the samples. The gels were stained in Instant Blue Coomassie Protein Stain and imaged using the ChemiDoc imaging system (Bio-Rad).

Dialysis

The fractions containing the protein were combined, and EDTA and DTT were added to a final concentration of 2 mM and 5 mM, respectively. The solution containing the protein was afterwards dialyzed overnight in 25 mM NaPi pH 7.5, 100 mM NaCl, 10% glycerol buffer at 4 °C.

Purification

As the SDS-PAGE indicated sufficient purity, size-exclusion chromatography (SEC) was not performed. The dialyzed solution was filtered through a 0.22 μ m vacuum filter. The concentration of the protein was determined by absorbance at 280 nm using a Nanodrop ($\epsilon_{280} = 32290 \text{ M}^{-1}\text{cm}^{-1}$, 28 kDa). The protein was aliquoted, frozen in liquid nitrogen and stored at -80 °C until use without further purification.

Production of A β ₁₋₄₂ peptide (NT*FISp-A β fusion protein)

Pre-culture

E. coli BL21 Gold (DE3) bacteria (New England BioLabs Inc.) containing pT7 plasmid were used for the expression Ab₄₂. From a glycerol stock of transformed cells, bacterial colonies were grown overnight on agar plates containing 70 μ g/mL kanamycin at 37 °C. A single colony was selected and used to inoculate the pre-culture LB medium containing kanamycin (70 μ g/mL). The pre-culture was incubated overnight at 37 °C, 180 rpm.

Main culture

For the main culture, 4 mL of the pre-culture were incubated independently in four sterile flasks each containing LB culture medium (400 mL), kanamycin (70 μ g/mL), chloramphenicol (30 μ g/mL). The cultures were incubated at 30 °C, 120 rpm until OD₆₀₀ ~ 0.8-0.9 was reached. The production of the NT*FISp-A β fusion protein was induced by adding IPTG (final concentration of 0.1 mM) individually to each flask and incubated at 20 °C, 120 rpm overnight.

Harvest and cell lysis

Cells were isolated by centrifugation at 5000xg for 20 minutes at 4°C. The supernatant was discarded and the cell pellet were frozen in liquid nitrogen and kept at -80°C until use. All subsequent purification steps were performed on ice. Cells were thawed on ice and resuspended in cold lysis buffer (20mM Tris-HCl pH 8, 8M urea, 40 mL per L of primary culture). The solution was then sonicated 3 times (amplitude: 70, pulse: 15 seconds, pause: 45 seconds). After sonication, the solutions were centrifuged at 21000xg for 15 minutes at 4 °C. The supernatants were separated from cells lysate pellet and filtered through 0.22 μ m vacuum filter.

IMAC purification

Affinity chromatography was performed using HiPrep Excel nickel columns (5 mL) on an ÄKTA Prime purification system. Two 5 mL columns connected to each other were used to increase the efficiency of the process. The protein was

loaded onto the column (3 mL/min flow), the discard was also collected and kept on ice (flow through) since part of the protein of interest may not have been retained on the column. The protein was eluted (3 mL/min flow, linear gradient to 100%), using a high imidazole concentration containing buffer (20 mM Tris-HCl pH 8, 8M urea, 300 mM imidazole). The same process was repeated for the flow through. SDS-PAGE was carried out to identify the fractions containing the NT*FISp-A β protein (~20 kDa). The fractions containing the protein were combined and its concentration was determined using a Nanodrop spectrophotometer (Thermo Fisher) ($\epsilon_{280} = 2980 \text{ M}^{-1}\text{cm}^{-1}$, 20 kDa).

Dialysis and TEV cleavage

The solution containing the protein dialyzed overnight in 20 mM Tris-HCl, pH 8 buffer at 4 °C. The Ab₁₋₄₂ peptide was cleaved from the NT*FISp protein by the specific TEV cleavage site at the N-terminal end of the A β sequence. The cleavage was carried out through dialysis by adding the TEV protease to the solution containing the fusion protein at a ratio of NT*FISp-A β : TEV 20:1 in 3 kDa pore size dialysis bags in Tris-HCl pH 8, 0.5 mM EDTA, 1 mM DTT buffer at 4 °C overnight. The cleavage was confirmed by SDS-PAGE. After dialysis, the solutions were centrifuged at 4700 rpm for 10 minutes at 4 °C to remove aggregates and the supernatant was lyophilized, frozen in liquid nitrogen and kept at -80 °C until use.

Purification

The lyophilized TEV cleavage product was resuspended in 6M Guanidine-HCl (10 mL) and kept at 4 °C for at least 30 minutes. Afterwards, the solution was loaded onto a Superdex 30 26/600 pg size exclusion column on an ÄKTA Pure protein purification system and eluted in 25 mM NaPi buffer pH 8.5, 0.2 mM EDTA. The fractions corresponding to the A β peptide were identified with SDS-PAGE and reunited. The protein concentration was calculated using the UV detector of the ÄKTA system using the integrated average of the eluted protein peak (concentration = average of peak area/0.2*1490). The protein was lyophilized and then frozen in liquid nitrogen and kept at -80 °C until use.

Mass Spectrometry

Ion mobility spectrometry was carried out on the Waters Vion IMS-QToF mass spectrometer, where 1 μ L of the purified protein sample was injected into the equipment with a maximum retention time of 7 minutes. The system was rinsed with 3 μ L of water between samples to minimize contamination between samples. The mass spectra were reconstructed using the MaxEnt algorithm in the MassLynx software according to the manual.

Preparation of monomeric A β

The lyophilized A β was dissolved in 6M guanidine-HCl and incubated for at least 30 minutes in order to dissolve any pre-formed aggregates. The peptide was purified by size exclusion chromatography using Superdex 200 Increase 10/300 GL column and eluted (manual injection, 0.5 mL/min flow, linear gradient to 100%) in 20 mM NaPi buffer pH 8, 0.2 mM EDTA for the validation of the kinetics of aggregation and 20 mM NaPi buffer pH 8, 0.2 mM EDTA + 1 % DMSO for the kinetics of aggregation in presence of compounds **65-124**. The protein concentration was calculated using the UV detector of the ÄKTA Pure system used for purification with the integrated average of the eluted protein peak (concentration (M) = average of peak area/0.2*1490).

A β_{1-42} aggregation kinetics validation

Solutions of different concentration (5 replicates per concentration) of monomeric A β (μ M) were prepared by diluting the peptide from the size exclusion chromatography in NaPi buffer, adding Thioflavin T (ThT) at a final concentration of 20 μ M. The samples were homogenized 8 times using a pipette. The samples were aliquoted into a 96-well half-area, low binding, transparent-bottom plate (Corning 3881) in final volume of 90 μ L and covered with an adhesive aluminum lid to minimize evaporation. The plate was then incubated in quiescent conditions in a plate reader (FLUROstar Optima, BGM) at 37°C for 12 hours. Each concentration was measured as five replicates. The half-life times and scalar exponent were analyzed using the AmyloFit free online platform ^[201]. Data analysis was performed following the protocol ^[200]. The data was then normalized from 0 to 1 by taking the average of the baseline and plateau values of the reaction. After normalization, the concentration of the aggregates can be observed as a function of time. An advanced basin-hopping algorithm was

applied to fit the experimental data into a protein aggregation model. The number of basin hops was set to 10 for the fit.

A β ₁₋₄₂ aggregation kinetics in the presence of compounds 65-124

Monomeric A β ₁₋₄₂ peptide was prepared as described below on the day of the experiment. The protein concentration was calculated using the UV detector of the ÄKTA system using the integrated average of the eluted protein peak (concentration (M) = average peak area/0.2*1490).

The aggregation assay was conducted in the same conditions described for kinetic validation. The data were analyzed using GraphPad Prism 10 (GraphPad Software). After manual removal of outlier replicates (n = 4), a non-linear regression model (Sigmoidal, 4PL, X is concentration) was applied, and the half-times for each compound were plotted and compared.

***In silico* predictions of ADME properties**

In silico prediction of ADMET-related parameters was performed using the freely available SwissADME web tool ^[94,95], which provides physicochemical profiling, lipophilicity estimation, water solubility prediction, permeability assessment and medicinal chemistry friendliness analysis based on validated computational models.

Future Perspectives

The overarching aim of this Ph.D. project was to design, synthesize, and carry out preformulation studies on new series of compounds with potential polypharmacological activity, focusing on two major therapeutic areas: neurodegenerative diseases, particularly Alzheimer's disease (AD), and cancer. The molecules were conceived as hybrid structures, developed through a fragment-based synthetic strategy starting from natural or nature-inspired antioxidant scaffolds.

Overall, the conclusions drawn from both sections highlight that the design and synthesis strategy successfully led to the identification of structurally well-defined compounds with promising biological profiles. Moreover, even in cases where the results were partial (Set I) or not fully optimal (e.g., cytosine–cinnamic acid hybrids), the studies proved valuable in establishing clear structure–activity relationships and in elucidating the contribution of the individual scaffolds to the overall biological effect.

Antiproliferative compounds

The antiproliferative studies presented in this thesis were intentionally designed as an initial biological filter to identify promising compound series characterized, first, by a favorable combination of molecular scaffolds and, second, by substitution patterns suitable for exploring structure–activity relationships within the chemical space surrounding the selected cores. The use of general antiproliferative screening as a first-line endpoint proved effective in highlighting scaffold combinations and substitutions capable of impairing cancer cell growth. Although a complete biological evaluation of all structural subsets has not yet been achieved (see Set I), comparative testing of representative compounds—such as the inclusion of compound 8 from Set I in Set II assays for comparison purposes—provided preliminary yet meaningful indications of the impact of molecular hybridization and substituent patterns on biological performance. Future work will therefore include completion and expansion of biological datasets on the whole Set I to enable more robust comparisons across the fully explored chemical space and to consolidate conclusions on the effectiveness of the design strategy.

Having identified compounds with encouraging antiproliferative profiles, subsequent studies will focus on elucidating their mechanisms of action. The identification of defined molecular targets represents a critical step toward the selection of true lead compounds. Integrated biochemical assays, target-based screening, and proteomic or transcriptomic analyses might be employed to determine whether the active derivatives interact with redox-regulating enzymes, cell-cycle controllers, or stress-response pathways. Once target hypotheses are established, computational approaches such as molecular docking, molecular dynamics simulations, and pharmacophore modeling could support structure-based optimization. These tools might guide refinement of substituent patterns on the coumarin and aromatic moieties, rationalize observed structure–activity relationships, and enable the design of new analogues with improved target engagement and drug-like properties.

Further biological investigations will also address the breadth and selectivity of anticancer activity. Expanded testing across diverse tumor cell lines will clarify whether the compounds display preferential activity toward specific cancer types, while parallel evaluation on healthy cells – at present explored on Set II only - will provide an early assessment of cytotoxic liability. Together, these studies will allow a transition from phenotypic screening to mechanism-driven lead optimization, enabling rational improvement of potency and selectivity.

In parallel, experimental determination of key physicochemical parameters, including lipophilicity, aqueous solubility, and metabolic stability, will further refine lead selection and help anticipate pharmacokinetic behavior.

From a translational perspective, formulation development constitutes a crucial next step for this sets of compounds characterized by a low-solubility profile, especially in aqueous solvents. The preformulation studies conducted on selected hydrazone derivatives demonstrated the feasibility of micellar encapsulation and confirmed that nonionic surfactants can enhance aqueous solubility of these intrinsically hydrophobic scaffolds. These findings indicate that solubility limitations typical of hydrazone-based compounds can be addressed through micellar systems. At this stage, micelles represent an enabling tool rather than a final dosage form, providing a crucial bridge toward the development of pharmaceutical formulations in aqueous environments and ultimately toward

obtaining the most appropriate dosage form for the specific application of these compounds.

Indeed, aqueous-based formulations are preferred due to their physiological compatibility, reduced toxicity risk, and more straightforward regulatory acceptance compared with organic-solvent systems. Moreover, they support advanced delivery technologies which are central to modern anticancer drug development.

Subsequently, future formulation studies will include systematic evaluation of drug encapsulation efficiency, loading capacity, stability, and release kinetics under physiologically relevant conditions. Stability assessments in simulated gastric and intestinal fluids, as well as in serum-containing media, will be required to establish formulation robustness and to predict *in vivo* performance. Further optimization of carrier composition may enable fine-tuning of particle size, surface characteristics, and release profiles, ultimately paving the way toward nanoformulations capable of improving bioavailability, reducing off-target exposure, and promoting controlled drug delivery to tumor tissues although *in vivo* pharmacokinetic and biodistribution studies will then be essential to verify whether micellar encapsulation effectively translates into improved systemic exposure and enhanced therapeutic efficacy.

Anti-neurodegeneration compounds

The neurodegeneration-oriented component of this thesis led to the development of hybrid scaffolds displaying promising multitarget profiles, including cholinesterase inhibition, antioxidant activity, and modulation of amyloid-related processes. These findings support the validity of the molecular hybridization strategy for Alzheimer's disease-oriented drug discovery. However, the current results represent an early discovery stage, and additional investigations are required to reinforce mechanistic understanding and translational relevance.

Future research will primarily focus on consolidating multitarget efficacy and translating enzymatic and biochemical findings into cellular proof-of-concept studies. For the coumarin-alicyclic amine hybrids and the feruloyl-phenothiazine, biological characterization will be expanded beyond enzyme inhibition assays. In particular, neuroprotection studies in neuronal cell models

exposed to A β -induced toxicity and oxidative stress will be essential to determine whether the observed enzymatic modulation translates into meaningful protection at the cellular level. Moreover, preliminary safety in neuronal and non-neuronal cell lines will be needed.

Computational modeling and molecular docking studies could complement experimental data by clarifying binding modes within cholinesterase active sites and rationalizing the influence of the basic nitrogen atom, linker length, and scaffold geometry on activity and selectivity. This integrated approach will support structure–activity relationship refinement and guide the design of optimized analogues with improved potency and balanced multitarget profiles.

A particularly critical future direction concerns validation of central nervous system accessibility. In silico ADME predictions indicate that several active compounds may possess favorable blood–brain barrier penetration properties. Nevertheless, computational estimates alone are insufficient to establish translational viability. Experimental assessment using in vitro BBB models—such as PAMPA-BBB assays and cell-based transwell systems employing brain endothelial monolayers—will be required to verify permeability, identify potential efflux mechanisms, and quantify transport efficiency. These studies will determine whether the compounds can reach therapeutically relevant concentrations within the CNS or whether dedicated formulation strategies would be necessary to enable effective brain delivery.

References

- [1] H. Sung, J. Ferlay, R. L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, "Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries" *CA. Cancer J. Clin.* **2021**, *71*, 209–249.
- [2] D. Hanahan, R. A. Weinberg, "Hallmarks of Cancer: The Next Generation" *Cell* **2011**, *144*, 646–674.
- [3] C. Holohan, S. Van Schaeybroeck, D. B. Longley, P. G. Johnston, "Cancer drug resistance: an evolving paradigm" *Nat. Rev. Cancer* **2013**, *13*, 714–726.
- [4] V. T. DeVita, T. S. Lawrence, S. A. Rosenberg, *DeVita, Hellman, and Rosenberg's Cancer: Principles & Practice of Oncology*, Lippincott Williams & Wilkins, **2008**.
- [5] F. Lumachi, D. A. Santeufemia, S. M. Basso, "Current medical treatment of estrogen receptor-positive breast cancer" *World J. Biol. Chem.* **2015**, *6*, 231–239.
- [6] C. Sawyers, "Targeted cancer therapy" *Nature* **2004**, *432*, 294–297.
- [7] A. Ribas, J. D. Wolchok, "Cancer immunotherapy using checkpoint blockade" *Science* **2018**, *359*, 1350–1355.
- [8] N. Vasan, J. Baselga, D. M. Hyman, "A view on drug resistance in cancer" *Nature* **2019**, *575*, 299–309.
- [9] V. Thomas, D. Giles, G. P. M. Basavarajaswamy, A. K. Das, A. Patel, "Coumarin Derivatives as Anti-inflammatory and Anticancer Agents" *Anticancer Agents Med. Chem.* **2017**, *17*, 415–423.
- [10] J. Grover, S. M. Jachak, "Coumarins as privileged scaffold for anti-inflammatory drug development" *RSC Adv.* **2015**, *5*, 38892–38905.
- [11] C. Seidel, M. Schnekenburger, C. Zwergel, F. Gaascht, A. Mai, M. Dicato, G. Kirsch, S. Valente, M. Diederich, "Novel inhibitors of human histone deacetylases: Design, synthesis and bioactivity of 3-alkenoylcoumarines" *Bioorg. Med. Chem. Lett.* **2014**, *24*, 3797–3801.
- [12] E. Konkoľová, M. Hudáčová, S. Hamul'áková, R. Jendželovský, J. Vargová, J. Ševc, P. Fedoročko, M. Kožurková, "Tacrine-Coumarin Derivatives as Topoisomerase Inhibitors with Antitumor Effects on A549 Human Lung Carcinoma Cancer Cell Lines" *Molecules* **2021**, *26*, 1133.
- [13] V. Flores-Morales, A. P. Villasana-Ruiz, I. Garza-Veloz, S. González-Delgado, M. L. Martinez-Fierro, "Therapeutic Effects of Coumarins with Different Substitution Patterns" *Molecules* **2023**, *28*, 2413.
- [14] K. C. Fylaktakidou, D. J. Hadjipavlou-Litina, K. E. Litinas, D. N. Nicolaides, "Natural and Synthetic Coumarin Derivatives with Anti-Inflammatory / Antioxidant Activities" *Curr. Pharm. Des.* **2004**, *10*, 3813–3833.
- [15] M. I. Hussain, Q. A. Syed, M. N. K. Khatkhat, B. Hafez, M. J. Reigosa, A. El-Keblawy, "Natural product coumarins: biological and pharmacological perspectives" *Biologia (Bratisl.)* **2019**, *74*, 863–888.
- [16] G. Kirsch, A. B. Abdelwahab, P. Chaimbault, "Natural and Synthetic Coumarins with Effects on Inflammation" *Molecules* **2016**, *21*, 1322.
- [17] A. Khanna, A. Narang, V. Thakur, K. Singh, N. Kumar, R. Kaur, Megha, A. Raj, M. Devi, Jyoti, R. Rana, A. Sharma, H. Kaur, P. Singh, S. Kaur, P. M. S. Bedi, "Design, synthesis, antibacterial evaluation, and molecular modelling studies of 1,2,3-triazole-linked coumarin-vanillin hybrids as potential DNA gyrase and topoisomerase IV inhibitors" *Bioorganic Chem.* **2025**, *164*, 108815.

- [18] S. Emami, S. Dadashpour, "Current developments of coumarin-based anti-cancer agents in medicinal chemistry" *Eur. J. Med. Chem.* **2015**, *102*, 611–630.
- [19] D. Trachootham, J. Alexandre, P. Huang, "Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?" *Nat. Rev. Drug Discov.* **2009**, *8*, 579–591.
- [20] C. F. Thorn, C. Oshiro, S. Marsh, T. Hernandez-Boussard, H. McLeod, T. E. Klein, R. B. Altman, "Doxorubicin pathways: pharmacodynamics and adverse effects" *Pharmacogenet. Genomics* **2011**, *21*, 440–446.
- [21] N.-K. V. Cheung, M. A. Dyer, "Neuroblastoma: developmental biology, cancer genomics and immunotherapy" *Nat. Rev. Cancer* **2013**, *13*, 397–411.
- [22] M. A. Jordan, L. Wilson, "Microtubules as a target for anticancer drugs" *Nat. Rev. Cancer* **2004**, *4*, 253–265.
- [23] B. A. Weaver, "How Taxol/paclitaxel kills cancer cells" *Mol. Biol. Cell* **2014**, *25*, 2677–2681.
- [24] J. D. Hayes, A. T. Dinkova-Kostova, K. D. Tew, "Oxidative Stress in Cancer" *Cancer Cell* **2020**, *38*, 167–197.
- [25] J. Alexandre, Y. Hu, W. Lu, H. Pelicano, P. Huang, "Novel action of paclitaxel against cancer cells: bystander effect mediated by reactive oxygen species" *Cancer Res.* **2007**, *67*, 3512–3517.
- [26] Y. Wu, J. Xu, Y. Liu, Y. Zeng, G. Wu, "A Review on Anti-Tumor Mechanisms of Coumarins" *Front. Oncol.* **2020**, *10*.
- [27] M. Koley, J. Han, V. A. Soloshonok, S. Mojumder, R. Javahershenas, A. Makarem, "Latest developments in coumarin-based anticancer agents: mechanism of action and structure–activity relationship studies" *RSC Med. Chem.* **2024**, *15*, 10–54.
- [28] Md. S. Al Hasan, Y. Emon, M. Y. Alshahrani, Md. Mizan, M. B. Uddin, A. M. W. Hasan, M. A. Sayeed, E. Mia, N. T. Yana, R. Hossan, M. T. Islam, "Coumarin derivatives as anticancer agents targeting PI3K-AKT-mTOR pathway: a comprehensive literature review" *Med. Oncol.* **2025**, *42*, 301.
- [29] N. Kurtanović, N. Tomašević, S. Matić, M. M. Mitrović, D. A. Kostić, M. Sabatino, L. Antonini, R. Ragno, M. Mladenović, "Human estrogen receptor α antagonists, part 2: Synthesis driven by rational design, *in vitro* antiproliferative, and *in vivo* anticancer evaluation of innovative coumarin-related antiestrogens as breast cancer suppressants" *Eur. J. Med. Chem.* **2022**, *227*, 113869.
- [30] A. Nikitjuka, M. Ozola, L. Jackevica, R. Bobrovs, R. Žalubovskis, "Exploration of 3,4-unsubstituted coumarins as thioredoxin reductase 1 inhibitors for cancer therapy" *Org. Biomol. Chem.* **2023**, *21*, 9630–9639.
- [31] Y. Shang, Q. Wei, Z. Sun, "Studying the cytotoxicity of coumarin–chalcone hybrids by a prooxidant strategy in A549 cells" *Monatshefte Für Chem. - Chem. Mon.* **2018**, *149*, 2287–2292.
- [32] S. S. Arya, J. E. Rookes, D. M. Cahill, S. K. Lenka, "Vanillin: a review on the therapeutic prospects of a popular flavouring molecule" *Adv. Tradit. Med.* **2021**, *21*, 1–17.
- [33] D. P. Bezerra, A. K. N. Soares, D. P. de Sousa, "Overview of the Role of Vanillin on Redox Status and Cancer Development" *Oxid. Med. Cell. Longev.* **2016**, *2016*, 9734816.
- [34] H. Nakamura, K. Takada, "Reactive oxygen species in cancer: Current findings and future directions" *Cancer Sci.* **2021**, *112*, 3945–3952.
- [35] J. P. Soni, R. Sood, J. Kumar in *Role Autophagy React. Oxyg. Species Cancer Treat. Princ. Curr. Strateg.* (Eds.: N. Mishra, R.K. Kaundal), Springer Nature Switzerland, Cham, **2024**, pp. 27–45.

- [36] C. Glorieux, S. Liu, D. Trachootham, P. Huang, "Targeting ROS in cancer: rationale and strategies" *Nat. Rev. Drug Discov.* **2024**, 23, 583–606.
- [37] M. Nigam, B. Punia, D. B. Dimri, A. P. Mishra, A.-F. Radu, G. Bungau, "Reactive Oxygen Species: A Double-Edged Sword in the Modulation of Cancer Signaling Pathway Dynamics" *Cells* **2025**, 14, 1207.
- [38] I. W. D. Hackh, C. Grant, R. Grant, *Grant & Hackh's Chemical Dictionary*, McGraw-Hill, **1987**.
- [39] T. Nasr, S. Bondock, M. Youns, "Anticancer activity of new coumarin substituted hydrazide–hydrazone derivatives" *Eur. J. Med. Chem.* **2014**, 76, 539–548.
- [40] R. H. Wiley, R. L. Clevenger, "Aldehyde Hydrazone Derivatives in Cancer Chemotherapy" *J. Med. Pharm. Chem.* **1962**, 5, 1367–1371.
- [41] H. Abdel-Aziz, T. Elsaman, M. Attia, A. Alanazi, "The Reaction of Ethyl 2-oxo-2H-chromene-3-carboxylate with Hydrazine Hydrate" *Molecules* **2013**, 18, 2084–2095.
- [42] J. R. Crowder, E. E. Gloverm, M. F. Grundon, H. X. Kaempfen, "Bisbenzylisoquinolines. Part 1V. The Preparation of Diaryl Ethers from Diaryliodonium Salts." **1963**, 4568–4585.
- [43] M. J. Ashton, D. C. Cook, G. Fenton, J.-A. Karlsson, M. N. Palfreyman, D. Raeburn, A. J. Ratcliffe, J. E. Souness, S. Thurairatnam, N. Vicker, "Selective Type IV Phosphodiesterase Inhibitors as Antiasthmatic Agents. The Syntheses and Biological Activities of 3-(Cyclopentyloxy)-4-methoxybenzamides and Analogs" *J. Med. Chem.* **1994**, 37, 1696–1703.
- [44] M. De Freitas Silva, C. Juliet Cristancho Ortiz, L. Ferreira Coelho, L. Pruccoli, B. Pagliarani, L. Pisani, M. Catto, G. Poli, T. Tuccinardi, F. Cardoso Vilela, A. Giusti-Paiva, M. Amaral Alves, H. M. Ribeiro De Souza, A. Tarozzi, V. Silva Gontijo, C. Viegas Jr., "Synthesis and pharmacological evaluation of novel N-aryl-cinnamoyl-hydrazone hybrids designed as neuroprotective agents for the treatment of Parkinson's disease" *Bioorganic Chem.* **2024**, 150, 107587.
- [45] G. Palla, G. Predieri, P. Domiano, "Conformational behaviour and E/Z isomerization of N-acyl and N-aroylhydrazones" *Tetrahedron* **1986**, 42, 3649–3654.
- [46] S. M. Landge, E. Tkatchouk, D. Benítez, D. A. Lanfranchi, M. Elhabiri, W. A. Goddard, I. Aprahamian, "Isomerization Mechanism in Hydrazone-Based Rotary Switches: Lateral Shift, Rotation, or Tautomerization?" *J. Am. Chem. Soc.* **2011**, 133, 9812–9823.
- [47] S. B. Kedare, R. P. Singh, "Genesis and development of DPPH method of antioxidant assay" *J. Food Sci. Technol.* **2011**, 48, 412–422.
- [48], "Developmental Therapeutics Program (DTP) - NCI," can be found under <https://dctd.cancer.gov/programs/dtp>(accessed 10 December 2025), **2025**.
- [49] J. I. Johnsen, C. Dyberg, M. Wickström, "Neuroblastoma—A Neural Crest Derived Embryonal Malignancy" *Front. Mol. Neurosci.* **2019**, 12, 9.
- [50] J. Nong, C. Su, C. Li, C. Wang, W. Li, Y. Li, P. Chen, Y. Li, Z. Li, X. She, Z. Yuan, S. Liu, C. Chen, Q. Liao, Y. Luo, B. Shi, "Global, regional, and national epidemiology of childhood neuroblastoma (1990–2021): a statistical analysis of incidence, mortality, and DALYs" *eClinicalMedicine* **2025**, 79, 102964.
- [51] K. I. Lundberg, D. Treis, J. I. Johnsen, "Neuroblastoma Heterogeneity, Plasticity, and Emerging Therapies" *Curr. Oncol. Rep.* **2022**, 24, 1053–1062.
- [52] T. Monclair, G. M. Brodeur, P. F. Ambros, H. J. Brisse, G. Cecchetto, K. Holmes, M. Kaneko, W. B. London, K. K. Matthay, J. G. Nuchtern, D. Von Schweinitz, T. Simon, S. L. Cohn, A. D. J. Pearson, "The International Neuroblastoma Risk Group

- (INRG) Staging System: An INRG Task Force Report” *J. Clin. Oncol.* **2009**, *27*, 298–303.
- [53] P. F. Ambros, I. M. Ambros, G. M. Brodeur, M. Haber, J. Khan, A. Nakagawara, G. Schleiermacher, F. Speleman, R. Spitz, W. B. London, S. L. Cohn, A. D. J. Pearson, J. M. Maris, “International consensus for neuroblastoma molecular diagnostics: report from the International Neuroblastoma Risk Group (INRG) Biology Committee” *Br. J. Cancer* **2009**, *100*, 1471–1482.
 - [54], “Neuroblastoma Treatment (PDQ®) - NCI,” can be found under <https://www.cancer.gov/types/neuroblastoma/hp/neuroblastoma-treatment-pdq>(accessed 10 September 2025), **2025**.
 - [55] N. O. Basta, G. C. Halliday, G. Makin, J. Birch, R. Feltbower, N. Bown, M. Elliott, L. Moreno, G. Barone, A. D. Pearson, P. W. James, D. A. Tweddle, R. J. McNally, “Factors associated with recurrence and survival length following relapse in patients with neuroblastoma” *Br. J. Cancer* **2016**, *115*, 1048–1057.
 - [56] R. C. Seeger, C. P. Reynolds, “Treatment of High-Risk Solid Tumors of Childhood with Intensive Therapy and Autologous Bone Marrow Transplantation” *Pediatr. Clin. North Am.* **1991**, *38*, 393–424.
 - [57] F. Salemi, W. Alam, M. S. Hassani, S. Z. Hashemi, A. A. Jafari, S. M. S. Mirmoeeni, M. Arbab, S. M. R. Mortazavizadeh, H. Khan, “Neuroblastoma: Essential genetic pathways and current therapeutic options” *Eur. J. Pharmacol.* **2022**, *926*, 175030.
 - [58] R. Colla, A. Izzotti, C. De Ciucis, D. Fenoglio, S. Ravera, A. Speciale, R. Ricciarelli, A. L. Furfaro, A. Pulliero, M. Passalacqua, N. Traverso, M. A. Pronzato, C. Domenicotti, B. Marengo, “Glutathione-mediated antioxidant response and aerobic metabolism: two crucial factors involved in determining the multi-drug resistance of high-risk neuroblastoma” *Oncotarget* **2016**, *7*, 70715–70737.
 - [59] B. Marengo, P. Monti, M. Miele, P. Menichini, L. Ottaggio, G. Foggetti, A. Pulliero, A. Izzotti, A. Speciale, O. Garbarino, N. Traverso, G. Fronza, C. Domenicotti, “Etoposide-resistance in a neuroblastoma model cell line is associated with 13q14.3 mono-allelic deletion and miRNA-15a/16-1 down-regulation” *Sci. Rep.* **2018**, *8*, 13762.
 - [60] A. H. Boulares, A. G. Yakovlev, V. Ivanova, B. A. Stoica, G. Wang, S. Iyer, M. Smulson, “Role of Poly(ADP-ribose) Polymerase (PARP) Cleavage in Apoptosis” *J. Biol. Chem.* **1999**, *274*, 22932–22940.
 - [61] M. M. Gaschler, B. R. Stockwell, “Lipid peroxidation in cell death” *Biochem. Biophys. Res. Commun.* **2017**, *482*, 419–425.
 - [62] S. Zanotti, B. Decaestecker, S. Vanhauwaert, B. De Wilde, W. H. De Vos, F. Speleman, “Cellular senescence in neuroblastoma” *Br. J. Cancer* **2022**, *126*, 1529–1538.
 - [63] J. Campisi, “Cellular senescence as a tumor-suppressor mechanism” **2001**.
 - [64] B. Liu, Z. Peng, H. Zhang, N. Zhang, Z. Liu, Z. Xia, S. Huang, P. Luo, Q. Cheng, “Regulation of cellular senescence in tumor progression and therapeutic targeting: mechanisms and pathways” *Mol. Cancer* **2025**, *24*, 106.
 - [65] S. Yamauchi, A. Takahashi, “Cellular senescence: mechanisms and relevance to cancer and aging” *J. Biochem.* **2025**, *177*, 163–169.
 - [66] J. Yang, M. Liu, D. Hong, M. Zeng, X. Zhang, “The Paradoxical Role of Cellular Senescence in Cancer” *Front. Cell Dev. Biol.* **2021**, *9*, 722205.
 - [67] C. A. Lipinski, “Drug-like properties and the causes of poor solubility and poor permeability” *J. Pharmacol. Toxicol. Methods* **2000**, *44*, 235–249.

- [68] K. T. Savjani, A. K. Gajjar, J. K. Savjani, "Drug Solubility: Importance and Enhancement Techniques" *Int. Sch. Res. Not.* **2012**, 2012, 195727.
- [69] P. Mladěnka, L. Applová, J. Patočka, V. M. Costa, F. Remiao, J. Pourová, A. Mladěnka, J. Karlíčková, L. Jahodář, M. Vopršalová, K. J. Varner, M. Štěrba, TOX-OER and CARDIOTOX Hradec Králové Researchers and Collaborators, "Comprehensive review of cardiovascular toxicity of drugs and related agents" *Med. Res. Rev.* **2018**, 38, 1332–1403.
- [70] R. Pinal, "Enhancing the Bioavailability of Poorly Soluble Drugs" *Pharmaceutics* **2024**, 16, 758.
- [71] A. Srivastava, H. Uchiyama, Y. Wada, Y. Hatanaka, Y. Shirakawa, K. Kadota, Y. Tozuka, "Mixed micelles of the antihistaminic cationic drug diphenhydramine hydrochloride with anionic and non-ionic surfactants show improved solubility, drug release and cytotoxicity of ethenzamide" *J. Mol. Liq.* **2019**, 277, 349–359.
- [72] S. Tambe, D. Jain, S. K. Meruva, G. Rongala, A. Juluri, G. Nihalani, H. K. Mamidi, P. K. Nukala, P. K. Bolla, "Recent Advances in Amorphous Solid Dispersions: Preformulation, Formulation Strategies, Technological Advancements and Characterization" *Pharmaceutics* **2022**, 14, 2203.
- [73] G. Nerli, L. M. D. Gonçalves, M. Cirri, A. J. Almeida, F. Maestrelli, N. Mennini, P. A. Mura, "Design, Evaluation and Comparison of Nanostructured Lipid Carriers and Chitosan Nanoparticles as Carriers of Poorly Soluble Drugs to Develop Oral Liquid Formulations Suitable for Pediatric Use" *Pharmaceutics* **2023**, 15, 1305.
- [74] A. Omari, R. Cao, Z. Zhu, X. Xu, "A comprehensive review of recent advances on surfactant architectures and their applications for unconventional reservoirs" *J. Pet. Sci. Eng.* **2021**, 206, 109025.
- [75] K. Nagaraj, S. Kamalesu, "State-of-the-art surfactants as biomedical game changers: unlocking their potential in drug delivery, diagnostics, and tissue engineering" *Int. J. Pharm.* **2025**, 676, 125590.
- [76] D. R. Perinelli, M. Cespi, N. Lorusso, G. F. Palmieri, G. Bonacucina, P. Blasi, "Surfactant Self-Assembling and Critical Micelle Concentration: One Approach Fits All?" *Langmuir* **2020**, 36, 5745–5753.
- [77] In *Des. Dev. New Nanocarriers*, William Andrew Publishing, **2018**, pp. 331–364.
- [78] X. Lu, M. Li, F. A. Arce, J. Ling, N. Setiawan, Y. Wang, X. Shi, H. R. Campbell, M. J. Nethercott, W. Xu, E. J. Munson, P. J. Marsac, Y. Su, "Mechanistic Investigation of Drug Supersaturation in the Presence of Polysorbates as Solubilizing Additives by Solution Nuclear Magnetic Resonance Spectroscopy" *Mol. Pharm.* **2021**, 18, 4310–4321.
- [79] B. Maity, A. Chatterjee, S. A. Ahmed, D. Seth, "Interaction of the Nonsteroidal Anti-inflammatory Drug Indomethacin with Micelles and Its Release" *J. Phys. Chem. B* **2015**, 119, 3776–3785.
- [80] P. Talele, S. Choudhary, N. Kishore, "Understanding thermodynamics of drug partitioning in micelles and delivery to proteins: Studies with naproxen, diclofenac sodium, tetradecyltrimethylammonium bromide, and bovine serum albumin" *J. Chem. Thermodyn.* **2016**, 92, 182–190.
- [81] BenchChem Technical Support Team **2025**.
- [82] G. L. Perlovich, V. P. Kazachenko, N. N. Strakhova, K.-J. Schaper, O. A. Raevsky, "Solubility and Transfer Processes of Some Hydrazones in Biologically Relevant Solvents" *J. Chem. Eng. Data* **2013**, 58, 2659–2667.
- [83] D. Ashagrie Tafere, M. Gebrezgiabher, F. Elemo, T. Sani, T. Belege Atisme, T. G. Ashebr, I. Nasser Ahmed, "Hydrazones, hydrazones-based coinage metal complexes, and their biological applications" *RSC Adv.* **2025**, 15, 6191–6207.

- [84] N. Azum, M. A. Rub, A. M. Asiri, W. A. Bawazeer, "Micellar and interfacial properties of amphiphilic drug–non-ionic surfactants mixed systems: Surface tension, fluorescence and UV–vis studies" *Colloids Surf. Physicochem. Eng. Asp.* **2017**, 522, 183–192.
- [85] A. A. Rana, A. Yusaf, S. Shahid, M. Usman, M. Ahmad, S. Aslam, S. A. Al-Hussain, M. E. A. Zaki, "Unveiling the Role of Nonionic Surfactants in Enhancing Cefotaxime Drug Solubility: A UV-Visible Spectroscopic Investigation in Single and Mixed Micellar Formulations" *Pharm. Basel Switz.* **2023**, 16, 1663.
- [86] N. Majumder, N. G Das, S. K. Das, "Polymeric micelles for anticancer drug delivery" *Ther. Deliv.* **2020**, 11, 613–635.
- [87] M. Lieber, B. Smith, A. Szakal, W. Nelson-Rees, G. Todaro, "A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells" *Int. J. Cancer* **1976**, 17, 62–70.
- [88] Ş. Comşa, A. M. Cîmpean, M. Raica, "The Story of MCF-7 Breast Cancer Cell Line: 40 years of Experience in Research" *Anticancer Res.* **2015**, 35, 3147–3154.
- [89] C. K. Osborne, R. Schiff, "Mechanisms of endocrine resistance in breast cancer" *Annu. Rev. Med.* **2011**, 62, 233–247.
- [90] W. D. Travis, E. Brambilla, A. G. Nicholson, Y. Yatabe, J. H. M. Austin, M. B. Beasley, L. R. Chirieac, S. Dacic, E. Duhig, D. B. Flieder, K. Geisinger, F. R. Hirsch, Y. Ishikawa, K. M. Kerr, M. Noguchi, G. Pelosi, C. A. Powell, M. S. Tsao, I. Wistuba, WHO Panel, "The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances Since the 2004 Classification" *J. Thorac. Oncol. Off. Publ. Int. Assoc. Study Lung Cancer* **2015**, 10, 1243–1260.
- [91] R. S. Herbst, D. Morgensztern, C. Boshoff, "The biology and management of non-small cell lung cancer" *Nature* **2018**, 553, 446–454.
- [92] J. Rotow, T. G. Bivona, "Understanding and targeting resistance mechanisms in NSCLC" *Nat. Rev. Cancer* **2017**, 17, 637–658.
- [93] S. N. Mokale, A. Begum, N. S. Sakle, V. R. Shelke, S. A. Bhavale, "Design, synthesis and anticancer screening of 3-(3-(substituted phenyl) acryloyl)-2H-chromen-2-ones as selective anti-breast cancer agent" *Biomed. Pharmacother.* **2017**, 89, 966–972.
- [94] A. Daina, O. Michielin, V. Zoete, "SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules" *Sci. Rep.* **2017**, 7, 42717.
- [95], "SwissADME," can be found under <https://www.swissadme.ch/>(accessed 5 December 2025), **2025**.
- [96] R. Verma, H. I. M. Boshoff, K. Arora, I. Bairy, M. Tiwari, V. G. Bhat, G. G. Shenoy, "Synthesis, antitubercular evaluation, molecular docking and molecular dynamics studies of 4,6-disubstituted-2-oxo-dihydropyridine-3-carbonitriles" *J. Mol. Struct.* **2019**, 1197, 117–133.
- [97] M. Tomita, K. Fujitani, A. Yoshiaki, "Cupric Oxide as an Efficient Catalyst in Ullmann Condensation Reaction" *Chem. Pharm. Bull.* **1965**, 13, 1341–1345.
- [98] J. Zhang, L. Zhao, C. Zhu, Z. Wu, G. Zhang, X. Gan, D. Liu, J. Pan, D. Hu, B. Song, "Facile Synthesis of Novel Vanillin Derivatives Incorporating a Bis(2-hydroxyethyl)dithioacetal Moiety as Antiviral Agents" *J. Agric. Food Chem.* **2017**, 65, 4582–4588.
- [99] S. Cardinal, P.-A. Paquet-Côté, J. Azelmat, C. Bouchard, D. Grenier, N. Voyer, "Synthesis and anti-inflammatory activity of isoquebecol" *Bioorg. Med. Chem.* **2017**, 25, 2043–2056.

- [100] M. K. Pandya, M. R. Chhasatia, N. D. Vala, T. H. Parekh, "Synthesis and characterization of 3-(4-aryl-5h-6,7,- dihydrocyclopenta[b]pyridin-2-yl), 3-(4-aryl-5,6,7,8- tetrahydroquinolin-2-yl) and 3-(5-hydroxy-biphenyl-3-yl) coumarins using piperidine as a catalyst" **2020**, *10*, 333–340.
- [101] W. Brand-Williams, M. E. Cuvelier, C. Berset, "Use of a free radical method to evaluate antioxidant activity" *LWT - Food Sci. Technol.* **1995**, *28*, 25–30.
- [102] E. Russo, A. Spallarossa, A. Comite, M. Pagliero, P. Guida, V. Belotti, D. Caviglia, A. M. Schito, "Valorization and Potential Antimicrobial Use of Olive Mill Wastewater (OMW) from Italian Olive Oil Production" *Antioxidants* **2022**, *11*, 903.
- [103] T. Aree, S. Jongrungruangchok, "Structure–antioxidant activity relationship of β -cyclodextrin inclusion complexes with olive tyrosol, hydroxytyrosol and oleuropein: Deep insights from X-ray analysis, DFT calculation and DPPH assay" *Carbohydr. Polym.* **2018**, *199*, 661–669.
- [104] B. Marengo, C. G. De Ciucis, R. Ricciarelli, A. L. Furfaro, R. Colla, E. Canepa, N. Traverso, U. M. Marinari, M. A. Pronzato, C. Domenicotti, "p38MAPK inhibition: a new combined approach to reduce neuroblastoma resistance under etoposide treatment" *Cell Death Dis.* **2013**, *4*, e589–e589.
- [105] S. Alfei, B. Marengo, G. Valenti, C. Domenicotti, "Synthesis of Polystyrene-Based Cationic Nanomaterials with Pro-Oxidant Cytotoxic Activity on Etoposide-Resistant Neuroblastoma Cells" *Nanomaterials* **2021**, *11*, 977.
- [106] V. Vichai, K. Kirtikara, "Sulforhodamine B colorimetric assay for cytotoxicity screening" *Nat. Protoc.* **2006**, *1*, 1112–1116.
- [107] World Health Organization, *Global action plan on the public health response to dementia 2017–2025*, World Health Organization, Geneva, **2017**.
- [108] A. Comas-Herrera, A. D. International, E. Aguzzoli, N. Farina, S. Read, S. Evans-Lacko, "World Alzheimer Report 2024: Global changes in attitudes to dementia" **2024**.
- [109] M. Esiri, "The basis for behavioural disturbances in dementia." *J. Neurol. Neurosurg. Psychiatry* **1996**, *61*, 303–304.
- [110] J. G. Greene, R. Smith, M. Gardiner, G. C. Timbury, "Measuring behavioural disturbance of elderly demented patients in the community and its effects on relatives: a factor analytic study" *Age Ageing* **1982**, *11*, 121–126.
- [111] P. Sweeney, H. Park, M. Baumann, J. Dunlop, J. Frydman, R. Kopito, A. McCampbell, G. Leblanc, A. Venkateswaran, A. Nurmi, R. Hodgson, "Protein misfolding in neurodegenerative diseases: implications and strategies" *Transl. Neurodegener.* **2017**, *6*.
- [112] D. M. Bowen, C. B. Smith, P. White, A. N. Davison, "Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies" *Brain* **1976**, *99*, 459–496.
- [113] D. M. Bowen, A. N. Davison, "Biochemical changes in the cholinergic system of the ageing brain and in senile dementia" *Psychol. Med.* **1980**, *10*, 315–319.
- [114] E. K. Perry, P. H. Gibson, G. Blessed, R. H. Perry, B. E. Tomlinson, "Neurotransmitter enzyme abnormalities in senile dementia" *J. Neurol. Sci.* **1977**, *34*, 247–265.
- [115] M. G. Savelieff, S. Lee, Y. Liu, M. H. Lim, "Untangling Amyloid- β , Tau, and Metals in Alzheimer's Disease" *ACS Chem. Biol.* **2013**, *8*, 856–865.
- [116] R. Singh, A. Panghal, K. Jadhav, A. Thakur, R. K. Verma, C. Singh, M. Goyal, J. Kumar, A. G. Namdeo, "Recent Advances in Targeting Transition Metals (Copper, Iron, and Zinc) in Alzheimer's Disease" *Mol. Neurobiol.* **2024**, *61*, 10916–10940.

- [117] M. Rosini, E. Simoni, A. Milelli, A. Minarini, C. Melchiorre, “Oxidative Stress in Alzheimer’s Disease: Are We Connecting the Dots?: Miniperspective” *J. Med. Chem.* **2014**, *57*, 2821–2831.
- [118] M. Alami, T. Fulop, K. Boumezough, A. Khalil, E. Zerif, H. Berrougui in *Biomark. Oxidative Stress Clin. Asp. Oxidative Stress* (Eds.: S. Andreescu, R. Henkel, A. Khelfi), Springer Nature Switzerland, Cham, **2024**, pp. 71–102.
- [119] Y. A. Hajam, R. Rani, S. Y. Ganie, T. A. Sheikh, D. Javaid, S. S. Qadri, S. Pramodh, A. Alsulimani, M. F. Alkhanani, S. Harakeh, A. Hussain, S. Haque, M. S. Reshi, “Oxidative Stress in Human Pathology and Aging: Molecular Mechanisms and Perspectives” *Cells* **2022**, *11*, 552.
- [120] P. Davies, A. J. F. Maloney, “Selective loss of central cholinergic neurons in alzheimer’s disease” *The Lancet* **1976**, *308*, 1403.
- [121] B. Li, J. A. Stribley, A. Ticu, W. Xie, L. M. Schopfer, P. Hammond, S. Brimijoin, S. H. Hinrichs, O. Lockridge, “Abundant Tissue Butyrylcholinesterase and Its Possible Function in the Acetylcholinesterase Knockout Mouse” *J. Neurochem.* **2000**, *75*, 1320–1331.
- [122] A. Nordberg, C. Ballard, R. Bullock, T. Darreh-Shori, M. Somogyi, “A review of butyrylcholinesterase as a therapeutic target in the treatment of Alzheimer’s disease” *Prim. Care Companion CNS Disord.* **2013**, *15*.
- [123] E. K. Perry, R. H. Perry, G. Blessed, B. E. Tomlinson, “Changes in Brain Cholinesterases in Senile Dementia of Alzheimer Type” *Neuropathol. Appl. Neurobiol.* **1978**, *4*, 273–277.
- [124] J. Hartmann, C. Kiewert, E. G. Duysen, O. Lockridge, N. H. Greig, J. Klein, “Excessive hippocampal acetylcholine levels in acetylcholinesterase-deficient mice are moderated by butyrylcholinesterase activity” *J. Neurochem.* **2007**, *100*, 1421–1429.
- [125] M. Mesulam, A. Guillozet, P. Shaw, B. Quinn, “Widely Spread Butyrylcholinesterase Can Hydrolyze Acetylcholine in the Normal and Alzheimer Brain” *Neurobiol. Dis.* **2002**, *9*, 88–93.
- [126] A. Ciro, J. Park, G. Burkhard, N. Yan, C. Geula, “Biochemical differentiation of cholinesterases from normal and Alzheimer’s disease cortex” *Curr. Alzheimer Res.* **2012**, *9*, 138–143.
- [127] A. L. Guillozet, M.-M. Mesulam, J. F. Smiley, D. C. Mash, “Butyrylcholinesterase in the life cycle of amyloid plaques” *Ann. Neurol.* **1997**, *42*, 909–918.
- [128] S. Darvesh, D. A. Hopkins, C. Geula, “Neurobiology of butyrylcholinesterase” *Nat. Rev. Neurosci.* **2003**, *4*, 131–138.
- [129] V. K. Ramanan, S. L. Risacher, K. Nho, S. Kim, S. Swaminathan, L. Shen, T. M. Foroud, H. Hakonarson, M. J. Huentelman, P. S. Aisen, R. C. Petersen, R. C. Green, C. R. Jack, R. A. Koeppe, W. J. Jagust, M. W. Weiner, A. J. Saykin, “APOE and BCHE as modulators of cerebral amyloid deposition: a florbetapir PET genome-wide association study” *Mol. Psychiatry* **2014**, *19*, 351–357.
- [130] T. P. J. Knowles, M. Vendruscolo, C. M. Dobson, “The amyloid state and its association with protein misfolding diseases” *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 384–396.
- [131] R. N. Rambaran, L. C. Serpell, “Abnormal protein assembly” *Prion* **2008**, *2*, 112–117.
- [132] G. G. Glenner, D. Ein, E. D. Eanes, H. A. Bladen, W. Terry, D. L. Page, “Creation of ‘Amyloid’ Fibrils from Bence Jones Proteins in vitro” *Science* **1971**, *174*.

- [133] G. G. Glenner, C. W. Wong, "Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein" *Biochem. Biophys. Res. Commun.* **1984**, *120*, 885–890.
- [134] J. A. Hardy, G. A. Higgins, "Alzheimer's Disease: The Amyloid Cascade Hypothesis" *Science* **1992**, *256*, 184–185.
- [135] E. Marcello, R. Epis, C. Saraceno, M. Di Luca in *Synaptic Plast. Dyn. Dev. Dis.* (Eds.: M.R. Kreutz, C. Sala), Springer, Vienna, **2012**, pp. 573–601.
- [136] L. Zuroff, D. Daley, K. L. Black, M. Koronyo-Hamaoui, "Clearance of cerebral A β in Alzheimer's disease: reassessing the role of microglia and monocytes" *Cell. Mol. Life Sci.* **2017**, *74*, 2167–2201.
- [137] A. Azargoonjahromi, "The duality of amyloid- β : its role in normal and Alzheimer's disease states" *Mol. Brain* **2024**, *17*, 44.
- [138] C. Duran-Aniotz, R. Morales, I. Moreno-Gonzalez, P. P. Hu, C. Soto, "Brains from non-Alzheimer's individuals containing amyloid deposits accelerate A β deposition in vivo" *Acta Neuropathol. Commun.* **2013**, *1*, 76.
- [139] W. Tsering, S. Prokop, "Neuritic Plaques — Gateways to Understanding Alzheimer's Disease" *Mol. Neurobiol.* **2024**, *61*, 2808–2821.
- [140] J. Mao, C. Wang, X. Li, R. Du, X. Zhang, R. Shen, A. Yang, X. Kou, "Study on the structure–activity relationships of natural γ -pyranone products and their derivatives with anti-AD activities focusing on metal chelation" *Mol. Divers.* **2025**.
- [141] S. A. Eagger, R. Levy, B. J. Sahakian, "Tacrine in Alzheimer's disease" *The Lancet* **1991**, *337*, 989–992.
- [142] P. B. Watkins, H. J. Zimmerman, M. J. Knapp, S. I. Gracon, K. W. Lewis, "Hepatotoxic Effects of Tacrine Administration in Patients With Alzheimer's Disease" *JAMA* **1994**, *271*, 992–998.
- [143] Y. Kawakami, A. Inoue, T. Kawai, M. Wakita, H. Sugimoto, A. J. Hopfinger, "The rationale for E2020 as a potent acetylcholinesterase inhibitor" *Bioorg. Med. Chem.* **1996**, *4*, 1429–1446.
- [144] S. L. Rogers, L. T. Friedhoff, "The Efficacy and Safety of Donepezil in Patients with Alzheimer's Disease: Results of a US Multicentre, Randomized, Double-Blind, Placebo-Controlled Trial" *Dementia* **1996**, *7*, 293–303.
- [145] S. L. Rogers, M. R. Farlow, R. S. Doody, R. Mohs, L. T. Friedhoff, Donepezil Study Group*, "A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease" *Neurology* **1998**, *50*, 136–145.
- [146] A. Enz, R. Amstutz, H. Boddeke, G. Gmelin, J. Malanowski, "Brain selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease" *Prog. Brain Res.* **1993**, *98*, 431–438.
- [147] M. Rösler, R. Anand, A. Cicin-Sain, S. Gauthier, Y. Agid, P. Dal-Bianco, H. B. Stähelin, R. Hartman, M. Gharabawi, "Efficacy and safety of rivastigmine in patients with Alzheimer's disease: international randomised controlled trial" *BMJ* **1999**, *318*, 633–638.
- [148] T. Thomsen, H. Kewitz, "Selective inhibition of human acetylcholinesterase by galanthamine in vitro and in vivo" *Life Sci.* **1990**, *46*, 1553–1558.
- [149] M. A. Raskind, E. R. Peskind, T. Wessel, W. Yuan, the Galantamine USA- Study Group, "Galantamine in AD" *Neurology* **2000**, *54*, 2261–2268.
- [150] G. K. Wilcock, S. Lilienfeld, E. Gaens, "Efficacy and safety of galantamine in patients with mild to moderate Alzheimer's disease: multicentre randomised controlled trial. Galantamine International-1 Study Group" *BMJ* **2000**, *321*, 1445–1449.

- [151] W. W. Fleischhacker, A. Buchgeher, H. Schubert, "Memantine in the treatment of senile dementia of the Alzheimer type" *Prog. Neuropsychopharmacol. Biol. Psychiatry* **1986**, *10*, 87–93.
- [152] M. S. el Nasr, B. Peruche, C. Roßberg, H.-D. Mennel, J. Krieglstein, "Neuroprotective effect of memantine demonstrated in vivo and in vitro" *Eur. J. Pharmacol.* **1990**, *185*, 19–24.
- [153] W. J. Deardorff, G. T. Grossberg, "A fixed-dose combination of memantine extended-release and donepezil in the treatment of moderate-to-severe Alzheimer's disease" *Drug Des. Devel. Ther.* **2016**, *10*, 3267–3279.
- [154] E. Yaghmaei, H. Lu, L. Ehwerhemuepha, J. Zheng, S. Danioko, A. Rezaie, S. A. Sajjadi, C. Rakovski, "Combined use of Donepezil and Memantine increases the probability of five-year survival of Alzheimer's disease patients" *Commun. Med.* **2024**, *4*, 99.
- [155] S. M. Hoy, "Lecanemab: First Approval" *Drugs* **2023**, *83*, 359–365.
- [156] C. Kang, "Donanemab: First Approval" *Drugs* **2024**, *84*, 1313–1318.
- [157] E. L. Ross, M. S. Weinberg, S. E. Arnold, "Cost-effectiveness of Aducanumab and Donanemab for Early Alzheimer Disease in the US" *JAMA Neurol.* **2022**, *79*, 478.
- [158] J. L. Cummings, A. H. Burstein, H. Fillit, "Alzheimer Combination Therapies: Overview and Scenarios" *J. Prev. Alzheimers Dis.* **2025**, *12*, 100328.
- [159] A. J. Claxton, J. Cramer, C. Pierce, "A systematic review of the associations between dose regimens and medication compliance" *Clin. Ther.* **2001**, *23*, 1296–1310.
- [160] A. Richter, S. E. Anton, P. Koch, S. L. Dennett, "The impact of reducing dose frequency on health outcomes" *Clin. Ther.* **2003**, *25*, 2307–2335.
- [161] A. Kabir, A. Muth, "Polypharmacology: The science of multi-targeting molecules" *Pharmacol. Res.* **2022**, *176*, 106055.
- [162] D. E. Edmondson, C. Binda in *Membr. Protein Complexes Struct. Funct.* (Eds.: J.R. Harris, E.J. Boekema), Springer, Singapore, **2018**, pp. 117–139.
- [163] F. Hubálek, J. Pohl, D. E. Edmondson, "Structural Comparison of Human Monoamine Oxidases A and B" *J. Biol. Chem.* **2003**, *278*, 28612–28618.
- [164] L. Emilsson, P. Saetre, J. Balciuniene, A. Castensson, N. Cairns, E. E. Jazin, "Increased monoamine oxidase messenger RNA expression levels in frontal cortex of Alzheimer's disease patients" *Neurosci. Lett.* **2002**, *326*, 56–60.
- [165] Y. Aluf, J. Vaya, S. Khatib, Y. Loboda, J. P. M. Finberg, "Selective inhibition of monoamine oxidase A or B reduces striatal oxidative stress in rats with partial depletion of the nigro-striatal dopaminergic pathway" *Neuropharmacology* **2013**, *65*, 48–57.
- [166] V. Poggetti, S. Salerno, E. Baglini, E. Barresi, F. Da Settimo, S. Taliani, "Carbonic Anhydrase Activators for Neurodegeneration: An Overview" *Molecules* **2022**, *27*, 2544.
- [167] B. G. Jang, S.-M. Yun, K. Ahn, J. H. Song, S. A. Jo, Y.-Y. Kim, D. K. Kim, M. H. Park, C. Han, Y. H. Koh, "Plasma carbonic anhydrase II protein is elevated in Alzheimer's disease" *J. Alzheimers Dis. JAD* **2010**, *21*, 939–945.
- [168] M. E. Solesio, P. M. Peixoto, L. Debure, S. M. Madamba, M. J. de Leon, T. Wisniewski, E. V. Pavlov, S. Fossati, "Carbonic anhydrase inhibition selectively prevents amyloid β neurovascular mitochondrial toxicity" *Aging Cell* **2018**, *17*, e12787.
- [169] P. Blandina, G. Provensi, M. B. Passani, C. Capasso, C. T. Supuran, "Carbonic anhydrase modulation of emotional memory. Implications for the treatment of cognitive disorders" *J. Enzyme Inhib. Med. Chem.* **2020**, *35*, 1206–1214.

- [170] In *Genes Environ. Alzheimers Dis.*, Academic Press, **2016**, pp. 239–266.
- [171] S. L. Cole, R. Vassar, “The Role of Amyloid Precursor Protein Processing by BACE1, the β -Secretase, in Alzheimer Disease Pathophysiology” *J. Biol. Chem.* **2008**, *283*, 29621–29625.
- [172] R. Yan, R. Vassar, “Targeting the β secretase BACE1 for Alzheimer’s disease therapy” *Lancet Neurol.* **2014**, *13*, 319–329.
- [173] M. G. Spillantini, M. Goedert, “Tau pathology and neurodegeneration” *Lancet Neurol.* **2013**, *12*, 609–622.
- [174] F. Plattner, M. Angelo, K. P. Giese, “The Roles of Cyclin-dependent Kinase 5 and Glycogen Synthase Kinase 3 in Tau Hyperphosphorylation” *J. Biol. Chem.* **2006**, *281*, 25457–25465.
- [175] R. Von Bernhardi, J. Eugenin, “Alzheimer’s Disease: Redox Dysregulation As a Common Denominator for Diverse Pathogenic Mechanisms” *Antioxid. Redox Signal.* **2012**, *16*, 974–1031.
- [176] M. J. Metcalfe, M. E. Figueiredo-Pereira, “Relationship Between Tau Pathology and Neuroinflammation in Alzheimer’s Disease” *Mt. Sinai J. Med. J. Transl. Pers. Med.* **2010**, *77*, 50–58.
- [177] M. T. Heneka, M. J. Carson, J. E. Khoury, G. E. Landreth, F. Brosseon, D. L. Feinstein, A. H. Jacobs, T. Wyss-Coray, J. Vitorica, R. M. Ransohoff, K. Herrup, S. A. Frautschy, B. Finsen, G. C. Brown, A. Verkhratsky, K. Yamanaka, J. Koistinaho, E. Latz, A. Halle, G. C. Petzold, T. Town, D. Morgan, M. L. Shinohara, V. H. Perry, C. Holmes, N. G. Bazan, D. J. Brooks, S. Hunot, B. Joseph, N. Deigendesch, O. Garaschuk, E. Boddeke, C. A. Dinarello, J. C. Breitner, G. M. Cole, D. T. Golenbock, M. P. Kummer, “Neuroinflammation in Alzheimer’s disease” *Lancet Neurol.* **2015**, *14*, 388–405.
- [178] C. Carvalho, P. I. Moreira, “Metabolic defects shared by Alzheimer’s disease and diabetes: A focus on mitochondria” *Curr. Opin. Neurobiol.* **2023**, *79*, 102694.
- [179] S. M. de la Monte, J. R. Wands, “Alzheimer’s disease is type 3 diabetes-evidence reviewed” *J. Diabetes Sci. Technol.* **2008**, *2*, 1101–1113.
- [180] D. Del Prete, F. Checler, M. Chami, “Ryanodine receptors: physiological function and deregulation in Alzheimer disease” *Mol. Neurodegener.* **2014**, *9*, 21.
- [181] L. Pisani, M. Catto, A. De Palma, R. Farina, S. Cellamare, C. D. Altomare, “Discovery of Potent Dual Binding Site Acetylcholinesterase Inhibitors via Homo- and Heterodimerization of Coumarin-Based Moieties” *ChemMedChem* **2017**, *12*, 1349–1358.
- [182] C. Brühlmann, F. Ooms, P.-A. Carrupt, B. Testa, M. Catto, F. Leonetti, C. Altomare, A. Carotti, “Coumarins Derivatives as Dual Inhibitors of Acetylcholinesterase and Monoamine Oxidase” *J. Med. Chem.* **2001**, *44*, 3195–3198.
- [183] A. Singh, S. Sharma, S. Arora, S. Attri, P. Kaur, H. Kaur Gulati, K. Bhagat, N. Kumar, H. Singh, J. Vir Singh, P. Mohinder Singh Bedi, “New coumarin-benzotriazole based hybrid molecules as inhibitors of acetylcholinesterase and amyloid aggregation” *Bioorg. Med. Chem. Lett.* **2020**, *30*, 127477.
- [184] S.-S. Xie, X.-B. Wang, J.-Y. Li, L. Yang, L.-Y. Kong, “Design, synthesis and evaluation of novel tacrine–coumarin hybrids as multifunctional cholinesterase inhibitors against Alzheimer’s disease” *Eur. J. Med. Chem.* **2013**, *64*, 540–553.
- [185] L. Piazzzi, A. Cavalli, F. Colizzi, F. Belluti, M. Bartolini, F. Mancini, M. Recanatini, V. Andrisano, A. Rampa, “Multi-target-directed coumarin derivatives: hAChE and BACE1 inhibitors as potential anti-Alzheimer compounds” *Bioorg. Med. Chem. Lett.* **2008**, *18*, 423–426.

- [186] C. Gnerre, M. Catto, F. Leonetti, P. Weber, P.-A. Carrupt, C. Altomare, A. Carotti, B. Testa, "Inhibition of Monoamine Oxidases by Functionalized Coumarin Derivatives: Biological Activities, QSARs, and 3D-QSARs" *J. Med. Chem.* **2000**, *43*, 4747–4758.
- [187] R. Orioli, F. Belluti, S. Gobbi, A. Rampa, A. Bisi, "Naturally Inspired Coumarin Derivatives in Alzheimer's Disease Drug Discovery: Latest Advances and Current Challenges" *Molecules* **2024**, *29*, 3514.
- [188] D. D. Soto-Ortega, B. P. Murphy, F. J. Gonzalez-Velasquez, K. A. Wilson, F. Xie, Q. Wang, M. A. Moss, "Inhibition of amyloid- β aggregation by coumarin analogs can be manipulated by functionalization of the aromatic center" *Bioorg. Med. Chem.* **2011**, *19*, 2596–2602.
- [189] A. Stefanachi, F. Leonetti, L. Pisani, M. Catto, A. Carotti, "Coumarin: A Natural, Privileged and Versatile Scaffold for Bioactive Compounds" *Molecules* **2018**, *23*, 250.
- [190] J. Cheung, M. J. Rudolph, F. Burshteyn, M. S. Cassidy, E. N. Gary, J. Love, M. C. Franklin, J. J. Height, "Structures of Human Acetylcholinesterase in Complex with Pharmacologically Important Ligands" *J. Med. Chem.* **2012**, *55*, 10282–10286.
- [191] R. T. Kareem, F. Abedinifar, E. A. Mahmood, A. G. Ebadi, F. Rajabi, E. Vessally, "The recent development of donepezil structure-based hybrids as potential multifunctional anti-Alzheimer's agents: highlights from 2010 to 2020" *RSC Adv.* **2021**, *11*, 30781–30797.
- [192] L. Pisani, R. M. Iacobazzi, M. Catto, M. Rullo, R. Farina, N. Denora, S. Cellamare, C. D. Altomare, "Investigating alkyl nitrates as nitric oxide releasing precursors of multitarget acetylcholinesterase-monoamine oxidase B inhibitors" *Eur. J. Med. Chem.* **2019**, *161*, 292–309.
- [193] B. Tasso, M. Catto, O. Nicolotti, F. Novelli, M. Tonelli, I. Giangreco, L. Pisani, A. Sparatore, V. Boido, A. Carotti, F. Sparatore, "Quinolizidinyl derivatives of bi- and tricyclic systems as potent inhibitors of acetyl- and butyrylcholinesterase with potential in Alzheimer's disease" *Eur. J. Med. Chem.* **2011**, *46*, 2170–2184.
- [194] L. Gu, Z. Guo, "Alzheimer's A β 42 and A β 40 peptides form interlaced amyloid fibrils" *J. Neurochem.* **2013**, *126*, 305–311.
- [195] E. R. LaVallie, E. A. DiBlasio, S. Kovacic, K. L. Grant, P. F. Schendel, J. M. McCoy, "A Thioredoxin Gene Fusion Expression System That Circumvents Inclusion Body Formation in the E. coli Cytoplasm" *Nat. Biotechnol.* **1993**, *11*, 187–193.
- [196] B. P. Austin, S. Nallamsetty, D. S. Waugh, "Hexahistidine-tagged maltose-binding protein as a fusion partner for the production of soluble recombinant proteins in Escherichia coli" *Methods Mol. Biol. Clifton NJ* **2009**, *498*, 157–172.
- [197] R. B. Kapust, J. Tözsér, T. D. Copeland, D. S. Waugh, "The P1' specificity of tobacco etch virus protease" *Biochem. Biophys. Res. Commun.* **2002**, *294*, 949–955.
- [198] A. Abelein, G. Chen, K. Kitoka, R. Aleksis, F. Oleskovs, M. Sarr, M. Landreh, J. Pahnke, K. Nordling, N. Kronqvist, K. Jaudzems, A. Rising, J. Johansson, H. Biverstål, "High-yield Production of Amyloid- β Peptide Enabled by a Customized Spider Silk Domain" *Sci. Rep.* **2020**, *10*, 235.
- [199] S. I. A. Cohen, S. Linse, L. M. Luheshi, E. Hellstrand, D. A. White, L. Rajah, D. E. Otzen, M. Vendruscolo, C. M. Dobson, T. P. J. Knowles, "Proliferation of amyloid- β 42 aggregates occurs through a secondary nucleation mechanism" *Proc. Natl. Acad. Sci.* **2013**, *110*, 9758–9763.

- [200] G. Meisl, J. B. Kirkegaard, P. Arosio, T. C. T. Michaels, M. Vendruscolo, C. M. Dobson, S. Linse, T. P. J. Knowles, “Molecular mechanisms of protein aggregation from global fitting of kinetic models” *Nat. Protoc.* **2016**, *11*, 252–272.
- [201], “AmyloFit - The Amyloid Aggregation Fitter,” can be found under <https://amylofit.com/amylofitmain/login/> (accessed 30 November 2025), **2025**.
- [202] C. Xue, T. Y. Lin, D. Chang, Z. Guo, “Thioflavin T as an amyloid dye: fibril quantification, optimal concentration and effect on aggregation” *R. Soc. Open Sci.* **2017**, *4*, 160696.
- [203] R. B. Kapust, D. S. Waugh, “Escherichia coli maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused” *Protein Sci.* **1999**, *8*, 1668–1674.
- [204] J. E. Tropea, S. Cherry, D. S. Waugh in *High Throughput Protein Expr. Purif.* (Ed.: S.A. Doyle), Humana Press, Totowa, NJ, **2009**, pp. 297–307.
- [205] N. A. Kuznetsova, O. L. Kaliya, “The photochemistry of coumarins” *Russ. Chem. Rev.* **1992**, *61*, 683–696.
- [206] S. A. Carvalho, L. O. Feitosa, M. Soares, T. E. M. M. Costa, M. G. Henriques, K. Salomão, S. L. De Castro, M. Kaiser, R. Brun, J. L. Wardell, S. M. S. V. Wardell, G. H. G. Trossini, A. D. Andricopulo, E. F. Da Silva, C. A. M. Fraga, “Design and synthesis of new (E)-cinnamic N-acylhydrazones as potent antitrypanosomal agents” *Eur. J. Med. Chem.* **2012**, *54*, 512–521.
- [207] C. J. C. Ortiz, C. M. Damasio, L. Pruccoli, N. F. Nadur, L. L. De Azevedo, I. A. Guedes, L. E. Dardenne, A. E. Kümmerle, A. Tarozzi, C. Viegas, “Cinnamoyl-N-Acylhydrazone-Donepezil Hybrids: Synthesis and Evaluation of Novel Multifunctional Ligands Against Neurodegenerative Diseases” *Neurochem. Res.* **2020**, *45*, 3003–3020.
- [208] H. Turkez, M. E. Arslan, J. N. Barboza, C. Y. Kahraman, D. P. de Sousa, A. Mardinoğlu, “Therapeutic Potential of Ferulic Acid in Alzheimer’s Disease” *Curr. Drug Deliv.* **2022**, *19*, 860–873.
- [209] A. V. Phadke, A. A. Tayade, M. P. Khambete, “Therapeutic potential of ferulic acid and its derivatives in Alzheimer’s disease—A systematic review” *Chem. Biol. Drug Des.* **2021**, *98*, 713–721.
- [210] Y. P. Singh, H. Rai, G. Singh, G. K. Singh, S. Mishra, S. Kumar, S. Srikrishna, G. Modi, “A review on ferulic acid and analogs based scaffolds for the management of Alzheimer’s disease” *Eur. J. Med. Chem.* **2021**, *215*, 113278.
- [211] E.-J. Wang, M.-Y. Wu, J.-H. Lu, “Ferulic Acid in Animal Models of Alzheimer’s Disease: A Systematic Review of Preclinical Studies” *Cells* **2021**, *10*, 2653.
- [212] E. Bramanti, L. Fulgentini, R. Bizzarri, F. Lenci, A. Sgarbossa, “ β -Amyloid Amorphous Aggregates Induced by the Small Natural Molecule Ferulic Acid” *J. Phys. Chem. B* **2013**, *117*, 13816–13821.
- [213] K. Ono, M. Hirohata, M. Yamada, “Ferulic acid destabilizes preformed β -amyloid fibrils in vitro” *Biochem. Biophys. Res. Commun.* **2005**, *336*, 444–449.
- [214] J.-J. Yan, J.-Y. Cho, H.-S. Kim, K.-L. Kim, J.-S. Jung, S.-O. Huh, H.-W. Suh, Y.-H. Kim, D.-K. Song, “Protection against β -amyloid peptide toxicity in vivo with long-term administration of ferulic acid” *Br. J. Pharmacol.* **2001**, *133*, 89–96.
- [215] X. Li, J. Zhang, H. Rong, X. Zhang, M. Dong, “Ferulic Acid Ameliorates MPP+/MPTP-Induced Oxidative Stress via ERK1/2-Dependent Nrf2 Activation: Translational Implications for Parkinson Disease Treatment” *Mol. Neurobiol.* **2020**, *57*, 2981–2995.

- [216] M. Benchekroun, L. Ismaili, M. Pudlo, V. Luzet, T. Gharbi, B. Refouvelet, J. Marco-Contelles, "Donepezil–Ferulic Acid Hybrids as Anti-Alzheimer Drugs" *Future Med. Chem.* **2015**, 7, 15–21.
- [217] A. T. Peedikayil, J. Lee, M. A. Abdelgawad, M. M. Ghoneim, M. E. Shaker, S. Selim, S. Kumar, S. Dev, H. Kim, B. Mathew, "Inhibitions of monoamine oxidases by ferulic acid hydrazide derivatives: synthesis, biochemistry, and computational evaluation" *Appl. Biol. Chem.* **2023**, 66, 66.
- [218] L. Fang, M. Chen, Z. Liu, X. Fang, S. Gou, L. Chen, "Ferulic acid–carbazole hybrid compounds: Combination of cholinesterase inhibition, antioxidant and neuroprotection as multifunctional anti-Alzheimer agents" *Bioorg. Med. Chem.* **2016**, 24, 886–893.
- [219] J. Mo, H. Yang, T. Chen, Q. Li, H. Lin, F. Feng, W. Liu, W. Qu, Q. Guo, H. Chi, Y. Chen, H. Sun, "Design, synthesis, biological evaluation, and molecular modeling studies of quinoline-ferulic acid hybrids as cholinesterase inhibitors" *Bioorganic Chem.* **2019**, 93, 103310.
- [220] J. Delay, P. Deniker, J. M. Harl, "Therapeutic use in psychiatry of phenothiazine of central elective action" *Ann. Med. Psychol. (Paris)* **1952**, 110, 112–117.
- [221] C. M. Wischik, P. C. Edwards, R. Y. Lai, M. Roth, C. R. Harrington, "Selective inhibition of Alzheimer disease-like tau aggregation by phenothiazines." *Proc. Natl. Acad. Sci.* **1996**, 93, 11213–11218.
- [222] J. Debord, L. Merle, J.-C. Bollinger, T. Dantoine, "Inhibition of Butyrylcholinesterase by Phenothiazine Derivatives" *J. Enzyme Inhib. Med. Chem.* **2002**, 17, 197–202.
- [223] M. Oz, D. E. Lorke, G. A. Petroianu, "Methylene blue and Alzheimer's disease" *Biochem. Pharmacol.* **2009**, 78, 927–932.
- [224] C. C. Boido, B. Tasso, V. Boido, F. Sparatore, "Cytisine derivatives as ligands for neuronal nicotine receptors and with various pharmacological activities" *Il Farm.* **2003**, 58, 265–277.
- [225] Y. S. Mineur, O. Somenzi, M. R. Picciotto, "Cytisine, a partial agonist of high-affinity nicotinic acetylcholine receptors, has antidepressant-like properties in male C57BL/6J mice" *Neuropharmacology* **2007**, 52, 1256–1262.
- [226] H. Rollema, A. Shrikhande, K. Ward, F. Tingley III, J. Coe, B. O'Neill, E. Tseng, E. Wang, R. Mather, R. Hurst, K. Williams, M. de Vries, T. Cremers, S. Bertrand, D. Bertrand, "Pre-clinical properties of the $\alpha 4\beta 2$ nicotinic acetylcholine receptor partial agonists varenicline, cytisine and dianicline translate to clinical efficacy for nicotine dependence" *Br. J. Pharmacol.* **2010**, 160, 334–345.
- [227] M. Cui, M. Xiao, L. Xu, Y. Chen, A. Liu, J. Ye, A. Hu, "Bioassay of ferulic acid derivatives as influenza neuraminidase inhibitors" *Archiv der Pharmazie* **2020**, 353, 1900174.
- [228] G. L. Ellman, K. D. Courtney, V. Andres, R. M. Featherstone, "A new and rapid colorimetric determination of acetylcholinesterase activity" *Biochem. Pharmacol.* **1961**, 7, 88–95.
- [229] R. Purgatorio, M. de Candia, M. Catto, M. Rullo, L. Pisani, N. Denora, A. Carrieri, A. A. Nevskaya, L. G. Voskressensky, C. D. Altomare, "Evaluation of Water-Soluble Mannich Base Prodrugs of 2,3,4,5-Tetrahydroazepino[4,3-b]indol-1(6H)-one as Multitarget-Directed Agents for Alzheimer's Disease" *ChemMedChem* **2021**, 16, 589–598.
- [230] M. Tonelli, M. Catto, B. Tasso, F. Novelli, C. Canu, G. Iusco, L. Pisani, A. D. Stradis, N. Denora, A. Sparatore, V. Boido, A. Carotti, F. Sparatore, "Multitarget Therapeutic Leads for Alzheimer's Disease: Quinolizidinyl Derivatives of Bi- and

- Tricyclic Systems as Dual Inhibitors of Cholinesterases and β -Amyloid (A β) Aggregation” *ChemMedChem* **2015**, *10*, 1040–1053.
- [231] Z. Radić, N. A. Pickering, D. C. Vellom, S. Camp, P. Taylor, “Three distinct domains in the cholinesterase molecule confer selectivity for acetyl- and butyrylcholinesterase inhibitors” *Biochemistry* **1993**, *32*, 12074–12084.
- [232] A. Saxena, A. M. Redman, X. Jiang, O. Lockridge, B. P. Doctor, “Differences in active-site gorge dimensions of cholinesterases revealed by binding of inhibitors to human butyrylcholinesterase” *Chem. Biol. Interact.* **1999**, *119–120*, 61–69.
- [233] G. C. González-Muñoz, M. P. Arce, B. López, C. Pérez, A. Romero, L. del Barrio, M. D. Martín-de-Saavedra, J. Egea, R. León, M. Villarroya, M. G. López, A. G. García, S. Conde, M. I. Rodríguez-Franco, “*N*-Acylaminophenothiazines: Neuroprotective agents displaying multifunctional activities for a potential treatment of Alzheimer’s disease” *Eur. J. Med. Chem.* **2011**, *46*, 2224–2235.
- [234] G. La Spada, D. V. Miniero, M. Rullo, M. Cipolloni, P. Delre, C. Colliva, M. Colella, F. Leonetti, G. M. Liuzzi, G. F. Mangiatordi, N. Giacchè, L. Pisani, “Structure-based design of multitargeting ChEs-MAO B inhibitors based on phenyl ring bioisosteres: AChE/BChE selectivity switch and drug-like characterization” *Eur. J. Med. Chem.* **2024**, *274*, 116511.
- [235] D. Harman, “Aging: A Theory Based on Free Radical and Radiation Chemistry” *J. Gerontol.* **1956**, *11*, 298–300.
- [236] M. Alami, T. Fulop, K. Boumezough, A. Khalil, E. Zerif, H. Berrougui in *Biomark. Oxidative Stress Clin. Asp. Oxidative Stress* (Eds.: S. Andreescu, R. Henkel, A. Khelfi), Springer Nature Switzerland, Cham, **2024**, pp. 71–102.
- [237] J. J. Rodríguez, H. N. Noristani, A. Verkhratsky, “The serotonergic system in ageing and Alzheimer’s disease” *Prog. Neurobiol.* **2012**, *99*, 15–41.
- [238] R. Hornedo-Ortega, G. Da Costa, A. B. Cerezo, A. M. Troncoso, T. Richard, M. C. Garcia-Parrilla, “In Vitro Effects of Serotonin, Melatonin, and Other Related Indole Compounds on Amyloid- β Kinetics and Neuroprotection” *Mol. Nutr. Food Res.* **2018**, *62*, 1700383.
- [239] A. Nous, S. Engelborghs, I. Smolders, “Melatonin levels in the Alzheimer’s disease continuum: a systematic review” *Alzheimers Res. Ther.* **2021**, *13*, 52.
- [240] L. P. Dolgacheva, V. P. Zinchenko, A. D. Nadeev, N. V. Goncharov, “Serotonergic Regulation in Alzheimer’s Disease” *Int. J. Mol. Sci.* **2025**, *26*, 5218.
- [241] E. Barresi, E. Baglini, V. Poggetti, J. Castagnoli, D. Giorgini, S. Salerno, S. Taliani, F. Da Settimo, “Indole-Based Compounds in the Development of Anti-Neurodegenerative Agents” *Molecules* **2024**, *29*, 2127.
- [242] V. Bártl, A. Dlabač, M. Protiva, “Tricyclic potential neuroleptics: 2-chloro-11-[4-(4-fluoroaralkyl)piperazino]-10,11-dihydrodibenzo[*b,f*]thiepins and related compounds” *Collect. Czechoslov. Chem. Commun.* **1980**, *45*, 3182–3189.
- [243] A. S. Mehanna, J. Y. Kim, “Design, synthesis, and biological testing of thiosalicylamides as a novel class of calcium channel blockers” *Bioorg. Med. Chem.* **2005**, *13*, 4323–4331.
- [244] P. Chen, J. Yang, Y. Zhou, X. Li, Y. Zou, Z. Zheng, M. Guo, Z. Chen, W.-J. Cho, N. Chattipakorn, W. Wu, Q. Tang, G. Liang, “Design, synthesis, and bioactivity evaluation of novel amide/sulfonamide derivatives as potential anti-inflammatory agents against acute lung injury and ulcerative colitis” *Eur. J. Med. Chem.* **2023**, *259*, 115706.
- [245] D. Panek, A. Więckowska, T. Wichur, M. Bajda, J. Godyń, J. Jończyk, K. Mika, J. Janockova, O. Soukup, D. Knez, J. Korabecny, S. Gobec, B. Malawska, “Design, synthesis and biological evaluation of new phthalimide and saccharin derivatives

- with alicyclic amines targeting cholinesterases, beta-secretase and amyloid beta aggregation” *Eur. J. Med. Chem.* **2017**, *125*, 676–695.
- [246] X. Li, X. Huang, Y. Zhao, Z. Zheng, M. Guo, Z. Chen, P. Chen, X. Li, J. Liao, M. Jiang, W.-J. Cho, Y.-C. Cho, R. Zeng, Q. Tang, G. Liang, “Design, synthesis and bioactivity evaluation of 4-hydroxycoumarin derivatives as potential anti-inflammatory agents against acute lung injury and colitis” *Eur. J. Med. Chem.* **2024**, *272*, 116487.
- [247] C. Antonello, S. Marciani Magno, O. Gia, F. Carlassare, F. Baccichetti, F. Bordin, “Diethylaminoalkyloxycoumarin and furocoumarin derivatives” *Il Farmaco* **1979**, *34*, 139–155.
- [248] L. Braconi, C. Riganti, A. Parenti, M. Cecchi, A. Nocentini, G. Bartolucci, M. Menicatti, M. Contino, N. A. Colabufo, D. Manetti, M. N. Romanelli, C. T. Supuran, E. Teodori, “Dual Inhibitors of P-gp and Carbonic Anhydrase XII (hCA XII) against Tumor Multidrug Resistance with Piperazine Scaffold” *Molecules* **2024**, *29*, 3290.
- [249] S.-S. Xie, X. Wang, N. Jiang, W. Yu, K. D. G. Wang, J.-S. Lan, Z.-R. Li, L.-Y. Kong, “Multi-target tacrine-coumarin hybrids: Cholinesterase and monoamine oxidase B inhibition properties against Alzheimer’s disease” *Eur. J. Med. Chem.* **2015**, *95*, 153–165.
- [250] L. N. Kulikova, R. Purgatorio, A. A. Beloglazkin, V. A. Tafeenko, R. G. Reza, D. D. Levickaya, S. Sblano, A. Boccarelli, M. de Candia, M. Catto, L. G. Voskressensky, C. D. Altomare, “Chemical and Biological Evaluation of Novel 1H-Chromeno[3,2-c]pyridine Derivatives as MAO Inhibitors Endowed with Potential Anticancer Activity” *Int. J. Mol. Sci.* **2023**, *24*, 7724.