shows extensive inflammatory infiltrates with high levels of cytokines that support the growth of CRC cells through the activation of STAT3 and NF-κB.[5] We previously reported the ability of rafoxanide to inhibit colon tumorigenesis in mice carrying a constitutional mutation in the adenomatous polyposis coli (*Apc*) gene (*Apc*$^{min/+}$ mice) and mimicking human sporadic CRC.[27] By comparing the expression of p-STAT3 Y705 and p-NF-κB/p65 S536 in nontumor and tumor tissues derived from colonic extracts of *Apc*$^{min/+}$ mice treated or not with rafoxanide, we showed that the antineoplastic effect of the drug was associated with reduced activation of STAT3 and NF-κB (Figure S3A), as well as with decreased levels of IL-6 and TNF-α (Figure S3B). These results were in line with those obtained in the experimental model of CAC, suggesting that rafoxanide could act on similar pathways to exert its antineoplastic effects in both mouse models of CRC. Next, we sought to determine the effects of rafoxanide on STAT3 and NF-κB activity in the CRC microenvironment. To this end, we compared the expression of p-STAT3 Y705 and p-NF-κB/p65 S536 in patient-derived sporadic CRC explants cultured with rafoxanide or vehicle (control). Immunohistochemistry showed robust activation of STAT3 and NF-κB in control CRC explants compared to those treated with the drug (Figure 3). It is noteworthy that rafoxanide treatment resulted in a reduction in cells positive for p-STAT3 Y705 and p-NF-κB/p65 S536 in transformed epithelial cells and tumor infiltrating cells (Figure 3). Taken together, these results suggest that rafoxanide-driven impairment of STAT3 and NF-κB activation in CRC could rely on a direct effect on the intracellular machinery governing the functionality of these transcription factors in cancer cells, a secondary effect due to inhibition of immune cell-derived STAT3/NF-κB activating factors in the tumor niche, or both.

3.3 | Rafoxanide impairs STAT3 and NF-κB activation in cultured CRC cells and in organoids derived from CRC patients

As a starting point to answer these questions, we initially investigated whether rafoxanide could directly hamper STAT3 and NF-κB activation in CRC cell lines. Treatment of HCT-116 and DLD1 cells with increasing doses (1.25, 2.5, and 5 μM rafoxanide) for 24 h markedly reduced p-STAT3 Y705 and, to a lesser extent, p-NF-κB/p65 S536 levels, in a dose-dependent manner in both cell lines (Figure 4A,B). It should be noted that rafoxanide treatment had no (in DLD1 cells) or minimal (in HCT-116 cells) effects on total STAT3 and p65 total proteins, indicating that the drug could interfere with the molecular machinery controlling the phosphorylation of the transcription factors mentioned above on these specific residues.

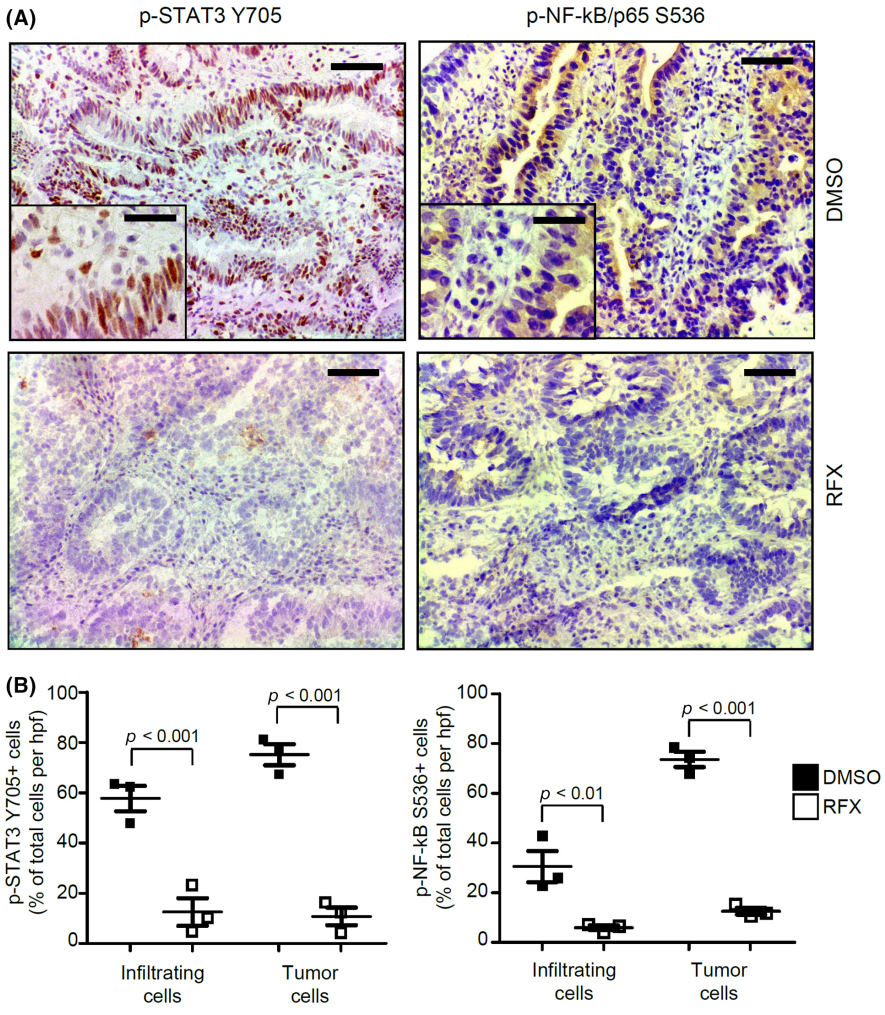


FIGURE 3 Rafoxanide (RFX) treatment reduces signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) activation in the epithelial and immune compartments of human colorectal cancer (CRC) explants. (A) Representative photographs of phosphorylated (p-)STAT3 Y705- and p-NF-κB/p65 S536-stained sections of freshly obtained human CRC explants treated with DMSO or 2.5 μM RFX for 12 h. Scale bars, 40 μm; 10 μm (insets). (B) Quantification of p-STAT3 Y705- and p-NF-κB/p65 S536-positive infiltrating and tumor cells in human CRC explants treated as indicated in (A). Data are presented as mean values of positive cells per high power field ± SEM of three independent experiments.

Time course studies confirmed the inhibitory effect of the drug on the expression of p-STAT3 Y705 and p-NF-κB/p65 S536 (Figure 4C). In particular, the decrease in p-STAT3 Y705 expression was already evident from 1 h of incubation with rafoxanide and preceded the inhibition of p-NF-κB/p65 S536 in DLD1 and, to a lesser degree, HCT-116 cells (Figure 4C).

To figure out how rafoxanide negatively affected STAT3 Y705 phosphorylation, we first investigated whether rafoxanide treatment could hinder the expression of JAK2, a key STAT3 Y705 targeting kinase.[10] However, we were unable to see any change in JAK2 expression after rafoxanide treatment in either HCT-116 or DLD1 cells (Figure S4). Further work indicated that the rafoxanide-mediated inhibitory effect on STAT3 Y705 phosphorylation was reverted by the addition of the protein tyrosine phosphatase inhibitor Na_3VO_4 (Figure 5A), suggesting a role for rafoxanide in modulating STAT3 dephosphorylation. The precise mechanism/s underlying the rafoxanide-driven p-STAT3 Y705 downregulation remains to be determined as our first results did not show any effect of rafoxanide in modulating the expression of SHP-1 or SHP-2, two key STAT3 tyrosine phosphatases (Figure 5B).[35]

Concerning the mechanism/s by which rafoxanide negatively regulates p-NF-κB activity, it is worth mentioning that multiple, distinct protein kinases that phosphorylate the transcriptionally most active NF-κB subunit p65 at S536 exist, such as IKKα, IKKβ, and CDK6,[36] with the latter requiring association with cyclins D1–D3 for full activation.[37] Rafoxanide treatment downregulated CDK6, but not IKKα or IKKβ, protein expression in HCT-116 and DLD1 cells (Figure 6). Furthermore, rafoxanide was recently proposed (through molecular docking studies) as a dual CDK4/CDK6 inhibitor,[38] and we have previously shown that rafoxanide treatment markedly reduced cyclin D1 expression in CRC cells.[27] These pieces of evidence suggest that the impairment of CDK6/cyclin D1 expression/activity might be the mechanism, or one of the mechanisms, by which the drug inhibits phosphorylation of NF-κB/p65 on S536 in CRC. To investigate whether rafoxanide was able to affect the proliferation and STAT3/NF-κB activation of human primary CRC cells, we took advantage of the generation and culture of intestinal organoids derived from CRC patients, which are able to faithfully recapitulate the molecular steps of disease evolution and the anatomical and functional hallmarks of the real organ.[39] Rafoxanide was added to organoids derived from CRC patients and the expression of Ki-67, p-STAT3 Y705, and p-NF-κB/p65 S536 was evaluated after 24 h by immunohistochemistry. Rafoxanide inhibited cell proliferation and, consistent with the results obtained in cultured CRC cells, STAT3/NF-κB activation in this experimental setting (Figure 7). Together, our data clearly show the ability of rafoxanide to directly inhibit STAT3 and NF-κB in CRC cells.

3.4 | Rafoxanide negatively affects STAT3/NF-κB activation and the production of protumorigenic cytokines in TILs

Beyond their direct effects in sustaining CRC cell proliferation and survival, STAT3 and NF-κB play an important role in shaping the immune microenvironment of CRC.[40,41] For example, STAT3 negatively regulates the T helper 1 (Th1)-type antitumor immune response and promotes Th17 cell differentiation,[42] while activation of NF-κB in immune cells contributes to the increased production of proinflammatory cytokines (such as TNFα, IL-1, and IL-6).[43] Thus, in subsequent studies, we investigated whether rafoxanide could modulate immune cell-related STAT3/NF-κB activity and protumorigenic signals in the CRC niche. Specifically, TILs isolated from explants derived from sporadic CRC patients were cultured in the presence of rafoxanide or DMSO (vehicle) for 16 h and the expression of p-STAT3 Y705 and p-NF-κB/p65 S536 was evaluated by immunoblotting. In parallel experiments, an aliquot of cells was used to evaluate changes in cell viability and cytokine production (that is, IFN-ɣ, IL-6, and TNF-α) by flow cytometry. Treatment with rafoxanide inhibited the activation of STAT3 and NF-κB in TILs (Figure 8A). This effect was associated with an increased frequency of CD3+ IFN-ɣ-producing cells, and with a decreased frequency of CD45+ IL-6- and TNF-α-producing cells (Figure 8B).

3.5 | Rafoxanide reduces TIL-derived culture supernatant-mediated cell proliferation and STAT3/NF-κB activation in CRC cells

Cytokine-producing immune/inflammatory cells contribute to the progression of CRC in part through the activation of STAT3/NF-κB signaling in transformed epithelial cells.[5] Finally, to address the biological relevance of the results observed in TILs after rafoxanide exposure, we investigated the effects of cell culture supernatants derived from DMSO- and rafoxanide-treated TILs on CRC cell proliferation and STAT3/NF-κB activation. Specifically, HCT-116 and DLD1 cells were cultured with cell-free supernatants derived from TILs treated with rafoxanide or DMSO (vehicle) for 16 h. As expected, TIL-derived supernatants robustly increased the expression of p-STAT3 Y705 and p-NF-κB/p65 S536, as well as cell proliferation in both HCT-116 and DLD1 cells (Figure 9). In particular, such responses were markedly hampered in cells cultured in the presence of cell-free supernatants derived from TILs treated with rafoxanide (Figure 9), which presented a reduced amount of IL-6 and TNF-α (Figure S5).

4 | DISCUSSION

In this study, we present data indicating that the anthelmintic drug rafoxanide is a powerful inhibitor of STAT3 and NF-κB activation in the CRC microenvironment.

We first reported that systemic administration of rafoxanide reduced the multiplicity and size of lesions in a mouse model that mimicked human CAC. In line with data from our previous report assessing the antineoplastic effects of rafoxanide in mice carrying a constitutional mutation in the *Apc* gene ($Apc^{min/+}$ mice) and mimicking sporadic CRC,[27] the drug exerted antimitogenic effects in transformed but not in normal colonic epithelial cells, further sustaining

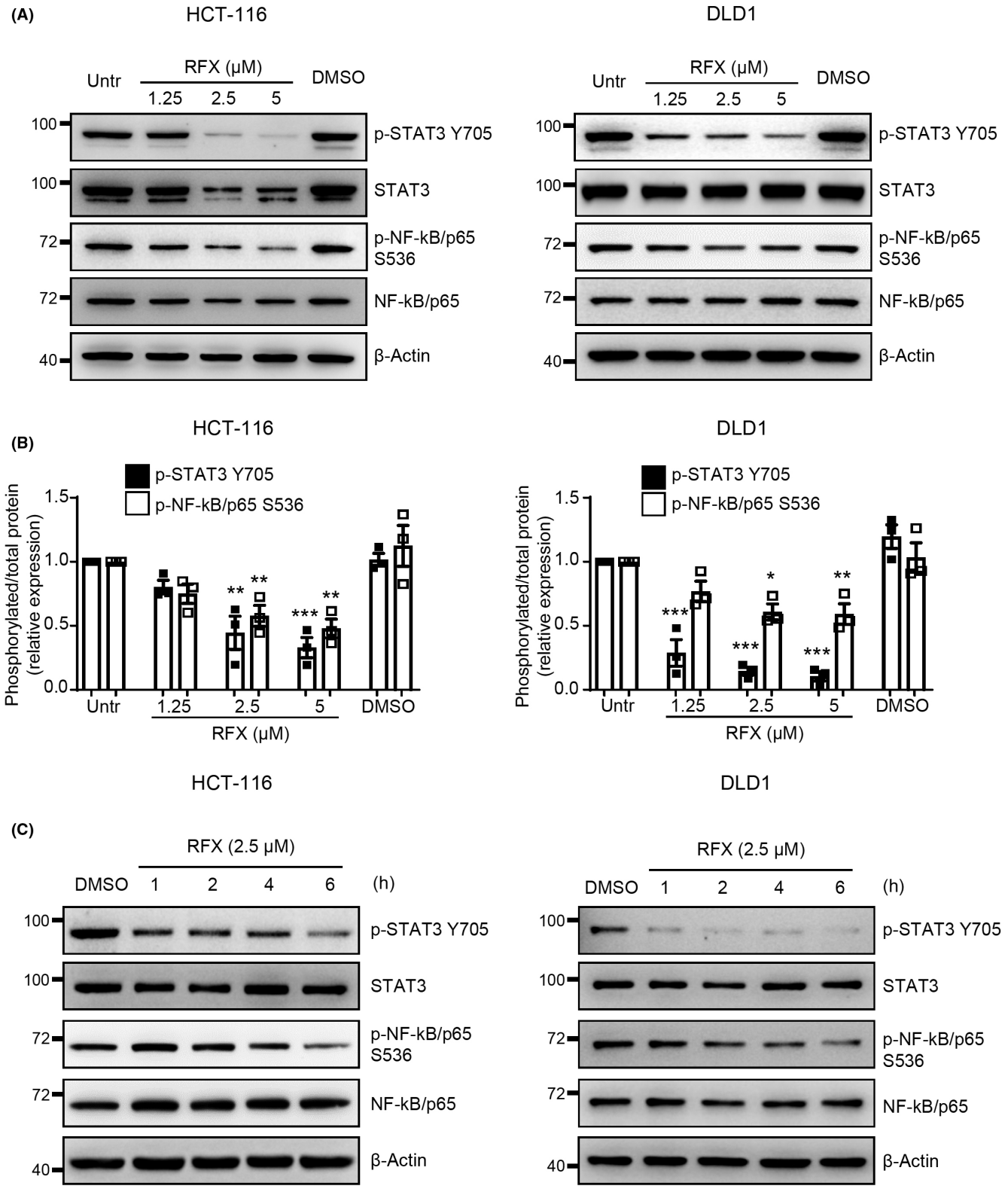


FIGURE 4 Treatment with rafoxanide (RFX) reduces the activation of signal transducer and activator of transcription 3 (STAT3) and nuclear factor-κB (NF-κB) in cultured colorectal cancer cells. (A) HCT-116 and DLD1 cells were left untreated (Untr) or treated with the indicated doses of RFX or DMSO for 24 h. Protein extracts were evaluated for phosphorylated (p-)STAT3 Y705, STAT3, p-NF-κB/p65 S536, and NF-κB/p65 by immunoblotting. β-Actin was used as a loading control. One of three independent experiments in which similar results were obtained is shown. (B) Quantitative analysis of the p-STAT3 Y705/STAT3 protein ratio and p-NF-κB/p65 S536/NF-κB/p65 protein ratio in total extracts of HCT-116 and DLD1 cells stimulated as indicated in (A). Values are the mean ± SEM of three independent experiments (DMSO- vs. RFX-treated cells). (C) HCT-116 and DLD1 cells were stimulated with DMSO or 2.5 μM RFX for the indicated time points. Protein extracts were evaluated for p-STAT3 Y705, STAT3, p-NF-κB/p65 S536 and NF-κB/p65 by immunoblotting. β-Actin was used as a loading control. One of three independent experiments in which similar results were obtained is shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

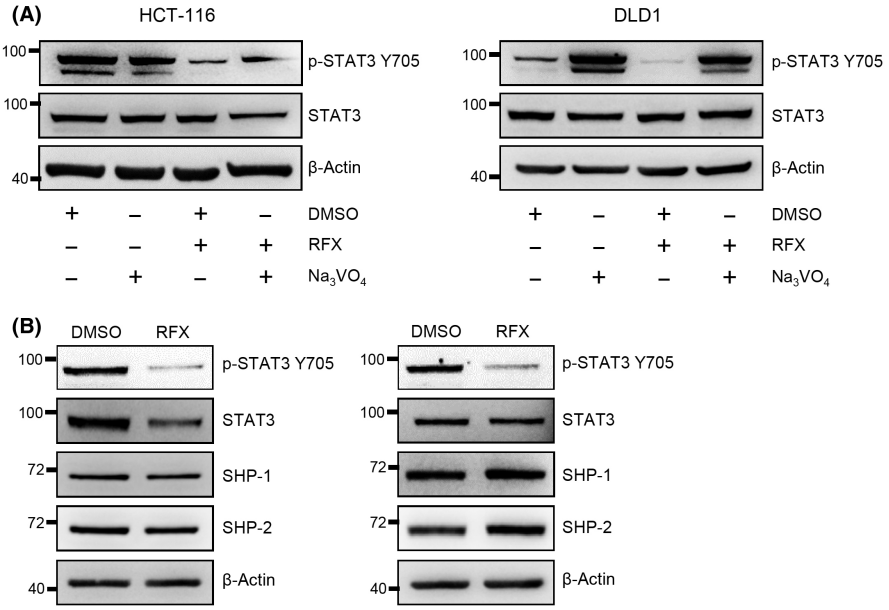
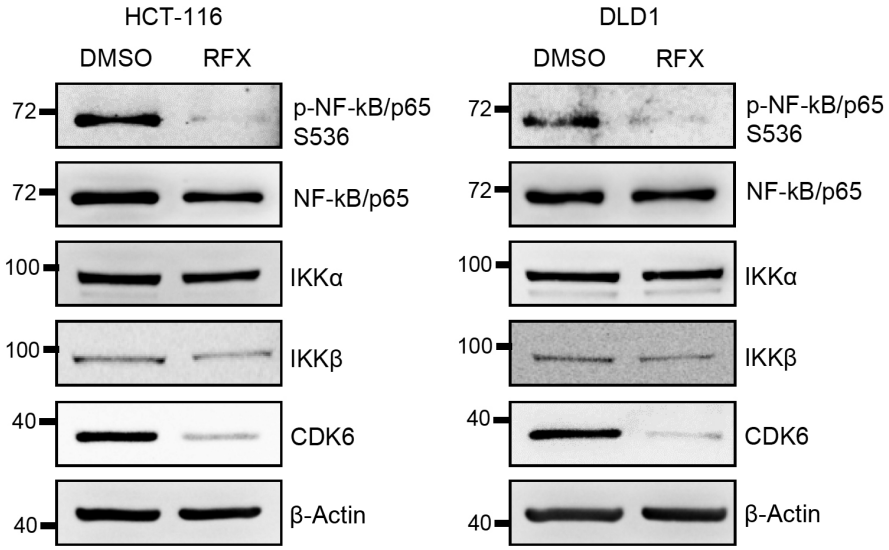


FIGURE 5 Role of protein tyrosine phosphatases (PTP) on rafoxanide-driven phosphorylated signal transducer and activator of transcription 3 (p-STAT3) Y705 downregulation. (A) Addition of the general PTP inhibitor Na_3VO_4 to HCT-116 and DLD1 cell cultures reduces the STAT3 Y705 dephosphorylation induced by rafoxanide (RFX). Cells were preincubated with Na_3VO_4 (used at 400 μM) for 1 h and then stimulated with DMSO or 2.5 μM RFX for 24 h. At the end, total extracts were prepared and the expression of p-STAT3 Y705 and STAT3 assessed by western blotting. β-Actin was used as loading control. One of three representative experiments in which similar results were obtained is shown. (B) RFX does not affect the expression of the key STAT3 targeting PTP SHP-1 and SHP-2. Total proteins extracted from HCT-116 and DLD1 cells treated with DMSO or RFX for 24 h were evaluated for p-STAT3 Y705, STAT3, SHP-1, and SHP-2 expression by western blotting. β-Actin was used as a loading control. One of three representative experiments in which similar results were obtained is shown.

FIGURE 6 Effect of rafoxanide (RFX) on phosphorylated nuclear factor-κB (p-NF-κB)/p65 S536 targeting kinases. Total proteins extracted from HCT-116 and DLD1 cells treated with DMSO or 2.5 μM RFX for 24 h were evaluated for p-NF-κB/p65 S536, NF-κB/p65, IKKα, IKKβ, and CDK6 expression by western blotting. β-Actin was used as a loading control. One of three representative experiments in which similar results were obtained is shown.



the evidence that rafoxanide preferentially interferes with biological pathways that support malignant cell growth in vivo. Consistent with our previous observations in *Apc*$^{min/+}$ mice, in our mouse model mimicking inflammation-associated colon tumorigenesis, rafoxanide would seem to be well tolerated, as no significant changes in bodyweight were observed in mice treated with the drug as compared to control mice. The apparent in vivo safety of rafoxanide is further supported by the evidence reported by Xiao et al. in a multiple myeloma xenograft model, showing no significant side-effects of the drug in mice receiving i.p. injections of rafoxanide at 15 mg/kg every other day for 14 days,[26] and, more recently, by Hu et al. in a non-small-cell lung cancer xenograft model, describing no obvious pathological damage in the liver or kidney as well as no significant changes in blood biochemical parameters related to liver or kidney function in mice treated with daily i.p. injections of rafoxanide at 15 mg/kg for 2 weeks.[24] However, further experimentation of the

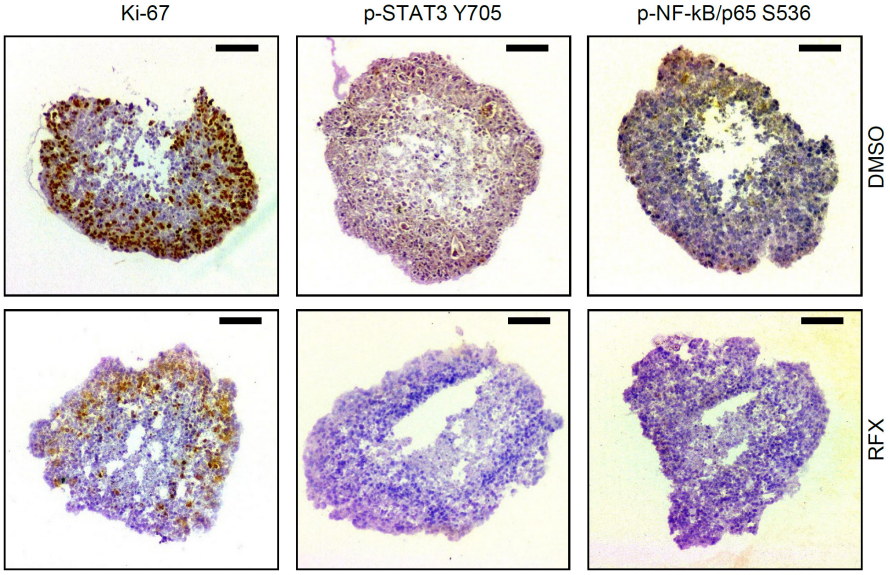


FIGURE 7 Treatment with rafoxanide (RFX) inhibits cell proliferation and signal transducer and activator of transcription 3 (STAT3)/nuclear factor-κB (NF-κB) activation in patient-derived colorectal cancer (CRC) organoids. Representative pictures of Ki-67, phosphorylated (p-) STAT3 Y705, and p-NF-κB/p65 S536-stained sections of CRC explant-derived organoids treated with either DMSO or 2.5 μM RFX for 24 h. One of three independent experiments in which similar results were obtained is shown. Scale bar, 200 μm.

dosage and long-term toxicity is needed and is currently ongoing to confirm the therapeutic potential and clinical benefit of rafoxanide. A reduced activation of STAT3/NF-κB was observed in tumor tissues from AOM/DSS treated mice that received rafoxanide compared to control mice, as well as a decrease in the expression of the immune cell-derived protumorigenic cytokines IL-6 and TNF-α, among the most potent activators of STAT3 and NF-κB in colonic epithelial cells, respectively,[5] and playing a key role in the development of CRC.[34] Taking advantage of the lysates derived from our previous study assessing the *Apc*$^{min/+}$ mice, we showed that the antineoplastic effect of the drug was associated with a reduced activation of STAT3/NF-κB and with decreased levels of IL-6 and TNF-α also in this experimental setting, raising the possibility that rafoxanide could affect the interplay between immune/inflammatory and malignant cells in the CRC niche independently of the presence of overt inflammation.

Our data produced in the AOM/DSS model are in line with the knowledge that blocking IL-6 or the downstream transcription factor STAT3 causes a significant suppression of CAC development in mouse models, indicating that STAT3 is indispensable for inflammation-associated colonic tumorigenesis.[44,45] However, our results in *Apc*$^{min/+}$ mice appear to contradict the paper by Oshima et al., which states that epithelial STAT3 is not necessary for intestinal tumor development in a Wnt activation-driven model of sporadic tumorigenesis.[46] This apparent discrepancy could be due to the fact that, different to the genetic ablation of STAT3, which was limited to the mouse intestinal epithelium,[46] the rafoxanide-driven STAT3 inhibition is not limited to the colon transformed epithelial cells but it likely extends to the immune cell compartment resulting in the modulation of both pro- and antitumor cytokines/factors. We previously reported that rafoxanide reduced transformed epithelial cell proliferation in sporadic CRC explants without substantially affecting the growth of normal colonic epithelial cells.[27] In the same context, to extend our in vivo observations to human CRC, we showed that rafoxanide negatively affected STAT3 and NF-κB activation. Notably, this effect was evident not only in transformed epithelial cells but also in tumor infiltrating cells, raising the possibility that rafoxanide could negatively impact STAT3 and NF-κB activity through a direct effect on cancer cells and/or by exerting a secondary inhibitory effect on immune cell-derived STAT3/NF-κB activating factors in the tumor microenvironment. Treatment of cultured CRC cells with rafoxanide revealed that the drug could directly affect STAT3/NF-κB phosphorylation/activation in cancer cells, in agreement with other studies reporting inhibitory properties of several anthelmintic drugs on STAT3 (i.e., pyrvinium, pyrvinium pamoate, niclosamide, albendazole, and flubendazole)[47–52] and NF-κB (e.g., niclosamide and flubendazole)[53,54] in different types of cancers (i.e., lung cancer, gastric cancer, CRC, glioblastoma, triple-negative breast cancer, and acute myelogenous leukemia). Although our initial results point to the involvement of STAT3 phosphatases in the downregulation of p-STAT3 Y705 driven by rafoxanide, more in-depth experimentation is needed to determine whether rafoxanide treatment might somehow modulate the activity of SHP-1 and/or SHP-2 and/or the expression/activity of other STAT3 Y705 targeting phosphatases (e.g., PTPRD, PTPRT, PTPRK).[35] To extend our observations to primary human cells, we generated patient-derived CRC organoids. Given their ability to recapitulate the anatomical and functional hallmarks of the real organ, organoids represent an additional step between 2D in vitro laboratory research and preclinical models and optimal systems for the screening of new drugs.[39] Of note, we showed that the culture of CRC organoids in the presence of rafoxanide resulted in a striking reduction in STAT3 and NF-κB activation as compared to the control.

The potential antitumor effects of various anthelmintic drugs are not limited to cytostatic and proapoptotic properties, as several studies highlighted their ability to modulate host immunity.[55] In particular, the activity of different subsets of immune cells can be influenced by some of these compounds (e.g., niclosamide and