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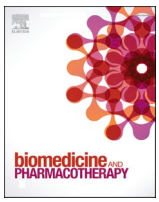

Rafoxanide sensitizes colorectal cancer cells to TRAIL-mediated apoptosis

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ABSTRACT

Colorectal cancer (CRC) remains a leading causes of cancer-related death in the world, mainly due to the lack of effective treatment of advanced disease. TNF-related apoptosis-inducing ligand (TRAIL)-driven cell death, a crucial event in the control of tumor growth, selectively targets malignant rather than non-transformed cells. However, the fact that cancer cells, including CRC cells, are either intrinsically resistant or acquire resistance to TRAIL, represents a major hurdle to the use of TRAIL-based strategies in the clinic. Agents able to overcome CRC cell resistance to TRAIL have thus great therapeutic potential and many researchers are making efforts to identify TRAIL sensitizers. The anthelmintic drug rafoxanide has recently emerged as a potent anti-tumor molecule for different cancer types and we recently reported that rafoxanide restrained the proliferation of CRC cells, but not of normal colonic epithelial cells, both in vitro and in a preclinical model mimicking sporadic CRC. As these findings were linked with the induction of endoplasmic reticulum stress, a phenomenon involved in the regulation of various components of the TRAIL-driven apoptotic pathway, we sought to determine whether rafoxanide could restore the sensitivity of CRC cells to TRAIL. Our data show that rafoxanide acts as a selective TRAIL sensitizer in vitro and in a syngeneic experimental model of CRC, by decreasing the levels of c-FLIP and survivin, two key molecules conferring TRAIL resistance. Collectively, our data suggest that rafoxanide could potentially be deployed as an anti-cancer drug in the combinatorial approaches aimed at overcoming CRC cell resistance to TRAIL-based therapies.

1. Introduction

Colorectal cancer (CRC) remains a leading causes of cancer-related deaths worldwide, mainly due to the lack of effective treatment for advanced disease [1]. Besides surgical intervention, chemotherapy, radiation therapy, and immunotherapy represent key CRC treatment modalities. Unfortunately, intrinsic and/or acquired resistance of CRC cells to therapies, the lack of efficacy of immunotherapeutics in many patients, as well as potential toxicities of current therapies, represent major limitations [2]. For these reasons, the identification of new anti-cancer agents with efficacy against CRC and lower toxicity in normal tissues is highly desirable.

Tumor necrosis factor-related apoptosis inducing ligand (TRAIL), also known as CD253, is a protein belonging to the Tumor necrosis factor (TNF) superfamily [3] that is capable to trigger programmed cell death after interaction with death receptors (DRs) [4]. Five homologous human receptors for TRAIL have been identified. Two of these receptors, DR4 (TRAIL-R1) and DR5 (TRAIL-R2), have cytoplasmic death domains and activate apoptotic pathways upon TRAIL binding [5]. Decoy receptor (DcR)1 and DcR2, also termed TRAIL-R3 and TRAIL-R4 respectively, are expressed on the cell surface but lack a functional intracellular death domain, thus competing with DR4/DR5 for TRAIL

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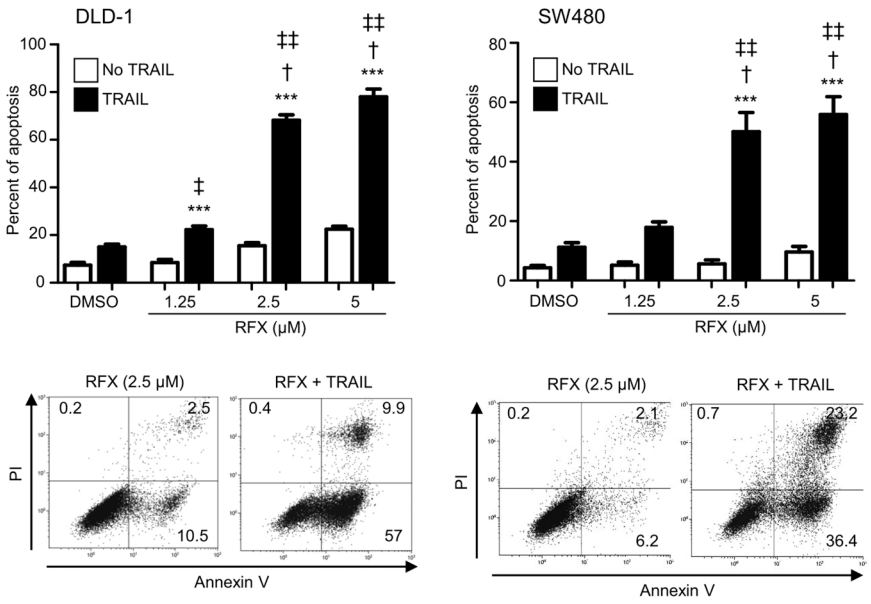

Fig. 1. Rafoxanide (RFX) sensitizes DLD-1 and SW480 cells to TRAIL-induced apoptosis. Cells were pre-incubated with DMSO (vehicle) or increasing doses of RFX for 8 h and then stimulated or not with 50 ng/ml TRAIL for further 24 h. The percentage of apoptosis was assessed by flow-cytometry. Data indicate mean ± SEM of 3 experiments (DMSO-treated cells vs. RFX + TRAIL-treated cells: ***, $P < 0.001$; TRAIL-treated cells vs. RFX + TRAIL-treated cells: †, $P < 0.001$; RFX-treated cells vs. RFX + TRAIL-treated cells: ‡, $P < 0.01$, ‡‡, $P < 0.001$). Bottom insets: representative dot-plots showing the percentages of AV- and/or PI-positive cells.

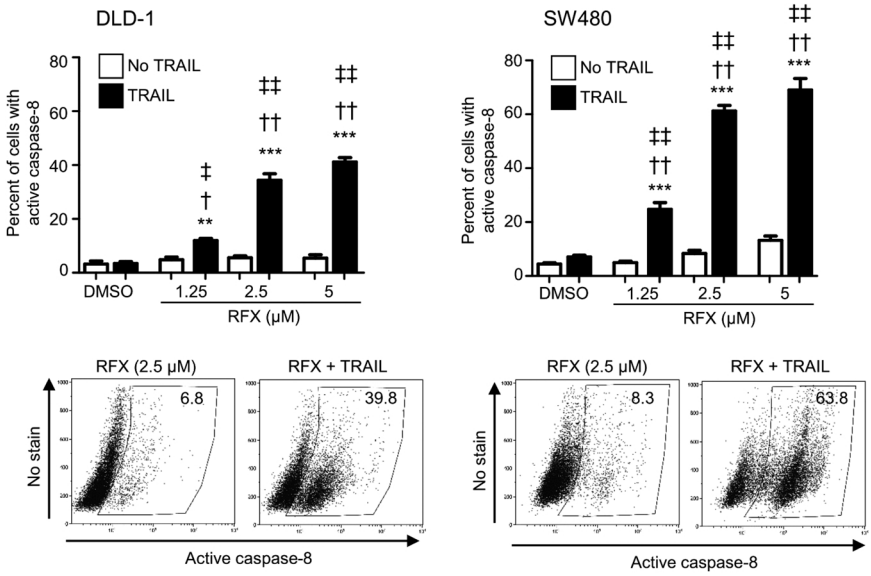

Fig. 2. The combination of rafoxanide (RFX) and TRAIL significantly enhances caspase-8 activation in DLD-1 and SW480 cells. Cells were pre-incubated with DMSO (vehicle) or increasing doses of RFX for 8 h and then stimulated or not with 50 ng/ml TRAIL for further 24 h. Caspase-8 activation was monitored by flow-cytometry. Data indicate mean ± SEM of 3 experiments (DMSO-treated cells vs. RFX + TRAIL-treated cells: **, $P < 0.01$, ***, $P < 0.001$; TRAIL-treated cells vs. RFX + TRAIL-treated cells: †, $P < 0.01$, ††, $P < 0.001$; RFX-treated cells vs. RFX + TRAIL-treated cells: ‡, $P < 0.05$, ‡‡, $P < 0.001$). Bottom insets: representative dot-plots showing the percentage of DLD-1 and SW480 cells expressing active caspase-8.

binding and conferring protection against TRAIL-driven apoptotic signal [5]. TRAIL can also bind a secreted, low-affinity receptor, termed osteoprotegerin, whose physiological relevance remains however ambiguous [6]. Interaction of TRAIL with DR4 and DR5 results in Caspase-8 activation through Fas-associated death domain (FADD) in the death-inducing signaling complex (DISC). Activated Caspase-8 can induce apoptosis by either directly activating terminal caspases (i.e., Caspase-3, −6 and −7) or through the intrinsic mitochondria-mediated apoptotic pathway [7]. Of note, TRAIL can specifically target malignant rather than non-transformed cells, likely due to the significantly higher expression of DcRs in the latter [8]. Such a selectivity, together with the ability, unlike most of chemotherapeutic drugs, to engage the apoptosis of neoplastic cells in a p53 independent manner [8,9], have suggested TRAIL as a safe and powerful candidate for cancer treatment. Indeed, preclinical studies have shown that soluble forms of TRAIL suppress the growth of human tumor xenografts, with no apparent systemic toxicity [10,11] and TRAIL-based strategies have been employed in clinical trials, some of which are still ongoing [12].

Despite the promising observations in experimental models, TRAIL therapy is less effective in certain cancer types due to the presence of intrinsic resistance mechanisms, thus representing a major limitation and a current challenge [13]. This has been, for example, documented in CRC patients [14]. Although the factor/s underlying the diminished susceptibility of cancer cells to TRAIL-induced apoptosis are not fully understood, downregulation of DRs (DR4 and DR5), upregulation of DcRs (DcR1 and DcR2) [4], overexpression of CASP8– and FADD-like apoptosis regulator (c-FLIP), as well as of downstream anti-apoptotic molecules – such as those belonging to Bcl-2 (e.g., Bcl-2, Bcl-xL) and IAP (e.g., XIAP, survivin) families – are the most commonly proposed TRAIL resistance mechanisms [13]. These observations, together with the demonstration that the above defects are reversible, suggest the need of a combinatorial approach to overcome the CRC cell resistance to TRAIL, and many researchers are making efforts to identify TRAIL sensitizers [15].

By studies of drug repositioning – defined as the investigation of existing drugs for the treatment of pathologies falling outside the scope

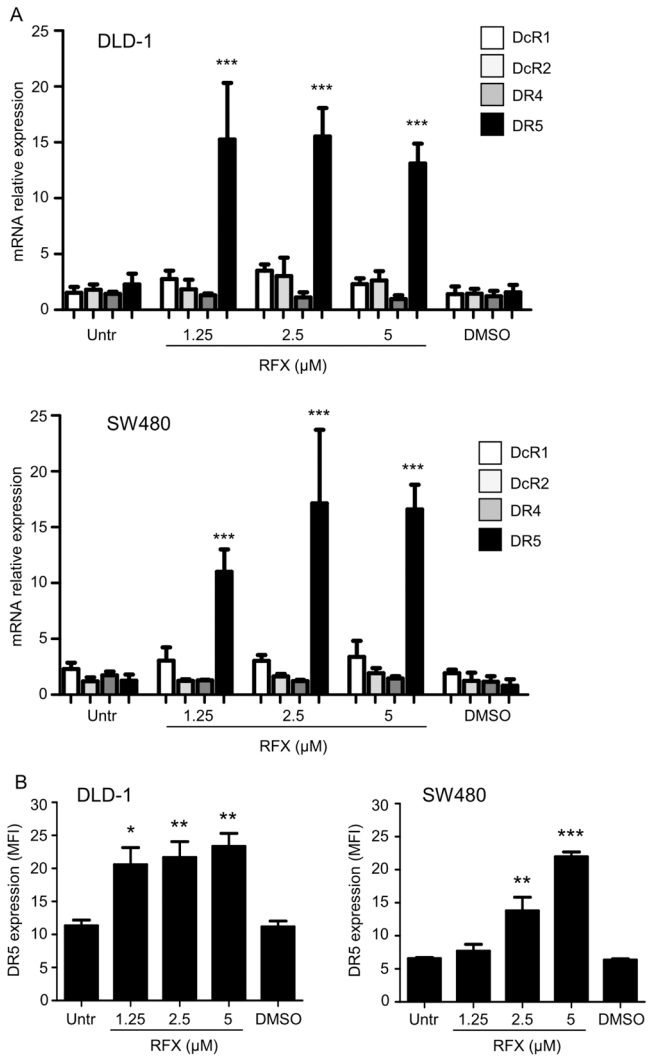

Fig. 3. Rafoxanide (RFX) enhances DR5 expression in DLD-1 and SW480 cells. A. Cells were either left untreated (Untr) or treated with increasing doses of RFX or DMSO (vehicle) for 24 h. DcR1, DcR2, DR4 and DR5 RNA transcripts were then evaluated by real-time PCR. Levels are normalized to β-actin. Values are mean ± SEM of 3 experiments (DR5 expression: DMSO vs RFX-treated cells, *** $P < 0.001$). B. Flow cytometric analysis of DR5 Mean Fluorescence Intensity (MFI) expression in DLD-1 and SW480 cells treated as indicated in A. Histograms indicate mean ± SEM of 3 experiments. DMSO vs RFX-treated cells, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of the original medical indication – the anthelmintic drug rafoxanide has recently emerged as a potent anti-tumor agent for different cancer types [16–19]. In particular, we reported that rafoxanide restrained the proliferation of CRC cells, but not of normal colonic epithelial cells, both in vitro and in a preclinical model mimicking sporadic CRC [20], and identified the drug as a *bona fide* immunogenic cell death (ICD) inducer [21].

As our above-mentioned findings were linked with the induction of endoplasmic reticulum (ER) stress, a phenomenon that has been involved in the regulation of the expression of various components of the TRAIL-driven apoptotic pathway [22–24], we sought to determine whether rafoxanide could restore the sensitivity of CRC cells to TRAIL.

2. Materials and methods

2.1. Animals

BALB/c mice were obtained from the Charles River Laboratories (Lodi, Italy) and maintained in filter-topped cages on autoclaved food and water at the University of Rome "Tor Vergata" animal facility (Rome, Italy). All animal experiments were approved by the animal ethics committee according to Italian legislation on animal experimentation [authorization 494/2017-PR, registered with the Italian Ministry of Health] and in compliance with European rules [EU Directive 2010/63/EU for animal experiments].

2.2. Patients and samples

Tissue samples were taken from the tumor area of 3 patients who underwent colon resection for sporadic CRC (all with TNM stages II–III) at the Tor Vergata University Hospital (Rome, Italy) and used for the generation of organoids. No patients received radiotherapy or chemotherapy before undergoing surgery. The human studies were approved by the local ethics committee (protocol n° R.S. 131.17) and each patient gave written informed consent. The study methodologies conformed to the standards set by the Declaration of Helsinki.

2.3. Cell cultures

Unless otherwise noted, reagents, chemicals, culture media and supplements were purchased from Sigma-Aldrich (Milan, Italy). The human CRC cell line HCT-116 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in McCoy's 5A medium supplemented with 10 % fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The human CRC cell lines DLD-1 and SW480, and the murine colon adenocarcinoma cell line CT26 were obtained from ATCC and maintained in RPMI 1640 medium supplemented with 10 % FBS and 1 % P/S. The human normal colonic epithelial cell line HCEC-1CT was obtained from EVERCYTE GmbH (Vienna, Austria) and maintained in ColoUp medium (EVERCYTE GmbH). Cell lines were authenticated by STR DNA fingerprinting using the PowerPlex 18D System kit according to the manufacturer's instructions (Promega, Milan, Italy). The STR profiles of all the cell lines matched the known DNA fingerprints. All the cells were used at a passage number between 10 and 25 and were routinely tested for mycoplasma contamination. Rafoxanide [PubChem CID: 31475] was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

To test the susceptibility of CRC and normal colonic epithelial cells to TRAIL, cells were incubated with increasing doses of recombinant TRAIL (12.5–100 ng/ml, human: cat.310–04, mouse: cat.315–19, Peprotech, London, UK) for 24 h. To evaluate whether rafoxanide sensitizes CRC cells to TRAIL-driven apoptosis and activates Caspase-8, cells were pre-incubated with increasing doses of rafoxanide (1.25–5 μM) for 8 h and then stimulated with TRAIL (12.5 and 50 ng/ml) for further 16–24 h. To evaluate the role of the proteasome pathway in the rafoxanide–mediated regulation of c-FLIP and survivin, cells were pre-incubated with the proteasome inhibitor lactacystin (10 μM) or DMSO for 30 min and then stimulated with rafoxanide for further 16 h.

2.4. Analysis and quantification of cell apoptosis and caspase-8 activation

To assess cell death and apoptosis, cells were stained with fluorescein isothiocyanate–conjugated Annexin V according to the manufacturer's instructions (Immunotools, Friesoythe, Germany) and 5 mg/ml propidium iodide (PI) for 20 min at 4 °C, and their fluorescence was measured using the FL-1 and FL-3 channels of Gallios (Beckman Coulter, Milan, Italy) flow-cytometer using Kaluza acquisition software. Viable cells were considered as AV-/PI- cells, apoptotic cells as AV+ /PI- cells, while secondary necrotic cells were characterized by AV+ /PI+ positive staining. EC_{50} values were calculated by Quest Graph EC50 Calculator, AAT Bioquest, Inc. (https://www.aatbio.com/tools/ec50-calculator). Caspase-8 activation was quantified by flow cytometry using the CaspGLOW™ Fluorescein Active Caspase-8 Staining Kit (Thermo Fisher

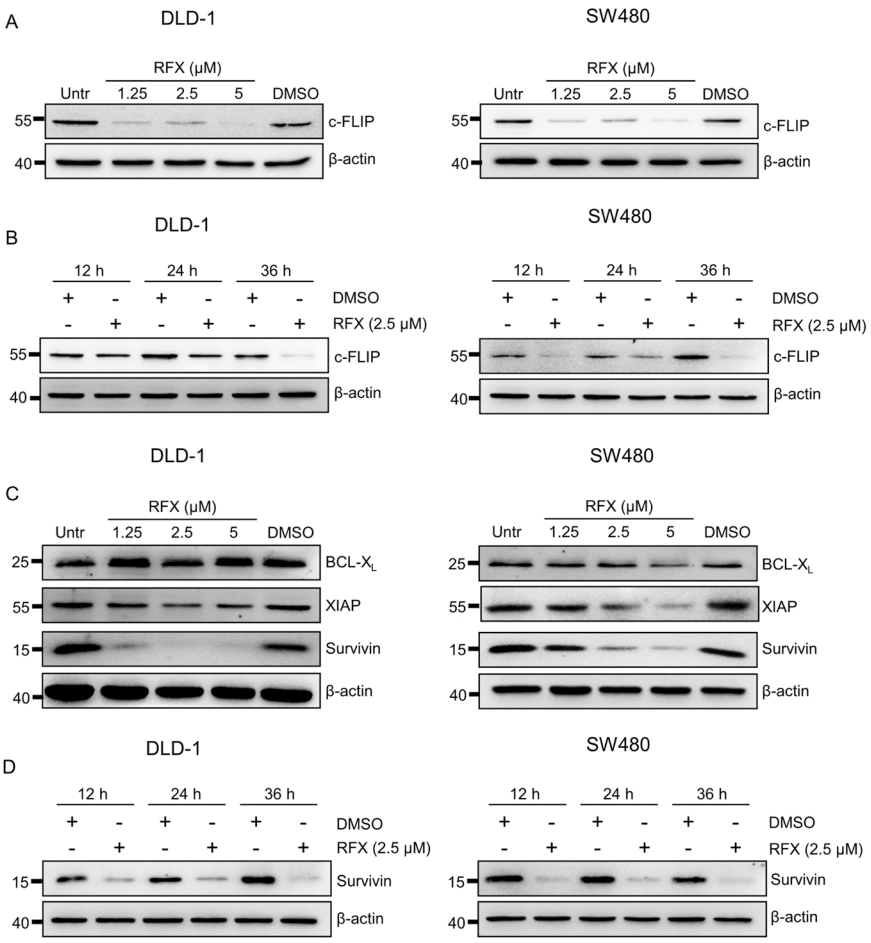

Fig. 4. Effect of rafoxanide (RFX) on the expression of TRAIL-related anti-apoptotic proteins in DLD-1 and SW480 cells. A. Total proteins from cells either left untreated (Untr) or treated with RFX or DMSO (vehicle) for 24 h were extracted and evaluated for c-FLIP expression by Western blotting. β-actin was used as loading control. One of 3 representative experiments where similar results were obtained is shown. B. Total proteins from cells treated with either 2.5 μM RFX or DMSO (vehicle) for the indicated time points were extracted and evaluated for c-FLIP expression by Western blotting. β-actin was used as loading control. One of 3 representative experiments where similar results were obtained is shown. C. Total proteins from cells treated as indicated in A were extracted and evaluated for Bcl-xL, XIAP and survivin by Western blotting. β-actin was used as loading control. One of 3 representative experiments where similar results were obtained is shown. D. Total proteins from cells treated as indicated in B were extracted and evaluated for survivin expression by Western blotting. β-actin was used as loading control. One of 3 representative experiments where similar results were obtained is shown.

Scientific, Milan, Italy) according to the manufacturer's instruction.

2.5. RNA extraction, cDNA preparation and Real-time PCR

Total RNA was extracted from cells by using Minikit Invitrogen™ PureLink™ RNA according to the manufacturer's instructions (Thermo Fisher Scientific). A constant amount of RNA (1 μg/sample) was reverse transcribed into cDNA, and 1 μL of cDNA per sample was then amplified by real-time PCR using SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA). Primers were as follows: DR4: FWD: 5′-CACAGCAATGGGAACATAGC-3′, REV: 5′-CAGGGACTTCTCTCTTCTTC-3′; DR5: FWD: 5′-GCCCCACAACAAAAGAGGTC-3′, REV: 5′-GGAGGTCATTCCAGTGAGTG- 3′; DcR1: FWD: 5´-AAAGTTCGTCGTCGTCATCG-3´; REV: 5 ´ACAGGCTCCAGTATGTTCTG-3 ´ , DcR2: FWD: 5 ´-AAGTTCCCCAGCAGACAGTG-3 ´; REV: 5 ´-GTACATAGCAGGCAAGAAGGC-3 ´; c-FLIP (long form), herein named c-FLIP: FWD: 5′-CGCTCAACAAGAACCAGTG-3′, REV: 5′-AGGGAAGTGAAGGTGTCTC-3′; and survivin: FWD: 5′-ACGACCCCATAGAGGAACATA-3′, REV: 5′ -CGCACTTTCTCCGCAGTTTC-3′. RNA expression was calculated relative to the housekeeping β-actin gene on the base of the ΔΔCt algorithm. β-actin: FWD: 5′-AAGATGACCCAGATCATGTTTGAGACC-3′, REV: 5′-AGCCAGTCCAGACGCAGGAT- 3′.

2.6. Analysis of DR4 and DR5 surface expression

Cells were stained with monoclonal anti-human phycoerythrin-conjugated DR4 or DR5 (Biolegend, San Diego, CA) or phycoerythrin-conjugated mouse control IgG1 (BD Biosciences, Heidelberg, Germany) and examined by flow cytometry. Positivity was defined as percentage of DR4- or DR5-positive cells and as mean fluorescence intensity (MFI) [25].

2.7. Western blotting and immunoprecipitation

Total proteins were extracted using the following lysis buffer: 10 mmol/L HEPES, 1 mmol/L EDTA pH 8.0, 60 mmol/L KCl, 0.2 % IGEPAL CA-630 (Nonidet P-40), 1 mmol/L sodium fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonylfluoride, and 1 mmol/L sodium orthovanadate, separated on an SDS-PAGE gel and transferred using a Trans-Blot Turbo apparatus (Bio-Rad Laboratories). Blots were then incubated with the following antibodies: caspase-8 and c-FLIP (ADI-AAM-118-E and ALX-804–961–0100, Enzo Life Sciences, Farmingdale, NY), Bcl-2 (sc-509), XIAP (sc-55550), Bcl-xL (sc-8392), survivin (sc-17779) (all from Santa Cruz Biotechnology) and β-actin (A-1978) followed by a secondary antibody conjugated to horseradish peroxidase. For immunoprecipitation studies, the protein lysates were prepared from DLD-1 and SW480 cells treated with either DMSO (vehicle) or 2.5 μM rafoxanide for 1–3 h. Proteins were immunoprecipitated with 2 μg of anti-ubiquitin (sc-8017, Santa Cruz Biotechnology) or control isotype for 1 h and then incubated with SureBeads™ protein G Magnetic Beads (Bio-Rad Laboratories) according with the manufacturer's instructions. The resulting immunoprecipitates were washed thoroughly 3 times with cold PBS-Tween 0.1 %, separated by SDS/PAGE, and immunoblotted for c-FLIP and survivin. For the input control, 5% of the amount of total proteins used in the pull-down assay were separated by SDS/PAGE, and immunoblotted for c-FLIP, survivin and β-actin. Membrane acquisition was performed in chemiluminescence with the ChemiDoc Imaging System (Bio-Rad Laboratories).

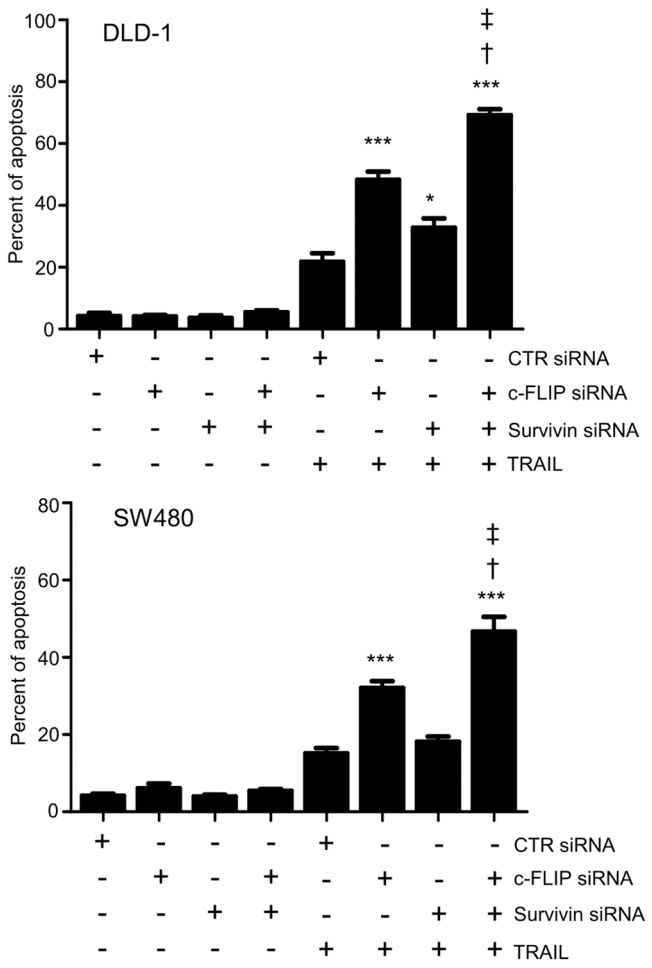

Fig. 5. Effect of c-FLIP and/or survivin knock-down on TRAIL-driven apoptosis in DLD-1 and SW480 cells. Cells transfected as indicated in the materials and methods section were treated or not with 50 ng/ml TRAIL for 24 h. Apoptosis was assessed by flow-cytometry. Data indicate mean ± SEM of 3 experiments (TRAIL-treated cells: CTR siRNA vs. c-FLIP and/or survivin siRNA, *, $P < 0.05$, ***, $P < 0.001$; c-FLIP siRNA vs. c-FLIP + survivin siRNA, †, $P < 0.001$; survivin siRNA vs. c-FLIP + survivin siRNA, ‡, $P < 0.001$).

2.8. DR5, c-FLIP and survivin knockdown by siRNA

To investigate the role of DR5, c-FLIP and survivin in TRAIL-mediated apoptosis, DLD-1 and SW480 cells were transfected with short interfering RNA (siRNA)s targeting DR5, c-FLIP and survivin (either alone or in combination, both from Santa Cruz Biotechnology), or control siRNA (Santa Cruz Biotechnology) using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according with the manufacturer's instructions.

2.9. Generation and culture of patient-derived CRC organoids

Surgically resected intestinal tumor tissues were washed in Hanks' balanced salt solution containing 1 % P/S and chopped into approximately 5 mm pieces. Tissue fragments were placed in a tube and incubated in Advanced DMEM/F12 medium (Thermo Fisher Scientific) containing 15 mM EDTA and rocked at 4 °C for 30 min. Large chunks of tissue were then removed and the remaining crypts centrifugated at 200 g for 3 min, embedded in matrigel (BD Biosciences) and seeded in a warmed 24-well plate. Matrigel was allowed to solidify for 30 min at 37 °C and overlaid with complete medium (IntestinCult OGM Human, #06010, StemCell Technologies, Cologne, Germany) supplemented with 10 μM of Y27632, a selective inhibitor of Rho-associated, coiled-coil containing protein kinase (ROCK) (#72302, StemCell Technologies). The entire medium was replaced every 3 days. The established organoids were then cultured with either DMSO or rafoxanide for 24 h. At the end, culture medium was removed and organoids were washed with PBS and incubated with organoid harvesting solution (Bio-Techne, Milan, Italy) for 1 h at 4 °C with gentle shaking. Released organoids from depolymerized matrigel were then collected and transferred into a cryomold containing Optimal Cutting Temperature (OCT), frozen and stored at − 80 °C.

2.10. Effect of rafoxanide and TRAIL on the in vivo growth of CT26-derived tumors

CT26-derived grafts were generated in co-housed 8–10-wk-old female BALB/c mice as previously described [26]. After 2 weeks, mice with similar tumor volume, determined by caliper measurements, were divided into 4 groups. The sham group received intraperitoneal (i.p.) injections of 10 % DMSO in PBS (vehicle) every other day starting from day 15, the second group received i.p. injections of 1 mg/kg/mouse recombinant mouse TRAIL (catalog no. 1121-TL/CF; Bio-Techne) dissolved in PBS every other day starting from day 15, the third group received i.p. injections of 7.5 mg/kg/mouse rafoxanide (in 10 % DMSO in PBS) every other day starting from day 15, and the last group received i.p. injections of rafoxanide and TRAIL at doses and time points indicated above. The dose of rafoxanide chosen for in vivo experiments (that is, 7.5 mg/kg), corresponding to 0.61 mg/kg rafoxanide for possible translation in humans [27] was selected in accordance with that currently used in veterinary treatment (i.e., 7.5–10 mg/kg) and in our previous in vivo experimental work [20]. The dose of TRAIL (1 mg/kg), was chosen based on that previously used in the same experimental tumor model in our previous work [24]. Mice were sacrificed at day 28. Tumors were excised, photographed, their volume calculated as previously described [26], and used for histochemistry experiments.

2.11. Histochemistry

Cryosections of CT26 cell-derived tumors were stained with primary antibodies directed against cleaved Caspase-3 (Asp175, Cell Signaling Technology). Hematoxylin eosin control-stained sections were prepared under identical histochemical conditions. Cryosections of CRC patient-derived organoids were stained with primary antibodies directed against c-FLIP (Enzo Life Sciences) and survivin (Santa Cruz Biotechnology). Positive cells were visualized using MACH4 Universal HRP-Polymer kit with DAB (Biocare Medical, Pacheco, CA). For immunofluorescence studies, cryosections of CT26 cell-derived tumors were fixed with paraformaldehyde (4 % final concentration), permeabilized with Triton X-100, and then blocked at room temperature with bovine serum albumine 1 %, Tween 0.1 %, and glycine 2 %. After overnight incubation at 4 °C with primary antibodies against c-FLIP and survivin (both from Santa Cruz Biotechnology), sections were incubated with a Alexa Fluor 568 and Alexa Fluor 488 secondary antibodies respectively (both from Thermo Fisher Scientific) for 1 h at room temperature. Slides were finally mounted using the prolong gold antifade reagent with DAPI (Thermo Fisher Scientific) Sections were analyzed by LEICA DMI4000 B microscope using LEICA application suite software (V4.6.2).

2.12. Statistical analysis

Data were analyzed using the two-tailed Student's t test for comparison between two groups or one-way ANOVA followed by Tukey's test for multiple comparisons. Significance was defined as p-values $<$ 0.05.

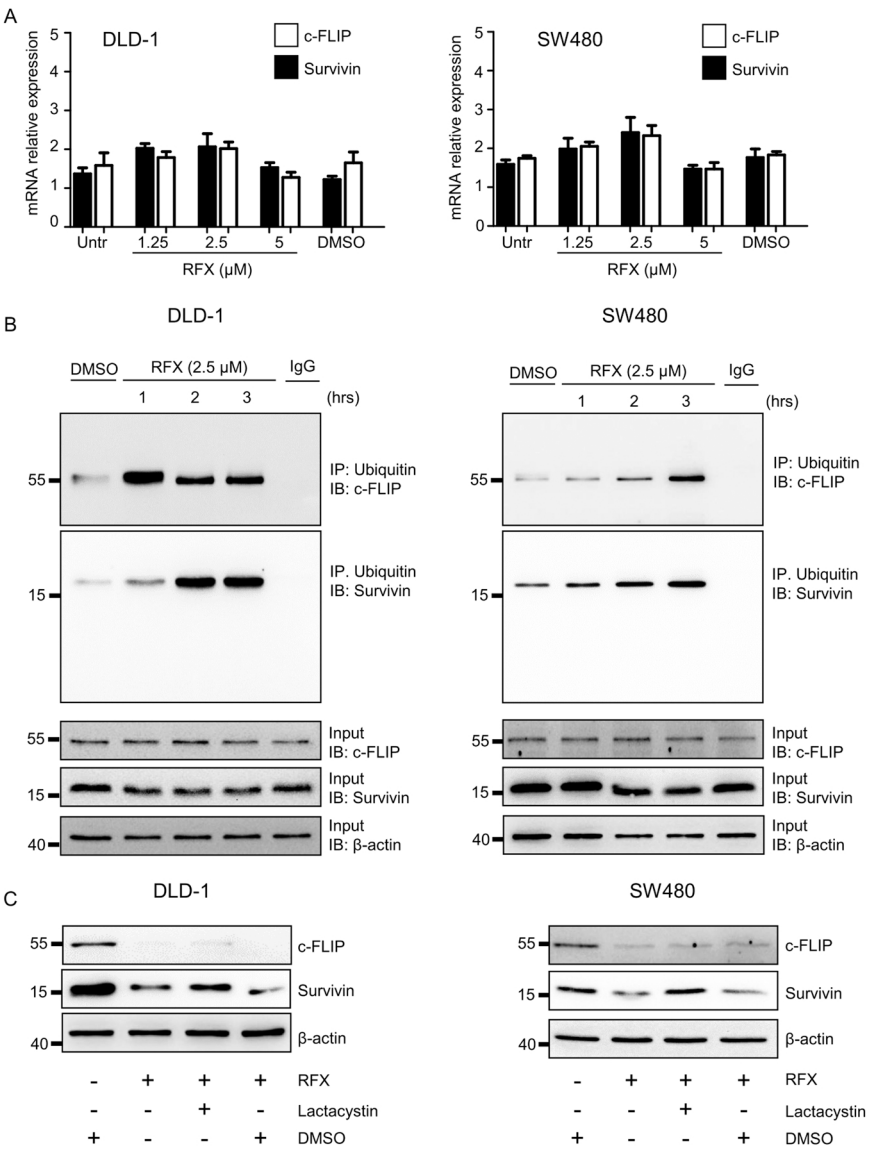

Fig. 6. Rafoxanide (RFX) promotes c-FLIP and survivin ubiquitination in DLD-1 and SW480 cells. A. RFX does not significantly affect c-FLIP and survivin RNA transcripts. Cells were either left untreated (Untr) or treated with increasing doses of RFX or DMSO (vehicle) for 24 h. c-FLIP and survivin RNA transcripts were then evaluated by real-time PCR. Levels are normalized to β-actin. Values are mean ± SEM of 3 experiments. B. Expression of ubiquitinated c-FLIP and survivin proteins in extracts of DLD-1 and SW480 treated with either DMSO (vehicle) or 2.5 μM RFX for 1–3 h. Proteins were subjected to immunoprecipitation (IP) with an anti-human ubiquitin or control isotype (IgG) antibody as indicated in the Materials and methods section and then to immunoblot (IB) analysis using anti-c-FLIP and anti-survivin antibodies. Input control was performed as indicated in the materials and methods section. One of 2 independent experiments in which similar results were obtained is shown. C. Effect of the proteasome inhibitor lactacystin on rafoxanide-mediated c-FLIP and survivin down-regulation. DLD-1 and SW480 cells were pre-incubated with either lactacystin (10 μM) or DMSO (vehicle) for 30 min and then treated or not with 2.5 μM rafoxanide for further 16 h. c-FLIP and survivin expression was assessed by Western blotting. One of 3 experiments in which similar results were obtained is shown.

3. Results

3.1. Rafoxanide sensitizes CRC but not normal colonic epithelial cells to TRAIL-mediated apoptosis

In preliminary studies we confirmed that CRC cells differently respond to TRAIL in terms of apoptosis [28]. TRAIL dose-dependently triggered HCT-116 cell apoptosis (EC_{50}: 18.47 ± 3.44 ng/ml) whereas DLD-1 and in particular SW480 cells were largely resistant against TRAIL-induced cell death (TRAIL EC_{50}: DLD-1: 44.55 ± 4.88 ng/ml, SW480: 46.08 ± 5.55 ng/ml) (Suppl. Fig. 1A-B). TRAIL did not affect the viability of normal colonic epithelial cells (i.e., HCEC-1CT cells) (Suppl. Fig. 1A-B) thus corroborating the notion of its cancer cell selectivity. Based upon these preliminary data, we selected the doses of 12.5 ng/ml (HCT-116) and 50 ng/ml TRAIL (DLD-1 and SW480) for the next studies. Initially we evaluated whether rafoxanide was able to enhance TRAIL-driven death in CRC cells. Pre-treatment of HCT-116, DLD-1 and SW480 cells with rafoxanide markedly increased the percentage of apoptosis following TRAIL treatment (Fig. 1 and Suppl. Fig. 2). Of note, rafoxanide, either alone or in combination with TRAIL, did not increase the fraction of apoptotic HCEC-1CT cells (Suppl. Fig. 2). The concentration of rafoxanide causing the maximal TRAIL sensitizing effect on CRC cells (i.e., 5 μM), strongly promoted TRAIL-mediated apoptosis in both DLD-1 and SW480 cell lines, with an EC_{50} of 2.1 ± 0.13 μM and 2.14 ± 0.19 μM respectively, without affecting cell viability as single treatment (EC_{50} of 2.53 ± 0.32 μM and 2.77 ± 0.18 μM respectively) (Fig. 1). Given the resistance of DLD-1 and SW480 cells to TRAIL, these cell lines were chosen to investigate the mechanisms by which rafoxanide increased TRAIL sensitivity.

3.2. Rafoxanide increases TRAIL-driven caspase-8 activation in CRC cells

As DR4/DR5-driven signals lead to activation of caspase-8, an enzyme involved in the TRAIL-dependent apoptotic pathway [29], we next monitored caspase-8 activation by flow-cytometry. The fraction of DLD-1 cells and SW480 cells expressing active caspase-8 as well as pro-caspase-8 protein levels were not significantly affected by either TRAIL or rafoxanide alone (Fig. 2 and Suppl. Fig. 3). Combination of rafoxanide and TRAIL significantly enhanced pro-caspase-8 cleavage and the fraction of active caspase-8–expressing CRC cells as compared with cells treated with single compounds (Fig. 2 and Suppl. Fig. 3). To dissect the mechanism by which rafoxanide enhances the sensitivity of CRC cells to TRAIL, we first investigated whether rafoxanide affected the expression of DRs and DcRs. Treatment of DLD-1 and SW480 cells with

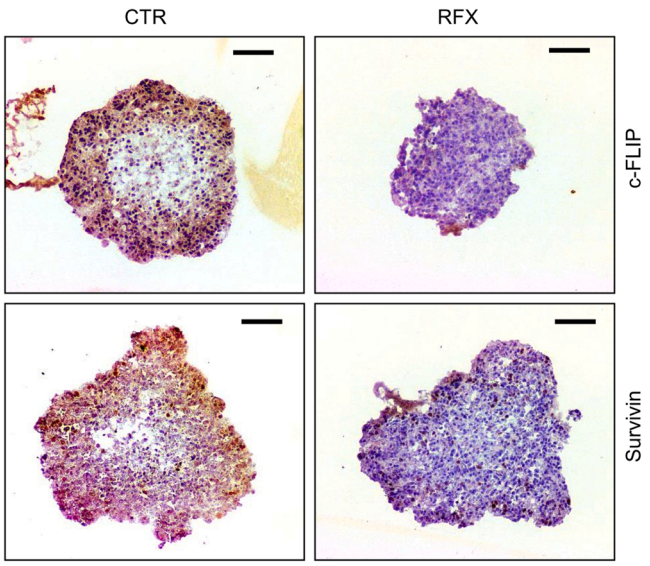

Fig. 7. Representative pictures of c-FLIP- and survivin-stained sections of CRC explant-derived organoids treated with either DMSO (CTR) or 2.5 μM rafoxanide (RFX) for 24 h. One of 3 independent experiments in which similar results were obtained is shown. The scale bars are 200 μm.

rafoxanide greatly enhanced the DR5 RNA transcripts (Fig. 3A). By contrast, no significant change in DcR1, DcR2 and DR4 RNA transcripts was seen in both DLD-1 and SW480 cells following rafoxanide exposure (Fig. 3A). Flow-cytometry analysis confirmed that rafoxanide increased DR5 (Fig. 3B) but not DR4 (not shown) surface expression in both DLD-1 and SW480 cells. To determine whether upregulation of DR5 by rafoxanide played a role in the sensitization of DLD-1 and SW480 cells to TRAIL, we tested the effect of rafoxanide + TRAIL on the apoptosis of cells transfected with DR5 siRNA. Knock-down of DR5 significantly abrogated the pro-apoptotic effect of rafoxanide and TRAIL on both cell lines (Suppl. Fig. 4). However, the fact that virtually all DLD-1 and SW480 cells (99.5 ± 0.2 and 98.4 ± 0.3 respectively) expressed membrane DR5 (Suppl. Fig. 5) even though they were largely resistant to TRAIL, strongly suggested the presence of interference in the caspase-8 activation and/or along the downstream TRAIL apoptotic pathway.

3.3. Rafoxanide negatively affects c-FLIP and survivin expression in CRC cells

At the DISC level, caspase-8 activation and apoptosis signaling is negatively regulated by c-FLIP [30]. c-FLIP is overexpressed in cancer cells and its suppression promotes caspase-8-dependent apoptosis both in vitro and in vivo and sensitizes these cells to TRAIL [31–33]. Rafoxanide strongly inhibited c-FLIP protein expression in both in DLD-1 and SW480 cells (Fig. 4A-B). Analysis of other anti-apoptotic intracellular proteins associated with TRAIL resistance in CRC cells (i.e., Bcl-2, Bcl-xL, XIAP and survivin) revealed that rafoxanide markedly reduced survivin protein expression in a dose-dependent fashion both in DLD-1 and SW480 cells (Fig. 4C). Time course studies confirmed the inhibitory effect of the drug on survivin (Fig. 4D). In contrast, rafoxanide slightly reduced Bcl-xL and XIAP in SW480 cells only when used at 5 μM whereas no relevant changes in the expression of such proteins were seen in rafoxanide-treated DLD-1 cells (Fig. 4C). Interestingly, we found a barely detectable expression of Bcl-2 in both cell lines (not shown). Altogether, these data argued against a major role of Bcl-2, Bcl-xL and XIAP in the rafoxanide-mediated TRAIL-apoptosis. Given these results and the experimental evidence indicating both c-FLIP and survivin to interfere with TRAIL-induced cell death in various cancer cells [24, 33–36], we hypothesized that c-FLIP and survivin could be involved in the rafoxanide-mediated TRAIL-induced apoptosis. To test such an assumption, we knocked-down c-FLIP and survivin, either alone or in combination, in DLD-1 and SW480 cells through siRNAs. Neither c-FLIP nor survivin knock-down influenced the effects of rafoxanide in promoting TRAIL-mediated DLD-1 and SW480 cell apoptotis (not shown) as well as the viability of such cells in the absence of TRAIL (Fig. 5 and Suppl. Fig. 6). Both cell lines became susceptible to TRAIL-induced apoptosis upon transfection with c-FLIP siRNA whereas survivin silencing only partially restored TRAIL sensitivity in DLD-1 but not in SW480 cells. Notably, contemporary transfection of cells with both siRNAs resulted in the higher fraction of apoptotic cells following TRAIL stimulation as compared with the single siRNAs alone (Fig. 5 and Suppl. Fig. 6).

To start dissecting the mechanism/s by which rafoxanide hampered c-FLIP and survivin levels in CRC cell lines, we investigated whether the drug could modulate *c-FLIP* and *SURVIVIN* gene transcription. Treatment of DLD-1 and SW480 cells with rafoxanide did not significantly affect both c-FLIP and survivin RNA transcripts, although a slight decrease in their levels was observed at the higher dose of the drug (i.e., 5 μM) in both cell lines (Fig. 6A). These results suggested a post-transcriptional control of c-FLIP and survivin expression by rafoxanide. Indeed, we next showed that rafoxanide enhanced the ubiquitination of both c-FLIP and survivin (Fig. 6B), an event followed by protein degradation via the proteasome machinery. To test whether the rafoxanide-mediated c-FLIP and survivin ubiquitination led to proteasome degradation, DLD-1 and SW480 cells were pre-incubated or not with the proteasome inhibitor lactacystin and then stimulated with either DMSO (vehicle) or rafoxanide. Survivin expression in protein lysates taken from cells receiving both lactacystin and rafoxanide was markedly higher as compared with lysates extracted from cells treated with either rafoxanide alone or rafoxanide plus DMSO (Fig. 6C). In contrast, no relevant changes in c-FLIP expression were observed regardless of whether rafoxanide treated DLD-1 and SW480 cells were pre-incubated or not with lactacystin (Fig. 6C).

3.4. Rafoxanide inhibits c-FLIP and survivin expression in CRC patient-derived organoids

To investigate whether rafoxanide was able to affect c-FLIP and survivin *ex vivo*, 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 [37]. Rafoxanide was added to CRC patient-derived organoids and c-FLIP and survivin expression was analyzed after 24 h by immunohistochemistry. Consistently with results obtained in cultured CRC cells, rafoxanide markedly reduced c-FLIP and survivin protein expression in this experimental setting (Fig. 7).

3.5. Rafoxanide synergizes with TRAIL in inhibiting the in vivo growth of CT26-derived tumors in mice

To translate these observations into mice, we assessed whether rafoxanide and TRAIL synergized in inducing apoptosis in a syngeneic experimental model of CRC in which tumors are generated by injecting the murine CRC cell line, CT26, into BALB/c mice. We previously reported that CT26 cells are resistant to TRAIL-induced apoptosis [24]. Initially, we showed that rafoxanide restored the susceptibility of cultured CT26 cells to TRAIL (Suppl. Fig. 7). Subsequently, mice with CT26-derived grafts were injected with TRAIL and/or rafoxanide. The graft tumor volume of mice receiving TRAIL alone did not significantly differ from that seen in sham mice. In contrast, mice treated with rafoxanide exhibited a significant decrease in the tumor volume as compared with sham (Fig. 8A). The tumor growth of mice receiving both rafoxanide and TRAIL was significantly reduced as compared with mice treated with rafoxanide alone (Fig. 8A). Mice treated with rafoxanide and TRAIL exhibited enhanced cell apoptosis (Fig. 8B). In line with the

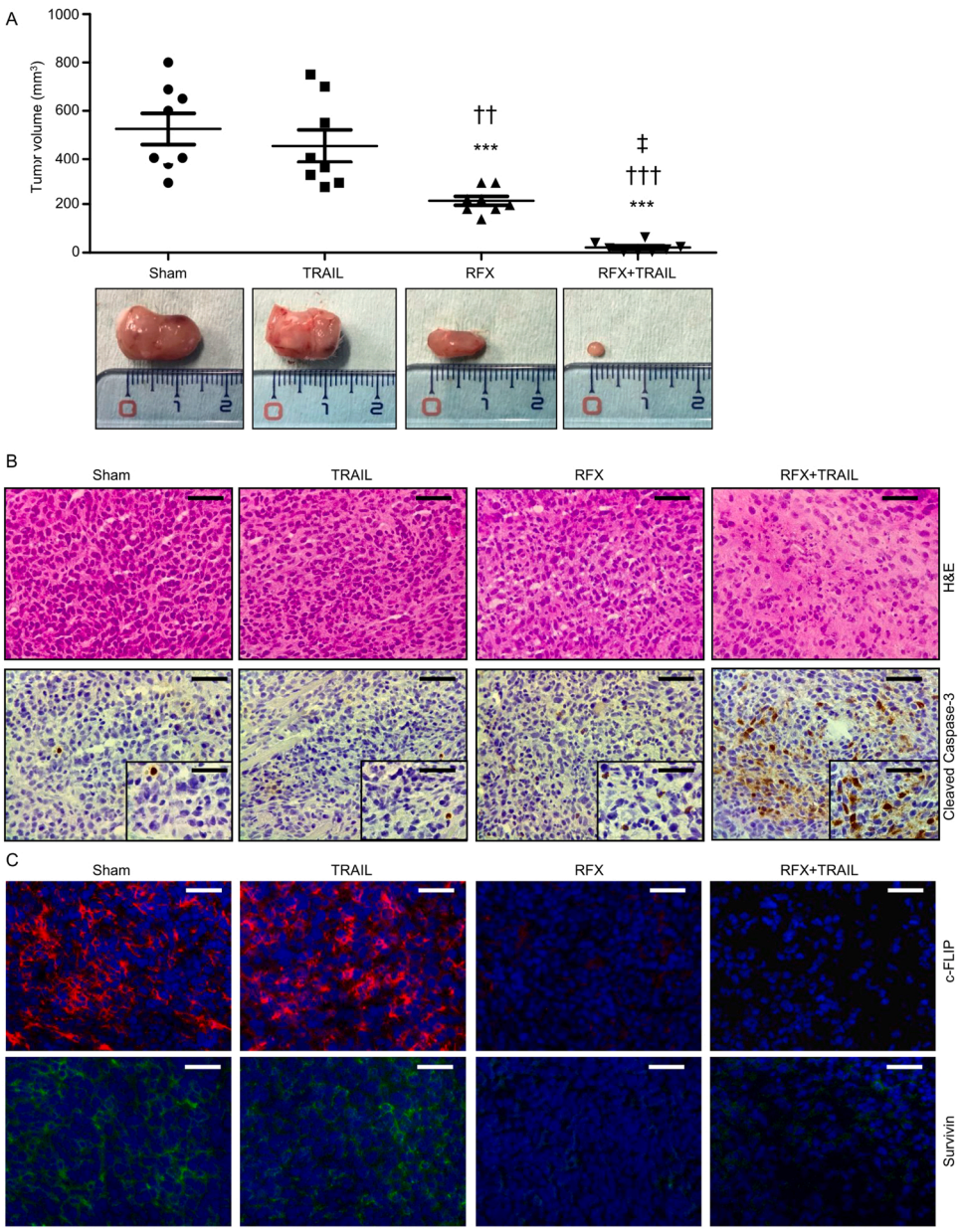

Fig. 8. Rafoxanide (RFX) synergizes with TRAIL in inhibiting the growth of CT26-derived tumors in mice. A. Representative histograms showing the tumor volume of CT26-derived grafts taken from sham mice and mice treated with TRAIL (1 mg/kg/mouse), RFX (7.5 mg/kg/mouse) or RFX+TRAIL, as described in the materials and methods section. Data indicate mean ± SEM of 2 independent experiments in which 4 mice per group were considered. Each point in the graph indicates the volume of CT26-derived tumors in a single mouse (Sham vs. RFX and RFX + TRAIL: *** $P < 0.001$; TRAIL vs. RFX and RFX + TRAIL ††, $P < 0.01$; †††, $P < 0.001$; RFX vs. RFX + TRAIL: ‡, $P < 0.05$.). Bottom insets: representative photographic images of grafts taken from mice treated as indicated above. B. Representative pictures of hematoxylin and eosin (H&E)- and cleaved caspase 3-stained sections of grafts taken from mice treated as indicated in A. The scale bars are 60 μm. The scale bars in the insets are 30 μm. One of 3 representative experiments is shown. C. Representative immunofluorescence pictures of c-FLIP and survivin-stained sections of grafts taken from mice treated as indicated in A. The scale bars are 60 μm. One of 3 representative experiments is shown.

in vitro observations in human CRC cell lines, grafts of mice treated with rafoxanide, either alone or in combination with TRAIL, showed c-FLIP and survivin reduction (Fig. 8C). Rafoxanide would seem well-tolerated as no significant changes in body weight were observed in mice treated with the drug, both alone and in combination with TRAIL, as compared with sham (not shown). Such observation is in line with our previous work [20] and with the evidence reported by Xiao et al. in a multiple myeloma xenograft model, showing no significant side effects of the drug in mice receiving intraperitoneal injections of 15 mg/kg rafoxanide every other day for 14 days [19].

4. Discussion

A major hurdle to the use of TRAIL-based therapeutics in the clinic is that cancer cells, including CRC cells, are either intrinsically resistant or acquire resistance to TRAIL [13,14]. Therefore, agents able to overcome CRC cell resistance to TRAIL have great therapeutic potential and could improve the way we manage patients with this neoplasia. In this study we present data indicating that rafoxanide restores the susceptibility of CRC cells, but not of normal colonic epithelial cells, to TRAIL-induced apoptosis. This action was evident in DLD-1 and SW480 cells, both possessing mutated *P53* and bearing *G13D* and *G12V* KRAS mutations respectively. Thus, the findings provide evidence for the potential use of rafoxanide as TRAIL sensitizer also in KRAS-mutated cancers, where TRAIL was found to miss its pro-apoptotic ability and even to promote tumor growth and invasion [38–40].

The TRAIL sensitizing effect of rafoxanide on CRC cells was associated with enhanced caspase-8 activation as well as with the increased expression and cell membrane localization of DR5, but not DR4. In this context, it is worth mentioning previous reports demonstrating that DR5 serves a greater role in apoptosis compared with that of DR4 [41,42] and indicating DR5 as the major determinant of TRAIL-driven cell death [24,43]. We also showed that almost all DLD-1 and SW480 cells expressed DRs on the cell membrane. These data were not surprising, as DR4 and DR5 overexpression has been reported in *KRAS* mutated CRCs [44], and pointed towards the presence of downstream TRAIL resistance mechanisms regulated by rafoxanide. Indeed, by analyzing the effects of the drug on the expression of several TRAIL pathway-related anti-apoptotic

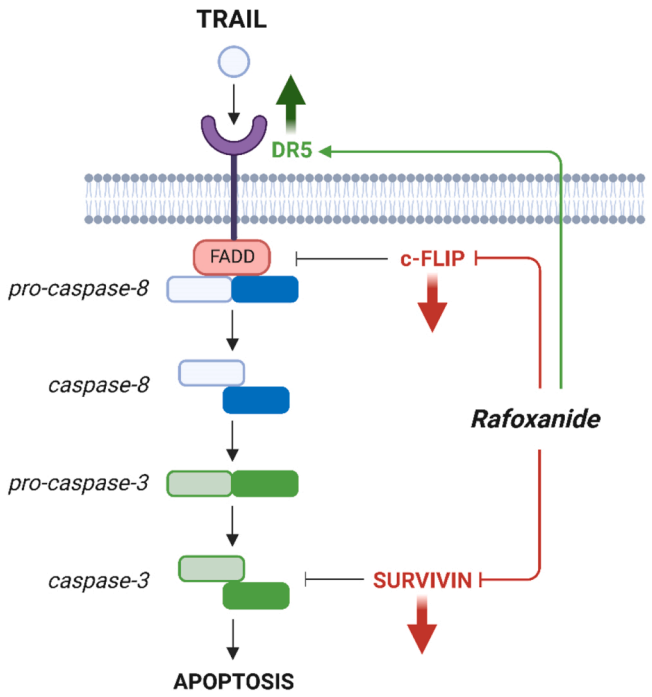

Fig. 9. Diagram of the putative molecular mechanisms underlying the TRAIL sensitizing activity of rafoxanide in colorectal cancer cells. "Created with BioRender.com.".

proteins, we observed a consistent, marked reduction of c-FLIP and survivin levels in both DLD-1 and SW480 cell lines upon rafoxanide exposure. Interestingly, we found a barely detectable expression of Bcl-2 in both cell lines thus arguing against a major role for this protein in conferring TRAIL resistance in our setting. These data are corroborated by accumulating studies indicating a progressive decrease in Bcl-2 levels during tumor progression and suggesting Bcl-2 to play less of a role in CRC survival and resistance [45]. Our observations are consistent with a large body of evidence showing that high c-FLIP and survivin expression occurring in different cancer types (including CRC) have strong inhibitory effects on TRAIL therapy [24,33–36] and suggest the modulation of these proteins as crucial events in the induction of CRC cell apoptosis following treatment with rafoxanide and TRAIL. In support of this hypothesis are functional experiments demonstrating that silencing of both c-FLIP and survivin in DLD-1 and SW480 cells in the presence of TRAIL resulted in a fraction of apoptotic cells similar to that observed in cells cultured with rafoxanide plus TRAIL. At least in this experimental setting, our results also pinpointed the main role of c-FLIP as compared to survivin in blocking the TRAIL signaling pathway, likely due to the upstream location and inhibitory activity of the former in the apoptotic cascade.

To investigate the mechanism/s by which rafoxanide negatively affected c-FLIP and survivin expression in CRC cells, we initially evaluated whether the drug reduced *c-FLIP* and *SURVIVIN* gene transcription. Dose response studies performed at 24 h revealed that both c-FLIP and survivin RNA transcripts were not substantially altered by rafoxanide treatment, in contrast with the dramatic down-regulation observed at the protein level in both cells lines at the same time point even at the lower dose of the drug (i.e., 1.25 μM). It is thus likely that regulation of c-FLIP and survivin by rafoxanide occurs at post-transcriptional level. Indeed, by immunoprecipitation and immunoblotting, we next showed that rafoxanide markedly increased c-FLIP and survivin ubiquitination in both DLD-1 and SW480 cells. Our data are consistent with previous studies showing that, in cancer cells, both c-FLIP and survivin can be polyubiquitinated and targeted for degradation by the ubiquitin/proteasome pathway [46–48]. Survivin but not c-FLIP protein expression was almost completely restored in rafoxanide-treated CRC cells cultured in the presence of the proteasome inhibitor lactacystin. A possible explanation for this latter result may rely on the presence of additional mechanisms other than proteasome-mediated degradation leading to a decrease of c-FLIP protein levels in CRC cells following rafoxanide treatment. In support of this view is a recent paper demonstrating the importance of protein synthesis inhibition in the down-regulation of c-FLIP expression upon ER stress induction in tumor cells [49]. As we previously reported that rafoxanide selectively induces ER stress in CRC cells [20], it is tempting to speculate that the negative effect of rafoxanide on c-FLIP protein expression may depend, at least in part, on a reduced activity of the protein synthesis machinery. As the ubiquitin antibody we used for immunoprecipitation experiments detects polyubiquitin chains linked by multiple lysine residues, we cannot tell whether the observed rafoxanide-driven protein ubiquitination occurs in a K48 or K63 manner. However, at least concerning survivin, the fact that lactacystin mostly abrogated the rafoxanide-driven protein down-regulation would point towards a K48-mediated ubiquitination. Future experimental efforts will be aimed at uncovering the precise mechanism/s involved in the rafoxanide-driven increase of c-FLIP and survivin ubiquitination (e.g, down-regulation of ubiquitin ligases, modulation of protein acetylases) [50] as well as the pathway/s underlying the rafoxanide-mediated down-regulation of the former in CRC cells.

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 lab research and preclinical models and optimal systems for the screening of new drugs [37]. Of note, we showed that culture of CRC organoids in the presence of rafoxanide resulted in a striking reduction of c-FLIP and survivin expression as compared to control.

Finally, we further strengthened our claims about the potential translational relevance of the TRAIL sensitizing effect of rafoxanide using an in vivo model of CRC. In this model, the combined therapy with rafoxanide and TRAIL resulted in a more pronounced tumor suppression and an increased cell apoptosis than that seen with single compounds. Consistently with our in vitro findings, the antineoplastic effects of rafoxanide associated with the downregulation of c-FLIP and survivin. In summary, we report the novel observation that rafoxanide acts as a selective TRAIL sensitizing agent against CRC cells in vitro and in vivo, likely by decreasing c-FLIP and survivin protein expression (Fig. 9).

Some limitations of the study to be considered in future experimental work are represented by the lack of comparison between the ability of rafoxanide to restore the TRAIL apoptotic cascade with that achieved by a positive control, the evaluation of rafoxanide as TRAIL sensitizing agent in additional preclinical models of CRC (preferably with lesions arising from the colonic tissue), and the need of more in-depth studies of dosage and long-term toxicity aimed at confirming the therapeutic potential and clinical benefit of the drug.

5. Conclusions

Collectively, our data suggest that rafoxanide could potentially be deployed as an anti-cancer drug in the combinatorial approaches aimed at overcoming CRC cell resistance to TRAIL-based therapies.

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CRediT authorship contribution statement

Federica Laudisi: Conceptualization, Investigation, Formal analysis, Methodology, Writing – original draft. **Teresa Pacifico:**

Investigation, Visualization, Writing – original draft. **Claudia Maresca:** Investigation. **Anderson Luiz-Ferreira:** Investigation. **Sara Antonelli:** Investigation. **Angela Ortenzi:** Investigation. **Alfredo Colantoni:** Investigation. **Antonio Di Grazia:** Investigation. **Eleonora Franzè:** Investigation. **Marco Colella:** Investigation. **Davide Di Fusco:** Investigation. **Giuseppe S. Sica:** Resources. **Ivan Monteleone:** Supervision, Funding acquisition. **Giovanni Monteleone:** Supervision, Funding acquisition. **Carmine Stolfi:** Conceptualization, Project administration, Supervision, Formal analysis, Validation, Visualization, Funding acquisition, Writing – review and editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Prof. Giovanni Monteleone reports a relationship with AbbVie Inc that includes: consulting or advisory.

Data availability

Data will be made available on request.

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None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113794.

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