SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 48: 205, 2022

Sulforaphane has an additive anticancer effect to FOLFOX in highly metastatic human colon carcinoma cells

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Received March 11, 2022; Accepted July 27, 2022

DOI: 10.3892/or.2022.8420

Abstract. Colorectal cancer (CRC) is one of the most common malignancies worldwide. Patients with CRC may need chemotherapy (CTx) in a neoadjuvant, adjuvant or palliative setting through the course of the disease. Unfortunately, its effect is limited by chemoresistance and chemotoxicity. Novel more effective and non-toxic CTx regimens are needed to further improve CRC treatment outcomes. Thus, the present study was designed to test the hypothesis that non-toxic sulforaphane (SF) is effective against CRC and has additive effects in combination with conventional 5-fluorouracil, oxaliplatin and folinic acid (FOLFOX) CTx *in vitro*. Highly metastatic human colon cancer cells, CX-1, and fibroblasts were treated with FOLFOX ± SF. Cell viability was assessed using an MTT assay. The level of apoptosis and the expression of apoptotic proteins were measured by TUNEL assay and quantitative PCR analysis. Aldehyde dehydrogenase isoform 1 (ALDH1) and multidrug resistance protein 2 (MRP2) levels were evaluated. The ability of cells to form spheroids was measured in three-dimensional cell culture. SF alone and in combination with FOLFOX effectively decreased the viability of the CX-1 cells, promoted apoptosis within the CX-1 cells, prevented cellular spheroid formation and decreased ALDH1 activity. However, SF promoted MRP2 expression and protein levels. In conclusion, SF together with conventional FOLFOX has additive anticancer effects against highly metastatic human CRC *in vitro*.

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Abbreviations: 5-FU, 5-fluorouracil; ALDH1, aldehyde dehydrogenase isoform 1; CRC, colorectal cancer; CTx, chemotherapy; FOL, folinic acid; MDR, multidrug resistance; MRP2, MDR protein 2; OX, oxaliplatin; SF, sulforaphane

Key words: sulforaphane, CRC, FOLFOX, CX-1, CTx

Introduction

Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-related deaths worldwide (1). Surgery remains the only potentially curative treatment option (2). However, almost 50% of surgical patients eventually relapse (3); therefore, systemic adjuvant chemotherapy (CTx) is needed to treat occult micrometastases for patients with advanced and high-risk tumors (3). Furthermore, CTx is the first-line option in cases of unresectable or metastatic CRC (4). Moreover, recent high-quality randomized control trials demonstrated the benefits of neoadjuvant CTx for advanced low rectal cancer (<5 cm from the anal verge) (5,6). Similarly, the focus on neoadjuvant CTx for colon cancer is increasing as well (7). Consequently, CTx already has a major role in modern CRC treatment and indications are for its expansion in the near future. Despite recent advances in the field of medical oncology, a significant proportion of patients undergoing current CTx regimens still face treatment failure

for chemoresistance or chemotoxicity (8). Thus, there is a need for novel, more effective and non-toxic CTx regimens to further improve the treatment outcomes of patients with CRC (8).

The phytochemical sulforaphane (SF), a major glucosinolate, is produced by the conversion of glucoraphanin by enzyme myrosinase, after intake of cruciferous vegetables such as broccoli, cabbage, cauliflower and kale (9,10). SF has an anti-tumorigenic effect against breast (11), prostate (12), bladder (13) and gastric (14) cancer *in vitro*. Furthermore, SF has low toxicity (14), thus it may be an attractive anticancer agent for the treatment of CRC. However, it remains unclear whether SF impairs the anti-tumorigenic potential of 5-fluorouracil (5-FU) and oxaliplatin (OX), both included in the conventional 5-FU, OX and folinic acid (FOLFOX) CTx for CRC. Also, some studies have raised caution about the safety of SF in the field of oncology, as it may play a role in the development of multidrug resistance (MDR) (15).

The present study was designed to test the hypothesis that SF works against CRC and has additive effects in combination with conventional CTx *in vitro*.

Materials and methods

Cell lines and drugs used in cell experiments. The highly metastatic human colon cancer CX-1 cell line (16) and human skin fibroblasts (both gifts from Professor Ingrid Herr, Division of Molecular OncoSurgery, Ruprecht-Karls-University, Heidelberg, Germany) were used for experiments. Cells were cultivated at 37˚C, in 5% CO_2, using Dulbecco's modified Eagle's medium-high glucose (PAA Laboratories GmbH; GE Healthcare) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH; GE Healthcare) and streptomycin (100 μg/ml)/penicillin (100 IU/ml) (both Biochrom AG).

5-FU (Pfizer Pharma GmbH), folinic acid (FOL) (Pfizer Pharma GmbH) and OX (Sanofi S.A.) were combined at a ratio of 2:20:1 in concentrations of 0.4, 4 and 0.2 μM, respectively, for cell experiments in order to represent FOLFOX CTx used in a human setting. A stock solution of SF (Calbiochem; Merck KGaA) was prepared in ethanol (99.8%; Carl Roth GmbH and Co. KG). Ethanol without SF was used as negative control. All samples used for the experiments had an equal final concentration of ethanol (<0.1%).

Cell viability assay. To determine the effects of SF and/or FOLFOX on CX1 and fibroblasts, cells were seeded into 96-well microplates at a density of $5x10^3$ per well and incubated for 24 h under standard conditions at 37˚C. Next, the media was changed to 20 μl culture medium supplemented with different concentrations of SF (2.5, 5, 10, 15 or 20 μM), FOLFOX or FOLFOX + SF (10 μM), and incubated for 48 h. For viability testing, the MTT assay was used as previously described (8,17).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). After cells had been treated for 24 h with SF (10 μM), FOLFOX or FOLFOX + SF (10 μM), mRNA was isolated using the RNeasy Mini Kit (Qiagen GmbH) and transcribed into cDNA using the First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH) according to the manufacturer's instructions. Custom-made primers for BAX (forward, 5'-GCA GATCATGAAGACAGGGG-3' and reverse, 5'-ACACTCGCT CAGCTTCTTGG-3') and BCL-2 (forward, 5'-GAACATTTC GGTGACTTCCG-3' and reverse, 5'-CCTGTTGATCATCCC TGGAG-3') were used, with GAPDH (forward, 5'-GACAGT CAGCCGCATCTTCT-3' and reverse, 5'-TTAAAAGCAGCC CTGGTGAC-3') as the endogenous control. MDR protein 2 (MRP2) primers (cat. no. QT00056294; Qiagen) were used with GAPDH (cat. no. QT01192646; Qiagen). qPCR was performed using a StepOne™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in triplicate. Briefly, qPCR was carried out for 40 cycles at 95˚C for 15 sec and extension at 60˚C for 60 sec. The fluorescent signal was measured at the end of the annealing phase of each cycle. The relative gene quantification was analyzed using the $^{\Delta\Delta}$Cq method described previously (18) using StepOne™ Software 2.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.).

TUNEL assay. After cells had been treated for 24 h with SF (10 μM), FOLFOX or FOLFOX + SF (10 μM), they were fixed in acetone (ROTIPURAN® ≥99.8%; Carl Roth GmbH and Co. KG) for 10 min at room temperature and DNA fragmentation was detected using an ApopTag® Peroxidase *in situ* Apoptosis Detection Kit according to the manufacturer's instructions (Merck KGaA). Briefly, fixed slides were prepared and incubated with 55 μl terminal deoxynucleotidyl transferase mixture in a humidified chamber at 37˚C for 1 h. Afterward, the slides were incubated with anti-digoxigenin conjugate for 30 min at room temperature, stained with diaminobenzidine peroxidase substrate (Vector Laboratories, Inc.) for 3 min at room temperature and counterstained with Mayer's hemalum solution (Merck Life Science UK, Ltd.) for 3 min at room temperature. Semi-quantitative analysis was performed by calculating the percentage of TUNEL-positive cells in 16 fields of view per condition under a light microscope.

Spheroid assay. For the spheroid assay, $5x10^3$ CX-1 cells were seeded in 12-well low-adhesion plates and cultured in NeuroCult® NS-A basal serum-free medium (Human) (Stemcell Technologies, Inc.) supplemented with 2 μg/ml heparin (Stemcell Technologies, Inc.), 20 ng/ml hEGF (R&D Systems, Inc.), 10 ng/ml hFGF-b (PeproTech, Inc.) and NeuroCult® NS-A proliferation supplements (Human) (Stemcell Technologies, Inc.) for 24 h at 37˚C. After cells had been treated with SF (1.25 μM), FOLFOX or FOLFOX + SF for 5 days, the number of spheroids in 15 fields of view of a self-made grid covering the well was counted. Cells of untreated spheroids were reseeded at $5x10^3$ cells/ml to evaluate the potential to form secondary and tertiary spheroids.

Aldehyde dehydrogenase isoform 1 (ALDH1) activity assay. To measure ALDH1 activity, $1x10^6$ CX-1 cells pre-treated with control, SF (10 μM), FOLFOX or FOLFOX + SF (10 μM) were exposed to 5 μl/ml Aldefluor substrate (Aldagen, Inc.) for 30 min at 37˚C. Pre-treatment with the ALDH1 inhibitor diethylamino-benzaldehyde (MilliporeSigma) for 30 min at 37˚C served as a negative control. Cells were analyzed by flow cytometry (FACScan; BD Biosciences) according

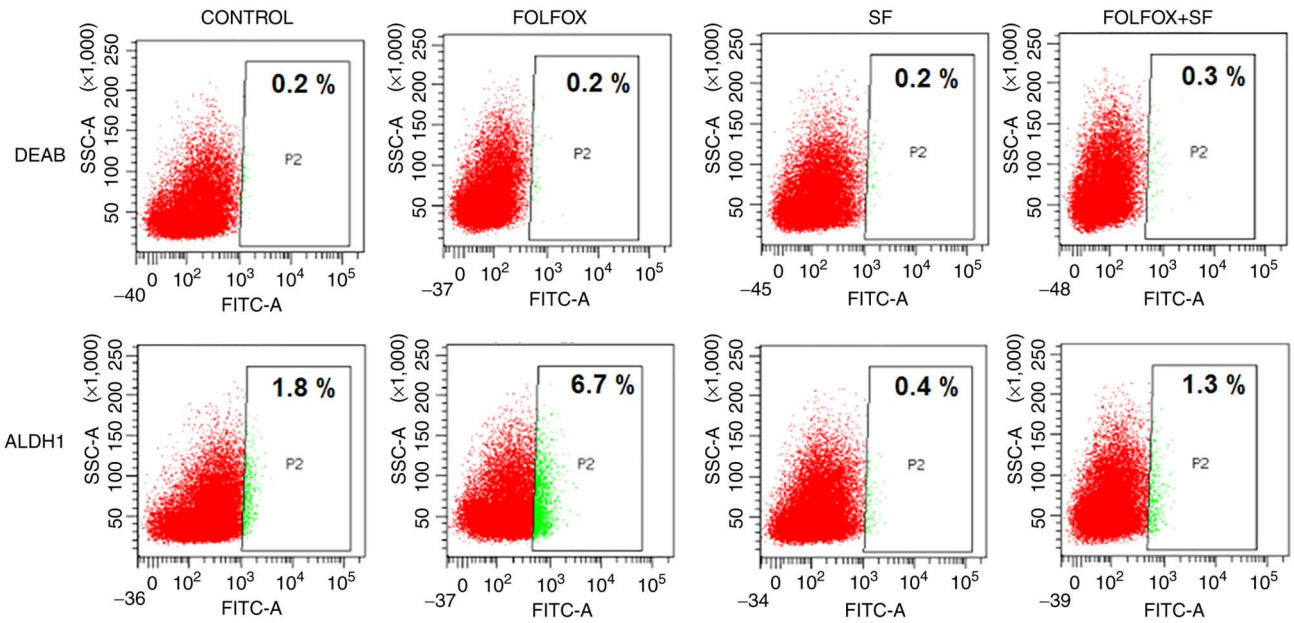

Figure 1. Representative flow cytometry dotplots from ALDH1 activity assay. SF, sulforaphane; DEAB, diethylamino-benzaldehyde; ALDH1, aldehyde dehydrogenase isoform 1.

to the manufacturer's instructions. Briefly, $1x10^6$ cells were incubated with Gammunex® (Talecris Biotherapeutics, Inc.) at 4˚C for 10 min to inhibit unspecific binding of antibodies. After washing with PBS/5% fetal calf serum, cells were incubated with unconjugated or fluorescein-isothiocyanate (FITC)-/phycoerythrin (PE)-conjugated primary antibody. After washing, cells were incubated with FITC- or PE-labeled secondary antibodies at 4˚C for 30 min to detect unconjugated primary antibody. PE-conjugated goat anti-rabbit IgG (cat. no. 554020; BD Pharmingen; BD Biosciences) or FITC-conjugated goat anti-mouse IgG (cat. no. 115-095-003; Jackson ImmunoResearch Laboratories, Inc.) were used as secondary antibodies. The data were analyzed using BD FACS Diva software (Becton, Dickinson and Company). PE- or FITC-labeled mouse IgG (BD Pharmingen; BD Biosciences) served as the isotype control. Gating was implemented based on negative control staining profiles. At least 10,000 cells were gated for each experiment. Representative flow cytometry dotplots are presented in Fig. 1.

Western blotting. Western blotting was performed as described previously (17). Briefly, after 48 h of cell culture incubation with treatment, cell lysates were prepared in RIPA buffer (MilliporeSigma) using a proteinase inhibitor cocktail (Roche Diagnostics GmbH). NuPAGE 4-12% Bis-Tris Gel (Novex; Thermo Fisher Scientific, Inc.) electrophoresis of 20 μg of each protein sample was performed using an XCell SureLock Mini-Cell module (Invitrogen; Thermo Fisher Scientific, Inc.) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc.) using an XCell IITM Blot Module (Invitrogen; Thermo Fisher Scientific, Inc.). Membranes were blocked in phosphate-buffered saline (PBS) +0.1% Tween with 5% BSA (SERVA Electrophoresis GmbH) at room temperature for 1 h and then incubated with primary antibodies at 4˚C for 12 h, followed by incubation with secondary antibodies for 1 h at room temperature. Films were developed using SuperSignal® West Pico chemiluminescent substrate (Thermo Fisher Scientific, Inc.) in a FUSION SL image acquisition system (Vilber Lourmat Deutschland GmbH). Restore™ western blot stripping buffer (Thermo Fisher Scientific, Inc.) was used where appropriate. Antibodies against MRP2 (cat. no. sc-518048; 1:200 dilution; Santa Cruz Biotechnology, Inc.) and β-actin (cat. no. A5441; 1:1,000 dilution; MilliporeSigma) were used as primary antibodies, and goat anti-mouse horseradish peroxidase-conjugated antibodies (cat. no. sc-2005; 1:2,000 dilution; Santa Cruz Biotechnology, Inc.) were used as secondary antibodies. Immunoblots were visualized and quantified by the FUSION SL imaging system (Vilber Lourmat Deutschland GmbH) and ImageJ (National Institutes of Health) software.

Immunofluorescence. For immunofluorescence analysis, $5x10^4$ CX-1 cells/chamber were seeded on four-chamber slides. After the cells had been treated with SF (10 μM), FOLFOX or FOLFOX + SF (10 μM) for 48 h, they were fixed in acetone (ROTIPURAN® ≥99.8%; Carl Roth GmbH and Co. KG) for 10 min at room temperature, blocked with 10% normal goat serum for 1 h at room temperature and incubated for 1 h at 37˚C with primary mouse anti-human monoclonal MRP2 antibody (Santa Cruz Biotechnology, Inc.), followed by washing in PBS and incubation with secondary goat anti-mouse polyclonal Cy2 antibody (cat. no. 115-225-146; Jackson ImmunoResearch Laboratories, Inc.) diluted with antibody diluent (Dako; Agilent Technologies, Inc.) to 1:20 and 1:200, respectively at 37˚C for 1 h. To counterstain cell nuclei, 4',6-diamidino-2-phenylindole (KPL, Inc.) was applied for 10 min at room temperature.

Statistical analysis. Statistical analysis was performed using SPSS v.25.0 (IBM Corp.). Data are presented as the median and interquartile range (IQR) unless stated otherwise. Differences between groups were analyzed using the non-parametric Mann Whitney U test or the Kruskal-Wallis test with Dunn's

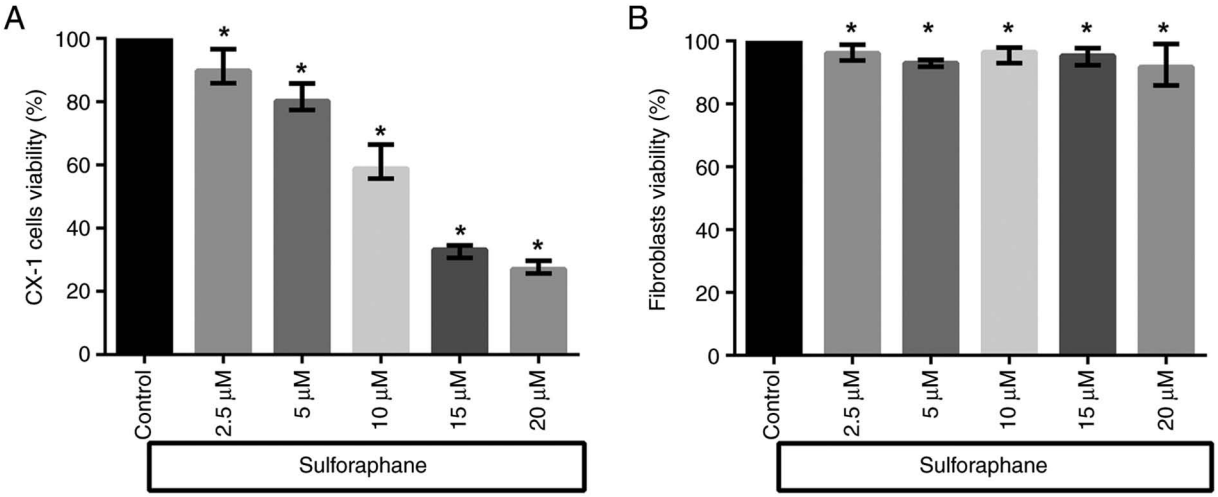

Figure 2. Impact of SF on the viability of colon cancer cells and fibroblasts. (A) CX-1 cell and (B) fibroblast viability were assessed by MTT assay after incubation with the indicated concentrations of SF. Results are presented as the median with interquartile range. *P<0.05. SF, sulforaphane.

post-hoc test. All statistical tests were two-sided. P<0.05 was considered to indicate a statistically significant difference.

Results

SF and FOLFOX impact on cell viability. SF significantly decreased the viability of the CX-1 cells in a dose-dependent manner to 89 (85-96), 80 (77-85), 58 (65-66), 33 (30-34) and 27% (25-29%) of the control at concentrations of 2.5, 5, 10, 15 and 20 μM, respectively (all P<0.05) (Fig. 2A). Similarly, SF had a significant negative impact on the viability of the fibroblasts at all concentrations, although it was slight compared with the impact of SF on the cancer cells. The highest tested concentration of SF (20 μM) reduced fibroblast viability by only 9% (1-15%) (P<0.05) (Fig. 2B). Ethanol, which was used as a solvent for SF, had no impact on cell viability (data not shown).

FOLFOX significantly decreased CX-1 cell viability to 70% (69-75%) of the control (P=0.002). Such an effect was similar as that achieved by 10 μM SF (compared with FOLFOX; P=0.240). The combination of FOLFOX + SF further decreased CX-1 cell viability to 49% (44-53%) of the control, and this combined treatment was significantly more effective than FOLFOX (P=0.002) or SF (P=0.002) alone (Fig. 3).

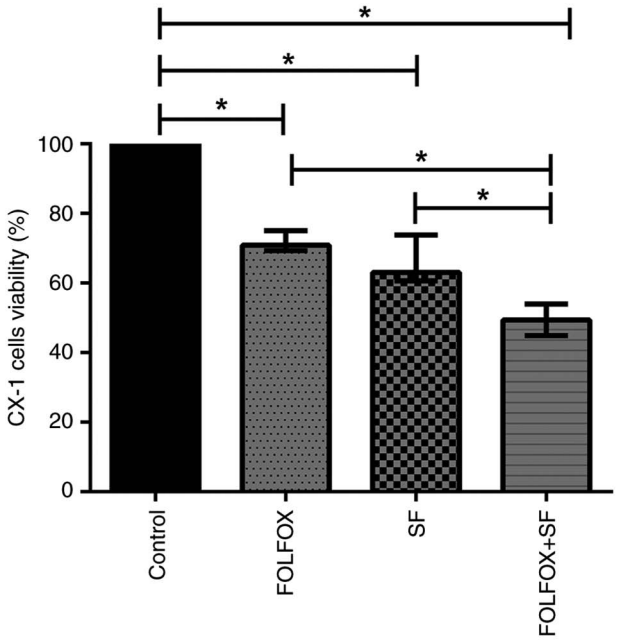

Figure 3. Impact of FOLFOX and SF on the viability of colon cancer cells. CX-1 cell viability was assessed by MTT assay after 48 h of incubation with FOLFOX ± SF. Results are presented as the median with interquartile range. *P<0.05. SF, sulforaphane; FOLFOX, 5-fluorouracil, oxaliplatin and folinic acid.

SF and FOLFOX impact on apoptosis in cells. Monotherapy with SF or FOLFOX increased the BAX/BCL-2 expression ratio up to ~1.5-fold higher compared with the control (Fig. 4A). The combination of FOLFOX + SF further increased the BAX/BCL-2 mRNA ratio to 3-fold higher compared with the control (Fig. 4A), and induced apoptosis in ~10% of CX-1 cells (Fig. 4B).

Spheroid formation. FOLFOX and SF significantly decreased the ability of the CX-1 cells to form spheroids to 57% (45-63%) (P=0.029) and 49% (40-57%) of the control (P=0.029). The combination of FOLFOX + SF further decreased the potential of the cells to form spheroids to 25% (20-34%) of the control (P=0.029). FOLFOX + SF was more effective than monotherapy using FOLFOX (P=0.029) or SF (P=0.029) (Fig. 5).

After cell-spheroids had been formed, they were dissociated and single cells were reseeded to evaluate serial spheroid formation capability. CX-1 cells were able to form secondary and tertiary spheroids.

ALDH1 activity. ALDH1 activity analysis showed that 1.3% (0.8-2.3%) of control-treated CX-1 cells were positive for ALDH1. SF reduced the number of positive cells by 4.3-fold to 0.3% (0.2-0.4%) (P=0.002). By contrast, FOLFOX increased

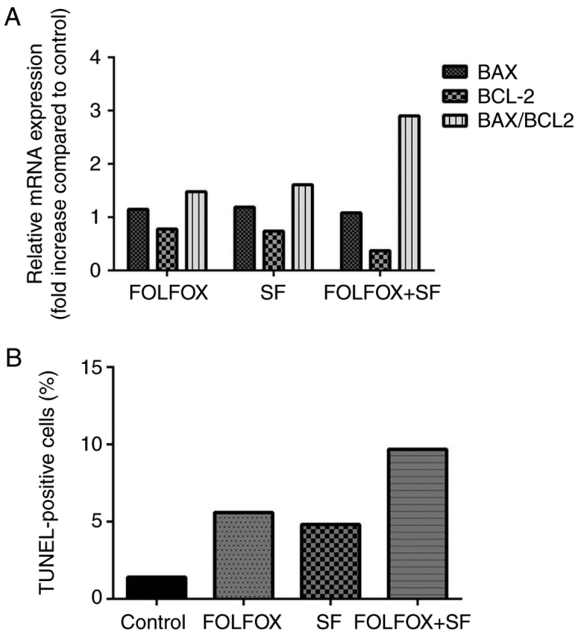

Figure 4. Impact of FOLFOX and SF on apoptosis in colon cancer cells. (A) Expression levels of the apoptosis-related genes BAX and BCL-2 were investigated in CX-1 cells after treatment with FOLFOX, SF or FOLFOX + SF. (B) The percentage of apoptotic cells after treatment with FOLFOX, SF or FOLFOX + SF was investigated by TUNEL assay in the colorectal cancer cells. SF, sulforaphane; FOLFOX, 5-fluorouracil, oxaliplatin and folinic acid.

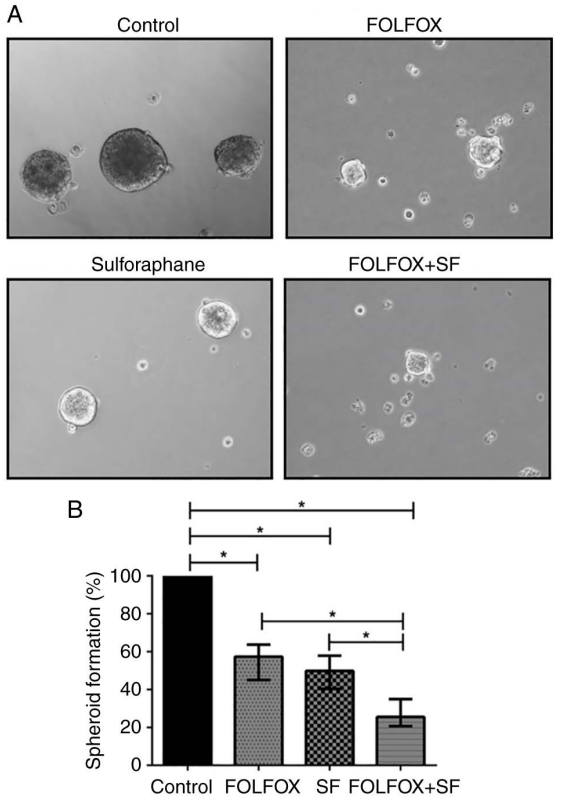

Figure 5. Impact of FOLFOX and SF on the ability of colon cancer cells to form spheroids. (A) Spheroid formation assay on CX-1 cells after treatment with FOLFOX, SF or FOLFOX + SF. Representative images of spheroids in each treatment group are shown. (B) FOLFOX and SF decreased the ability of the CX-1 cells to form spheroids, although combined treatment with FOLFOX + SF was the most effective. Results are presented as the median with interquartile range. *P<0.05. SF, sulforaphane; FOLFOX, 5-fluorouracil, oxaliplatin and folinic acid.

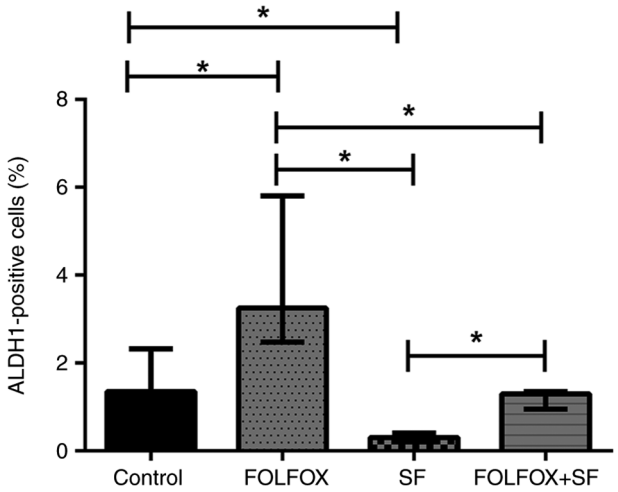

Figure 6. Impact of FOLFOX and SF on ALDH1 activity in colon cancer cells. ALDH1 activity in CX-1 cells was measured by flow cytometry after the cells were treated with FOLFOX, SF or FOLFOX + SF. Results are presented as the median with interquartile range. *P<0.05. SF, sulforaphane; FOLFOX, 5-fluorouracil, oxaliplatin and folinic acid.

ALDH1 activity by 2.4-fold compared with the control (P<0.001). SF alleviated the FOLFOX-induced increase, and the number of ALDH1-positive cells in the FOLFOX + SF group [1.3% (0.9-1.3%)] was similar to that in the control group (P=0.699) (Fig. 6).

MRP2 expression. Monotherapy with FOLFOX or SF upregulated MRP2 mRNA expression in CX-1 cells by 2.8-fold (2.0- to 3.2-fold) (P=0.001) and 7.8-fold (7.5- to 8.3-fold) compared with the control (P=0.001). Combined treatment with FOLFOX + SF further upregulated MRP2 expression to 8.7-fold (8.4-8.8-fold) higher compared with the control (P<0.05) (Fig. 7).

Discussion

In the present study, the anti-tumorigenic effect of SF alone and in combination with FOLFOX on highly metastatic colon cancer cells (CX-1) was investigated, to the best of our knowledge, for the first time. The results of the study showed that SF alone and in combination with FOLFOX effectively decreased the viability of the CX-1 cells, promoted apoptosis within the CX-1 cells, prevented cellular spheroid formation and decreased ALDH1 activity.

FOLFOX is a standard CTx regimen for CRC (19,20), but it has some significant adverse effects, including hepatotoxicity, nephrotoxicity, neurotoxicity and cardiotoxicity (8,21-24). Such toxicity together with chemoresistance limits the FOLFOX therapeutic success rate (25,26), thus novel and less toxic anticancer agents are needed. As aforementioned, SF is considered a new promising candidate drug due to its documented anticancer properties against breast (11), prostate (12), bladder (13) and gastric cancer. The present study demonstrated a similar anticancer effect of SF against colon cancer *in vitro*. SF decreased CX-1 cell viability and promoted apoptosis in these cells. Furthermore, SF at concentrations of up to 20 μM was only slightly toxic for non-malignant cells

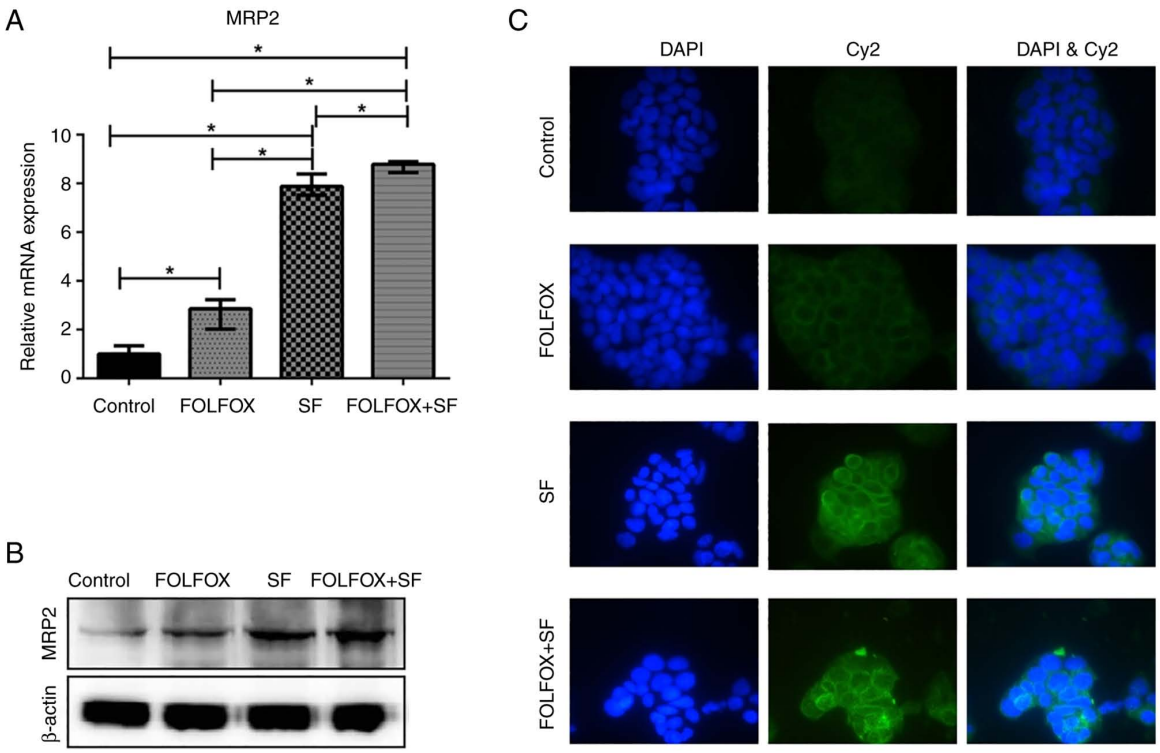

Figure 7. Impact of FOLFOX and SF on MRP2. After CX-1 cells were treated with FOLFOX, SF or FOLFOX + SF, the expression of the MRP2 gene was measured by (A) reverse transcription-quantitative PCR and (B) western blot assays. (C) Representative images of immunofluorescence staining at x40 magnification in each treatment group are shown. *P<0.05. SF, sulforaphane; MRP2, multidrug resistance protein 2; DAPI, 4',6-diamidino-2-phenylindole; FOLFOX, 5-fluorouracil, oxaliplatin and folinic acid.

(fibroblasts), and such results confirmed previous observations by Kallifatidis *et al* The safety of SF has been shown in a human setting as well. A recent pilot study demonstrated that a daily intake of 508 μmol SF did not cause serious adverse effects in patients with advanced pancreatic cancer (27,28).

Furthermore, the present study demonstrated that SF was effective when combined with conventional FOLFOX CTx. Such a combination was more effective in decreasing colon cancer cell viability than either of the drugs used separately. The possibility of effectively combining SF with OX (29) or 5-FU (30) has been shown in previous *in vitro* studies. However, to the best of our knowledge, the present study was the first to show the efficacy of SF in combination with FOLFOX CTx, which is used in routine clinical practice. Separately, both treatments, SF and FOLFOX, induce CRC cell death via the apoptotic pathway (31,32). The results of the present study show effectively that both therapies cooperatively potentiate apoptosis, as apoptotic cell number in the FOLFOX + SF group in the TUNEL assay was significantly higher than that in groups treated with monotherapy. Furthermore, FOLFOX + SF impacted the expression of the pro-apoptotic BAX and anti-apoptotic BCL-2 genes (33). The balance between these two genes impacts the sensitivity of cells to apoptotic stimuli (34,35). Lower levels of this ratio may lead to the resistance of cancer cells to apoptosis, thus promoting tumor progression and aggressiveness (36). The present study showed that FOLFOX achieves the highest BAX/BCL-2 ratio when conventional treatment is supplemented with SF. Therefore, the data suggest, that SF may potentiate FOLFOX-induced apoptotic cancer cell death.

Recently, three-dimensional cell culture systems have gained increasing attention in the field of drug discovery due to the benefits of providing more physiologically relevant information and more predictive data for *in vivo* testing compared with conventional two-dimensional cell cultures (37,38). SF has a known, suppressive effect on spheroid formation in pancreatic and breast cancer cells (39,40). Thus, the present study investigated the impact of SF and FOLFOX on CX-1 cell spheroid formation, and showed that each therapy alone decrease spheroid formation. However, a combination of FOLFOX + SF was more effective to inhibit the formation of colonospheres than either of the drugs used as monotherapy. Furthermore, the present study experiments demonstrated that CX-1 cancer cells were able to form colonospheres and sustain spheroid formation during serial passages. This indicates the ability of CX-1 cells to self-renew, which is a hallmark of cancer stem-like cell (CSC) theory (41). CSCs account for a minor population in a tumor, but are closely associated with its metastatic potential and recurrence after primary treatment, as these cells are chemoresistant (42). ALDH1 activity is considered a marker of CSCs in various malignancies, including CRC (43,44). Also, increased ALDH1 activity in cancer cells indicates their resistance to chemotherapeutic agents (45,46). Thus, inhibition of ALDH1 sensitizes CRC cells to CTx (47). The present study showed that monotherapy with FOLFOX increased ALDH1 activity in CX-1 cells, but that SF prevented such an increase. Therefore, we hypothesize that resistance to conventional CTX may be decreased by combining FOLFOX with SF.

Certain studies suggest that dietary components that modulate detoxification systems, such as SF, should be studied carefully before being recommended for use during CTx (15,48). This is since these compounds may have additional influences on the disposition of chemotherapeutic drugs. Several previous studies have shown that SF increases the MRP2 protein level in various cancer cell lines, including colorectal cancer and hepatocellular carcinoma cell lines (15,49). MRP2 is involved in the development of MDR, one of the major obstacles to the success of current cancer therapy (15). MDR is a result of the fact that different cytotoxic agents share the same efflux system (15). MDR transporters, which include P-glycoprotein, MRP1 and MRP2, physiologically are the cellular self-defense systems against toxic compounds and aid in cancer prevention. Although, at the same time, in a number of different tumors, such as esophageal and blader cancer, they are upregulated and result in clinical drug resistance (15,50,51). The present study showed that MRP2 levels were increased by conventional FOLFOX, SF or a combination of the two drugs. However, such upregulation does not prevent CRC cells from cytotoxicity and death, as shown in the results of the current study. Furthermore, SF added to conventional CTx potentiated its antitumorigenic properties.

The present study has several limitations. First, the experiments were performed in only one CRC cell line, so future experiments should verify these results in other similar cells. Second, the study did not investigate the mechanism by which SF promotes apoptosis and interacts with FOLFOX. Third, this was an *in vitro* study, and *in vivo* studies are needed to confirm the antitumorigenic effect and safety of SF before clinical trials can be conducted. Despite these limitations, the present study managed to show the potential benefits of SF with or without FOLFOX against CX-1 CRC cells and provide the knowledge necessary for future investigations in the field.

In conclusion, the present study demonstrated that SF alone and in combination with FOLFOX effectively decreased the viability of CX-1 cells, promoted apoptosis within CX-1 cells, prevented cellular spheroid formation and decreased ALDH1 activity. SF together with conventional FOLFOX has additive anticancer effects against highly metastatic human CRC. While SF has no toxic effects on non-cancer cells, fibroblasts, subsequent experimental studies are warranted to assess its value *in vivo*.

Acknowledgments

The authors wish to thank Ms. Elvyra Mohr from Heidelberg University Hospital (Heidelberg, Germany) for providing technical support and advice while performing the experiments.

Funding

This study was funded by the internal funds of the Department of General and Transplant Surgery, Heidelberg University Hospital, Heidelberg, Germany.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AC and DS contributed equally to this work. KS and PS conceived and designed the study. AC, DSi, DSc, GK, HB, AC and IH performed the experiments, the literature review and the critical revision of the study. AC, DS and AB performed data analysis and visualization. AC, DSi, DSc, GK, HB, AC, IH and AB prepared the manuscript. KS and PS revised the manuscript. AC and DS confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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