

Inhibitory Effects of 5-Fluorouracil and Oxaliplatin on Human Colorectal Cancer Cell Survival Are Synergistically Enhanced by Sulindac Sulfide

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Abstract. *Background:* COX inhibitors appear to be promising agents in combination with cytostatics in the treatment of colorectal carcinoma (CRC). The aim of this study was to compare growth inhibitory effects of cytostatics (5-fluorouracil, 5-FU; oxaliplatin) and COX inhibitor sulindac sulfide (an active metabolite of sulindac), given alone or in combination, on several CRC cell lines. *Materials and Methods:* A series of human CRC cell lines were incubated with various combinations of the test drugs used in concentrations from 3 to 200 μ M. The cell survival was assessed by MTT assay. Isobolograms and median effect method of Chou and Talalay were used to assess the nature and quantitative aspects of interaction observed between studied drugs. Cell cycle progression and apoptosis were measured using flow cytometric methods. In addition, growth inhibitory effects of studied agents on CRC cell lines were compared with a normal (mouse fibroblast) cell line. *Results:* Sulindac sulfide synergistically potentiated the inhibitory effects of 5-FU and oxaliplatin on CRC survival, parallel to the induction of apoptosis. A dose reduction effect for synergistic activity of sulindac sulfide with studied cytostatics (in the range of 5- to 14-fold, when compared to single agent) suggested that the inhibitory effect of cytostatics on CRC survival may be obtained at low doses. In addition, sulindac sulfide appeared to be more specific against CRC cells than normal cells. *Conclusion:* It was apparent that combination of 5-FU or oxaliplatin with sulindac sulfide results in a powerful inhibition of growth of colorectal carcinoma cells *in vitro*, which may be more specific for cancer than normal cells.

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The standard therapy for colorectal carcinoma (CRC) is based on cytostatics such as 5-fluorouracil (5-FU) and/or oxaliplatin (1, 2), with limited therapeutic success. Non-steroidal anti-inflammatory drugs (NSAIDs), especially sulindac and celecoxib, inhibit the progression of premalignant polyps into CRC (3, 4). On this basis, celecoxib was registered as a treatment for familial adenomatous polyposis (FAP) (5, 6) and later introduced as an adjuvant agent for the treatment of CRC (7, 8).

Recent evidence indicates that inhibition of angiogenesis by sulindac sulfide (an active metabolite of sulindac) is a secondary phenomenon to its strong pro-apoptotic activity: (i) sulindac sulfide did not specifically interact with an angiogenic cascade and exerted antiangiogenic effect on matured blood vessels; (ii) sulindac sulfide stimulated caspase-3 activity in matured blood vessels (9); and (iii) sulindac sulfide stimulated extracellular and mitochondrial pathways of apoptosis in cultured endothelial cells (10). It was already postulated by Piazza *et al.* that induction of apoptosis primarily accounts for the growth inhibitory properties of sulindac metabolites in colon cancer cells (11). The strong induction of apoptosis suggests that sulindac sulfide may potentiate the inhibitory effect of 5-FU and/or oxaliplatin on the growth of CRC cells. In order to assess such a possibility, the effect of combinations of sulindac sulfide with 5-FU or oxaliplatin on survival of human CRC cells of various cell lines was studied *in vitro*.

Materials and Methods

Cell cultures and drug treatment. Colo-205, SW48 and HT-29 human colorectal cancer cell lines (CRC cell lines) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were grown in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 5% (v/v) heat inactivated fetal bovine serum (FBS; Gibco), 2 mM glutamax (Gibco), 100 units/ml penicillin, 100 μ g/ml streptomycin and 250 ng/ml amphoterycin (Gibco). Balb/c 3T3, a normal mouse fibroblast cell line, was obtained from ATCC and was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat inactivated bovine serum (Gibco), 2 mM glutamax, 100 units/ml penicillin, 100 μ g/ml

streptomycin and 250 ng/ml amphotericin. Cells were cultured at 37°C in a humidified atmosphere including 5% CO₂. Cells were incubated with drugs for 24, 48 and 72 h. For the Colo-205 line, both floating and attached cells were harvested for subsequent analysis.

Drugs. The following drugs were studied: sulindac sulfide, oxaliplatin and 5-FU (Sigma, St. Louis, MO, USA). The concentrations of the studied drugs were in the range from 3 to 200 µM. The drugs were dissolved in 100% dimethylsulfoxide (DMSO; Sigma) and then diluted in the media for experiments. In all experiments, control cells were incubated with DMSO alone. The final concentration of DMSO was maintained at 0.2%.

MTT assay. This assay relies on the ability of viable cells to reduce a yellow tetrazolium salt (MTT; Sigma) metabolically to a purple formazan product. This reaction takes place when mitochondrial reductase enzymes are active.

Cells were grown in 96-well plates (1×10⁴/200 µl/well). After incubation with the reagents, the medium was removed and the cells were treated with 50 µl of MTT for 4 h at 37°C. Subsequently, 150 µl of solubilization solution (10% SDS) were added and the mixture incubated at 37°C overnight. The solubilized formazan product was spectrophotometrically quantified with the help of a microtiter plate reader, Power Wave XS (Bio Tek, Winooski, VT, USA), at 570 nm.

Analysis of drug interaction. Cells of the different CRC cell lines were simultaneously incubated for 72 hours with 5-FU, or oxaliplatin with sulindac sulfide, or with each agent alone. The nature of the interactions between studied drugs was analyzed with the help of isobologram (12) and median effect methods described by Chou and Talalay (13, 14).

The isobolograms were constructed by determining the doses of different drugs causing a defined fractional cell kill effect (Fa). The obtained doses, for each drug alone, were plotted on x- and y-axes and the line of additivity was created by joining both points. The observed dose combination of two agents, giving the defined fraction of killed cells, located near or on the line of additivity (achieved for a particular effect, e.g. 50%) represents an additive drug-drug interaction, whereas dose combinations falling below or above this line represent synergism or antagonism, respectively (12).

The combination index (CI) method is a mathematical and quantitative evaluation of a two-drug pharmacological interaction. Using data from the cytotoxicity experiments and CalcuSyn ver. 2.0 software (Biosoft, Cambridge, UK), CI values were generated over a range of Fa levels from 0.05 to 0.95 (5-95% cell kill). CIs of <1 indicate synergism (the smaller the value, the greater the degree of synergy), CIs equal to 1 indicate additivity, and CIs>1 indicate antagonism. Data generated from the CI method were used to quantify the dose-reduction index (DRI) for the combination of two drugs. The DRI represents the fold-decrease of each individual agent when two drugs are used in combination as opposed to alone to achieve a particular Fa. Each CI or DRI ratio represented here is the mean value derived from at least five independent experiments.

Flow cytometric analysis of cell cycle. Cells (~1×10⁶) were resuspended in 4 ml of 80% ethanol (-20°C) and incubated at -20°C for 24 h, washed twice in phosphate-buffered saline (PBS), and stained with 50 µg/ml propidium iodide (PI) and 100 µg/ml RNase in 0.1% PBST solution (PBS supplemented with 0.1% Triton®×100) for 30 min in the dark at room temperature. The

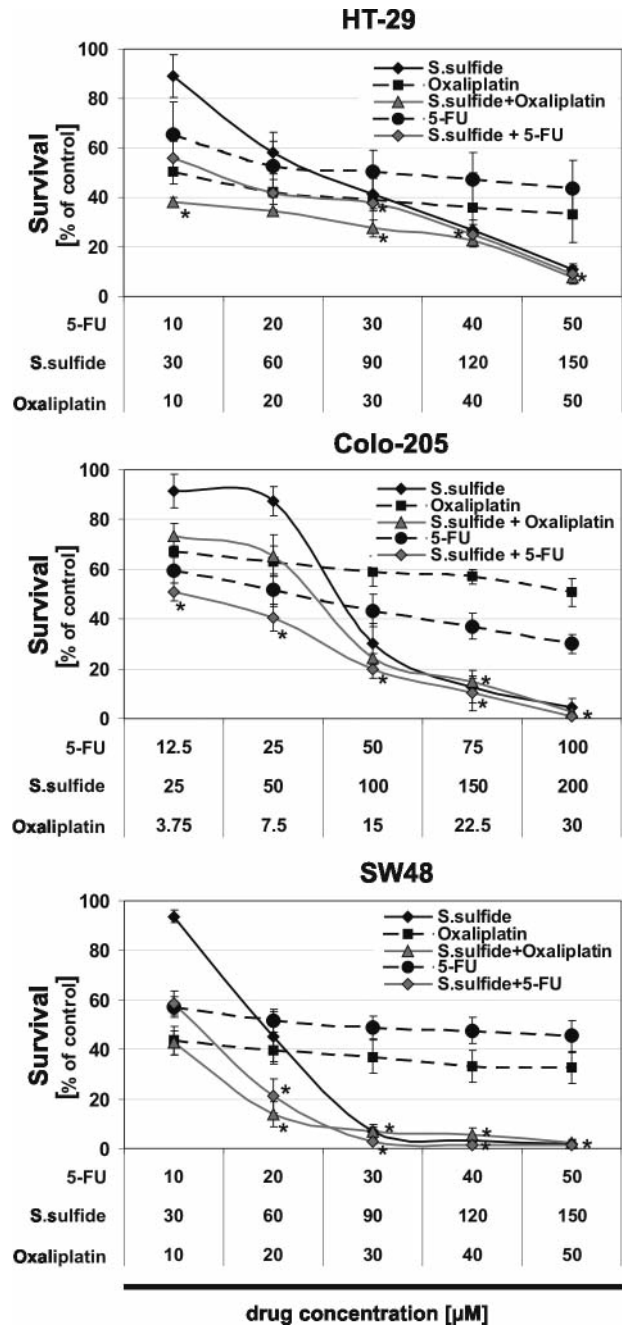


Figure 1. Effects of sulindac sulfide (S. sulfide), 5-fluorouracil (5-FU) and oxaliplatin (concentrations in µM given below figures) on survival of colorectal cancer cells of HT-29, Colo-205 and SW48 cell lines (as percent of control (DMSO)). Drugs were applied singly or in combination for 72 h. Each bar represents the mean±SD (n=5); *p<0.05, for comparison with 5-FU or oxaliplatin.

samples were then measured using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The DNA histograms were analyzed using ModFit LT V3.0 software (BD Biosciences).

Table I. IC_{50} values of the studied drugs for inhibition of growth of various cell lines (cells were incubated with drugs for 72 h).

Drugs	IC_{50} (μM)				SI		
	Balb/c 3T3	Colo-205	SW48	HT-29	Balb/c 3T3/ Colo-205	Balb/c 3T3/SW48	Balb/c 3T3/HT-29
S. sulfide	1195 \pm 176	70 \pm 7	57 \pm 8	71 \pm 8	17.1	21	17
5-FU	13 \pm 3	31 \pm 5	27 \pm 5	20 \pm 3	0.4	0.5	0.7
Oxaliplatin	73 \pm 7	34 \pm 6	7 \pm 1	9 \pm 0.4	2.1	10.4	8.1

IC_{50} values (concentrations required to reduce the viability of cells by 50% as compared with the control cells) were computed using linear or non-linear regression (three parametric Hill function) ($R^2 > 0.9$). They are presented as mean \pm SD from at least five independent experiments. SI (selectivity index) represents inhibition of survival of Balb/c 3T3 cells *versus* different CRC cell line cells (ratio of respective IC_{50} values).

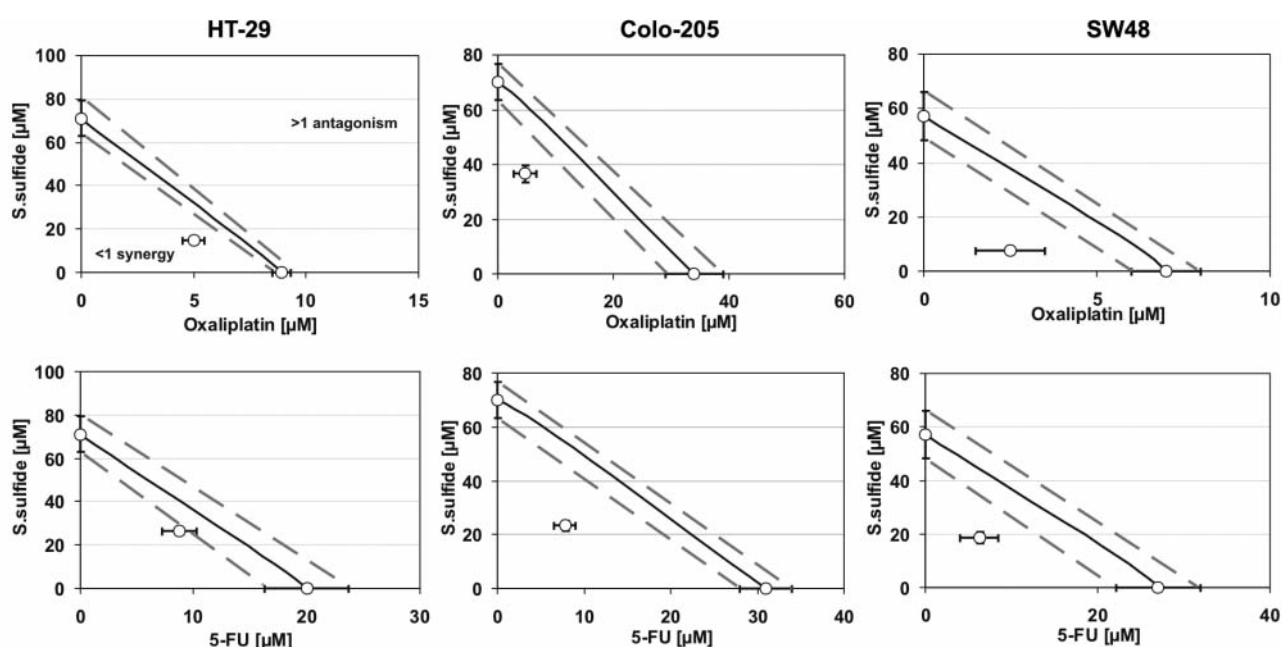


Figure 2. Isobolograms describing the interaction of sulindac sulfide with 5-fluorouracil (5-FU) or oxaliplatin. The isobolograms were constructed by connecting the IC_{50} values of sulindac sulfide with the IC_{50} of 5-FU or oxaliplatin. The black heavy lines indicate the theoretical line of additivity. Results (means of five experiments) below the additive line indicate synergism and those above the additive line antagonism.

Flow cytometric analysis of apoptosis. Apoptosis was measured, according to the manufacturer's instructions, using an annexin V-FITC kit (BD Biosciences). The cells were collected after treatment, washed twice with PBS and centrifuged. The cell pellet was resuspended in ice-cold binding buffer. The annexin V-FITC and PI solutions were added to the cell suspension and mixed gently. The samples were then incubated for 15 min in the dark before flow cytometric analysis.

Statistical analysis. Data were presented as mean values \pm standard deviation (SD). Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA) followed by *posthoc* Tukey's test (Statistica, StatSoft, USA). Value of $p < 0.05$ (marked with asterisks) was assumed as statistically significant.

Results

Growth studies. Sulindac sulfide inhibited survival of colorectal CRC cells, in all lines studied, as measured by inhibition of mitochondrial dehydrogenase activity (MTT assay). The inhibitory effects on cells survival were dose and time dependent. The strongest inhibition, approaching 100%, after 72 h incubation, was observed with 70 to 200 μM of sulindac sulfide (Figure 1). The correlation coefficient between dose and inhibition of cell survival was greater than 0.9 on CRC cells. Inhibition of cell survival in various CRC cell lines by sulindac sulfide and cytostatics tested (5-FU and

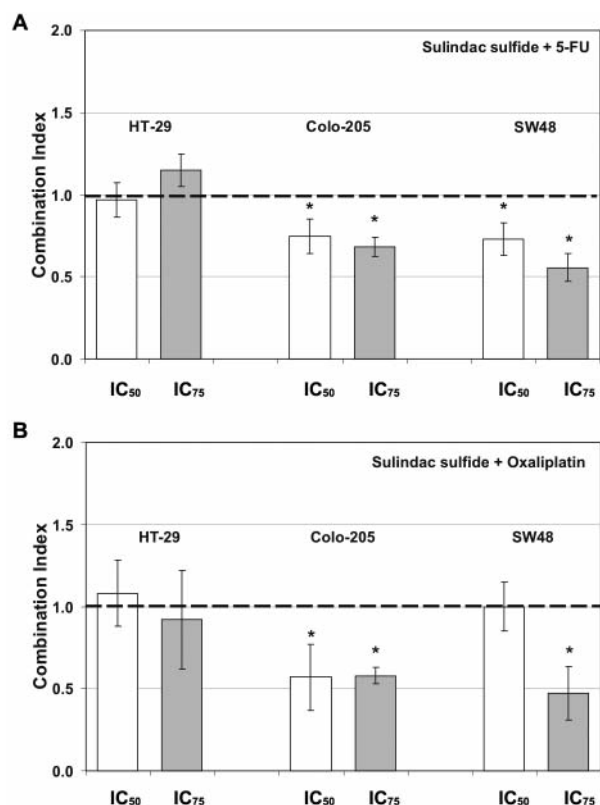


Figure 3. Mean values of the combination index at the affected fractions of 50% (IC₅₀) and 75% (IC₇₅) when 5-fluoruracil (5-FU) or oxaliplatin was combined with sulindac sulfide in HT-29, Colo-205 and SW48 cells. Mean IC₅₀ and IC₇₅±SD (n=5) are shown. A CI value significantly less than 1 indicates synergism, a CI not significantly different from 1 indicates addition, and a CI significantly higher than 1 indicates antagonism; *p<0.05.

oxaliplatin) was compared with inhibition of survival of Balb/c 3T3 cells (IC₅₀ values are shown in Table I). IC₅₀ of sulindac sulfide for Balb/c 3T3 was extrapolated from the dose-response curve as at the maximal dose and time of incubation tested, IC₅₀ did not reach statistical significance.

The SI (selectivity index) obtained by calculating ratios of IC₅₀ for Balb/c 3T3 versus each CRC cell line studied is shown in Table I (value above 1 indicates greater inhibition of cancer cells survival than normal cells). The results show that sulindac sulfide was several times more selective against cancer cells than against normal cells as compared with 5-FU and oxaliplatin.

Interactions of sulindac sulfide with cytostatics (5-FU or oxaliplatin). Figure 1 shows survival of various CRC cells (as % of control) in the presence of cytostatics and sulindac sulfide applied singly or in combination. Sulindac sulfide potentiated the inhibitory effects of both studied cytostatics on CRC cells. The influence of sulindac sulfide on the effects of 5-FU and oxaliplatin was dose dependent (Figure 1).

The nature of the interaction between sulindac sulfide and the studied cytostatics on the survival of CRC cells in various cell lines was analyzed with the help of isobolograms and median effects method of Chou and Talalay (13, 14). Isobolograms describing the interactions between sulindac sulfide with cytostatics (5-FU and oxaliplatin) are shown in Figure 2. Isobolograms indicated that interactions between sulindac sulfide and 5-FU or oxaliplatin are synergistic, with the exception of interaction with 5-FU on HT-29 cell line.

Analysis performed with the help of the median effect method revealed that combination of sulindac sulfide and cytostatics produced synergistic or additive effects depending on the range of cell kill level (Fa). Synergism was statistically significant in the Colo-205 cell line at inhibition levels of 50 and 75% (for both cytostatics), achieving CI values from 0.57 to 0.8, and in the SW48 cell line at inhibition levels of 50 (only for 5-FU) and 75% (for both cytostatics), with CI values in the range from 0.47 to 0.73. In HT-29 cells, the combination of sulindac sulfide with cytostatics resulted in additive interaction (CI values of 0.92-1.08 and 1.0-1.15 at 50% and 75% inhibition levels, respectively) (Figure 3).

For combinations of sulindac sulfide with 5-FU or oxaliplatin, the DRI parameter was calculated. This index, above the value of 1, indicates a synergistic interaction. In cells exposed to combination of sulindac sulfide and oxaliplatin, the concentration of cytostatic needed to achieve a 75% reduction in cell proliferation was reduced ~5-fold (Colo-205), ~14-fold (SW48) and ~11-fold (HT-29), when compared with single agent treatment (Table II).

Effect of combined treatment with sulindac sulfide and cytostatics on cell cycle progression and induction of apoptosis. Analysis of DNA content in cells, with the help of flow cytometry, revealed that 5-FU, significantly (p<0.05) increased the percentage of the cells in the S-phase in all CRC lines studied, and this effect was significantly potentiated by combination of 5-FU with sulindac sulfide (Figure 4A). Oxaliplatin significantly increased the cell fraction in the G2/M phase in the Colo-205 cell line and this effect was significantly potentiated by sulindac sulfide (Figure 4B). The effects of oxaliplatin on the cell cycle were less pronounced than in the case of 5-FU and their modifications by sulindac sulfide were also modest.

Annexin V/PI analysis was performed to confirm apoptosis induction, which could be responsible for the synergistic effect observed after the treatment of CRC cells with the combined treatment. The average percentage of apoptotic cells increased significantly after exposure to all agents applied alone as compared with the control (p<0.02). However, combinations of studied agents produced the

Table II. Dose reduction index (DRI) values for drug combination schedules after 72 h of simultaneous treatment.

Cell line	Drug I	Drug II	Molar ratio	DRI±SD at Fraction affected level			
				50%		75%	
				I	II	I	II
HT-29	Sulindac sulfide	5-FU	3:1	2.5±0.6*	1.50±0.5	-	3.6±1.0
		Oxaliplatin	3:1	N/D	N/D	1.2±0.3	11.0±5.0
Colo 205	Sulindac sulfide	5-FU	2:1	2.14±0.3*	4.29±0.6*	1.8±0.3*	6.9±1.7*
		Oxaliplatin	6.7:1	1.54±0.5	3.51±1.1*	1.5±0.2*	4.8±2.0
SW48	Sulindac sulfide	5-FU	3:1	1.93±0.3*	3.6 ±1.0	2.0±0.4*	38.0±3.1
		Oxaliplatin	3:1	3.25±1.8	1.2 ±0.1*	2.9±1.2*	13.5±3.5*

Data are expressed as means±SD (n>3). *p<0.05. N/D not determined. The DRI determines the magnitude of dose reduction allowed for each drug when given in synergistic combination, as compared with the concentration of a single agent that is needed to achieve the same effect. 25, 50 and 75% indicates selected values of fraction affected level.

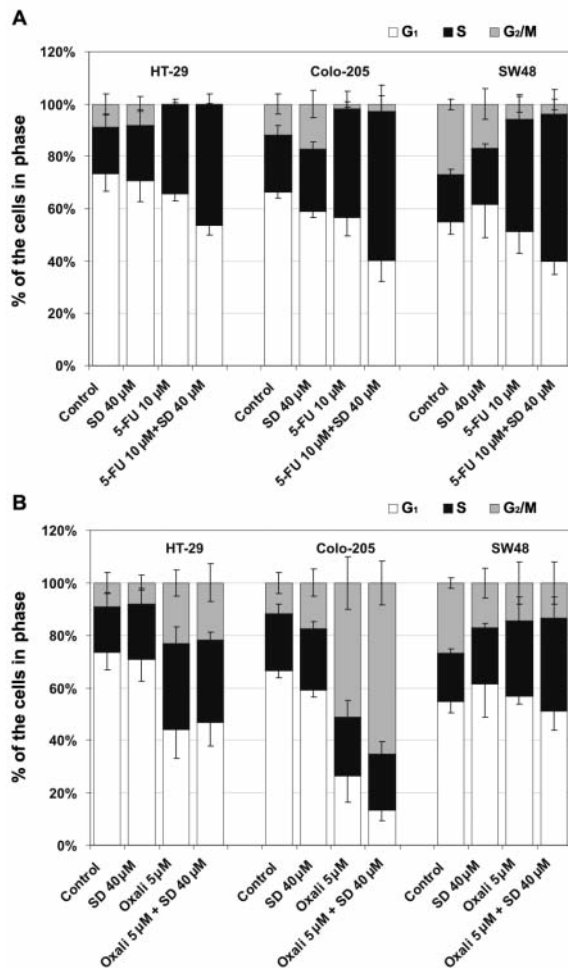


Figure 4. Changes in cell cycle progression in HT-29, Colo-205 and SW48 cells after 72 h treatment with cytostatics and sulindac sulfide (S. sulfide) applied alone or in combination. Each bar represents the mean±SD (n≥4). The data obtained from FACS were analyzed using ModFit LT V3.0 software to determine the percentage of cells in each phase of the cell cycle. A, Combination of S. sulfide with 5-FU; B, combination of S. sulfide with oxaliplatin.

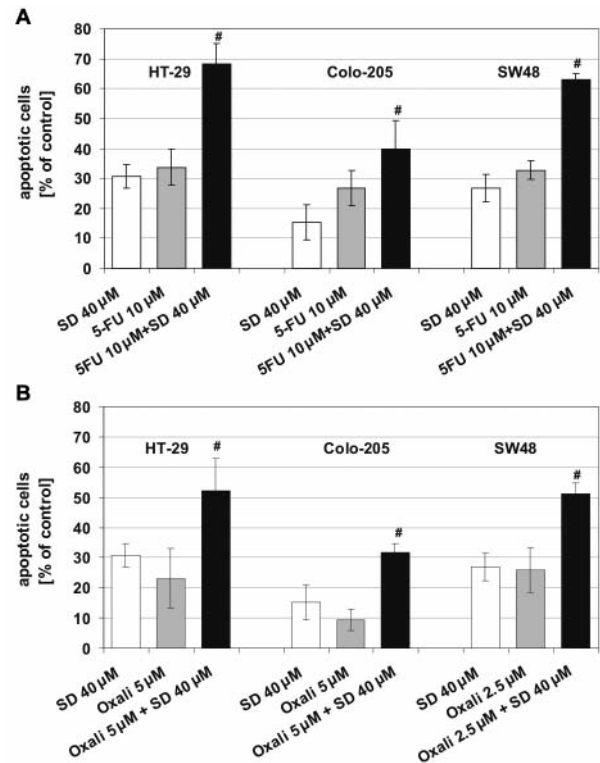


Figure 5. Induction of apoptosis in HT-29, Colo-205 and SW48 cells after 72 h treatment with 5-fluorouracil or oxaliplatin (Oxali) alone or in combination with sulindac sulfide (S. sulfide). Each bar represents the mean±SD (n≥4). #p<0.05, for comparison with cytostatics. A, Combination of SD with 5-FU; B, combination of S. sulfide with oxaliplatin.

greatest increase in apoptotic cells in all of three cell lines tested (HT-29, Colo-205 and SW48). Apoptosis induced by combination treatment was 2.1-fold higher for sulindac sulfide and 5-FU combination in HT-29 cells, and 3-fold higher for sulindac sulfide and oxaliplatin in Colo-205 cells, compared with the cytostatics applied alone (Figure 5).

Discussion

The most important finding of this work is the demonstration, for the first time, that sulindac sulfide (an active metabolite of sulindac) used in combination with cytostatics potentiated the inhibitory effects of 5-FU and oxaliplatin on survival of studied human CRC cells. The studied agents given alone induced the inhibition of growth of CRC cells of Colo-205, HT-29 and SW48 cell lines with the strongest inhibition observed after incubation of cell lines, with sulindac sulfide (Figure 1). Addition of sulindac sulfide to 5-FU or oxaliplatin greatly potentiated inhibitory effects of both cytostatics, however, the value of such interaction could only be estimated by isobolograms. The isobolograms, constructed on the basis of IC₅₀ values, indicated the synergistic interaction between sulindac sulfide and both studied cytostatics, 5-FU and oxaliplatin (Figure 2).

Highly synergistic interactions resulting from the addition of sulindac sulfide to 5-FU or oxaliplatin on the studied CRC cell lines (with the exception of one case: interaction between sulindac sulfide and 5-FU on HT-29 line, Figure 2) were quantitatively analyzed according to the method of Chou and Talalay. The obtained CI at 75% inhibition level was, in the case of sulindac sulfide and 5-FU between 0.56-0.73, and in the case of sulindac sulfide and oxaliplatin between 0.47-0.58 (in both cases significantly below 1) for Colo-205 and SW48 (Figure 3). These ranges are regarded as synergism (15).

The inhibition of CRC cell growth by combinations of sulindac sulfide with cytostatics was accompanied by cell cycle arrest (Figure 4). Combination of sulindac sulfide and 5-FU induced cell cycle arrest in the S-phase, in all lines studied, while the effect of oxaliplatin on the cell cycle was not very cell specific. Combination of sulindac sulfide and oxaliplatin affected the cell cycle in the G2/M phase in Colo-205 and in the S-phase in SW48 cells, compared to drugs applied alone (Figure 4B). Induction of cell cycle arrest may result from limitation of the ability of the cells to repair damaged DNA and/or uncoupling DNA from RNA and disturbances of protein synthesis, which may direct the cell to the apoptotic pathway.

Indeed, addition of sulindac sulfide to cytostatics led to at least a two-fold potentiation of apoptosis (Figure 5) (with the exception of sulindac sulfide and 5-FU interaction on Colo-205 that amounted to 1.48) in comparison to cytostatics given alone. This powerful potentiation of apoptosis by sulindac sulfide is not surprising as by itself it has strong pro-apoptotic potential (16). Thus, when studied cytostatics and sulindac sulfide are applied simultaneously they may mutually enhance their activity against CRC cells. Sulindac sulfide-dependent induction of Bax protein and activity of caspase-8, -9 and -3 enzymes (16) may enhance Bax/Bak-dependent mitochondrial pathway induced by 5-FU or oxaliplatin (17, 18).

The synergistic effects of sulindac sulfide with cytostatics and the relatively low cytotoxicity exerted by sulindac sulfide towards normal fibroblast (as shown by the SI ratio for IC₅₀ Balb/c 3T3 / IC₅₀ CRC cell lines, see Table I) makes sulindac sulfide a good candidate for adjuvant therapy. The DRI calculated for the combinations of sulindac sulfide with 5-FU and oxaliplatin indicated that the doses of the cytostatics could be reduced in the range of ~4- to 38-fold (5-FU) and ~5- to 14-fold (oxaliplatin), depending on the cell line (Table II), suggesting that the same inhibitory effect on CRC cell survival may be obtained at lower doses of cytostatics. Furthermore, the concentrations of cytostatics and sulindac sulfide used in the present work are achievable *in vivo* and the unique pharmacokinetic properties of sulindac (formation and concentration of sulindac sulfide in the colon) (19-22) make the combined treatment of sulindac and cytostatics very attractive. Such treatment would limit side-effects against normal cells, but remains far more effective against cancer cells than the efficacy of classical cytostatics alone. Thus, the obtained results warrant further investigation in animal models and clinical trials.

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