Sequence-dependent effect of a cyclooxygenase-2 inhibitor on topoisomerase I inhibitor and 5-fluorouracil-induced cytotoxicity of colon cancer cells

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Selective cyclooxygenase-2 (COX-2) inhibitors have been found to induce anti-proliferative and apoptotic activity in many cancer cells. However, interaction between COX-2 inhibitors and other chemotherapeutic agents remains to be determined. We investigated the interactive effects of a selective COX-2 inhibitor, etodolac, in combination with 5-fluorouracil (5-FU) or SN-38 (active metabolite of irinotecan) on colon cancer cell lines, HT29 and SW620, in simultaneous and sequential administration schedules. Isobologram analysis demonstrated that etodolac in combination with 5-FU or SN-38 according to a simultaneous schedule resulted in only an additive effect; however, synergism was achieved in a sequential schedule. Apoptosis induction in both cell lines was also significantly increased after sequential treatment with etodolac followed by either 5-FU or SN-38 compared to that after simultaneous treatment with etodolac and either 5-FU or SN-38. Our study suggests apoptosis-inducing synergism resulted from administration of etodolac and either 5-FU or SN-38 sequentially, but not simultaneously. *Anti-Cancer Drugs* 15:287–294 © 2004 Lippincott Williams & Wilkins.

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Introduction

Mounting evidence indicates that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the incidence of colorectal cancer (CRC) in human and experimental animals [1,2]. The anti-carcinogenic effects were believed to be associated with inactivation of the cyclooxygenase (COX) enzyme, specifically COX-2 activity. Furthermore, histopathological studies of tumor specimens reveal that high COX-2 expression can be correlated with increased tumor invasiveness in lung cancer [3] and, thus, with poor prognosis in CRC [3–5]. In a previous study on tissues from colon cancer patients, we found that the COX-2 expression level was significantly higher in the primary tumor compared to normal mucosa and even higher in the metastatic tumor tissue, implying that COX-2 may play a significant role not only in the initiation phase, but also in the malignant progression of human CRCs [6]. The growth-retardation and apoptosis-inducing effects of COX-2-selective inhibitors can also be demonstrated in nude mice bearing xenografts of either gastric or head/neck squamous cell cancer [7,8]. Furthermore, the apoptosis-inducing effect of COX-2 inhibitors was more profound in CRC cells with a higher invasive potential. Inactivation of the COX-2 gene could dramatically reduce both the number and size of polyps in a murine model of familial adenomatous polyposis (FAP) [9]. In addition, similar cancer prevention capability is inherent in a COX-2 gene knockout mouse model [10]. Results from animal genetic and pharmacological studies open up the possibility of cancer prevention in FAP patients by inactivation of the COX-2 enzyme. COX-2 inhibitors were found to be capable of inhibiting cell growth, inducing cell-cycle arrest and apoptosis in cancer cells [11,12]. In addition, the COX-2-selective inhibitor, etodolac, was capable of inducing apoptosis in CRC cell lines, especially in those with high invasive potential [13].

For advanced CRC, chemotherapy is currently the major therapeutic modality. Chemotherapy for advanced CRCs is an immense challenge, since most advanced disease patients eventually succumb to the cancer. Therefore, enhancing chemotherapeutic efficacy by combining it with agents acting through different tumor-suppression mechanisms, and by administration according to an optimal schedule, demands continued exploration. 5-Fluorouracil (5-FU), in use for more than 50 years, remains the backbone chemotherapeutic agent for advanced or metastatic CRC, although it produces a response rate of only around 11% [14]. New drugs like irinotecan, in combination with 5-FU, have resulted in great progress in the treatment of advanced CRCs [15].

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Irinotecan, an inhibitor of DNA topoisomerase I, is extensively metabolized in the liver to various metabolites. It is cleaved enzymatically by carboxylesterases to form SN-38, with cytotoxic activity 100–1000 times greater than that of its parent drug [16]. Thus, we used SN-38 in this study to evaluate their interactive effects. Interaction between a COX-2 inhibitor and anti-cancer drugs is of great interest and clinically relevant; however, the resultant effect of combination of a COX-2 inhibitor and 5-FU or SN-38 has been variable [17–19]. Therefore, optimization of drug interaction according to administration sequence may be important and clinically relevant.

The main purpose of this study is to investigate the interaction between a COX-2 inhibitor, etodolac, with known effective chemotherapeutic agents, 5-FU and SN-38, on the cytotoxicity of CRC cells, specifically to explore and identify an optimal administration sequence. The results of this study may offer a basis for developing effective combination chemotherapeutic regimen which incorporate a COX-2 inhibitor into 5-FU or irinotecan-containing regimen for CRC.

Materials and methods

Cell lines and culture methods

The ATCC/HTB38 HT29 and SW620 colon cancer cell lines were obtained from ATCC (Rockville, MD). All cell lines were maintained in a humidified atmosphere of 5% CO_2 and 95% air in minimal essential medium (MEM) (Sigma, St Louis, MO), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM of L-glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin sulfate (all from Life Technologies, Grand Island, NY). For the cytotoxicity studies, cells were seeded at a fixed seeding density in 180 µl of medium into 96-well microtiter plates and incubated overnight at 37°C in a 5% CO_2 atmosphere in order to allow for recovery from trypsinization prior to starting the MTT assay.

Drugs

Etodolac (Wyeth-Ayerst Research, Radnor, PA) [20] was first dissolved in 80% (v/v) ethanol as a stock solution and diluted to appropriate concentration with MEM before each experiment. SN-38 (Aventis Pharma, France), the active metabolite of CPT-11 [21], was dissolved in DMSO (1 mg/ml) as stock solution. 5-FU (ICN, Costa Mesa, CA) was prepared at 10 mg/ml as a stock solution.

Study design and MTT assay

For the MTT assay, HT29 and SW620 were treated with etodolac in combination with either 5-FU or SN-38 in a concurrent or sequential schedule. For concurrent schedules, the tested cells in 96-well plates were treated with etodolac and either 5-FU or SN-38 simultaneously at a series of predetermined concentrations. After a 24-h incubation with the drugs, the medium containing drugs was washed off and replaced with fresh medium. The cells were then subjected to MTT assay after a further 48-h incubation. For the sequential schedule, the tested cells in 96-well plates were treated with etodolac at serial concentrations for 24 h, the etodolac was then washed off, followed by treatment with either 5-FU or SN-38, in graded concentrations for another 24 h. The drugs were then washed off to be replaced with fresh medium. The cells were then subjected to the MTT assay after a further 48-h incubation.

In the cytotoxicity assays for both HT29 and SW620 lines, etodolac was tested at 8–10 interval concentrations ranging from 0.0 to 4.0 mM; 5-FU from 0.0 to 10.0 µg/ml and SN-38 from 0.0 to 200×10^{-9} M.

For either the SW620 or HT29 cell line, 2500 cells were seeded in each well of 96-well plates 16 h before drug administration. For each concentration combination, 24 wells were assayed. Three independent experiments were repeated.

Cell survival was determined by a MTT colorimetric assay [22]. The concentration of 50% viability inhibition (IC_{50}) value was defined as the drug concentration at which 50% reduction in absorbance occurred.

Isobologram

Dose–response interactions between etodolac and 5-FU or between etodolac and SN-38 were evaluated using the isobologram method of Steel and Peckham [23]. The theoretical basis of the isobologram method and the procedure for making the isobologram has been described elsewhere [24–26]. The combination data points would fall near the mode I (hetero-addition) line if the agents were additive by independent mechanisms. If the agents were additive by similar mechanisms, their data points would fall near the mode II (iso-addition) lines.

According to the dose–response curves of etodolac and either 5-FU or SN-38, 'an envelope of additivity' was constructed. Data points were used to determine the interactive effects of *in vitro* drug combinations according to either a concurrent or sequential schedule. When the data points of drug combination fall within the area surrounded by the three lines (envelope of additivity), the combination was regarded as additive. When the data points fall to the left of the envelope, the combination was regarded as having a supra-additive effect (synergism). When the points fall to the right of the envelope, but within the square or on the line of the square, the combination was regarded as having a sub-additive effect, i.e. the combination was superior or equal to a single agent, but was less than additive. When the points fell outside the square, the combination was regarded as

having a protective effect, i.e. the combination was inferior to a single agent in cytotoxicity. Both sub-additive and protective effects were regarded as being antagonistic.

Apoptosis assays

The interactive effects of drugs on HT29 and SW620 cells were also evaluated for apoptosis induction with the Annexin-V assay. HT29 and SW620 cells were treated with 0.5 mM of etodolac in combination with either 0.5 μg/ml of 5-FU or 10×10^{-9} M of SN-38 according to the simultaneous or the sequential schedule as mentioned above. After each treatment, the supernatant in each culture was gently removed, and adherent cells were detached by trypsinization and further Annexin-V binding. After each treatment, 0.5 to 1×10^6 cells were collected, washed twice with phosphate-buffered saline (PBS) and resuspended in 0.5 ml of Annexin-V binding buffer (2.5 mmol/l $CaCl_2$, 20 mmol/l HEPES, pH 7.4, and 140 mmol/l NaCl) containing fluorescein isothiocyanate (FITC)-conjugated Annexin-V and propidium iodide (PI) (10 μg/ml) (R&D Systems, Minneapolis, MN) for 15 min in ice. Annexin-V and PI staining are determined by flow cytometry on a FACScan (Becton Dickinson, San Jose, CA). The apoptosis-induction effect was quantified by Annexin-V assay. The Annexin-V^+/PI^- (early apoptotic) population represents apoptotic cells, while Annexin-V^+/PI^+ population represents necrotic cells.

Statistical analysis

The results of drug interaction derived from the isobologram were analyzed as described previously [27]. If the mean value of the observed data was equal to the predicted maximum or minimum value or fell between the predicted maximum and minimum values, the combination was regarded as having an additive effect. If the mean value of the observed data was smaller than that of the predicted minimum values or larger than that of the predicted maximum values, the combinations were considered to have a synergistic or antagonistic effect respectively. Significance was determined by the Wilcoxon signed-rank test. Probability value $(p) \leq 0.05$ was considered significant. If the observed data is smaller than the predicted minimum, but with $p > 0.05$, the combination will be regarded as having an additive effect rather than a synergistic effect. Similarly, if the observed data is larger than the predicted maximum, but with $p > 0.05$, the combination will still be regarded as having an additive effect. All statistical analyses were performed using the SPSS 11.0 software program.

Results

Dose–response interaction between etodolac and 5-FU or SN-38

The dose–response curves of etodolac in combination with 5-FU or SN-38 in HT29 (A) and SW620 (B) cells are shown in Figure 1. HT29 or SW620 cells were exposed to etodolac in combination with 5-FU or SN-38 concurrently (upper rows in Fig. 1A and B) or sequentially (lower rows in Fig. 1A and B). Cell viability was measured using the MTT assay and was plotted as a percentage of the control (untreated cells). Each isobologram was generated based on such dose–response curves.

Isobologram analysis

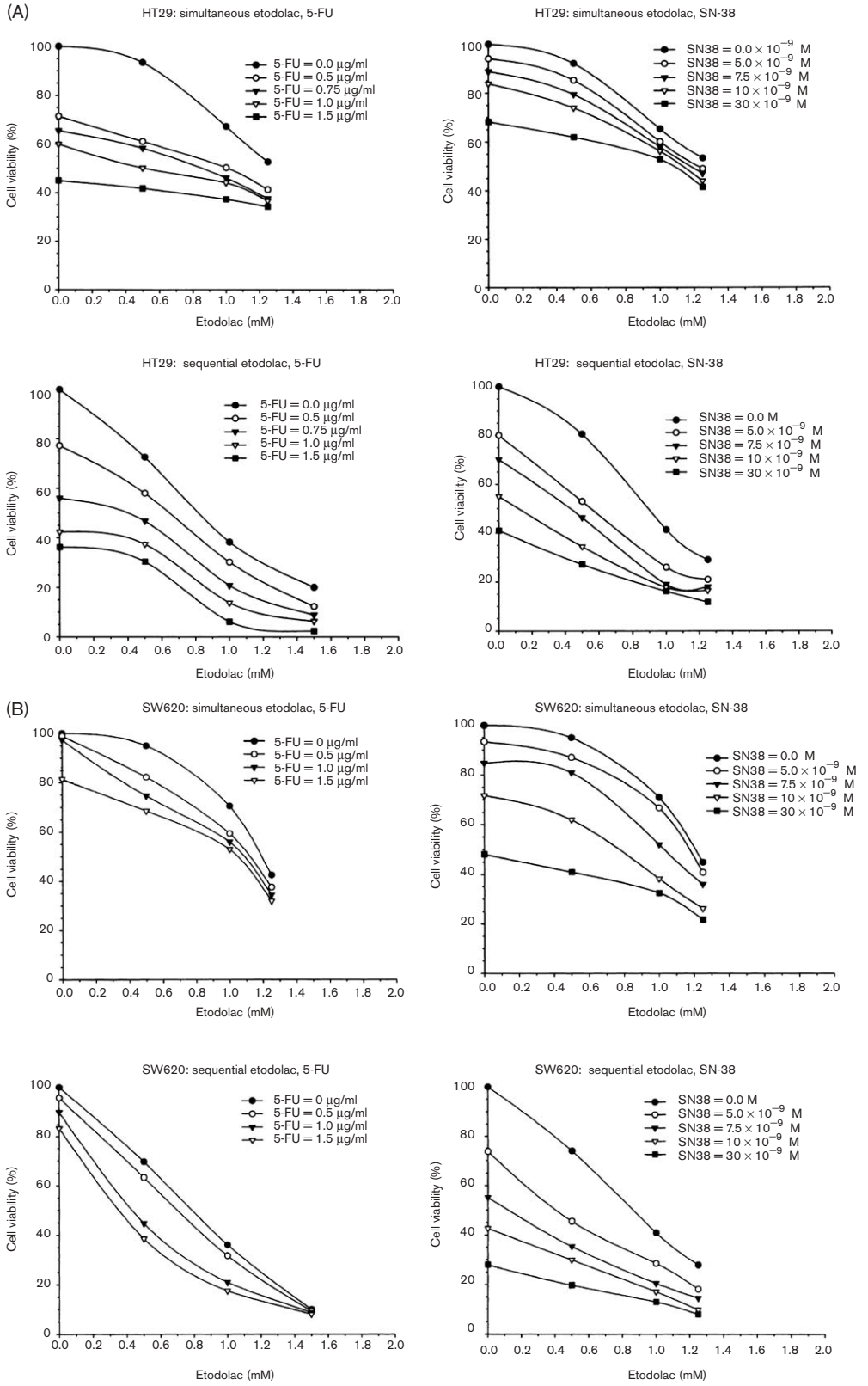
The isobolograms for etodolac in combination with 5-FU in HT29 and in SW620 cells according to the simultaneous schedule are shown in the left upper Figure 2(A and B, respectively). Nearly all data points of iso-effect dose combinations for etodolac and 5-FU administered simultaneously were located in the area of 'additive envelopes' and implied an additive effect. The mean values of the observed data were larger than the predicted minimum values and smaller than the predicted maximum values (Table 1), corroborating an additive effect. In contrast, in the left lower part of Figure 2(A and B), nearly all data points of iso-effect dose combinations for etodolac and 5-FU administered sequentially in HT29 and SW620 cells fell in the area of supra-additivity. These mean observed iso-effective dose were significantly smaller than the predicted minimum values (Table 1), corroborating a synergistic effect.

The isobolograms for etodolac in combination with SN-38 in HT29 and in SW620 cells according to the concurrent schedule are shown in the right upper portion of Figure 2(A and B, respectively). Most data points of iso-effect dose combinations for etodolac and SN-38 administered sequentially were located near the mode II boundary of the additive envelope and implied an additive or a sub-additive effect. The mean values of the observed data were larger than the predicted maximum values; however, not significantly enough to imply an antagonistic effect, but this implies an additive effect (Table 1). In contrast, in the right lower portion of Figure 2(A and B), nearly all data points of iso-effect dose combinations for etodolac and SN-38 administrated sequentially in HT29 and SW620 cells fall in the area of supra-additivity. These mean observed iso-effective doses were significantly smaller than the predicted minimum values (Table 1), corroborating a synergistic effect.

Induction of apoptosis by etodolac in combination with 5-FU or SN-38 administered simultaneously or sequentially

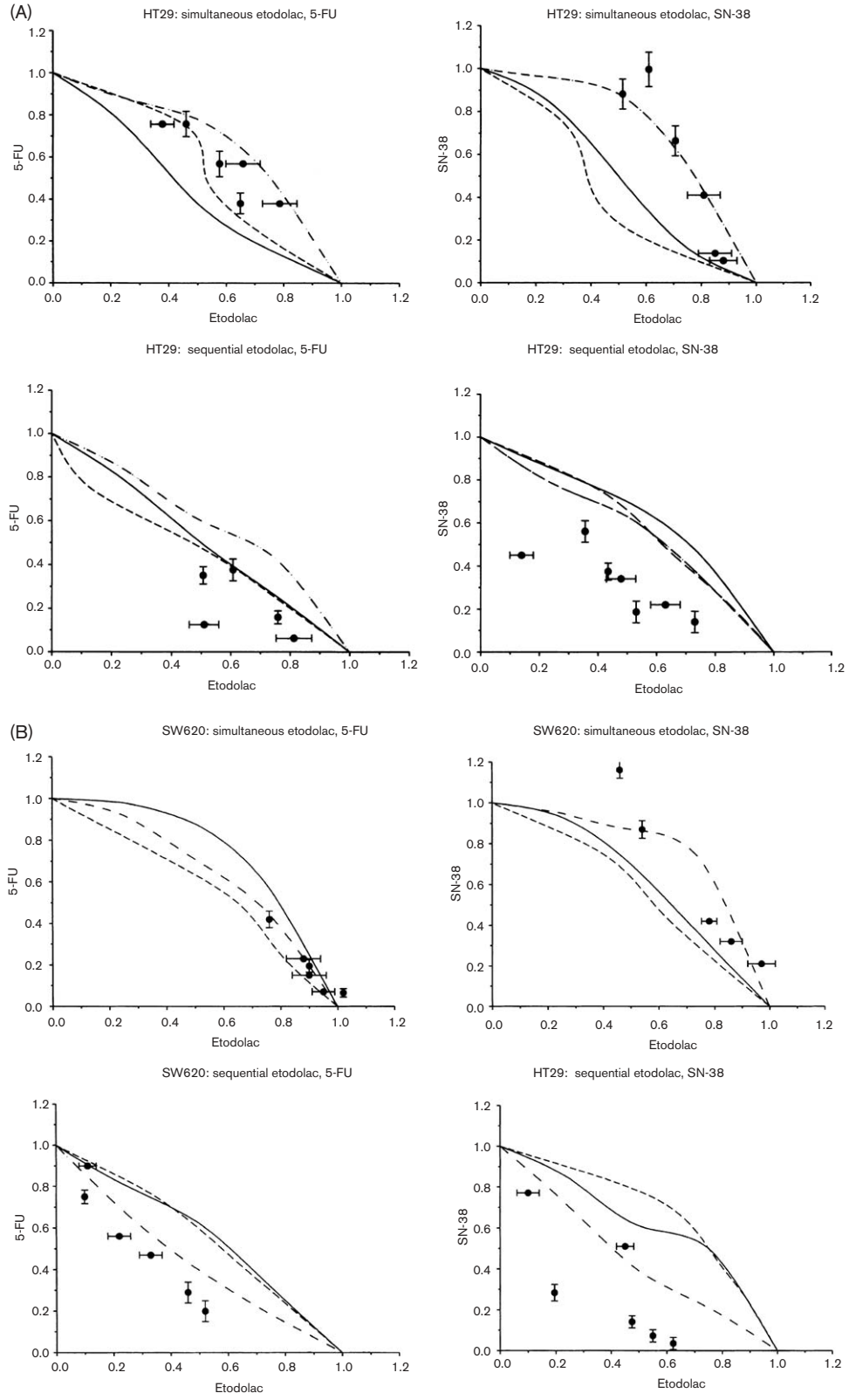
As shown in Figure 3, apoptosis was induced by etodolac in combination with 5-FU or SN-38 administered either concurrently or sequentially. Compared to treatment with a single agent, the apoptotic percentage increased in both HT29 and SW620 cells when they were treated with etodolac in combination with 5-FU or SN-38, regardless of administration following a simultaneous or sequential schedule. Furthermore, the apoptotic percentage induced by sequential exposure to etodolac and then 5-FU or SN-

Fig. 1



Dose–response curves of etodolac in combination with 5-FU or SN-38 in HT29 (A) and SW620 (B) cells. Cells were exposed to the two indicated drugs concurrently [upper rows in (A) and (B)] and sequentially [lower rows in (A) and (B)]. Cell viability was measured using the MTT assay and was plotted as a percentage of the control (cell without exposure to drugs). The concentrations of etodolac are shown on the abscissa, and those of 5-FU and SN-38 on the intercept in each figure. Each point represents the mean of three independent experiments.

Fig. 2



Isobolograms of etodolac in combination with 5-FU (left column) or SN-38 (right column) according to simultaneous (upper row) or sequential (lower row) schedules for the HT29 (A) and the SW620 (B) cells. The 'additive envelopes' were bounded with both mode I (solid line) and mode II (dotted and dash lines) lines. Data are presented as means ± SE for at least three independent experiments. For both HT29 and SW620, the data obtained according to the simultaneous schedule fell mainly in the area of additivity; while those according to sequential schedule, mainly in the area of supra-additivity.

Table 1 **Mean values of observed data, predicted minimum and maximum values of etodolac in combination with 5-FU or SN-38**

	Schedule	Cell line	Observed data[a]	Predicted minimum[b]	Predicted maximum[c]	Effect
Simultaneous	etodolac + 5-FU	HT29	0.59	0.38	0.69	additive
		SW620	0.90	0.86	0.93	additive
Sequential	etodolac → 5-FU	HT29	0.64	0.79	0.88	synergism ($p=0.043$)
		SW620	0.29	0.40	0.55	synergism ($p=0.046$)
Simultaneous	etodolac + SN-38	HT29	0.73	0.40	0.65	additive ($p=0.917$, for antagonism)
		SW620	0.72	0.48	0.64	additive ($p=0.892$, for antagonism)
Sequential	etodolac → SN38	HT29	0.47	0.76	0.82	synergism ($p=0.018$)
		SW620	0.40	0.66	0.84	synergism ($p=0.046$)

[a]Mean value of observed data.
[b]Mean value of the predicted minimum values for an additive effect.
[c]Mean value of the predicted maximum values for an additive effects.

38 was significantly increased compared to that by concurrent exposure to etodolac and 5-FU/or SN-38.

Discussion

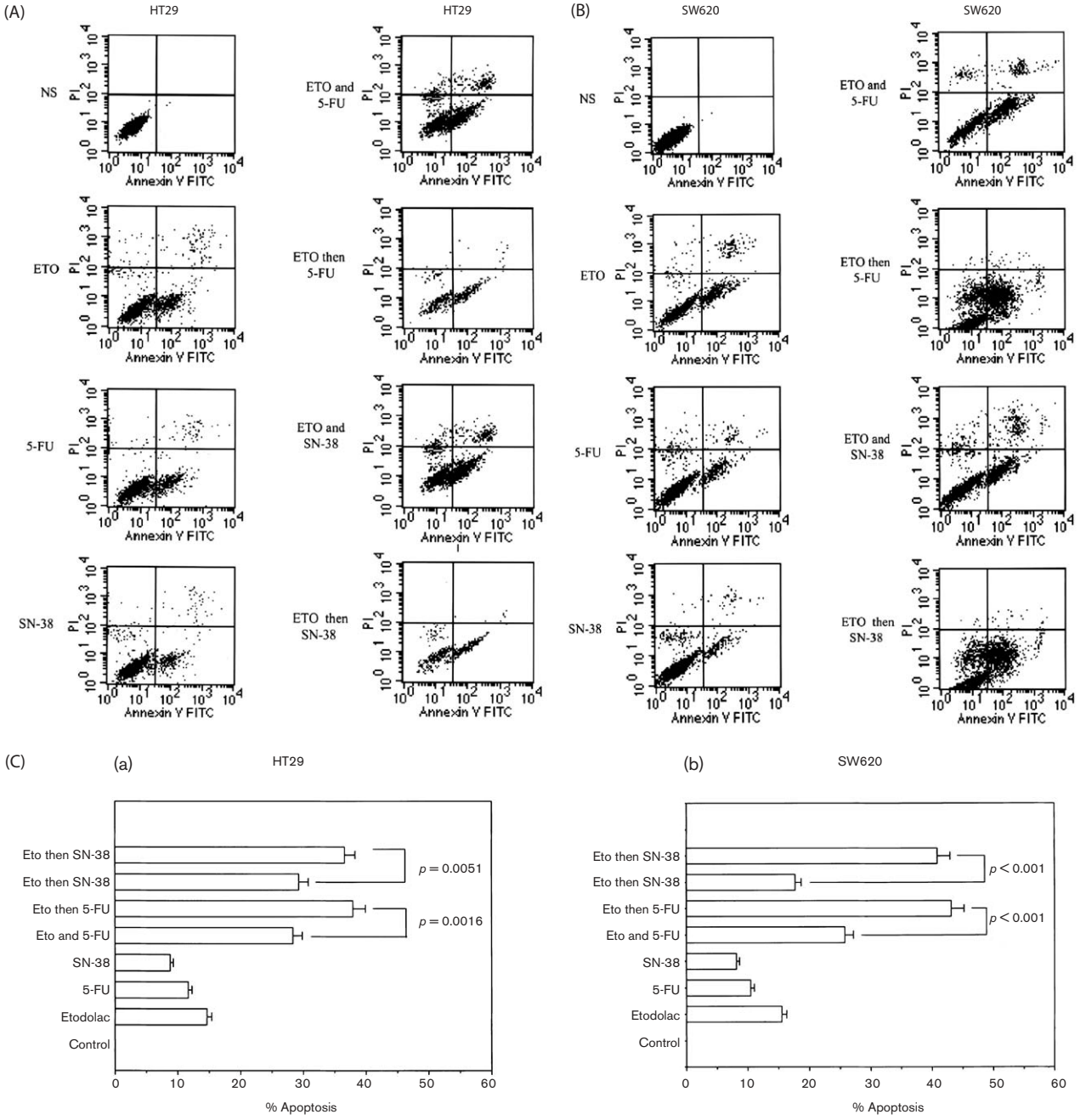
COX-2 inhibitors alone have not only demonstrated their capability to induce cell-growth retardation and apoptosis in many types of cancer cells from *in vitro* experiments, but have also been reported to have the ability to reduce disease progression in FAP patients, and inhibit growth and metastatic potential of colon cancer cells both in animal models and *in vitro* studies [13,15,28]. Thus, this raises the question whether it is possible to incorporate this anti-inflammatory drug to enhance the efficacy of chemotherapy regimen currently used for the treatment of cancer patients.

In this study, we have demonstrated that the cytotoxic effect of etodolac, a COX-2 inhibitor, in combination with 5-FU or SN-38 to be schedule-dependent, favoring a sequential schedule. We have also shown that sequential exposure to etodolac followed by either 5-FU or SN-38 was more effective than concurrent exposure. Our data may provide a preclinical rationale in scheduling combination drug therapy for CRC, optimizing tumor control efficacy in patients receiving long-term NSAIDs or COX-2 inhibitors, and also in patients undergoing chemotherapy.

Agents for use in combination therapy should be at least as effective as monotherapy and have different mechanisms of cytotoxic action with non-overlapping toxicity profiles. The anti-tumor effect of NSAIDs probably manifests itself through the induction of cell death in neoplastic tissues. COX-2-selective inhibitors may induce growth retardation and apoptosis of gastric [29] and head/neck squamous cancer [8] implanted as xenografts in nude mice. The effect of inhibition on COX enzyme, especially the COX-2 form, is thought to be the principle mechanism by which NSAIDs induce apoptosis. COX-related mediators are likely to function through signal transduction pathways, like the MEK/ERK pathway, leading to an ultimate regulatory pathway of apoptosis [30]. The apoptosis-inducing mechanism of COX inhibitors are thus different from those of the traditional chemotherapeutic agents, such as thymidylate synthase blocker 5-FU or topoisomerase I-targeting drug CPT-11, which exert their cytotoxic effect either by direct inhibition of DNA synthesis or enzyme-mediated DNA damage. However, when etodolac and 5-FU/SN-38 were applied simultaneously on HT29 and SW620 cells, as shown in Figures 2(A and B), the data points are closer to the mode II line than to mode I line in the isobolograms. This implies that the cytotoxic mechanisms by which COX-2 inhibitors and 5-FU or SN-38 interact may be similar, at least in some unknown aspects. We suspect that the COX-2 inhibitor and 5-FU/SN-38 probably induce overlapping signals leading to apoptosis or affect cell-cycle regulation. This may contribute to the additive, but not synergistic, results of simultaneous exposure to etodolac in combination with 5-FU or SN-38.

It is interesting that although we have demonstrated a supra-additive effect in sequential administration of etodolac followed by either 5-FU or CPT-11, only an additive effect has been noted in the simultaneous administration regimen of etodolac with either 5-FU or SN-38. This may result from a multi-factorial interaction; amongst these factors, we are most impressed by the cell-cycle dependence. 5-FU is a thymidine synthase inhibitor and is well known to be S phase dependent. The cytotoxicity of topoisomerase I inhibitor (e.g. SN-38) is also S phase specific, because the collision of advancing replication forks with inhibitor–topoisomerase I–DNA complexes results in DNA damage and this may activate an S checkpoint response [31,32]. However, COX-2 inhibitor was reported in some cases to induce cell-cycle arrest and induce cell accumulation in G_0/G_1 phase [12,33]. The cell-cycle arrest induced by COX-2 inhibitors may thus attenuate the cytotoxicity of 5-FU and SN-38 when the COX-2 inhibitor is given concurrently. On the other hand, the COX-2 inhibitor alone may induce apoptosis (Fig. 3) or, in some cases, when apoptosis does not actually occur, it may sensitize some HT29 or SW620 colon cancer cells to be apoptotic. When

Fig. 3



Quantification of apoptotic cells of exponentially growing HT29 (A) and SW620 (B) cells by Annexin-V assay. Right lower quadrant in each small figure represents the population of apoptotic cells with low intensity of propidium iodide, but high Annexin-V expression. The control representing cells without exposure to drugs is shown in the first row, left column of (A) or (B). Cells treated with the indicated single drug are also shown in the left column. In the right column, Annexin assays are used to determine apoptotic cells treated simultaneously with etodolac and 5-FU (first row) or with etodolac and SN-38 (third row); and sequentially with etodolac being followed by 5-FU (second row) and with etodolac being followed by SN-38 (fourth row). Apoptotic percentage of HT29 (C, a) and SW620 (C, b) cells treated with the indicated protocols are presented. Both HT29 and SW620 cells showed significant increments ($p<0.01$) in apoptotic percentage when treated with sequential schedules compared to those treated with simultaneous schedules. Data are presented as mean values (columns) and standard errors (bars) of at least three independent experiments.

administrated in sequence, tumor cells, after the withdrawal of the COX-2 inhibitor, re-enter the cell cycle in synchronization and are sensitized to the second apoptosis-inducing agent. This is a probable mechanism by which a sequential COX-2 inhibitor followed by 5-FU or SN-38 exhibits a synergistic effect.

In summary, we have demonstrated that there is synergism between a selective COX-2 inhibitor, etodolac, in combination with either 5-FU or CPT-11 on colon cancer cell lines, and that the synergism is sequence dependent. Our findings provide a guideline for administration of combination chemotherapeutic drugs for CRC patients, including for those on long-term prescription of a COX-2 inhibitor. In addition, our results may provide a preclinical rationale and model for integration of a COX-2 inhibitor with chemotherapeutic agents in CRC patients.

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