The role of protein kinase C in the synergistic interaction of safingol and irinotecan in colon cancer cells

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Abstract. Colon cancer represents one of the most common solid tumors in adults. Although 5-fluorouracil (5-FU) and irinotecan have been frequently administered in colon cancer patients, low response rates to these single drug therapies were reported. It is therefore imperative to search for new targeted combination therapies that are effective. In this study, we investigated the anti-cancer effect of safingol as a single agent or in combination with irinotecan using HT-29 and LS-174T colon cancer cells as our *in vitro* models. As a single agent, safingol was more potent than irinotecan and 5-FU, with IC_{50} values of 2.5±1.1 μM and 3.4±1.0 μM achieved in HT-29 and LS-174T cells, respectively. However, protein kinase C (PKC) was not inhibited with concentrations of safingol which could induce substantial cell kill. The combination of safingol/irinotecan at 1:1 molar ratio was found to be additive in HT-29 cells (CI=0.94) and synergistic in LS-174T cells (CI=0.68), and resulted in concentration- and time-dependent down-regulation of p-PKC and p-MARCKS. The drug effect of the safingol/irinotecan combination was further modulated in the presence of a PKC stimulator (phorbol 12-myristate 13-acetate) or a PKC inhibitor (staurosporine). Furthermore, the 1:1 safingol/irinotecan combination inhibited the adhesion of colon cancer cells to the extracellular matrix 4-h post-treatment. Taken together, modulation of the PKC pathway could be a possible molecular basis for the observed synergism of the safingol/irinotecan combination, and these results demonstrate the therapeutic potential of this drug combination in colon cancer treatment.

Introduction

Colon cancer is one of the most frequently diagnosed cancers, and it is a leading cause of cancer-related mortality worldwide (1). Anti-cancer drugs such as irinotecan and 5-fluorouracil (5-FU) have been routinely administered, often with curative intent, to eradicate circulating cancer cells. Irinotecan is a topoisomerase I inhibitor, whereby inhibition of this enzyme results in stabilization of the cleavable complex, breakage of DNA strands, failure of replication and ultimately cell death (2-4). 5-FU inhibits thymidylate synthase, an enzyme essential for the synthesis of pyrimidines required for DNA replication (5). Nevertheless, response rates of colon cancer patients treated with these two standard cytotoxic drugs remain <40% when these drugs are used as monotherapies, with patients subsequently becoming resistant (6,7). Therefore, it is imperative to identify other drugs or drug combinations, especially those which can target particular signaling pathways to provide alternative therapeutic options to effectively eliminate the cancer cells and improve the survival of colon cancer patients.

Bioactive lipid molecules are receiving increasing attention due to their emerging role in the pathogenesis of human disorders including cancer, inflammation, neurological, immune and metabolic disorders (8,9). Safingol [(2S, 3S)-2-amino-1,3-octadecanediol], a bioactive lipid, is a saturated analog of sphingosine (10-12). It acts as a competitive inhibitor of sphingosine kinase (SK) that prevents the formation of sphingosine-1-phosphate (S1P) (10,13), and in turn, inhibits cell proliferation, invasion and angiogenesis (8,13). Another suggested mechanism by which safingol could possibly act is by modulating protein kinase C (PKC) pathway, whereby safingol displaces phorbol dibutyrate from its lipid binding site on the regulatory domain of PKC (12,14). As a result, PKC-regulated processes such as cell survival, proliferation, differentiation and invasion could be inhibited (15,16).

When used in combination with other anti-cancer drugs, safingol demonstrated synergism with fenretinide, 1-ß-D-arabinofuranosylcytosine (ara-C), cisplatin, vinblastine, and mitomycin-C in a variety of tumor cell lines (11,17-22). More importantly, safingol has been demonstrated in a resistant cell line, MCF-DOXR (20), and in a pilot study (11) that it exhibited synergistic effects with doxorubicin without dose-limiting toxicity. Currently, safingol in combination with cisplatin is under phase I clinical trial for patients with advanced solid tumors, in which preliminary data showed promising activity in refractory adrenocortical cancer (23).

While safingol has been previously shown to potentiate the anti-cancer effect of irinotecan in colon cancer cells (6), the

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molecular mechanism underlying the potentiating effect has not been explored. Since the PKC pathway plays an important role in colon cancer cell proliferation, adhesion and migration (24,25), we hypothesize that the enhanced cancer cell kill by a combination of safingol and irinotecan could be mediated through the PKC pathway. In this study, we probed the changes in the expression levels of phosphorylated PKC and its downstream substrate MARCKS, as well as the ability of colon cancer cells to attach to extracellular matrix upon exposure to safingol/irinotecan combination. We also analyzed the drug effect of this combination in the presence of modulators of PKC activity, and used the median effect principle described by Chou and Talalay (26) to give a quantitative representation of the pharmacological interaction between safingol and irinotecan.

Materials and methods

Reagents. Safingol was purchased from Sigma (St. Louis, MO, USA) and dissolved in ethanol. Stock solutions of 2 mg/ml were stored at -20˚C and freshly diluted with medium to the appropriate concentrations before use in experiments. Irinotecan hydrochloride trihydrate 20 mg/ml (Aventis Pharma, UK) and 5-FU 50 mg/ml (Mayne Pharma, Australia) were obtained from the National University Hospital, Singapore. All other chemicals used in the study were purchased from Sigma Chemical Company unless otherwise stated.

Cell cultures. Two human colorectal cancer cell lines, LS-174T and HT-29, were purchased from American Type Culture Collection (Manassas, USA). Stock cultures of both cancer cell lines were maintained as monolayer in 75-cm^2 tissue culture flasks (Iwaki, Japan). LS-174T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) while HT-29 in Iscove's modified Dulbecco's medium (IMDM). All media were supplemented with 10% v/v fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA), 0.3 g/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cell lines were maintained in a humidified, 5% CO_2 incubator at 37˚C and sub-cultured twice weekly using 0.25%v/v trypsin/EDTA (Gibco, Grand Island, NY, USA). All experiments were performed using cells in exponential growth phase from passage 5-20 post-thawing from frozen stock.

Cell viability assay. Viability of the cancer cells following drug treatment was determined using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, cells were plated at 5,000 per well in 96-well plates and allowed to adhere overnight before exposure to safingol, irinotecan or 5-FU alone or as fixed molar ratio combinations of safingol/irinotecan at 1:1 (safingol/irinotecan concentrations are 0.313:0.313, 0.625:0.625, 1.25:1.25, 2.5:2.5, 5.0:5.0, 10.0:10.0 μM), 1:4 (safingol/irinotecan concentrations are 0.078:0.313, 0.156:0.625, 0.313:1.25, 0.625:2.5, 1.25:5.0, 2.5:10.0 μM) and 4:1 (safingol/irinotecan concentrations are 0.156:0.039, 0.313:0.078, 0.625:0.156, 1.25:0.313, 2.5:0.625, 5.0:1.25 μM). In some experiments, 100 nM of phorbol 12-myristate 13-acetate (PMA) or 50 nM of staurosporine was added 1 h before the addition of drugs. The plates were then incubated for 72 h at 37˚C. At the end of the incubation, 50 μl MTT (1 mg/ml in media) was added to cells and incubated for 4 h. Subsequently, the MTT-containing medium was removed, and the purple formazan precipitate was solubilized in DMSO. Absorbance (λ=570 nm) was measured in a microtiter plate reader (Tecan, Infinite M2000). All assays were done at least 3 times unless otherwise stated.

Cancer cell viability was calculated based on the absorbance readings using the following equation: viability = $[(Abs_{test} - Abs_{blank})/(Abs_{vehicle\ control} - Abs_{blank})] \times 100\%$, where Abs_{test}, Abs_{blank}, $Abs_{vehicle\ control}$ represent the absorbance readings from the drug-treated wells, the medium only wells and the vehicle control wells, respectively. Using the median effect principle described by Chou and Talalay (26), the potency of the drugs could be reflected by the median dose (D_m) values which were estimated using CalcuSyn 3.0 software (Biosoft, Cambridge, UK). With respect to drug combination experiments, results obtained from the MTT assay were used to compute the combination index (CI) using the following equation, with the assumption that the drug combinations were mutually exclusive: $CI = [(D)_1 / (Dx)_1] + [(D)_2 / (Dx)_2]$, where $(D)_1$ and $(D)_2$ are the concentrations of drug 1 and drug 2, respectively, that inhibit x% in the actual experiment when they are used in combination. $(D_x)_1$ and $(D_x)_2$ are the concentrations of drug 1 and drug 2, respectively, that inhibit x% in the actual experiment when they are used as single agents. Having a CI of <1, ~1 or >1 is indicative of a synergistic, additive or antagonistic interaction for a drug combination.

Cell cycle analysis. Flow cytometric analysis was performed to determine the distribution of cells in the various phases of the cell cycle using propidium iodide (PI) to stain for nuclear DNA as described previously (27). Briefly, $1x10^7$ cells were harvested, fixed with ice-cold 70% v/v ethanol, and stored overnight at -20˚C followed by staining with PI staining buffer (1 mg/ml RNase A, 0.1% v/v Triton X-100, 50 μg/ml PI in PBS) at 37˚C for 15 min. The samples were subsequently kept on ice for 1 h before analysis. Stained samples were analyzed on the Dako flow cytometer model CyAn™ ADP, and 10,000 events were collected. Data were plotted with Summit V4.3 Build 2445 software. The percentage of cells in the sub-G1/G0 phase represented the apoptotic fraction.

Western blotting. HT-29 and LS-174T cells were exposed to safingol alone, irinotecan alone or the combination of the two drugs for the specified concentrations and duration. In some experiments, cells were pretreated with PMA for 1 h before the addition of drugs. After drug treatment, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold cell lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% w/v NP-40, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium vanadate, 10% v/v glycerol, 1X protease inhibitor cocktail) just before use. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Proteins were separated by electrophoresis carried out on 1.5-mm thick, 7.5% sodium dodecyl sulfate-polyacrylamide gels using Bio-Rad's Powerpac™ HC Apparatus (Bio-Rad Laboratories, Singapore) and electroblotted onto nitrocellulose membranes in ice-cold transfer buffer (39 mM glycine, 48 mM Tris base,

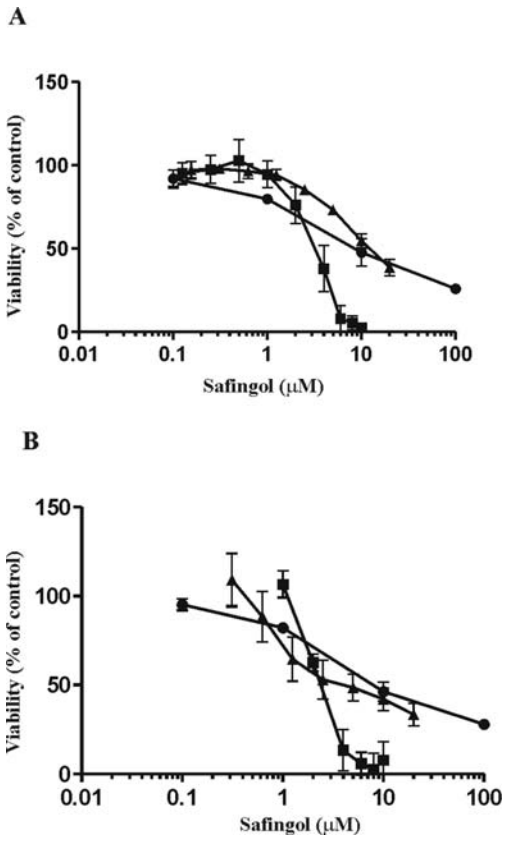

Figure 1. Effects of safingol (■), irinotecan (▲) or 5-FU (●) on the viability of (A) HT-29 and (B) LS-174T cells. Cells were treated with the indicated concentrations of drugs for 72 h. Cellular viability was assessed using MTT assay. Results shown are means ± SEM from 3 independent experiments.

20% v/v methanol). The membranes were subsequently blocked with 5% w/v bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline with 0.1% v/v Tween-20 (TBS/T) for 2 h. The membranes were then probed with primary antibodies overnight at 4˚C. Primary rabbit polyclonal p-PKC (pan) (γThr514) and p-MARCKS (Ser152/156) antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and diluted with TBS-Tween (TBS/T) in 1:1000 ratio containing 5%w/v BSA. Membranes were probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Pierce Biotechnology, Rockford, IL, USA) for another hour, followed by detection with chemiluminescence Supersignal® West Dura Extended Duration Substrate (Pierce Biotechnology). ß-actin was included as the loading control.

Cell adhesion assay. In brief, cells were plated at $6x10^5$ per well in 6-well plates and allowed to adhere overnight. The next day, cells were exposed to safingol, irinotecan or combinations of safingol/irinotecan at 1:1 molar ratio for 4 h in serum-free condition. At the end of the drug treatments, equal number of viable cells was seeded onto a 24-well plate which was precoated with basement membrane matrix gel (BD Biosciences, MA) and allowed to attach for 24 h at 37˚C. At the end of the incubation, media was aspirated and the non-adhered cells were washed thrice with PBS. Morphology of cells was photographed using Leica light microscope. Lastly,

Table I. IC_{50} values of safingol, irinotecan and 5-FU in HT-29 and LS-174T colon cancer cell lines.

Cell line	Safingol (μM)	Irinotecan (μM)	5-FU (μM)
HT-29	2.5±1.1	14±2.5[a]	23±7.3[a]
LS-174T	3.4±1.0	7.3±2.3[a]	35±13[a]

Cell viability was determined using MTT assay and subsequently analyzed by Calcusyn 3.0 software to estimate IC_{50} values. Each reported value is the mean ± SEM from 3 independent experiments. All r-values were ≥0.9. [a]$p<0.05$, significantly different from safingol-treated group.

400 μl of premixed WST-1 cell proliferation reagent (Clontech Laboratories, Inc.) was added and absorbance was measured at 440 nm. Percentage of cell adhesion was calculated using the following equation: % cell adhesion = $(Abs_{test}/Abs_{vehicle\ control})$ x 100%.

Statistical analysis. All data values are reported as mean ± standard error of the mean (SEM). Statistical differences were determined using the one-way analysis of variance (ANOVA) with Newman-Keuls test used for post-hoc multiple comparisons. $P<0.05$ were considered to be statistically significant.

Results

Effect of safingol, irinotecan or 5-FU as single agents in colon cancer cells. The biological effect of safingol on HT-29 and LS-174T colon cancer cell lines was compared to that of irinotecan and 5-FU, the two cytotoxic drugs commonly used in colon cancer therapy. Cell viability was determined using MTT assay. After drug exposure of 72 h, the viability of HT-29 and LS-174T cells was reduced by safingol, irinotecan and 5-FU in a concentration-dependent manner (Fig. 1). In order to compare the potency of safingol, irinotecan and 5-FU in the two cell lines, the IC_{50} value of each drug in the respective cell line was estimated and summarized in Table I. Among the three drugs, the IC_{50} values for safingol were the lowest, with 2.5±1.1 μM and 3.4±1.0 μM achieved in HT-29 and LS-174T cells, respectively. This translates to a 5.6-fold and a 2.1-fold improvement in potency in HT-29 and LS-174T cells, respectively, when safingol activity was compared to that of irinotecan. Similarly, a 9.2- and 10-fold improvement in potency was observed in HT-29 and LS-174T cells, respectively, when safingol activity was compared to that of 5-FU. Of note, these IC_{50} values of safingol could be achieved in the bloodstream according to a pilot phase I study (11).

Effect of fixed ratio combinations of safingol and irinotecan. As irinotecan showed relatively poor treatment response rates when administered as a single agent, it has been combined with safingol previously, using specific concentrations of the two agents in an attempt to enhance the anti-cancer effect (6). In our study, fixed molar ratio combinations of safingol and

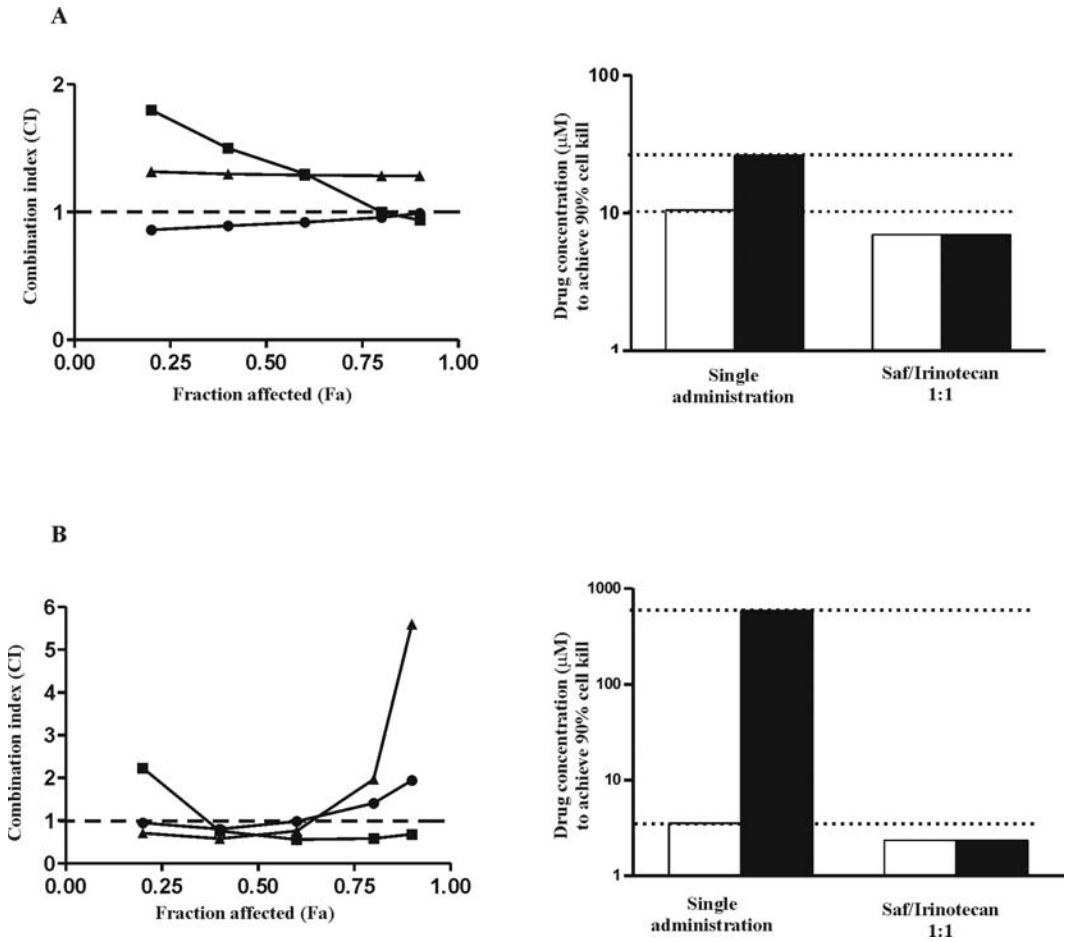

Figure 2. Effects of safingol on irinotecan cytotoxicity in (A) HT-29 and (B) LS-174T cells. Left panel: Combination index was plotted as a function of fraction affected (Fa) for safingol/irinotecan at 1:1 (■), 1:4 (▲) and 4:1 (●) molar ratio in the colon cancer cell lines. Cell viability was assessed using MTT assay and effective concentrations were analyzed using Calcusyn 3.0 software. CI value of <1 is synergistic, ~1 is additive and >1 is antagonistic. Right panel: The drug concentrations required to achieve 90% cell kill of safingol (white bars) and irinotecan (black bars) used alone or at 1:1 fixed molar ratio are shown.

Table II. Combination indices of safingol/irinotecan combinations administered in different molar ratios in colon cancer cell lines.

	Combination index (CI)	
Combination	LS-174T	HT-29
Safingol/Irinotecan		
4:1	1.9	0.99
1:1	0.68	0.94
1:4	5.6	1.3

Results from MTT viability assay were pooled from 3 independent studies and used to compute the CI values using Calcusyn 3.0 software. The reported CI values were based on the drug concentration to achieve 90% cell kill. CI <1, ~1 or >1 denotes a synergistic, additive or antagonistic interaction of the combination, respectively. All r-values were ≥0.9.

irinotecan were evaluated over a range of concentrations, and specifically, the two drugs were administered at molar ratios of 4:1, 1:1 and 1:4. The measure of synergy between the two drugs was determined by the CI value derived from the median effect principle described by Chou and Talalay using the software CalcuSyn 3.0 (26).

The plots of CI against fraction affected (Fa) for the various fixed ratio combinations of safingol/irinotecan in HT-29 and LS-174T cells are presented in Fig. 2. It is important to evaluate if the drug combination is synergistic at maximum cancer cell kill; thus, the CI values were estimated at an effect level of 90% cell kill for the various ratios and are summarized in Table II. The 1:1 molar ratio of safingol/irinotecan appeared to be the most promising, with a CI value of 0.68 in LS-174T cells indicating synergy and a CI value of 0.94 in HT-29 cells indicating additivity. Furthermore, safingol/irinotecan at 1:1 molar ratio allowed substantial dose reduction when used in combination as compared to the administration of the individual agents. Specifically, the concentrations to achieve 90% cell kill in HT-29 and LS-174T cells for irinotecan were reduced by 3.7- and 250-fold, respectively, and those for safingol were reduced by 1.5-fold in both of the cell lines (Fig. 2). On the contrary, safingol/irinotecan at 1:4 molar ratio showed strong antagonism in both cell lines, and the combination at 4:1 molar ratio showed antagonism in LS-174T cells and additive effect in HT-29 cells. These results are not unexpected as treatment outcome

Table III. Percentage of cells in various phases of the cell cycle after treatment with safingol, irinotecan or safingol/irinotecan (1:1) for 24 and 48 h.

	sub-G1/G0	G1	S	G2/M
HT-29 24 h treatment				
Control	1.73±0.1%	50.7±1.48%	26.0±2.92%	22.1±1.76%
10 μM Safingol	7.65±1.31%[a]	37.3±2.65%[a]	19.9±3.62%	35.7±3.65%
10 μM Irinotecan	2.11±0.37%	4.91±0.23%[a]	39.9±12.3%	53.8±12.3%[a]
Saf/Irinotecan (1:1)	10.9±1.71%[a]	13.8±1.69%[a]	27.6±2.57%	48.4±3.14%
HT-29 48 h treatment				
Control	2.24±0.16%	56.9±4.20%	23.4±4.67%	18.1±0.79%
10 μM Safingol	14.3±0.35%[a]	38.6±2.63%[a]	19.6±1.43%	28.3±3.20%
10 μM Irinotecan	13.2±2.85%[a]	7.19±0.69%[a]	19.3±4.43%	49.2±12.5%[a]
Saf/Irinotecan (1:1)	23.2±3.99%[a]	17.7±0.74%[a]	28.6±1.25%	31.0±2.36%
LS-174T 24 h treatment				
Control	4.74±0.25%	65.4±2.72%	17.7±1.99%	12.8±0.74%
10 μM Safingol	32.7±7.09%[a]	43.5±5.72%[a]	12.3±0.18%	12.0±1.32%
10 μM Irinotecan	5.57±1.17%	18.4±1.65%[a]	28.9±5.14%[a]	47.8±4.46%[a]
Saf/Irinotecan (1:1)	38.6±4.02%[a]	22.4±1.90%[a]	17.2±0.49%	22.0±2.76%
LS-174T 48 h treatment				
Control	8.84±1.81%	65.4±1.94%	14.5±1.29%	11.0±2.30%
10 μM Safingol	41.8±7.03%[a]	39.1±4.45%[a]	11.5±0.67%	6.35±2.14%
10 μM Irinotecan	9.87±0.88%	18.0±1.85%[a]	32.3±4.25%[a]	37.6±3.81%[a]
Saf/Irinotecan (1:1)	57.7±3.24%[a]	18.8±2.03%[a]	12.1±0.39%	14.3±3.36%

Cell cycle analysis of HT-29 and LS-174T cells after 24 and 48 h treatment with 10 μM safingol, 10 μM irinotecan or 10 μM safingol + 10 μM irinotecan (1:1 molar ratio). Each reported value represents the mean ± SEM from 3 independent experiments. [a]$p<0.05$, significantly different from control group.

of drug combinations could be highly dependent on the ratios of the individual agents (28).

Effect of safingol/irinotecan at 1:1 molar ratio on the cell cycle status of colon cancer cells. Propidium iodide staining was performed on cells treated with safingol or irinotecan alone or the drug combination, and the results are presented in Table III. Three observations could be made. First, as the exposure time was increased from 24 to 48 h, treatments with either the individual drugs (10 μM safingol or 10 μM irinotecan) or the combination (10 μM safingol + 10 μM irinotecan) exhibited increases in the apoptotic cell fraction as indicated by the percentage in the sub-G1/G0 phase. Second, it is of note that the cell cycle profiles of irinotecan-treated cells were different from those of safingol-treated cells. Irinotecan induced significant increases in the percentage of cells in the G2/M phase, which is consistent with previous findings (29,30), whereas safingol induced significant increases in the apoptotic cell fraction without significantly affecting G2/M phase. These results suggest that safingol and irinotecan acted differently on the cell cycle and in the induction of cell death. Third, comparing the apoptotic cell fraction of single agent treatment with that of the 1:1 molar ratio of the drug combination, the results indicated that the 1:1 safingol/irinotecan combination was additive in HT-29 cells and synergistic in LS-174T cells, which are consistent with the cell viability data presented in Fig. 1.

Role of PKC in mediating the cytotoxic effect of safingol and safingol/irinotecan combination. Since the PKC pathway plays an important role in colon cancer cell proliferation, adhesion and migration (24,25), it is of interest to investigate the role of PKC in mediating the cytotoxic effect of the safingol/irinotecan combination. The expression levels of phosphorylated PKC and its downstream substrate, MARCKS (20), were probed by immunoblotting after treatment with safingol or irinotecan alone or the drug combination at 1:1 molar ratio (Fig. 3). Unexpectedly, the expression levels of p-PKC and p-MARCKS in HT-29 and LS-174T cells were only slightly reduced by 10 μM safingol after 72 h of exposure (Fig. 3A), despite the finding that safingol is a PKC inhibitor which binds to the regulatory domain of the kinase (12,14). While substantial cell kill could be achieved with 10 μM safingol (Fig. 1 and Table III), this effect may not be mediated through modulation of PKC activity. On the contrary, safingol/irinotecan at 1:1 molar ratio substantially reduced the expression levels of p-PKC and p-MARCKS in HT-29 and LS-174T cells (Fig. 3A). Furthermore, the reduction of p-PKC and p-MARCKS expression levels was found to be dependent on the concentration of the drug combination (Fig. 3B) and the exposure time (Fig. 3C).

To further investigate the role of PKC in the synergistic effect of 1:1 safingol/irinotecan combination, modulators of PKC activity were used on LS-174T cells, which included the stimulator phorbol 12-myristate 13-acetate (PMA) (31)

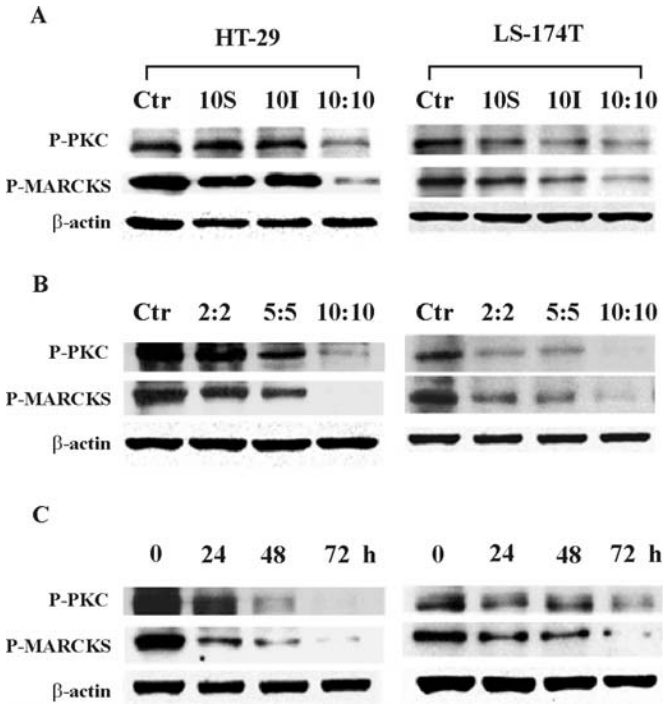

Figure 3. The effects of safingol, irinotecan or safingol/irinotecan (1:1) on the phosphorylation of PKC and MARCKS in HT-29 and LS-174T cells. Protein lysates were assayed by Western blotting and ß-actin was used as the loading control. (A) HT-29 and LS-174T cells were treated with 10 μM safingol, 10 μM irinotecan or 10 μM safingol + 10 μM irinotecan (1:1 molar ratio) for 72 h. (B) Concentration-dependent down-regulation of p-PKC and p-MARCKS. Values indicated in this panel represent the molar concentrations (in μM) of safingol and irinotecan when given at 1:1 ratio. (C) Time-dependent down-regulation of p-PKC and p-MARCKS. The concentration of safingol and irinotecan used in the 1:1 combination was 10 μM for each drug. All blots shown are representative of 3 independent experiments.

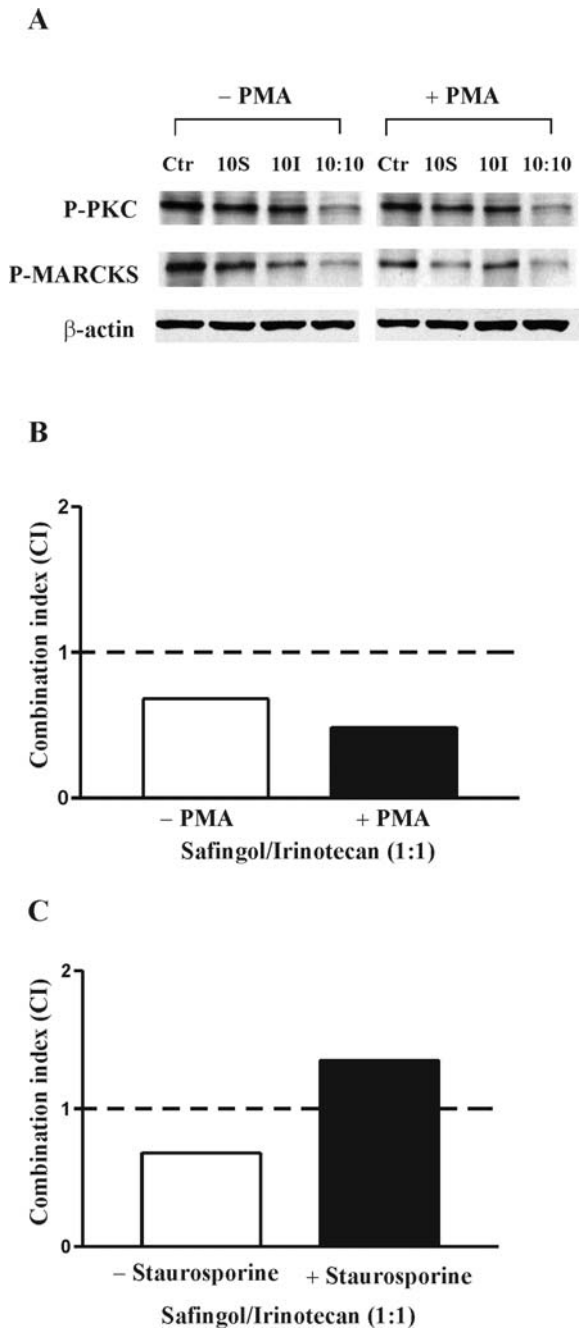

Figure 4. Effects of safingol, irinotecan or safingol/irinotecan (1:1) with or without phorbol 12-myristate 13-acetate (PMA) stimulation in LS-174T cells. (A) Phosphorylation of PKC and MARCKS with or without PMA stimulation. Protein lysates were assayed by Western blotting and ß-actin was used as the loading control. All blots shown are representative of 3 independent experiments. (B) Combination index at 90% cell kill of safingol/irinotecan (1:1) with (black bars) or without (white bars) PMA stimulation. (C) Combination index at 90% cell kill of safingol/irinotecan (1:1) with (black bars) or without (white bars) staurosporine. Cell viability was assessed using MTT assay and effective concentrations were used to compute the combination indices using Calcusyn 3.0 software. CI value of <1 is synergistic, ~1 is additive, and >1 is antagonistic.

and the inhibitor staurosporine (32). Treatment with safingol alone or irinotecan alone provided modest reduction of p-PKC and p-MARCKS expression levels when the colon cancer cells were stimulated with PMA (Fig. 4A). In contrast, treatment with the 1:1 safingol/irinotecan combination reduced substantially the phosphorylation of PKC and of MARCKS. Cell viability in the presence of PMA stimulation was determined for various drug treatments, and CI values were determined based on the viability data. In LS-174T cells, the CI value for the combination was reduced from 0.68 (no PMA) to 0.48 (with PMA), indicating an increase in synergistic activity under PKC stimulation (Fig. 4B). In contrast, the presence of 50 nM staurosporine, which was effective in inhibiting PKC activity (data not shown), could modulate the outcome of the 1:1 safingol/irinotecan combination from a synergistic effect to an antagonistic effect, as reflected by the increase in CI value from 0.68 (no staurosporine) to 1.35 (with staurosporine) (Fig. 4C).

Effect of safingol/irinotecan at 1:1 molar ratio on colon cancer cell adhesion. As activation of PKC is reported to mediate cell adhesion and migration (24,33,34), the effect of the 1:1 safingol/irinotecan combination on inhibiting colon cancer cell adhesion to extracellular matrix was investigated using a Matrigel-based *in vitro* assay. After 4 h of treatment with the 1:1 safingol/irinotecan combination, significant decreases in the percentage of HT-29 and LS-174T cells adhered to the Matrigel could be observed (Fig. 5). This observation is consistent with the ability of the drug combination to modulate PKC activity and downstream signaling.

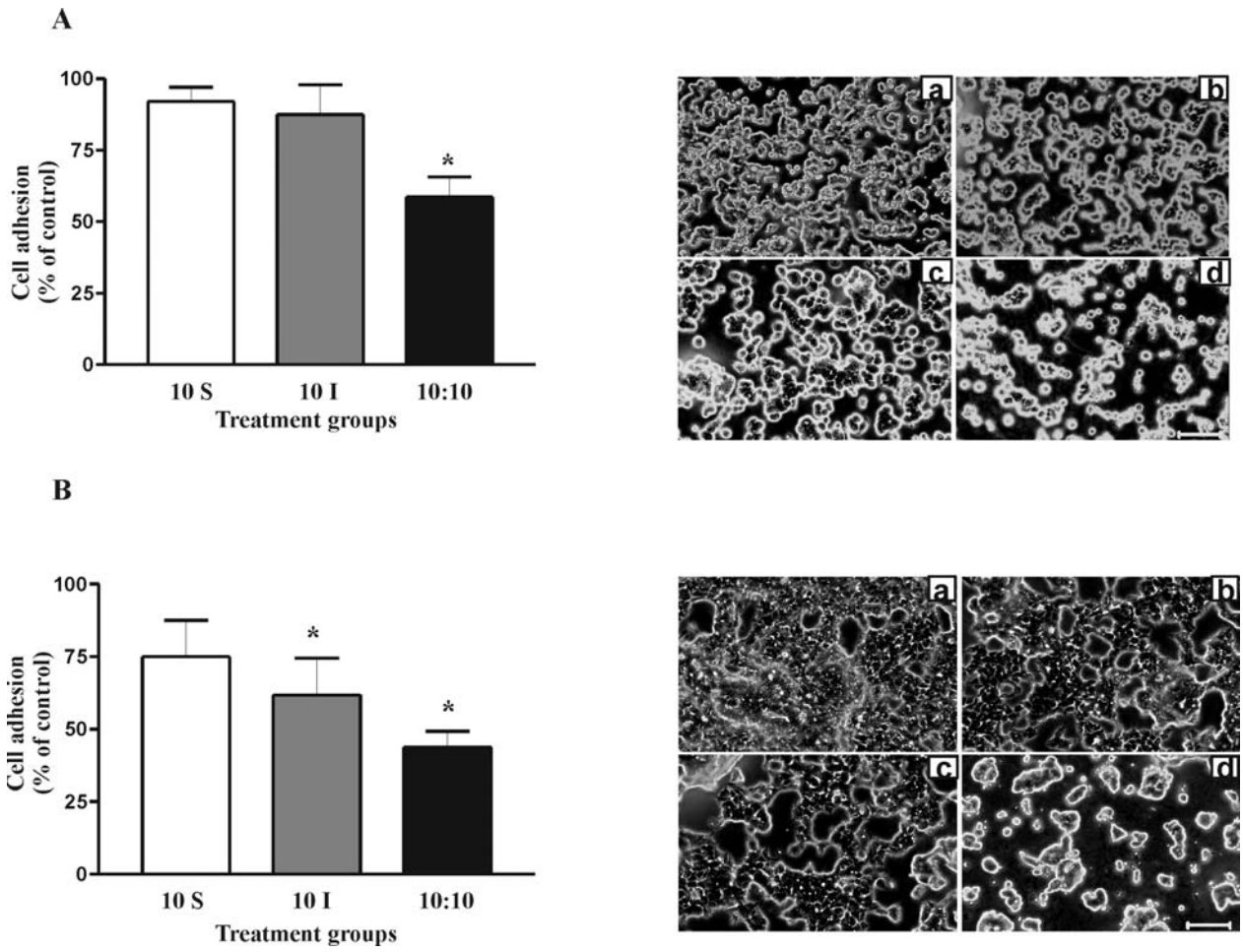

Figure 5. Effects of safingol, irinotecan or safingol/irinotecan (1:1) on cell adhesion in (A) HT-29 and (B) LS-174T cells. Cells were treated with 10 μM safingol, 10 μM irinotecan or 10 μM safingol + 10 μM irinotecan (1:1 molar ratio) for 4 h and allowed to adhere to Matrigel for 24 h. Number of cells adhered were determined using WST-1 cell proliferation reagent. Each reported value represents the mean ± SEM from 3 independent experiments. $^{*}p<0.05$, significantly different from control group. Right panel: Representative images of cells taken under a light microscope. (a) Control, (b) 10 μM safingol, (c) 10 μM irinotecan, (d) 10 μM safingol + 10 μM irinotecan (1:1 molar ratio). Scale bar (50 μm) shown in (d) applies to all images.

Discussion

In the present study, the anti-cancer activity of safingol as a single agent was compared to that of two commonly used chemotherapeutic drugs, irinotecan and 5-FU, in the colon cancer cell lines, HT-29 and LS-174T. Our results demonstrated that safingol as a single agent was more potent than irinotecan or 5-FU monotherapy. However, from a clinical point of view, combinations of chemotherapeutics are often used to treat cancer patients to reduce the risk of developing resistance. If a particular anti-cancer drug combination is synergistic, it would further offer the benefit of reducing the dose and subsequent treatment-related toxicities, yet without compromising efficacy. Safingol is being evaluated not as monotherapy but in combination with cisplatin in a phase I clinical trial for treating solid tumors (23). Thus, in this study, safingol was combined with irinotecan in various fixed molar ratios to determine if this drug combination was synergistic in colon cancer.

As compared to previous studies that have reported the role of safingol as a PKC inhibitor (12,14,20), our results demonstrated only modest inhibitory effect on PKC and MARCKS phosphorylation when colon cancer cells were exposed to 10 μM safingol. However, substantial cell kill could be observed in our current study at this concentration of safingol, as demonstrated by MTT viability data and flow cytometry analyses. It is possible that exposure to high concentrations of safingol (40-50 μM) could lead to effective inhibition of PKC activity and phosphorylation of MARCKS (20,35), and that safingol could have inhibited PKC without interfering its phosphorylation site (20). Our results, on the other hand, are comparable with those reported by Hoffmann *et al* (17), whereby the IC_{50} values of safingol in six cell lines of squamous cell carcinomas of head and neck ranged from 3.8-8.6 μM, similar to those obtained here. More importantly, previously reported serum levels of safingol that could be achieved in human (C_{max} of 3.44 μM) compared well with the *in vitro* concentrations used here (11). Thus, our data suggest that PKC inhibition may not be the primary molecular effect of safingol monotherapy, which is in line with observations from Hoffmann *et al* that no relationship existed between PKC activity and sensitivity to safingol in squamous cell carcinomas of head and neck (17). However, subtle differences in the sensitivity to safingol among the individual PKC isoforms might be possible. More recent studies have shed new light onto the molecular mechanism

of safingol, which could act as an inhibitor of sphingosine kinase (23) or as an inducer of autophagy (36). Further studies on the molecular effects brought about by safingol treatment are warranted, which would contribute to the selection of appropriate anti-cancer agents for effective combination treatment strategies.

Protein kinase C has emerged as an important target for anti-cancer drug development, and numerous small molecule inhibitors and anti-sense molecules have been developed and entered into clinical trials (37-39). Given the important role of PKC in tumorigenesis, cell proliferation, and drug resistance development, it is of interest to explore the role of PKC in mediating the synergistic effect of safingol/irinotecan combination given at 1:1 molar ratio. This combination effectively inhibited the phosphorylation of PKC and MARCKS in a concentration- and time-dependent manner, suggesting that the inhibition of PKC pathway could be responsible for the synergism of the safingol/irinotecan combination. Moreover, the drug combination effect could be further modulated by the use of either a PKC stimulator or a PKC inhibitor. The drug combination could also inhibit cell adhesion to extracellular matrix. This is important because the ability of cancer cells to extravasate and intravasate is greatly controlled by their attachment to basement membrane and extracellular matrix (40). A recent study by Noda *et al* (41) showed that 25-30% of oral squamous cell carcinoma cells were unable to adhere to growth surface after treated with 25 μM safingol for 3-6 h. In our study, with a 4-h treatment of safingol/irinotecan 1:1 combination (at 10 μM for each drug), 40-55% of colon cancer cells were unable to adhere to the extracellular matrix. This finding suggests the potential of the safingol/irinotecan drug combination in preventing tumor cell adhesion and subsequently invasion and metastasis.

More importantly, the concentrations of safingol and irinotecan used in the current study to effect synergism in killing colon cancer cells could be achieved clinically. One of the key advantages in using synergistic drug combinations is the potential to minimize toxicities from the individual drugs, and it is also desirable if the toxicities of the individual drugs are non-overlapping. The primary toxicity of safingol in human/animals are intravascular hemolysis and hepatotoxicity (12,42), whereas the dose-limiting toxic effect of irinotecan is diarrhea (4). Thus, the combination of safingol and irinotecan would exhibit non-overlapping toxicity profiles. Furthermore, the concentration of irinotecan could be reduced by 3.7- to 250-fold when combined with safingol in 1:1 molar ratio. This demonstrates the therapeutic potential of safingol/irinotecan combination which could allow substantial dose reduction of irinotecan and thus minimizing the dose-limiting diarrhea observed with the clinical use of irinotecan (4). One challenge remains with the use of such fixed ratio drug combinations, whereby the synergistic drug ratio may not be achieved at the site of action due to the differences in the pharmacokinetic behavior of the individual drugs (43). Recently, the use of drug delivery systems such as liposomes or polymeric nanoparticles could co-deliver and maintain synergistic drug ratios of therapeutic agents at the tumor site (28). Improved therapeutic efficacy could be achieved through such sophisticated formulations in animal models, and phase I/II clinical trials have begun to evaluate these novel formulations (44,45). As treatment outcomes might greatly depend on the ratio of the two drugs, advanced delivery technology could help in the realization of the therapeutic potential of synergistic drug combinations whereby the biodistribution of the drug combination could be synchronized through the delivery system.

In summary, our current study provides supporting evidence to the therapeutic potential of safingol, which has been shown to act synergistically with a number of conventional chemotherapeutics (11,17-22,37). The effectiveness and synergism of safingol/irinotecan at 1:1 molar ratio could be attributed to the down-regulation of the PKC pathway. These results suggest that the inhibition of PKC by the safingol/irinotecan combination is an attractive and effective strategy to enhance colon cancer cell killing.

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