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ORIGINAL ARTICLE

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Phosphatidylcholine-associated nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit DNA synthesis and the growth of colon cancer cells in vitro

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Abstract The use of NSAIDs or COX-2 inhibitors for chemoprevention of colorectal cancer has been suggested for patients at high risk for this disease. However, the gastrointestinal side effects of traditional NSAIDs which consist of bleeding and ulceration, and the cardiovascular effects of COX-2 inhibitors may limit their usefulness. In preclinical studies, our laboratory has shown that the addition of phosphatidylcholine (PC) to the NSAIDs aspirin (ASA) or ibuprofen (IBU) results in a NSAID–PC with fewer GI side effects and also maintained or enhanced analgesic, anti-pyretic and anti-inflammatory efficacy over the unmodified NSAID. Because NSAID–PCs have not been tested for anti-cancer activity, in the present study, ASA–PC and IBU–PC were tested on the SW-480 human colon cancer cell line. SW-480 cells were incubated in media containing 1–5 mM NSAID or NSAID–PC for 2 days. Measurements were made of cell number, cell proliferation (DNA synthesis), and manner of cell death (necrosis and apoptosis). ASA and IBU reduced cell number in a dose-dependent manner with IBU showing a greater potency than ASA. The association of PC to the NSAID resulted in greater reductions of cell number for both NSAIDs. Furthermore, the NSAID–PC formulation had significantly greater efficacy and potency to inhibit cellular DNA synthesis than the unmodified NSAID. PC alone at the doses and times used had no effect on cell number in this cell line, but did have a small effect to reduce DNA synthesis. None of the drugs had a clear effect on cell death by necrosis. Only IBU and IBU–PC caused cell death by apoptosis in SW-480 cells. We conclude that NSAID–PCs have activity to impede the growth of colon cancer cells in vitro, which is due, in major part, to a marked reduction in DNA synthetic activity of these cells. This growth inhibitory effect appears to be independent of COX-2 activity, since it is known that SW-480 cells do not have this inducible COX isoform. Due to its greater efficacy in this model system, IBU–PC should be further evaluated as a chemopreventive agent that is safer for the GI tract than unmodified NSAID.

Keywords NSAID · Phosphatidylcholine · Colon cancer · Aspirin · Ibuprofen

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Introduction

Clinical and laboratory studies have linked the chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin with a reduced incidence of colon cancer [10, 13, 19, 25, 27]. The mechanism by which NSAIDs may prevent colon cancer is not entirely clear, but may be related to the drug's ability to inhibit cyclooxygenase (COX) and possibly the COX-2 isoform, which is elevated in many cancers [7, 18], or to other more direct cellular and/or membrane actions [3, 11, 18, 23, 26, 33, 34].

One important limiting factor on the use of NSAIDs in the prevention of colon cancer is the side effect of NSAIDs directed at the gastrointestinal tract which causes bleeding and ulceration in susceptible individuals when given chronically [6, 9]. To avoid these undesirable side effects, the use of COX-2 inhibitor drugs which are generally safer for the stomach, has shown promise [24]. However, recent studies linking chronic COX-2 inhibitor usage, especially at high doses necessary for anti-cancer activity, to an increased risk of potentially fatal cardiovascular events [8], has left the future of COX-2 inhibitor drugs in an uncertain position.

In an attempt to improve the GI safety of traditional NSAIDs, a new class of NSAIDs has been developed in which the drug has been associated (non-covalently) with a phosphatidylcholine (PC) molecule. This results in an "NSAID–PC" with little or no associated GI

erosions and/or bleeding in both rodents and clinical trials [1, 15]. In addition, NSAID–PCs have the same or greater efficacy to relieve pain, fever and inflammation than unmodified NSAIDs, which may be due to their greater membrane permeability and/or increased potency to inhibit COX-2 activity [16, 17].

NSAID–PCs have not been tested for anti-cancer activity. Therefore, in the current study, Aspirin-PC and Ibuprofen-PC, in comparison with the unmodified NSAID, were investigated for growth inhibition effects on a widely used human colon cancer cell line, SW-480, which does not express COX-2 [23]. By using this COX-2 deficient cell line, the necessity of this enzyme for drug activity could be assessed.

Materials and methods

Cell culture

SW-480 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and from The Texas Gulf Coast Digestive Diseases Center (Houston, TX, USA). SW-480 cells were maintained in Dulbecco's modified Eagle medium with high glucose, 10% fetal bovine serum, penicillin and streptomycin. Cells were incubated in 24-well tissue culture plates for 2 days in the absence (control) and presence of 1–5 mM aspirin (ASA), ibuprofen (IBU), ASA–PC, IBU–PC, or PC (Phospholipon 90G from Nattermann International GMBH, Cologne, Germany) The phospholipid suspensions were prepared by dissolving a weighed amount of PC in chloroform, drying under nitrogen gas, and resuspending in liquid by sonication for 20 min in a bath sonicator (Laboratory Supplies Co. Inc., Hicksville, NY, USA). For PC alone, the lipid was resuspended in phosphate buffered saline. For the NSAID–PC complex (PLx Pharma Inc., Houston, TX, USA), the lipid was resuspended in solubilized NSAID in normal saline at pH 4 for ASA and pH 6.5 for IBU, followed by adjustment to pH 7. The PC and NSAID were always prepared at equimolar concentrations. NSAIDs were solubilized in saline by forming the sodium salt and then adjusting the pH. Drugs were prepared at a 10*times* concentration and diluted in tissue culture medium containing 1% fetal bovine serum before adding to the cell cultures.

Cell number

An estimate of the number of viable cells was assessed by the MTT assay in which 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetra-zolium bromide (MTT, Sigma Chemical Co., St Louis, MO, USA) was added to the media of cells for 2 h (0.5 mg/ml final concentration). The colored product that formed was extracted into a solvent (90% isopropanol, 0.2% sodium dodecyl sulfate, and 0.01N HCl) and its absorbance was read at 570 nm.

Cell DNA synthesis

The rate at which cells proliferated was estimated by addition of ^{3}H-thymidine (1 μCi/ml) to culture media for the final 5 h of incubation. Cells were then rinsed, incubated with 10% cold trichloroacetic acid for 30 min, and the radiolabelled DNA was dissolved in 0.5 N sodium hydroxide for scintillation counting.

Cell death by necrosis

An estimate of necrosis was determined by measurement of the cytosolic enzyme lactate dehydrogenase (LDH) that was released into the culture medium (LDH kit from Thermo Electron Corp., Louisville, CO, USA).

Cell death by apoptosis

The level of apoptosis was estimated by measurement of caspase 3/7 activity formed in the cells using the ApoONE kit (Promega Corp., Madison, WI, USA) Apoptosis was also estimated by determination of mononucleosomes and oligonucleosomes with the Cell Death Detection ELISA^{plus} kit from Roche Applied Science (Indianapolis, IN, USA).

Calculations

Individual experiments were performed with three or four replicates at each point and values were calculated as a percentage of the untreated control cells. Each study was repeated at least twice and the results were combined. Differences between treatment groups were analyzed by analysis of variance and the Fisher LSD test.

Results

Cell number of SW-480 cells

Aspirin alone dose dependently reduced the cell numbers of SW-480 cells with relatively modest, but significant inhibition at 2.5 and 5 mM (Fig. 1**a**) Ibuprofen alone was more toxic to the colon cancer cells, showing ∽50% inhibition at 2.5 mM (Fig. 1**b**). PC alone did not significantly affect cell number. The combinations of ASA–PC and IBU–PC both significantly reduced cell number over either the NSAID or PC alone at most of the doses tested.

Cell DNA synthesis in SW-480 cells

Aspirin alone reduced DNA synthesis in a dose-dependent manner, similar to its effect on cell number (Fig. 2**a**). Ibuprofen greatly reduced DNA synthesis

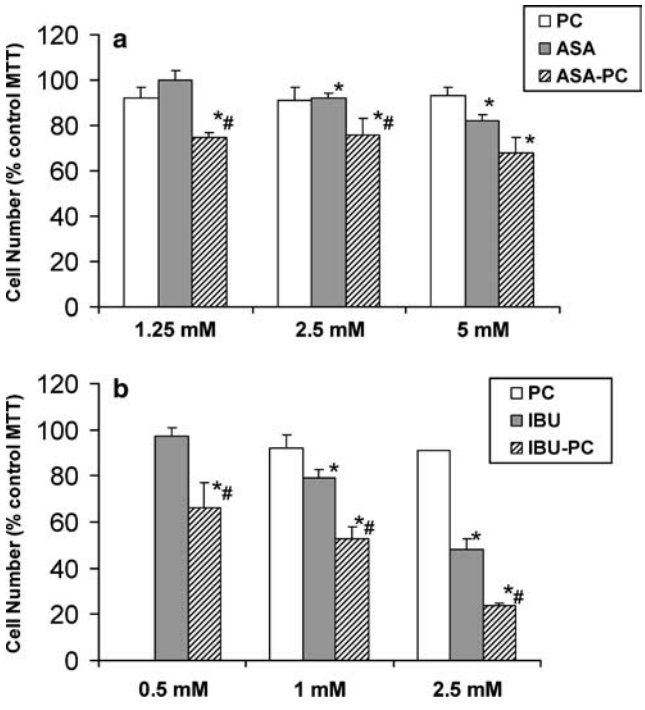

Fig. 1 Effect of NSAIDs, NSAID–PCs, and PC on cell number of SW-480 cells. Cells were incubated for 2 days with test agents (a, ASA; b, IBU) as described in the Methods, and assayed for MTT spectrophotometrically. Values were calculated as a percent of the control ± standard error. There were 3–4 replicates per group with all studies repeated at least twice. (*$P < 0.05$ versus control; #$P < 0.05$ versus NSAID alone; control = 100 ± 2%)

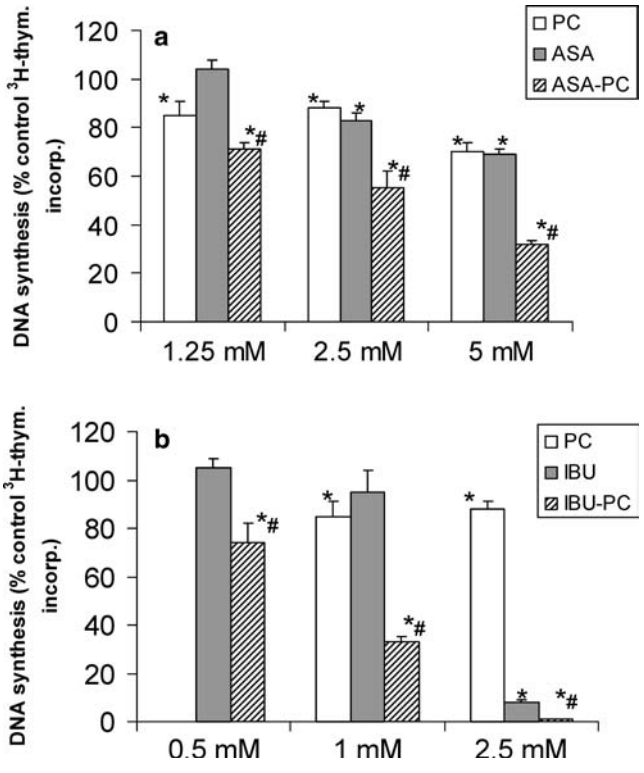

Fig. 2 Effect of NSAIDs, NSAID–PCs, and PC on DNA synthesis in SW-480 cells. Cells were incubated with test agents for 2 days (a, ASA; b, IBU), with ^{3}H-thymidine added for the last five hours. After extraction and counting of the radioactivity, results were calculated as average CPM per well and converted to a percentage of the untreated control. (*$P < 0.05$ versus control; #$P < 0.05$ versus NSAID alone; control = 100 ± 1.5%)

with >60% and >90% inhibition at 1 and 2.5 mM, respectively (Fig. 2**b**). PC alone had a modest, but significant, effect to inhibit DNA synthesis with 30% inhibition at 5 mM. The combinations of ASA–PC and IBU–PC both significantly reduced DNA synthesis over either the NSAID or PC alone when administered at an equivalent dose.

Cell death by necrosis in SW-480 cells

Neither aspirin, ibuprofen, PC, ASA–PC or IBU–PC had a consistent effect on LDH release as a measure of necrotic cell death when administered at a dose of 2.5 mM or higher (Table 1). As a positive control of the test system, the bile acid and detergent, sodium deoxycholate, was incubated with these cells and it produced significant LDH release at a concentration of 0.7 mM.

Table 1 Effect of NSAIDs, NSAID–PCs, and PC on LDH release in SW-480 cells

Treatment	Value (% control)
Control	100 ± 7
ASA 5 mM	88 ± 5
ASA–PC 5 mM	90 ± 4
IBU 2.5 mM	187 ± 43
IBU–PC 2.5 mM	168 ± 30
PC 5 mM	96 ± 3
DOC 0.7 mM	1950 ± 250

LDH was measured in the media of cells exposed to the indicated agents for 2 days.Deoxycholate (DOC) was tested as a positive control.

Cell death by apoptosis in SW-480 cells

Aspirin and ASA–PC, when administered at doses of 5 mM, had no consistent effect on cell death by apoptosis as detected through assay of caspase 3/7 and by nucleosome measurement (data not shown). In contrast, ibuprofen alone and IBU–PC induced apoptosis when caspase 3/7 was measured (Fig. 3**a**). The major response was seen at the highest dose of 2.5 mM. There was no consistent enhancement of IBU–PC over that seen with IBU alone. In agreement with these effects of IBU and IBU–PC, it was also found by the detection of apoptosis by nucleosome assay that IBU and IBU–PC induced apoptosis only at 2.5 mM (Fig. 3**b**).

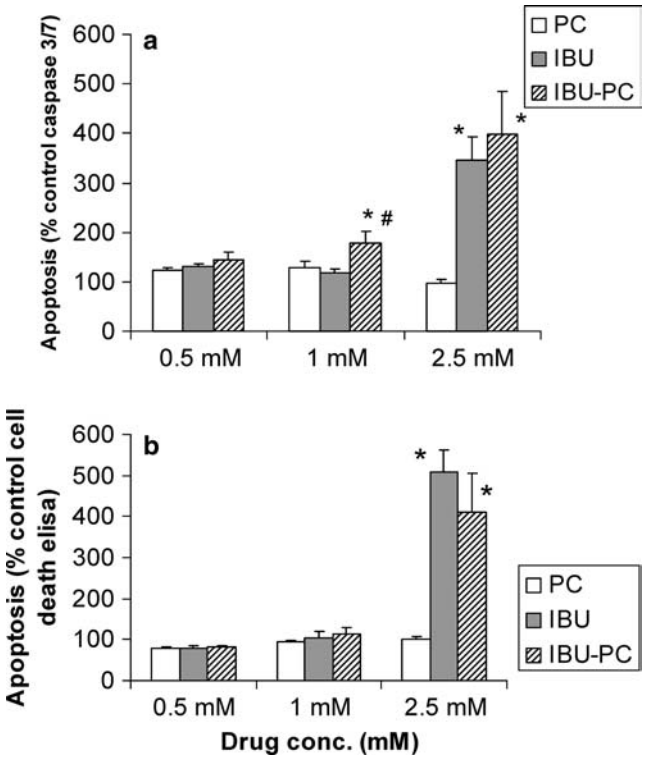

Fig. 3 Effect of ibuprofen, IBU–PC and PC on cell death by apoptosis in SW-480 cells. Cells were incubated with test agents for 2 days and then the cells were assayed for caspase 3/7 with the APOone kit (**a**) and mononucleosomes and oligonucleosomes with an ELISA (**b**) Values are expressed as a percent of control. (*$P < 0.05$ versus control; #$P < 0.05$ versus NSAID alone; control = 100 ± 4%)

Discussion

Our results which document that aspirin and ibuprofen inhibit the growth and proliferation of colon cancer cells are consistent with other reports in the literature using NSAIDs in cell culture systems [14, 23, 32, 33]. However, little of this work has been done with ibuprofen, and our results from this in vitro work suggest that it is a much more potent anti-cancer agent than may have been previously appreciated. Ibuprofen has also shown in vivo chemopreventive activity in the rat azoxymethane-induced carcinogenesis model [28, 29] and in a preliminary report using a mouse syngeneic colon cancer model [31].

In our hands, ibuprofen reduced SW-480 cell numbers to a greater extent and at a lower concentration than did aspirin. The IC_{50} for IBU in our system was ~2.5 mM, whereas it was >>5 mM for ASA. This finding is similar to that of Hixson et al. [14] who reported a 3–4 fold greater IC_{50} for ASA, compared to IBU on another human colon cancer cell line. Other investigators who have tested ASA on SW-480 cells have also reported requirements of 5–10 mM for at least 50% inhibition of cell growth [20, 30, 33, 34]. Thus, although ASA is recommended for chemoprevention of colon cancer in man, it is much less potent than IBU in ours and others' model systems. It is possible that IBU is a better choice for this indication, but such a determination will have to wait for further validation through appropriate animal model testing.

The mechanism by which IBU reduced cell number in our studies appeared to be primarily by inhibiting DNA synthesis, with almost complete inhibition occurring by 2.5 mM. ASA, in contrast, would require >5 mM for 50% inhibition. So again, IBU exhibited a more potent growth inhibitory effect on the colon cancer cells.

Investigations of cell death for mechanisms to explain NSAID inhibition of growth revealed that neither ASA nor IBU affected necrosis as detected by LDH release. This negative finding is consistent with the lack of any reports on this manner of cell death by NSAIDs. However, many NSAIDs have been reported to induce apoptosis, and as a consequence, we investigated this cell death mechanism. It was found that ibuprofen did enhance apoptotic activity as assessed by both an increase in caspase 3/7 activity and formation of nucleosomes, with the greatest effect at 2.5 mM. Thus, a portion of the inhibitory action of IBU on growth of SW-480 cells at the highest dose may be due to the promotion of cell death by apoptosis. It should be noted that the anti-neoplastic effect of IBU and specifically IBU–PC at lower doses (0.5 and 1 mM), cannot be explained by promoting the cell death pathway. In addition, we did not observe an effect of ASA on apoptosis at any of the doses tested (1.25–5 mM), a response noted by some investigators [11, 33, 34], but not by others [23, 32]. It is possible that ASA in our system may have induced an apoptotic response if higher concentrations were used or longer times of incubation were utilized.

PC alone did not reduce SW-480 cell numbers during the time interval tested here, although PC did modestly inhibit DNA synthesis. It is likely that a longer incubation period would eventually result in fewer cells compared to the control. That PC could have growth inhibitory activity on colon cancer cells has also been reported by Awad et al. [2] who noted that polyunsaturated PC (30 μM for 12 days) had greater growth inhibitory activity than saturated PC. This is in agreement with the use of Phospholipon 90G in our studies, which is a polyunsaturated PC. These authors further showed that this action may be related to the inhibition of phosphoinositide-specific phospholipase C and a subsequent effect on protein kinase C activation. In other cell lines (immortalized keratinocytes and vascular smooth muscle cells), polyunsaturated PC (0.8–4 mM for 1–2 days) has also been associated with suppressed cell proliferation and increased membrane fluidity [4, 21]. Thus, PC alone has at least modest effects to directly inhibit cell growth, which may help to explain why a diet rich in unsaturated lipids appears to be beneficial at reducing cancer risk.

A major finding of this paper is the observation that the combinations of ASA–PC and IBU–PC both exhibited significantly enhanced growth inhibition over

the NSAID alone on the SW-480 cells, as seen by reduced cell number. The IBU–PC combination gave the greatest overall reduction in cell growth, with a 1 mM concentration giving the same inhibition as 2.5 mM IBU alone. Similar to the NSAIDs alone, the NSAID–PCs also appeared to act primarily through inhibition of DNA synthesis, with significant reductions of ^{3}H-thymidine incorporation at every concentration. IBU–PC also had an apoptotic effect that contributed to its enhancement of growth inhibition. It thus appears that the NSAID–PCs act in a manner similar to the NSAIDs alone to reduce cancer cell growth in vitro, but with enhanced activity, particularly in their inhibitory effect on DNA synthesis.

An explanation for the enhanced efficacy of NSAID–PCs in this system remains to be determined. Previous in vivo studies with ASA–PC suggested that superior COX-2 inhibition may have contributed to the enhanced efficacy [17]. However, the growth inhibitory action of the NSAIDs and NSAID–PCs in the current studies appeared to have a component that was independent of COX-2, as the drugs were active on a COX-2 deficient cell line. A number of COX-independent mechanisms have been proposed for NSAIDs [3, 11, 18, 30, 33, 34] and will be of interest to investigate in the future. It is also possible that COX-2 inhibitory activity of these drugs may contribute to the total growth inhibitory effect and may become apparent when in vivo testing is performed.

The concentrations in this study at which the NSAIDs and NSAID–PCs inhibited DNA synthesis and cell proliferation in vitro (with the latter agent being more potent) were in the 0.5–5 mM range, a value which is high compared to the reported blood levels in pharmacokinetic studies (i.e., 0.1–0.2 mM [5, 12, 22]). However, our inhibitory concentrations are similar to those reported by other investigators using similar tissue culture systems [14, 23, 30]. Additionally, it is possible that luminal concentrations of drug within the intestine after oral dosing with delayed-release formulations may be considerably higher than blood levels, and may be closer to the effective in vitro concentrations. It is also known that in vitro studies may not be entirely predictive of in vivo responses. Certainly, factors such as absorption, metabolism and elimination of drug can vary between in vivo and cell culture systems, complicating comparisons. Finally, the cell culture line used in these studies did not possess COX-2 activity, an enzyme whose activity in vivo may be a major component of both cancer potential and a target for NSAID therapeutic action.

In conclusion ASA–PC and IBU–PC exert a growth inhibition to colon cancer cells that is more efficacious than the NSAID alone. These NSAID–PCs act, in major part, by inhibiting DNA synthesis and proliferation of the colon cancer cells. Since IBU–PC appears to be particularly effective at inhibiting the growth of colon cancer cells, with both anti-proliferative and apoptotic properties, it warrants further study as a potential chemopreventive agent with superior growth inhibitory activity and GI safety.

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