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Chemoprevention with phosphatidylcholine non-steroidal anti-inflammatory drugs *in vivo* and *in vitro*

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Abstract. The chemopreventive activity of non-steroidal anti-inflammatory drugs (NSAIDs), particularly aspirin, has been well demonstrated in preclinical and clinical studies. However, the primary side effect from this class of drug is gastrointestinal (GI) bleeding, which has limited the widespread use of NSAIDs for the prevention of cancer. The development of GI-safer NSAIDs, which are associated with phosphatidylcholine (PC) may provide a solution to this therapeutic problem. In the present study, the efficacy of two NSAIDs, aspirin and indomethacin, were compared using murine colon cancer cell line MC-26. Each NSAID was assessed alone and in combination with PC, using *in vitro* and *in vivo* systems. The results reveal that the PC-associated NSAIDs had a significantly higher degree of protection against cancer cell growth compared with the unmodified NSAIDs. It was also observed that Aspirin-PC and Indomethacin-PC prevented the metastatic spread of cancer cells in a syngeneic mouse model. These results support the potential use of PC-NSAIDs for the chemoprevention of colorectal cancer.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs that in addition to providing analgesic, antipyretic, and anti-inflammatory effects, also possess chemopreventive actions against the development of a number of cancers in both animal models and humans (1,2). Even though the molecular mechanism of this anti-neoplastic effect is not completely understood, there has been increasing interest in the chemopreventive activity of NSAIDs due to their demonstrated ability to reduce the incidence and severity of various cancers based upon clinical outcome studies (3-5). In particular, colorectal cancer incidence rates are reduced in persons who consume daily aspirin or ibuprofen (6-8). We previously reported that aspirin and a novel aspirin derivative which is associated with phosphatidylcholine (Aspirin-PC) to provide protection of the gastrointestinal (GI) tract against aspirin-induced injury, are both effective cancer-preventing agents in an animal model of colon cancer (9). That model consists of using the colon carcinogen azoxymethane (AOM) along with the colon inflammatory agent dextran sodium sulfate (DSS) to produce colitis-associated pre-neoplastic aberrant crypts in the colon (10) that are blocked by aspirin or Aspirin-PC treatments. While this chemically-induced colon cancer model provides good evidence of chemopreventive activity, and has been used by others for screening chemopreventive agents (11-13), it is not the sole model for testing anticancer agents. In order to further test the potential chemopreventive activity of Aspirin-PC, we decided to use another animal model, which directly tests the ability of drugs to inhibit the growth of cancer cells *in vivo*. In this model, tissue culture-grown murine colon cancer cells (MC-26) will be inoculated into the mouse splenic capsule and allowed to grow for 4 weeks prior to collection of splenic (primary tumor) and hepatic (metastatic) tissues for analysis of cancer nodule growth (14). Not only does this model allow for screening of cancer growth and metastatic spread, but it has the added advantage that mouse cells are used in the mouse (syngeneic) and no immunosuppression is required. In addition, MC-26 cells in culture can be used to study the ability of test drugs to inhibit cancer cell growth. Previous investigators showed that the NSAID ibuprofen is effective at blocking cancer growth in this model (15).

Indomethacin is another NSAID that has previously been reported to have anti-neoplastic activity at low doses in both rodents (16,17) and humans (18,19). Accordingly, we performed *in vitro* studies to compare the growth-inhibitory effect of the PC-associated aspirin and indomethacin, vs. unmodified NSAIDs on MC-26 colon cancer cells. Also, these drugs were tested in the *in vivo* MC-26 mouse model system.

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Abbreviations: NSAID, non-steroidal anti-inflammatory drug; PC, phosphatidylcholine; GI, gastrointestinal; AOM, azoxymethane; DSS, dextran sodium sulfate; TXB_2, thromboxane B2; COX, cyclooxygenase; PGE_2, prostaglandin E_2

Key words: aspirin, indomethacin, colorectal cancer, chemoprevention, non-steroidal anti-inflammatory drug

Materials and methods

Test drugs. For cell culture, aspirin was purchased from Rhodia and indomethacin was from Spectrum Chemical (Gardena, CA, USA). For the animal study, aspirin (uncoated) was purchased from Walgreens (Deerfield, IL, USA). Aspirin-PC and Indomethacin-PC were prepared as described below for the cell culture and animal studies.

We used established procedures to prepare our PC-associated test drug formulations for cell culture and intragastric dosing (20,21). For cell culture, the Aspirin-PC stock solution was prepared as described previously (9). Briefly, the aspirin was firstly dissolved in the serum-free culture medium at 10 mmol/l and then combined with an equimolar amount of purified soy phosphatidylcholine/PC (S-100; Lipoid LLC, Newark, NJ, USA), which was previously dissolved in chloroform and then blown dry under nitrogen. The tubes were then sonicated at room temperature in a bath-type sonicator for 20 min until a homogenous suspension was obtained (Fig. 1 for the chemical structures of aspirin, indomethacin and soy PC). In the animal experiments, we used different procedures preparing Aspirin-PC as described previously (9,22). To make Indomethacin-PC stock for both cell culture and animal study, 8 gram of indomethacin (acid form) and 16 gram of Lipoid S-100 were subsequently dissolved into 60 ml of Acetone (Thermo Fisher Scientific, Inc., Fair Lawn, NJ, USA) in a 500-ml flat bottom round flask in 40˚C water bath. Then the flask was connected to a rota-vaporator and vacuum-processed for 14-16 h to remove the acetone. Finally, the Indomethacin-PC was collected in a brown glass jar and kept at 4˚C. To prepare the Indomethacin-PC solution for oral administration, the drug was weighed, and deionized distilled water added to a glass vial to the desired concentration and sonicated for 20 min at room temperature.

Cell culture. Murine colon cancer cells (MC-26) were obtained from the NIH National Cancer Institute. The cell line was cultured in suggested growth medium with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Tests for mycoplasma were negative and were conducted with the MycoAlert Mycoplasma Detection Kit from Lonza (Rockland, ME, USA). This cell line is known to express COX-2 (14).

MC-26 cells were preincubated with the drugs at a concentration range from 0 to 1.0 mmol/l (aspirin/Aspirin-PC) or 0 to 50 μmol/l (indomethacin/Indomethacin-PC) for 15 min to promote optimal exposure to our test-drugs, prior to pipetting the cells onto 48-well plates at a density of $2x10^3$ cells/well, and cultured at 37˚C in a mixture of 5% CO_2 and 95% air. The cells were then cultured in the above growth medium in the presence and absence of the test drug formulations for 8 days with one medium change on the 4th day, at which time the culture medium was collected into 1.5 ml Eppendorf tubes and centrifuged at high speed for 10 min. Then the supernatant was collected for prostaglandin (PGE_2) assay as a measure of COX-2 activity. Cells on day 8 were used for the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay as a measure of cell number as outlined below.

MTT assay. MTT (purchased from Sigma-Aldrich; Merck KGaA) was added to the culture media of cells at a final concentration of 0.5 mg/ml for 4 h. The purple formazan product was then extracted into a solvent (90% isopropanol, 0.2% sodium dodecyl sulfate, and 0.01 mol/l HCl) which was then collected from the wells and read at an absorbance of 570 nm, as previously described (22).

Animal study. Young adult (20-24 g) male BALB/c mice were supplied by Harlan Laboratories, Inc. (Envigo, Indianapolis, IN, USA) and housed in the Center for Laboratory Animal Medicine and Care (CLAMC) facility at The University of Texas Health Science Center at Houston (UTHealth). Mice were maintained in accordance and compliance with policies approved by the Animal Welfare Committee (AWC), the Institutional Animal Care and Use Committee (IACUC) for The University of Texas Health Science Center at Houston (UTHealth). This facility is approved by the PHS and AAALAC.

On day one, mice were anesthetized and subjected to a laparotomy under isoflurane anesthesia as previously described (14,15,23), in order to inoculate their splenic capsules with $2x10^5$ cells/ml, 0.1 ml per mouse. Following the method of Yao *et al* cited above, immediately after cancer cell implantation, the mice were randomly divided into five treatment groups and treated once daily orally with vehicle (saline), aspirin (20 mg/kg), Aspirin-PC (20 mg ASA+20 mg PC/kg), indomethacin (2 mg/kg), or Indomethacin-PC (2 mg indomethacin + 4 mg PC/kg) and this treatment was continued daily for 28 days. A non-cancer group was also included as control. Thereafter, the mice were sacrificed and tissues were collected for analysis of tumor growth (spleen weight), possible GI injury due to NSAID (hematocrit), and metastatic cancer cell spread (liver weight and nodule number), plus fecal hemoglobin was assessed for evidence of GI bleeding, serum levels of Thromboxane B_2 (TXB_2) were assayed as a measure of NSAID pharmacologic action (inhibition of platelet COX-1 activity), and spleen tissue was assayed for PGE_2 as a measure of COX-2 activity.

Fecal hemoglobin analysis. Fecal hemoglobin (Hb) was monitored by collecting the fecal droppings at regular intervals from the bedding and storing them at -20˚C until the day of analysis. The feces were weighed, and then distilled water was added at a 1:10 feces (g): Water (ml) ratio. After standing for 1 h, the feces were disrupted into a homogenous suspension by vortexing for 2 min and then the Hb analyzed by a previously described method (24).

ELISAs. The animal serum was analyzed by using the thromboxane B_2 EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's specifications. Blood of individual mice was collected at the end of the experiment under terminal anesthesia following a protocol for cardiac puncture, and serum was separated within 1 h following blood collection by centrifugation at 500 x g for 10 min, and then aliquoted and stored at -80˚C for subsequent testing at a 1/200 dilution.

The MC-26 cell medium collected on day 4 of culture, and the excised animal spleen tissue were analyzed by using the Prostaglandin E_2 EIA kit (Cayman Chemical) according to the manufacturer's specifications. Splenic tumor tissues were

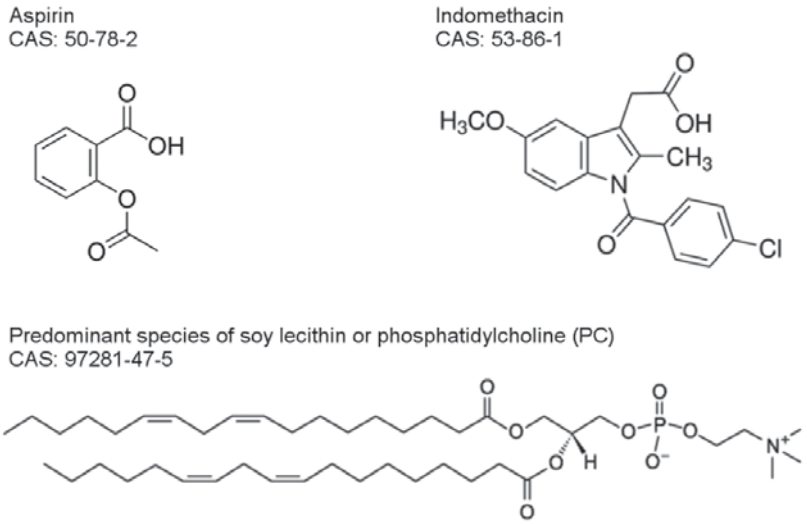

Figure 1. Chemical structures of aspirin, indomethacin and soy phosphatidylcholine.

homogenized in methanol, followed by SPE (C18) purification as suggested by the manufacturer's instruction. The final extract was resuspended in buffer and tested.

Statistics. Statistical analyses were performed using the statistics application StatView 5.01 (SAS Institute Inc., Cary, NC, USA). Values are expressed as the mean ± standard error of the mean, and were evaluated by ANOVA followed by Fisher's PLSD test. A two-tailed value of $P<0.05$ was considered to indicate statistically significant differences.

Results

In vitro effects of test drugs on MC-26 colon cancer cells in culture. The effects of our test drugs on the growth of MC-26 colon cancer cell line were examined over an 8-day culture period (Fig. 2A and B). Aspirin alone had an inhibitory effect on the growth of the cells only at the highest concentration of 1 mmol/l, while the PC complexed aspirin, Aspirin-PC, showed significant inhibition at the much lower concentration of 25 μmol/l (Fig. 2A). All of the Aspirin-PC concentrations gave significantly lower cell growth than the comparable doses of aspirin alone. In comparison, the other tested NSAID, indomethacin, was much more potent than aspirin with a significant inhibition of the cancer cell growth at a concentration of 20 μmol/l, and Indomethacin-PC was inhibitory at an even lower concentration of 8 μmol/l (Fig. 2B). The Indomethacin-PC concentrations of 8-50 μmol/l were significantly more effective at cell growth inhibition than comparable doses of indomethacin alone.

The expression of PGE_2 in culture medium (Fig. 2C and D) did not parallel the effects on cell growth for either NSAID. Aspirin alone (Fig. 2C) had no apparent effect on PGE_2 levels, while Aspirin-PC was significantly inhibitory at concentrations of 0.4-1 mmol/l, which was higher than the level that inhibited cell growth. In contrast, indomethacin alone (Fig. 2D) was a potent inhibitor of PGE_2, even at the lowest concentration tested. Once again, Indomethacin-PC was even more potent than the unmodified NSAID, with significantly lower levels of PGE_2 than indomethacin at every concentration. These concentrations of both indomethacin and Indomethacin-PC that inhibited PGE_2 levels were considerably lower than the concentrations that affected cell growth.

MC-26 colon cancer cell implantation mouse study. As described above, the MC-26 colon cancer study in mice was terminated after four weeks of cancer cell implantation and animal dosing. This time allowed for greater cancer cell growth as evidenced by spleen weights in vehicle-treated mice (20 mg/g body weight), compared to that of the non-cancer control mice (3.3 mg/g body weight). Treatment with indomethacin, Indomethacin-PC or Aspirin-PC gave clear and significant reductions ($P<0.05$) in splenic tumor nodules and spleen weights (Fig. 3A). However, aspirin alone was not protective in this model at the dose tested. Since a previous unpublished animal study showed PC alone had no effect on cancer cell growth in this model, we did not include a PC alone treatment group in this experiment.

An analysis of liver tissue revealed the presence of a number of metastatic tumor nodules (Fig. 3B), which tended to be reduced by treatment with indomethacin, Indomethacin-PC or Aspirin-PC, but not aspirin alone, similar to the spleen weight results. However, there were too few liver nodules to see a significant difference and the liver organs weights did not show differences either (not shown).

Assessments of GI bleeding showed no differences between treatment groups, with hematocrits in a normal range of 0.43 to 0.47 and fecal hemoglobin also showing minimal alterations (0.62 to 0.88 mg Hb/g feces) (Table I).

To verify that the NSAIDs used in this study were pharmacologically active, serum was analyzed for COX-1 activity by measurement of TXB_2 formed from platelets during blood clotting. Fig. 3C shows 80-90% inhibition of TXB_2 by all treatments, including aspirin alone, supporting the NSAIDs' ability to inhibit prostaglandin formation, a primary action of this class of drugs. It was noted that there was no difference between TXB_2 levels in non-cancer controls and vehicle (cancer) controls, suggesting there are no cancer-driven differences in platelet counts and/or activity. This lack of a difference was confirmed by platelet counts in a sampling

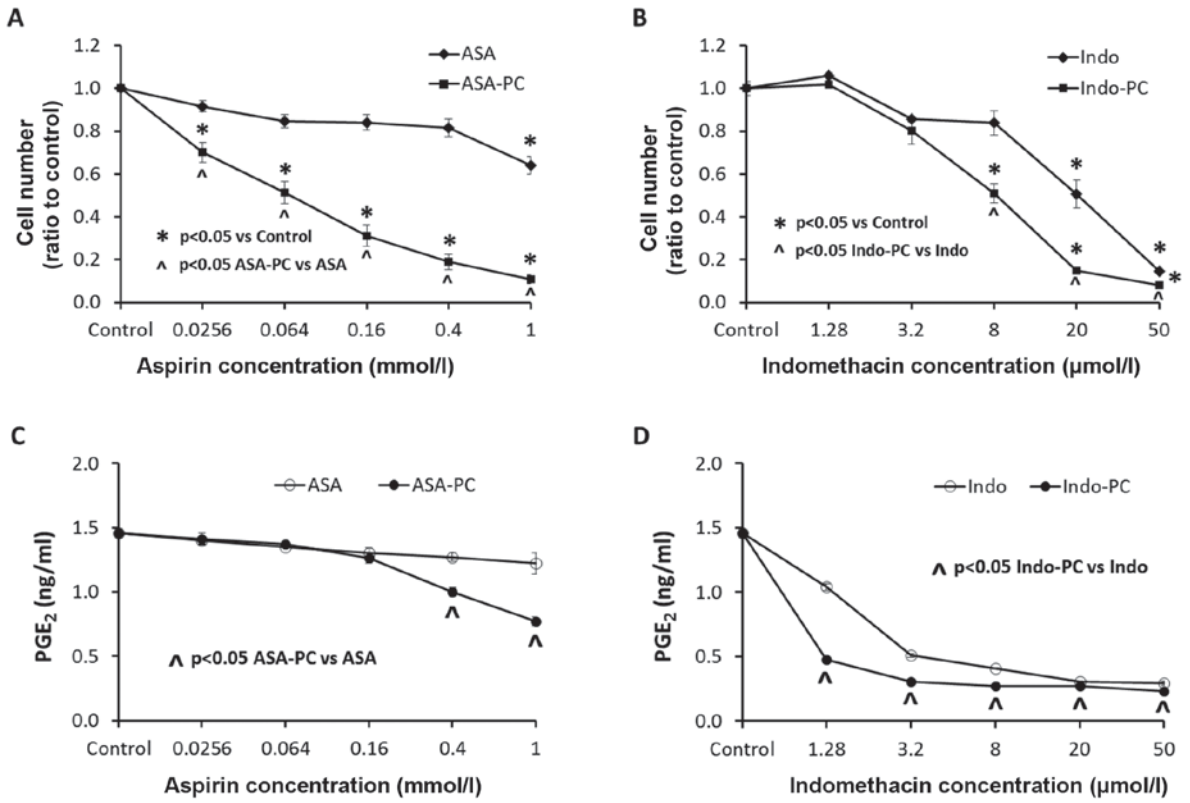

Figure 2. *In vitro* effects of test drugs on MC-26 colon cancer cells. Test drugs were incubated with cells at (A and C) 0-1 mmol/l for aspirin and Aspirin-PC or (B and D) 0-50 mmol/l for indomethacin and Indomethacin-PC. Cell growth was analyzed by (A and B) MTT assay and (C and D) PGE_2 in the medium by ELISA. All experiments were repeated three times and significant differences are indicated.

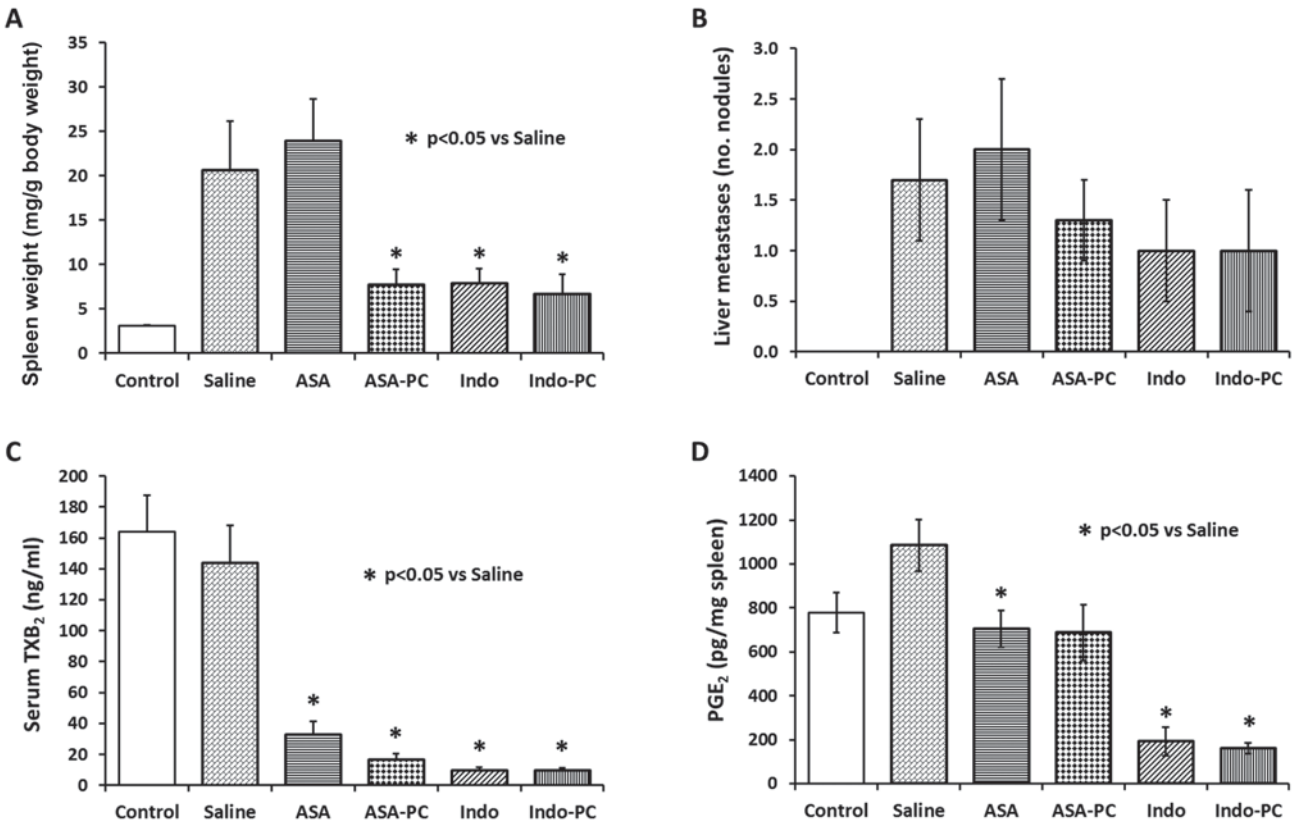

Figure 3. Effect of test drugs on MC-26 cancer cell implantation mouse model system. Immediately after cancer cell inoculation into the splenic capsule, the mice were randomly grouped and administered with saline (vehicle), aspirin, Aspirin-PC, indomethacin, or Indomethacin-PC for 28 days, daily at the following NSAID doses: 20 mg/kg ASA, 20 mg/kg ASA + 20 mg/kg PC, 2 mg/kg Indo, and 2 mg/kg Indo + 4 mg/kg PC, respectively. A non-cancer group was also included as control. Values are: (A) spleen weight; (B) liver metastases; (C) serum TXB_2; and (D) spleen PGE_2. *P<0.05 vs. saline group.

Table I. Measures of gastric bleeding in mice.

Treatment group	Hematocrit	Fecal hemoglobin (mg/g feces)
Control	0.43±0.02	0.62±0.04
Saline	0.44±0.02	0.64±0.03
ASA	0.45±0.02	0.85±0.07
ASA-PC	0.47±0.01	0.88±0.06
Indo	0.44±0.04	0.61±0.04
Indo-PC	0.44±0.02	0.79±0.05

At the end of the study, blood was collected for hematocrit analysis as a measure of gastric bleeding. Samples of fecal pellets were also collected for analysis of hemoglobin content as a second measure of gastric bleeding. Values are expressed as the mean ± standard error of the mean for each group. N=9-10/group.

of animals where measured values were between 366 to $634x10^3/\mu l$.

Spleen tissue levels of PGE_2 in Saline-treated cancer controls (Fig. 3D) were elevated over non-cancer Controls (~35%), but not significantly so, by the infiltration of cancer cells, as the Saline group was not different from Control (P=0.0708). There were apparent reductions of PGE_2 by all test NSAIDs, with indomethacin and Indomethacin-PC inducing the greatest inhibition (~84%). Aspirin and Aspirin-PC gave similar reductions of PGE_2 (~36% vs. saline-treated controls), although Aspirin-PC just missed the level of significance (P=0.0545).

Discussion

As briefly mentioned earlier, aspirin and related NSAIDs have been demonstrated to possess chemopreventive/anti-cancer activity against colorectal cancers and a number of other cancers, reducing both the incidence and cancer-related mortality (1,2). Most of this clinical evidence is based upon outcome studies, demonstrating a link between NSAID consumption and risk of developing cancer (3-8). However, there have been several published prospective studies demonstrating chemopreventive efficacy of aspirin and celecoxib and colorectal cancer (25-27), as well as a pilot clinical study demonstrating that indomethacin-treatment can significantly increase length of survival of patients with advanced cancer (19).

Previous *in vitro* testing of NSAIDs and PC-NSAIDs in our laboratory has shown that aspirin and ibuprofen are effective at inhibiting the growth of the human colon cancer cell line SW480 which involves inhibition of DNA synthesis (28). Both PC-NSAIDs were more effective than the unmodified NSAID. Aspirin and Aspirin-PC were also shown to be effective against MC-26 and Caco-2 (human colon cancer) cell lines when cultured in the presence of washed platelets which involves epithelial-mesenchymal transition (EMT) (9). We also reported that indomethacin and Indomethacin-PC (21), but not aspirin or ibuprofen +/- PC (28), can promote apoptosis. Others have described a variety of actions to explain the anti-neoplastic actions of NSAIDs, many involving COX inhibition (1,2).

Our *in vitro* studies show that both of the PC-NSAIDs were more potent than their parent NSAID at inhibiting cancer cell growth. This direct action of the Aspirin-PC and Indomethacin-PC is an important distinction and supports their further development for chemoprevention of colon cancer. However, our attempt to explain a possible mechanism related to COX inhibitory activity was not consistent for both NSAIDs. Aspirin-PC suppressed cell growth at a much lower concentration than that at which it inhibited PGE_2 produced by COX (Fig. 2C vs. A). In contrast, Indomethacin-PC inhibited COX at a lower concentration than it needed to inhibit cell growth (Fig. 2D vs. B). Thus, the action of Indomethacin-PC, but not Aspirin-PC, could be explained, only in part, by COX inhibition.

The *in vivo* chemopreventive/anti-cancer effects of NSAIDs likely involve even more complicated mechanisms than seen with *in vitro* work. There are numerous reports to support a role for COX-2 overexpression in solid cancers, and specific COX-2 inhibitors have found use clinically in some of those cancers (29). Because MC-26 cells possess COX-2, they have been used in the MC-26 animal model to test the anticancer activity of specific COX-2 inhibitor drugs such as NS-398 (23) and rofecoxib (14), both of which displayed significant chemopreventive activity. In addition, our laboratory has proposed that blood platelets (which possess COX-1), which are elevated in some cancers such as ovarian cancer, may provide a means for cancer cells to migrate and invade distant organs (22). In an AOM/DSS mouse colon cancer model, we previously showed an increased number of circulating platelets that was reduced following aspirin or Aspirin-PC treatment (9). However, in the current cancer cell implantation animal model there was no indication of elevated platelet counts in the cancer controls. Consistent with this data, there was no increase in TXB_2 between non-cancer controls and MC-26 injected controls. Nevertheless, all of our test NSAIDs were very effective in significantly reducing thromboxane levels by >80%. However, COX-independent mechanisms have also been proposed to explain the anticancer actions of NSAIDs (30), and investigations into a role for microRNAs may offer a means to understand these mechanisms (31).

Testing of aspirin and indomethacin compounds in the MC-26 model revealed that aspirin alone at the dose tested was not effective at limiting cancer cell growth in the spleen, while Aspirin-PC provided significant reductions in spleen weight. Further, indomethacin alone showed a significant effect that was equaled by Indomethacin-PC. This result with indomethacin is consistent with a report that indomethacin in the drinking water was able to suppress tumor growth with the MC-26 model (32). No previous reports of aspirin use in this model were found. Thus, both of these PC-associated NSAIDs gave clear protection against cancer cell growth in the spleen. Regarding metastatic spread to the liver in this model, there was considerable variability seen in controls, so that the small reductions seen with the PC-NSAIDs were not significant, although the effects were consistent with the splenic size reductions.

Measures of GI bleeding in the MC-26 colon cancer model including hematocrit and fecal hemoglobin did not reveal any

signs of adverse effects at the doses of drugs used. The drugs were all administered orally for 28 days, which was enough time for GI bleeding to be manifest, but none occurred that we could detect.

The doses of drugs used here were sufficient to see anti-cancer activity and COX-1 inhibition (inhibition of TXB_2 formation), indicating they were pharmacologically active doses. However, we cannot attribute the chemopreventive action solely to COX-1 inhibition, as aspirin alone demonstrated that property, but did not display anti-cancer activity in the syngeneic colon cancer mouse model employed in the current study.

Similarly, the anticancer actions may be related partly but not fully, to COX-2 inhibition (ie, anti-inflammatory dose) based on current knowledge of dose effects. The human equivalent to the mouse aspirin dose of 20 mg/kg is 96 mg for a 60 kg person, or about the dosage of a baby aspirin (81-100 mg). This mouse dose of aspirin/Aspirin-PC was able to minimally inhibit COX-2 to some extent (see Fig 3D), but not fully, which is consistent with our previous reports that 30 and 40 mg/kg reduced GI prostaglandin E_2 (PGE_2) by 66 and 80%, respectively (24,33). Yet only Aspirin-PC, and not aspirin, was effective at preventing growth of cancer cells *in vivo*.

The human equivalent to the mouse indomethacin dose of 2 mg/kg is 9.6 mg for a 60 kg person, which is well below the maximum recommended daily (anti-inflammatory) dose of 150-200 mg per day for treatment of gout or bursitis. However, the mouse dose of indomethacin in our study was able to almost fully inhibit COX-2 as seen from the data presented in Fig. 3D, where splenic levels of PGE_2 were reduced by >80% in mice treated with either indomethacin or Indomethacin-PC. We cannot rule out the possibility that indomethacin or Indomethacin-PC may have a COX-2 inhibitory component as part of their anticancer mechanism.

It is notable that all of these animal drug doses were effective at lower levels than are generally associated with their use for pain and inflammation in man. While it is possible that anti-inflammatory doses of NSAIDs may differ between mouse and human, it is also possible that non-COX mechanisms are involved with this cancer model. This possibility is also supported by the finding that a COX-2 prostaglandin (ie, PGE_2) was not elevated over control in cancer tissues tested with the MC-26 model. This finding underscores that animal models represent various aspects of human cancer, and that multiple models are needed to elucidate a more complete picture of anticancer activity. While the mechanistic basis of the anti-neoplastic action of PC-NSAIDs remains to be fully elucidated, it is clear that PC-NSAIDs, notably Aspirin-PC and Indomethacin-PC may provide an effective and potentially GI-safer alternative for colon cancer chemoprevention and possibly treatment.

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Availability of data and materials

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

Author's contributions

LML directed the full project, reviewed the results and wrote the paper with EJD. TP was responsible for all the animal experiments, including animal surgery, animal dosing and collecting the tissue and blood samples following euthanasia and measuring the fecal hemoglobin. DF did all the cell culture studies, prepared PC-NSAIDs for the *in vitro* studies and performed thromboxane and prostaglandin analyses by ELISA. EJD prepared MC-26 cells to be injected into the mice, prepared the PC-associated drugs for animal studies, directed the animal studies, analyzed the data and was the lead writer of the paper.

Ethics approval and consent to participate

Mice were maintained in accordance and compliance with policies approved by the Animal Welfare Committee, the Institutional Animal Care and Use Committee (IACUC) for The University of Texas Health Science Center at Houston (UTHealth), meeting NIH Guide for the Care and Use of Laboratory Animals. The institution's animal facility is approved by the PHS and AAALAC.

Consent for publication

Not applicable.

Competing interests

LML is a co-founder and shareholder in PLx Pharma Inc., which is developing PC-NSAIDs for commercial use. The remaining co-authors have no competing interests to report.

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