Antitumor and Antiangiogenic Effects of Aspirin-PC in Ovarian Cancer

Yan Huang1,2, Lenard M. Lichtenberger3, Morgan Taylor1, Justin N. Bottsford-Miller1, Monika Haemmerle1, Michael J. Wagner4, Yasmin Lyons1, Sunila Pradeep1, Wei Hu1, Rebecca A. Previs1, Jean M. Hansen1, Dexiong Fang5, Piotr L. Dorniak1, Justyna Filant1, Elizabeth J. Dial1, Fangrong Shen1, Hiroto Hatakeyama1, and Anil K. Sood1,5,6

Abstract

To determine the efficacy of a novel and safer (for gastrointestinal tract) aspirin (aspirin-PC) in preclinical models of ovarian cancer, in vitro dose–response studies were performed to compare the growth-inhibitory effect of aspirin-PC versus aspirin on three human (A2780, SKOV3ip1, and HeyA8) and a mouse (ID8) ovarian cancer cell line over an 8-day culture period. In the in vivo studies, the aspirin test drugs were studied alone and in the presence of a VEGF-A inhibitor (bevacizumab or B20), due to an emerging role for platelets in tumor growth following antiangiogenic therapy, and we examined their underlying mechanisms. Aspirin-PC was more potent (vs. aspirin) in blocking the growth of both human and mouse ovarian cancer cells in monolayer culture. Using in vitro model systems of ovarian cancer, we found that aspirin-PC significantly reduced ovarian cancer growth by 50% to 90% (depending on the ovarian cell line). The efficacy was further enhanced in combination with Bevacizumab or B20. The growth-inhibitory effect on ovarian tumor mass and number of tumor nodules was evident, but less pronounced for aspirin and the VEGF inhibitors alone. There was no detectable gastrointestinal toxicity. Both aspirin and aspirin-PC also inhibited cell proliferation, angiogenesis, and increased apoptosis of ovarian cancer cells. In conclusion, PC-associated aspirin markedly inhibits the growth of ovarian cancer cells, which exceeds that of the parent drug, in both cell culture and in mouse model systems. We also found that both aspirin-PC and aspirin have robust antineoplastic action in the presence of VEGF-blocking drugs. Mol Cancer Ther; 15(12); 2894–904. ©2016 AACR.

Introduction

Over the past 50 years, there has been a growing awareness that aspirin consumption on a regular basis is associated with a reduced incidence and mortality of many cancers afflicting no less than 20 tissues (1–3). The strongest evidence is based on large meta-analyses (>100,000 subjects), following health professionals and nurses for over 10 to 20 years, where it has been reported that cancer incidence is reduced by 25% to 40% in subjects regularly taking low-dose (75–325 mg) aspirin on a daily basis, with the most compelling evidence acquired for colorectal cancer. These findings have resulted in a subcommittee of the US Preventive Services Task Force (USPSTF), recently recommending that individuals ages 50 to 69 years old take low-dose aspirin for 10 years or longer to prevent colorectal cancer, with the proviso they consult their physician beforehand, as aspirin can cause gastrointestinal (GI) ulceration and bleeding which can develop into life-threatening hemorrhage in susceptible individuals (http://www.uspreventiveservicestaskforce.org/Page/Document/draft-recommendation-statement/aspirin-to-prevent-cardiovascular-disease-and-cancer). Rothwell and colleagues also reported convincing data for nine other cancers where chronic use of low-dose aspirin was associated with a statistically significant reduction of cancer incidence and mortality (4, 5). It was also striking that the same group reported that aspirin use even when initiated after cancer diagnosis could have a significant impact by reducing the metastatic spread of the primary cancer and prolong cancer patient survival as much as 2-fold (4, 5).

Ovarian cancer is the leading cause of gynecological cancer-related mortality. Following initial response to therapy, there is frequently rapid emergence of drug resistance (6, 7). In a recent study, we also demonstrated that platelets play an important role in tumor regrowth following withdrawal of anti-VEGF therapy (8). Though prior studies have mostly focused on associating

1Department of Gynecological Oncology and Reproductive Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas. 2Department of Gynecological Oncology, Fudan University Shanghai Cancer Center, Shanghai, P.R. China. 3Department of Integrative Biology and Pharmacology, The University of Texas Health Science Center at Houston, McGovern Medical School, Houston, Texas. 4Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas. 5Center for RNA Interference and Non-Coding RNAs, The University of Texas MD Anderson Cancer Center, Houston, Texas. 6Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, Texas.

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Current address for F. Shen: Department of Obstetrics and Gynecology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, P.R. China.

Y. Huang and L.M. Lichtenberger are co-first authors of this article.

Corresponding Author: Anil K. Sood, MD Anderson Cancer Center, 1515 Holcombe Blvd Unit 1962, Houston, TX 77030. Phone: 713-745-5266; Fax: 713-792-7586; E-mail: asood@mdanderson.org
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Aspirin use to GI cancer, a number of clinical outcome studies have recently been reported that ovarian cancer is particularly sensitive to chronic consumption of aspirin, with ovarian cancer incidence being significantly reduced in regular users of low-dose aspirin (9, 10), though other studies reported aspirin and NSAID use did not provide an effect that reached statistical significance (11). In addition, similar to both colorectal (5) and breast cancers (12), there is evidence that aspirin use even after diagnosis can reduce the recurrence of ovarian cancer and possibly prolong patient survival (9).

As indicated by the USPSTF recommendations, aspirin use has been limited, due to the drug’s adverse event profile, which like other NSAIDs can cause potentially serious GI ulceration and bleeding, especially if taken at high anti-inflammatory doses. Evidence suggests that NSAIDs induce GI injury by both inhibiting epithelial cyclooxygenase (COX) activity and thereby depleting the tissue of “cytoprotective” prostaglandins and by a direct cytotoxic effect on the GI mucosa by compromising cellular (plasmalemmal and mitochondrial) extracellular/mucus function, thereby disrupting both barrier function and electron transport/ATP generation of the tissue (13, 14). The chemical association of NSAIDs with membrane and extracellular phospholipids has been of substantial interest to us (14–17), leading to the development of a family of phosphatidylcholine (PC)-associated NSAIDs (PC-NSAID; refs. 16, 18–22). The formulation that has undergone most rigorous preclinical and clinical testing is aspirin-PC, in which aspirin is formulated with PC-enriched soy lecithin (called PL2200 Aspirin) and has been awarded a new drug approval (NDA, #203697) by the FDA.

Based upon the encouraging results in the literature on the chemopreventive activity of aspirin, we have previously performed an in vitro study on colon cancer cell lines, demonstrating that PC-associated aspirin had clear potential to inhibit cell growth, with a suggestion that the complex may be more potent than the parent drug (23). Here, we examined the biological effects of aspirin-PC formulation in ovarian cancer models alone and in combination with anti-VEGF mAbs.

Materials and Methods

Cell culture

We purchased the human ovarian cancer cell lines HeyA8, SKOV3ip1, and A2780 and the murine ovarian cancer cell line ID8 from the MD Anderson Characterized Cell Line Core Facility, which supplies authenticated cell lines. All experiments were performed with cell lines at 60% to 80% confluence. The HeyA8, SKOV3ip1, and A2780 cells were cultured in RPMI1640 medium supplemented with 10% (SKOV3ip1 and A2780) or 15% (HeyA8) FBS and 0.5% gentamicin, and incubated at 37°C in a mixture of 5% CO2 and 95% air. ID8 cells were cultured in DMEM medium containing 5% FBS, 1x insulin-transferrin-selenium (ITS), and 0.5% gentamicin. The tumor cells were free of pathogenic murine viruses and Mycoplasma (assayed by M.A. Bioproducts).

To study the effects of our aspirin test formulations on cell growth, at subculture each of the ovarian cancer cell lines was preincubated with the aspirin test drugs at a concentration range from 0 to 1.0 mmol/L for 15 minutes, prior to pipetting the cells onto a 48-well plates at a density of 2 × 105 cells/well, and cultured at 37°C in a mixture of 5% CO2 and 95% air. The cells were then cultured in the above growth medium in the presence and absence of the aspirin test formulations (at the same concentration, as used during the preincubation period) for 8 days (changing the medium with fresh medium on the 4th day). At the completion of the growth period, the number of viable cells/well was assessed using the established MTT method, which provides a linear relationship between the colored reaction product and cell number (23). Briefly, this entails adding MTT (3(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma Aldrich Chemical Co.) to the media of cells at a final concentration of 0.5 mg/ml for 4 hours. The solution containing the resultant purple-colored 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.

Tube-forming assay

Matrigel (12.5 mg/ml) was thawed at 4°C, and 50 μl was added to each well (μ-Slide Angiogenesis, Ibidi, cat. no. 81506) and allowed to solidify for 20 minutes at 37°C. The wells with immortalized human vascular endothelial cells (RF24; 8,000 per well), which had previously been treated with or without Aspirin-PC or aspirin at 0.4 mmol/L for 4 or 8 days, were then incubated for 6 hours at 37°C (which we have determined is sufficient time for tube formation to be complete). Experiments were performed in triplicate, using a previously described method (24). Employing an Olympus IX81-inverted microscope, images per well were taken at ×100 magnification. The amount of fully formed tubes per image was quantified.

Drugs and antibodies

Aspirin (uncoated) for the animal studies was purchased from Walgreens. Aspirin-PC was prepared as described below for the culture and animal studies. Bevacizumab (Genentech/Roche) is a humanized mAb that inhibits VEGF-A. Anti-VEGF antibody B20 (Genentech) is a cross-species reactive, function-blocking mAb targeting both human and murine VEGF-A. The antibodies used in immunofluorescence (IF) and immunohistochemistry were CD31 (BD Pharmingen), Ki67 (Neomarkers Inc.), cleaved caspase-3 (Biocare Medical LLC), CA-9 (Novus Biologicals), horse-radish peroxidase–conjugated goat anti-rabbit immunoglobulin G (Serotec Harlan Bioproducts for Science, Inc.), biotinylated goat anti-rabbit secondary antibody (Biocare Medical), and Alexa Fluor 594–conjugated anti-rat antibody (Jackson Immunoresearch Laboratories).

Aspirin test formulations

We used established procedures to prepare our PC-associated aspirin test formulations for cell culture and intragastric dosing (18, 21). For cell culture, to prepare the drug stock solution, aspirin was 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, provided by Lipoid Inc., GE), 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 minutes until a homogenous suspension was obtained (see Supplementary Fig. S1 for the chemical structures of aspirin and soy PC). For animal dosing, we followed the procedure established for preparing aspirin-PC for oral administration. This involved adding equal concentrations of
protonated aspirin (Rhodia) to PC-enriched soy lecithin (P35SB, provided by Lipoid Inc.) which was prewarmed to 37°C under constant stirring until a homogenous clear suspension was formed. This suspension was made weekly and stored at room temperature in the dark until immediately before animal dosing at which time the drug solution at 5 mg aspirin/mL was made with water to the desired concentration, and sonicated in a bath-type sonicator as outlined above prior to intra gastric administration to the mice with a feeding needle. The aspirin comparator formulations were prepared at the equivalent concentration, in either the culture medium or vehicle (water) for the in vitro and in vivo studies, respectively.

ELISA

Blood samples were allowed to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2,000 × g. Serum samples were diluted 1:50 for TGFβ1 and 1:10 for TXB₂ measurement. Total TGFβ1 in serum was measured after converting latent TGFβ1 to active TGFβ1 by acidification (10-minute incubation at room temperature with 1 mol/L HCl for serum, followed by neutralization by adding the same volume of 1.2 mol/L NaOH in 0.5 mol/L HEPES) with a sandwich ELISA specific for the activated form of TGFβ1 (R&D Systems). TXB₂ was measured by a competitive ELISA kit (R&D Systems).

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. At this time, the feces were allowed to thaw to room temperature, weighed, and then distilled water was added at a 1 g:10 mL feces:water ratio. After standing for 1 hour, the feces were disrupted into a homogenous suspension by vortexing for 2 minutes and then the Hb analyzed by a previously described method (19).

Animal experiments

Female athymic nude mice (NCr-nu) and C57BL/6 mice were purchased from Taconic Farms. HeyA8 and SKOV3ip1 cells were injected into the intraperitoneal cavity of NCr-nu mice (at a concentration of 2.5 × 10⁶ cells/0.2 mL for HeyA8 and 1 × 10⁶ cells/0.2 mL for SKOV3ip1 cells) per mouse as described in previous publications using the orthotopic mouse model of ovarian cancer (25–27). ID8 cells at a concentration of 2 × 10⁶ cells/0.2 mL per mouse were injected into the intraperitoneal cavity of C57BL/6 mice. All mouse studies were approved by the Institutional Animal Care and Use Committee and were carried for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the US Public Health Service Policy on Human Care and Use of Laboratory Animals. In most of the in vivo studies, the mice were randomly divided into six treatment groups: untreated control, aspirin (20 mg/kg intragastrically, dissolving the drug in water, daily), Aspirin-PC (20 mg aspirin/kg intragastrically, dissolving the drug in water, daily), Bev (Bevacizumab, 6.25 mg/kg intraperitoneally, dissolving the drug in PBS, twice weekly), B20 (5 mg/kg intraperitoneally, dissolving the drug in PBS, twice weekly), or aspirin or aspirin-PC plus Bev or B20. Treatment was initiated 7 to 10 days after cancer cell injection into the intraperitoneal cavity. After 3 to 4 weeks of treatment, the mice were sacrificed, and total body weight, total blood, tumor location and weight, and number of tumor nodules were recorded. Tumor specimens were preserved in either optimum cutting temperature medium (Miles Inc.; for frozen slides), or fixed in formalin (for paraffin slides) for further analysis.

Immunohistochemical and IF staining

Paraffin-embedded tumor tissue samples were incubated with Ki67 (1:400) or cleaved caspase-3 (1:100). Staining with rat monoclonal anti-mouse CD31 (1:800; BD Pharmingen) was performed on the frozen sections using IF staining using a previously described technique (28). After incubation overnight at 4°C, Alexa Fluor 594–conjugated anti-rat antibody (1:1,000) was incubated for 1 hour at room temperature. After the samples were washed with PBS, they were incubated with DAPI (Invitrogen) for 10 minutes at room temperature. Mounted samples were visualized using a fluorescent microscope (Leica Microsystems CMS GmbH) with the appropriate filter (original magnification, ×200). Two independent investigators evaluated immunohistochemical staining. Expression was scored as intensity (1 = no/focal, 2 = weak, 3 = moderate, 4 = strong), ordinal ranking of percent positive cells (0 = none, 1%–25%, 2% = 26%–50%, 3% = 51%–75%, 4% = 76%–100%), and an overall score calculated as the intensity and ordinal ranking of percent positive cells.

To detect hypoxia in the collected ovarian tumor tissues, we examined carbonic anhydrase-9 (CA-9) expression using a previously described immunohistochemical staining and scoring method (29). Briefly, this entailed incubating paraffin tumor tissue sections with anti-rabbit CA-9 antibody (1:500, cat. no. NB100-417, Novus Biologicals) at 4°C overnight. After washing the slides with PBS, tumor sections were incubated with a biotinylated goat anti-rabbit secondary antibody (cat.no. G6020H; Biocare Medical) followed by incubation with Streptavidin-HRP (cat.no. HP604H; Biocare Medical). Signal development was done using DAB. CA-9 tumor expression was scored by multiplying grade of intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong) with percentage of positive cells (0 = no positive cells, 1% < positive cells, 2% = 10%–50% positive cells, 3% = 51%–80% positive cells, 4% ≥80% positive cells; range, 0–12).

High Performance Liquid Chromatography analysis

Aspirin and salicylic acid from culture media were analyzed by High Performance Liquid Chromatography (HPLC) using a previously described method (30). Briefly, this entailed extracting the samples in acetonitrile (1 part media: 3 parts acetonitrile), followed by centrifugation with 10 μL samples supplemented with a Model 2707 autosampler onto an Agilent Zorbax 300 SBC18 column (at 45°C) using a Model 1525 binary HPLC pump with mixtures of 50 mmol/L phosphoric acid/acetonitrile (80%/20% initially), followed by a 20%/80% mixture. Detection was through a Model 2489 UV detector at 230 nm. The salicylic acid and aspirin concentrations were determined by comparing the elution peaks with that of purified standards of acetylsalicylic and salicylic acid.

Statistical analyses

Statistical analyses were performed using the Statistical Package for Social Science software version 18.0 (SPSS, Inc.). Continuous variables were compared using a Student t test (for 2 groups) or analysis of variance (for all groups) if the data were normally distributed. For nonparametric distributions, the Mann–Whitney U test (for 2 groups) was used.
A P value < 0.05 with two-tailed testing was deemed statistically significant.

**Results**

**In vitro effects of aspirin and aspirin-PC on the growth of ovarian cells in culture**

We first examined the effects of our aspirin test drugs on the growth of four ovarian cancer cell lines (Fig. 1A–D). Aspirin had only a modest-to-negligible inhibitory effect on the growth of these cell lines within the dose range studied, with A2780 and SKOV3ip1 only showing a response at the highest doses tested and HeyA8 human and ID8 mouse cells showing a clear response at doses of ≥0.16 mmol/L. Of particular note, all four cell lines showed a more prominent growth-inhibitory response to aspirin-PC at most of the doses tested over the 8-day culture period. It was determined that this growth-inhibitory effect was not attributable to our aspirin test formulations interfering with the initial attachment of the cells to the surface of the culture plate, as the cell density and viability were not affected by either aspirin or aspirin-PC after incubation periods of ≤4 days. We also determined by HPLC that aspirin (when added to the medium as either unmodified aspirin or Aspirin-PC) was stable (<10% breakdown to salicylate) in over a 4-day culture period, after which it was exchanged for fresh aspirin-containing medium (data not shown).

**In vivo effects of aspirin-PC, aspirin, and VEGF inhibitors in a mouse model of ovarian cancer**

On the basis of our in vitro studies, we next examined the in vivo efficacy of our aspirin test drugs alone and in combination with VEGF-A antagonists on the growth of ovarian tumors using established orthotopic and syngeneic mouse model systems. Aspirin-PC (at a dose of 20 mg aspirin/kg) alone and in combination with a VEGF-A inhibitor (either Bev or B20) resulted in a significant inhibition of the growth of HeyA8 and SKOV3ip1 ovarian tumors; similar results were noted with the syngeneic ID8 model (Fig. 2A–C) and the number of tumor nodules (Fig. 2D–F). Of particular note was the observation that the highly significant growth-inhibitory action of our aspirin test drugs (aspirin and aspirin-PC) was equivalent to or exceeded that of the VEGF inhibitor, whereas the combination of the two classes of drugs induced a further reduction in both tumor mass and number of tumor nodules that was appreciably lower than either test agent alone.

In two of the animal experiments, we compared the inhibitory activity of aspirin-PC versus aspirin on the growth of human (HeyA8) and mouse (ID8) ovarian cancer cells in the presence...
and absence of Bev or B20, respectively. As demonstrated in Fig. 2B, C, E, and F, aspirin-PC and aspirin both significantly inhibited the growth of both human and mouse ovarian tumors ($P < 0.01$), with the PC-associated aspirin tending to have a greater growth-inhibitory effect than the unmodified NSAID.

In the HeyA8 model, treatment with unmodified aspirin significantly reduced ovarian tumor growth [76.4% reduction in tumor weight vs. control ($P < 0.01$) and 69.1% decrease in the number of tumor nodules vs. control ($P < 0.05$; Fig. 2B and E)]. Aspirin-PC also resulted in profound antitumor activity [86.4% reduction in tumor weight vs. control ($P < 0.01$) and 79.4% decrease in the number of tumor nodules vs. control ($P < 0.01$; Fig. 2B and E)]. In this study, Bev also exhibited significant antitumor activity [63.6% reduction in tumor weight vs. control ($P < 0.01$) and 58.8% decrease in the number of tumor nodules vs. control ($P < 0.05$; Fig. 2B and E)]. Most interestingly, the combination of aspirin plus Bev resulted in a large reduction in tumor weight and tumor nodules, compared with either aspirin or Bev alone ($P < 0.05$ tumor weight and number of nodules of combo vs. Bev; with the tumor weight and number of nodules of the combo vs. aspirin not reaching statistical significance having $P$ values of 0.06 and 0.14, respectively). This potentially synergistic interaction with the VEGF inhibitor was also observed for aspirin-PC, as the combination of aspirin-PC plus Bev resulted in a large reduction in tumor weight (96.4% vs. control; $P < 0.01$) and tumor nodules (85.3% vs. control; $P < 0.01$; Fig. 2B and E). In our statistical analysis, it was determined that the combination of aspirin-PC plus Bev resulted in a significant reduction in tumor weight and nodule number, compared with aspirin-PC or Bev alone ($P < 0.05$). In the HeyA8 study, there are no significant differences between aspirin and aspirin-PC alone and/or in combination with the VEGF inhibitor.

In the ID8 model, aspirin treatment significantly reduced ovarian tumor growth [49.2% reduction in tumor weight vs. control ($P < 0.01$) and 42.9% decrease in the number of tumor nodules ($P < 0.05$; Fig. 2C and F)]. Aspirin-PC also possessed highly significant antitumor activity [72.3% reduction in tumor weight vs. control ($P < 0.01$) and 73.3% decrease in the number of tumor nodules vs. control ($P < 0.01$; Fig. 2C and F)]. The B20 treatment on its own exhibited profound antitumor activity [73.8% reduction in tumor weight vs. control ($P < 0.01$) and 73.9% decrease in the number of tumor nodules ($P < 0.01$; Fig. 2C and F)]. In our statistical analysis, it was determined that the combination of aspirin-PC plus Bev resulted in a significant reduction in tumor weight and number of nodules, compared with aspirin-PC or Bev alone ($P < 0.01$). In the HeyA8 study, there are no significant differences between aspirin and aspirin-PC alone and/or in combination with the VEGF inhibitor.

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nodules vs. control ($P < 0.01$; Fig. 2C and F). The combination of aspirin plus B20 resulted in an even greater reduction in tumor weight (86.2% vs. control; $P < 0.01$) and tumor nodules (85.1% vs. control; $P < 0.01$; Fig. 2C and F). Similar to the effects of aspirin ± VEGF inhibitor, the combination of aspirin-PC plus B20 resulted in a large reduction in tumor weight (96.9% vs. control; $P < 0.01$) and tumor nodules (91.9% vs. control; $P < 0.01$; Fig. 2C and E). In our statistical analysis, it was determined that the combination of aspirin plus B20 resulted in a significant reduction in tumor weight, and nodule number compared with aspirin or B20 alone ($P < 0.05$). Further, the combination of aspirin-PC plus B20 resulted in a significant reduction in tumor weight and number of nodules, compared with aspirin-PC or B20 alone ($P < 0.05$). Of particular note, aspirin-PC demonstrated significantly greater anti-tumor activity than plain aspirin (45.5% reduction in tumor weight and 53.3% decrease in the number of tumor nodules ($P < 0.05$)). Lastly, aspirin-PC plus B20 demonstrated significantly greater antitumor activity than unmodified aspirin plus B20 (77.8% reduction in tumor weight; $P < 0.01$).

Effect of aspirin-based treatment on tumor vessels, cell proliferation, and apoptosis

To examine the biological effects of our test agents alone and in combination, we examined tumor specimens from the HeyA8 mouse model for markers of proliferation (Ki67), apoptosis (cleaved caspase-3), and microvessel density (MVD: CD31). Representative images of Ki67-immunohistochemical staining are presented in Fig. 3A (top panel). We observed that rates of proliferation were significantly decreased in the treatment groups over the control group ($P < 0.01$). In addition, the combination of aspirin plus Bev resulted in a large reduction in Ki67 expression (37.3% vs. aspirin, $P < 0.01$; 57.4% vs. Bev, $P < 0.01$; Fig. 3B). Further, the combination of aspirin-PC plus Bev resulted in a large reduction in Ki67 expression (29.6% vs. aspirin-PC, $P < 0.01$; 61.2% vs. Bev, $P < 0.01$; Fig. 3B). It was also determined that there was a significant difference between aspirin and aspirin-PC in Ki67 expression ($P < 0.05$).

The rates of apoptosis were modestly increased in the treatment groups over cleaved caspase-3 levels of the control group ($P < 0.01$). Interestingly, the combination of aspirin plus Bev
resulted in a large increment in cleaved caspase-3 expression (65.3% vs. aspirin, \( P < 0.01 \); 71.3% vs. Bev, \( P < 0.01 \); Fig. 3C).

The combination of aspirin-PC plus Bev also resulted in a large increase in the expression of cleaved caspase-3 which significantly exceeded the effects of either agent alone (67.9% vs. aspirin-PC, \( P < 0.01 \); 72.3% vs. Bev, \( P < 0.01 \); Fig. 3C).

We observed that the number of tumor microvessels scored in fixed/IFC (CD31)-stained ovarian tumor tissue was significantly decreased in the treatment groups over in the vehicle-treated control group (\( P < 0.01 \)). In addition, the combination of aspirin or aspirin-PC plus Bev resulted in a large reduction in MVD, thus providing evidence that cojoint therapy of aspirin drugs with a VEGF-A inhibitor is more effective in blocking angiogenesis in ovarian tumor tissue than either agent alone (\( P < 0.01 \); Fig. 3D).

We also determined that serum levels of total TGF\( \beta \)1 in the ID8 mouse model were significantly decreased in the treatment groups over values of the control group (\( P < 0.01 \)). In addition, the combination of aspirin plus B20 resulted in a further reduction in serum levels of total TGF\( \beta \)1 (25.3 \pm 3.0 vs. 60.1 \pm 5.4, \( P < 0.01 \); 25.3 \pm 3.0 vs. 57.7 \pm 2.0, \( P < 0.01 \); Fig. 4A). A similar synergistic interaction was recorded for the combination of aspirin-PC plus Bev, which resulted in a large reduction in serum TGF\( \beta \)1 levels (25.3 \pm 3.0 vs. 60.1 \pm 5.4, \( P < 0.01 \); 25.3 \pm 3.0 vs. 57.7 \pm 2.0, \( P < 0.01 \); Fig. 4A).

Serum levels of total TGF\( \beta \)1 and TXB\( \beta \)

We examined serum level of total TGF\( \beta \)1 and TXB\( \beta \) in the HeyA8 and the ID8 mouse models. In the first analysis, we observed that serum levels of total TGF\( \beta \)1 in the HeyA8 mouse model were significantly decreased in the treatment groups over those measured in the vehicle-treated control group (\( P < 0.01 \)). In addition, the combination of aspirin plus Bev resulted in a large reduction in serum levels of total TGF\( \beta \)1 \( \left( \frac{25.5}{\text{C}24} \right) \right. \text{vs. } \left( \frac{77.2}{\text{C}5.3}, \left( \frac{25.5}{\text{C}5.3} \right) \right._{5.3}, \left( \frac{P < 0.01}{\text{C}5.3} \right) \text{vs. } \left( \frac{57.7}{\text{C}2.0}, \left( \frac{P < 0.01}{\text{C}2.0} \right) \right. \text{vs. } \left( \frac{57.7}{\text{C}2.0}, \left( \frac{P < 0.01}{\text{C}2.0} \right) \) Fig. 4A).

It was determined that both aspirin and aspirin-PC significantly reduced (\( P < 0.01 \)) serum thromboxane (TXB\( \beta \)) levels >70% in the HeyA8 and ID8 mouse models of ovarian cancer, and this profound antiplatelet effect was maintained when the
The therapeutic utility of aspirin has expanded from reduction of fever, inflammation, and pain to the treatment and/or reducing the risk of the development of cardiac disease, stroke thrombosis, diabetes, neurological disease, and cancer-afflicting numerous tissues (3, 32), which is the focus of the current study. As discussed earlier, the evidence supporting the association of aspirin consumption and a reduction in the incidence and mortality is most compelling for colorectal cancer which has led the USPSTF to recommend long-term usage of low-dose aspirin to prevent this common form of cancer. Beyond its use as an effective chemopreventive agent, there is also evidence that aspirin usage may reduce the metastatic spread of the disease suggesting its possible adjunctive use in cancer chemotherapy (5, 12).

Although the evidence supporting the use of aspirin as a chemopreventive agent for cancer and specifically colorectal cancer is compelling, aspirin usage has not been recommended to the general public because of the drugs’ GI side effects, notably gastroduodenal ulcers and bleeding, which can be life-threatening in susceptible individuals (33–35). This concern has led to the development of PC-associated aspirin and related NSAIDs that have been demonstrated to be less injurious to the gastroduodenal mucosa in both animal studies (16, 18–20) and short-term clinical trials (21, 22) due to its ability to preserve the surface hydrophobic barrier properties of the tissue. Animal studies also indicated that this novel family of lipidic NSAIDs maintained their therapeutic activity to reduce fever, inflammation, and in the case of aspirin, platelet inhibition, with the suggestion that the PC-associated drug may have more pronounced therapeutic efficacy and potency than the parent NSAID (18, 30, 36). We have also reported that PC-associated aspirin is effective in reducing the growth rate of colon cancer cells in culture (23), and have recently completed a series of (unpublished observations) studies, demonstrating the efficacy of chronic dosing of aspirin-PC to reduce tumor mass in a rodent model of colon cancer. Because of the enormous clinical potential of a GI-safer aspirin, PLx Pharma has

### Table 1. Body weight and fecal Hb measurements from in vivo studies

| SKOV3i3p cells/nude mice | Control 23.25 ± 0.71 | Aspirin-PC 22.74 ± 0.62 | Bev 22.42 ± 0.87 | Aspirin-PC/Bev 21.65 ± 0.45 |
| ID8 cells/C57BL-6 mice | Control 23.65 ± 0.62 | Aspirin 21.64 ± 0.32 | Aspirin-PC 21.08 ± 0.52 | B20 20.00 ± 0.13 |
| | Aspirin/B20 19.77 ± 0.16 | Aspirin-PC/B20 19.63 ± 0.22 |
| | Hey A8 cells/nude mice | Control 26.44 ± 0.53 | Aspirin 25.15 ± 0.65 | Aspirin-PC 24.99 ± 0.62 |
| | Aspirin-PC/Bev 24.40 ± 0.67 |

NOTE: Body weight in grams at the termination of the study is expressed as the mean ± SEM for n = 10 mice per group. Fecal Hb was measured from pooled samples collected from group cages, and is expressed as mg Hb per gram of feces for each group. For comparison, these Hb values are considered baseline and are much lower than values typically obtained from animals chronically treated with a higher-dose NSAID that induces GI bleeding (i.e., 2.5 to 15 mg Hb/g feces).
Our in vivo studies also revealed that both aspirin and aspirin-PC inhibited cell growth and the growth of microvessels associated with the ovarian tumor tissue, with both inhibitory actions augmented by coadministration of a VEGF inhibitor. An unexpected but very interesting finding was that apoptotic activity which was modestly elevated by the aspirin test drugs was markedly increased when administered in combination with Bev, although the VEGF-A inhibitor had little effect in promoting cell death on its own.

The rationale for testing our aspirin test drugs in combination with anti-VEGF drugs was based upon our prior work that demonstrated a key role for platelets in stimulating tumor growth following antiangiogenesis [8]. In addition to the known effect of aspirin on activation of platelets, it has been also reported that most NSAIDs, including aspirin at doses above the COX–1 inhibitory antiplatelet dose, have the capacity to inhibit COX–2-mediated prostacyclin production, which appears to be involved in the mechanism of hypoxia-induced angiogenic response in endothelial cells [39–41]. We therefore evaluated local hypoxia in the tumor microenvironment by assessing CA-9 expression, an established marker of tissue hypoxia [29, 31], and demonstrated evidence of CA-9 expression in tumor-bearing controls, which was further exacerbated by aspirin, an effect which was partially reversed in mice treated with aspirin-PC. Thus, our results suggest a COX-dependent inflammatory response in endothelial cells and associated cells supporting the tumor. Interestingly, the hypoxic response was exacerbated by aspirin, possibly due to the drug’s ability to reduce microvessel growth, whereas aspirin-PC had a lesser effect on tissue hypoxia, which may suggest its use with other antineoplastic agents as tissue hypoxia is thought to be a marker of resistance to chemotherapy [31].

Although we did not observe evidence of increased GI bleeding when aspirin and anti-VEGF agents were used in combination in our experiments, proper cautionary measures should be taken during clinical development.

In support of the possible role of the platelet in these growth responses, we also observed that both aspirin test drugs blocked platelet activation, as demonstrated by the drugs significantly inhibiting by >70% circulating thromboxane (presumably due to irreversible COX-1 inhibition) and by a comparable amount, release of the platelet growth factor, TGFβ1, into the blood. It also should be noted that the aspirin dose (20 mg/kg) administered to the mice in all three studies is comparable with low-dose (75–100 mg) aspirin, and neither aspirin-PC nor the unmodified NSAID induced evidence of GI injury and/or bleeding (see Table 1). Thus, these results suggest the clinical potential of using low-dose Aspirin-PC or aspirin as an adjunctive therapy with chemotherapeutic agents and notably VEGF inhibitors, which may be particularly useful for patients with later-stage ovarian cancer that are not candidates for surgical intervention and are refractory to most chemotherapeutic approaches.

The mechanism by which aspirin-PC and aspirin block the growth of ovarian cancer tissue has yet to be elucidated. The concept that COX-2 is overexpressed in most tumor tissue and drives unregulated cell proliferation and reduced apoptotic activity [3, 42] clearly may not apply to the effect of our test drugs on ovarian cancer that preferentially overexpress COX-1 (43–45). Nor does it explain why aspirin which is a weak COX-2 inhibitor has profound chemopreventive activity on ovarian cancer, while the more selective coxibs are less effective. Our
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cell culture results suggest a direct effect of aspirin on cell growth, likely due to COX-1 inhibition at doses of $\geq 0.4$ mmol/L. It is also possible that the aspirin test formulations may have promoted apoptotic activity of the ovarian cancer cells. However, our previous findings on colon cancer cell lines demonstrated that aspirin had no direct effect on promoting cell death at aspirin concentrations up to 2.5 mmol/L (23). Under in vivo conditions, it is likely that aspirin will be rapidly converted to salicylic acid in circulation, due to the short half-life of the drug in circulation (which has been estimated at 7–12 minutes in rodents) as a consequence of the presence of deacetyl hydrolase enzymatic pathway in plasma (46), giving the acetylated drug limited opportunity to inhibit ovarian tissue COX-1. In support of this, we have (unpublished observations) evidence that aspirin levels in circulation in rodents ranged between 5 and 10 μmol/L at 10 to 20 minutes after intragastric dosing of aspirin at a $5 \times$ higher concentration (100 mg/kg) than that used in the current study, falling to undetectable levels thereafter.

One likely mechanism that aspirin could block the growth and potential metastatic spread of ovarian cancer cell is by irreversibly blocking platelet activation via its well-known ability to acetylate and thereby inactivate platelet COX-1 (47, 48). Indeed, this would be consistent with the compelling evidence that late-stage cancer patients, including those with ovarian cancer, have elevated platelet counts (thrombocytosis) that result in a much higher risk for developing deep vein thrombosis than the general population (27, 49). As indicated earlier, we and others have indeed reported that platelets promote cancer cell proliferation, in part by promoting epithelial–mesenchymal transition (EMT), as well as promoting the translocation from the primary tumor into circulation and ultimately from the blood to tissue sites where metastatic growth and angiogenesis occur, a process facilitated by the local release of platelet-derived VEGF, TGFβ, and PDGF (7, 25, 27). Thus, aspirin’s unique action to irreversibly inactivate platelets (via acetylation of COX-1) may be a very powerful mechanism to block the progression of cancer, a concept that has been considered by others (3, 5, 50). In support of this possibility, Cho and colleagues (25) and Stone and colleagues (27) used the orthotopic mouse model to demonstrate that the proliferation of human ovarian tissue can be promoted by the infusion of fresh platelets. Furthermore, mouse platelets have been reported to extravasate into the ovarian tumor tissue, as demonstrated with a specific immunohistochemical stain using an antibody directed against a platelet surface protein and genetically engineered fluorescently labeled platelets (27). Future studies will be directed at determining if the platelet indeed is the primary target of aspirin’s antineoplastic action and if PC association provides an advantage for aspirin to target and inactivate the platelet.

Disclosure of Potential Conflicts of Interest

L.M. Lichtenberger is a consultant/advisory board member for Plx Pharma Inc. L.M. Lichtenberger and E.J. Dial have ownership interest (including patents) in Plx Pharma Inc. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: Y. Huang, L.M. Lichtenberger, J.N. Bottsford-Miller, A.K. Sood


Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Huang, L.M. Lichtenberger, M. Taylor, M. Haemmerle, M.J. Wagner, D. Fang, E.J. Dial, A.K. Sood

Writing, review, and/or revision of the manuscript: Y. Huang, L.M. Lichtenberger, M. Haemmerle, M.J. Wagner, Y. Lyons, W. Hu, R.A. Previs, J.M. Hansen, D. Fang, P.L. Dormia, E.J. Dial, A.K. Sood

Administrative, technical, or material support (i.e., reporting and organizing data, constructing databases): Y. Huang, L.M. Lichtenberger, M. Taylor, W. Hu, F. Shen, A.K. Sood

Study supervision: Y. Huang, L.M. Lichtenberger, A.K. Sood

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