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Phospho-aspirin (MDC-22) prevents pancreatic carcinogenesis in mice

George Mattheolabakis¹, Ioannis Papayannis¹, Jennifer Yang², Brandon M. Vaeth², Ruixue Wang², Jela Bandovic³, Nengtai Ouyang¹, Basil Rigas^{1,5}, and Gerardo G. Mackenzie^{1,2,4}

¹Department of Medicine, Stony Brook University, Stony Brook, NY, 11794-8175

²Department of Family, Population and Preventive Medicine, Stony Brook University, Stony Brook, NY, 11794-8175

³Department of Pathology, Stony Brook University, Stony Brook, NY, 11794-8175

⁴Stony Brook Cancer Center, Stony Brook University, Stony Brook, NY, 11794-8175

⁵Medicon Pharmaceuticals Inc, Setauket, NY 11733

Abstract

Pancreatic cancer (PC) is a deadly disease with a dismal 5-year survival rate of <6%. The currently limited treatment options for PC underscore the need for novel chemopreventive and therapeutic agents. Accumulating evidence indicates that aspirin use is associated with a decreased risk of PC. However, the anticancer properties of aspirin are restricted by its gastrointestinal toxicity and its limited efficacy. Therefore, we developed phospho-aspirin (PA; MDC-22), a novel derivative of aspirin, and evaluated its chemopreventive efficacy in preclinical models of PC. PA inhibited the growth of human PC cell lines 8–12 fold more potently than aspirin; based on the 24-h IC₅₀ values. In a Panc-1 xenograft model, PA, at a dose of 100 mg/kg/d ×5/wk for 30 days, reduced tumor growth by 78% (p<0.01 vs vehicle control). Furthermore, PA prevented pancreatitis-accelerated acinar-to-ductal metaplasia in mice with activated Kras. In *p48-Cre;Kras^{G12D}* mice, cerulein treatment (6 hourly injections 2×/wk × 3 wks) led to a significant increase in ductal metaplasia, replacing the majority of the exocrine compartment. Administration of PA 100 mg/kg/d ×5/wk for 21 days (starting on the first day of cerulein injection) inhibited the acinar-to-ductal metaplasia, reducing it by 87% (p<0.01, vs cerulein-treated control). PA appeared to be safe, with the animals showing no signs of toxicity during treatment. Mechanistically, PA inhibited EGFR activation in PC, an effect consistently observed in PC cells, primary acinar explants and in vivo. In conclusion, our findings indicate that PA has strong anticancer efficacy in preclinical models of PC, warranting its further evaluation.

Corresponding author: Gerardo G. Mackenzie, Ph.D., Department of Family, Population and Preventive Medicine, Stony Brook University; HSC, T17-080; Stony Brook, NY 11794-8173. Phone: (631) 444-9537; Fax: (631) 444-9553. Gerardo.Mackenzie@stonybrookmedicine.edu.

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Keywords

phospho-aspirin; pancreatic cancer prevention; EGFR; ERK; aspirin; pancreatic carcinogenesis

Introduction

Pancreatic cancer (PC) is a complex and highly lethal disease with a five-year survival of <6.7% (1). Despite improved treatment modalities during the last decade, current chemotherapy and radiation therapy regimens offer minimal or no help (2). Thus, there is an urgent need to develop novel prevention strategies against PC.

An impressive body of evidence supports the notion that chemoprevention has the potential to be a major component in the control of cancer, including PC (2, 3). The development of successful chemoprevention strategies appears essential for individuals who are at high risk of developing PC, including people with premalignant lesions, such as certain pancreatic cysts, and those with inherited factors (4). Subjects at a higher risk of developing PC include those with: a history of Peutz-Jeghers syndrome, familial breast cancer gene, hereditary pancreatitis and familial atypical multiple mole melanoma (2). Other significant risk factors that contribute to the development of PC include tobacco smoking, chronic pancreatitis, type II diabetes and obesity. These individuals constitute a population in need for effective strategies for PC prevention, with the development of novel chemopreventive agents being a critical component.

The epidermal growth factor receptor (EGFR) is a key molecular determinant of PC. Aberrant activation of EGFR plays an important role in pancreatic carcinogenesis via the sustained initiation of downstream cascades that promote cell survival and proliferation (5, 6). Moreover, EGFR plays a critical role in Kras-driven tumorigenesis and the loss of EGFR may completely abrogate the development of pancreatic intraepithelial neoplasia (5, 6). Given its essential role in pancreatic carcinogenesis, the EGFR signaling pathway is an attractive target for chemoprevention (7).

Aspirin, the most widely used nonsteroidal anti-inflammatory drug (NSAID) in the world (8, 9), has a significant antineoplastic effect (10, 11). For example, aspirin has been formally documented to be a chemopreventive agent against colon cancer (12, 13). Systematic review, meta-analysis and case-control studies support the notion that a daily high-dose aspirin regimen may reduce risk of developing PC (14, 15). However, the anticancer properties of aspirin are restricted by its gastrointestinal toxicity and its limited efficacy. In recent years, our laboratory has developed novel agents for cancer prevention and treatment. One of them, phospho-aspirin (PA; MDC-22; Fig. 1A) consists of aspirin chemically modified at its carboxylic group, the moiety accounting for its gastrointestinal toxicity (16). Indeed, PA demonstrated a much improved gastrointestinal safety profile compared to aspirin and is more efficacious in the treatment of breast cancer than aspirin (17, 18). In addition, a key molecular target of PA is EGFR (18).

In light of these findings and the critical role played by EGFR in pancreatic carcinogenesis (5, 6), in this study we assessed the efficacy of PA in the prevention of PC. PA was much

more potent than aspirin in inhibiting PC cell growth, reduced human PC growth in subcutaneous xenograft models and prevented pancreatic carcinogenesis in a transgenic mouse model of PC. Mechanistically, the antineoplastic effect of PA was mediated by the inhibition of EGFR activation.

Materials and Methods

Reagents

PA was provided by Medicon Pharmaceuticals, Inc., (Setauket, NY). Aspirin was from Sigma-Aldrich (St. Louis, MO). For cell culture studies, we prepared 400 mM and 1M stock solutions of PA and ASA in DMSO, respectively. All general solvents and reagents were of HPLC grade or of the highest grade commercially available.

Cell culture

Human PC cell lines (Panc-1, MIA PaCa-2, AsPC-1, HPAF-II, CFPAC-1 and BxPC-3) were from the American Type Culture Collection (ATCC; Manassas, VA), which characterizes them using cytogenetic analysis. We have not authenticated these cell lines. These cells were grown as monolayers in the specific medium and conditions suggested by ATCC. All the cell lines were characterized for cell morphology and growth rate and passaged in our laboratory less than 6 months after being received.

Cell viability assay

It was determined using an assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT), as previously described (19).

Clonogenic assay

It was performed as previously described (20). Briefly, MIA PaCa-2 and Panc-1 cells, plated in 6-well plates (1,000 cells/well), were treated with various concentrations of PA or aspirin for 24h. Following treatment, media was replaced and cells grown in the absence of the drugs for 7 days, with their media replaced every 3 days. The cells were then stained with 1% crystal violet in Borate Buffer saline (0.1 M, pH 9.3) and 0.02% ethanol. Following lysis, the absorbance was read at 570 nm.

Cytokinetic analysis

Following treatment with PA, apoptosis was assayed by staining with Annexin V-FITC and propidium iodide (PI) and analyzing the fluorescence intensities by FACScaliber (BD Bioscience); Cell proliferation was assayed by 5-bromo-2'-deoxyuridine (BrdU) incorporation, and cell cycle by flow cytometry; all as described (21).

Plasmid transfections

EGFR plasmid was purchased from Addgene (Cambridge, MA). The transient transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

Immunoblotting

After treatment, total cell fractions were obtained, and Western blots were performed as described (22). Antibodies, were obtained from Cell Signaling Technology (Beverly, MA), except β -actin (Sigma-Aldrich; Saint Louis, MO).

Immunofluorescence

Panc-1 cells, seeded in chamber slide dishes, were pre-incubated for 60 min in the presence of PA $1 \times IC_{50}$, erlotinib 40 nM or equivalent volumes of DMSO. Following stimulation with EGF 10 ng/ml for 15 minutes, Panc-1 cells were fixed with paraformaldehyde (4% w/v in PBS). In a second experiment, Panc-1 cells, MIA PaCa-2 cells and AsPC-1 cells were treated with PA $1 \times IC_{50}$ for 4h. Immunostaining was performed for p-EGFR (Cat # ab40815; Abcam, Cambridge, UK). After PBS washing, sections were incubated with an Alexa Fluor 488-linked secondary antibody. After staining, slides were mounted in VECTASHIELD with DAPI and photographed under a Nikon ECLIPSE 90i microscope with a digital camera.

Determination of prostaglandin E₂

Prostaglandin E₂ (PGE₂) levels in the cell culture media were determined with an immunoassay (Cayman Chemical; Ann Arbor, MI). Briefly, 1.5×10^6 Panc-1 cells were pre-incubated with various concentrations of PA or ASA for 30 min followed by treatment with the calcium ionophore A23187 5 μ M for 3h.

Pancreatic Epithelial Explants Culture

Pancreatic epithelial explants from 6–8 week-old *Kras^{LSL-G12D/+};Ptf1a^{Cre/+}* mice (referred to hereinafter as *Kras^{G12D}*) were established following previously published protocols (22). Briefly, following isolation, the cellular suspension was plated on plates pre-coated with 50 μ g/ml of bovine collagen type I. The next day, cells were either left untreated or treated with PA 200 μ M for 5 days. Media were changed on days 1 and 3. For quantification, acinar explants were seeded in triplicates; cell clusters were counted from at least 3 optical fields/well. Immunofluorescence of acinar explants and the resulting metaplasia was performed as described above and the images were examined under a Zeiss LSM510 meta-confocal microscope.

Animal studies

All animal studies were approved by our Institutional Animal Care and Use Committee.

Efficacy studies in pancreatic xenograft models

Female Balb/C nude mice (Charles River Laboratories, Wilmington, MA) were inoculated subcutaneously into each of their flanks with 2.5×10^6 Panc-1 or MIA PaCa-2 cells in Matrigel (BD Biosciences, Franklin Lakes, NJ). Once the tumor reached approximately 150–200 mm³, animals were randomized into the control group, which received corn oil, and the treatment groups, which received orally PA 100 mg/kg in corn oil (n=7/group, once daily 5x/wk), until the end of the study. The dose of PA represents <20% of its maximum tolerated dose, which provides an ample therapeutic window (17, 18). Tumor volume was calculated as previously described (23, 24). At the end of the experiments, animals were

euthanized by CO₂ asphyxiation and tumor weights were measured after their careful resection. Tumor tissue was collected for analysis.

Pancreatitis-accelerated carcinogenesis in mice with activated Kras

In the first study, six week old *KrasG12D* mice (C57Bl/6J background) were injected intraperitoneally with saline or cerulein (250 µg/kg hourly, once per day for three consecutive days). Cerulein-injected mice were divided into vehicle or PA treatment groups (n=5/group). PA 100 mg/kg was given orally once a day, starting on the day of the first cerulein injection. Three days after the last cerulein injection (day 6 of treatment), mice were euthanized, serum was isolated and the pancreas was excised and fixed in formalin or snap frozen for further analysis.

In the second study, two month old *KrasG12D* mice were injected intraperitoneally with cerulein (50 µg/kg hourly, ×6/d, 2d/wk, ×3 wks). Cerulein-injected mice were divided in vehicle or PA treatment groups (n=8/group). PA 100 mg/kg was given 5d/wk by oral gavage for three weeks, starting on the day of the first cerulein injection. On day 21, mice were euthanized and the pancreas was excised and fixed in formalin and processed for morphological studies.

Histological analysis

Pancreatic tissues were fixed in 10% neutral-buffered formalin for 24 h and then processed and embedded in paraffin according to standard protocols. Tissue sections (4 μ m) stained with hematoxylin and eosin (H&E) were evaluated histologically by two pathologists, blinded to the identity of the samples. The presence and extent of pancreatic ductal metaplasia were scored based on the degree of epithelial stratification and nuclear atypia according to histopathology criteria, and expressed as the percent of lesion over the total pancreas area. Briefly, acinar-to-ductal metaplasia was defined as the conversion of acinar cells into ductal cells. This change was characterized by the dilatation of the acinus or group of acini and the formation of a duct like structure with visible lumen lined by flat epithelial cells. (25, 26).

Immunohistochemical analysis

Immunohistochemical staining for Ki-67 (Cat # sc-7907, Santa Cruz Biotechnology, Santa Cruz, CA), PCNA (sc-15402; Santa Cruz Biotechnology, Santa Cruz, CA), α -amylase (Cat # 3796, Cell Signaling Technology (Beverly, MA) and p-EGFR (Cat # ab40815; Abcam, Cambridge, UK) was performed on human PC xenograft tissue samples or mouse pancreatic samples, as previously described (27).

Statistical analysis

Results are expressed as Mean±SEM. Differences between groups were determined by one-factor analysis of variance followed by Tukey's test for multiple comparisons. $p < 0.05$ was statistically significant.

Results

PA inhibits the growth of human PC cells in culture and in human PC xenografts

Our initial goal was to establish the potency of PA in inhibiting the growth of PC cells in culture. We treated a panel of six human PC cell lines with or without escalating concentrations of PA (25–800 μ M) for 24 h, and compared the potency of PA to that of aspirin, its parent compound. As shown in Fig. 1B, PA reduced PC cell growth concentration-dependently in all six of the human PC cell lines tested. Furthermore, PA inhibited the growth of human PC cells more potently than aspirin (Fig. 1B and Fig. S1). Indeed, PA inhibited cell growth more potently than aspirin in a panel of six human PC cell lines. The potency enhancement ranged between 5 and 8-fold in these PC cell lines (Fig. 1B and Fig. S1A).

We next examined the effect of PA and ASA on colony formation using two pancreatic cancer cell lines. PA inhibited colony formation in a concentration-dependent manner and was significantly superior to ASA (Fig. 1C and Fig. S1B). For example, PA 400 μ M significantly ($p<0.01$) reduced colony formation by 80% and 59% in MIA PaCa-2 and Panc-1 cells, respectively; whereas ASA, at the same concentration, had no significant effect on colony formation. In both cell lines, the difference in potency between PA and ASA groups was significant ($p<0.01$), with PA being at least 2-fold more potent.

To assess the anticancer potential of PA *in vivo*, we employed heterotopic (subcutaneous) PC xenografts in nude mice. Initially, we evaluated the *chemotherapeutic* effect of PA on subcutaneous Panc-1 xenografts. As shown in Fig. 1D, PA 100 mg/kg, once daily 5d/wk, significantly inhibited Panc-1 xenograft growth starting on day 11 of treatment until the end of the study ($p<0.01$ for all time points). At sacrifice (day 30 of treatment), the tumor volume ($mean\pm SEM$ for this and all subsequent values) of vehicle was 460.2 ± 83.7 mm³ and that of PA was 183.9 ± 38.8 mm³, representing a 78% tumor growth inhibition compared to vehicle control ($p<0.01$).

To rule out a cell line-specific effect, we evaluated the chemotherapeutic effect of PA in subcutaneous MIA PaCa-2 PC xenografts. PA (100 mg/kg, p.o., 5d/wk) also potently inhibited MIA PaCa-2 xenograft growth, resulting in a 53.1% tumor growth inhibition compared to control ($p<0.01$; Fig. 1E).

In these animal studies PA was well tolerated, with the mice showing no weight loss or other signs of toxicity during treatment. This is consistent with our previous findings in rats and mice (17, 18), documenting an enhanced safety of PA compared to aspirin.

PA inhibits the growth of human PC through a strong cytokinetic effect

PA inhibited tumor growth through a potent cytokinetic effect. *In vitro* treatment of AsPC-1 cells with PA for 24h led to a concentration-dependent induction of apoptosis (Fig. 2A). Of note, PA induced a minimal amount of necrosis (less than 2.5%, Fig. 2A). This was also observed in Panc-1, MIA PaCa-2 and HPAF-II cells, where PA $1.5\times IC_{50}$ induced apoptosis by 5.0, 3.6 and 2.7-fold, respectively (Fig. 2B and Figure S2A). Furthermore, in Panc-1 cells, treatment with PA reduced time-dependently the levels of full-length caspase-3 (Fig.

S2B). We then evaluated if PA affected the levels of survivin, an anti-apoptotic protein highly expressed in PC (28). Treatment of Panc-1 cells with PA reduced survivin levels in a time-dependent manner (Fig. S2B).

In addition, PA inhibited the G₁ to S cell cycle phase transition (Fig. 2C), and inhibited cell proliferation in AsPC-1 cells by 32% (data not shown), as also observed *in vivo*. In the heterotopic Panc-1 xenografts, PA significantly ($p<0.05$) inhibited cell proliferation by 35%, as shown by PCNA immunohistochemical staining (Fig. 2D).

Signaling effects of PA

The tumor suppressor p53, frequently mutated in human PC, has a central function in cell cycle regulation, DNA repair, cellular senescence, and apoptosis. Thus, we evaluated the effect of PA on p53 acetylation, which is critical for its stability and transcriptional activity (29). In MIA PaCa-2 cells, PA induced p53 acetylation concentration-dependently at two distinct lysine (K379 and K382) residues (Fig. S3A). *In vivo*, PA induced p53 acetylation at the K382 residue in Panc-1 xenografts (Fig. S3B), suggesting that PA is an inducer of p53 acetylation *in vitro* and *in vivo*. We also observed a time-dependent induction of p21, a downstream target of p53 after treatment with $1.5\times\text{IC}_{50}$ PA, which may contribute to the cell cycle arrest (Fig. S3C).

By analogy to ASA, a COX inhibitor, we investigated the effects of PA on the COX pathway, examining the effect of PA and ASA on the production of PGE₂ by Panc-1 cells following stimulation with the calcium ionophore A23187 (30). While PA failed to alter significantly the production of PGE₂ by these cells, ASA, as expected, completely inhibited it at both concentrations tested (Fig. S4), suggesting that PA exerts its effect COX-independently.

PA inhibits EGFR activation in human PC cells and xenografts

EGFR expression is known to correlate with the progression of many types of cancer, including PC (5, 6), and is considered a therapeutic target. We have recently shown that EGFR is a key molecular target of PA in breast cancer (18). Thus, we investigated the contribution of this pathway to the anti-cancer effect of PA in PC.

We initially evaluated the expression of EGFR in a panel of four PC cells. While Panc-1, AsPC-1 and MIA PaCa-2 cells express higher levels of phosphorylated EGFR, no EGFR phosphorylation was detected in HPAF-II cells (Fig. S5). In PC cell lines, PA inhibited both constitutive and EGF-stimulated EGFR activation, decreasing EGFR phosphorylation. For example, in MIA PaCa-2 cells, PA inhibited EGFR phosphorylation in a concentration-dependent manner, becoming evident at $1\times\text{IC}_{50}$ (Fig. 3A). In addition, PA reduced EGFR phosphorylation in Panc-1, MIA PaCa-2 and AsPC-1 cells by 35%, 78% and 81% ($p<0.01$ vs control for all), respectively, as evidenced by immunofluorescence (Fig. 3B and Fig. S6). Moreover, in Panc-1 cells, PA prevented the EGF-stimulated increase in EGFR phosphorylation, as evidenced by western blot and immunofluorescence (Fig. 3C–D). The effect was comparable to that of erlotinib, an EGFR inhibitor used clinically (Fig. 3C). This observation was confirmed *in vivo*, where PA reduced EGFR phosphorylation by 65% in the Panc-1 xenografts compared to controls ($p<0.05$, Fig. 3E).

Because EGFR activates ERK during pancreatic tumorigenesis (5), we evaluated whether PA treatment leads to an inhibition of ERK activation. In Panc-1 cells, PA reduced ERK phosphorylation in a concentration-dependent manner (Fig. 3F).

To determine whether EGFR is a key molecular target for PA in PC, we evaluated the effect of PA on cell death in MIA PaCa-2 cells transiently transfected with an EGFR-overexpressing plasmid (Fig. 3G). EGFR overexpression suppressed the induction of apoptosis by PA. After 24h of incubation with PA $1.5 \times \text{IC}_{50}$, the annexin V(+) fraction in mock transfected MIA PaCa-2 cells was increased by 6.8-fold. In contrast, PA induced apoptosis by 3-fold in cells that overexpressed EGFR (Fig. 3G).

PA reduces pancreatitis-associated tumorigenesis by inhibiting EGFR activation

To better dissect the effect of PA in PC progression, we next used the *KrasG12D* mouse model, which reproducibly shows metaplasia beginning at ~8 weeks of age, with progression to pancreatic ductal adenocarcinoma at ~1 year (31).

Earlier studies have shown that cerulein treatment affect EGFR expression in *KrasG12D* mice (5). For this purpose, we treated six week-old *KrasG12D* mice with 250 µg/kg cerulein, a known inducer of pancreatic inflammation, daily for 3 days, followed by 3 days of recovery (Fig. 4A). Serum amylase and lipase levels, markers of acute pancreatic injury, were elevated after 1h of cerulein injections compared with *KrasG12D* mice. PA failed to prevent acute pancreatic injury (Fig. S7). As expected, enzyme levels returned to basal levels in both control and PA-treated groups at the end of the treatment.

Using this model, we examined whether PA could inhibit EGFR expression prior to rampant epithelial morphogenesis (Fig. 4B). Cerulein treatment of *KrasG12D* mice induced higher active EGFR [EGFR (pY1068)] expression. PA treatment reduced EGFR phosphorylation levels (Fig 4B). The reduction in EGFR was associated with a significant reduction in pERK1/2 levels, consistent with what was observed in vitro (Fig. 4C).

In this model, we also evaluated whether PA could inhibit early pancreatitis-dependent, acinar cell-derived tumorigenesis. With cerulein treatment, *KrasG12D* mice showed significant replacement of normal pancreatic tissue with fibrotic, inflamed tissue while the majority of epithelium was replaced by metaplasia (Fig. 4C). PA-treated mice were almost completely protected from this dramatic transition (Fig. 4C), and retained mostly phenotypically normal tissue with only rare PAS+ ductal metaplasia. PA-treated pancreata retained amylase staining, an acinar marker (32), and had significantly fewer proliferating cells, as determined by immunohistochemistry for PCNA (Fig. 4C).

PA prevents pancreatitis-accelerated ductal metaplasia in mice with activated Kras

Since chronic pancreatitis is a significant risk factor for PC (33, 34), we investigated whether PA inhibits Kras-driven pancreatic carcinogenesis in the setting of pancreatitis induced by cerulein. For this purpose, we treated two month-old *KrasG12D* mice with cerulein (50 µg/kg; 6 hourly injections 2×/wk for 3 weeks) to induce pancreatitis. Concomitant with pancreatitis induction and continuing for the following 3 weeks, mice were treated with PA 100 mg/kg/d ×5/wk or vehicle (Fig. 5A). Histologic analysis showed a significant increase in

ductal metaplasia and mucin metaplasia lesions in *KrasG12D* mice treated with cerulein, significantly replacing the exocrine compartment (Fig. 5B–C). In control mice, this effect was accompanied by lower expression of amylase, and an increase in cell proliferation. PA treatment prevented cerulein-induced acinar-to-ductal metaplasia by 87% ($p<0.01$), an effect associated with 4-fold increased ($p<0.05$) amylase expression (Fig. 5B–C).

In this setting, p-EGFR levels in PA-treated pancreata were reduced by 69% ($p<0.05$), compared to controls (Fig. 5B–C). PA treatment also reduced p-ERK levels by 79% ($p<0.05$) and decreased cell proliferation (Ki-67 expression) by 67%, compared to cerulein-treated controls (Fig. 5B–C).

To confirm if PA prevents acinar-to-ductal metaplasia, we isolated pancreatic acinar explants from *KrasG12D* mice, which spontaneously transdifferentiate into duct cells when embedded in collagen, and treated them without or with PA for 5 days (Fig. S8). As expected on day 5 in culture, untreated control generated numerous metaplastic structures. Treatment with PA reduced by 46% ($p<0.01$) the ability of acinar cell explants to generate metaplastic structures (Fig. 6A), suggesting that PA prevents acinar-to-ductal metaplasia in vivo and in vitro.

Given that EGFR activation increases during ductal transdifferentiation (5, 6), we examined whether PA could inhibit EGFR phosphorylation in the resulting ductal metaplasia structures isolated from *KrasG12D* mice. At day 5, EGFR phosphorylation was strongly positive in untreated ductal structures, but was reduced following treatment with PA (Fig. 6B).

Discussion

Successful chemoprevention approaches could be a key component in the management of PC in individuals who are at high risk, including people with premalignant lesions, such as certain pancreatic cysts, and those with inherited factors. These patients at risk would benefit from the development of effective strategies for PC prevention; e.g. the development of new chemopreventive agents. Our data demonstrate that PA effectively inhibits PC in preclinical models. Underlying this effect are the substantial inhibition of cell proliferation, induction of apoptosis and the inhibition of cell cycle transition. A significant inhibition of EGFR activation by PA appears to be an important component of its mechanism of action.

PA appears to satisfy two critical requisites for any chemopreventive agent: improved safety and enhanced efficacy. We have previously documented on the superior safety of PA, clearly demonstrating that is far safer than aspirin in mice and rats (17, 18). Regarding its efficacy, we showed that PA is a strong chemopreventive agent. The chemopreventive effect of PA in PC is: a) broad, encompassing both PC xenografts and genetic engineered mouse models; and b) potent, as evidenced by the robust growth inhibition achieved in Panc-1 and MIA PaCa-2 xenografts, and the strong inhibition of acinar-to-ductal metaplasia in the *KrasG12D* mice. It is noteworthy that the doses used for PA are lower than 10% of its maximum tolerated dose.

It is becoming increasingly clear that sustained metaplasia is as an early precursor to malignant transformation in several organs, including the pancreas (35). Pancreatic acinar-

to-ductal metaplasia can result from pancreatic injury or Kras activation, and is an early step in pancreatic cancer progression by triggering the development of serious lesions, such as PanIN or pancreatic ductal adenocarcinoma (35). A previous study on patients with duct-like metaplasia induced by chronic pancreatitis demonstrated a 16-fold increase in the relative risk for pancreatic ductal adenocarcinoma, increasing to 50-fold in patients with familial chronic pancreatitis (36). PA was able to profoundly suppress acinar-to-ductal metaplasia, a precursor of the preneoplastic PanIN lesions (37), in vivo and in primary acinar explants, indicating that PA might be an effective chemopreventive agent for PC.

The role of EGFR as a therapeutic target in PC is complex. In metastatic PC patients, anti-EGFR therapy has only a modest survival effect (38, 39); however, EGFR appears to be a key molecular determinant of pancreatic tumorigenesis (5, 6). Aberrant activation of EGFR plays an important role in pancreatic carcinogenesis via the sustained initiation of downstream cascades that promote cell survival and proliferation. Moreover, EGFR plays a critical role in Kras-driven tumorigenesis and the loss of EGFR has been shown to completely abrogate PanIN development (5, 6). Various studies have shown that EGFR is generally involved in post-transformation functions in *Ras*-mutated tumors. In addition, concomitant pancreatic activation of oncogenic Kras and EGFR signaling leads to accelerated formation of high-grade preneoplastic lesions and carcinoma (5, 6, 40). Moreover, endogenous EGFR signaling is required to maintain the critical threshold of Ras activity required for tumorigenesis (41).

Given its essential role in pancreatic carcinogenesis, this EGFR signaling pathway is an attractive target for chemoprevention (7). Our work identified EGFR as a key molecular target of PA. PA is a potent inhibitor of EGFR phosphorylation *in vitro* and *in vivo*. Inhibition of EGFR phosphorylation inhibited the ERK pathway, and this was accompanied with a decrease in cell proliferation. Consistent with our findings, the EGFR inhibitor erlotinib has been shown to inhibit ERK phosphorylation in PC (42). Furthermore, overexpression of EGFR in PC cells conferred substantial drug resistance to PA, suggesting that EGFR-dependent signaling is a key molecular target of PA. At this time, however, we cannot rule out the possibility that PA exerts an anti-inflammatory effect and that the inhibition of EGFR may be the result of such reduced inflammation.

In conclusion, our work indicates that PA has a strong chemopreventive effect against PC in preclinical models, and indicates EGFR as a critical molecular target. Although more work is needed, at a clinical level, the use of a PA may offer a safer preventive option for those patients at higher risk of developing PC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Phospho-aspirin inhibits the growth of PC cells and xenografts

A: PA's chemical structure. **B:** Cell growth was determined in AsPC-1, BxPC-3, Panc-1 and MIA PaCa-2 cells after treatment with escalating concentrations of PA or aspirin (ASA) for 24 h. Results are expressed as % control. **C:** PA inhibits colony formation concentration-dependently in MIA PaCa-2 and Panc-1 cells. Aspirin (ASA) 400 μM had no effect. Results are expressed as % control. * $p < 0.01$, compared to control group. # $p < 0.01$, compared to ASA. **D:** Chemotherapeutic effect of PA on subcutaneous Panc-1 xenografts in nude mice; * $p < 0.01$, compared to vehicle control group; $n = 12-14$ tumors/group. **E:** Chemotherapeutic

effect of PA on subcutaneous MIA PaCa-2 xenografts in nude mice. All values are mean $\pm$ SEM; *p<0.01, vs. control.

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Fig. 2. Phospho-aspirin exerts a strong cytokinetic effect on PC cells

A: AsPC-1 cells treated with PA for 24 h were stained with Annexin V/propidium iodide, and the percentage of apoptotic cells was determined by flow cytometry. **B:** Panc-1 cells treated with PA for 24 h were stained with Annexin V/propidium iodide, and the percentage of apoptotic cells was determined by flow cytometry. **C:** PA blocks the G₁/S cell cycle phase transition after 24 h treatment in human AsPC-1 PC cells, determined by flow cytometry following propidium iodide staining. **D:** The percentage of proliferating cells in vehicle or PA-treated Panc-1 xenografts were determined by PCNA staining. Representative images

and the quantification of PCNA expression in tumor sections. All values are mean $\pm$ SEM;
* $p < 0.05$, vs. control.

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Fig. 3. Phospho-aspirin inhibits EGFR phosphorylation in PC cells and xenografts

A: Immunoblots for EGFR and phosphorylated EGFR (p-EGFR) in MIA PaCa-2 cells treated with PA for 2h. **B:** Immunofluorescence staining for p-EGFR in Panc-1 cells treated or untreated with PA ($1 \times IC_{50}$) for 4h. EGF (10 ng/ml) stimulation for 15 min was used as positive control. Representative images in each group from three independent experiments are shown (original magnification, $\times 20$). **C:** Immunofluorescence staining for p-EGFR in Panc-1 cells pre-treated with PA ($1 \times IC_{50}$) or Erlotinib (40 nM) for 1h, followed by EGF (10 ng/ml) stimulation for 30 min. Representative images in each group from three independent

experiments are shown (original magnification, $\times 20$). (*Right*) immunofluorescence intensity in each group were quantified. **D:** Immunoblots for EGF-stimulated EGFR and p-EGFR in Panc-1 cells treated with PA as indicated. **E:** Panc-1 xenografts tumor lysates were analyzed for EGFR and p-EGFR by immunoblotting. Loading control: β -actin. Each lane represents a different tumor sample. Bands were quantitated and results expressed as percent control for each protein; $*p < 0.05$ vs. control. **F:** Immunoblots for ERK and p-ERK after treatment of Panc-1 cells with PA for 6h. **G:** EGFR overexpression rescued PC cells from apoptosis induced by PA. Effect of PA on apoptosis from EGFR overexpressed MIA PaCa-2 cells or its mock control. Western blot confirms EGFR overexpression.

Fig. 4. Phospho-aspirin inhibits EGFR phosphorylation in *KrasG12D* mice

A: Cerulein treatment protocol. **B:** IHC for p-EGFR in vehicle or PA treated mice. Scale bar = 100μm for primary micrographs, 20μm for insets. **C:** The following stains were performed: H&E (×4); scale bars, 200μm; and PAS (×10); scale bars, 100μm. **Bottom:** IHC for Amylase (×10), PCNA and p-ERK, (20×); scale bars, 50μm. **Right panel:** The percent of ductal metaplasia per total pancreas area, PAS (+), amylase (+), PCNA and p-ERK (+) staining per field were quantified and expressed as the mean±SEM (**p* < 0.05, for all).

Fig. 5. Phospho-aspirin protects against acute pancreatitis-accelerated ductal metaplasia formation

A: Cerulein treatment protocol. **B:** The following stains were performed: H&E ($\times 4$); scale bars, 200 μ m. IHC for amylase ($\times 10$); scale bars, 100 μ m. IHC for Ki-67 and p-EGFR ($20\times$); scale bars, 50 μ m. **C:** The extent of ductal metaplasia lesions was quantified and expressed as percent of ductal metaplasia per total pancreas area. Furthermore, the percent of amylase positive area, p-EGFR, Ki-67 and p-ERK1/2 positive staining per field were quantified and expressed as the mean $\pm$ SEM (* $p < 0.05$, for all).

Fig. 6. Phospho-aspirin prevents metaplasia formation in primary acinar explants

A: Acinar cell explants isolated from pancreata of 6–8 week old *KrasG12D* mice were left untreated (control) or treated with PA 200 μ M for 5 days. (*Left*) Phase contrast images of explant cultures on day 5 ($\times 10$). (*Right*) The number of ductal structures/well; * $p < 0.01$, compared to vehicle control. **B:** Immunofluorescence staining for p-EGFR and cytokeratin-19 was performed in the resulting metaplasia on day 5. EGF (10 ng/ml) = positive control. Representative images from three independent experiments are shown (original magnification, $\times 20$).