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 Rigas1,4, and Gerardo G. Mackenzie1,2,5

Abstract

Pancreatic cancer is a deadly disease with a dismal 5-year survival rate of <6%. The currently limited treatment options for pancreatic cancer underscore the need for novel chemopreventive and therapeutic agents. Accumulating evidence indicates that aspirin use is associated with a decreased risk of pancreatic cancer. However, the anticancer properties of aspirin are restricted by its gastrointestinal toxicity and its limited efficacy. Therefore, we developed phospho-aspirin (MDC-22), a novel derivative of aspirin, and evaluated its chemopreventive efficacy in preclinical models of pancreatic cancer. Phospho-aspirin inhibited the growth of human pancreatic cancer cell lines 8- to 12-fold more potently than aspirin; based on the 24-hour IC50 values. In a Panc-1 xenograft model, phospho-aspirin, at a dose of 100 mg/kg/d 5 times per week for 30 days, reduced tumor growth by 78% (P < 0.01 vs. vehicle control). Furthermore, phospho-aspirin prevented pancreatitis-accelerated acinar-to-ductal metaplasia in mice with activated Kras. In p48-CreKrasG12D mice, cerulein treatment (6 hourly injections two times per week for 3 weeks) led to a significant increase in ductal metaplasia, replacing the majority of the exocrine compartment. Administration of phospho-aspirin 100 mg/kg/day five times per week 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). Phospho-aspirin appeared to be safe, with the animals showing no signs of toxicity during treatment. Mechanistically, phospho-aspirin inhibited EGFR activation in pancreatic cancer, an effect consistently observed in pancreatic cancer cells, primary acinar explants and in vivo. In conclusion, our findings indicate that phospho-aspirin has strong anticancer efficacy in preclinical models of pancreatic cancer, warranting its further evaluation.

Introduction

Pancreatic cancer is a complex and highly lethal disease with a 5-year survival of <6.7% (1). Despite improved treatment modalities during the last decade, current chemotherapy and radiotherapy regimens offer minimal or no help (2). Thus, there is an urgent need to develop novel prevention strategies against pancreatic cancer. An impressive body of evidence supports the notion that chemoprevention has the potential to be a major component in the control of cancer, including pancreatic cancer (2, 3). The development of successful chemoprevention strategies appears essential for individuals who are at high risk of developing pancreatic cancer, including people with premalignant lesions, such as certain pancreatic cysts, and those with inherited factors (4). Subjects at a higher risk of developing pancreatic cancer 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 pancreatic cancer include tobacco smoking, chronic pancreatitis, type II diabetes, and obesity. These individuals constitute a population in need for effective strategies for pancreatic cancer prevention, with the development of novel chemopreventive agents being a critical component.

EGFR is a key molecular determinant of pancreatic cancer. 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 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 the risk of developing pancreatic cancer (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 (MDC-22; Fig. 1A), consists of aspirin chemically modified at its carboxylic group, the moiety accounting for its gastrointestinal toxicity (16). Indeed, phospho-aspirin demonstrated a much improved gastrointestinal safety profile compared with aspirin and is more efficacious in the treatment of breast cancer than aspirin (17, 18). In addition, a key molecular target of phospho-aspirin 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 phospho-aspirin in the prevention of pancreatic cancer. Phospho-aspirin was much more potent than aspirin in inhibiting pancreatic cancer cell growth, reduced human pancreatic cancer growth in subcutaneous xenograft models, and prevented pancreatic carcinogenesis in a transgenic mouse model of pancreatic cancer. Mechanistically, the antineoplastic effect of phospho-aspirin was mediated by the inhibition of EGFR activation.

Materials and Methods

Reagents

Phospho-aspirin was provided by Medicon Pharmaceuticals, Inc. Aspirin was from Sigma-Aldrich. For cell culture studies, we prepared 400 mmol/L and 1 mol/L stock solutions of phospho-aspirin and aspirin in DMSO, respectively. All general solvents and reagents were of high-performance liquid chromatography grade or of the highest grade commercially available.

Cell culture

Human pancreatic cancer cell lines (Panc-1, MIA PaCa-2, AsPC-1, HPAF-II, CFPAC-1, and BxPC-3) were from the ATCC, 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 passed 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 (MTT), as described previously (19).

Clonogenic assay

Clonogenic assay was performed as described previously (20). Briefly, MIA PaCa-2 and Panc-1 cells, plated in 6-well plates
(1,000 cells/well), were treated with various concentrations of phospho-aspirin or aspirin for 24 hours. After treatment, media were replaced and cells grew 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 mol/L, pH 9.3) and 0.02% ethanol. After lysis, the absorbance was read at 570 nm.

**Cytokinetic analysis**

After treatment with phospho-aspirin, apoptosis was assayed by staining with Annexin V-FITC and propidium iodide (PI) and analyzing the fluorescence intensities by FACSCalibur (BD Biosciences); Cell proliferation was assayed by 5-bromo-2'-deoxyuridine (BrdUrd) incorporation, and cell cycle by flow cytometry; as described previously (21).

**Plasmid transfections**

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

**Immunoblotting**

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

**Immunofluorescence**

Panc-1 cells, seeded in chamber slide dishes, were preincubated for 60 minutes in the presence of phospho-aspirin 1xIC50 or erlotinib 40 nmol/L or equivalent volumes of DMSO. After stimulation with EGF 10 ng/mL, for 1 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 phospho-aspirin 1xIC50 for 4 hours. Immunostaining was performed for phospho-EGFR (catalog no. ab40815; Abcam). 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). Briefly, 1.5 x 10^6 Panc-1 cells were preincubated with various concentrations of phospho-aspirin or aspirin for 30 minutes followed by treatment with the calcium ionophore A23187 5 μmol/L for 3 hours.

**Pancreatic epithelial explants culture**

Pancreatic epithelial explants from 6- to 8-week-old KrasLSL-G12D/+;Ptf1aCre/+ mice (referred to hereinafter as KrasG12D) were established following previously published protocols (22). Briefly, after isolation, the cellular suspension was plated on plates precoated with 50 μg/mL of bovine collagen type I. The next day, cells were either left untreated or treated with phospho-aspirin 200 μmol/L 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) were inoculated subcutaneously into each of their flanks with 2.5 x 10^6 Panc-1 or MIA PaCa-2 cells in Matrigel (BD Biosciences). Once the tumor reached approximately 150–200 mm^3, animals were randomized into the control group, which received corn oil, and the treatment groups, which received phospho-aspirin 100 mg/kg orally in corn oil (n = 7/group, once daily 5 times per week), until the end of the study. The dose of phospho-aspirin represents <20% of its MTD, which provides an ample therapeutic window (17, 18). Tumor volume was calculated as described previously (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, Male and female 6-week-old KrasG12D mice (C57Bl/6 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 phospho-aspirin treatment groups (n = 5/group). Phospho-aspirin 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, Male and female 2-month-old KrasG12D mice were injected intraperitoneally with cerulein (50 μg/kg hourly, 6 times per day, 2 days per week, for 3 weeks). Cerulein-injected mice were divided in vehicle or phospho-aspirin treatment groups (n = 8/group). Phospho-aspirin 100 mg/kg was given 5 days per week by oral gavage for 3 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 morphologic studies.

**Histologic analysis**

Pancreatic tissues were fixed in 10% neutral-buffered formalin for 24 hours and then processed and embedded in paraffin according to standard protocols. Tissue sections (4 μm) 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 on the basis of 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 (catalog no. sc-7907; Santa Cruz Biotechnology), proliferating cell nuclear antigen (PCNA; sc-15402; Santa Cruz Biotechnology), α-amylase (catalog no. 3796; Cell Signaling Technology), and p-EGFR (catalog no. ab40815; Abcam) was performed on human pancreatic cancer xenograft tissue samples or mouse pancreatic samples, as described previously (27).

Statistical analysis

Results are expressed as mean ± SEM. Differences between groups were determined by one-factor ANOVA followed by Tukey test for multiple comparisons. P < 0.05 was statistically significant.

Results

Phospho-aspirin inhibits the growth of human pancreatic cancer cells in culture and in human pancreatic cancer xenografts

Our initial goal was to establish the potency of phospho-aspirin in inhibiting the growth of pancreatic cancer cells in culture. We treated a panel of six human pancreatic cancer cell lines with or without escalating concentrations of phospho-aspirin (25–800 μmol/L) for 24 hours, and compared the potency of phospho-aspirin to that of aspirin, its parent compound. As shown in Fig. 1B, phospho-aspirin reduced pancreatic cancer cell growth concentration-dependently in all six of the human pancreatic cancer cell lines tested. Furthermore, phospho-aspirin inhibited the growth of human pancreatic cancer cells more potently than aspirin (Fig. 1B and Supplementary Fig. S1). Indeed, phospho-aspirin inhibited cell growth more potently than aspirin in a panel of six human pancreatic cancer cell lines. The potency enhancement ranged between 5- and 8-fold in these pancreatic cancer cell lines. Phospho-aspirin for 24 hours led to a concentration-dependent decrease in colony formation using two pancreatic cancer cell lines. Phospho-aspirin induced a minimal amount of necrosis (<2.5%; Fig. 2A). This was also observed in Panc-1, MIA PaCa-2, and HPAF-II cells, where phospho-aspirin 1.5xIC50 induced apoptosis by 5.0-, 3.6-, and 2.7-fold, respectively (Fig. 2B and Supplementary Fig. S2A). Furthermore, in Panc-1 cells, treatment with phospho-aspirin reduced time-dependently the levels of full-length caspase-3 (Supplementary Fig. S2B). We then evaluated whether phospho-aspirin affected the levels of survivin, an antiapoptotic protein highly expressed in pancreatic cancer (28). Treatment of Panc-1 cells with phospho-aspirin reduced survivin levels in a time-dependent manner (Supplementary Fig. S2B).

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

Signaling effects of phospho-aspirin

The tumor suppressor p53, frequently mutated in human pancreatic cancer, has a central function in cell-cycle regulation, DNA repair, cellular senescence, and apoptosis. Thus, we evaluated the effect of phospho-aspirin on p53 acetylation, which is critical for its stability and transcriptional activity (29). In MIA PaCa-2 cells, phospho-aspirin induced p53 acetylation concentration-dependently at two distinct lysines (K379 and K382) residues (Supplementary Fig. S3A). In vivo, phospho-aspirin induced p53 acetylation at the K382 residue in Panc-1 xenografts (Supplementary Fig. S3B), suggesting that phospho-aspirin 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 1.5xIC50 phospho-aspirin, which may contribute to the cell-cycle arrest (Supplementary Fig. S3C).

To rule out a cell line–specific effect, we evaluated the chemotherapeutic effect of phospho-aspirin in subcutaneous MIA PaCa-2 pancreatic cancer xenografts. Phospho-aspirin (100 mg/kg, orally, 5 days per week) also potently inhibited MIA PaCa-2 xenograft growth, resulting in a 53.1% tumor growth inhibition compared with control (P < 0.01; Fig. 1E).

In these animal studies, phospho-aspirin 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 phospho-aspirin compared with aspirin.

Phospho-aspirin inhibits the growth of human pancreatic cancer through a strong cytokinetic effect

Phospho-aspirin inhibited tumor growth through a potent cytokinetic effect. In vitro treatment of AsPC-1 cells with phospho-aspirin for 24 hours led to a concentration-dependent induction of apoptosis (Fig. 2A). Of note, phospho-aspirin induced a minimal amount of necrosis (<2.5%; Fig. 2A). This was also observed in Panc-1, MIA PaCa-2, and HPAF-II cells, where phospho-aspirin 1.5xIC50 induced apoptosis by 5.0-, 3.6-, and 2.7-fold, respectively (Fig. 2B and Supplementary Fig. S2A). Furthermore, in Panc-1 cells, treatment with phospho-aspirin reduced time-dependently the levels of full-length caspase-3 (Supplementary Fig. S2B). We then evaluated whether phospho-aspirin affected the levels of survivin, an antiapoptotic protein highly expressed in pancreatic cancer (28). Treatment of Panc-1 cells with phospho-aspirin reduced survivin levels in a time-dependent manner (Supplementary Fig. S2B).

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

Signal transduction

The tumor suppressor p53, frequently mutated in human pancreatic cancer, has a central function in cell-cycle regulation, DNA repair, cellular senescence, and apoptosis. Thus, we evaluated the effect of phospho-aspirin on p53 acetylation, which is critical for its stability and transcriptional activity (29). In MIA PaCa-2 cells, phospho-aspirin induced p53 acetylation concentration-dependently at two distinct lysines (K379 and K382) residues (Supplementary Fig. S3A). In vivo, phospho-aspirin induced p53 acetylation at the K382 residue in Panc-1 xenografts (Supplementary Fig. S3B), suggesting that phospho-aspirin 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 1.5xIC50 phospho-aspirin, which may contribute to the cell-cycle arrest (Supplementary Fig. S3C).

To rule out a cell line–specific effect, we evaluated the chemotherapeutic effect of phospho-aspirin in subcutaneous MIA PaCa-2 pancreatic cancer xenografts. Phospho-aspirin (100 mg/kg, orally, 5 days per week) also potently inhibited MIA PaCa-2 xenograft growth, resulting in a 53.1% tumor growth inhibition compared with control (P < 0.01; Fig. 1E).

In these animal studies, phospho-aspirin 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 phospho-aspirin compared with aspirin.

Phospho-aspirin inhibits the growth of human pancreatic cancer through a strong cytokinetic effect

Phospho-aspirin inhibited tumor growth through a potent cytokinetic effect. In vitro treatment of AsPC-1 cells with phospho-aspirin for 24 hours led to a concentration-dependent induction of apoptosis (Fig. 2A). Of note, phospho-aspirin induced a minimal amount of necrosis (<2.5%; Fig. 2A). This was also observed in Panc-1, MIA PaCa-2, and HPAF-II cells, where phospho-aspirin 1.5xIC50 induced apoptosis by 5.0-, 3.6-, and 2.7-fold, respectively (Fig. 2B and Supplementary Fig. S2A). Furthermore, in Panc-1 cells, treatment with phospho-aspirin reduced time-dependently the levels of full-length caspase-3 (Supplementary Fig. S2B). We then evaluated whether phospho-aspirin affected the levels of survivin, an antiapoptotic protein highly expressed in pancreatic cancer (28). Treatment of Panc-1 cells with phospho-aspirin reduced survivin levels in a time-dependent manner (Supplementary Fig. S2B).

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

Signal transduction

The tumor suppressor p53, frequently mutated in human pancreatic cancer, has a central function in cell-cycle regulation, DNA repair, cellular senescence, and apoptosis. Thus, we evaluated the effect of phospho-aspirin on p53 acetylation, which is critical for its stability and transcriptional activity (29). In MIA PaCa-2 cells, phospho-aspirin induced p53 acetylation concentration-dependently at two distinct lysines (K379 and K382) residues (Supplementary Fig. S3A). In vivo, phospho-aspirin induced p53 acetylation at the K382 residue in Panc-1 xenografts (Supplementary Fig. S3B), suggesting that phospho-aspirin 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 1.5xIC50 phospho-aspirin, which may contribute to the cell-cycle arrest (Supplementary Fig. S3C).

By analogy to aspirin, a COX inhibitor, we investigated the effects of phospho-aspirin on the COX pathway, examining the effect of phospho-aspirin and aspirin on the production of PGE2 by Panc-1 cells following stimulation with the calcium ionophore A23187 (30). While phospho-aspirin failed to alter significantly the production of PGE2 by these cells, aspirin, as expected, completely inhibited it at both concentrations tested.
Phospho-aspirin inhibits EGFR activation in human pancreatic cancer cells and xenografts

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

We initially evaluated the expression of EGFR in a panel of four pancreatic cancer 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 pancreatic cancer cell lines, phospho-aspirin inhibited both constitutive and EGF-stimulated EGFR activation, decreasing EGFR phosphorylation. For example, in MIA PaCa-2 cells, phospho-aspirin inhibited EGFR phosphorylation in a concentration-dependent manner, becoming evident at 1xIC50 (Fig. 3A). In addition, phospho-aspirin 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 Supplementary Fig. S6). Moreover, in Panc-1 cells, phospho-aspirin prevented the EGF-stimulated increase in EGFR phosphorylation, as evidenced by Western blot analysis and immunofluorescence (Fig. 3C and D). The effect was comparable with that of erlotinib, an EGFR inhibitor used clinically (Fig. 3C). This observation was confirmed in vivo, where phospho-aspirin reduced EGFR phosphorylation by 65% in the Panc-1 xenografts compared with controls (P < 0.05; Fig. 3E).

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

To determine whether EGFR is a key molecular target for phospho-aspirin in pancreatic cancer, we evaluated the effect of phospho-aspirin 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 phospho-aspirin. After 24 hours of incubation with phospho-aspirin 1.5xIC50, the annexin V(+) fraction in mock-transfected MIA PaCa-2 cells was increased by 6.8-fold. In contrast, phospho-aspirin induced apoptosis by 3-fold in cells that overexpressed EGFR (Fig. 3G).

Phospho-aspirin reduces pancreatitis-associated tumorigenesis by inhibiting EGFR activation

To better dissect the effect of phospho-aspirin in pancreatic cancer progression, we next used the KrasG12D mouse model,
which reproducibly shows metaplasia beginning at approximately 8 weeks of age, with progression to pancreatic ductal adenocarcinoma at approximately 1 year (31).

Earlier studies have shown that cerulein treatment affects EGFR expression in KrasG12D mice (5). For this purpose, we treated 6-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 1 hour of cerulein injections compared with KRasG12D mice. Phospho-aspirin failed to prevent acute pancreatic injury (Supplementary Fig. S7). As expected, enzyme levels returned to basal levels in both control and phospho-aspirin–treated groups at the end of the treatment.

Using this model, we examined whether phospho-aspirin could inhibit EGFR expression prior to rampant epithelial morphogenesis (Fig. 4B). Cerulein treatment of KrasG12D mice induced higher active EGFR [EGFR (pY1068)] expression. Phospho-aspirin 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 \textit{in vitro} (Fig. 4C).

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

\textbf{Phospho-aspirin prevents pancreatitis-accelerated ductal metaplasia in mice with activated \textit{Kras}}

As chronic pancreatitis is a significant risk factor for pancreatic cancer (33, 34), we investigated whether phospho-aspirin inhibits \textit{Kras}-driven pancreatic carcinogenesis in the setting of pancreatitis induced by cerulein. For this purpose, we treated 2-month-old \textit{KrasG12D} mice with cerulein (50 \textit{mg/kg}; 6 hourly injections for 2 times per week for 3 weeks) to induce pancreatitis. Concomitant with pancreatitis induction and continuing for the following 3
weeks, mice were treated with phospho-aspirin 100 mg/kg/d for 5 times per week 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 and C). In control mice, this effect was accompanied by lower expression of amylase, and an increase in cell proliferation. Phospho-aspirin 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 and C).

In this setting, p-EGFR levels in phospho-aspirin–treated pancreata were reduced by 69% ($P < 0.05$), compared with controls (Fig. 5B and C). Phospho-aspirin treatment also reduced p-ERK levels by 79% ($P < 0.05$) and decreased cell proliferation (Ki-67
inhibition of cell-cycle transition. A significant inhibition of cell proliferation, induction of apoptosis, and the loss of EGFR have been shown (original magnification, ×20).

**Figure 6.** Phospho-aspirin (PA) prevents ductal metaplasia formation in primary acinar explants. A, acinar cell explants isolated from pancreata of 6- to 8-week-old KrasG12D mice were left untreated (control) or treated with phospho-aspirin 200 μmol/L for 5 days. Phase contrast images of explant cultures on day 5 (×10; left). The number of ductal structures/well: *P < 0.01, compared with vehicle control (right). 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, ×20).

We have previously documented the superior safety of phospho-aspirin, clearly demonstrating that it is far safer than aspirin in mice and rats (17, 18). Regarding its efficacy, we showed that phospho-aspirin is a strong chemopreventive agent. The chemopreventive effect of phospho-aspirin in pancreatic cancer is: (i) broad, encompassing both pancreatic cancer xenografts and genetic engineered mouse models; and (ii) 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 phospho-aspirin are lower than 10% of its MTD.

It is becoming increasingly clear that sustained metaplasia is 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). Phospho-aspirin 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 phospho-aspirin might be an effective chemopreventive agent for pancreatic cancer.

The role of EGFR as a therapeutic target in pancreatic cancer is complex. In metastatic pancreatic cancer 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 posttransformation 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 phospho-aspirin. Phospho-aspirin 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 pancreatic cancer (42). Furthermore, overexpression of EGFR in pancreatic cancer cells conferred substantial drug resistance to phospho-aspirin, suggesting that EGFR-dependent signaling is a key molecular target of phospho-aspirin. At this time, however, we cannot rule out the possibility that phospho-aspirin 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 phospho-aspirin has a strong chemopreventive effect against pancreatic cancer in preclinical models, and indicates EGFR as a critical molecular target. Although more work is needed, at a clinical level, the use of a phospho-aspirin may offer a safer preventive option for those patients at higher risk of developing pancreatic cancer.

References


Grant Support

This work was supported by the Stony Brook Cancer Center and NIH grants R21CA185209 (to G.G. Mackenzie) and R01CA154172 (to B. Rigas). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 17, 2015; revised April 21, 2016; accepted April 22, 2016; published OnlineFirst May 2, 2016.


Phospho-Aspirin (MDC-22) Prevents Pancreatic Carcinogenesis in Mice

George Mattheolabakis, Ioannis Papayannis, Jennifer Yang, et al.