Effect of Cromolyn on S100P Interactions With RAGE and Pancreatic Cancer Growth and Invasion in Mouse Models

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Background: We previously found that S100P, a member of the S100 protein family, is expressed in more than 90% of pancreatic tumors and is associated with tumor growth and invasion. In the current study, we investigated the ability of the antiallergy drug, cromolyn, to block S100P function.

Methods: Interactions between cromolyn and S100P were investigated using a drug affinity column and by examining cromolyn’s effects on coimmunoprecipitation of S100P and receptor for advanced glycation end-products (RAGE). The effects of cromolyn on cell growth, invasion, and nuclear factor-κB (NFκB) activity of pancreatic cancer cells with (BxPC-3 and MPanc-96) and without (Panc-1) endogenous S100P were investigated by cell proliferation assay, by cell invasion assay, and by luciferase reporter gene assay, respectively. The effects of cromolyn on tumor growth in vivo were investigated in three orthotopic models (n = 20 mice per model) by administration of cromolyn (5 mg/kg body weight, daily) with and without gemcitabine (125 mg/kg body weight, biweekly), the drug currently used to treat pancreatic cancer. Tumor growth was assayed by reporter gene expression. All statistical tests were two-sided.

Results: S100P was retained on a cromolyn affinity column. Cromolyn blocked the coimmunoprecipitation of S100P and RAGE. In vitro, cromolyn (100 μM) inhibited S100P-stimulated Panc-1 cell proliferation (S100P, mean = 0.93 U, versus S100P + cromolyn, mean = 0.56 U, difference = 0.37 U; 95% confidence interval [CI] = 0.24 to 0.49 U; P = .001, n = 3), invasion (S100P, mean = 58.0%, versus S100P + cromolyn, mean = 9.4%, difference = 48.6%; 95% CI = 38.8% to 58.8%; P<.001, n = 3), and NFκB activity (S100P, mean = 14 460, versus S100P + cromolyn, mean = 7360 photons/s, difference = 7100 photons/s; 95% CI = 3689 to 10 510 photons/s; P = .005, n = 3). In vivo, cromolyn inhibited tumor growth in mice bearing tumor with endogenous S100P (BxPC-3: control, mean = 1.6 × 10⁹ photons/s, versus cromolyn, mean = 4.4 × 10⁸ photons/s, difference = 1.2 × 10⁹ photons/s; 95% CI = 6.2 × 10⁸ to 1.6 × 10⁹ photons/s; P<.001, n = 5; MPanc-96: control, mean = 1.1 × 10¹⁰ photons/s, versus cromolyn, mean = 4.8 × 10⁸ photons/s, difference = 6.2 × 10⁹ photons/s; 95% CI = 1.9 × 10⁸ to 1.0 × 10¹⁰ photons/s; P = .009, n = 5) and increased the effectiveness of gemcitabine (BxPC-3: gemcitabine, mean = 9.2 × 10⁸ photons/s, versus combination, mean = 1.8 × 10⁸ photons/s, difference = 7.4 × 10⁸ photons/s; 95% CI = 4.5 × 10⁸ to 1.0 × 10⁹ photons/s; P<.001; MPanc-96: gemcitabine, mean = 4.1 × 10⁸ photons/s, versus combination, mean = 2.0 × 10⁹ photons/s, difference = 2.1 × 10⁹ photons/s; 95% CI = 4.4 × 10⁸ to 3.8 × 10⁹ photons/s; P<.001). However, cromolyn had no effect on growth of tumors lacking S100P (Panc-1).

Conclusion: Cromolyn blocks S100P, prevents activation of RAGE, inhibits tumor growth, and increases the effectiveness of gemcitabine in experimental models. [J Natl Cancer Inst 2006;98:1806–18]

Despite recent advances in understanding the biology of pancreatic cancer and molecular alterations in tumor pathogenesis, pancreatic cancer remains an oncologic challenge, with a 5-year
Pancreatic adenocarcinoma is arguably the most lethal of all cancers, with more than 95% of patients diagnosed with the disease dying from it, more than half within 6 months. In the United States, it ranks fourth among the leading causes of cancer death, accounting for more than 30,000 deaths annually (1). There is no effective therapy for pancreatic cancer other than early resection, but only a small percentage of patients are good candidates for surgery. Gemcitabine is the current conventional chemotherapy for pancreatic cancer, and it provides meager benefits (2). Combinations of gemcitabine with radiation or with other cytotoxic agents have also proven disappointing.

Because of the poor response to these standard forms of therapy, recent efforts have focused on the application of novel, biologically targeted agents aimed at well-known cancer mechanisms. Examples of these approaches include compounds that target vascular endothelial growth factor receptors, e.g., bevacizumab; the epidermal growth factor receptor (EGFR), e.g., cetuximab; the EGFR-activating tyrosine kinase, e.g., erlotinib and gefitinib; and K-ras, e.g., farnesyl transferase inhibitor tipifarnib. However, most of the early clinical trials with the newer agents have shown no (2,3) or only very modest (4) survival advantage compared with standard gemcitabine treatment. Clearly, new targets and therapeutic approaches are needed for this disease. Therapeutic target development requires identification of novel molecules, validation of their functional importance, understanding their mechanisms of action, and strategies for intervention.

S100P has recently been found to be overexpressed in pancreatic (5–7), breast (8), and lung (9) cancers. S100P is a 95-amino acid member of the S100 family of proteins (10). S100P is functionally important for pancreatic cancer cell growth and survival, in that we previously observed that levels of cellular S100P affect the rate of tumor growth in vivo and resistance of pancreatic cancer cells against 5-fluorouracil treatment in vitro (11). In colon cancer cell lines, S100P levels are associated with resistance to chemotherapy (12). In lung cancer, S100P levels are associated with decreased patient survival (9). S100P is also associated with increased metastasis and decreased patient survival in breast cancer (8). Taken together, the evidence suggests that overexpressed S100P may increase tumor growth and metastasis and decrease patient survival. If so, blocking S100P function might improve responses to therapeutic treatments.

The mechanisms of action of S100P have recently been elucidated in studies with pancreatic cancer cells. We found that S100P is secreted by pancreatic cancer cells and acts extracellularly through interactions with a cell surface protein receptor for advanced glycation end-products (RAGE) (13). RAGE is a multiligand receptor that interacts with a variety of molecules, including advanced glycation end-products, S100 molecules (S100B, S100A12, S100P), amyloid, and amphoterin (14). RAGE participates in a number of important pathologic processes, including Alzheimer’s disease, diabetes, inflammation, and cancer (14). Activation of RAGE by S100P stimulates several cellular signaling pathways, including the Mitogen-activated protein (MAP) kinase pathway νκB (NFκB) pathways (13). NFκB signaling may be of particular importance because basal NFκB activity is elevated in the majority of pancreatic cancers (15) and elevated NFκB activity is associated with increased resistance to therapies (16,17). Therefore, interventions that block the ability of S100P to activate RAGE may provide therapeutic benefit.

In the current study, we focused on the effects of cromolyn (disodium 1,3-bis-[2-carboxylatochromon-5-yl]oxy]-2-hydroxypropane), an anti-allergie compound (18) that has previously been shown to bind specifically to other members of the S100 protein family (S100A1, S100A12, S100A13) (19–21). We examined the interactions of cromolyn with S100P and its effects on S100P activation of RAGE. We then measured the consequences of these effects on cell proliferation, invasion, NFκB activity, and responses to gemcitabine using three pancreatic cancer cell lines BxPC-3 and MPanc-96 (which express endogenous S100P) and Panc-1 (which do not express S100P) and mouse models of pancreatic adenocarcinoma using these cell lines.

Materials and Methods

Cell Culture and Treatment

Panc-1 and BxPC-3 pancreatic adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). MPanc-96 pancreatic adenocarcinoma cell lines were originally established by Dr Timothy J. Eberlein (St Louis, MO) (22). BxPC-3 cells were cultured in RPMI-1640 with 10% fetal bovine serum (FBS). Panc-1 and MPanc-96 cells were routinely cultured in Dulbecco’s modified Eagle medium with 10% FBS. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

Cromolyn (sodium salt) was purchased from Sigma (St Louis, MO) as a sterile white powder in glass vials and stored at room temperature. For in vitro experiments, a stock solution of cromolyn (1 mM) was prepared in culture media. For in vivo experiments, cromolyn was dissolved at 50 mg/mL of phosphate-buffered saline (PBS; 0.025 M Na₂HPO₄, 0.025 M NaH₂PO₄ in 0.87% of NaCl). The reconstituted solution was clear and colorless. Platelet-derived growth factor (PDGF) was purchased from Sigma and reconstituted in sterile-filtered 4 mM HCl containing 0.1% bovine serum albumin to prepare a stock solution of 10 μg/mL, aliquotted, and stored in –20 °C.

S100P Expression and Purification

S100P protein was expressed and purified, as described previously (13). Briefly, full-length human S100P cDNA (NM_005980) was cloned into the pTrcHis2 vector (Invitrogen, Carlsbad, CA), and S100P expression was induced in vector-transformed bacteria by adding 1 mM of isoprpyl-1-thio-β-d-galactopyranoside. Histagged S100P was purified using a probond resin column, according to the manufacturer’s instructions (Invitrogen). Briefly, the bacterial lysate containing S100P was loaded into the column and incubated for 60 minutes using gentle agitation to allow S100P to bind with the resin. Nonspecific proteins were removed with wash buffer (50 mM NaH₂PO₄, 0.5 M NaCl, 20 mM imidazole, pH 8.0), and S100P was eluted using elution buffer (50 mM NaH₂PO₄, 0.5 M NaCl, 250 mM imidazole, pH 8.0) and stored at –80 °C with 5% sterile glycerol. The purity of the S100P protein was approximately 95%, as indicated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting. The purified S100P was found to be free from endotoxin (lipopolysaccharide [LPS]) contamination, as indicated by a Limulus amebocyte gel formation assay using Gram-negative bacteria LPS as a standard (Cambrex, Walkersville, MD). Proteins isolated from noninduced bacteria were used as an additional

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negative control. Before use in cell culture, S100P was diluted in culture medium for use at the indicated concentrations.

**S100P Binding to Cromolyn Using Affinity Chromatography**

Cromolyn was coupled to TOYOPEARL resin, as described previously (21). Briefly, 0.1 g of cromolyn was dissolved in 1 mL of N,N-dimethylformamide and added to 7 mL (5 g wet mass) AF-TOYOPEARL. Next, 0.5 g of N-ethyl-N-(3-dimethylamino-propyl) carbodiimide hydrochloride, suspended in 10 mL of N,N-dimethylformamide, was added to the cromolyn + AF-TOYOPEARL slurry. The pH was adjusted to 5.0, and the slurry was incubated with gentle shaking for 48 hours at 25 °C. As a negative control, a column was prepared by blocking the amino group of AF-TOYOPEARL with sodium acetate and acetic anhydride. Purified His-tagged S100P was applied to the cromolyn-coupled and control columns, which had been equilibrated previously with equilibration buffer (20 mM Tris–HCl, 0.5 mM CaCl₂, pH 7.5). The columns were then washed once with 15 mL of wash buffer (20 mM Tris–HCl, 0.2 mM CaCl₂, pH 7.5) to remove unbound proteins, and bound proteins were eluted with 15 mL of elution buffer (20 mM Tris–HCl, 0.5 mM CaCl₂, pH 7.5). The eluted protein fraction was concentrated using a protein concentration column YM-3 (Millipore, Bedford, MA), separated by 15% SDS–PAGE analysis, and stained with 0.1% Coomassie blue.

**Coimmunoprecipitation of S100P and Receptor for Advanced Glycation End-products**

For coimmunoprecipitation experiments, BxPC-3 cell lysates were incubated in the absence or presence of cromolyn (100 μM) at 4 °C overnight. S100P was immunoprecipitated using a mouse monoclonal anti-S100P antibody (Transduction Laboratories, San Diego, CA) for 6 hours at 4 °C and IgG-immobilized beads (Pierce Biotechnology, Inc, Rockford, IL). Antibody-associated proteins were electrophoresed on 10% polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in PBS/5% milk overnight at 4 °C. RAGE was detected using a goat polyclonal anti-RAGE antibody (1:200, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and S100P was detected using a polyclonal anti-S100P antibody (1:50, R&D Systems, Minnepolis, MN) by immunoblotting, as described (13). Briefly, membranes were incubated in primary antibody for 1 hour at room temperature followed by incubation with horseradish peroxidase–labeled secondary antibody for 30 minutes at room temperature. After a thorough wash in Tris-buffered saline-Tween buffer (TBS-1 M Tris–HCl, pH 8.3, 3 M NaCl, 0.1% Tween-20), antibody–protein complexes were detected by using a chemiluminescent substrate (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). The intensity of the band was estimated (as density units) using densitometry (GS-250 Molecular Imaging System, Bio-Rad Laboratories, Richmond, CA). The experiment was repeated three times.

**Cell Growth Studies**

Growth of Panc-1 and BxPC-3 pancreatic adenocarcinoma cells was analyzed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, WI), according to the manufacturer’s directions. Purified S100P (100 nM final concentration) with or without cromolyn (100 μM final concentration) was added to Panc-1 cells. Only cromolyn (0–100 μM) was added to BxPC-3 cells, which express endogenous S100P. For both cell models, 1.0 × 10⁵ cells per well were plated in 96-well culture dishes. Cells were treated with S100P, PDGF, or cromolyn, or a combination, followed immediately by treatment with either S100P or PDGF. MTS (20 μL per well) was added to cells at various times, and the mixture was incubated for 1 hour at 37 °C. Samples were then read at 490 nm (as OD units, U) on a uQuant-Microplate Spectrophotometer (Bio-Tek Services Inc, Richmond, VA). The assay was performed three times in triplicate.

**Development of Stable Cell Lines**

To study pancreatic cancer cell NFκB activity, we developed a lentivirus NFκB luciferase reporter gene construct. The NFκB luciferase reporter gene was excised and isolated from the pNFκB-xB vector (Clonetec, Mountain View, CA) and cloned into the lentiviral vector FG9 (gift from Dr Xiao-Feng Qin, Department of Immunology, M. D. Anderson Cancer Center), replacing the CMV–LTR and UbIC promoters (23), to form Lenti-NFκB-luc. Lentiviral NFκB vector was cotransfected with packaging constructs pRSVREV, pMDLg/pRRE, and the VSV-G expression plasmid pCMVGV, and lentivirus was produced in 293T cells by the calcium transfection method, as previously described (23). Lenti-NFκB-luc was titrated, and Panc-1 cells, which lack endogenous S100P, and BxPC-3 cells, which possess high levels of endogenous S100P, were each infected with Lenti-NFκB-luc virus (25 μL viral supernatant/mL of medium) mixed with polybrene (4 μg/mL medium). Functional validation of NFκB reporter activity was conducted in vitro using tumor necrosis factor-α (20 ng/mL, Sigma) as a positive control.

To study pancreatic cancer growth in vivo, we developed a lentiviral luciferase construct (without NFκB). The luciferase coding sequence was isolated from the pGL-3 vector (Promega) and cloned into the lentiviral vector FG9 behind the CMV–LTR and UbIC promoters to form the luciferase-expressing lentivirus (Lenti-luc). Viral particles were produced as described above using packaging vectors. Cells were infected with Lenti-luc virus (25 μL viral supernatant/mL of medium) mixed with polybrene (4 μg/mL medium). Luciferase expression was confirmed in 0 × 10⁵–10 × 10⁵ cells per well in a 24-well plate by measuring the light emission after adding luciferin (150 μg/mL) using the IVIS system (Xenogen Corp, Alameda, CA), and emitted light was directly proportional to cell number.

**Flow Cytometry to Measure Apoptosis**

Standard propidium iodide (PI) staining by the hypotonic lysis method was used for apoptosis studies. Apoptosis was induced in 1.0 × 10⁵ BxPC-3 cells by treatment with gemcitabine (10 μM), with or without cromolyn. After 48 hours, the cells were detached from culture dishes by incubation in 0.05% trypsin–EDTA, washed once with cold PBS, then incubated for 30 minutes in 500 μL of hypotonic solution (0.1% sodium citrate, 0.1% Triton X-100, 100 μg/mL RNase, and 50 μg/mL PI), and analyzed by flow cytometry (EPICS XL, Beckman...
Cromolyn (Fig. 1, A) has been shown to interact with several S100 proteins, as indicated by the binding of S100 molecules to a cromolyn affinity column (19–21). To determine whether

The antitumorigenic capability of the drug cromolyn was assessed in 4-week-old male CB17 scid mice (n = 20) carrying orthotopic tumors of BxPC-3, MPanc-96, and Panc-1 cells stably expressing a Lenti-luc reporter. All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center. All mice were maintained in a sterile environment. Cages, bedding, food, and water were all autoclaved. All mice were maintained on a daily 12-hour light/12-hour dark cycle, according to the institutional animal welfare guidelines.

Bioluminescence imaging was conducted using a cryogenically cooled IVIS 100 imaging system coupled to a data acquisition computer running Living Image Software (Xenogen Corp). Before imaging, mice were placed in an acrylic chamber, anesthetized with 1.5% isoflurane–air mixture, and injected intraperitoneally with 15 mg/mL of luciferin potassium salt in PBS at a dose of 150 mg/kg body weight. A digital gray scale image of each mouse was acquired, followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the mouse. Tumor volume was quantified as the sum of all detected photons within the region of the tumor per second. At the end of experiment, the mice were killed, and tumors were surgically removed and weighed. After the primary tumors were removed, cancer cell dissemination and metastasis were visualized using IVIS imaging, and metastatic colonies were counted. Subsequently, tissues were fixed with formaldehyde, and histology was used to verify the accuracy of the bioluminescence data.

**Statistical Analysis**

Data presented are the means and 95% confidence intervals (CIs) of the three or more independent experiments. For in vitro experiments and in vivo studies on tumor growth, comparisons between groups were made using a two-tailed two-sample (unpaired) Student’s t test. For the in vivo experiments of NFκB activity, a two-tailed (paired) Student’s t test was used. Differences for which P value was less than .05 were considered statistically significant.

**RESULTS**

**Drug Affinity Chromatography**

Cromolyn (Fig. 1, A) has been shown to interact with several S100 proteins, as indicated by the binding of S100 molecules to a cromolyn affinity column (19–21). To determine whether...
S100P also interacted with cromolyn, a drug-coupled carrier was prepared by dehydrative attachment of cromolyn to a hydrophilic polymer with amino groups, AF-TOYOPEARL resin. A negative control column of AF-TOYOPEARL resin lacking cromolyn was also prepared. Purified recombinant S100P was added to the columns, and the columns were washed extensively. Bound protein was then eluted with an EGTA-containing buffer. The cromolyn affinity column (Fig. 1, B, lane 3), but not the control column (Fig. 1, B, lane 2), retained S100P, indicating a specific interaction.

Fig. 1. Effect of cromolyn on the interaction of S100P with receptor for advanced glycation end-products (RAGE) and cancer cell growth, survival, and invasiveness in vitro. A) Chemical structure of cromolyn. B) Cromolyn was coupled with the amino group of AF-amino TOYOPEARL to generate a cromolyn affinity column. A negative control column was prepared by blocking the amino group. Purified S100P was added to both columns, which were then washed extensively. Protein was eluted from the columns using EGTA and subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis and staining with Coomassie blue. Lane 1, molecular weight markers; lane 2, eluate from the control column; lane 3, eluate from the cromolyn column (arrow, S100P). The gel shown is one of three independent experiments.

D) Panc-1 pancreatic adenocarcinoma cells (1.0 × 10^3 cells per well) were cultured in the presence (circles) or absence (squares) of S100P (100 nM) and with (solid) or without (open) cromolyn (100 μM), and cell proliferation was analyzed at 24, 48, and 72 hours. Means and 95% confidence intervals of three independent experiments performed in triplicate are shown. *P = .001, S100P alone versus S100P + cromolyn.

E) Panc-1 cells were plated at 1.0 × 10^3 cells per well and treated with platelet-derived growth factor (PDGF) (10 ng/mL) with or without cromolyn (100 μM), and cell proliferation was analyzed after 48 hours. Means and 95% confidence intervals of three independent experiments performed in triplicate are shown. *P = .004, PDGF, and **P = .022, cromolyn + PDGF, versus control.

F) BxPC-3 cells were plated at 1.0 × 10^3 cells per well and treated with 0, 1, 10, and 100 μM of cromolyn, and cell proliferation was analyzed after 48 hours. Means and 95% confidence intervals of three independent experiments performed in triplicate are shown. *P = .002, †P < .001 versus control.

G) BxPC-3 cells were treated with cromolyn (10 μM) with (+) or without (−) gemcitabine (10μM), and apoptosis was analyzed after 48 hours by flow cytometry. *P < .001 versus control; †P < .001 versus gemcitabine. Means and 95% confidence intervals of three independent experiments performed in triplicate are shown. Two-tailed two-sample (unpaired) Student’s t tests were used to determine P values.
Effect of Cromolyn on Interaction of S100P With Receptor for Advanced Glycation End-products and Cancer Cell Growth, Survival, and Invasiveness In Vitro

Our previous studies indicated that S100P coimmunoprecipitates with RAGE (11,13). To determine the influence of cromolyn on this interaction, lysates from BxPC-3 cells were immunoprecipitated with a mouse monoclonal anti-S100P antibody in the presence or absence of cromolyn. The immunoprecipitated proteins were subjected to immunoblotting with an anti-RAGE antibody (Fig. 1, C). RAGE was identified in the precipitate, confirming the interaction between S100P and RAGE. Inclusion of cromolyn (100 μM) resulted in statistically significant reduction in the coimmunoprecipitation of S100P and RAGE (control, mean = 34,040 density units, versus cromolyn, mean = 8410 density units, difference = 25,640 density units; 95% CI = 18,641 to 32,638 density units; P < .001; Fig. 1, C), suggesting that cromolyn interfered with the interaction. In contrast, cromolyn had no effect on the total amount of S100P immunoprecipitated (Fig. 1, C), indicating that cromolyn did not interfere with the interaction between S100P and the monoclonal antibody and that equal amounts of protein were loaded on the gel.

To determine whether inhibiting the interaction between S100P and RAGE would influence cancer cell growth, we tested the effects of cromolyn on pancreatic cancer cells in vitro. We examined the effects of cromolyn both on cells that lack endogenous S100P (Panc-1) and those that express high levels of endogenous S100P (BxPC-3). Cromolyn treatment alone had no effect on cell proliferation of Panc-1 cells, indicating a lack of nonspecific toxic effects (Fig. 1, D). However, cromolyn completely blocked the effects of exogenous S100P treatment (S100P, mean = 0.93 MTS units, versus S100P + cromolyn [100 μM], mean = 0.56 MTS units, difference = 0.37 MTS units; 95% CI = 0.25 to 0.49 MTS units; P = .001; Fig. 1, D). To further investigate the specificity of cromolyn’s effects, we examined PDGF stimulation of Panc-1 cell proliferation in the presence of cromolyn. As expected, PDGF statistically significantly increased Panc-1 cell growth (control, mean = 0.5 MTS units, versus PDGF, mean = 0.7 MTS units, difference = 0.2 MTS units; 95% CI = 0.1 to 0.3 MTS units; P = .004; Fig. 1, E). Cromolyn neither had inhibitory effect on basal growth of Panc-1 cells nor did it inhibit growth stimulation caused by PDGF (Fig. 1, E). Likewise, cromolyn did not influence serum-stimulated Panc-1 cell proliferation (data not shown). These data suggest that cromolyn does not have nonspecific effects on Panc-1 cell growth. In contrast, in BxPC-3 cells, cromolyn inhibited basal rates of cell proliferation in a concentration-dependent manner (0 μM, mean = 1.2 MTS units, versus 10 μM, mean = 0.7 MTS units, difference = 0.5 MTS units; 95% CI = 0.3 to 0.7 MTS units; P = .002; 100 μM, mean = 0.6 MTS units, difference = 0.6 MTS units; 95% CI = 0.4 to 0.8 MTS units; P < .001; Fig. 1, F). These data suggest that autocrine activation of RAGE by S100P contributes to basal cell proliferation in this cancer cell line. Similar results were found with MPanc-96 cells, which also express endogenous S100P (data not shown). Thus, cromolyn was able to block the effects of both exogenous and endogenous S100P on pancreatic cancer cell proliferation.

We previously found that S100P provides a survival advantage for pancreatic cancer cells (11). Therefore, we examined whether cromolyn would influence the responsiveness of BxPC-3 cells to gemcitabine-induced apoptosis. As expected, treatment with gemcitabine resulted in statistically significant cell death (control, mean = 3.7%, versus gemcitabine, mean = 31.0%, difference = 27.3%; 95% CI = 23.8% to 30.8%; P < .001; Fig. 1, G). In contrast, cromolyn was not toxic to the cells and had no statistically significant effect on BxPC-3 cell apoptosis (Fig. 1, G). However, in combination with gemcitabine, cromolyn statistically significantly increased cell death (gemcitabine, mean = 30.8%, versus combination, mean = 42.6% of cells were apoptotic, difference = 11.6%; 95% CI = 7.8 to 15.4%; P = .001; Fig. 1, G).
We previously found that S100P increases migration and invasion of pancreatic cancer cells (11). Therefore, we sought to determine the effects of cromolyn on pancreatic cancer cell invasion with matrigel assays. In Panc-1 cells, which do not express S100P, cromolyn did not reduce basal cell invasion. However, cromolyn did block the effects of exogenous S100P on Panc-1 cell invasion (S100P, mean = 58.0%, versus S100P + cromolyn, mean = 9.4% of cells invaded, difference = 48.6%; 95% CI = 38.8% to 58.8%; P < .001; Fig. 2, A and B). By contrast, in BxPC-3 cells, which express S100P endogenously; cromolyn inhibited basal cell invasiveness in a concentration-dependent manner (0 μM, mean = 19.3%, versus 10 μM, mean = 9.3% of cells invaded, difference = 10.0%; 95% CI = 4.5% to 15.5%; P = .008; 100 μM, mean = 7.7%, difference = 11.7%; 95% CI = 8.2% to 15.2%; P < .001; Fig. 2, C and D). Likewise, cromolyn also inhibited basal cell invasiveness of MPanc-96 cells (data not shown). These data further indicate that cromolyn can inhibit both endogenous and exogenous S100P actions.

Effect of Cromolyn on NFκB Activity In Vitro and In Vivo

We showed previously that S100P stimulates NFκB activity in a dose- and time-dependent manner (11,13). To determine whether cromolyn inhibited this response, we examined the effects of cromolyn on S100P activation of an NFκB luciferase reporter gene construct in both Panc-1 and BxPC-3 cells in vitro. In Panc-1 cells, which lack endogenous S100P, cromolyn had no effect on basal NFκB activity (Fig. 3, A). However, S100P increased NFκB promoter activity in Panc-1 cells in a concentration-dependent manner (0 nM, mean = 3358 photons/s, versus 1 nM, mean = 6902 photons/s, difference = 3544 photons/s; 95% CI = 852 to 6235 photons/s, P = .022; 10 nM, mean = 8758 photons/s, difference = 5400 photons/s; 95% CI = 3968 to 6832 photons/s; P < .001; 100 nM, mean = 14,460 photons/s, difference = 11,100 photons/s; 95% CI = 7771 to 14430 photons/s; P < .001) and cromolyn (100 μM) inhibited this effect (100 nM of S100P alone, mean = 14,460 photons/s, versus combination, mean = 7360 photons/s, difference = 7100 photons/s; 95% CI = 3689 to 10,510 photons/s; P = .005). In BxPC-3 cells, which express endogenous S100P, cromolyn inhibited basal NFκB activity in a concentration-dependent manner (0 μM, mean = 1.4 × 10^6 photons/s, versus 10 μM, mean = 9.4 × 10^5 photons/s, difference = 4.7 × 10^5 photons/s; 95% CI = 2.6 × 10^5 to 6.7 × 10^5 photons/s; P = .003; 100 μM, mean = 8.2 × 10^5 photons/s, difference = 5.9 × 10^5 photons/s; 95% CI = 5.1 × 10^5 to 6.6 × 10^5 photons/s; P < .001; Fig. 3, B).

To determine whether cromolyn treatment could also reduce NFκB activity in vivo, we examined NFκB activity of BxPC-3 cells stably expressing an NFκB luciferase reporter construct that had been transplanted orthotopically into the pancreas of nude mice (n = 4). After 1 week, NFκB luciferase activity was determined before (0 time point) and at 24 and 48 hours after a single dose of cromolyn (5 mg/kg body weight by ip injection). Cromolyn administration reduced basal NFκB activity by at least 80% 24 hours after injection (0 hour, mean = 9.9 × 10^6 photons/s, versus 24 hours, mean = 1.3 × 10^6 photons/s, difference = 8.6 × 10^6 photons/s; 95% CI = 5.1 × 10^6 to 1.4 × 10^7 photons/s; P = .005; Fig. 3, C). At 48 hours after cromolyn injection, NFκB activity returned to control levels. These data indicate that cromolyn inhibits, both in vitro and in vivo, basal NFκB levels of pancreatic cancer cells that express endogenous S100P.
Based on our previous observations of the effects of S100P on pancreatic cancer growth in vivo (11), we further wished to analyze the effects of cromolyn on tumors developed in vivo in immunodeficient mice. For this purpose, pancreatic cancer cell lines BxPC-3, MPanc-96, and Panc-1 (n = 20 mice per cell line) were stably transfected with the Lenti-luc vector and were implanted orthotopically into the pancreas. After 1 week, tumor development was confirmed, and the mice carrying each tumor cell line were divided into four treatment groups (n = 5 mice per group) having equal mean tumor size (Figs. 4, A; 5, A; and 6, A). One group of mice was then treated biweekly with a submaximal...
concentration of gemcitabine (125 mg/kg body weight by ip injection). A second group was treated with a daily injection of cromolyn (5 mg/kg body weight by ip injection). A third group was treated with both biweekly gemcitabine and daily cromolyn. The control group was treated daily with vehicle. Treatments were continued for 5 weeks, and the effects on the tumor burden and metastasis were analyzed by weekly bioluminescence imaging (Figs. 4, B; 5, B; and 6, B). Gemcitabine strongly reduced the tumor burden relative to controls, in both the BxPC-3 (control, mean = $1.6 \times 10^9$ photons/s, versus gemcitabine, mean = $9.2 \times 10^8$ photons/s, difference = $6.8 \times 10^8$ photons/s; 95% CI = $1.8 \times 10^8$ to $1.1 \times 10^9$ photons/s; $P = .013$; Fig. 4, C) and the MPanc-96 (control, mean = $4.1 \times 10^9$ photons/s, versus gemcitabine, mean = $9.4 \times 10^8$ photons/s; 95% CI = $4.3 \times 10^8$ to $9.4 \times 10^8$ photons/s; $P < .001$; Fig. 5, C) models. Cromolyn also statistically significantly reduced tumor burden.

Fig. 5. Effect of cromolyn on MPanc-96 tumor growth and metastasis in vivo. MPanc-96 cells stably expressing the firefly luciferase gene were injected orthotopically into 4-week-old male CB17 scid mice. A) Bioluminescence imaging was used to estimate tumor volume after 1 week. Mice were divided into four groups of five mice each with an equivalent mean tumor size between groups. B) One group was treated with water (control), one group received gemcitabine biweekly (125 mg/kg body weight biweekly by intraperitoneal injection), one group was administered cromolyn daily (5 mg/kg body weight daily by intraperitoneal injection), and the final group was given the combination of cromolyn and gemcitabine. All groups were retreated for another 5 weeks. Bioluminescence imaging was done weekly to assess the tumor growth. C) At the end of the experiment, the volumes of primary tumors were quantified. *$P < .001$, †$P = .009$, ‡$P < .001$ versus control; $\ddagger P = .02$ versus gemcitabine. D) Metastasis to the liver was assessed after the removal of the primary tumor. *$P = .014$, †$P = .017$, ‡$P = .001$ versus control. E) Metastasis to the lung was also assessed. *$P = .03$, †$P = .013$, ‡$P = .012$ versus control. F) Weight of mice at the end of experiment is indicated. Means and 95% confidence intervals from two independent experiments are shown (n = 20). Two-tailed two-sample (unpaired) Student’s t tests were used to determine $P$ values.
in the BxPC-3 (control, mean = $1.6 \times 10^9$ photons/s, versus cromolyn, mean = $4.4 \times 10^8$ photons/s; difference = $1.2 \times 10^9$ photons/s; 95% CI = $6.2 \times 10^8$ to $1.6 \times 10^9$ photons/s; $P < .001$; Fig. 4, C) and MPanc-96 (control, mean = $1.1 \times 10^{10}$ photons/s, versus cromolyn, mean = $4.8 \times 10^9$ photons/s; difference = $6.2 \times 10^9$ photons/s; 95% CI = $1.9 \times 10^9$ to $1.0 \times 10^{10}$ photons/s; $P = .009$; Fig. 5, C) models, and this effect was similar in extent to that of gemcitabine. In combination, gemcitabine and cromolyn reduced tumor burden to a greater extent than gemcitabine alone in both the BxPC-3 (gemcitabine, mean = $9.2 \times 10^8$ photons/s, versus combination, mean = $1.8 \times 10^8$ photons/s, difference = $7.4 \times 10^8$ photons/s; 95% CI = $4.5 \times 10^8$ to $1.0 \times 10^9$ photons/s; $P < .001$; Fig. 4, C) and the MPanc-96 (gemcitabine, mean = $4.1 \times 10^9$ photons/s, versus combination, mean = $2.0 \times 10^9$ photons/s, difference = $2.1 \times 10^9$ photons/s; 95% CI = $4.4 \times 10^8$ to $3.8 \times 10^9$ photons/s; $P < .001$; Fig. 5, C) models. In contrast to these effects

Fig. 6. Effect of cromolyn on Panc-1 tumor growth and metastasis in vivo. Panc-1 cells stably expressing the firefly luciferase gene were injected orthotopically into 4-week-old male CB17 scid mice. A) Bioluminescence imaging was utilized to estimate tumor volume after 1 week. Mice were divided into four groups of five mice each with an equivalent mean tumor size between groups. B) One group was treated with water (control), one group received gemcitabine biweekly (125 mg/kg body weight biweekly by intraperitoneal injection), one group was administered cromolyn daily (5 mg/kg body weight daily by intraperitoneal injection), and the final group was given the combination of cromolyn and gemcitabine. All groups were treated for another 5 weeks. Bioluminescence imaging was done weekly to assess the tumor growth. C) At the end of the experiment, the volumes of primary tumors were quantified. D) Metastasis to the liver was assessed after the removal of the primary tumor. E) Metastasis to the lung was also assessed. F) Weight of mice at the end of experiment is indicated. * $P = .024$ versus control. Means and 95% confidence intervals from two independent experiments are shown (n = 20). Two-tailed two-sample (unpaired) Student’s t tests were used to determine $P$ values.
on pancreatic cancer cells that express endogenous S100P, cromolyn treatment did not reduce tumor development or increase the effectiveness of gemcitabine in a model involving Panc-1 cells, which do not express S100P (Fig. 6, A–C).

We also analyzed the effects of these treatments on tumor metastasis. In the BxPC-3 in vivo model, gemcitabine treatment alone did not reduce metastasis in either lung or liver compared with that in control mice. Cromolyn treatment statistically significantly reduced lung (control, mean = 5.5 × 10⁸ photons/s, versus cromolyn, mean = 1.6 × 10⁸ photons/s; 95% CI = 2.3 × 10⁸ to 5.4 × 10⁸ photons/s; P = .013; Fig. 5, E) but not liver metastasis in this model. However, the combination of cromolyn and gemcitabine reduced metastasis in both lung and liver. For example, in the liver, the combination reduced metastasis by more than 90% (control, mean = 5.8 × 10⁸ photons/s, versus combination, mean = 4.2 × 10⁷ photons/s; 95% CI = 1.0 × 10⁸ photons/s; P = .014; Fig. 4, D) compared with control. In the lung, the combination reduced metastasis to a greater extent than gemcitabine alone (gemcitabine, mean = 3.9 × 10⁸ photons/s, versus combination, mean = 3.6 × 10⁷ photons/s; 95% CI = 2.1 × 10⁸ to 5.0 × 10⁸ photons/s; P = .001; Fig. 4, E).

In the MPanc-96 in vivo model, gemcitabine treatment reduced both liver (control, mean = 9.4 × 10⁹ photons/s, versus gemcitabine, mean = 1.2 × 10⁹ photons/s; 95% CI = 2.2 × 10⁸ to 1.4 × 10⁹ photons/s; P = .015; Fig. 5, D) and lung (control, mean = 5.9 × 10⁷ photons/s, versus gemcitabine, mean = 5.0 × 10⁷ photons/s, 95% CI = 5.4 × 10⁷ photons/s; P = .03; Fig. 5, E) metastases. Cromolyn also reduced liver (control, mean = 9.4 × 10⁸ photons/s, versus cromolyn, mean = 2.2 × 10⁸ photons/s, 95% CI = 1.6 × 10⁸ to 1.2 × 10⁸ photons/s; P = .017; Fig. 5, D) and lung metastases (control, mean = 5.9 × 10⁷ photons/s, versus cromolyn, mean = 3.2 × 10⁶ photons/s; 95% CI = 1.5 × 10⁷ to 9.7 × 10⁶ photons/s; P = .013; Fig. 5, E) in the MPanc-96 model. Thus, in the MPanc-96 model, the combination of gemcitabine with cromolyn was not greater than either drug alone. In the Panc-1 in vivo model, cromolyn, gemcitabine, or the combination did not reduce liver or lung metastasis (Fig. 6, D and E).

As an indication of overall toxicity, we evaluated the effects of the treatments on body weight at the end of the experiment. Gemcitabine treatment caused a small but statistically significant decrease in body weight in the BxPC-3 (control, mean = 31.1 g, versus gemcitabine, mean = 27.2 g, difference = 3.9 g; 95% CI = 1.0 to 6.7 g; P = .013; Fig. 4, F) and Panc-1 (control, mean = 32.0 g, versus gemcitabine, mean = 27.2 g, difference = 4.8 g; 95% CI = 0.82 to 8.7 g; P = .024; Fig. 6, F) tumor models. However, daily injections of cromolyn had no effect on body weight for up to 6 weeks in any of the three models (Figs. 4, F; 5, F; and 6, F). The combination of cromolyn with gemcitabine reduced body weight in the BxPC-3 cell model, but this effect was not greater than the effect of gemcitabine alone (Fig. 4, F). The combination of cromolyn and gemcitabine did not affect body weight in either the MPanc-96 or Panc-1 models (Figs. 5, F and 6, F).

**Discussion**

In the current study, we identified the drug cromolyn as a small molecule inhibitor of S100P activation of RAGE. Cromolyn bound to S100P, inhibited S100P interactions with RAGE, and decreased S100P-mediated increases in cancer cell growth, survival, and invasiveness in vitro. Cromolyn also inhibited basal activity of the NFkB pathway in vitro and in vivo in pancreatic cancer cells with endogenous S100P. These mechanisms likely explain the observed inhibition of tumor growth and the ability of cromolyn to increase the effectiveness of gemcitabine to eliminate pancreatic cancer cells in mouse models. Together, these data support the further investigation of cromolyn as a possible treatment for pancreatic cancer.

Cromolyn is widely used for the prophylactic treatment of allergic asthma (18). Cromolyn is commonly considered a mast cell stabilizer based on its ability to prevent secretion from some mast cells. However, the specific mechanisms of cromolyn’s actions on mast cells are uncertain. An attractive model for the actions of cromolyn on mast cells is based upon its ability to interact with a component of a regulated Ca²⁺ channel and prevent Ca²⁺ entry and mast cell secretion (24). However, there are several observations that do not fit this model. First, not all mast cells are inhibited by cromolyn. Cromolyn interferes with secretion specifically in rat peritoneal mast cells but not rat intestinal mucosal mast cells (25,26). Second, the inhibitory effects of cromolyn are not Ca²⁺ dependent in either mast cells (27) or in macrophages (28). Other suggested targets of cromolyn actions have included moesin (29), C1 channels (30), protein kinase c (31), and nucleotide diphosphate kinase (32). However, cromolyn is impermeant to cells, and therefore, interactions with intracellular molecules are unlikely to account for its biologic activity. Recently, cromolyn has been shown to bind with high affinity to Ca²⁺-binding molecules belonging to the S100 family. Although it is unclear whether interactions with S100 molecules influence the actions of cromolyn on mast cells, this interaction may explain the results of cromolyn observed in the current investigation of effects on pancreatic cancer.

Few studies have investigated the effects of cromolyn on cancer. One study reported that cromolyn induced clotting and increased hypoxia in a murine model of breast cancer (33). In another study (34), cromolyn treatment was shown to greatly suppress the carcinogen induction of tumors in rats. In both these studies, the suggestion was made that the anticancer effects of cromolyn were likely due to an inhibitory effect on mast cells. However, neither study provided direct evidence for a role of mast cells or showed that cromolyn had inhibited mast cell function. The current data suggest that the previously unappreciated ability of cromolyn to interact with S100 proteins may help explain its ability to inhibit cancer.

S100 molecules are small (9–12 kDa) calcium-binding proteins that display 30%–50% homology within the family. There are at least 19 members of the S100 family, and most map closely together on chromosome 1q21, with the exception of S100P, which is located on 4p16 (10). Cromolyn has previously been found to bind S100 proteins A1, B, A12, and A13 (19–21). Together with the current observation concerning S100P, it seems likely that cromolyn binds to structural features common to many of the S100 family members. S100 proteins are involved in the regulation of a number of cellular processes. Some of these molecules also have roles in inflammatory responses (35). Recently, interest has been growing in the involvement of S100 proteins in cancer because of their differential expression in a variety of tumors (5,9,12,36). The expression of several S100 proteins has previously been observed in pancreatic tumors in profiling studies.
We previously found that the S100 proteins A2, A4, A5, A6, A8, A9, A10, A11, A13, and A14 were expressed in both chronic pancreatitis and pancreatic cancer samples. In contrast, the S100P isoform was highly expressed only in pancreatic cancer (5). Because several forms of S100 are expressed in pancreatic cancer cells, we cannot rule out the other possibility that other S100 proteins are also secreted by pancreatic cancer cells and may participate in the autocrine regulation of growth and survival. However, our previous data indicated that specific silencing of S100P in pancreatic cancer cell lines was sufficient to dramatically reduce growth, survival, and invasiveness in vitro and tumor growth in vivo (11). Therefore, the simplest explanation for our observations is that cromolyn binds S100P and inhibits its activity.

Abundant evidence supports the hypothesis that the major effects of cromolyn observed on pancreatic cancer cells in the current study were mediated specifically by interactions with S100P. Cromolyn blocked the effects of exogenously added S100P, but not PDGF or serum, on pancreatic cancer cell functions. Furthermore, in pancreatic cancer cells with high endogenous levels of S100P, cromolyn inhibited basal rates of proliferation, invasion, NFκB promoter activity, and resistance to cytotoxic drugs in vitro and tumor growth and resistance to gemcitabine in vivo. In contrast, in pancreatic cancer cells that do not express endogenous S100P, cromolyn did not affect cell function in vitro or tumor development in vivo. Cromolyn also had no effect on proliferation or migration of other cells that lack endogenous S100P, including NIH3T3 cells (data not shown). Therefore, although there may be other targets of cromolyn, it appears that S100P is the most important target with regard to pancreatic cancer.

We have previously shown that the actions of S100P are mediated by activation of RAGE. In particular, either a short-peptide RAGE antagonist or a blocking anti-RAGE antibody was able to block the interaction between S100P and RAGE and S100P-stimulated cell functions (13). In the current study, we found that cromolyn interfered with the interaction of S100P with RAGE, as indicated by a reduction in the level of communoprecipitated S100P and RAGE complexes. Furthermore, cromolyn specifically and potently inhibited S100P functions similar to other methods of blocking RAGE activation. Thus, the possible explanation for the effects of cromolyn on pancreatic cancer involves its ability to block activation of RAGE by S100P.

NFκB activity is high in the majority of pancreatic cancers (15), in which it mediates antiapoptotic signaling (16). Inhibition of NFκB has been shown to improve the effectiveness of cytotoxic agents in pancreatic cancer cells (17). We observed a concentration-dependent stimulation of NFκB activity by exogenous S100P in Panc-1 cells, as expected, and cromolyn completely inhibited S100P stimulation. We also observed that cromolyn reduced basal NFκB activity in BxPC-3 pancreatic cancer cells, which have high levels of endogenous S100P. This inhibition of basal NFκB activity was measured both in vitro and in vivo using bioluminescence imaging. To our knowledge, this report is the first to directly show the effectiveness of a treatment to reduce NFκB activity in pancreatic cancer cells in vivo. The observed ability of cromolyn to reduce basal NFκB activity was associated with its ability to increase the effectiveness of treatments with the cytotoxic chemotherapeutic agent, gemcitabine. However, further studies will be necessary to determine the role of NFκB activity inhibition in the anticancer effects of cromolyn.

There is a growing body of evidence that, in addition to its effects on cancer cell growth, S100P may also play an important role in cancer metastasis. We previously reported that silencing of S100P by siRNA resulted in a reduction in invasiveness in vitro and metastasis in vivo (11). A recent study in breast cancer found an association between S100P levels and metastasis in animal models and also with reduced patient survival (8). Furthermore, in that study, S100P genetic transfer to nonmetastatic rat breast cancer cell lines induced dramatic metastatic behavior. Thus, we were interested to determine the effects of cromolyn on metastasis in pancreatic cancer models. We observed that cromolyn inhibited the invasiveness of pancreatic cancer cells that express endogenous S100P in vitro. Furthermore, cromolyn reduced the total volume of distant metastases in two orthotopic tumor models developed with cells that express endogenous S100P. Cromolyn also increased the effectiveness of gemcitabine treatments to reduce the total volume of metastases in these models. However, analysis of the numbers of metastases, rather than total volume, did not indicate any statistically significant effect of cromolyn (data not shown). Thus, in the current study the influence of cromolyn appears to be primarily on cancer cell growth rather than directly on metastasis. Further experiments will be necessary to fully determine the effect of cromolyn on metastasis.

This study has potential limitations. One issue is the specificity of cromolyn. This study indicates that cromolyn can inhibit the interaction of S100P and RAGE in pancreatic cancer cells in vitro. However, cromolyn may have other targets that are important in vivo. Pancreatic cancer cells secrete S100P, and cromolyn may block S100P’s interactions with other cells in the tumor microenvironment. Furthermore, cromolyn may interact with other molecules, e.g., other members of the S100 family or other unidentified targets. The identities and relative importance of all the cromolyn targets that might affect pancreatic cancer remain unknown. Another limitation in the current study is the assessment of tumor growth in vivo. To be useful clinically, a therapy must inhibit the growth of existing tumors. Future studies will be needed to determine whether cromolyn alone can cause tumor regression or if it can increase the effectiveness of gemcitabine or other cytotoxic therapies in this regard.

In summary, the current results confirm and extend our previous studies indicating that S100P is an important factor that contributes to the aggressive nature of pancreatic cancer. Furthermore, the current study identifies the antiallergic drug cromolyn as a useful inhibitor that can bind with S100P and block its ability to activate RAGE and induce responses in cell and animal models. Treatment with cromolyn in combination with gemcitabine strongly inhibited pancreatic tumor growth and metastasis in two independent mouse models of pancreatic cancer. Thus, cromolyn treatment in combination with gemcitabine should be tested to improve patient outcome with this deadly aggressive cancer. Whether cromolyn may also improve responses to cytotoxic treatments in other cancers would also appear to be worth further investigation.

**References**


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