BASIC RESEARCH

Potential use of the anti-inflammatory drug, sulfasalazine, for targeted therapy of pancreatic cancer

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ABSTRACT

Pancreatic cancer is an aggressive, drug-resistant disease; its first-line chemotherapeutic, gemcitabine, is only marginally effective. Intracellular depletion of glutathione, a major free-radical scavenger, has been associated with growth arrest and reduced drug resistance (chemosensitization) of cancer cells. In search of a new therapeutic approach for pancreatic cancer, we sought to determine whether specific inhibition of the plasma membrane x_c^- cystine transporter could lead to reduced uptake of cysteine, a key precursor of glutathione, and subsequent glutathione depletion. Sulfasalazine (approximately 0.2 mmol/L), an anti-inflammatory drug with potent x_c^--inhibitory properties, markedly reduced L-[^{14}C]-cystine uptake, glutathione levels, and growth and viability of human MIA PaCa-2 and PANC-1 pancreatic cancer cells *in vitro.* These effects were shown to result primarily from inhibition of cystine uptake mediated by the x_c^- cystine transporter and not from inhibition of nuclear factor κB activation, another property of sulfasalazine. The efficacy of gemcitabine could be markedly enhanced by combination therapy with sulfasalazine both *in vitro* and in immunodeficient mice carrying xenografts of the same cell lines. No major side effects were observed *in vivo.*

The results of the present study suggest that the x_c^- transporter plays a major role in pancreatic cancer by sustaining or enhancing glutathione biosynthesis, and as such, represents a potential therapeutic target. Sulfasalazine, a relatively nontoxic drug approved by the U.S. Food and Drug Administration, may, in combination with gemcitabine, lead to more effective therapy of refractory pancreatic cancer.

KEY WORDS

Pancreatic cancer, x_c^- cystine transporter, sulfasalazine, cystine, cysteine, glutathione, gemcitabine resistance, NFκB

1. INTRODUCTION

Pancreatic cancer is one of the most aggressive and drug-resistant cancers and the fourth-leading cause of cancer-related deaths in North America [1,2]. It is characterized by a lack of detectable symptoms in early stages of the disease, leading to late diagnosis and rapid development into metastatic, drug-resistant cancer [2]. Although gemcitabine (GEM) is the first-line chemotherapeutic for pancreatic cancer, it increases survival only marginally; most cases, if not all, are palliative [3]. It is therefore crucial to identify new therapeutic targets and approaches for treatment of this deadly disease.

Glutathione is a major antioxidant considered essential for protection of cells from oxidative stress. It also plays an important role as a detoxifier and is known to underlie drug resistance [4,5]. Oxidative stress is particularly generated in cancer cells because of their relatively high metabolism, and glutathione depletion has been suggested as a therapeutic approach for a variety of cancers, in particular to reduce resistance to conventional anticancer agents [5–7].

Intracellularly, glutathione is present mainly in a reduced form—that is, as a linear tripeptide thiol [consisting of glutamate, cysteine, and glycine (GSH)] with a short half-life. Cysteine is a rate-limiting precursor of GSH, and sustenance of adequate intracellular cysteine levels is critical for maintaining adequate GSH levels, cell growth, and viability [4]. For some cancers, this sustenance depends on uptake of cysteine or cystine (the oxidized form of the amino acid) from the microenvironment, as indicated by the cystine/cysteine growth requirements of cell lines derived from lymphomas, gliomas, and pancreatic cancers, among others [8–12].

Evidence is increasing to suggest that the x_c^- plasma membrane cystine/glutamate antiporter plays an important role in the maintenance of intracellular cysteine/cystine levels by mediating direct uptake of cystine and *in vivo* cysteine supply by stromal cells (for example, activated macrophages, fibroblasts). Such somatic cells can use the

x_c^- transporter to pick up cystine (the predominant extracellular form), reduce the cystine intracellularly to cysteine, and secrete surplus cysteine (which neighbouring cancer cells can readily take up using the ubiquitous ASC transport system)[13]. This particular growth-promoting function of stromal cells has been well established using co-cultures of fibroblasts and lymphoma cells that do not express a cystine transporter: Acting as feeder layers, the fibroblasts supply cysteine essential for growth of the lymphoma cells[14,15]. In view of these mechanisms, inhibition of the x_c^- transporter, leading to cystine/cysteine starvation and subsequent glutathione depletion, has been proposed as a potential therapeutic approach in a variety of cancers[7–13].

We recently observed that, in pancreatic ductal adenocarcinoma tissue from patients, the x_c^- transporter was overexpressed relative to normal pancreatic tissue from the same patients. We also obtained evidence that human pancreatic cancer cell lines, such as MIA PaCa-2 and PANC-1, critically depend on cystine uptake via the x_c^- transporter for growth and viability and that GEM resistance in PANC-1 cells is associated with elevated x_c^- expression[11]. Together, these findings suggest that specific inhibition of the x_c^- transporter, aimed at glutathione depletion (with subsequent growth arrest and reduced drug resistance of target cells), could provide a new approach for targeted therapy of pancreatic cancer.

In the present study, we targeted the x_c^- cystine transporter to examine the effect on growth and GEM resistance in MIA PaCa-2 and PANC-1 cells *in vitro* and as xenografts in immunodeficient mice. As an x_c^- inhibitor, we used sulfasalazine, a well-established anti-inflammatory drug with potent x_c^- inhibitory properties, which is also relatively non-toxic[9,16].

2. MATERIALS AND METHODS

2.1 Materials, Animals, Cultures and Cell Viability and Proliferation Assay

Chemicals, dyes, solvents, and solutions were obtained from Sigma–Aldrich Canada (Oakville, ON) unless otherwise indicated. The BC Cancer Research Centre Animal Resource Centre, BC Cancer Agency, Vancouver, bred the 8- to 10-week-old male Rag-2M mice used in the study. Animal care and experiments were carried out in accordance with the guidelines of the Canadian Council on Animal Care. The human pancreatic cancer cell lines MIA PaCa-2 and PANC-1 were originally obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and were maintained as monolayer cultures in minimum essential medium containing 0.1 mmol/L cystine (StemCell Technologies, Vancouver, BC), supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, U.S.A.) and 3.6 g/L glucose, as previously described[11].

Cell proliferation and viability were determined by neutral red uptake assays using 96-well plates initially containing about 1000 cells per well. After a 4-hour incubation of treated cells with 100 μL 0.0025% neutral red dye in culture medium, intracellular neutral red dye was measured by absorbance at 550 nm using a VersaMax microplate reader **(Molecular Devices, Sunnyvale, CA, U.S.A.)**, as previously described[11].

2.2 Drug Preparations, x_c^- Function, and Glutathione Assays

Gemcitabine (Eli Lilly and Company, Indianapolis, IN, U.S.A.) was dissolved in 0.9% NaCl (33 mmol/L) for *in vitro* studies and in phosphate-buffered saline [PBS (12 mg/mL)] for *in vivo* studies. Sulfasalazine solutions were freshly prepared and used as previously described[9]. Cystine uptake activity of the Na^+-independent x_c^- transporter in cultures was measured using a buffer solution free of Na^+ ions (to exclude the contribution of Na^+-dependent transporters), supplemented with 112 nmol/L L-[^{14}C]-cystine [300 mCi/mmol (Perkin Elmer and Analytical Sciences, Waltham, MA, U.S.A.)] in the presence or absence of 1 μmol/L non-labelled amino-acid competitors (L-glutamate, L-cystine), a non-competitor (L-leucine), sulfasalazine (0.2 mmol/L) or 2-mercaptoethanol [2-ME (66 μmol/L)] for 20 minutes at 37°C, as previously described[11]. Total glutathione (GSH+GSSG) levels were measured using the ApoGSH GSH Colorimetric Detection Kit (BioVision, Mountain View, CA, U.S.A.) following the manufacturer's recommendations.

2.3 Reporter Assay for Nuclear Factor κB

To determine nuclear factor κB (NFKB) activity, MIA PaCa-2 or PANC-1 cells were transfected with NFKB luciferase reporter constructs. In 24-well plates, cells were plated at 4000 cells per well and incubated overnight. They were then co-transfected overnight with 300 ng/well pGL3-empty luciferase reporter vector (control) or pGL3-NFKB promoter luciferase reporter (Promega, Madison, WI, U.S.A.) and 7.5 ng/well pRL-cytomegalovirus [internal control vector (Promega)] using 3 μL/well of ExGen 500 *in vitro* transfection reagent (Fermentas, Burlington, ON), according to the manufacturer's recommendations.

One day post transfection, the medium was removed, and the cells were incubated for 3 hours with 10 ng/mL tumour necrosis factor α (TNFα—to induce NFKB activity) or with culture medium (control). The medium was then removed, and the cells were further incubated for 24 hours with various concentrations of sulfasalazine or MSG, with or without 2-ME (66 μmol/L), after which the cells were washed, and NFKB luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega), following the manufacturer's recommendations.

2.4 *In Vivo* Drug Testing

PANC-1 or MIA PaCa-2 cells (3×10^6 cells/100 μL) were injected subcutaneously into both dorsal flanks of Rag-2M mice. The developing xenografts were allowed to grow for about 3 weeks, until the tumours reached an average volume of approximately 50 mm³. The mice were randomized into control and treatment groups (6 mice per group). For 14 days (a period considered long enough to develop significant differences in tumour volume between treated and untreated animals), the mice were treated with PBS (control), sulfasalazine (250 mg/kg intraperitoneally twice daily), GEM (120 mg/kg intraperitoneally once weekly), or a combination of sulfasalazine and GEM. The GEM dosage was obtained from other studies [17]; the sulfasalazine dosages used were previously found to be effective for rats [9]. Calliper measurements were used to assess tumour volume pre- and post-treatment, using the formula

volume (mm³) =
0.52 × length × width × height (all mm).

The mice were provided with food and water *ad libitum,* and their health was monitored daily for signs of stress, including weight loss and abnormal behaviour.

2.5 Statistical Analysis

The Student *t*-test was used to determine statistical significance. Results at $p \leq 0.05$ were considered significant.

3. RESULTS

3.1 Role of the x_c^- Transporter in Cystine Uptake: Effect of Sulfasalazine

As shown in Figure 1(A), uptake of L-[¹⁴C]-cystine by MIA PaCa-2 and PANC-1 cells was markedly inhibited—to about the same extent (approximately 60%–80%)—by L-glutamate and L-cystine, basic substrates of the x_c^- cystine/glutamate antiporter featuring similar affinities for the transporter [18]. In contrast, L-leucine, a non-substrate, had no effect on L-[¹⁴C]-cystine uptake [Figure 1(A)]. The data show that, under the Na⁺-free conditions used, uptake of cystine by both cell lines was primarily mediated by the x_c^- transporter.

Sulfasalazine, used under the same conditions, also markedly decreased uptake of L-[¹⁴C]-cystine by the cells (approximately 60%–70%), similar to glutamate and cystine, indicating that its reduction of L-[¹⁴C]-cystine uptake reflected an ability to markedly inhibit the function of the x_c^- transporter, as previously established in other systems [9]. Furthermore, sulfasalazine did not affect messenger RNA expression of the xCT and 4F2hc subunits of the x_c^- transporter in the two cell lines (data not shown)—supporting existing evidence that the drug
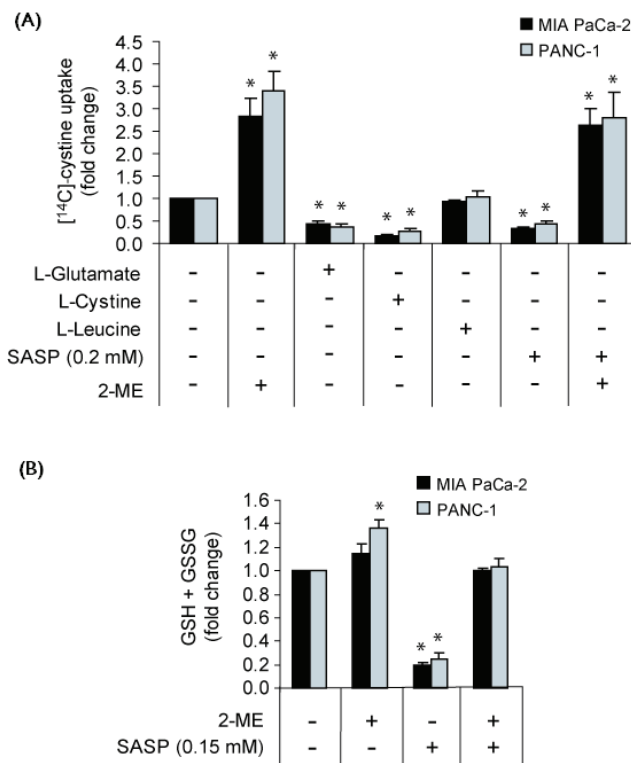

FIGURE 1   Effect of sulfasalazine (SASP) on x_c^- function and glutathione levels of MIA PaCa-2 and PANC-1 cells. (A) L-[¹⁴C]-cystine 112 nmol/L uptake by cells incubated for 24 hours under Na⁺-free conditions with SASP 0.2 mmol/L in the absence or presence of 2-mercaptoethanol (2-ME) 66 μmol/L, competitive substrate (1 μmol/L L-glutamate or L-cystine), or non-substrate (1 μmol/L L-leucine); (B) Intracellular total glutathione (GSH+GSSG) in cells incubated for 24 hours in maintenance medium (containing cystine approximately 0.1 mmol/L) with or without SASP 0.15 mmol/L and 2-ME 66 μmol/L in the combinations indicated. Data represent the mean ± standard error from three independent experiments. mM = mol/L. * $p \leq 0.05$.

inhibits the function of the transporter rather than its expression.

Figure 1(A) also shows that, in cultures containing 2-ME 66 μmol/L (that is, control + 2-ME and sulfasalazine + 2-ME), L-[¹⁴C]-cystine uptake was much higher than it was in counterpart cultures containing no 2-ME. These results were expected on the basis of the well-established cystine uptake–enhancing ability of 2-ME which, at 50–100 μmol/L, allows cellular uptake of cystine via a transport system not normally used for cystine uptake. Thus, interaction of 2-ME and cystine has been reported to produce a mixed disulfide of 2-ME and cysteine that cells can take up using the L transport system; the disulfide is then split intracellularly into cysteine and 2-ME. The cysteine accumulates in the cells, but the 2-ME escapes rapidly back into the medium to again react with cystine for a continued supply of cysteine to the cells [19].

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3.2 Effect of Sulfasalazine on Glutathione Levels

MIA PaCa-2 and PANC-1 cells were incubated in maintenance medium for 24 hours with 0.15 mmol/L sulfasalazine in the absence and presence of 2-ME 66 μmol/L. As shown in Figure 1(B), sulfasalazine led to a marked reduction of the glutathione levels (70%–80%), which could be completely prevented by inclusion in the medium of cystine uptake-enhancing 2-ME 66 μmol/L. At this concentration, 2-ME does not affect the chemical structure of sulfasalazine, as indicated by high-performance liquid chromatography of sulfasalazine samples treated with the thiol for extended periods (Buckley AR and colleagues. University of Cincinnati Academic Health Center. Personal communication) [12]. Taken together, the results in Figure 1 indicate that sulfasalazine-induced inhibition of x_c^--mediated cystine uptake can, within 24 hours, lead to a major reduction of the glutathione levels in the two cell lines.

3.3 Growth-Inhibitory Effect of Sulfasalazine

Sulfasalazine inhibited *in vitro* growth of MIA PaCa-2 and PANC-1 cell lines (measured using neutral red uptake assay) at half maximal inhibitory concentrations of 0.01 mmol/L and 0.05 mmol/L respectively (data not shown), indicating that the PANC-1 cells were less sensitive to the drug. Sulfasalazine at 0.2 mmol/L, a patient-tolerated plasma concentration [20], markedly decreased cell viability of MIA PaCa-2 and PANC-1 cell lines—that is, by 87% and 80% respectively [Figure 2(A)]. This growth inhibition could be largely prevented by including 2-ME 66 μmol/L in the culture medium [Figure 2(A)]. The sulfasalazine-induced growth arrest therefore appears to be primarily attributable to inhibition of cystine uptake. As shown in Figure 2(B), sulfasalazine-induced loss of cell viability manifested itself after about 48 hours. It may be noted that the concentration of cystine used in the culture medium (about 0.1 mmol/L) approximates that observed in human blood plasma [21].

3.4 Effect of Sulfasalazine on NFκB Activation: Relationship to x_c^--Inhibitory and Growth-Inhibitory Activity

Sulfasalazine is also known to inhibit activation of the NFκB transcription factor, and its growth-inhibitory activity in certain experimental systems has been related to NFκB inhibition [22] as distinct from cystine starvation [9,10]. We therefore sought to determine whether growth arrest of the two cell lines by sulfasalazine [Figure 2(A)] also involved inhibition of NFκB activation.

As shown in Figure 3(A), a 24-hour incubation of MIA PaCa-2 cells (transfected with NFκB luciferase constructs) with sulfasalazine 0.2 mmol/L reduced NFκB activity by about 40%–50% for both TNFα-treated
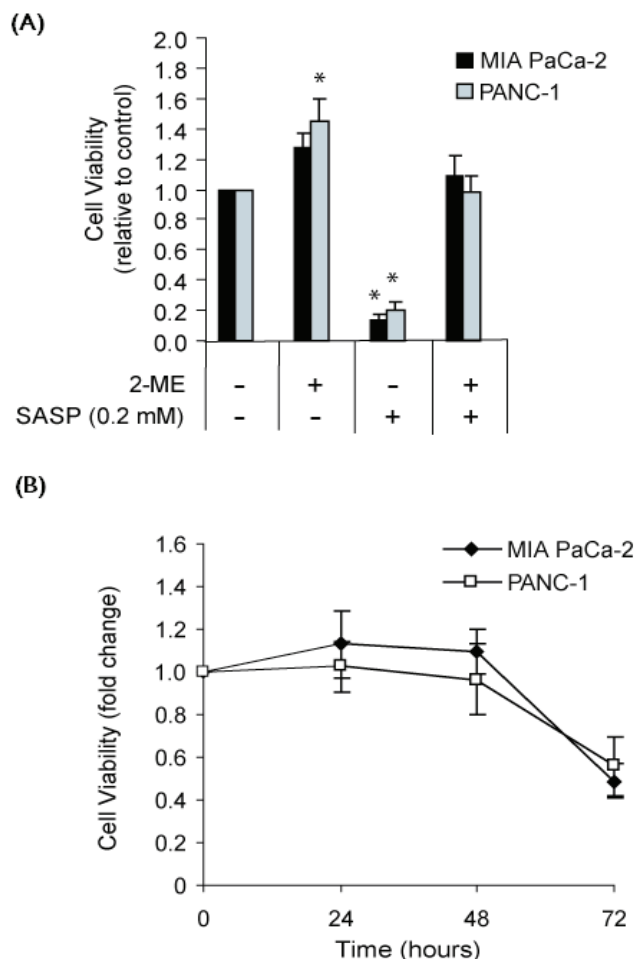

FIGURE 2   *Effect of sulfasalazine (SASP) on growth and cell viability of MIA PaCa-2 and PANC-1 cell cultures in maintenance medium (containing cystine approximately 0.1 mmol/L) as measured by neutral red uptake assay. (A) Viability of cells incubated for 72 hours with SASP 0.2 mmol/L and 2-mercaptoethanol (2-ME) 66 μmol/L in the combinations indicated. (B) Proliferation of cells incubated with SASP 0.2 mmol/L for 24, 48, and 72 hours. Data represent the mean ± standard error from three independent experiments. mM = mol/L. * p ≤ 0.05.*

and -untreated cells. Inclusion of 2-ME 66 μmol/L in the culture medium did not prevent the sulfasalazine-induced decline in NFκB activity. Similar results were obtained with PANC-1 cells [Figure 3(B)]. These data contrast with those showing sulfasalazine-induced inhibition of cystine uptake [about 60%–70%, Figure 1(A)], reduction of glutathione levels [70%–80% after 24 hours, Figure 1(B)], and population growth [Figure 2(A)], all of which could be prevented by 2-ME 66 μmol/L because of enhancement of cystine uptake [Figure 1(A)].

Taken together, the results (Figures 1–3) show that cultures containing both sulfasalazine 0.2 mmol/L and 2-ME 66 μmol/L exhibited normal glutathione levels and growth despite markedly reduced NFκB activity. This finding strongly suggests that the growth
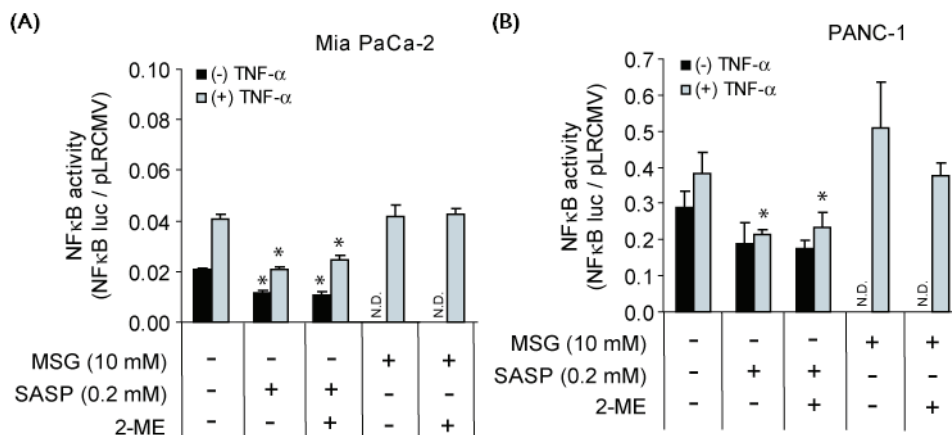
FIGURE 3   Activity of nuclear factor κB (NFκB) in MIA PaCa-2 and PANC-1 cells as affected by sulfasalazine (SASP) and monosodium gluta-mate (MSG). Activity of NFκB was measured by NFκB-promoter luciferase assay in (A) MIA PaCa-2 cells and (B) PANC-1 cells, untreated or pretreated with tumour necrosis factor α (TNFα) for 3 hours to induce NFκB activation, followed by a 24-hour incubation with SASP 0.2 mmol/L or MSG 10 mmol/L in maintenance medium in the absence or presence of 2-mercaptoethanol 66 μmol/L. Data represent the mean ± standard error from three independent experiments. N.D. = not determined; mM = mol/L. * p < 0.05 with respect to control.

inhibition of the two cell lines by sulfasalazine 0.2 mmol/L [Figure 2(A)] was not based on inhibition of NFκB activation. Furthermore, as shown in Figure 3, NFκB activation in the two cell lines was not inhibited by MSG 10 mmol/L, which is a highly specific and rapid inhibitor of x_c^--mediated cystine uptake [8,9]; and, as previously found, MSG at 4 mmol/L fully arrested growth of MIA-PaCa-2 and PANC-1 cell cultures [11]. The lack of effect of MSG on NFκB activation indicates that the reduction in NFκB activity by sulfasalazine 0.2 mmol/L was not related to its interference with cystine uptake—that is, the function of the x_c^- transporter.

3.5 Effect of Sulfasalazine on GEM Sensitivity of Cells *In Vitro* and *In Vivo*

As shown in Figure 4(A), incubation with sulfasalazine 0.1 mmol/L alone for just 24 hours (0–24 h) did not affect survival of the cell lines as measured 72 hours later by neutral red uptake assay (at hour 96); in contrast, treatment with GEM 10 nmol/L alone (24–96 h) caused significant cell death in the MIA PaCa-2 cell line, but much less in the PANC-1 cell line, previously shown to be highly resistant to GEM [11] [Figure 4(A)]. The lack of effect of 24-hour treatment with sulfasalazine alone (ended by removal of the drug-containing medium) shows that the cells, which were still viable at hour 24 [Figure 2(B)], can recover following removal of the drug. Importantly, greatly reduced cell survival was evident in cultures of both cell lines that had been subjected to a 24-hour treatment with sulfasalazine 0.1 mmol/L (0–24 h), followed by a 72-hour treatment with GEM 10 nmol/L (24–96 h). The growth of the MIA PaCa-2 cell line was essentially abrogated and the relatively GEM-resistant PANC-1 line was greatly affected by the combined sequential
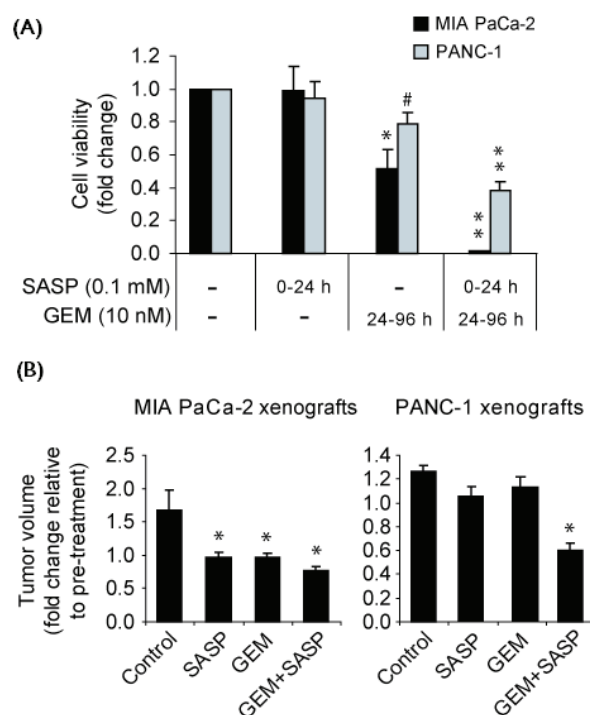

FIGURE 4   Sulfasalazine (SASP)–induced sensitization of MIA PaCa-2 and PANC-1 cells to gemcitabine (GEM) in vitro and in vivo. (A) Cell viability, as measured by neutral red uptake assay, of cells incubated in vitro for 96 hours in maintenance medium while subjected to treatment with no drugs (control); SASP 0.1 mmol/L (0–24 h); GEM 10 nmol/L (24–96 h); and SASP 0.1 mmol/L (0–24 h) followed by GEM 10 nmol/L (24–96 h). Data represent the mean ± standard error from three independent experiments. mM = mol/L. # p ≤ 0.05, * p ≤ 0.01, and ** p < 0.001. (B) Tumour volumes of subcutaneous xenografts in four groups of Rag-2M mice (each n = 6) after 2-weeks of treatment with intraperitoneal injections of phosphate-buffered saline (control, twice daily), SASP 250 mg/kg (twice daily), GEM 120 mg/kg (once weekly), or GEM+SASP. * p < 0.05.

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use of the two drugs [Figure 4(A)]. The results indicate that treatment with sulfasalazine can increase pancreatic cancer cell sensitivity to GEM *in vitro*.

We next determined whether sulfasalazine could enhance pancreatic cancer cell sensitivity to GEM *in vivo*. To that end, mice bearing actively growing MIA PaCa-2 and PANC-1 subcutaneous xenografts were treated for 2 weeks with PBS (control), sulfasalazine alone, GEM alone, or sulfasalazine and GEM in combination. All treatments were administered by intraperitoneal injection, particularly aiming to avoid intra-intestinal cleavage of sulfasalazine to sulfapyridine and 5-amino-salicylic acid [16], both of which lack x_c^--inhibitory activity [9]. Xenografts of MIA PaCa-2 treated with either sulfasalazine alone (twice daily) or GEM alone (once weekly) showed marked inhibition of tumour growth. Significantly greater inhibition was found when the two drugs were used in combination [Figure 4(B)]. Xenografts of the GEM-resistant PANC-1 cells, receiving the same protocol, were relatively resistant to treatment with GEM alone and also to sulfasalazine alone; in contrast, therapy with combined sulfasalazine and GEM resulted in much enhanced growth inhibition [Figure 4(B)]. These treatments in the mice did not lead to major side effects. The data indicate that sulfasalazine can act as an anticancer drug and as a chemosensitizing agent *in vivo*.

4. DISCUSSION

Pancreatic cancer is characterized by aggressive metastatic behaviour and resistance to conventional chemotherapy [1,2]. Development of novel, more effective approaches—particularly those aimed at overcoming drug resistance—is therefore of critical importance. In a previous study, we obtained evidence suggesting that pancreatic cancer growth and viability could critically depend on uptake of cystine/cysteine from the micro-environment and that the x_c^- cystine/glutamate antiporter played a major role in uptake of the amino acid [11]. In the present study, the function of the x_c^- transporter in pancreatic cancer cells was further examined, particularly in relation to sulfasalazine, which, as previously observed with lymphoma cells [9], acted as an x_c^- inhibitor in both MIA PaCa-2 and PANC-1 cell cultures. The high sulfasalazine-induced inhibition of L-[^{14}C]-cystine uptake by cells in Na$^+$-free medium was similar to that found for cystine and glutamate, two highly specific x_c^- inhibitors [Figure 1(A)]. The substantial sulfasalazine-induced decreases in glutathione levels and in growth and viability, which were specifically based on cystine uptake inhibition [because they could be prevented by 2-ME–enhanced cystine uptake, Figures 1(B) and 2(A)], indicate that the x_c^- transporter is indeed an important mediator of cystine uptake essential for the growth and viability of both studied pancreatic cancer cell lines.

Growth inhibition resulting from glutathione depletion has been observed in a variety of experimental systems, including those involving pancreatic cancer [23] and glioma [10] cells, and can be overcome by inclusion in the culture medium of glutathione ethyl ester, a membrane-permeable form of glutathione [10]. The sulfasalazine-induced growth inhibition of MIA PaCa-2 and PANC-1 cell cultures [Figure 2(A)] was likely also attributable to marked reductions in the levels of glutathione, as reported for sulfasalazine-treated glioma cells [10]. Thus sulfasalazine-induced loss of cell viability started after about 48 hours of incubation [Figure 2(B)], at a time when glutathione levels had been severely reduced for at least 24 hours [Figure 1(B)]. Moreover, enhancement of cellular cystine uptake by 2-ME 66 μmol/L prevented reductions both in cell viability [Figure 2(A)] and in glutathione level [Figure 1(B)]. *In vivo,* sulfasalazine on its own also inhibited growth of pancreatic cancer cell xenografts, in particular xenografts of MIA PaCa-2 cells [Figure 4(A)], consistent with the greater sensitivity of those cells to sulfasalazine *in vitro*. It may be noted that sulfasalazine treatment of xenografts of human glioma and lung cancer cells has been reported to lead to a reduction both of tumour growth and of glutathione content [10,12].

Growth arrest of cancer cells by sulfasalazine has also been reported to stem from inhibition of NFKB, a transcription factor considered to be a major protector against apoptosis [24]. Sulfasalazine 0.2 mmol/L indeed reduced NFKB activity in the two studied cell lines by 40%–50% (Figure 3), but the reduced NFKB activity could not account for the sulfasalazine-induced growth arrest. Although inclusion of 2-ME 66 μmol/L in the culture medium did not prevent reduction of NFKB activity (Figure 3), it did allow for normal growth in the presence of the drug [Figure 2(A)]. That finding, and the additional finding that MSG, a specific x_c^- inhibitor, did not affect NFKB activity (Figure 3), suggests that the action of sulfasalazine on NFKB activity was not directly related to interference with x_c^- transporter function. Inhibition of x_c^- function and inhibition of NFKB activation appear to represent two distinct mechanisms of action of sulfasalazine. Taken together, the results show that growth arrest of pancreatic cancer cells by sulfasalazine used at relatively low, patient-tolerated concentrations (for example, 0.2 mmol/L), is primarily attributable to inhibition of x_c^- transporter function rather than to inhibition of NFKB activation. Support for this conclusion has come from a recent publication reporting that sulfasalazine inhibits the growth of primary brain tumours independent of NFKB [25].

Glutathione depletion has also been linked to chemosensitization of various cell types [4,5,7,26], which, as indicated by the present study, may also include pancreatic cancer cells. Thus MIA PaCa-2 and GEM-resistant PANC-1 cells, both showing glutathione

depletion after a 24-hour treatment with sulfasalazine [Figure 1(B)], were more sensitive *in vitro* to GEM when pretreated with sulfasalazine than when no pre-treatment occurred [the MIA PaCa-2 cells in particular, Figure 4(A)]. Sulfasalazine-induced sensitization was also apparent *in vivo* and markedly reduced the GEM resistance of the PANC-1 cell xenografts [Figure 4(B)]. It is not clear why the MIA PaCa-2 cells *in vivo* were not as responsive to sulfasalazine-induced sensitization as they were *in vitro* (Figure 4).

The basis for the sulfasalazine-induced sensitization of pancreatic cancer cells to GEM is not clearly understood. Apparently, GEM neither forms conjugates with glutathione, nor is it detoxified by this tripeptide thiol. However, there is evidence that GEM can cause reactive oxygen intermediates (ROIS) in pancreatic cancer cells [27]. Elimination of such ROIS by glutathione could contribute to the drug resistance of the cells. In such a case, sulfasalazine-induced reduction of glutathione levels could lead to reduced drug resistance.

Sensitization of pancreatic cancer cells by sulfasalazine has also been reported by other researchers. Enhancement of etoposide sensitivity, for example, has been investigated in relation to sulfasalazine as an inhibitor of NFκB activity [28]. Drug resistance has been linked to elevated glutathione levels, but it has also been associated with constitutive NFκB activity [24], and chemosensitization by sulfasalazine could involve not only glutathione depletion but also NFκB inhibition.

5. CONCLUSIONS

The results of the present study suggest that the x_c^- transporter plays a major role in pancreatic cancer as a mediator of cystine uptake for the biosynthesis of glutathione important for cell growth, viability, and drug resistance. As such, the x_c^- transporter represents a potential therapeutic target in this disease. Sulfasalazine, a relatively nontoxic drug approved by the U.S. Food and Drug Administration, may, in combination with GEM, lead to more effective therapy of refractory pancreatic cancer.

6. CONFLICT OF INTEREST DISCLOSURES

7. ACKNOWLEDGMENTS

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