

Targeting the sphingolipid metabolism to defeat pancreatic cancer cell resistance to the chemotherapeutic gemcitabine drug

Julie Guillermet-Guibert,¹ Lise Davenne,¹ Dimitri Pchejetski,² Nathalie Saint-Laurent,¹ Leyre Brizuela,² Céline Guilbeau-Frugier,³ Marie-Bernadette Delisle,³ Olivier Cuvillier,² Christiane Susini,¹ and Corinne Bousquet¹

¹INSERM U858, I2MR, IFR31, ²CNRS, Institut de Pharmacologie et de Biologie Structurale, UMR5089, and ³Service d'Anatomie-Pathologique, Rangueil Hospital, Toulouse, France

Abstract

Defeating pancreatic cancer resistance to the chemotherapeutic drug gemcitabine remains a challenge to treat this deadly cancer. Targeting the sphingolipid metabolism for improving tumor chemosensitivity has recently emerged as a promising strategy. The fine balance between intracellular levels of the prosurvival sphingosine-1-phosphate (S1P) and the proapoptotic ceramide sphingolipids determines cell fate. Among enzymes that control this metabolism, sphingosine kinase-1 (SphK1), a tumor-associated protein overexpressed in many cancers, favors survival through S1P production, and inhibitors of SphK1 are used in ongoing clinical trials to sensitize epithelial ovarian and prostate cancer cells to various chemotherapeutic drugs. We here report that the cellular ceramide/S1P ratio is a critical biosensor for predicting pancreatic cancer cell sensitivity to gemcitabine. A low level of the ceramide/S1P ratio, associated with a high SphK1 activity, correlates with a robust intrinsic pancreatic cancer cell chemoresistance toward gemcitabine. Strikingly, increasing the ceramide/S1P ratio, by using pharmacologic (SphK1 inhibitor

or ceramide analogue) or small interfering RNA-based approaches to up-regulate intracellular ceramide levels or reduce SphK1 activity, sensitized pancreatic cancer cells to gemcitabine. Conversely, decreasing the ceramide/S1P ratio, by up-regulating SphK1 activity, promoted gemcitabine resistance in these cells. Development of novel pharmacologic strategies targeting the sphingolipid metabolism might therefore represent an interesting promising approach, when combined with gemcitabine, to defeat pancreatic cancer chemoresistance to this drug. [Mol Cancer Ther 2009;8(4):809–20]

Introduction

Pancreatic cancer ranks as the fifth leading cause of cancer-related death in western countries. The only curative treatment of pancreatic cancer is a surgical resection that is possible in only 10% to 15% of cases and unfortunately poorly improves patient survival (5% after 5 years). Conventional chemotherapy is relatively ineffective, this cancer being one of the most drug-resistant tumors (1). The use of the pyrimidine antimetabolite gemcitabine (2',2'-difluorodeoxycytidine, an analogue of deoxycytidine) as adjuvant chemotherapeutic drug for pancreatic cancer has emerged over the past decade. However, the primary benefit includes a palliation of disease symptoms but no significant survival benefit (2). Therefore, a rational strategy for future drug development is to identify new molecular targets to improve the clinical outcomes of this disease.

Sphingolipids have recently emerged as potent second messenger molecules controlling cellular responses to various prosurvival or stress stimuli. Ceramide, sphingosine, and sphingosine-1-phosphate (S1P) are interconvertible lipids that mostly compose the sphingolipid metabolism. Ceramide and sphingosine levels are up-regulated on cell treatment with different cytokines, anticancer drugs, and other stress-causing agonists and in turn mediate cell growth arrest and apoptosis via the regulation of various signaling pathways and subsequent caspase activation (3). On the contrary, S1P, a further metabolite of ceramide, is a growth promoter and survival factor, acting by up-regulating several antiapoptotic pathways including phosphatidylinositol 3-kinase or nuclear factor- κ B (NF- κ B; ref. 4). During cellular metabolism, ceramide is converted into sphingosine that, in turn, is phosphorylated by a sphingosine kinase (SphK; two isoforms exist, SphK1 and SphK2) to form S1P. Importantly, phosphorylation of sphingosine is a rate-limiting step in the sphingolipid metabolism; thus, the activity of SphK is crucial for maintaining the balance between proapoptotic and prosurvival signaling lipids. Consistently, we have introduced the concept of the "sphingolipid

Received 6/24/08; revised 11/19/08; accepted 1/2/09.

Grant support: Association pour la Recherche Contre le Cancer grant 3899, Ligue Nationale Contre le Cancer grant RAB08004BBA, Canceropole Grand Sud-Ouest grant RPT08004BBA, and University Paul Sabatier de Toulouse grant CR27 A01BQR-2007; French government fellowship (J. Guillermet-Guibert); Lilly Laboratories, Club Français du Pancréas, and Ligue Nationale Contre le Cancer fellowships (L. Davenne); and Association Etudes et Recherches Urologiques (D. Pchejetski).

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Note: J. Guillermet-Guibert, L. Davenne, and D. Pchejetski contributed equally to this work.

Requests for reprints: Corinne Bousquet, INSERM U858, I2MR, IFR31, Cancer Department, CHU Rangueil, Bât L3, 1 avenue Jean Poulhès, 31432 Toulouse, France. Phone: 33-5-61-32-36-02; Fax: 33-5-61-32-24-03. E-mail: corinne.bousquet@inserm.fr

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doi:10.1158/1535-7163.MCT-08-1096

biostat" whereby the dynamic balance between intracellular S1P versus sphingosine and ceramide levels, and the signaling pathways that control this balance, are critical factors that determine whether a cell survives or dies (5). SphK1 plays a critical role in the regulation of this balance toward proliferation and survival (6). SphK1 is a tumor-associated enzyme whose expression is increased in various human (7) and mice (8) tumors. Strikingly, anti-SphK1 therapies [anti-SphK1-based small interfering RNA (siRNA) or pharmacologic inhibition methods] have proven their efficacy to kill some cancer cell lines whether or not they are sensitive to conventional chemotherapy or radiotherapy, making this enzyme a very appealing candidate for anticancer therapy (7, 9, 10). Consistently, antibodies targeting S1P have been recently shown to have a significant antineoplastic potential (11), and clinical trials using inhibitors of SphK1 in combination with chemotherapeutic treatments are ongoing for chemotherapy-resistant ovarian cancers and hormone-refractory prostate cancers. Recently, we and other groups have correlated resistance to anticancer therapies of several cancer cell lines with a defect in both ceramide production and/or SphK1 inhibition (9, 12, 13). In these conditions, the concept that SphK1 inhibition might sensitize resistant cancer cells to these inefficient therapies has emerged.

The role of sphingolipid metabolites and SphK1 in pancreatic cancer development and progression is unknown. The present study was therefore undertaken to investigate whether a dysregulation of the sphingolipid biostat in pancreatic cancer cells is involved in their resistance toward conventional chemotherapies and whether affecting this fine balance reverts this resistance.

Materials and Methods

Cell Lines

Human pancreatic cancer cells BxPC-3 and Panc-1 were cultured at 37°C in humidified air and 5% CO₂ in DMEM supplemented with 7.5% FCS, 2 mmol/L L-glutamine, 5 units/mL streptomycin/penicillin, 250 ng/mL amphotericin B (Invitrogen), and 2.5 µg/mL Plasmocin (Invivogen). FLAG epitope-tagged human wild-type SphK1 cDNA (6) or pcDNA-3 empty vector were used for stable transfection of BxPC-3 cells by PEI reagent (Euromedex). Pools of stable transfectants were selected with 600 µg/mL G418 (Invivogen).

Reagents

Gemcitabine (Gemzar) was obtained from Lilly Laboratories. [γ -³²P]ATP was purchased from Perkin-Elmer and silica gel TLC (Partisil LK6D) plates were from Whatman International. SphK1 inhibitor SKI and *Escherichia coli* diacylglycerol kinase were obtained from Calbiochem. C2-ceramide was purchased from Sigma.

Cell Growth Assay

Mock- and SphK1-transfected BxPC-3 cells were plated in 35 mm diameter dishes (10⁵ per dish) in DMEM containing 7.5% serum. Cells were cultured for 48 h and cell proliferation was measured by cell counting using Coulter ZI counter model (Coultronics France).

Cell Viability Assay

Cells were grown (10⁴ per well, 96-well dish) and treated as indicated. Mitochondrial viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) colorimetric assay.

RNA Interference

Transient gene silencing of human SphK1 was done using double-stranded SphK1 siRNA 5'-GGGCAAGGCCUUG-CAGCUCd(TT)-3' and 5'-GAGCUGCAAGGCCUUGCCCCd(TT)-3' or control siRNA 5'-UUCUCCGAACGUGUCAC-GUd(TT)-3' and 5'-ACGUGACACGUUCGGAGAAd(TT)-3' (Qiagen; ref. 14) using Jet SI (Polyplus Transfection) according to the manufacturer's instructions.

Preparation of Whole-Cell Extracts and Western Blot Analysis

Cells were lysed in 50 mmol/L Tris-HCl (pH 7.4)/100 mmol/L NaCl/1 mmol/L EDTA/1.5% CHAPS/1 mmol/L DTT/protease inhibitors (Complete EDTA-free cocktail; Roche). After a rotative incubation of 15 min at 4°C, samples were centrifuged at 10,000 × g for 10 min at 4°C. Soluble proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane (Pall). After probing with cleaved caspase-3 antibody (Cell Signaling) and a horseradish peroxidase-conjugated secondary antibody (ImmunoPure goat anti-rabbit IgG; Pierce), the protein bands were detected by enhanced chemiluminescence (Pierce). Blotting with β -tubulin antibody (monoclonal anti- β -tubulin antibody; Sigma) was used as a loading control.

SphK1 mRNA Level Quantification by Real-time Quantitative Reverse Transcription-PCR

Total RNA was extracted with the RNeasy Kit (Qiagen) according to the manufacturer's instructions. After DNase treatment, total RNA were reverse transcribed using random hexamer primers (Fermentas). Resulting cDNAs were used in a real-time quantitative PCR using SYBR Green as a dye (Applied Biosystems) and specific primers for 18S rRNA (forward 5'-AAACGGCTACCACATCCAAG-3' and reverse 5'-CCTCCAATGGATCCTCGTTA-3') and SphK1 (forward 5'-CTGGCAGCTTCCTTGAACCAT-3' and reverse 5'-TGTGCAGAGACAGCAGGTTCA-3'). PCR primer efficiencies were measured by preparing a standard curve for each primer pair (efficiency >85%). Target gene expression was normalized using the 18S rRNA as control. Samples incubated without reverse transcriptase were used as negative template controls.

SphK1 Assay and Dosages of Sphingolipids

SphK1 activity was done as described previously (15). Briefly, cells were harvested and lysed by freeze-thawing in buffer A [20 mmol/L Tris (pH 7.4), 20% glycerol, 1 mmol/L β -mercaptoethanol, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 40 mmol/L β -glycerophosphate, 15 mmol/L NaF, 10 µg/mL leupeptin, aprotinin and soybean trypsin inhibitor, 1 mmol/L phenylmethylsulfonyl fluoride, and 0.5 mmol/L 4-deoxy pyridoxine]. SphK activity was determined in the presence of 50 µmol/L

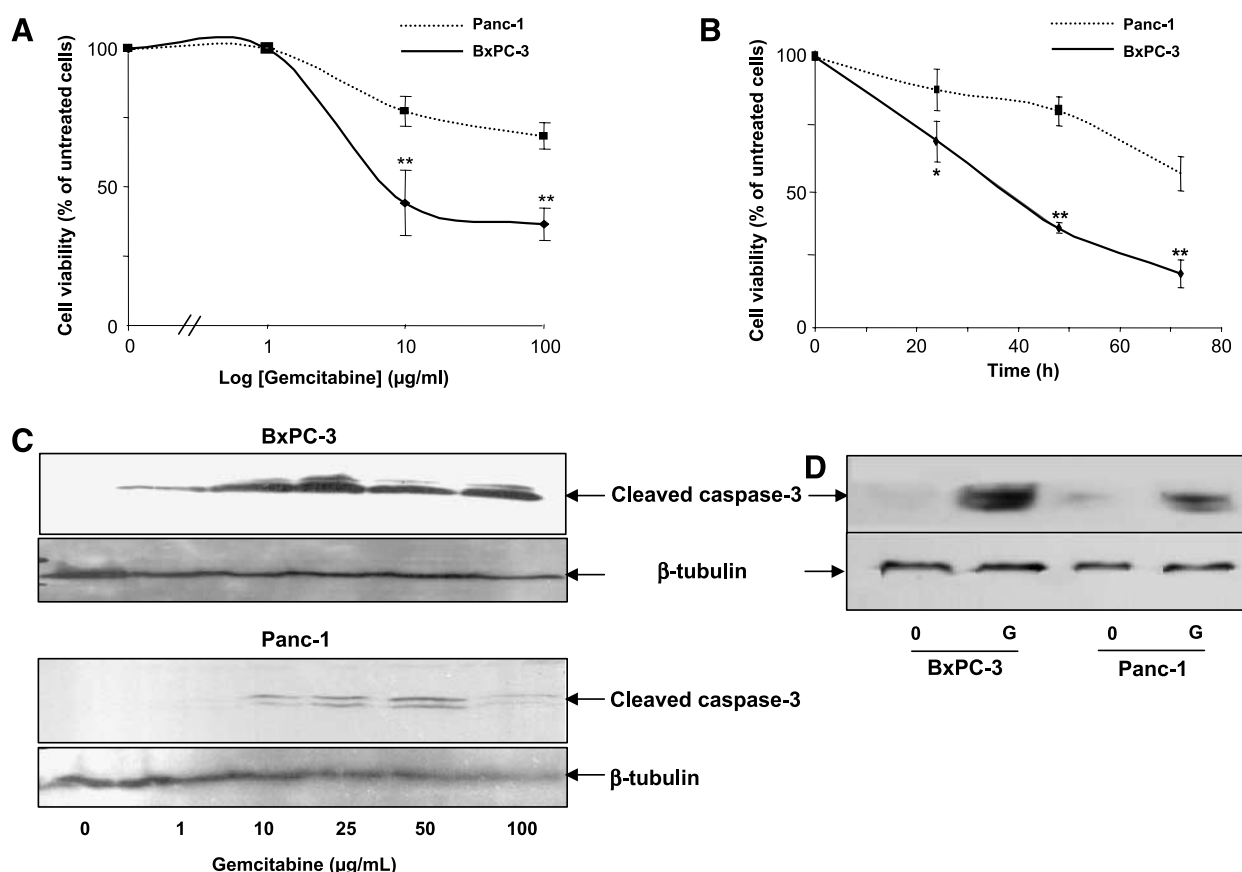


Figure 1 Gemcitabine differentially regulates cell viability and caspase-3 cleavage in BxPC-3 and Panc-1 cells. BxPC-3 and Panc-1 cells were incubated in the presence of gemcitabine (G; 0–100 µg/mL) for 48 h (A, C, and D) or 100 µg/mL gemcitabine for the indicated times (B). A and B, cell viability as measured in a MTT assay. Representative of at least three independent experiments, expressed as 100% of the respective untreated cells. *, $P < 0.05$; **, $P < 0.01$, gemcitabine-treated Panc-1 versus BxPC-3 cells. C and D, immunoblots using an anti-cleaved caspase-3 antibody (top) or an anti-β-tubulin antibody (bottom). Representative of three independent experiments. Expression of β-tubulin was used as an internal loading control.

sphingosine, 0.25% Triton X-100, and [γ - 32 P]ATP (10 µCi, 1 mmol/L) containing 10 mmol/L MgCl₂. Radiolabeled S1P was separated by TLC on silica gel G60 with 1-butanol/ethanol/acetic acid/water (80:20:10:20, v/v) and visualized by autoradiography. SphK 1-specific activity was expressed as pmol S1P formed/min/mg protein.

Mass amounts of ceramide were measured by the *E. coli* enzyme diacylglycerol kinase. [γ - 32 P]ceramide 1-phosphate was extracted, resolved from other reaction products by TLC using chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, v/v) as developing solvent, and quantified by liquid scintillation counting. Intracellular S1P content was measured as described by Edsall and Spiegel (16). Briefly, buffer C [200 mmol/L Tris-HCl (pH 7.4), 75 mmol/L MgCl₂ in 2 mol/L glycine (pH 9.0)] was added to the aqueous phase of Folch extraction (1:6, v/v) and S1P was dephosphorylated by addition of 50 units/sample alkaline phosphatase (BD Biosciences) for 30 min at 37°C. Reaction was stopped by addition of concentrated HCl and organic phase containing sphingosine was separated and evaporated. Evaporated sphingosine was resuspended in "SphK" buffer with 0.25% Triton X-100. Sphingosine was converted

into S1P by addition of cytosolic extracts of SphK1 and [γ - 32 P]ATP (10 Ci, 1 mmol/L) containing 10 mmol/L MgCl₂.

Immunohistochemistry

High-density (207 cores) multiple pancreatic cancer and normal pancreatic tissue array (US Biomax, Euromedex) was used for immunohistochemistry with a rabbit anti-SphK1 antibody (1:200; gift from Dr. Pitson, Institute of Medical and Veterinary Science; ref. 17).

Statistical Analysis

Statistical analysis was done by unpaired *t* test. All values are mean $\pm$ SE.

Results

Panc-1 Cells Are More Resistant Than BxPC-3 Cells to Gemcitabine-Induced Cell Death

To explore whether pancreatic cancer cell resistance toward the chemotherapeutic drug gemcitabine correlates with a dysregulation of the sphingolipid metabolism, the two human pancreatic cancer BxPC-3 and Panc-1 cell lines were chosen for their relative resistance to gemcitabine. A cell viability assay using increasing concentrations of

gemcitabine (0–100 $\mu\text{g/mL}$) for 48 h showed a greater resistance to gemcitabine of Panc-1 than BxPC-3 cells (Fig. 1A). A time-course treatment of both cell lines using a high dose of gemcitabine (100 $\mu\text{g/mL}$) confirmed greater resistance of Panc-1 than BxPC-3 cells to gemcitabine (Fig. 1B). Gemcitabine cytotoxicity in pancreatic cancer cells results from its proapoptotic action, acting on the cleavage and activation of the executioner caspase-3 (18). Therefore, caspase-3 cleavage was explored in both BxPC-3 and Panc-1 cells treated with increasing concentrations of gemcitabine (Fig. 1C). Whereas cleavage of caspase-3 was visualized with a gemcitabine concentration as low as 1 $\mu\text{g/mL}$ in BxPC-3 cells, a 10-fold higher concentration was necessary to detect this cleavage in Panc-1 cells. Furthermore, caspase-3 was more potently cleaved by 100 $\mu\text{g/mL}$ gemcitabine treatment in BxPC-3 compared with Panc-1 cells (Fig. 1D), confirming the difference in responsiveness to gemcitabine in terms of cell death activation of both cell lines.

Intracellular Ceramide/S1P Ratio Correlates with Pancreatic Cancer Cell Responsiveness to Gemcitabine

Alterations in the balance between the proapoptotic ceramide and oncogenic S1P sphingolipids have been reported in cancer cells. Furthermore, the ratio between these two

lipids has been shown to determine cell fate and to be possibly an indicator of chemoresistance (19). Ceramide and S1P levels, as well as SphK1 activity, were therefore measured in BxPC-3 and Panc-1 cells (Fig. 2). At the basal level (without addition of gemcitabine), ceramide concentration was 2.5-fold lower (Fig. 2A) and S1P 1.8-fold higher (Fig. 2B) in Panc-1 compared with BxPC-3 cells. As a consequence, the ceramide/S1P ratio was 2.2 ± 0.4 -fold lower in Panc-1 than in BxPC-3 cells (Fig. 2C). SphK1, which is overexpressed and/or excessively activated in many cancers, critically affects the sphingolipid ceramide/S1P rheostat by allowing the production of S1P from sphingosine, which is itself a product of degradation of ceramide by a ceramidase. SphK1 activity was assessed in both BxPC-3 and Panc-1 cells. As shown in Fig. 2D, SphK1 activity was increased by 2 ± 0.4 -fold in Panc-1 versus BxPC-3 cells. However, no difference between both cell lines was observed in terms of SphK1 mRNA expression as assessed by real-time quantitative reverse transcription-PCR (Fig. 2E). We therefore hypothesized that Panc-1 cell resistance to gemcitabine might result from a dysregulation of the sphingolipid ceramide/S1P ratio, which might be the consequence of an excess of SphK1 activation in these cells balancing this ratio toward S1P production. This hypothesis

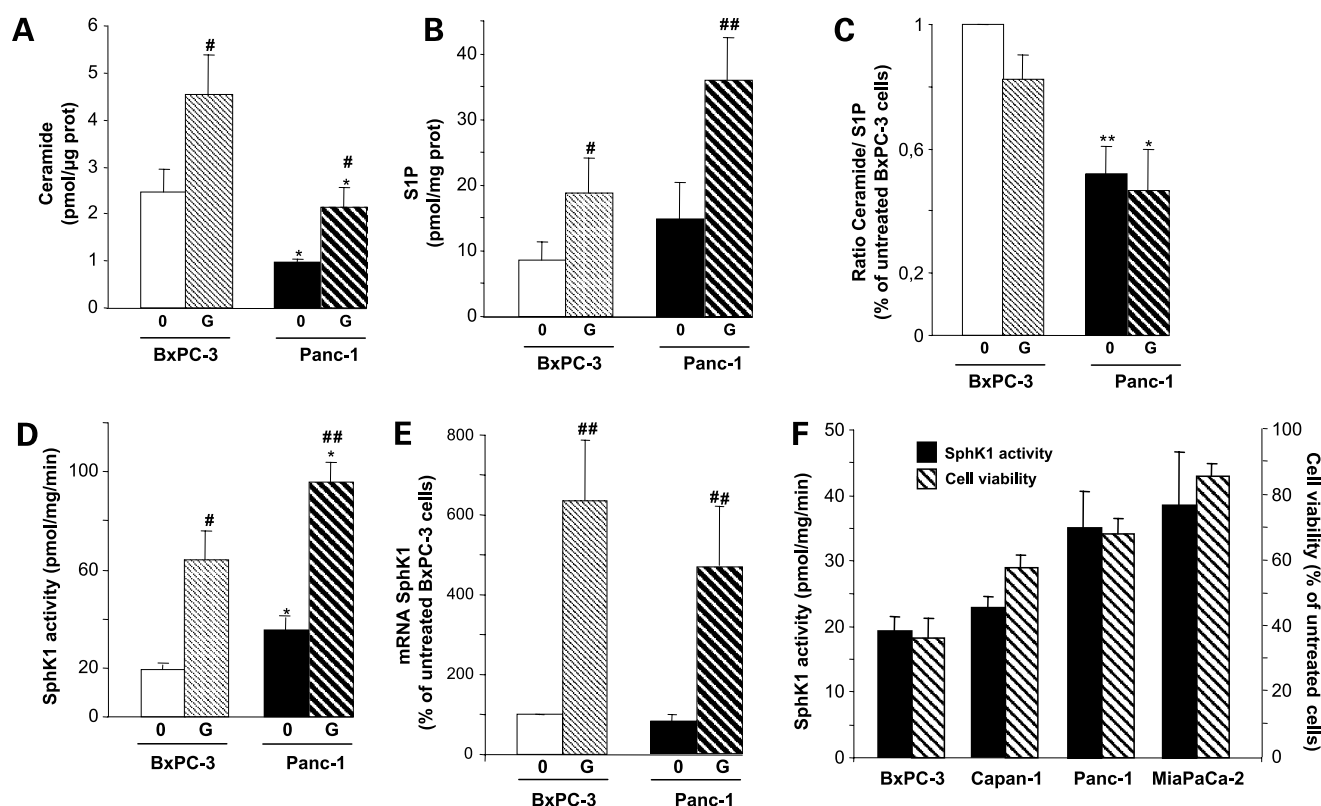


Figure 2 Differences in the sphingolipid metabolism between pancreatic cancer cell lines. **A** to **E**, BxPC-3 and Panc-1 cells were treated or not for 48 h with 100 $\mu\text{g/mL}$ gemcitabine. Ceramide (**A**) and S1P (**B**) levels and SphK1 activity (**D**) were assessed as described in Materials and Methods. Representative of four independent experiments. Ceramide/S1P ratios (**C**) are expressed as normalized with the ratio observed in untreated BxPC-3 cells (ratio = 1). **E**, SphK1 mRNA levels were quantified by quantitative reverse transcription-PCR and results are expressed as normalized with mRNA level observed in untreated BxPC-3 cells (100%). Representative of four independent experiments. **F**, BxPC-3, Capan-1, Panc-1, and MiaPaCa-2 cells were treated or not for 48 h with 100 $\mu\text{g/mL}$ gemcitabine, and SphK1 activity (left Y axis) and cell viability (MTT assay; right Y axis) were assessed. *, $P < 0.05$; **, $P < 0.01$, Panc-1 versus BxPC-3 cells. #, $P < 0.05$; ##, $P < 0.01$, gemcitabine-treated versus untreated cells.

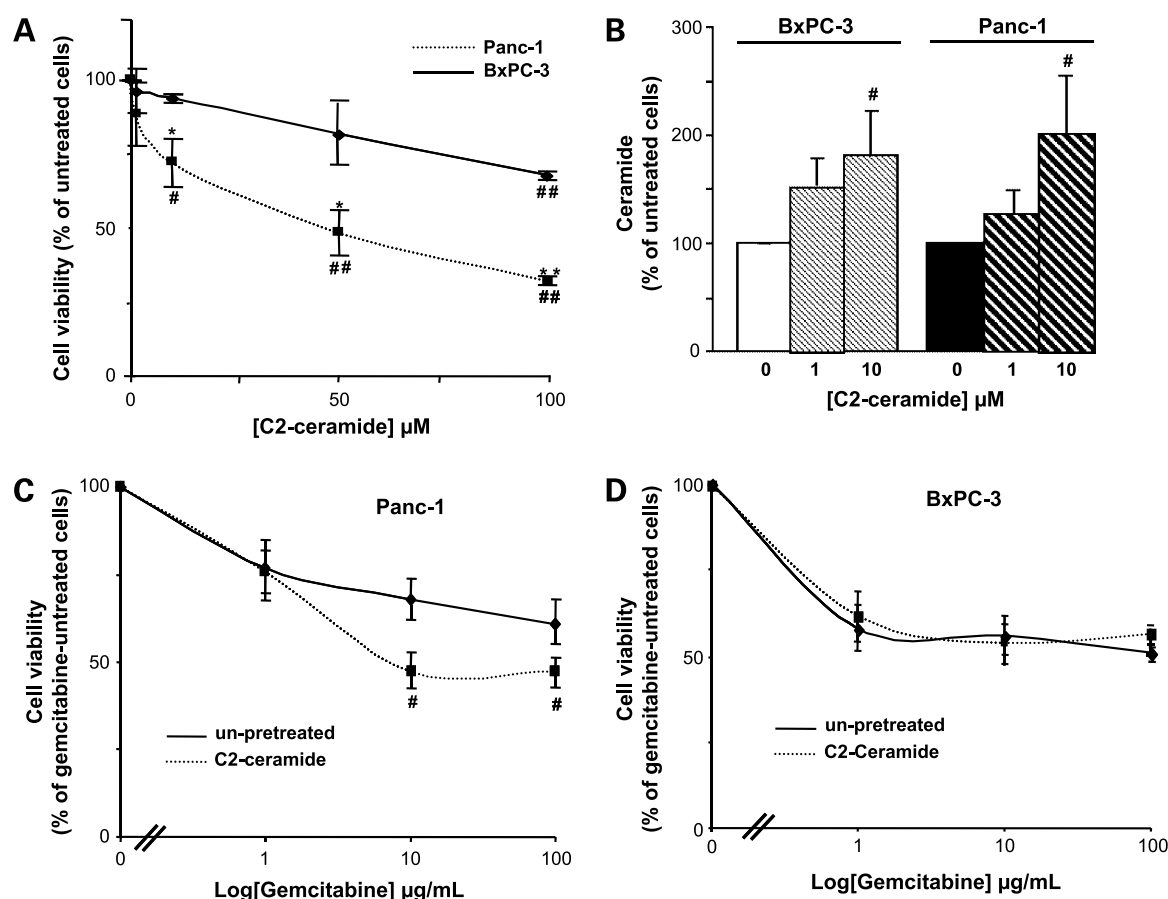


Figure 3 Cell treatment with exogenous ceramide (C2-ceramide) inhibits BxPC-3 and Panc-1 cell survival and sensitizes Panc-1 cells to gemcitabine. **A** and **B**, BxPC-3 and Panc-1 cells were treated for 48 h with C2-ceramide (0–100 μ M/L). Cell viability was assessed by MTT assay (**A**) and ceramide levels (**B**) were assessed as described. Results are expressed as 100% of the respective untreated cells. Representative of three independent experiments. Panc-1 (**C**) and BxPC-3 (**D**) cells were pretreated or not for 24 h with 1 μ M/L C2-ceramide and then treated for 48 h with gemcitabine (0–100 μ g/mL). C2-ceramide treatment increased intracellular ceramide levels from 1.73 ± 0.37 to 2.51 ± 0.22 pmol/ μ g proteins in BxPC-3 cells and from 0.82 ± 0.03 to 1.06 ± 0.06 pmol/ μ g proteins in Panc-1 cells. Cell viability was assessed by MTT assay. Results are expressed as 100% of the gemcitabine-untreated cells. Representative of four independent experiments. *, $P < 0.05$; **, $P < 0.01$, Panc-1 versus BxPC-3 cells. #, $P < 0.05$; ##, $P < 0.01$, C2-ceramide pretreated versus non-pretreated cells.

was confirmed by correlating pancreatic cancer cell resistance to gemcitabine with SphK1 activity in two additional human pancreatic cancer cell lines, including Capan-1 and MiaPaCa-2 (Fig. 2F). Interestingly, pancreatic cancer cell resistance to gemcitabine correlated with SphK1 activity; the higher was this SphK1 activity, the more resistant (and the more viable) were the cells to gemcitabine treatment.

Gemcitabine Does Not Affect the Sphingolipid Ceramide/S1P Ratio in Pancreatic Cancer Cells

Cancer cells that have aberrant ceramide metabolism (no ceramide generation) in response to chemotherapy might acquire resistance to these drugs (20). We therefore investigated whether ceramide metabolism is differently affected by gemcitabine treatment in BxPC-3 and Panc-1 cells. Cell treatment with 100 μ g/mL gemcitabine similarly increased ceramide concentrations by 1.9 ± 0.1 - and 2.2 ± 0.3 -fold in BxPC-3 and Panc-1 cells, respectively (Fig. 2A), which could therefore not account for the difference in cell responsiveness to gemcitabine observed between both cell lines. Sur-

prisingly, gemcitabine treatment also increased, to a similar extent in both BxPC-3 and Panc-1 cells, S1P concentrations (by 2.3 ± 0.1 - and 2.7 ± 0.3 -fold, respectively; Fig. 2B), SphK1 activity (by 3.3 ± 0.3 - and 2.8 ± 0.3 -fold, respectively; Fig. 2D), and SphK1 mRNA expression (by 6.3 ± 1.5 - and 6 ± 1.8 -fold, respectively; Fig. 2E). As a consequence, the sphingolipid ceramide/S1P ratio was not significantly affected in both BxPC-3 and Panc-1 cells by gemcitabine and was also lower (2.4 ± 0.8 -fold) in gemcitabine-treated Panc-1 than BxPC-3 cells ($P < 0.01$; Fig. 2C).

These results suggest that difference in gemcitabine cell resistance observed between the two human pancreatic cancer BxPC-3 and Panc-1 cell lines is not a consequence of a different gemcitabine response on the sphingolipid metabolism.

Up-Regulating Ceramide Concentration Sensitizes Panc-1 Cells to Gemcitabine-Induced Cell Death

We then investigated whether the difference in cell resistance to gemcitabine observed between Panc-1 and BxPC-3

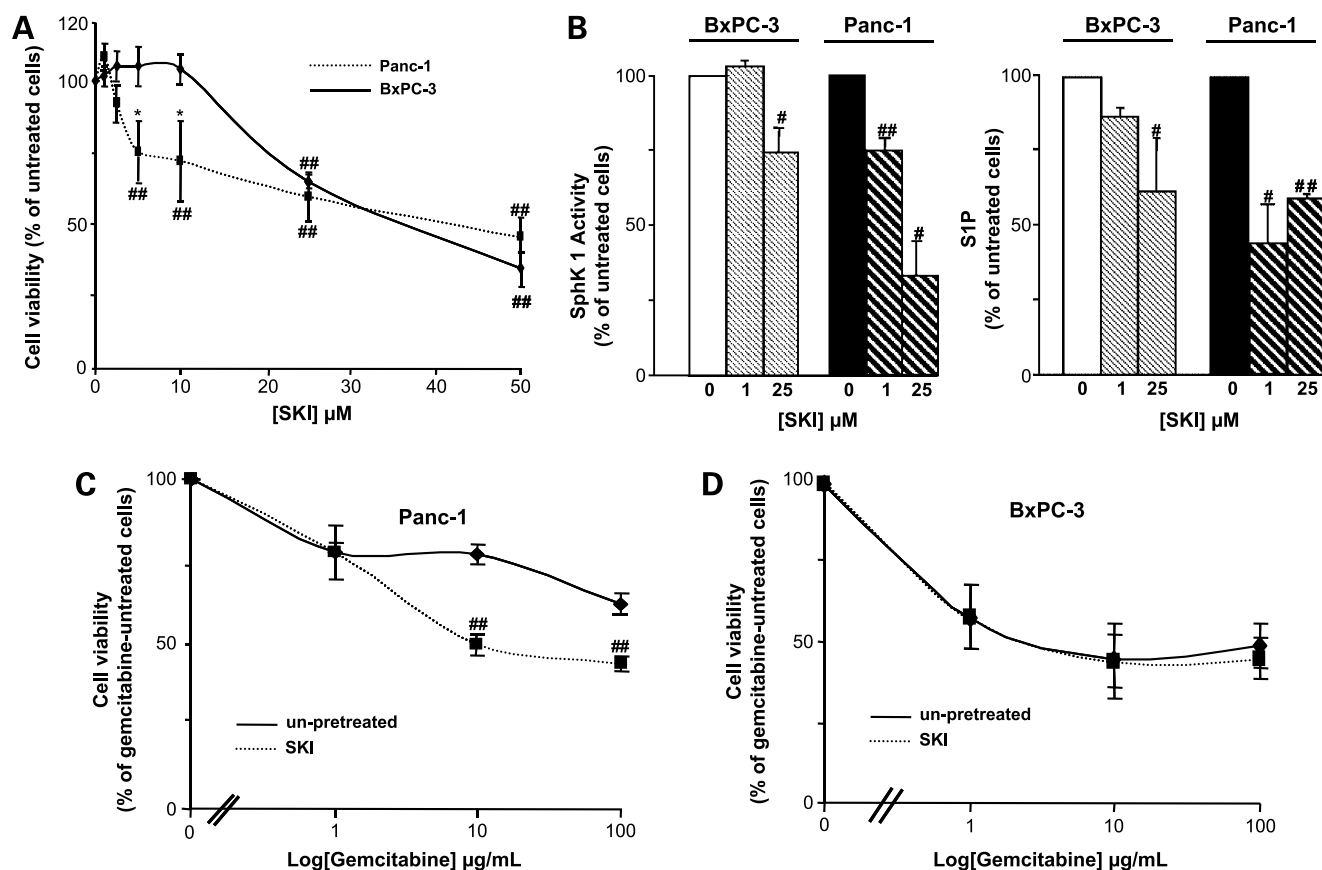


Figure 4 Cell treatment with a SKI inhibits BxPC-3 and Panc-1 cell survival and sensitizes Panc-1 cells to gemcitabine. **A** and **B**, BxPC-3 and Panc-1 cells were treated with SKI (0–50 μ M) for 72 h. Cell viability was assessed by MTT assay (**A**) and SphK1 activity and S1P levels (**B**) were assessed as described. Results are expressed as 100% of the respective untreated cells. Representative of three independent experiments. Panc-1 (**C**) and BxPC-3 (**D**) cells were pretreated or not for 24 h with 1 μ M SKI and then treated for 48 h with gemcitabine (0–100 μ g/mL). SKI treatment decreased intracellular S1P levels from 11.74 ± 2 to 10.11 ± 0.31 pmol/mg proteins in BxPC-3 cells and from 20.46 ± 1.69 to 9.07 ± 2.78 pmol/mg proteins in Panc-1 cells. Cell viability was assessed by MTT assay. Results are expressed as 100% of the gemcitabine-untreated cells. Representative of four independent experiments. *, $P < 0.05$; **, $P < 0.01$, Panc-1 versus BxPC-3 cells. #, $P < 0.05$; ##, $P < 0.01$, SKI pretreated versus non-pretreated cells.

cells results from an intrinsic difference in the sphingolipid metabolism. To this goal, we asked first whether up-regulating the ceramide/S1P ratio affects pancreatic cancer cell resistance to gemcitabine. BxPC-3 and Panc-1 cells were treated with increasing concentrations of a cell-permeable synthetic short-chain ceramide analogue, C2-ceramide (*N*-acetyl-sphingosine). Synthetic short-chain ceramide analogues have been reported to trigger cell death in cancer cells and to be sometimes active on drug-resistant cancer cells (21). Interestingly, pancreatic cancer cell sensitivity to gemcitabine and C2-ceramide was inversely correlated (Fig. 3A). Whereas a concentration as low as 10 μ M/L C2-ceramide significantly decreased Panc-1 cell survival, a 10-fold higher concentration of this analogue (100 μ M/L) was necessary to significantly and comparatively affect BxPC-3 cell viability while inducing a potent inhibition of Panc-1 cell survival. As expected, this C2-ceramide-induced cell death was associated with an increase in ceramide concentration in both cell lines (Fig. 3B) while having no effect on SphK1 activity or on S1P concentration (data not

shown). To test whether increasing the ceramide concentration in pancreatic cancer cells affects their responsiveness to gemcitabine, both BxPC-3 and Panc-1 cells were pretreated with a low concentration of C2-ceramide (1 μ M/L) and then treated with increasing concentrations of gemcitabine (0–100 μ g/mL). In C2-ceramide pretreated Panc-1, but not BxPC-3, cell survival was significantly decreased by gemcitabine compared with the non-pretreated cells and reached levels of cell viability observed, for the same concentrations of gemcitabine, in gemcitabine-treated BxPC-3 cells whether or not pretreated with C2-ceramide (compare Fig. 3C and D). These results indicate that up-regulating the ceramide/S1P ratio by increasing ceramide concentration in the gemcitabine-resistant Panc-1 cells sensitizes these cells to this chemotherapeutic drug cytotoxicity.

Down-Regulating SphK1 Activity Sensitizes Panc-1 Cells to Gemcitabine-Induced Cell Death

To further investigate whether up-regulating the ceramide/S1P ratio affects pancreatic cancer cell sensitivity to gemcitabine, SphK1 activity was inhibited by using either

a commercial pharmacologic inhibitor (SKI; Fig. 4) or an anti-SphK1-based RNA interference method (Fig. 5A-C).

As observed with the C2-ceramide analogue, at concentrations of SKI up to 25 $\mu\text{mol/L}$, Panc-1 cells were more sensitive than BxPC-3 cells to SKI-induced cell death. At concentrations of $\geq 25 \mu\text{mol/L}$, SKI was equally potent to inhibit cell survival in both cell lines (Fig. 4A). SphK1 activity and S1P concentration were assessed to check the efficacy of SKI, which confirmed a significant inhibition by SKI of SphK1 activity and S1P concentrations in both BxPC-3 and Panc-1 cells, whereas ceramide concentrations were not affected (data not shown; Fig. 4B). To test whether inhibiting SphK1 activity and consequently decreasing S1P concentration in pancreatic cancer cells affects their sensitivity to gemcitabine, both BxPC-3 and Panc-1 cells were pretreated with a low concentration of SKI (1 $\mu\text{mol/L}$) and then treated with increasing concentrations of gemcitabine (0-100 $\mu\text{g/mL}$; Fig. 4C and D). SKI

pretreatment did not affect BxPC-3 cell sensitivity to gemcitabine (Fig. 4D), which is consistent with an absence of inhibition of SphK1 activity or S1P concentration in BxPC-3 cells with this low dose of SKI (Fig. 4B). By contrast, 1 $\mu\text{mol/L}$ SKI decreased SphK1 activity and S1P levels in Panc-1 cells (Fig. 4B), which is consistent with a significant gemcitabine-mediated decrease of cell survival of these SKI-pretreated Panc-1 cells (Fig. 4C). In these conditions, Panc-1 cell survival reached levels of cell viability observed, for the same concentrations of gemcitabine, in gemcitabine-treated BxPC-3 cells whether or not pretreated with SKI (compare Fig. 4C and D). These results further confirm that up-regulating the ceramide/S1P ratio by down-regulating S1P concentration in the gemcitabine-resistant Panc-1 cells sensitizes these cells to this chemotherapeutic drug.

This hypothesis was further assessed by using a RNA interference method to inhibit SphK1 activity (Fig. 5A-C).

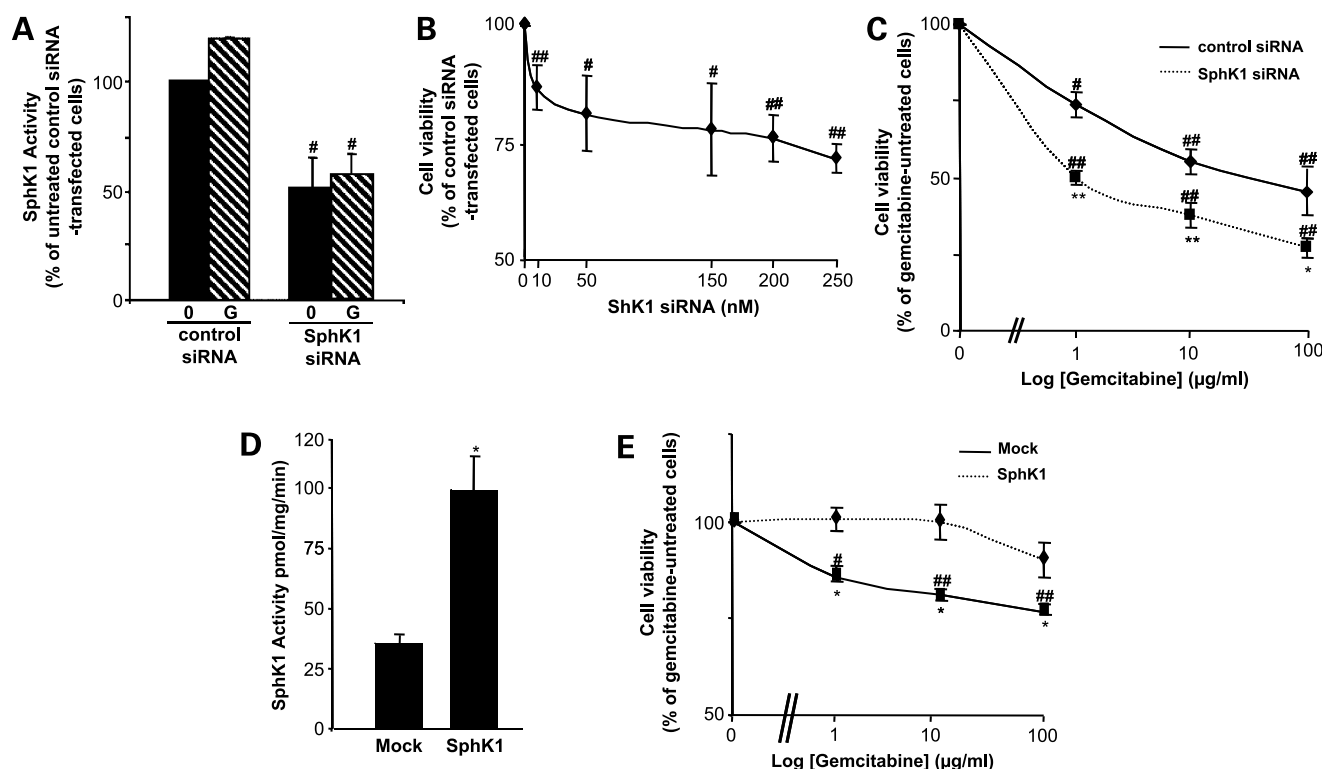


Figure 5 Decreasing or, conversely increasing, SphK1 activity sensitized, or rendered Panc-1 cells more resistant, respectively, to gemcitabine. **A** to **C**, Panc-1 cells were transfected with a SphK1 or control siRNA. **A**, SphK1 activity was assessed on Panc-1 cells transfected for 48 h with 100 nmol/L SphK1 or control siRNA and then treated or not for 48 h with 100 $\mu\text{g/mL}$ gemcitabine. Results are expressed as the percentage of the SphK1 activity measured in gemcitabine-untreated control siRNA-transfected cells (100%). Representative of three independent experiments. **B**, Panc-1 cells were transfected with increasing concentrations of SphK1 or control siRNA (0-250 nmol/L). Cell viability was assessed at 96 h of culture by MTT assay. Results are expressed as the percentage of the cell viability observed in SphK1 versus control siRNA-transfected cells for the same concentration of siRNA used and are normalized as 100% of cell survival observed in control siRNA-transfected cells. Representative of five independent experiments. **C**, Panc-1 cells were transfected for 48 h with 50 nmol/L SphK1 or control siRNA and then treated for 48 h with gemcitabine (0-100 $\mu\text{g/mL}$). Cell viability was assessed by MTT assay. Results are expressed as the percentage of the cell viability observed in the respective gemcitabine-untreated control or SphK1-transfected cells (100%). Representative of six independent experiments. #, $P < 0.05$; ##, $P < 0.01$, SphK1 versus control siRNA-transfected Panc-1 cells. **D** and **E**, Panc-1 cells were transfected with a SphK1 cDNA. **D**, efficient SphK1 cDNA transfection in Panc-1 cells was checked by measuring SphK1 activity in mock- and SphK1-transfected Panc-1 cells. Representative of three independent experiments. **E**, mock- and SphK1-transfected Panc-1 cells were treated for 48 h with gemcitabine (0-100 $\mu\text{g/mL}$). Cell viability was assessed by MTT assay. Results are expressed as 100% of the respective gemcitabine-untreated control or SphK1-transfected cells. Representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$, SphK1 versus mock-transfected cells. #, $P < 0.05$; ##, $P < 0.01$, gemcitabine-treated versus untreated cells.

Efficacy of the SphK1 siRNA treatment was confirmed because, whether or not treated with gemcitabine, Panc-1 cells transfected with the SphK1 siRNA showed a significant decrease of the SphK1 activity (~2-fold) compared with control siRNA-transfected cells (Fig. 5A). Gemcitabine-resistant Panc-1 cells were then transfected with increasing concentrations (0-250 nmol/L) of the SphK1 or control siRNA, and SphK1 siRNA-transfected Panc-1 cell viability was measured as a percent of the control siRNA-transfected cells (Fig. 5B). A concentration as low as 10 nmol/L of SphK1 siRNA significantly inhibited Panc-1 cell survival, which was dose-dependently affected by the SphK1 siRNA treatment. Then, we investigated whether inhibiting SphK1 activity in Panc-1 cells sensitizes these cells to gemcitabine. Cell viability of SphK1 versus control siRNA-transfected Panc-1 cells was significantly decreased for all gemcitabine concentrations used (Fig. 5C). However, when using this same RNA interference approach to inhibit SphK1 activity in BxPC-3 cells, no cell sensitization to gemcitabine was obtained (data not shown), which is consistent with the absence of SKI-mediated BxPC-3 cell sensitization to gemcitabine (Fig. 4D). These results indicate that up-regulating the ceramide/S1P ratio by silencing SphK1 through either pharmacologic inhibition or RNA interference in the gemcitabine-resistant Panc-1 cells sensitizes these cells to this chemotherapeutic agent.

Up-Regulating SphK1 Activity Renders Panc-1 and BxPC-3 Cells More Resistant to Gemcitabine-Induced Cell Death

To investigate whether up-regulating SphK1 activity in pancreatic cancer cells might render them (more) resistant to gemcitabine, Panc-1 (Fig. 5D and E) and BxPC-3 (Fig. 6A-C) cells were transfected with the SphK1 cDNA. These cells showed an increase of SphK1 activity (Figs. 5D and 6A) and S1P concentration (data not shown) compared with mock cells. Strikingly and consistently with the results we observed in Fig. 2B and D, cell treatment with gemcitabine enhanced SphK1 activity and S1P concentration (data not shown) in both mock- and SphK1-transfected cells (Fig. 6A). We then explored whether increasing SphK1 activity affects Panc-1 and BxPC-3 cell survival and sensitivity to gemcitabine. SphK1-transfected BxPC-3 cells proliferated faster than mock cells (Fig. 6B), and both Panc-1 and BxPC-3 cells were more resistant to gemcitabine as assessed in a cell survival assay whereby cell viability of SphK1 versus mock-transfected cells was significantly increased on gemcitabine treatment (Figs. 5E and 6C for Panc-1 and BxPC-3 cells, respectively). These results indicate that down-regulating the ceramide/S1P ratio by increasing SphK1 activity (and as a consequence S1P level) in both Panc-1 and BxPC-3 cells renders these cells more resistant to this chemotherapeutic drug.

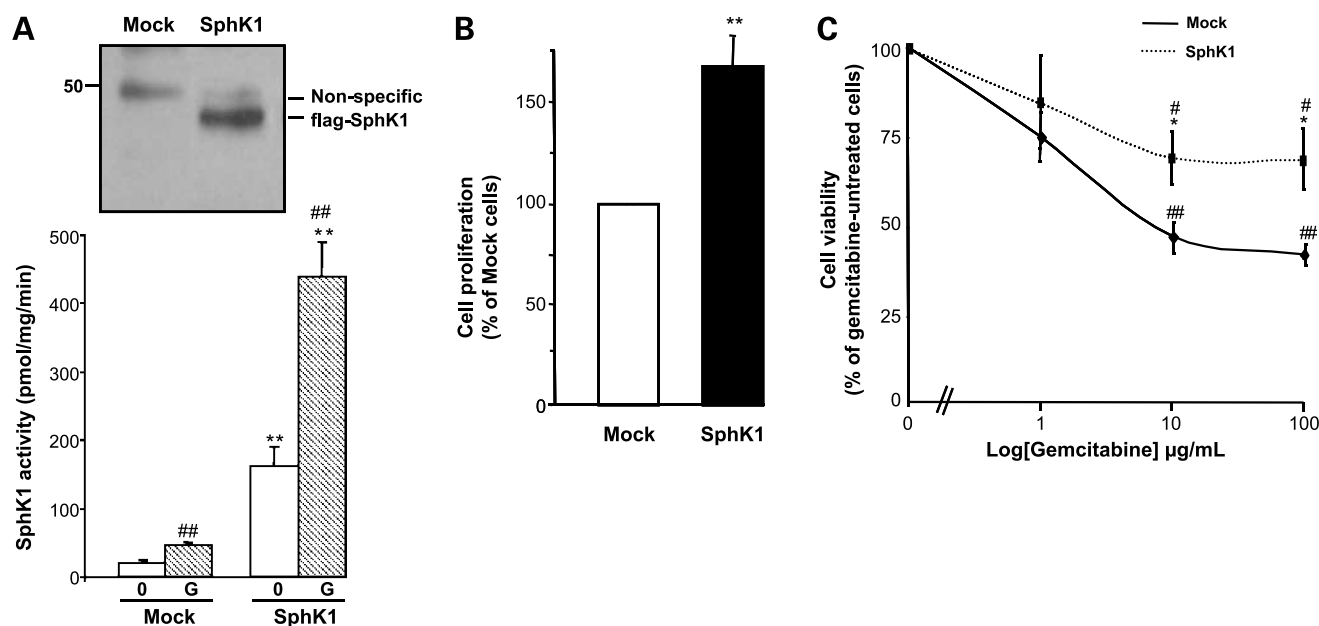


Figure 6 Increasing SphK1 activity sensitized BxPC-3 cells to gemcitabine. **A** to **C**, BxPC-3 cells were transfected with a SphK1 cDNA. **A**, efficient FLAG-tagged SphK1 cDNA transfection in BxPC-3 cells was checked by Western blot using an anti-FLAG antibody, and SphK1 activity was measured in mock- and SphK1-transfected BxPC-3 cells treated or not with 100 μg/mL gemcitabine. Representative of five independent experiments. **B**, mock- and SphK1-transfected BxPC-3 cell proliferation was assessed after 48 h of cell culture. Results are expressed as 100% of mock-transfected cells. Representative of 10 independent experiments. **C**, mock- and SphK1-transfected BxPC-3 cells were treated for 48 h with gemcitabine (0-100 μg/mL). Cell viability was assessed by MTT assay. Results are expressed as 100% of the respective gemcitabine-untreated control or SphK1-transfected cells. Representative of five independent experiments. *, $P < 0.05$; **, $P < 0.01$, SphK1 versus mock-transfected cells. #, $P < 0.05$; ##, $P < 0.01$, gemcitabine-treated versus untreated cells.

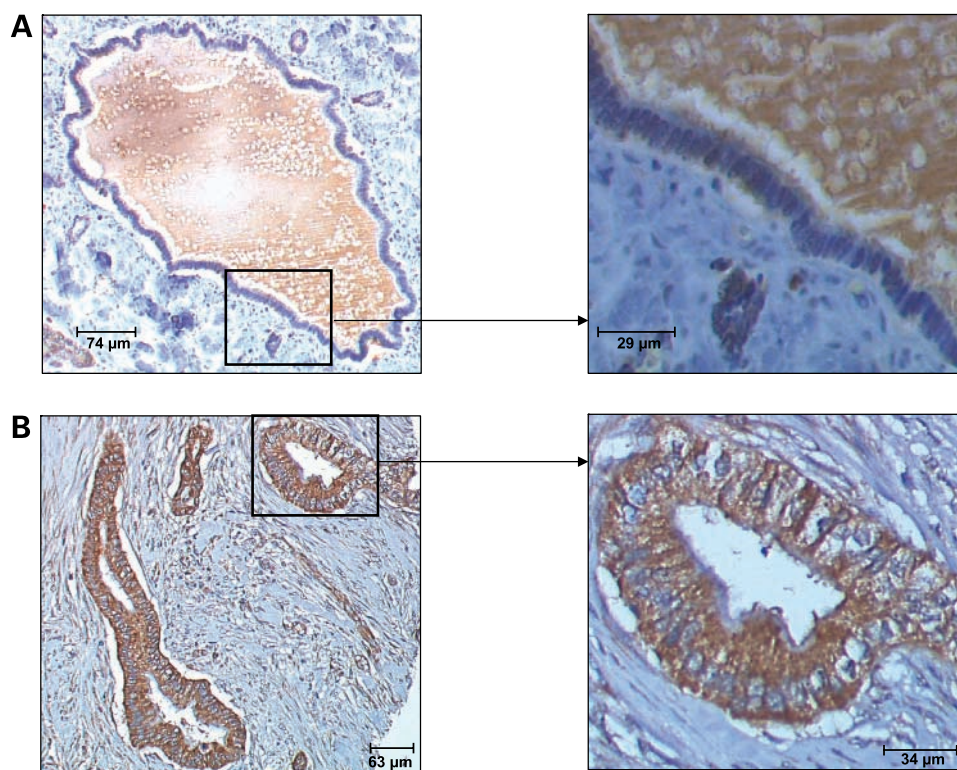


Figure 7 SphK1 expression is up-regulated in pancreatic adenocarcinoma ductal lesions versus normal ductal epithelium. Immunohistochemistry was done with the anti-SphK1 antibody using a high-density tissue array of normal pancreas (autopsy; $n = 10$; **A**) and pancreatic adenocarcinoma ($n = 60$; **B**). **A**, a major normal duct whereby ductal cells show only a faint staining for SphK1. **B**, an adenocarcinoma lesion whereby a robust immunostaining is present in all ductal cells.

SphK1 Expression Is Up-Regulated in Pancreatic Adenocarcinoma Ductal Lesions

Our results indicate that pancreatic cancer cell resistance to gemcitabine results from a dysregulation of the sphingolipid ceramide/S1P ratio. An excess of SphK1 activation in these cells, balancing this ratio toward S1P production, is shown to correlate with pancreatic cancer cell resistance to gemcitabine (Fig. 2F). SphK1 expression was therefore investigated by immunohistochemistry in pancreatic tissue arrays including normal pancreas (10 cases) and pancreatic adenocarcinoma (60 cases; Fig. 7A and B). Whereas a faint immunostaining for SphK1 was observed in ductal cells of normal pancreas, a robust immunostaining was present in ductal cells of all adenocarcinoma. Up-regulation of SphK1 expression in pancreatic cancer therefore represents a biologically relevant change strengthening that a dysregulation of the sphingolipid biostat in pancreatic cancer cells is possibly involved in their resistance toward the conventional gemcitabine chemotherapy.

Discussion

Our data provide novel breakthrough emphasizing the role of the sphingolipid metabolism in pancreatic cancer cell chemoresistance. Indeed, we here show that the intrinsic cellular ceramide/S1P ratio is a critical biosensor for predicting pancreatic cancer cell resistance to the chemotherapeutic gemcitabine drug. Strikingly, increasing this ratio improves cell sensitivity.

Compelling evidences suggest that ceramide is a critical determinant of cancer cell apoptotic response in response to many cytotoxic agents including chemotherapeutic drugs (22, 23). Alternatively, enhanced expression and/or activity of enzymes involved in the metabolism of ceramide, including SphK1 that produces the antiapoptotic S1P sphingolipid and decreases ceramide levels, contributes to intrinsic or acquired drug resistance of cancer cells (24, 25). Targeting the sphingolipid metabolism for improving tumor chemosensitivity has therefore emerged as a novel therapeutic approach.

Our results indicate that gemcitabine efficiently induces ceramide levels in pancreatic cancer cells independently on their degree of resistance to this drug. Consistently, generation of ceramide in response to gemcitabine, in the resistant Panc-1 cells, was shown to result from the activation of the acid sphingomyelinase, which hydrolyzes sphingomyelin to ceramide (26). Surprisingly, gemcitabine also increased, independent of pancreatic cancer cell sensitivity to this drug, the expression and activity of SphK1, which resulted in an increase of S1P production. As a consequence, the net ceramide/S1P ratio was overall not affected by this drug. The most resistant Panc-1 cells have indeed higher basal and gemcitabine-stimulated S1P levels than sensitive cells. Conversely, the most sensitive BxPC-3 cells have higher basal and gemcitabine-stimulated ceramide levels than resistant cells. This result indicated that the difference in gemcitabine sensitivity observed between resistant Panc-1 and

sensitive BxPC-3 cells does not result from a difference in the gemcitabine response but rather from an intrinsic difference in their cellular ceramide/S1P ratio. A threshold phenomenon of this ceramide/S1P ratio might be operative, which determines pancreatic cancer cell sensitivity or resistance to gemcitabine. Consistently, SphK1 activity, which critically controls this balance toward the production of S1P, was shown to correlate with pancreatic cancer cell resistance to gemcitabine, as we here show in four different human pancreatic cancer cell lines.

Interestingly, SphK1 mRNA is up-regulated in various human tumors compared with normal tissues (27). SphK1 protein expression was here shown to be specifically and robustly up-regulated in pancreatic adenocarcinoma ductal lesions compared with normal pancreatic ductal epithelium, confirming a specific dysregulation of the sphingolipid metabolism at the cancerous ductal cell level.

Interestingly, gemcitabine was here reported to increase SphK1 expression and activity in pancreatic cancer cells. By contrast, we showed previously that, in acute myeloid leukemic or prostatic cancer cells, chemotherapeutic drugs inhibit SphK1 activity, which was required and predictive for an efficient drug cytotoxicity (9, 14). Although mechanisms for gemcitabine-mediated increase of SphK1 expression and activity in pancreatic cancer cells are unknown, this induction may explain why the majority of human pancreatic cancers become resistant to gemcitabine on treatment with this drug. By up-regulating S1P levels, gemcitabine counteracts ceramide production also induced by this same drug, and the net ceramide/S1P ratio is maintained below the threshold whereby apoptosis could be initiated. In addition, it has recently been reported in leukemia cells that doxorubicin also up-regulates SphK1 expression and S1P production and excretion. It was suggested that this secreted S1P could serve as a "come-and-get-me" signal for scavenger cells to engulf chemotherapy-induced apoptotic cells to prevent necrosis (28).

Importantly, we have shown here that, by affecting the ceramide/S1P ratio through pharmacologic and molecular approaches, one can sensitize or render pancreatic cancer cell more resistant to gemcitabine. Both SphK1 inhibition by the SphK1 inhibitor SKI, which reduced S1P generation, and addition of the exogenous C2-ceramide analogue to cells increased the ceramide/S1P ratio, resulting in a decrease of Panc-1 cell viability, as reported for other cancer cells (3). More interestingly, a low (1 $\mu\text{mol/L}$) SKI or C2-ceramide concentration, which did not affect by themselves cell viability but decreased S1P generation or increased intracellular ceramide level, respectively, sensitized resistant Panc-1 cells to gemcitabine. However, higher concentrations of these pharmacologic drugs (up to 10 $\mu\text{mol/L}$) failed to sensitize these cells to gemcitabine (data not shown) probably because these doses already potentially reduced by themselves cell viability. These results are consistent with a previous report showing that addition of exogenous sphingomyelin (which generates cellular ceramide through the activity of a sphingomyelinase) also sensitized Panc-1 cells to gemcitabine (26). In further support of the critical role of

SphK1 in pancreatic cancer cell resistance to gemcitabine, we showed that molecular approaches to either down-regulate SphK1, with specific siRNA, or up-regulate it by overexpressing this protein sensitized Panc-1 cells or rendered BxPC-3 and Panc-1 cells more resistant to gemcitabine, respectively.

Chemotherapy using gemcitabine is now the standard treatment for advanced pancreatic cancer, although most human pancreatic cancer cells are resistant to this drug, resulting in therapeutic failure. Precise mechanisms for pancreatic cancer cell resistance to gemcitabine are not well understood, and gene expression profiles in correlation with cell sensitivity have been proposed. These multiple genes encode for proteins involved both in the regulation of survival or apoptotic machinery, including Src tyrosine kinase (29), c-Met tyrosine kinase receptor (30), protein kinase B/Akt and NF- κ B (31), focal adhesion kinase (32), integrin-linked kinase (33), inhibitor of apoptosis proteins (34), anti-apoptotic Bcl-2 or Bcl-xL (35), and p8 proteins (36), or in phenotypic alterations including epithelial-to-mesenchymal transition and reexpression of stem cell markers (30). Nucleoside transporters, including hENT1, and cellular enzymes, including dCK, RRM1, and RRM2, which regulate gemcitabine transport and metabolism, respectively, are also important determinants for gemcitabine cytotoxicity and clinical efficacy in pancreatic cancer (37-40). In an effort to reconcile these data with a sphingolipid-dependent mechanism underlying pancreatic cancer cell sensitivity to gemcitabine, we can assume that because sphingolipids, including ceramide, are components of complex lipid/membrane rafts networks, they function as nodes within webs of signaling, regulating adhesive, cytoskeletal, proliferative, and apoptotic pathways. Interestingly, cell exposition to stimuli that ultimately leads to increased intracellular ceramide levels induces the formation of a specific subset of rafts, named "cer-raft" and enriched with ceramide and death signaling molecules including death ligands/receptors. As a consequence, the second subset of raft, named "chol-raft" and enriched with cholesterol and receptor tyrosine kinase family members, is displaced. Consequently, "start" signals to apoptosis are immediately triggered, at the expense of receptor tyrosine kinase-induced growth signals, which are inhibited. One can therefore understand why higher intrinsic or drug-induced intracellular ceramide levels may facilitate apoptosis signals. The sphingolipid metabolism has therefore been clearly proposed as a promising target for new treatment approaches that modulate major cell-fate decisions in cancer as well as stem cells (41).

In addition, one can speculate that the activity of membrane drug transporters, including the hENT1 nucleoside transporter whose expression in pancreatic tumors correlates with clinical outcome of patients treated with gemcitabine (38), might be affected by plasma membrane lipid-raft composition and therefore by an increase of ceramide levels. Controversial results have been provided concerning the role of the P-glycoprotein pump-efflux system in pancreatic cancer cell resistance to gemcitabine. Nevertheless, an overexpression of SphK1 activity in brain

tumor-derived endothelial cells has been reported to contribute to the acquisition and maintenance of the multidrug resistance phenotype through a stimulation of P-glycoprotein transport activity (42), establishing a direct link between sphingolipid metabolism and multidrug resistance phenotype.

Furthermore, it has been shown that ceramide and SphK1/S1P have opposite actions on the phosphatidylinositol 3-kinase/Akt cell survival pathway, whose excessive activation is, however, critically involved in pancreatic cancer cell resistance to gemcitabine (31, 35). Ceramide is indeed able, by directly promoting the recruitment into lipid raft of phosphatase and tensin homologue (43) and/or by indirectly activating ceramide-activated protein phosphatase 2A (43, 44), to inhibit specifically this pathway, conducting to NF- κ B inactivation and to decreased Bcl-xL expression. Conversely, both SphK1 and, indirectly, S1P by acting in an autocrine loop via its S1PR/Edg receptors are able to stimulate this same pathway (45, 46). Interestingly, treatment of the resistant Panc-1 cells with exogenous C2-ceramide or with the SphK1 inhibitor SKI dose-dependently decreased NF- κ B activity and also sensitized these cells to gemcitabine when used at low (1 μ mol/L) concentration (data not shown). These results suggest that up-regulating the ceramide/S1P ratio might sensitize pancreatic cancer cells to gemcitabine at least partly through an inhibition of NF- κ B activity.

In conclusion, our results provide new insights regarding mechanisms for pancreatic cell resistance to gemcitabine and identify the ceramide/S1P ratio as a biosensor of gemcitabine sensitivity in pancreatic cancer cells. Targeting the sphingolipid metabolism might be of critical interest for improving pancreatic cancer chemosensitivity to gemcitabine. Our data indeed indicate that cell treatment with low doses of a ceramide analogue or of a SphK1 inhibitor, which both increase the ceramide/S1P ratio, improves pancreatic cancer cell sensitivity to gemcitabine. Therefore, combining gemcitabine with drugs targeting the sphingolipid metabolism might represent a novel promising approach to defeat therapeutic failure using gemcitabine as a single chemotherapeutic drug.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. T. Levade and coworkers (INSERM U858, I2MR) for helpful comments and suggestions.

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Julie Guillermet-Guibert, Lise Davenne, Dimitri Pchejetski, et al.

Mol Cancer Ther 2009;8:809-820.

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