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Abstract

This is the first report of sphingosine 1-phosphate lyase (SPL) protein expression and enzymatic activity in human neoplasm. This enzyme drives irreversible degradation of sphingosine 1-phosphate (S1P), a bioactive lipid associated with resistance to therapeutics in various cancers, including prostate adenocarcinoma. In fresh human prostatectomy specimens, a remarkable decrease in SPL enzymatic activity was found in tumor samples, as compared to normal adjacent tissues. A significant relationship between loss of SPL expression and higher Gleason score was confirmed in tissue microarray analysis. Moreover, SPL protein expression and activity were inversely correlated with those of sphingosine kinase-1 (SphK1), the enzyme producing S1P. SPL and SphK1 expressions were independently predictive of aggressive cancer on tissue microarray, supporting the relevance of S1P in prostate cancer. In human C4-2B and PC-3 cell lines, silencing SPL enhanced survival after irradiation or chemotherapy by decreasing expression of proteins involved in sensing and repairing DNA damage or apoptosis, respectively. In contrast, enforced expression of SPL sensitized cancer cells to irradiation or docetaxel by tilting the ceramide/S1P balance towards cell death. Interestingly, the S1P degradation products failed to sensitize to chemo- and radiotherapy supporting the crucial role of ceramide/S1P balance in cancer. Of note, the combination of SPL enforced expression with a SphK1 silencing strategy by further decreasing S1P content made prostate cancer cells even more sensitive to anticancer therapies, suggesting that a dual strategy aimed at stimulating S1P lyase and inhibiting SphK1 could represent a future approach to sensitize cancer cells to cancer treatments.
Introduction

Sphingosine 1-phosphate (S1P) has emerged as a key lipid mediator that promotes tumor cell proliferation, survival, migration and angiogenesis (1, 2). S1P tissue level is low and kept under control through a delicately equilibrium between its synthesis and its degradation (1). It has been suggested that the balance between the levels of S1P and its metabolic precursors ceramide and sphingosine provides a rheostat mechanism that decides whether a cell dies (via ceramide or sphingosine) or proliferates and survives (via S1P) (3). So far, most studies have focused on sphingosine kinase-1 (SphK1), the oncogenic enzyme converting sphingosine into the growth-promoting S1P (4). SphK1 activity is stimulated by a wide array of agonists (e.g., growth factors, hormones) to generate S1P, which then can act either extracellularly, by binding to dedicated receptors to drive paracrine or autocrine signaling cascades, or intracellularly (5). In addition, recent findings obtained on tumor tissues from patients indicate that SphK1 represents a potential prognostic marker and a viable target for therapy (6), including in prostate cancer (7). The reduction of S1P levels by SphK1 inhibition increases the efficacy of chemotherapy and radiotherapy (8, 9) while addition of exogenous S1P protects cancer (3) and non-cancer cells (10). Surprisingly, little attention has been paid to the S1P lyase (SPL), the sole enzyme that can decrease levels of intracellular S1P by irreversible cleavage into hexadecenal and ethanolamine phosphate (11). Recent studies - mostly conducted in non-cancer cells - have shown that ectopic expression of SPL results in increased sensitivity to stress including serum starvation (12), chemotherapy (13, 14) and irradiation (15). More importantly, in vivo subcutaneous implant of SPL-/- murine embryonic fibroblasts resulted in tumor formation suggesting a tumor suppressive capacity for SPL (16) in opposition to the oncogenic role of SphK1.
(17). Despite the fact that mRNA SPL was found downregulated in human colorectal carcinomas (14), the evidence of protein expression and enzymatic activity changes in human cancer tissues that would implicate SPL is lacking.

In this study, we aimed to analyze the expression and enzymatic activity of SPL in tissue microarrays and fresh human prostate cancer specimens. We report for the first time that both SPL expression and activity are downregulated in cancerous tissues, and establish a significant relationship between loss of SPL expression and prostate cancer aggressiveness. In addition, we find an opposite relationship between the levels of SPL and SphK1 protein expression and enzymatic activity. Poorly-differentiated cancers exhibited low SPL and high SphK1 expression pointing out the potential influence of S1P in prostate carcinogenesis. Based on this original clinical information, the potential role of SPL in regulating sensitivity/resistance to various anticancer therapies (radiotherapy, chemotherapy) was investigated in canonical human PC-3 and C4-2B prostate cancer cell lineages. We suggest that a dual strategy aimed at stimulating S1P lyase and inhibiting SphK1 could represent a future approach to sensitize cancer cells to cancer treatments.
Materials and Methods

Chemicals and reagents

Culture medium, serum, and antibiotics were from Invitrogen (Villebon sur Yvette, France). \([\gamma-^{32P}]ATP, \text{Serine L-[}^{3}\text{H (G)}, \text{d-erythro-[}^{3}\text{H}\text{] sphingosine and [methyl-}^{3}\text{H]}\text{thymidine were from Perkin-Elmer (Courtaboeuf, France). Silica gel 60 TLC plates were from VWR (Fontenay sous Bois, France). Sphingosine was from Biomol (Plymouth Meeting, PA). MTT, umbelliferone were from Sigma (Saint-Quentin Fallavier, France). Brain ceramide extract and d-erythro-S1P were from Avanti Polar Lipids (Alabaster, AL).}

Cell models

PC-3 and C4-2B cell lines were from DSMZ (Braunschweig, Germany) and Viromed (Minnetonka, MN), respectively. C4-2B is a castration-resistant prostate cancer cell line isolated from the bone metastasis of a mouse xenograft inoculated with C4-2 cells, a subline of LNCaP cells. Cells were cultured in RPMI 1640 containing 10% FBS at 37°C in 5% CO\(_2\) humidified incubators. GFP-tagged wild-type human S1P lyase cDNA (12) was used for stable transfection using Lipofectamine reagent (Invitrogen, Saint Aubin, France). Stable clones of human S1P lyase-GFP fusion protein or pcDNA 3.0 vector were generated in PC-3 and selected with 0.5 mg/ml G418. Empty vector- and S1P lyase-transfected cells were designated as PC-3/Neo, PC-3/SPL1 and PC-3/SPL2, respectively. Cell lines were routinely verified by the following tests: morphology check by microscope, growth curve analysis, and mycoplasma detection (MycoAlert; Lonza, Basel, Switzerland). All experiments were started with low-passaged cells (< 15 times).
Cell viability and proliferation assays

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay (18). The ³H-thymidine cell proliferation assay was carried out as previously reported (19).

Radiation survival determination

Survival after irradiation was defined as the ability of the cells to maintain their clonogenic capacity and to form colonies. Cells were plated from exponentially growing cell culture at 1,000-1,500 cells/well in 6 well-plates 18 h before irradiation. Single doses ranging from 2 to 6 Gy were delivered. Colonies were fixed and stained with crystal violet (2 mg/ml in 150 mM NaCl) for 20 min. Survival clones were counted 6 days after irradiation (20).

RNA interference experiments

For the siRNA experiments, 21 nucleotide complementary RNAs with symmetrical 2 nucleotide 3' overhangs were obtained from Sigma to the following regions of human SPL: bases 409–429 (SPL1a), bases 656–676 (SPL1b) or bases 1653–1673 (SPL1c). Human SphK1-specific siRNA has been previously reported (21, 22) and aleatory sequence scrambled siRNA was from Eurogentec (Angers, France). Transfections were carried out using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Western blot analysis and antibodies

Mouse anti-S1P lyase (Sigma), mouse anti-γH2AX (clone JBW301; Millipore, Billerica, MA), rabbit anti-caspase-3 (clone 8G10; Cell Signalling, Danvers, MA), rabbit anti-PARP
(Cell Signalling, Danvers, MA), mouse anti-GFP (clone 1GF-2A3; Euromedex, Souffelweyersheim, France) were used as primary antibodies. Proteins were visualized by an enhanced chemiluminescence detection system (Pierce, Brebières, France) using anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (Bio-Rad, Hercules, CA). Equal loading of protein was confirmed by probing the blots with the mouse anti-tubulin (clone DM1A; Santa Cruz, Heidelberg, Germany) antibody. Densitometry quantitation was determined using Image J software (NIH, Bethesda, MD).

**X-ray irradiation**

Irradiation was carried out in a Faxitron Rx-650 irradiator (Faxitron X-ray Corporation, Lincolnshire, IL, USA) at a dose rate of 0.48 Gy/min.

**S1P lyase activity**

S1P lyase activity determination was conducted as described (23) with little modifications. The assay was carried out in 96-well plates. The incubation mixture consisted of 65 µl of cell lysate or prostate tissue sample (8-10 mg protein/ml), 15 µl of coumarinic sphinganine 1-phosphate analogue substrate solution (200 µM final concentration), 25 mM Na₃VO₄ (5 µl, 0.5 M), 25 mM NaF (5 µl, 0.5 M) and 0.25 mM pyridoxal phosphate (5 µl, 5 mM), 0.5 mM EDTA (5 µl, 10 mM), 2 mM DTT (2 µl, 0.1 M). The cells lysates were harvested by trypsinization, washed with PBS 1X and resuspended in 0.5 M potassium phosphate (PP) buffer pH 7.4 (8-10 mg protein/ml). The prostate tissue samples were disrupted directly in the PP buffer with the help of an Ultraturrax homogenizer (T25 basic). The cell or tissue suspension was frozen by immersing the tube in liquid nitrogen and then it was thawed at 25 °C (water bath). After gentle mixing, the freeze-thaw cycle was repeated five times. For the substrate
solution, 5 µl of a 4 mM stock solution in 0.005N HCl in MeOH were taken, the solvent was removed under nitrogen and the substrate was resuspended with 20 µl of PBS 1X and sonicated for 1 min. The plate containing the final reaction mixture was incubated at 37 °C for 18 hours in the dark, and after this time the reaction was stopped with MeOH (50 µl/well) and 100 mM Glycine/NaOH buffer (100 µl/well). After one hour in the dark, the fluorescence intensity was measured at 355/460 nm. Upon cleavage of the coumarinic sphinganine 1-phosphate analogue substrate by SPL, an aldehyde is produced, which then undergoes subsequent β-elimination to release the fluorescent product umbelliferone and acrolein. A calibration curve was made with umbelliferone in the 0.01-100 µM concentration range prepared in the same solution.

**SphK1 and serine palmitoyltransferase (SPT) activities**

The determination of SphK1 and SPT activities were conducted as previously reported (24, 25).

**Mass measurement of ceramide, sphingosine and S1P**

The protocols for measurement of ceramide, sphingosine and S1P have been described in detail previously (9, 26).

**Tissue procurement**

S1P lyase pattern of expression in prostate tissue was studied in specimens obtained from our institution tissue biorepository after IRB approval and informed consent. They consisted of thirteen consecutive patients undergoing laparoscopic radical prostectomies performed for clinical T1c–T2c with at least two positive biopsies from January to March 2011.
Immunochemistry

Tissue microarray (TMA) sections representative of prostate cancer (n=88 in duplicates) were used to report SphK1 and S1P lyase expression in relation to cancer differentiation. All sections were deparaffinized, hydrated, boiled with 10 mM of citrate buffer (pH 6) for 30 min, treated with 0.3% H₂O₂ for 5 min, pre-incubated in blocking solution (1% BSA in PBS) for 10 min at room temperature and incubated with the primary antibody (anti-SphK1 [27] diluted 1:100 or anti-S1P lyase (Sigma) diluted 1:100) for 4 h at 4 °C. The sections were then washed with PBS and processed with the two-step EnVision+ HRP DAKO system (DAKO, Carpinteria, CA). Tumor Gleason sum was recorded for each individual microarray and as proposed by Rubin to report immunohistochemical results in prostate cancer (28), the expression was scored as negative (1), faint/equivocal (2), moderate (3) or strong (4). The pattern of S1P lyase expression was recorded as diffuse cytoplasmic, luminal or both.

Statistical analysis

The statistical significance of differences between the means was evaluated using the unpaired Student’s t or the one-way analysis of variance (ANOVA) tests. All statistical tests were two-sided, and the level of significance was set at P < 0.05. Calculations were done using Instat 3 (GraphPad Software, San Diego, CA). For immunohistochemistry studies, the relationship between Gleason Sum (GS ≤ 3+4 vs. GS ≥ 4+3) and S1P lyase and SphK1 expression (negative and faint/equivocal vs. moderate or strong) was analysed by Pearson's test. Statistical significance was set at a P value < 0.05, all reported values are two-sided. To investigate whether S1P lyase and SphK1 patterns of expression were independently predictive of Gleason score we then conducted a logistic regression analysis with immunohistochemistry variables as dependent variables and
Gleason score as independent variable.
Results

S1P lyase expression and enzymatic activity are downregulated in cancer versus non-cancer tissue in patients, and associated with tumor grade

S1P lyase (SPL) expression was restricted to the epithelial layer, with no staining evidenced in the stromal compartment of the gland (Fig 1A). Diffuse and intense cytoplasmic expression of SPL (Fig 1A), reinforced at the luminal aspect of the gland was observed in non-cancer glands (strong expression, expression score 4, *). Expression in cancer was strikingly different and related to grade. While well-differentiated cancers (Gleason score ≤3+4, Fig 1B) showed conserved pattern of expression (moderate expression, expression score 3, *), poorly-differentiated cancer (Gleason score ≥4+3) were characterized by equivocal (Fig 1C, expression score 2) or abrogated cytoplasmic expression (Fig 1D, expression score 1). The pattern of expression of SphK1 has been previously reported from the same TMA (7). Semi-quantitative analysis (Table 1) evaluated the relationship between Gleason score and SPL and SphK1 expressions. Conserved patterns of SPL expression were associated with lower Gleason scores as compared to negative or equivocal SPL expression (P=0.00003). On the other hand, strong SphK1 expression correlated with higher Gleason scores (P =0.019) than negative or equivocal SphK1 staining. The independent value of SPL and SphK1 expression was then sought by logistic regression analysis on a subset of 139 spots where SPL and SphK1 expressions were both available. SPL loss (P=0.002) and SphK1 upregulation (P <0.01) were shown to be independently predictive of aggressive cancer. As shown in Fig 2B, low S1P lyase with high SphK1 expression was associated with high Gleason score (85% of the TMA spots were poorly-differentiated cancers and 15% well-differentiated cancers), whereas low Gleason score cancers exhibited the
opposite pattern. To further look for a relationship between SPL and SphK1 expressions, we quantified SPL and SphK1 enzymatic activities in fresh prostatic tissues obtained from thirteen consecutive patients undergoing radical prostatectomy. SPL activity was markedly diminished in tumor samples (Fig 2B) accounting for a statistically significant ($P=0.0004$) 30% decrease in cancer tissue as compared to the individual non-cancer counterpart. Using the same extracts, SphK1 enzymatic activity was found 2-fold upregulated in cancer (Fig 2C), in line with our previously published data (7). Of note, concordant results were found from protein expression (Fig 2A) and enzymatic activity experiments (Fig 2B and C), in human prostate cancer tissues. Collectively, these results establish that the S1P producing SphK1 enzyme is overexpressed in cancer whereas the S1P degrading SPL enzyme is underexpressed, highlighting the importance of S1P in prostate cancer.

**Modulating S1P lyase activity impacts S1P metabolism**

First, downregulation of SPL was conducted by siRNA strategy with three different siRNAs tested and validated by assessing protein expression and enzymatic activity. The siSPL1b induced a 50% decrease in SPL expression (Fig 3A) and enzymatic activity (Fig 3B) in both PC-3 and C4-2B cell lines. The combination of the three sequences (SPL1a+SPL1b, SPL1a+SPL1c, SPL1b+SPL1c, SPL1a+SPL1b+SPL1c) did not improve the efficacy of the transfection (data not shown). The silencing of SPL activity was accompanied by a significant rise in intracellular S1P content (Fig 3C). Second, stable overexpression of SPL was carried out in PC-3 cells. As shown in Fig 4A, transfection efficiency was verified in two different clones (PC-3/SPL1 and PC-3/SPL2). As anticipated, SPL enzymatic activity was clearly enhanced (Fig 4B) and S1P content significantly reduced yet not to the same extent (Fig 4B). The content in pro-apoptotic
ceramide was significantly augmented (Fig 4B), possibly as a consequence of the stimulation of serine palmitoyltransferase (SPT) (Fig 4B), a key enzyme in de novo pathway of ceramide generation. Interestingly, the content of pro-apoptotic sphingosine (Fig 4B) was reduced while SphK1 activity was increased in PC-3 overexpressing SPL (Fig 4B). These data indicate that PC-3/SPL cells may struggle to survive to the enforced degradation of S1P and generation of ceramide by enhancing S1P production, through increased SphK1 activity as confirmed by the decrease in sphingosine, its substrate. However, this adaptive pro-survival mechanism is not sufficient enough as an important pool of S1P (about 30%, Fig 4B) is degraded due to overexpression of S1P lyase. Further analysis of SPL overexpressing PC-3 cells indicated a different behaviour compared to PC-3/Neo control cell line. PC-3 overexpressing-SPL cell lines showed a reduced (about 30%) proliferation rate (Fig 4C) as compared to PC-3/Neo cells, likely a consequence of a higher basal rate of apoptosis (Suppl. Fig 1), in line with their relative changes in ceramide and S1P levels (Fig 4B).

**Opposite effects of S1P lyase downregulation and overexpression with regard to irradiation**

As compared to the scrambled RNA-control counterparts, the knockdown of SPL activity by RNA-interference strongly enhanced survival after irradiation in PC-3 (Fig 5A) and C4-2B (Suppl. Fig 2A) cells. The radioprotective effect of SPL inhibition was due to S1P, as its addition to the culture medium of PC-3 and C4-2B wild-type cells before irradiation mimicked a radiation-resistant phenotype (Fig 5A and Suppl. Fig 2B). In contrast, invalidation of SphK1 by siRNA (21) rendered cells more sensitive to irradiation in PC-3 (Fig 5A) and C4-2B cells (Suppl. Fig 2A). Although irradiation causes a variety of lesions in DNA, double-strand breaks (DSB) are principally responsible for
radiation lethality. One early marker of DSB is the phosphorylation of H2AX, which is usually referred to as γ-H2AX. 2 Gy of irradiation induced H2AX phosphorylation in scrambled-RNAi transfected PC-3 (Fig 5B) and C4-2B (Suppl Fig 2C) cells. In SPL-silenced cells, irradiation-induced H2AX phosphorylation was decreased suggesting less DNA damage in these cells (Fig 5B and Suppl Fig 2C). Conversely, the silencing of SphK1 in PC-3 (Fig 5B) and C4-2B (Suppl Fig 2C) cells caused a robust increase of γ-H2AX.

The survival capability of SPL-overexpressing cell lines after irradiation was significantly reduced as compared to PC-3/Neo control cells (Fig 5C). PC-3/SPL1 cells showed an increased γ-H2AX expression before and after 2-Gy X-irradiation as well as a delay in the recovery of the basal level expression (Fig 5D). These results were confirmed by immunofluorescence staining and FACS (data not shown) and similar results were obtained with the PC-3/SPL2 cell line (data not shown). Importantly, the combination of SPL enforced expression with a siRNA strategy against SphK1 by further lowering S1P content (data not shown) rendered PC-3 cells even more sensitive to irradiation (Fig 5C).

**Chemotherapy-induced cell death is inhibited by S1P lyase knock-down and potentiated by S1P lyase overexpression**

Docetaxel is the current standard of care for the treatment of metastatic prostate cancer. As compared to the siScramble-pretreated PC-3 or C4-2B cells, the silencing of SPL significantly inhibited the loss of cell viability observed after treatment with increasing doses of docetaxel (Fig 6A). In prostate cancer cells, docetaxel-induced cell death involves the processing of caspases particularly the executioner caspase-3, an event associated with the activation of poly(ADP-ribose) polymerase (PARP), a canonical caspase-3 substrate (29). In scrambled-RNAi transfected PC-3, a diminution of both 32
kDa unprocessed caspase-3 and 116 kDa unprocessed PARP expression during treatment with docetaxel was found (Fig 6C). However, no activation of caspase-3 and PARP were observed in SPL-silenced cells (Fig 6C), consistent with inhibition of caspase-3 activity and the notion that S1P inhibits activation of caspases that cleave poly(ADP-ribose) polymerase (30). In contrast, overexpression of SPL in PC-3 cells markedly diminished cell viability of cells treated with docetaxel (Fig 6B), and amplified activation of both caspase-3 and PARP processing (Fig 6D). In line with the data obtained with radiotherapy, the combination of SPL enforced expression with a siRNA strategy against SphK1 rendered PC-3 cells even more sensitive to irradiation (Fig 6B).
Discussion

Because of the importance of S1P in cancer (1, 2), considerable interest has developed in understanding the function of the enzymes implicated in its metabolism. Concordant studies have shown that SphK1, responsible for S1P synthesis, is instrumental to cancer promotion, progression and resistance to treatment in vitro and in vivo, notably in prostate cancer (9, 19, 21, 22, 31). In complement to preclinical literature, we recently reported the relationship between increased SphK1 activity and relevant clinical features in human prostate cancer resection specimens confirming a central role for the SphK1/S1P signaling in prostate cancer (7). S1P is irreversibly degraded by SPL generating two products, ethanolamine phosphate and a long-chain aldehyde (32). Previous findings showed the downregulation of SPL at the mRNA level in human colorectal carcinomas (14) and in Min mouse intestinal adenomas, suggesting that SPL loss may correlate with and/or contribute to intestinal carcinogenesis (14). In this study we establish that both protein expression and enzymatic activity of SPL are downregulated in cancer versus non-cancer tissue in prostate cancer patients, and associated with tumor grade. Statistical analyses show that loss of SPL expression is significantly associated with aggressive cancers (high Gleason score). More importantly, SPL loss of function is accompanied by a concomitant increase in SphK1 expression. Aggressive poorly-differentiated cancers exhibit low SPL and high SphK1 expression whereas well-differentiated cancers display the opposite pattern. These data are confirmed in fresh human prostatectomy specimens where significant decrease in SPL enzymatic activity and increase in SphK1 activity are found. The opposite relationship between the levels of SPL and SphK1 protein expression and enzymatic activity therefore supports the importance of S1P in prostate cancer. In colon cancer, although
SPL mRNA expression was found to be down-regulated in 8 out of 10 samples, Saba and collaborators did not find that SphK1 was differentially expressed in tumors (14). To the best of our knowledge, we report for the first time such inverse relationship between enzymes playing antagonistic effects on the level of S1P. Our data therefore warrant further clinical investigations of paired SphK1 and SPL expression in other tumor locations.

The role of SPL in regulating sensitivity/resistance to chemotherapeutics has been investigated in several systems. The sensitizing effect to diverse agents (platinum salts, doxorubicin and etoposide) associated with SPL overexpression in *Disctyostelium*, HEK293 and lung cancer A549 cells has been reported previously (13, 33). In line with these studies, we found that enforced expression of SPL potentiates the response to docetaxel, the standard chemotherapy in prostate cancer, whereas SPL silencing prevents cell death. With regard to irradiation, we demonstrate that silencing SPL enhances survival after irradiation by decreasing expression of γ-H2AX as well as DNA-PKcs-P and ATM-P (data not shown), proteins involved in the sensing and repairing of DNA double-strand breaks. As expected, enforced expression of SPL sensitizes cancer cells to irradiation. Our findings are in line with earlier studies demonstrating the radioprotective capability of S1P notably in vivo (10, 34). The pro-survival effect of SPL silencing could indeed be attributed to S1P accumulation, as this effect was mimicked by addition of exogenous S1P to prostate cancer cells. Importantly, the combination of SPL overexpression with a SphK1 silencing by further decreasing S1P content make prostate cancer cells even more sensitive to irradiation or to chemotherapy, suggesting that a dual strategy aimed at stimulating S1P lyase and inhibiting SphK1 could represent a future approach to potentiate sensitization of cancer cells. In contrast, the silencing of SphK2 isoform activity (which is minor in the PC-3 and C4-2B prostate cancer cell
models as compared to SphK1 activity) did not sensitize to docetaxel nor to radiotherapy (Suppl. Fig. 3).

The sensitization induced by SPL overexpression is likely to be attributed to its effect on the ceramide/S1P balance (increased ceramide and decreased S1P) rather than increased formation of SPL reaction products. We found that SPL overexpression is associated with increased ceramide and decreased sphingosine and S1P levels. We also report that cancer cells overexpressing SPL may struggle to survive to the SPL-mediated degradation of pro-survival S1P and generation of pro-apoptotic ceramide by enhancing S1P production through increased SphK1 activity that could explain the decrease in sphingosine we observed. We suggest a mechanistic linkage between SphK1 and SPL activities. If the overexpression of SPL is accompanied with increased SphK1 activity (Fig. 4), it appears that overexpression of SphK1 is also associated with higher SPL activity (data not shown). Similarly, the silencing of SphK1 induces a decrease in SPL activity, whereas the silencing of SPL triggers inhibition of SphK1 activity (data not shown). Overall, the silencing or the enforced expression of one enzyme impacts the activity of the other one, suggesting a compensatory mechanism between SphK1 and SPL exists to maintain a certain level of S1P to ensure cell survival.

Contradictory results about the mechanism of ceramide production under enforced expression of SPL have been reported in the literature. Using the same HEK293 cell model, Reiss et al. (12) demonstrated a de novo synthesis of ceramide whereas Kumar et al. ruled out this mechanism (15). Our results suggest a de novo synthesis through serine palmitoyltransferase activation. Additional data are needed to help clarify these divergent results. With regard to the reaction products of SPL, they are unlikely to account for the sensitizing properties of SPL. Interconvertible derivatives of S1P
degradation product hexadecenal, namely hexadecanol and palmitate, although inducing a limited loss of cell viability by themselves, do not sensitize to irradiation nor to chemotherapy (Suppl. Fig 4).

Overall, our clinical observations showing that SPL is down-regulated in prostate cancer while SphK1 is up-regulated suggests that a therapeutic approach combining the inhibition of S1P production associated with a stimulation of its degradation should have a favorable therapeutic index, notably in combination with radio- or chemotherapy.
Acknowledgments

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References


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Table 1. Semi-quantitative analysis of the relationship between SPL or SphK1 expression and Gleason score in prostate cancer TMA.
Figure legends

Figure 1: Representative patterns of S1P lyase expression in normal and cancer glands

TMA and high power magnifications (X100 and X400) of normal glands showing a restricted SPL expression to the epithelial layer (A), well-differentiated Gleason score 5 prostate cancer (B) and poorly-differentiated Gleason score 8 (C) and 9 (D) cancers.

Figure 2: An inverse relationship exists between S1P lyase (SPL) and SphK1 protein expression and enzymatic activity in human prostate cancer

A. Semi-quantitative analysis of the relationship between combined SPL and SphK1 expression and Gleason score. Staining intensity was scored as low (sum of negative and faint/equivocal) and high (sum of moderate and strong). The n value represents the number of spots for which both SphK1 and SPL expression were evaluated (total n=139). SPL (B) and SphK1 (C) activities were quantified in 13 individual pairs of cancer and non-cancerous lesions (see Material and Methods section, tissue procurement). Median SPL activities were 6.0 (95% CI: 5.2-6.2) and 4.0 (95% CI: 3.1-4.2) pmol/min/mg of protein in non-cancerous and tumor samples, respectively (P < 0.0001). Median SphK1 activities were 88 (95% CI: 71-129) and 259 (95% CI: 223-289) pmol/min/mg of protein in non-cancerous and tumor samples, respectively (P < 0.0001).

Figure 3: S1P lyase (SPL) inhibition by RNA silencing increases S1P levels in PC-3 and C4-2B prostate cancer cells

A. PC-3 (upper panel) or C4-2B (bottom panel) cells were untransfected or transfected with 25 or 75 nM of different siSPL (siSPL1a, siSPL1b, siSPL1c) or 75 nM scrambled siRNA (siScramble) for 72h. Cell lysates were assayed for SPL expression by western
blot analysis. Equal loading of protein was monitored using antibody to α-tubulin.

Similar results were obtained in three independent experiments. S1P lyase enzymatic activity (B) and S1P content (C) were quantified in PC-3 (left panel) or C4-2B (right panel) cells untransfected or transfected with 75 nM siScramble or siSPL1b for 72h. Columns, mean of at least 3 independent experiments; bars, SEM. ***, P < 0.001.

Figure 4: S1P lyase-enforced expression in PC-3 cells shifts the sphingolipid metabolism toward pro-apoptotic ceramide and reduces cell proliferation

A, S1P lyase expression in two different clones (PC-3/SPL1 and PC-3/SPL2) of PC-3 cells was analyzed by western blotting using an anti-GFP antibody. Equal loading of protein was monitored using antibody to α-tubulin. Representative images of GFP expression in PC-3/SPL1 and PC-3/SPL2 cells (inset). B, SPL activity, S1P content, SphK1 activity, ceramide content, SPT activity and sphingosine content were quantified from extracts obtained from PC-3/Neo, PC-3/SPL1 and PC-3/SPL2. Columns, mean of at least 4 independent experiments; bars, SEM. The two-tailed P values between the means are: *, P < 0.05; **, P < 0.01; ***, P < 0.001. C, cell proliferation was assessed with 3H-thymidine incorporation assay. Points, mean of at least 3 independent experiments; bars, SEM.

Figure 5: The modulation of S1P content in PC-3 prostate cancer cells triggers sensitivity or resistance to irradiation

A, PC-3 cells were transfected with 75 nM siScramble, 75 nM siSPL1b or 33 nM siSphK1 for 72h (left panel) or treated with 5 µM exogenous S1P for 30 min then removed (right panel). Cells were then irradiated from 2 to 6 Gy and survival clones were counted 6 days later. Data are expressed as the percentage of survival fraction compared to non-
irradiated cells. Points, mean of at least 4 independent experiments; bars, SEM. The two-tailed $P$ values between the means of siScramble and siSphK1 or siSPL1b treated cells (left panel) or the means of untreated and S1P-treated cells (right panel) are: *, $P < 0.05$; **, $P < 0.01$. B, cell lysates from PC-3 cells treated with siScramble, siSPL1b or siSphK1 before irradiation (2 Gy) and recovered at different times were analyzed for $\gamma$-H2AX expression. Equal loading of protein was monitored using antibody to $\alpha$-tubulin. Similar results were obtained in three independent experiments. C, PC-3/Neo and two different clones of PC-3 cells overexpressing SPL (PC-3/SPL1 and PC-3/SPL2) were transfected or not with siScramble (not shown as non-transfected or siScramble transfected cell lines reacted in a similar fashion) or siSphK1 for 72h. Cells were then irradiated from 2 to 6 Gy and survival clones were counted 6 days later. Data are expressed as the percentage of survival fraction compared to non-irradiated cells. Points, mean of at least 3 independent experiments; bars, SEM. The two-tailed $P$ values between the means of PC-3/Neo and the other experimental conditions are: **, $P < 0.01$; ***, $P < 0.001$. D, cell lysates from PC-3/Neo and PC-3/SPL1 cells recovered at indicated times were analyzed for $\gamma$-H2AX expression. Equal loading of protein was monitored using antibody to $\alpha$-tubulin. Similar results were obtained in three independent experiments.

Figure 6: The modulation of S1P content in prostate cancer cells alters sensitivity to chemotherapy through caspase signaling

A, PC-3 (left panel) and C4-2B (right panel) cells were transfected with control siRNA (siScramble, 75 nM, 72h), or siRNA directed to S1P lyase (SPL1b, 75 nM, 72h) then treated with the indicated concentrations of docetaxel for 72h, and cell viability was assessed by MTT. B, PC-3/Neo and two different clones of PC-3 cells overexpressing SPL (PC-3/SPL1 and PC-3/SPL2) were transfected or not with siScramble (not shown as
non-transfected or siScramble transfected cell lines reacted in a similar fashion) or siSphK1 for 72h. Cells were then treated with the indicated concentrations of docetaxel during 72h and cell viability was assessed by MTT. Data are expressed as the percentage of cell viability compared to untreated cells. Points, mean of at least 4 independent experiments; bars, SEM. The two-tailed P values between the means are: * P < 0.05; **, P < 0.01; ***, P < 0.001. C. Cell lysates from PC-3 wild-type cells transfected with control siRNA (siScramble, 75 nM, 72h), or siRNA directed to S1P lyase (SPL1b, 75 nM, 72h) then incubated for the indicated times with 20 nM docetaxel were analyzed for full-length caspase-3 (32 kDa) and PARP (116 kDa) expression. D. Cell lysates from PC-3/Neo and PC-3/SPL1 cells treated for the indicated times with 20 nM docetaxel were analyzed for full-length caspase-3 (32 kDa) and PARP (116 kDa) expression. Equal loading of protein was monitored using antibody to α-tubulin. Similar results were obtained in three independent experiments.
Figure 2

A. 

Gleason score ≤ 3+4

Gleason score ≥ 4+3

B. 

SPL activity (pmol/min/mg protein)

C. 

SphK1 activity (pmol/min/mg protein)

n=27 n=16 n=68 n=28
Figure 3

A.  

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<tr>
<th>Treatment</th>
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<th>C4-2B SPL activity (pmol/min/mg protein)</th>
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<td>1.2</td>
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<td>siSPL1a (25 nM)</td>
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<td>siSPL1a (75 nM)</td>
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B.  

<table>
<thead>
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<th>Treatment</th>
<th>SPL activity (pmol/min/mg protein)</th>
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<tr>
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<td>siScramble (75 nM)</td>
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<td>siSPL1b (25 nM)</td>
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C.  

<table>
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<th>S1P (pmol/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>No treatment</td>
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<td>[Graph with bars]</td>
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<tr>
<td>siSPL1b (25 nM)</td>
<td>[Graph with bars]</td>
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</tbody>
</table>

*** = significant difference
**Figure 4**


B. 

- SPL activity (pmol/min/mg protein)
- Ceramide (pmol/mg protein)
- SphK1 activity (pmol/min/mg protein)
- Sphingosine (pmol/pmol phospholipids)

C. Cell proliferation (%)

PC-3/Neo, PC-3/SPL1, PC-3/SPL2

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<th>PC-3/SPL2</th>
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* * * ** *** ** *** ** **
Figure 5

A.  
- siScramble  - siSPL1b  - siSphK1  

Survival Fraction (%)  

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B.  
- siScramble  - siSPL1b  - siSphK1  

γH2AX -  

α-tubulin -  

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C.  

Survival Fraction (%)  

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<td>100</td>
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<tr>
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</tr>
<tr>
<td>6</td>
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D.  
- PC-3/Neo  - PC-3/SPL1  

γH2AX -  

α-tubulin -  

<table>
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<tr>
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<td>1.9</td>
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<tr>
<td>24h</td>
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** * ***
**Figure 6**

**A.**

Bar graphs showing cell viability (% of untreated cells) for PC-3 and C4-2B cell lines treated with different concentrations of Docetaxel (nM) over time. The x-axis represents Docetaxel concentrations (5, 10, 20 nM), and the y-axis represents cell viability (% of untreated cells).

**B.**

Bar graphs showing cell viability (% of untreated cells) for PC-3/Neo, PC-3/SPL1, and PC-3/SPL2 cell lines treated with Docetaxel (nM) over time. The x-axis represents Docetaxel concentrations (5, 10, 20 nM), and the y-axis represents cell viability (% of untreated cells).

**C.**

Western blots showing expression levels of Caspase-3 (32 kDa) and α-tubulin (116 kDa) in the siScramble and siSPL1b conditions for PC-3 and C4-2B cell lines. The x-axis represents time (0, 24, 48h), and the y-axis represents protein expression levels.

**D.**

Western blots showing expression levels of Caspase-3 (32 kDa), α-tubulin (116 kDa), and PARP (116 kDa) in the PC-3/Neo and PC-3/SPL1 conditions. The x-axis represents time (0, 24, 48h), and the y-axis represents protein expression levels.
Molecular Cancer Therapeutics


Leyre Brizuela, Isabelle ADER, Catherine MAZEROLLES, et al.

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