**Therapeutic Discovery** 

Molecular Cancer Therapeutics

# First Evidence of Sphingosine 1-Phosphate Lyase Protein Expression and Activity Downregulation in Human Neoplasm: Implication for Resistance to Therapeutics in Prostate Cancer

Leyre Brizuela<sup>1,2</sup>, Isabelle Ader<sup>1,2</sup>, Catherine Mazerolles<sup>1,2,3</sup>, Magalie Bocquet<sup>1,2</sup>, Bernard Malavaud<sup>1,2,3</sup>, and Olivier Cuvillier<sup>1,2</sup>

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 with normal adjacent tissues. A significant relationship between loss of SPL expression and higher Gleason score was confirmed in tissue microarray (TMA) 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 TMA, 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 toward 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 SPL, and inhibiting SphK1 could represent a future approach to sensitize cancer cells to cancer treatments. Mol Cancer Ther; 11(9); 1841-51. ©2012 AACR.

# 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 delicate 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; ref. 3). So far, most studies have focused on sphingosine kinase-1 (SphK1),

Corresponding Author: Olivier Cuvillier, CNRS, Institut de Pharmacologie et de Biologie Structurale (IPBS), CNRS UMR 5089, 205 route de Narbonne, BP 64182, 31077 Toulouse Cedex 4, France. Phone: 33-561-17-55-13; Fax: 33-561-17-58-71; E-mail: olivier.cuvillier@inserm.fr

doi: 10.1158/1535-7163.MCT-12-0227

©2012 American Association for Cancer Research.

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), whereas addition of exogenous S1P protects cancer (3) and noncancer 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 noncancer 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<sup>-/-</sup> murine embryonic fibroblasts resulted in tumor formation, suggesting a tumor suppressive capacity for SPL (16) in opposition to the oncogenic

Authors' Affiliations: <sup>1</sup>CNRS, Institut de Pharmacologie et de Biologie Structurale; <sup>2</sup>Université de Toulouse, UPS, IPBS; and <sup>3</sup>Hôpital Rangueil, Service d'Urologie et de Transplantation Rénale, Toulouse, France

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

www.aacrjournals.org

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 (TMA) 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. On the basis of 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 SPL, 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. [ $\gamma$ -<sup>32</sup>P]ATP, serine L-[<sup>3</sup>H (G)], D-erythro-[3-<sup>3</sup>H] sphingosine, and [methyl-<sup>3</sup>H] thymidine were from Perkin-Elmer. Silica gel 60 TLC plates were from VWR. Sphingosine was from Biomol. MTT, umbelliferone were from Sigma. Brain ceramide extract and D-erythro-S1P were from Avanti Polar Lipids.

#### Cell models

PC-3 and C4-2B cell lines were from DSMZ and Viromed, 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% CO2 humidified incubators. GFPtagged wild-type human SPL cDNA (12) was used for stable transfection using Lipofectamine reagent (Invitrogen). Stable clones of human SPL-GFP fusion protein or pcDNA 3.0 vector were generated in PC-3 and selected with 0.5 mg/mL G418. Empty vector- and SPL-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). All experiments were started with low-passaged cells (<15 times).

## **Cell viability and proliferation assays**

Cell viability was determined by MTT bromide assay (18). The <sup>3</sup>H-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 to 1,500 cells per well in 6 well-plates 18 hours 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 mmol/L NaCl) for 20 minutes. 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. Transfections were carried out using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

#### Western blot analysis and antibodies

Mouse anti-SPL (Sigma), mouse anti- $\gamma$ H2AX (clone JBW301; Millipore), rabbit anti-caspase-3 (clone 8G10; Cell Signalling), rabbit anti-PARP (Cell Signalling), mouse anti-GFP (clone 1GF-2A3; Euromedex) were used as primary antibodies. Proteins were visualized by an enhanced chemiluminescence detection system (Pierce) using anti-rabbit or anti-mouse horseradish peroxidase (HRP)–conjugated IgG (Bio-Rad). Equal loading of protein was confirmed by probing the blots with the mouse anti-tubulin (clone DM1A; Santa Cruz) antibody. Densitometry quantitation was determined using ImageJ software (NIH).

# **X-ray irradiation**

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

## **SPL** activity

SPL 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  $\mu$ L of cell lysate or prostate tissue sample (8–10 mg protein/mL), 15  $\mu$ L of coumarinic sphinganine 1-phosphate analog substrate solution (200  $\mu$ mol/L final concentration), 25 mmol/L Na<sub>3</sub>VO<sub>4</sub> (5  $\mu$ L, 0.5 mol/L), 25 mmol/L NaF (5  $\mu$ L, 0.5 mol/L), and 0.25 mmol/L pyridoxal phosphate (5  $\mu$ L, 5 mmol/L), 0.5 mmol/L EDTA (5  $\mu$ L, 10 mmol/L), 2 mmol/L DTT (2  $\mu$ L, 0.1 mol/L). The cells lysates were harvested by trypsinization, washed with PBS 1X, and resuspended in 0.5 mol/L potassium phosphate buffer pH7.4 (8–10 mg protein/mL). The prostate tissue samples were disrupted directly in the potassium phosphate 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 5 times. For the substrate solution, 5 µL of a 4 mmol/L stock solution in 0.005 N 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 minute. 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  $\mu$ l/well) and 100 mmol/L 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 analog substrate by SPL, an aldehyde is produced, which then undergoes subsequent  $\beta$ -elimination to release the fluorescent product umbelliferone and acrolein. A calibration curve was made with umbelliferone in the 0.01 to 100  $\mu$ mol/L concentration range prepared in the same solution.

#### SphK1 and serine palmitoyltransferase activities

The determination of SphK1 and serine palmitoyltransferase (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**

SPL pattern of expression in prostate tissue was studied in specimens obtained from our institution tissue biorepository after Institutional Review Board approval and informed consent. They consisted of 13 consecutive patients undergoing laparoscopic radical prostatectomies conducted for clinical T1c–T2c, with at least 2 positive biopsies from January to March 2011.

## Immunochemistry

TMA sections representative of prostate cancer (n = 88in duplicates) were used to report SphK1 and SPL expression in relation to cancer differentiation. All sections were deparaffinized, hydrated, boiled with 10 mmol/L of citrate buffer (pH 6) for 30 minutes, treated with 0.3% H<sub>2</sub>O<sub>2</sub> for 5 minutes, preincubated in blocking solution (1% bovine serum albumin in PBS) for 10 minutes at room temperature and incubated with the primary antibody [anti-SphK1; ref. 27) diluted 1:100 or anti-SPL (Sigma) diluted 1:100] for 4 hours at 4°C. The sections were then washed with PBS and processed with the 2-step EnVision+ HRP DAKO system (Dako). 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 SPL 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 *t* or the one-way ANOVA tests. All statistical tests were 2-sided, and the level of significance was set at *P* < 0.05. Calculations were done using Instat 3 (GraphPad Software). For immunohistochemistry studies, the relationship between Gleason Sum (GS  $\leq$  3 + 4 vs. GS  $\geq$  4 + 3) and SPL and SphK1 expression (negative and faint/equivocal vs. moderate or strong) was analyzed by Pearson test. Statistical significance was set at *P* value less than 0.05, all reported values are 2-sided. To investigate whether SPL 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

# SPL expression and enzymatic activity are downregulated in cancer versus noncancer tissue in patients and associated with tumor grade

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 noncancer glands (strong expression, expression score 4, \*). Expression in cancer was strikingly different and related to grade. Whereas well-differentiated cancers (Gleason score  $\leq 3$ + 4, Fig. 1B) showed conserved pattern of expression (moderate expression, expression score 3, \*), poorly differentiated cancer (Gleason score  $\geq 4 + 3$ ) 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). Semiquantitative 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 with 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, in which 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 SPL 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

www.aacrjournals.org



Figure 1. Representative patterns of SPL expression in normal and cancer glands. TMA and high power magnifications (×100 and ×400) of normal glands showing a restricted SPL expression to the epithelial layer (A), welldifferentiated Gleason score 5 prostate cancer (B), and poorly differentiated Gleason score 8 (C) and 9 (D) cancers.

enzymatic activities in fresh prostatic tissues obtained from 13 consecutive patients undergoing radical prostectactomy. 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 with the individual noncancer 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 established 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 SPL activity impacts S1P metabolism

First, downregulation of SPL was conducted by siRNA strategy with 3 different siRNAs tested and validated by assessing protein expression and enzymatic activity. The siSPL1b induced a 50% decrease in SPL expression

	Gleason score ⊴3+4	Gleason score ≥4+3	Р
SPL expression			
Negative or faint/equivocal	13 (27.7%)	73 (63.5%)	0.00003
Moderate or strong	34 (72.3%)	42 (36.5%)	
	n = 47	<i>n</i> = 115	
SphK1 expression			
Negative or faint/equivocal	27 (65.9%)	39 (41.4%)	0.019
Moderate or strong	14 (34.1%)	55 (58.6%)	
	<i>n</i> = 41	n = 94	

Table 1. Semiguantitative analysis of the relationship between SPL or SphK1 expression and Gleason

Mol Cancer Ther; 11(9) September 2012 1844

**Molecular Cancer Therapeutics** 



Figure 2. An inverse relationship exists between SPL and SphK1 protein expression and enzymatic activity in human prostate cancer. A, semiquantitative 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 noncancerous lesions (see Material and Methods section, tissue procurement). Median SPL activities were 6.0 (95% Cl, 5.2-6.2) and 4.0 (95% Cl, 3.1-4.2) pmol/min/mg of protein in noncancerous and tumor samples, respectively (P < 0.0001). Median SphK1 activities were 88 (95% Cl, 71-129) and 259 (95% Cl, 223-289) pmol/min/mg of protein in noncancerous and tumor samples, respectively (\*\*\*, P < 0.0001).

(Fig. 3A) and enzymatic activity (Fig. 3B) in both PC-3 and C4-2B cell lines. The combination of the 3 sequences (SPL1a+SPL1b, SPL1a+SPL1c, SPL1b+SPL1c, and 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 2 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 proapoptotic ceramide was significantly augmented (Fig. 4B), possibly as a consequence of the stimulation of SPT (Fig. 4B), a key enzyme in de novo pathway of ceramide generation. Interestingly, the content of proapoptotic sphingosine (Fig. 4B) was reduced, whereas SphK1 activity was increased in PC-3 overexpressing SPL (Fig. 4B). These data indicated 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 prosurvival mechanism is not sufficient enough as an important pool of S1P (about 30%, Fig. 4B) is degraded because of overexpression of SPL. Further analysis of SPL-overexpressing PC-3 cells indicated a different behavior compared with PC-3/Neo control cell line. PC-3–overexpressing SPL cell lines showed a reduced (about 30%) proliferation rate (Fig. 4C) as compared with PC-3/Neo cells, likely a consequence of a higher basal rate of apoptosis (Supplementary Fig. S1), in line with their relative changes in ceramide and S1P levels (Fig. 4B).

# **Opposite effects of SPL downregulation and overexpression with regard to irradiation**

As compared with 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 (Supplementary Fig. S2A) 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 Supplementary Fig. S2B). In contrast, invalidation of SphK1 by siRNA (21) rendered cells more sensitive to irradiation in PC-3 (Fig. 5A) and C4-2B cells (Supplementary Fig. S2A). 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  $\gamma$ -H2AX. Two Gy of irradiation induced H2AX phosphorylation in scrambled RNAi-transfected PC-3 (Fig. 5B) and C4-2B (Supplementary Fig. S2C) cells. In SPL-silenced cells, irradiation-induced H2AX

www.aacrjournals.org

Brizuela et al.



Figure 3. SPL inhibition by RNA silencing increases S1P levels in PC-3 and C4-2B prostate cancer cells, A. PC-3 (top) or C4-2B (bottom) cells were untransfected or transfected with 25 or 75 nmol/L of different siSPL (siSPL1a, siSPI 1b, and siSPI 1c) or 75 nmol/L scrambled siRNA (siScramble) for 72 hours. 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 3 independent experiments. SPL enzymatic activity (B) and S1P content (C) were quantified in PC-3 (left) or C4-2B (right) cells untransfected or transfected with 75 nmol/L siScramble or siSPL1b for 72 hours. Columns, mean of at least 3 independent experiments; bars, SEM. \*\*\*, *P* < 0.001.

phosphorylation was decreased, suggesting less DNA damage in these cells (Fig. 5B and Supplementary Fig. S2C). Conversely, the silencing of SphK1 in PC-3 (Fig. 5B) and C4-2B (Supplementary Fig. S2C) cells caused a robust increase of  $\gamma$ -H2AX.

The survival capability of SPL-overexpressing cell lines after irradiation was significantly reduced as compared with PC-3/Neo control cells (Fig. 5C). PC-3/SPL1 cells showed an increased  $\gamma$ -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 fluorescence-activated cell sorting (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 SPL knockdown and potentiated by SPL overexpression

Docetaxel is the current standard of care for the treatment of metastatic prostate cancer. As compared with the siScramble-pretreated PC-3 or C4-2B cells, the silencing of SPL significantly inhibited the loss of cell viability

1846 Mol Cancer Ther; 11(9) September 2012



**Figure 4.** SPL-enforced expression in PC-3 cells shifts the sphingolipid metabolism toward proapoptotic ceramide and reduces cell proliferation. A, SPL expression in 2 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  $\alpha$ -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 2-tailed *P* values between the means are \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001. C, cell proliferation was assessed with <sup>3</sup>H-thymidine incorporation assay. Points, mean of at least 3 independent experiments; bars, SEM.

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 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 PARP (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 an 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 2 products, ethanolamine phosphate and a long-chain aldehyde (32). Previous

www.aacrjournals.org

Brizuela et al.



**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 nmol/L siSPL1b, or 33 nmol/L siSPhK1 for 72 hours (left) or treated with 5 µmol/L exogenous S1P for 30 minutes then removed (right). 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 with nonirradiated cells. Points, mean of at least 4 independent experiments; bars, SEM. The 2-tailed *P* values between the means of siScramble and siSphK1 or siSPL1b-treated cells (left) or the means of untreated and S1P-treated cells (right) 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 3 independent experiments. C, PC-3/Neo and 2 different clones of PC-3 cells overexpressing SPL (PC-3/SPL1 and PC-3/SPL2) were transfected or not with siScramble (not shown as nontransfected or siScramble transfected cell lines reacted in a similar fashion) or siSphK1 for 72 hours. 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 with nonirradiated cells. Points, mean of at least 3 independent experiments; bars, SEM. The 2-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/Neo and the other experimental conditions are \*\*, *P* < 0.01; \*\*\*\*, *P* < 0.001. D, cell lysates from PC-3/Neo and PC-3/Neo and 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 l

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 carcinogen-

esis (14). In this study, we establish that both protein expression and enzymatic activity of SPL are downregulated in cancer versus noncancer tissue in prostate cancer patients and associated with tumor grade. Statistical

**Molecular Cancer Therapeutics** 



#### Sphingosine 1-Phosphate Lyase and Prostate Cancer

**Figure 6.** The modulation of S1P content in prostate cancer cells alters sensitivity to chemotherapy through caspase signaling. A, PC-3 (left) and C4-2B (right) cells were transfected with control siRNA (siScramble, 75 nmol/L, 72 hours) or siRNA directed to SPL (SPL1b, 75 nmol/L, 72 hours), then treated with the indicated concentrations of docetaxel for 72 hours, and cell viability was assessed by MTT. B, PC-3/Neo and 2 different clones of PC-3 cells overexpressing SPL (PC-3/SPL1 and PC-3/SPL2) were transfected or not with siScramble (not shown as nontransfected or siScramble-transfected cell lines reacted in a similar fashion) or siSphK1 for 72 hours. Cells were the treated with the indicated concentrations of docetaxel during 72 hours, and cell viability was assessed by MTT. Data are expressed as the percentage of cell viability compared with untreated cells. Points, mean of at least 4 independent experiments; bars, SEM. The 2-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 were transfected to SPL (SPL1b, 75 mm/L, 72 hours), then inclubated for the indicated times with 20 nmol/L docetaxel and 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 were treated for the indicated times with 20 nmol/L docetaxel and were analyzed for full-length caspase-3 (32 kDa) and PARP (116 kDa) expression. Equal loading of protein was monitored using antibody to  $\alpha$ -tubulin. Similar results were obtained in 3 independent experiments.

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 in which 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 downregulated in 8 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 show that silencing SPL enhances survival after irradiation by decreasing expression of  $\gamma$ -H2AX as well as DNA-PKcs-P and ATM-P (data not shown), proteins involved in the sensing and repairing of DNA DSBs. As expected, enforced expression of SPL sensitizes cancer cells to

www.aacrjournals.org

irradiation. Our findings are in line with earlier studies showing the radioprotective capability of S1P notably in vivo (10, 34). The prosurvival 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 SPL 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 with SphK1 activity) did not sensitize to docetaxel nor to radiotherapy (Supplementary Fig. S3).

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 prosurvival S1P and generation of proapoptotic 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 seems 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 and colleagues (12) showed a *de novo* synthesis of ceramide, whereas Kumar and colleagues ruled out this mechanism (15). Our results suggest a *de novo* synthesis through SPT activation. Additional data are

References

- 1. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nat Rev Mol Cell Biol 2003;4:397–407.
- Pyne NJ, Pyne S. Sphingosine 1-phosphate and cancer. Nat Rev Cancer 2010;10:489–503.
- Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind S, et al. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature 1996;381:800–3.
- Cuvillier O. Downregulating sphingosine kinase-1 for cancer therapy. Expert Opin Ther Targets 2008;12:1009–20.
- Pitson SM. Regulation of sphingosine kinase and sphingolipid signaling. Trends Biochem Sci 2011;36:97–107.

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 (Supplementary Fig. S4).

Overall, our clinical observations showing that SPL is downregulated in prostate cancer, whereas SphK1 is upregulated 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.

#### **Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

# Authors' Contributions

Conception and design: L. Brizuela, I. Ader, O. Cuvillier

**Development of methodology:** L. Brizuela, I. Ader, B. Malavaud Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Brizuela, I. Ader, C. Mazerolles, M. Bocquet, B. Malavaud

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Brizuela, I. Ader, C. Mazerolles, M. Bocquet, O. Cuvillier

Writing, review, and/or revision of the manuscript: L. Brizuela, C. Mazerolles, B. Malavaud, O. Cuvillier

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Brizuela, C. Mazerolles Study supervision: L. Brizuela, C. Mazerolles, O. Cuvillier

#### Acknowledgments

The authors thank Dr. Saba (Children's Hospital Oakland Research Institute, Oakland, CA) for helpful advices and for providing GFP-tagged wild-type and mutant human SPL cDNA; Dr. Casas and Dr. Fabriàs (Institut de Química Avançada de Catalunya, Barcelona, Spain) for providing us SPL substrate; and Ms. Mahéo (CHU Rangueil, Toulouse, France) for technical expertise.

#### Grant Support

O. Cuvillier was supported by the Association pour la Recherche sur le Cancer (ARC), the Ligue Nationale contre le Cancer (Equipe Labellisée LIGUE 2011), the Institut National du Cancer (INCa), and the Association pour la Recherche sur les Tumeurs de Prostate (ARTP).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 7, 2012; revised June 6, 2012; accepted June 20, 2012; published OnlineFirst July 10, 2012.

- Cuvillier O. Sphingosine kinase-1–a potential therapeutic target in cancer. Anticancer Drugs 2007;18:105–10.
- Malavaud B, Pchejetski D, Mazerolles C, de Paiva GR, Calvet C, Doumerc N, et al. Sphingosine kinase-1 activity and expression in human prostate cancer resection specimens. Eur J Cancer 2010;46: 3417–24.
- Pchejetski D, Doumerc N, Golzio M, Naymark M, Teissie J, Kohama T, et al. Chemosensitizing effects of sphingosine kinase-1 inhibition in prostate cancer cell and animal models. Mol Cancer Ther 2008;7:1836–45.
- Pchejetski D, Boehler T, Brizuela L, Sauer L, Doumerc N, Golzio M, et al. FTY720 (Fingolimod) sensitizes prostate cancer cells to

1850 Mol Cancer Ther; 11(9) September 2012

#### Sphingosine 1-Phosphate Lyase and Prostate Cancer

radiotherapy by inhibition of sphingosine kinase-1. Cancer Res 2010; 70:8651–61.

- **10.** Morita Y, Perez GI, Paris F, Miranda SR, Ehleiter D, Haimovitz-Friedman A, et al. Oocyte apoptosis is suppressed by disruption of the acid sphingomyelinase gene or by sphingosine-1-phosphate therapy. Nat Med 2000;6:1109–14.
- van Veldhoven PP, Mannaerts GP. Sphingosine-phosphate lyase. Adv Lipid Res 1993;26:69–98.
- Reiss U, Oskouian B, Zhou J, Gupta V, Sooriyakumaran P, Kelly S, et al. Sphingosine-phosphate lyase enhances stress-induced ceramide generation and apoptosis. J Biol Chem 2004;279:1281–90.
- Min J, Van Veldhoven PP, Zhang L, Hanigan MH, Alexander H, Alexander S. Sphingosine-1-phosphate lyase regulates sensitivity of human cells to select chemotherapy drugs in a p38-dependent manner. Mol Cancer Res 2005;3:287–96.
- 14. Oskouian B, Sooriyakumaran P, Borowsky AD, Crans A, Dillard-Telm L, Tam YY, et al. Sphingosine-1-phosphate lyase potentiates apoptosis via p53- and p38-dependent pathways and is downregulated in colon cancer. Proc Natl Acad Sci U S A 2006;103: 17384–9.
- Kumar A, Oskouian B, Fyrst H, Zhang M, Paris F, Saba JD. S1P lyase regulates DNA damage responses through a novel sphingolipid feedback mechanism. Cell Death Dis 2011;2:e119.
- Colie S, Van Veldhoven PP, Kedjouar B, Bedia C, Albinet V, Sorli SC, et al. Disruption of sphingosine 1-phosphate lyase confers resistance to chemotherapy and promotes oncogenesis through Bcl-2/Bcl-xL upregulation. Cancer Res 2009;69:9346–53.
- Xia P, Gamble JR, Wang L, Pitson SM, Moretti PA, Wattenberg BW, et al. An oncogenic role of sphingosine kinase. Curr Biol 2000;10: 1527–30.
- Cuvillier O, Mayhew E, Janoff AS, Spiegel S. Liposomal ET-18-OCH(3) induces cytochrome c-mediated apoptosis independently of CD95 (APO-1/Fas) signaling. Blood 1999;94:3583–92.
- Dayon A, Brizuela L, Martin C, Mazerolles C, Pirot N, Doumerc N, et al. Sphingosine kinase-1 is central to androgen-regulated prostate cancer growth and survival. PLoS One 2009;4:e8048.
- 20. Ader I, Muller C, Bonnet J, Favre G, Cohen-Jonathan E, Salles B, et al. The radioprotective effect of the 24 kDa FGF-2 isoform in HeLa cells is related to an increased expression and activity of the DNA dependent protein kinase (DNA-PK) catalytic subunit. Oncogene 2002;21:6471–9.
- Pchejetski D, Golzio M, Bonhoure E, Calvet C, Doumerc N, Garcia V, et al. Sphingosine kinase-1 as a chemotherapy sensor in prostate adenocarcinoma cell and mouse models. Cancer Res 2005;65: 11667–75.

- Ader I, Brizuela L, Bouquerel P, Malavaud B, Cuvillier O. Sphingosine kinase 1: a new modulator of hypoxia inducible factor 1alpha during hypoxia in human cancer cells. Cancer Res 2008;68:8635–42.
- Bedia C, Camacho L, Casas J, Abad JL, Delgado A, Van Veldhoven PP, et al. Synthesis of a fluorogenic analogue of sphingosine-1-phosphate and its use to determine sphingosine-1-phosphate lyase activity. Chembiochem 2009;10:820–2.
- 24. Brizuela L, Dayon A, Doumerc N, Ader I, Golzio M, Izard JC, et al. The sphingosine kinase-1 survival pathway is a molecular target for the tumor-suppressive tea and wine polyphenols in prostate cancer. FASEB J 2010;24:3882–94.
- Dickson RC, Lester RL, Nagiec MM. Serine palmitoyltransferase. Methods Enzymol 2000;311:3–9.
- Mitra P, Payne SG, Milstien S, Spiegel S. A rapid and sensitive method to measure secretion of sphingosine-1-phosphate. Methods Enzymol 2007;434:257–64.
- Pitson SM, Moretti PA, Zebol JR, Lynn HE, Xia P, Vadas MA, et al. Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. EMBO J 2003;22:5491–500.
- Rubin MA, Zhou M, Dhanasekaran SM, Varambally S, Barrette TR, Sanda MG, et al. alpha-Methylacyl coenzyme A racemase as a tissue biomarker for prostate cancer. JAMA 2002;287:1662–70.
- 29. Mediavilla-Varela M, Pacheco FJ, Almaguel F, Perez J, Sahakian E, Daniels TR, et al. Docetaxel-induced prostate cancer cell death involves concomitant activation of caspase and lysosomal pathways and is attenuated by LEDGF/p75. Mol Cancer 2009;8:68.
- 30. Cuvillier O, Rosenthal DS, Smulson ME, Spiegel S. Sphingosine 1phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. J Biol Chem 1998;273:2910–6.
- Nava VE, Cuvillier O, Edsall LC, Kimura K, Milstien S, Gelmann EP, et al. Sphingosine enhances apoptosis of radiation-resistant prostate cancer cells. Cancer Res 2000;60:4468–74.
- 32. Stoffel W, Assmann G. Metabolism of sphingosine bases. XV. Enzymatic degradation of 4t-sphingenine 1-phosphate (sphingosine 1phosphate) to 2t-hexadecen-1-al and ethanolamine phosphate. Hoppe Seylers Z Physiol Chem 1970;351:1041–9.
- 33. Min J, Stegner AL, Alexander H, Alexander S. Overexpression of sphingosine-1-phosphate lyase or inhibition of sphingosine kinase in *Dictyostelium discoideum* results in a selective increase in sensitivity to platinum-based chemotherapy drugs. Eukaryot Cell 2004;3:795–805.
- Paris F, Perez GI, Fuks Z, Haimovitz-Friedman A, Nguyen H, Bose M, et al. Sphingosine 1-phosphate preserves fertility in irradiated female mice without propagating genomic damage in offspring. Nat Med 2002;8:901–2.

www.aacrjournals.org



# **Molecular Cancer Therapeutics**

# First Evidence of Sphingosine 1-Phosphate Lyase Protein Expression and Activity Downregulation in Human Neoplasm: Implication for Resistance to Therapeutics in Prostate Cancer

Leyre Brizuela, Isabelle Ader, Catherine Mazerolles, et al.

Mol Cancer Ther 2012;11:1841-1851. Published OnlineFirst July 10, 2012.

Updated versionAccess the most recent version of this article at:<br/>doi:10.1158/1535-7163.MCT-12-0227Supplementary<br/>MaterialAccess the most recent supplemental material at:<br/>http://mct.aacrjournals.org/content/suppl/2012/07/10/1535-7163.MCT-12-0227.DC1.html

Cited articles	This article cites 34 articles, 14 of which you can access for free at: http://mct.aacrjournals.org/content/11/9/1841.full.html#ref-list-1
Citing articles	This article has been cited by 3 HighWire-hosted articles. Access the articles at: http://mct.aacrjournals.org/content/11/9/1841.full.html#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.