Sphingosine-1-Phosphate Lyase Regulates Sensitivity of Human Cells to Select Chemotherapy Drugs in a p38-Dependent Manner

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Abstract
Resistance to cisplatin is a common problem that limits its usefulness in cancer therapy. Molecular genetic studies in the model organism Dictyostelium discoideum have established that modulation of sphingosine kinase or sphingosine-1-phosphate (S-1-P) lyase, by disruption or overexpression, results in altered cellular sensitivity to this widely used drug. Parallel changes in sensitivity were observed for the related compound carboplatin but not for other chemotherapy drugs tested. Sensitivity to cisplatin could also be potentiated pharmacologically with dimethylsphingosine, a sphingosine kinase inhibitor. We now have validated these studies in cultured human cell lines. HEK293 or A549 lung cancer cells expressing human S-1-P lyase (hSPL) show an increase in sensitivity to cisplatin and carboplatin as predicted from the earlier model studies. The hSPL-overexpressing cells were also more sensitive to doxorubicin but not to vincristine or chlorambucil. Studies using inhibitors to specific mitogen-activated protein kinases (MAPK) show that the increased cisplatin sensitivity in the hSPL-overexpressing cells is mediated by p38 and to a lesser extent by c-Jun NH2-terminal kinase MAPKs. p38 is not involved in vincristine or chlorambucil cytotoxicity. Measurements of MAPK phosphorylation and enzyme activity as well as small interfering RNA inhibition studies show that the response to the drug is accompanied by up-regulation of p38 and c-Jun NH2-terminal kinase and the lack of extracellular signal-regulated kinase up-regulation. These studies confirm an earlier model proposing a mechanism for the drug specificity observed in the studies with Dictyostelium and support the idea that the sphingosine kinases and S-1-P lyase are potential targets for improving the efficacy of cisplatin therapy for human tumors. (Mol Cancer Res 2005;3(5):287–96)

Introduction
Cisplatin [cis-diaminedichloroplatinum (II)] is a widely used drug for the treatment of non-Hodgkin’s lymphoma, small cell and non–small cell lung cancers, and testicular, ovarian, head and neck, esophageal, and bladder cancers (1). However, resistance to the drug, whether pre-existing or acquired during treatment, continues to be a major obstacle to its efficacy. Cisplatin acts by causing DNA damage, which in turn initiates a cascade of events that result in cell death. Resistance to the drug can result from pre-damage events, such as changes in drug accumulation in the cell or drug inactivation. Alternatively, resistance can result from post-damage events, such as increased repair of the damaged DNA, or altering the signal transduction cascade leading to death (1, 2). Identifying the many pathways and molecules that are involved in the cellular response to cisplatin and other drugs will identify new molecular targets that may be exploited to increase the efficacy of the drugs. Given the difficulty and expense of producing new anticancer drugs, improving the usefulness of existing drugs is important.

A search for cisplatin-resistant mutants in the model organism Dictyostelium discoideum identified the gene that encodes the enzyme sphingosine-1-phosphate (S-1-P) lyase, which catalyzes the degradation of S-1-P to phosphoethanolamine and hexadecenal, as involved in modulating the response of cells to cisplatin (3). S-1-P is a bioactive lipid with multiple regulatory roles in the cell (4), and it has been suggested that it is the relative level of S-1-P to sphingosine or ceramide that determines a cell’s fate: elevated levels of ceramide lead to cell death and elevated levels of S-1-P will result in cell proliferation (5, 6). We hypothesized that an alteration in the sphingolipid balance, as a result of inactivation of S-1-P lyase, was the cause for the increased resistance of the S-1-P lyase–null mutant cells to cisplatin. Subsequent genetic studies in D. discoideum have firmly established the centrality of this pathway in controlling sensitivity to cisplatin in this organism (7, 8). As predicted, overexpression of the S-1-P lyase had the opposite effect of the null mutation and resulted in increased sensitivity to cisplatin. Disruption of the two sphingosine kinases (the enzymes that

Received 12/2/04; revised 3/9/05; accepted 3/18/05.
Grant support: NIH grants GM53929, CA95872 (S. Alexander and H. Alexander), and CA57530 (M.H. Hanigan) and Flemish Fonds voor Wetenschappelijk Onderzoek G.0405.02 (P.P. Van Veldhoven).
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Mol Cancer Res 2005;3(5). May 2005
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synthesize S-1-P from sphingosine and ATP) caused increased sensitivity to the drug, whereas overexpression of the sphingosine kinase enzyme resulted in decreased sensitivity. Pharmacologically, we have shown that addition of S-1-P resulted in increased resistance, whereas treatment with dimethylsphingosine, an inhibitor of sphingosine kinase (9), potentiated sensitivity to cisplatin, suggesting that cotreatment with both drugs may be useful clinically. Taken together, the results show that manipulation of the expression/activation of the S-1-P lyase and the sphingosine kinase enzymes directly influenced cisplatin efficacy.

The most surprising finding of all these studies in *D. discoideum* was that the effect of altering the S-1-P lyase and sphingosine kinase levels was specific for cisplatin and the related platinum drug carboplatin, whereas sensitivity to other chemotherapeutic drugs, such as etoposide, 5-fluoro-2'-deoxyuridine, or doxorubicin, was unchanged. S-1-P and ceramide have been shown to regulate opposing mitogen-activated protein kinases (MAPK). S-1-P up-regulates the survival MAPK/extracellular signal-regulated kinase (ERK) 1/2 and down-regulates the death kinase p38, whereas ceramide has the opposing effects on ERK1/2 and p38 (10). Thus, we asked whether the increase in sensitivity of the S-1-P lyase-overexpressing cells to cisplatin stems from the up-regulation of p38 or from the lack of up-regulation of the ERK1/2 and whether this can account for the preferential response to platinum compounds.

In the current study, we extend our previous studies in *D. discoideum* to human cells. We show that overexpression of the human S-1-P lyase (hSPL) results in increased sensitivity to the chemotherapeutic drug cisplatin and the related compound carboplatin as was shown for *D. discoideum*. In human cells, the increase in sensitivity also extends to doxorubicin but not to vincristine and chlorambucil. We further show that the increased sensitivity to cisplatin in the hSPL-expressing cells is mediated by the death MAPK p38 but not by the ERK1/2 or the phosphatidylinositol 3-kinase (PI3K) survival enzymes and that this accounts for the observed drug specificity.

**Results**

**Expression of the hSPL Gene in HEK293 and A549 Cells**

HEK293 cells were stably transfected with the pVB003 hSPL-FLAG construct or empty vector and selected for G418 resistance. Western analysis (Fig. 1A) confirms the expression of the fusion protein in the hSPL-overexpressing HEK293 cells. When grown in DMEM, the hSPL-overexpressing cells make 6-fold more S-1-P lyase than the vector control. When the cells are grown in minimal medium (Opti-MEM), the difference is even more pronounced, with >13-fold overexpression (Fig. 1B). The immunofluorescent staining pattern of the hSPL-transformed HEK293 cells suggests that the protein is associated with the endoplasmic reticulum, which agrees with experiments showing colocalization of hSPL with the known endoplasmic reticulum marker KDEL in Chinese hamster ovary cells (11), and with calreticulin in HeLa cells (12). Comparison of the immunofluorescent staining and phase images of >500 cells in the hSPL-overexpressing cell line indicated that 65% of the cells are overexpressing hSPL and that the expression level was reasonably constant among the brightly positive cells (data not shown). Figure 1D and E shows the Western analysis and immunofluorescent staining of transiently transfected A549 cells as described in A, E. Immunofluorescent staining of transiently transfected hSPL-overexpressing A549 cells as described in C.

**hSPL Regulates Growth Rate**

Comparison of the growth rates of the hSPL-overexpressing HEK293 cell line and the control cell line (Fig. 2) shows that both cell lines grow at identical rates in medium with full serum, which contains S-1-P (13). In contrast, both cell lines have lower growth rates in serum-free Opti-MEM medium, with the hSPL over-expressing cells being the most affected. The growth rate of both lines is increased to equal levels by the addition of S-1-P to the minimal medium. The growth rate

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4 P.P. Van Veldhoven, unpublished data.
in the S-1-P-supplemented serum-free medium is less than that in full serum presumably because of the lack of other serum factors. The decrease in growth rate is consistent to what was observed in *D. discoideum* cells overexpressing S-1-P lyase (7).

**hSPL Overexpression Results in Increased Drug Sensitivity**

Control and hSPL-overexpressing HEK293 cells were assayed for sensitivity to a range of concentrations of each of five drugs (Fig. 3A). The hSPL-overexpressing cells show increased sensitivity to both cisplatin and carboplatin, although carboplatin is less cytotoxic. The hSPL-overexpressing cells also showed increased sensitivity to doxorubicin. The increases in sensitivity are apparent throughout the concentration range. In contrast, no change in sensitivity was observed to vincristine or chlorambucil. The fact that only 65% of the HEK293 are overexpressing the hSPL transgene makes the result even more striking.

The increased sensitivity to cisplatin was not the result of altered uptake of the drug, because there is no difference in the uptake of the drug between the control and the hSPL-overexpressing cells (Fig. 3C). This agrees with measurements of cisplatin uptake made in *D. discoideum* cells overexpressing or lacking the S-1-P lyase or the sphingosine kinase genes, where there was no difference in uptake between the mutants and the parental controls (8).

To test whether the specificity profile to the drugs was cell type specific, we studied the response of A549 lung cancer cell line transiently transfected with the *hSPL* gene to the same drugs (Fig. 3B). The results shows that the hSPL-overexpressing A549 cells are more sensitive to cisplatin, carboplatin, and doxorubicin but not to the other drugs. Although the A549 cells are generally less sensitive to the drugs, the effect of hSPL overexpression is not unique to one cell type.

**Pharmacologic Modulation of S-1-P Metabolism in HEK293 Cells Alters Sensitivity to Cisplatin**

Treatment of the cells with the sphingosine kinase inhibitor dimethylsphingosine was predicted to mimic the phenotype of the hSPL-overexpressing cells (7, 8). Figure 4A shows that dimethylsphingosine has a dose-dependent toxic effect on the control HEK293 cells. Moreover, when given together, dimethylsphingosine dramatically increases the sensitivity of the cells to cisplatin. The level of killing is greater than the sum of the cell killing from cisplatin alone and dimethylsphingosine alone. When hSPL-overexpressing cells are treated with dimethylsphingosine, both the effect of dimethylsphingosine alone and the effect of dimethylsphingosine plus cisplatin are even more pronounced. This is presumably because the cells already have reduced levels of S-1-P due to the increased hSPL activity. Indeed, a decrease in the basal levels of S-1-P and sphingosine in HEK293 cells overexpressing the hSPL has been documented (14).

Addition of S-1-P increased the resistance of control HEK293 cells and hSPL-overexpressing HEK293 cells to cisplatin in a dose-dependent manner. Predictably, because of the increased hSPL activity in those cells, the effect is not as profound in the overexpressing cells as in the control cells (Fig. 4B). Adding S-1-P to dimethylsphingosine-treated cells reversed the effect of dimethylsphingosine (data not shown).

The effect of extracellular S-1-P likely is mediated through a G-protein-coupled receptor, because in the presence of pertussis toxin (an inhibitor of G, subunits) S-1-P failed to increase the resistance to cisplatin in either control or hSPL-overexpressing cells (Fig. 4C). Interestingly, although pertussis toxin alone had no effect on the viability of the control cells, it increased the sensitivity of the cells to cisplatin. This may indicate that there is cycling of S-1-P out of the cell and that the presence of pertussis toxin blocks its binding to the S-1-P receptor. The effect of pertussis toxin is even more apparent in the hSPL-overexpressing cells.

**Increase in Cisplatin Sensitivity Due to the hSPL Overexpression Is Mediated by p38**

S-1-P is known to regulate the MAPKs that in turn regulate cell survival or cell death (10). To determine if the increased sensitivity of the overexpressing strain to cisplatin resulted from up-regulation of p38 or from the lack of up-regulation of the ERK1/2 kinase, we treated the control and hSPL-overexpressing cells with specific inhibitors of MAPKs and determined the response of the cells to cisplatin.

Inhibiting the survival enzymes ERK1/2 (Fig. 5A) did not result in a statistically significant increase in sensitivity to cisplatin in either the control or the hSPL-overexpressing cells. Similarly, inhibition of the survival enzyme P38K did not alter the response of either cell line to cisplatin (Fig. 5B). The simultaneous inhibition of both ERK1/2 and P38K enzymes (Fig. 5C) resulted in an increased sensitivity to cisplatin. However, because both the control and the hSPL-overexpressing cell lines responded to the same extent, it suggests that the effect may be the result of a general inhibition of cell survival (see also Fig. 5F and G below). It also suggests that these enzymes can compensate for each other.
In contrast, inhibition of p38 had a significant effect on the response to cisplatin. Cotreatment of the control and hSPL-overexpressing cells with the p38 inhibitor resulted in an increase in resistance to cisplatin in both cell lines (Fig. 5D). The effect was more pronounced in the hSPL-overexpressing line in which the p38 inhibition restored resistance to the level seen in the control cells.

The stress-activated MAPK c-Jun NH2-terminal kinase (JNK) is also regulated by cellular levels of S-1-P (10). However, the inhibition of JNK (Fig. 5E) resulted in only a slight increase of resistance to cisplatin in both the control and the hSPL-overexpressing lines. These findings suggest that the primary mediator of cell death in response to cisplatin is p38.

To further test the specificity of the above findings for cisplatin, the effect of these inhibitors was examined with vincristine and chlorambucil, whose action was not influenced by the overexpression of S-1-P lyase (Fig. 3). As observed with cisplatin, simultaneous inhibition of both ERK and PI3K increased the sensitivity of both control and hSPL-overexpressing cells to vincristine (Fig. 5F) and chlorambucil (Fig. 5G) although to a lesser extent than with cisplatin. These data imply again that inhibiting both ERK1/2 and PI3K decreases the ability of a cell to survive drug toxicity in a nonspecific manner and that it was independent of the level of S-1-P lyase. Remarkably, inhibition of p38, which had a significant effect on the response to cisplatin, did not alter the sensitivity of the cell
lines to either vincristine (Fig. 5H) or chlorambucil (Fig. 5I). This clearly shows that p38 does not modulate the response to all drugs and explains the observed specificity of the response to those drugs that function through p38.

**Overexpression of hSPL Results in an Increase in the Level of Activated p38 in Response to Cisplatin**

Pharmacologic inhibition of MAPKs using specific inhibitors indicated that the effect of hSPL expression on cisplatin sensitivity was mediated primarily through p38. This suggested that stimulation with cisplatin would result in a concomitant change in the level of activation of the p38 protein. Western analysis, using antibodies to both the total and the phosphorylated forms of several MAPKs, shows that cisplatin treatment of both control and hSPL-overexpressing HEK293 cells causes the level of phospho-p38 to increase. The increase occurs by 30 minutes and is maintained through 6 hours, with a greater increase in the hSPL-overexpressing cells (Fig. 6A). The level of total p38 is constant for both cell lines.

Inhibition of JNK exhibited a smaller effect on cisplatin sensitivity than inhibitors of p38 (compare Fig. 5E and D, respectively). Figure 6B shows that both the 54-kDa and the 46-kDa forms of JNK are phosphorylated in response to cisplatin, but the response occurs later than that seen for p38 (i.e., no response at 30 minutes). The increase in phosphorylation is slightly greater in the hSPL-overexpressing cells. The phosphorylation is greater for the 46-kDa form than for the 54-kDa form. Interestingly, the level of total 54-kDa protein remains constant, whereas the level of 46-kDa protein increases as a result of cisplatin treatment.

In contrast, examination of ERK1/2 phosphorylation status (Fig. 6C) shows an opposing response where the phosphorylation of the 44- and 42-kDa forms of ERK is more pronounced in the control cells than in the hSPL-overexpressing cells. Indeed, in the overexpressing cells, the ERK1/2 phosphorylation seems to go down in the first 30 minutes and return to basal level at 6 hours. The level of total p44 and p42 ERK is constant for both cell lines. Western analyses of tubulin protein (Fig. 6D) confirmed equal protein loading for each time point.

**Sensitivity to Cisplatin Correlates with Increased Enzyme Activity of p38**

The pharmacologic studies and the MAPK phosphorylation data strongly support the idea that p38 plays a central role in modulating the effect of hSPL expression on cisplatin sensitivity. To measure this directly, we tested the kinase activity of p38 in control and hSPL-overexpressing cells after drug treatment. The results shown in Fig. 7 confirm that the increased sensitivity of the overexpressing cells correlates with increased p38 enzyme activity in both time- and concentration-dependent manner. The *in vivo* toxicity experiments in this study were done in the range of 1 to 10 μmol/L cisplatin, and these experiments are carried out for 3 days. To be able to measure the immediate, short-term, response in the *in vitro* p38 kinase assay, it is necessary to perform these experiments at higher doses of cisplatin. These conditions are based on the sensitivities of the assay and have been documented previously for *in vitro* p38 kinase assays (15, 16). Therefore, hSPL-overexpressing or control cells were treated with 50 and 250 μmol/L cisplatin for 30 minutes and 3 hours and were then

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**FIGURE 4.** Pharmacologic modulation of S-1-P metabolism affects the cells response to cisplatin. Cells were treated with 0, 1, 2, and 5 μmol/L dimethylsphingosine (DMS; A), 0, 1, 2, and 5 μmol/L S-1-P (B), and 1 μmol/L S-1-P and 100 ng/mL pertussis toxin (C) for 1 hour before the addition of 3 μmol/L cisplatin. Survival was measured at 72 hours of incubation using the MTS assay.

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Mol Cancer Res 2005;3(5). May 2005

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analyzed for p38 activity by monitoring the phosphorylation of the p38 target protein activating transcription factor-2 (ATF-2). The level of active p38 enzyme activity in the control cells increased 1.03- and 1.3-fold over the basal level after 30 minutes of treatment with 50 or 250 µmol/L cisplatin, respectively, and 1.3- and 4.0-fold over the basal level after 3 hours of treatment. This agrees with the data in Fig. 6 and the studies of Losa et al. (16), showing that p38 is phosphorylated in response to cisplatin. In contrast, the level of p38 activity in the hSPL-overexpressing cells increased 1.15- and 1.65-fold over basal level after 30 minutes and 4.2- and 7.8-fold after 3 hours of treatment with cisplatin. Thus, it is clear that the phosphorylation of p38 (Fig. 6A) accurately reflects the activation of the enzyme and that p38 activity is greatly enhanced in the hSPL-overexpressing cells.

Small Interfering RNA Inhibition of p38 Reverses the Effects of hSPL Overexpressing on Cisplatin Sensitivity

To establish further the role of p38 in the response to cisplatin, we tested the effect of p38 small interfering RNA (siRNA) inhibition. Fig. 8C shows that p38 expression is greatly reduced in both control and hSPL-overexpressing HEK293 cells transfected with p38 siRNA. Transfection efficiency was >90% based on a control transfection with fluorescein-conjugated siRNA (data not shown). As above, the hSPL-overexpressing cells are more sensitive than the control cells to cisplatin, but this increased sensitivity is reversed completely in the p38 siRNA-transfected hSPL-overexpressing cells (Fig. 8A and B).

Discussion

Previous studies using D. discoideum cells showed that manipulating the level of the enzymes that directly metabolize S-1-P alters the response of cells to cisplatin. Deleting the S-1-P lyase gene or overexpressing the sphingosine kinase increased resistance to cisplatin, whereas deleting the sphingosine kinase genes or overexpressing the S-1-P lyase resulted in increased sensitivity to cisplatin (3, 7, 8). The results strongly support the idea that the level of S-1-P directly influences drug sensitivity. Interestingly, there was a strong bias toward platinum-based drugs with parallel changes in sensitivity toward both cisplatin and carboplatin. There were no previous reports linking the enzymes responsible for S-1-P metabolism with cisplatin resistance. The present study extends these findings to human cells (HEK293 and A549 lung cancer) and confirms that these enzymes represent potential new molecular targets for modulating the efficacy of cisplatin in treating human cancer. More than a mere confirmation of previous studies in D. discoideum, this study shows that this genetically tractable model organism is effective in identifying new drug targets and emphasizes the usefulness of nonmammalian model systems in cancer research.

FIGURE 5. Effect of MAPK inhibitors on drug sensitivity in control and hSPL-overexpressing HEK293 cells. Left, control cells; right, hSPL-overexpressing cells. Cells were treated with 0, 1, 3, and 9 µmol/L cisplatin (A–E), 0, 0.05, 0.1, and 0.5 µmol/L vincristine (F and H), or 0, 10, 20, and 50 µmol/L chlorambucil (G and I) in the presence of the following inhibitors: 50 µmol/L MAPK kinase inhibitor PD98059 (A), 10 µmol/L PI3K inhibitor LY294002 (B), 50 µmol/L MAPK kinase inhibitor and 10 µmol/L PI3K inhibitors (C), 10 µmol/L p38 inhibitor SB203580 (D), 10 µmol/L JNK inhibitor SP600125 (E), 50 µmol/L MAPK kinase inhibitor and 10 µmol/L PI3K inhibitors (F and G), and 10 µmol/L p38 inhibitor (H and I). *, P ≤ 0.05; **, P ≤ 0.001.
FIGURE 6. hSPL expression in HEK293 cells affects the levels of each panel. By scanning the films and the corresponding bar graph are presented below of all the samples (D). The levels of phosphorylation were quantified by stripping and reprobed with anti-tubulin antibodies to confirm equal loading.

The level of S-1-P lyase in the HEK293 hSPL-overexpressing cells was increased 13-fold over the endogenous level and properly localized in the endoplasmic reticulum. These cells showed growth inhibition in minimal medium, which was reversed by addition of S-1-P. These results are consistent with those of Reiss et al. (14). The hSPL-overexpressing cells were more sensitive to cisplatin, carboplatin, and doxorubicin but not to vincristine or chlorambucil. Parallel studies on hSPL-overexpressing A549 lung cancer cells showed the same pattern of drug sensitivity, indicating that the effect is not specific to one cell type. The increase in sensitivity to doxorubicin seen in these studies is likely a reflection of the biology of human cells, as it was not observed in D. discoideum (7).

Nevertheless, the overexpression of hSPL did not result in an across the board increase in drug sensitivity. Atomic absorption spectrophotometry measurements of platinum levels did not indicate altered cisplatin uptake as the proximal cause of the change in sensitivity. These results agree with those in D. discoideum where cisplatin uptake was normal in S-1-P lyase overexpressor and null cells and in sphingosine kinase overexpressor and null cells. Taken together, these results indicate that S-1-P acts through a mechanism not directly associated with plasma membrane transporters or pumps.

We proposed previously a model where the balance between ERK and p38, which reflects the balance between the basal levels of S-1-P and ceramide (10), ultimately would control sensitivity to cisplatin. The model was based on the genetic studies in D. discoideum (7, 8), where modulating the enzymes of S-1-P metabolism altered the response of cells to chemotherapeutic drugs in a specific manner, and on recent reports that suggest that cisplatin exerts its cytotoxic effect through the downstream activation of the MAPK p38 and the down-regulation of the ERK1/2 MAPK (16-18). Here, we present three types of evidence—genetic (through the use of stable and transient overexpression cell lines), pharmacologic (through the use of inhibitors), and biochemical (by directly measuring enzyme activity)—to support this model. These data show that p38 signaling seems to be the main pathway, which mediates cellular viability in response to changes in sphingolipid levels. We show that overexpression of the S-1-P lyase results in a significantly higher level of p38 enzyme activity, which in turn results in an increased sensitivity to cisplatin. Inhibition of p38 by siRNA in the overexpressor line reversed this increased sensitivity to that observed with the control cells. The model then argues that in normal cells the balance between the bioactive lipids allows for activation of p38 to a certain level in response to cisplatin, resulting in a corresponding level of cell death. In cells overexpressing the S-1-P lyase, where the basal level of S-1-P is much lower than in normal cells, the activation of p38 is more robust and the level of cisplatin-mediated cell death is correspondingly higher.

The study by Reiss et al. (14) showed that the overexpression of S-1-P lyase in HEK293 cells resulted in a diminished basal level of sphingosine and S-1-P as well as an increase in the intracellular levels of ceramide. These findings agree with the findings in this study because ceramide does activate p38 (19). Thus, the combination of lower S-1-P in the cells, as a result of S-1-P lyase overexpression, and a concomitant increase in ceramide result in shifting the balance between the opposing MAPKs, which ultimately influences the response of the cells to drugs that act through the activation of p38.

The involvement of p38 in the response of cells of nontumor origin to cisplatin raises the question of preferential toxicity of cisplatin for tumor cells (20) and suggests ways to protect nontumor cells from the drug. For example, cisplatin shows significant nephrotoxicity in humans and different mechanisms have been implicated (21-23). The specific inhibition of hSPL (and/or activation of sphingosine kinase) in the kidney might reduce the toxicity and allow cisplatin to be used at a higher dose that is more effective in killing tumor cells.

S-1-P levels in cells are regulated by the activity of the sphingosine kinases, S-1-P phosphatase and the S-1-P lyase.
Previous studies suggested that the sphingosine kinase acts as an oncogene (24), and elevated levels of sphingosine kinase have been reported in a variety of tumors (25, 26). The S-1-P lyase is located on chromosome 10q21. Several cytogenetic studies have shown that this region of chromosome 10 is deleted or rearranged in a variety of different cancers (27-29). Direct evidence for a tumor-suppressing function for S-1-P lyase is still missing, although there is ample evidence that S-1-P modulates the immune response (30, 31) and as a consequence probably modulates sensitivity to cancer. The data in the current study linking the level of this enzyme to cisplatin sensitivity strengthen this correlation. Moreover, we suggest that deletion of this chromosomal region in some tumors may result in cells that are more resistant to cisplatin-based therapy. This information then could be used clinically to establish alternative chemotherapy regimens. The next important step will be to test the usefulness of modulating sphingolipids in xenograft studies in animals. Local administration of enzyme inhibitors to tumors, or tumor-specific expression of the relevant enzymes, should show the usefulness of S-1-P lyase and sphingosine kinase as targets to improve the efficacy of cisplatin in chemotherapy.

Materials and Methods

Reagents

Cisplatin, carboplatin, doxorubicin, chlorambucil, vincristine, S-1-P, pertussis toxin, geneticin, mouse anti-tubulin antibody, and mouse anti-FLAG M2 monoclonal antibody were from Sigma Chemical Co. (St. Louis, MO). N,N-dimethylsphingosine, sphingosine, and d-erythro-S-1-P were from Biomol (Plymouth Meeting, PA). MAPK kinase inhibitor PD98059, p38 inhibitor SB203580, JNK inhibitor SP600125, and PI3K inhibitor LY294002 were from Calbiochem (La Jolla, CA). DMEM, Opti-MEM, fetal bovine serum, MEM nonessential amino acids, and LipofectAMINE 2000 were from Invitrogen (Carlsbad, CA). Antibodies to MAPKs and phosphorylated MAPKs and Nonradioactive p38 Assay kit were from Cell Signaling Technology (Beverly, MA). BCA protein determination assay and horseradish peroxidase–conjugated anti-mouse IgG were from Pierce (Rockford, IL). Alexa 488–conjugated goat anti-mouse IgG antibody was from Molecular Probes (Eugene, OR), d-erythro-[4,5-3H]dihydrosphingosine-1-phosphate and unlabeled d-erythro-dihydrosphingosine-1-phosphate were from American Radiolabeled Chemicals (St. Louis, MO).

Recombinant hSPL

A recombinant clone was generated as follows. An amplicon, covering the 5’ end of the hSPL cDNA (base -177 to 286), was

![Figure 7](image_url)  
**FIGURE 7.** p38 enzyme activity. The hSPL-overexpressing and control cell lines were treated with 0, 50, or 250 μmol/L cisplatin for 30 minutes (A) or 3 hours (B). Protein (250 μg) from each sample was incubated with anti-phospho-p38 antibody–conjugated beads overnight at 4°C. p38 kinase activity was assayed by phosphorylation of the substrate protein activating transcription factor-2 (ATF-2) and visualized with anti-phospho–(ATF-2) antibodies. Results were quantified and bar diagrams, depicting fold activation over nontreated samples, are presented in C and D, respectively.

![Figure 8](image_url)  
**FIGURE 8.** siRNA inhibition of p38 confirms that hSPL acts through p38 to control sensitivity to cisplatin. Sensitivity to cisplatin in vector control (A) and hSPL-overexpressing HEK293 cells (B) following siRNA inhibition of p38. ○, p38 siRNA; ●, nontransfected cells. C. Western analysis. Lanes 1 to 4, level of p38; lanes 5 to 8, level of the control ATP citrate lyase.
obtained by PCR on a human liver 5’ stretch plus λgt11 cDNA library (Clontech, Palo Alto, CA; ref. 32). This was used as a primer together with hSPL15r (5’-GGAATTCGTTCATG-GTCTATCTTGGAG) and ESI44070 as template to generate a full-length hSPL cDNA. After a second PCR on this full-length cDNA with hSPL17f (5’-CGAGATCTCTAGCAGACCT-TCTGATG) and hSPL15r as primers, the amplicon was treated with dATP/Taq DNA polymerase, ligated into pCRII-Topo (Invitrogen), and transfected in Top10F’ Escherichia coli. Plasmids from selected clones were treated with Bgl II and EcoRI and the insert was subcloned into Bam HI/EcoRI–restricted pCMV-Tag2B (Stratagene, La Jolla, CA) to generate pVB003 vector (11).

Cell Lines and Transfection

The human embryonic kidney cell line (HEK293) or the lung cancer cell line (A549) were cultured at 37°C, 5% CO2 in DMEM, 10% fetal bovine serum or in serum-free Opti-MEM medium. For generating stable cell lines, cells were seeded in 75 mm2 flasks and transfected with 20 μl medium. For generating stable cell lines, cells were seeded in DMEM, 10% fetal bovine serum or in serum-free Opti-MEM. After 24 hours, cells were washed twice with Opti-MEM and then grown in the DMEM, 10% fetal bovine serum. After 24 hours, cells were transfected with Bgl II and EcoRI and the insert was subcloned into Bam HI/EcoRI–restricted pCMV-Tag2B (Stratagene, La Jolla, CA) to generate pVB003 vector (11).

Growth Measurements

Cells (5 x 10^3 per well) were plated in six-well plates in DMEM, 10% fetal bovine serum. After 24 hours, cells were washed twice with Opti-MEM and then grown in the indicated medium. At indicated times, cells were washed with PBS, trypsinized, counted three times in Beckman-Coulter Z1 Particle Counter (Hialeah, FL), and the results were averaged. The data represent the average of three wells per time point.

S-1-P Lyase Assay

Cells (1 x 10^5) were harvested and washed in cold lysis buffer (0.25 mol/L sucrose, 5 mmol/L MOPS, 1 mmol/L EDTA, 1 mmol/L DTT, protease inhibitors). Pellets were lysed in 80 μl lysis buffer, 0.5% Triton X-100 and diluted to 0.1% Triton X-100 with lysis buffer, and 40 μl were used per assay. Protein concentration was determined using BCA protein assay. The enzyme assay was done as described (33) using D-erythro-[4,5-3H]dihydrosphingosine-1-phosphate as substrate, and the activity is expressed as pmol/min/mg protein.

Drug Treatment and Cytotoxicity Assay

Exponentially growing cells were seeded 1 day before the experiment in Opti-MEM medium and then treated with various concentrations of indicated drugs. The use of Opti-MEM is necessary for drug assays because serum contains S-1-P (34) and because cisplatin binds to serum albumin (34, 35). Stock solutions were prepared in 3 mmol/L NaCl, 1 mmol/L NaPO4 (pH 6.5) or in DMSO (not to exceed a final concentration of 0.2% DMSO). For experiments using combinations of cisplatin and other reagents, cells were preincubated with the various agents 1 hour before the addition of cisplatin. Viability was determined using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, CellTititer 96 Aqueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI) according to the manufacturer’s instructions. Percent survival was calculated as the absorbance ratio of treated to untreated cells. In experiments where inhibitors were used, the effect of the inhibitor alone was compared with the untreated cells, but the effect of cisplatin in the presence of the inhibitor was compared with that of cells with inhibitor alone. Each experimental treatment was done in six replicates per point and each experiment was repeated twice. Results were expressed as mean ± SD. Data presented were analyzed with Student’s t test. Ps < 0.05 were accepted as a statistically significant difference compared with controls (*, P ≤ 0.05; **, P ≤ 0.001).

siRNA Inhibition of p38

A SignalSilence Pool p38 MAPK siRNA kit (Cell Signaling Technology) was used according to the manufacturer’s specifications. p38 MAPK siRNAs (10 nmol/L) were transfected into stable hSPL-overexpressing and control cells. A target-specific anti-p38 antibody was used to confirm the silencing of p38 MAPK expression. A non-target-specific antibody to ATP citrate lyase was used to control for equal loading and to monitor the specificity of p38 MAPK siRNA. A fluorescein-labeled nontarget siRNA control was used to monitor the transfection efficiency.

p38 Enzyme Activity

hSPL-overexpressing and control cells were grown in six-well plates to 80% confluence and then treated with 50 and 250 μmol/L cisplatin for 30 minutes and 3 hours. Cultures were harvested in non-denaturing buffer, lysed by sonication, and centrifuged to remove cell debris. Protein concentration was determined by BCA. Extract (250 μg) was used for each assay of p38 activity by monitoring the phosphorylation of the p38 target protein AFT-2, using the Nonradioactive p38 MAPK Assay kit, according to the manufacturer’s instructions. All assays were done using the same amount of protein extract, as determined by BCA, and was confirmed by probing a parallel set of samples with anti-tubulin antibodies.

Western Analysis

Cells were cultured in six-well plates, washed three times with ice-cold PBS, scraped into PBS, and collected by centrifugation. Pellets were washed three times in ice-cold PBS and lysed by sonication in buffer containing 62 mmol/L Tris-HCl (pH 6.8), 2% SDS, 5 mmol/L DTT, 1 mmol/L EDTA, and protease inhibitors. Protein concentration was determined by BCA, and protein (20 μg) was assayed as described (8). Loading of equal protein samples was confirmed by stripping the membranes and reprobing with anti-tubulin antibodies.
All antibodies were used according to the manufacturer’s instructions. Quantitation of Western analysis was done by scanning the blots in high resolution on a flat bed scanner and quantifying the bands using MetaMorph 4.6r9 program.

**Immunofluorescence Microscopy**

Cells (3 × 10^6 per well) were grown in six-well plates for 48 hours on sterile glass coverslips. The cells were washed three times with ice-cold PBS, fixed in 3.7% paraformaldehyde for 30 minutes at 4 °C, and washed three times with PBS. Cells were permeabilized with PBS, 0.1% Triton X-100 for 10 minutes at room temperature and blocked for 1 hour with PBS, 1% bovine serum albumin, and 5% normal goat serum. Cells were incubated for 1 hour at room temperature with a 1:200 dilution of mouse anti-FLAG monoclonal antibody and washed five times in PBS followed by 1-hour incubation with the appropriate anti-mouse IgG Alexa 488–conjugated secondary antibody. The cells were washed with PBS and examined with an Olympus 1X-70 microscope fitted with a MRC-600 confocal laser (Bio-Rad Laboratories, Hercules, CA).

**Intracellular Platinum Concentration**

Stably transfected HEK293 cells in six-well plates were exposed in triplicate to 0 to 100 μmol/L cisplatin for indicated times. After cisplatin treatment, cells were rapidly trypsinized, washed twice with ice-cold PBS, and pelleted at 4 °C. The platinum concentration was determined as described using atomic absorption spectrophotometry (8).

**Acknowledgments**

We thank Andy Stegner, Priya Sridevi, and Bandhana Katoch for assistance and suggestions. We also thank Mayandi Sivaguru of the Molecular Cytology Core.

**References**

Molecular Cancer Research

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Junxia Min, Paul P. Van Veldhoven, Lei Zhang, et al.


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