Upregulation of ABC transporters contributes to chemoresistance of sphingosine 1-phosphate lyase-deficient fibroblasts

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Abstract  Sphingosine 1-phosphate (S1P) is an extra- and intracellular mediator that regulates cell growth, survival, migration, and adhesion in many cell types. S1P lyase is the enzyme that irreversibly cleaves S1P and thereby constitutes the ultimate step in sphingolipid catabolism. It has been reported previously that embryonic fibroblasts from S1P lyase-deficient mice (Sgpl1−/−-MEFs) are resistant to chemotherapy-induced apoptosis through upregulation of B cell lymphoma 2 (Bcl-2) and Bcl-2-like 1 (Bcl-xL). Here, we demonstrate that the transporter proteins Abcc1/MRP1, Abcb1/MDR1, Abca1, and spinster-2 are upregulated in Sgpl1−/−-MEFs. Furthermore, the cells efficiently sequestered the substrates of Abcc1 and Abcb1, fluo-4 and doxorubicin, in subcellular compartments. In line with this, Abcb1 was localized mainly at intracellular vesicular structures. After 16 h of incubation, wild-type MEFs had small apoptotic nuclei containing doxorubicin, whereas the nuclei of Sgpl1−/−-MEFs appeared unchanged and free of doxorubicin. A combined treatment with the inhibitors of Abcb1 and Abcc1, zosuquidar and MK571, respectively, reversed the compartmenalization of doxorubicin and rendered the cells sensitive to doxorubicin-induced apoptosis. It is concluded that upregulation of multidrug resistance transporters contributes to the chemoresistance of S1P lyase-deficient MEFs.—Ihlefeld, K., H. Vienken, R. F. Claas, K. Blankenbach, A. Rudowski, M. ter Braak, A. Koch, P. P. Van Veldhoven, J. Pfeilschifter, and D. Meyer zu Heringdorf. Upregulation of ABC transporters contributes to chemoresistance of sphingosine 1-phosphate lyase-deficient fibroblasts. J. Lipid Res. 2015. 56: 60–69.

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Intrinsic or acquired chemoresistance is one of the major problems of modern tumor therapy. Chemoresistance of tumor cells can be caused by several mechanisms such as induction of drug metabolism, upregulation of multidrug transporters, modification of drug targets, cell cycle arrest, regulation of DNA replication and repair, and modulation of apoptosis (1). About two decades of research now have demonstrated that sphingolipids play a role in the regulation of cell survival, apoptosis, and chemoresistance, with general consensus identifying ceramide as a proapoptotic and sphingosine 1-phosphate (S1P) as an antiapoptotic mediator (2, 3). According to the sphingolipid biostat model, the equilibrium between S1P and ceramide regulates cell fate decisions (4). Ceramide and S1P are metabolically interconverted by ceramidases and sphingosine kinases (SphKs), phosphatases and ceramide synthases, respectively (5). S1P acts as an agonist at specific G protein-coupled receptors, termed S1P1–5, to regulate cell cycle growth, migration, and cell-cell contacts and thereby modulates lymphocyte emigration from lymphatic tissues, angiogenesis, vascular barrier function, tissue homeostasis, and inflammation (6, 7). In addition, several intracellular activities of S1P have been described recently, where this mediator was delivered by SphK1 or SphK2 directly to a target protein, for example histone deacetylases (HDACs), tumor necrosis factor receptor-associated factor-2, mitochondrial prohibitin-2, or β-site amyloid precursor protein cleaving enzyme-1 [reviewed in Maceyka et al. (6)]. In contrast to these apparently direct interactions of SphKs with S1P target proteins, intracellularly generated S1P has to be transported across the plasma membrane to be able to activate its specific G protein-coupled receptors (8). Among

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Abbreviations: [Ca2+]i, intracellular free Ca2+ concentration; ER, endoplasmic reticulum; HDAC, histone deacetylase; MEF, mouse embryonic fibroblast; S1P, sphingosine 1-phosphate; SphK, sphingosine kinase; TSA, trichostatin A.

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the transporter proteins that have been shown to export S1P, there are several members of the ABC transporter family such as the multidrug resistance-related protein Abcc1, the cholesterol transporter Abca1, and the breast cancer resistance protein Abcg2 (9–11). While these ABC transporters either do not regulate plasma concentrations of S1P or are redundant with respect to this activity (12), plasma S1P is regulated by the non-ABC transporter-related transport protein spinster-2, which is probably a specific S1P transporter (13, 14).

While the role of ABC transporters in chemoresistance of cancers is widely recognized (1, 8, 15), the role of the enzymes that catalyze S1P formation and degradation in cancer growth and survival appears to be less clear. SphK1 is upregulated in many cancers, and cancer cells might have a “non-oncogene addiction” for this enzyme (16); however, a new highly potent SphK1 and SphK1/2 inhibitors failed to inhibit cancer cell proliferation and growth of tumor xenografts in mice (17, 18). Recently, also S1P lyase, which is an endoplasmic reticulum (ER) resident enzyme that cleaves S1P irreversibly and catalyzes the ultimate step in sphingolipid catabolism, has been connected with cancer growth and chemoresistance (19–21). Early studies have shown that overexpression of S1P lyase rendered the cells sensitive to apoptosis induced by serum deprivation or chemotherapeutic agents, while siRNA-induced knockdown diminished apoptosis at baseline and in response to chemotherapy (22–24). Recent studies show that S1P lyase is downregulated in human colon cancers (24), human melanoma cell lines (19), and human prostate cancers (21). Fibroblasts lacking S1P lyase were able to form colonies in soft agar and to induce tumors in immunocompromised mice (19). In human prostate cancers, S1P lyase expression and activity was inversely correlated with clinical malignancy scores, and cell death of prostate cancer cell lines, induced by irradiation or chemotherapy, was reduced by S1P lyase knockdown and potentiated by S1P lyase overexpression (21).

Although a low expression of S1P lyase thus appears to be favorable for proliferation and survival of cancer cells, the presence of the enzyme is essential for development and survival of the mammalian organism as a whole. Thus, a full knockout of S1P lyase in the mouse induced growth retardation and early death after a few weeks, accompanied by severe organ damage, immunosuppression, a major disturbance of lipid homeostasis, and a proinflammatory phenotype [for review, see Aguilar and Saba (20)]. On the other hand, mouse embryonic fibroblasts (MEFs) from S1P lyase-deficient mice (Sgpl1−/−MEFs) proliferated without retardation and, in the absence of serum, even better than MEFs from wild-type mice (19). The resistance of Sgpl1−/−MEFs to apoptosis induced by doxorubicin and etoposide was ascribed to upregulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL, because a combined knockdown of both proteins with siRNA improved the sensitivity of the cells (19). In our own studies on these S1P lyase-deficient fibroblasts, we observed that S1P accumulated by ~5-fold compared with wild-type cells when whole cells were extracted and ~50-fold in nuclear preparations (25, 26). Furthermore, we observed a reduced HDAC activity and downregulation of class I HDACs, which contributed to the disturbed Ca2+ homeostasis with enhanced Ca2+ storage and elevated basal intracellular free Ca2+ concentration ([Ca2+]i) in these cells (26).

The starting point of our present study was the hypothesis that this accumulation of S1P in S1P lyase-deficient MEFs may induce counterregulatory mechanisms such as upregulation of S1P secretion, which would then keep cytosolic S1P at a normal level while nuclear pools of S1P probably have no access to the export mechanisms. Therefore, we studied the expression and functionality of the transporters that have been implicated in S1P export, i.e., spinster-2, Abcc1, Abca1, and Abcg2, and also of the multidrug resistance protein Abcb1, which is known to be regulated by SphK and S1P (27). We demonstrate that several of the transporters were upregulated in Sgpl1−/−MEFs and caused a sequestration of the ABC transporter substrates fluo-4 and doxorubicin. Furthermore, we show that the compartmentalization of doxorubicin contributes significantly to the chemoresistance of these cells. These results link S1P lyase to the regulation of multidrug transporters and suggest that this activity, in addition to S1P’s ability to interfere with apoptotic signaling pathways, plays an important role in cancer cell chemoresistance.

MATERIALS AND METHODS

**Materials**

Fluo-4/AM, tetramethylrhodamine, ER-Tracker Blue-White DPX, LysoTracker Red DND-99, and Hoechst 33342 were obtained from Molecular Probes/Invitrogen (Invitrogen GmbH, Karlsruhe, Germany). Doxorubicin, MK571, probenecid, verapamil, staurosporine, and fatty acid-free BSA were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Trichostatin A (TSA) was from Calbiochem/Merck Millipore (Darmstadt, Germany), and zosquidar was from Selleck Chemicals LLC (Houston, TX). All other chemicals were from previously described sources (25, 26).

**Cell culture**

Embryonic fibroblasts from S1P lyase-deficient and corresponding wild-type mice had been prepared as described previously (25). The cells were cultured in DMEM/F12 supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin, and 10% FCS in a humidified atmosphere of 5% CO2/95% air at 37°C. If not stated otherwise, the cells were kept in serum-free medium overnight before experiments.

**Measurements of S1P and sphingosine in cellular supernatants**

The cells were seeded onto 3.5 cm dishes and grown to near confluence. They were kept in serum-free medium supplemented with 1 mg/ml fatty acid-free BSA in the absence or presence of 10 µM sphingosine for 16 h. Thereafter, the cellular supernatans (1.5 ml) were collected and centrifuged for 10 min at 1,800 x g and 4°C. The supernatans were transferred into fresh tubes and supplemented with 1.2 ml methanol containing 20 ng/ml v-erythro-C17-sphingosine and 20 ng/ml v-erythro-C17-SIP (Avanti Polar Lipids Inc., Alabaster, AL) as internal standards. Then 35 µl 1 M HCl, 70 µl 10% KCl, and 2 ml chloroform...
were added, and after thorough mixing and centrifugation, the organic phase was collected. The aqueous phase was reextracted two times with chloroform, and the organic phases were combined and dried down. The lipids were redissolved in 200 μl DMSO containing 2% HCl, and LC-MS/MS was performed as previously described (28). The cell pellets were washed, scraped into lysis buffer, and subjected to protein measurements.

Measurements of \(^{[3}H\)SIP and \(^{[3}H\)phosphoinosine in cells and supernatants

The cells were seeded onto 3.5 cm dishes and grown to near confluence. Before experiments, they were kept for 16 h either in serum-free medium supplemented with 10 mg/ml fatty acid-free BSA or in medium containing 10% FCS. Labeling was performed in the respective media for 2 h with 0.5 μCi/ml \(^{[3}H\)phosphoinosine. Then, the cells were washed twice and incubated for a further 4 h either in serum-free medium supplemented with 10 mg/ml fatty acid-free BSA or in medium containing 10% FCS. Thereafter, the cellular supernatants (1 ml) were collected, and 1 ml methanol, 70 μl 10% KCl, 35 μl 1 M HCl, and 2 ml chloroform were added. Cell monolayers were washed with ice-cold PBS and scraped into 1 ml methanol. The dishes were washed with 1 ml methanol, and 1.6 ml of high salt solution (0.74% KCl, 0.04% CaCl₂, 0.034% MgCl₂), 35 μl 1 M HCl, and 2 ml chloroform were added. Lipid extraction was performed as described for the LC-MS/MS measurements. The dried samples were redissolved in 50 μl methanol and separated by TLC with 1-butanol:acetic acid:water 3:1:1. Arachidonic acid containing S1P and sphingosine, respectively, were identified with nonradioactive standard samples and scraped off the TLC plates, and radioactivity was quantified by liquid scintillation counting. Separate dishes were used for protein measurements.

Fluorescence microscopy

For microscopic analysis, the cells were cultured on 8-well chambered coverslides (µ-slide; ibidi GmbH, Martinsried, Germany) coated with poly-L-lysine. If not stated otherwise, the cells were fixed with formaldehyde and stained with anti-p-glycoprotein antibody (Sigma-Aldrich Chemie GmbH) followed by Alexa-Fluor 555-conjugated anti-mouse antibody (Invitrogen GmbH). The fluorescence was monitored by confocal microscopy as described previously (28).

Immunocytochemistry

The subcellular distribution of Abcb1 was analyzed by immunocytochemistry. Cells grown on 8-well chambered coverslides were fixed with formaldehyde and stained with anti-p-glycoprotein antibody (Sigma-Aldrich Chemie GmbH) followed by Alexa-Fluor 555-conjugated anti-mouse antibody (Invitrogen GmbH). The fluorescence was monitored by confocal microscopy as described above using the 543 nm excitation laser line and a 575–630 nm emission band pass filter.

PCR

mRNA was isolated from serum-starved MEFs with TRIZOL (Sigma-Aldrich Chemie GmbH). cDNA was prepared with the RevertAid first strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany). Real-time PCR was performed with the Applied Biosystems 7500 Fast Real-Time PCR System. Probes, primers, and the reporter dyes 6-FAM and VIC were from Applied Biosystems (Darmstadt, Germany). The cycling conditions were 95°C for 15 min (1 cycle), followed by 95°C for 15 s and 60°C for 1 min (40 cycles). mRNA expression levels were analyzed by the ΔΔCt method with GAPDH as reference.

Western blotting

Cell lysates were separated by SDS gel electrophoresis and blotted onto polyvinylidene difluoride membranes. Blots were stained with antibodies directed against Abcc1 (Abcam, Cambridge, UK), caspase-3 (Cell Signaling Technology, Danvers, MA), or β-actin (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and analyzed with HRP-conjugated secondary antibodies using the ECL system (GE Healthcare, Freiburg, Germany).

Cell viability assays

In the first set of experiments, Sgpl1\(^{-/-}\) and Sgpl1\(^{-/-}\)-MEFs were seeded onto 96-well plates and grown to near confluence. The cells were incubated with doxorubicin, staurosporine, or vehicle for 16 h in serum-free medium, and cell viability was analyzed with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer’s instructions (CellTiter 96 NonRadioactive Cell Proliferation Assay; Promega, Mannheim, Germany). In the second set of experiments, MEFs were seeded onto 24-well plates, grown to near confluence, and pretreated with ABC transporter inhibitors for 1 h before addition of the chemotherapeutic agents. Cell viability was analyzed with the sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium (XTT) assay (Cell Proliferation Assay XTT; AppliChem, Darmstadt, Germany).

Data analysis and presentation

Averaged data are means ± SEM from the indicated number (n) of independent experiments or means ± SD from a representative experiment performed with n replicates. Graphical presentation and statistical analysis were performed with Prism-5 (GraphPad Software, San Diego, CA). Microscopic images are representative for at least three similar experiments and were edited using the LSM Image Browser or the ZEN software (http://www.zeiss.com/micro).

RESULTS

Because S1P accumulated in S1P lyase-deficient MEFs, we hypothesized that the cells would try to counteract this by upregulating S1P secretion. Therefore, we analyzed the expression of the multidrug transporters Abcc1 (= MRPI), Abca1, and Abcg2, which all have been associated with S1P extrusion, of Abcb1 (= MDR1 or p-glycoprotein), which is known to be regulated by S1P metabolism (27), and of spinster-2, which appears to be a specific S1P transporter (13, 14). Indeed, the mRNA levels of Abcc1 and Abca1 were upregulated by 3- to 5-fold in Sgpl1\(^{-/-}\)-MEFs, while Abcg2 remained unaltered (Fig. 1A). Of the two transcripts of Abcb1, Abcb1a was strongly induced by ~10-fold, while Abcb1b was upregulated by ~2-fold (Fig. 1A). Spinster-2 mRNA was induced by ~3-fold in Sgpl1\(^{-/-}\)-MEFs (Fig. 1A). On the protein level, both the unglycosylated form at ~130 kDa and the glycosylated mature form at ~170 kDa of Abcc1
were upregulated in Sgpl1−/−-MEFs (Fig. 1B). The expression of the mature form was quantified by densitometry and found to be enhanced by ~2-fold (Fig. 1B). Because it is known that HDAC inhibitors can upregulate multidrug resistance transporters in tumor cells, and because HDAC activity and expression of class I HDACs were decreased in Sgpl1−/−-MEFs, we analyzed whether a treatment with the pan-HDAC inhibitor TSA induced an upregulation of the transporters in wild-type cells. However, TSA did not upregulate Abcc1, Abcb1, or Abca1 in either Sgpl1+/+ or Sgpl1−/−-MEFs (data not shown). In contrast, TSA strongly induced the expression of spinster-2 by ~10-fold in both Sgpl1+/+ and Sgpl1−/−-MEFs (Fig. 1A). Spinster-2 expression thus appears to be regulated by histone acetylation.

Because the transporters that could potentially transport S1P were upregulated in Sgpl1−/−-MEFs, we analyzed whether S1P was elevated in the supernatants of these cells. However, although total S1P levels were ~5-fold higher in S1P lyase-deficient MEFs (25), the concentrations of S1P in the supernatants were similar in wild-type and lyase-deficient MEFs (Fig. 2A). This is in agreement with a previous report (29). In addition, the concentrations of sphingosine in the supernatants were similar in both cell types (Fig. 2A), although total cellular sphingosine levels were ~2-fold higher in the knockout MEFs (25). Moreover, treatment with 10 µM external sphingosine overnight to further challenge S1P metabolism in the lyase-deficient MEFs did not significantly elevate S1P in the supernatants, and although sphingosine was generally higher in supernatants of the treated cells, there was again no difference between Sgpl1+/+ and Sgpl1−/−-MEFs (Fig. 2A). Finally, to confirm the results of the LC-MS/MS measurements, we labeled the cells with [3H]sphingosine and analyzed the secretion of [3H]S1P and [3H]sphingosine in the presence of either 10 mg/ml BSA or 10% FCS as potential acceptors of the secreted lipids. As shown in Fig. 2B, there was no enhanced secretion of [3H]S1P or [3H]sphingosine even under these conditions, although both lipids accumulated strongly in S1P lyase-deficient MEFs.

To further address the functional significance of ABC transporter upregulation, we analyzed next whether Sgpl1−/−-MEFs were able to extrude the substrate of Abcc1, fluo-4. However, we observed only a slightly diminished fluo-4 loading in Sgpl1−/−-MEFs, while it was evenly distributed throughout the cytosol and nuclei of wild-type cells (Fig. 3). Two different cellular staining patterns of fluo-4 were observed in Sgpl1−/−-MEFs, a rather punctuate and a rather reticular staining pattern, respectively (Fig. 4). The fluo-4-containing organelles did not colocalize with tetramethylrhodamine, the mitochondrial marker (Fig. 4A). However, the reticular staining pattern overlapped with an ER tracker, while the punctuate staining pattern colocalized with a lysosomal tracker (Fig. 4B, C). It has to be taken into account that these MEFs are polyclonal cell populations. Therefore, the two different staining patterns could represent two main different cell types.

It has been shown previously that Sgpl1−/−-MEFs are resistant to chemotherapy-induced apoptosis, in particular to apoptosis induced by etoposide and doxorubicin (19). Doxorubicin is a substrate of Abcb1 and Abcc1, but the
the transporter was indeed only partially localized at the plasma membrane and instead was situated mainly at intra-cellular punctuate compartments (Fig. 6B). Finally, we analyzed the influence of a combined pretreatment with the specific Abcb1 inhibitor zosuquidar and the Abcc1 inhibitor MK571. Again, this treatment effectively prevented the nuclear exclusion of doxorubicin by $\text{Sgpl1}^{-/-}$-MEFs, and some but not all cells showed small condensed nuclear staining.

previous study did not find a difference in cellular uptake of doxorubicin between $\text{Sgpl1}^{+/+}$- and $\text{Sgpl1}^{-/-}$-MEFs (19). Therefore, we wondered whether doxorubicin was not extruded but rather compartmentalized in the knockout MEFs, and we analyzed the subcellular distribution of doxorubicin by monitoring its autofluorescence by confocal microscopy. As shown in Fig. 5, doxorubicin, which intercalates into the DNA, initially localized to the nuclei in both $\text{Sgpl1}^{+/+}$- and $\text{Sgpl1}^{-/-}$-MEFs; only minor staining outside the nuclei was observed after 1 h. However, after 16 h of incubation, there was a major difference between the two cell types. Doxorubicin was still bound to the DNA in wild-type MEFs, which had small condensed nuclei suggestive of apoptosis. In contrast, doxorubicin was completely excluded from the nuclei of $\text{Sgpl1}^{-/-}$-MEFs and strongly compartmentalized in predominantly punctuate structures (Fig. 5). Double staining revealed that there was a partial overlap between the compartments that accumulated fluo-4 and doxorubicin, respectively (data not shown). To verify that doxorubicin compartmentalization was caused by ABC transporters localized on cellular organelles, we applied an inhibitor of organic anion transporters, probenecid; the Abcc1 inhibitor MK571; and the nonspecific Abcb1 inhibitor verapamil (Fig. 6A). Of these inhibitors, only verapamil was able to partially prevent doxorubicin compartmentalization. As shown in Fig. 6A, some of the knockout MEFs that had been treated with doxorubicin in the presence of verapamil had small condensed nuclei containing doxorubicin, suggestive of apoptosis, while other cells kept doxorubicin compartmentalized but at the same time could not fully exclude doxorubicin from their nuclei (Fig. 6A). These observations suggest that Abcb1 at least partially accounted for doxorubicin compartmentalization in $\text{Sgpl1}^{-/-}$-MEFs. Staining of the cells with an antibody against Abcb1 revealed that the transporter was indeed only partially localized at the plasma membrane and instead was situated mainly at intra-cellular punctuate compartments (Fig. 6B). Finally, we analyzed the influence of a combined pretreatment with the specific Abcb1 inhibitor zosuquidar and the Abcc1 inhibitor MK571. Again, this treatment effectively prevented the nuclear exclusion of doxorubicin by $\text{Sgpl1}^{-/-}$-MEFs, and some but not all cells showed small condensed nuclear staining.

Fig. 2. No enhanced export of S1P from $\text{Sgpl1}^{-/-}$-MEFs. A: Analysis by MS. The cells had been treated with vehicle or 10 µM sphingosine as indicated in serum-free medium containing 1 mg/ml fatty acid-free BSA for 16 h. The content of S1P and sphingosine in the cell culture supernatants was determined by LC-MS/MS, divided by the cellular protein content and normalized to untreated wild-type cells (means ± SEM; n = 4–7). B: Analysis after labeling with $[^3\text{H}]$sphingosine. The cells had been incubated for 2 h with $[^3\text{H}]$sphingosine in either serum-free medium containing 10 mg/ml BSA or in medium containing 10% FCS, as indicated. After washing and further incubation for 4 h in the respective media, the lipids were extracted from cells and supernatants, separated by TLC and quantified by liquid scintillation counting (means ± SD from a representative experiment; n = 3; * P < 0.05, ** P < 0.01, *** P < 0.001 in t-test comparing $\text{Sgpl1}^{+/+}$- and $\text{Sgpl1}^{-/-}$-MEFs).

Fig. 3. Compartmentalization of fluo-4 in $\text{Sgpl1}^{-/-}$-MEFs. Fluo-4-loaded MEFs were analyzed by confocal laser scanning microscopy. The cells were incubated with 4 µM fluo-4/AM for ~30 min, washed, and analyzed within the next 1 h. Shown are representative images.
proapoptotic agent staurosporine reduced the viability of wild-type cells by more than 75% at 10 nM and more than 90% at 30 nM, while the viability of Sgpl1−/−-MEFs was reduced by 50% at 10–500 nM, and the chemoresistance of the S1P lyase-deficient cells was overcome at 1 µM of staurosporine (Fig. 8A). A combined inhibition of Abcc1 and Abcb1 by pretreatment with zosuquidar plus MK571 had no further influence on reduction of cell viability by doxorubicin or staurosporine in wild-type MEFs (Fig. 8B). Furthermore, doxorubicin-induced caspase-3 cleavage was minimally influenced, if at all, and staurosporine-induced caspase-3 cleavage was not influenced by the treatment with the transporter inhibitors in these cells (Fig. 8C). In contrast, in Sgpl1−/−-MEFs, the treatment with zosuquidar plus MK571 rendered the cells sensitive to doxorubicin (Fig. 8B). Similarly, the minor caspase-3 cleavage induced by the doxorubicin treatment in Sgpl1−/−-MEFs was strongly augmented.

Finally, we analyzed whether the observed upregulation of the multidrug transporters contributed to the resistance of Sgpl1−/−-MEFs against chemotherapy-induced apoptosis by measuring the influence of ABC transporter inhibitors on cell viability and caspase-3 cleavage. As shown in Fig. 8A, the viability of wild-type MEFs was strongly reduced by incubation for 16 h with 1 µM doxorubicin, while Sgpl1−/−-MEFs were not significantly affected by this treatment, in agreement with the results of the microscopic analysis described above and with the data of Colié et al. (19). Incubation for 16 h with the proapoptotic agent staurosporine reduced the viability of wild-type cells by more than 75% at 10 nM and more than 90% at ≥30 nM, while the viability of Sgpl1−/−-MEFs was reduced by ~50% at 10–500 nM, and the chemoresistance of the S1P lyase-deficient cells was overcome at 1 µM of staurosporine (Fig. 8A). A combined inhibition of Abcc1 and Abcb1 by pretreatment with zosuquidar plus MK571 had no further influence on reduction of cell viability by doxorubicin or staurosporine in wild-type MEFs (Fig. 8B). Furthermore, doxorubicin-induced caspase-3 cleavage was minimally influenced, if at all, and staurosporine-induced caspase-3 cleavage was not influenced by the treatment with the transporter inhibitors in these cells (Fig. 8C). In contrast, in Sgpl1−/−-MEFs, the treatment with zosuquidar plus MK571 rendered the cells sensitive to doxorubicin (Fig. 8B).

Fig. 4. Characterization of fluo-4-accumulating subcellular compartments. Sgpl1−/−-MEFs were loaded with 4 µM fluo-4/AM for ~30 min and stained with 1 µM tetramethylrhodamine, 500 nM ER-Tracker Blue-White DPX, or 500 nM LysoTracker Red DND-99 as indicated. The images show cells with a predominantly reticular (upper panels in A and B) or a predominantly punctuate (lower panels in A and B, and C) fluo-4 staining pattern, respectively. The circles in C mark a region in which the colocalization of punctuate fluo-4 staining with lysosomes is particularly obvious.

Fig. 5. Compartmentalization of doxorubicin in Sgpl1−/−-MEFs. The cells were incubated for 1 h or 16 h with 1 µM doxorubicin and washed, and the nuclei were stained with 1 µM Hoechst 33342. Shown are typical microscopic images of Sgpl1+/+ and Sgpl1−/−-MEFs, respectively.
Taken together, these data suggest that a large part of doxorubicin resistance of S1P lyase-deficient MEFs was caused by upregulation of the multidrug transporters Abcc1 and Abcb1, while the sensitivity to staurosporine-induced apoptosis was not dependent on Abcc1 or Abcb1 in these cells.

**DISCUSSION**

For more than 20 years, ceramide and S1P have been implicated in regulation of cell survival, apoptosis, and chemoresistance, and yet surprisingly little is known about the mechanisms by which the biostat works on the molecular level. While G protein-coupled S1P receptors account for activation of survival signaling pathways by extracellular S1P, such as protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) or ERK, the direct targets involved in antiapoptotic signaling by intracellular S1P are less clear (2, 3, 30). Recently, a direct activation of the proapoptotic effector molecules BAK and BAX by S1P and hexadecenal, respectively, has been demonstrated in a reconstitution system with purified mitochondria (31); however, this mechanism can explain the occasionally proapoptotic but not the widespread antiapoptotic effect of S1P, although it might be in line with an antiapoptotic role of S1P lyase knockout with reduced formation of hexadecenal. Indeed, the product of the S1P lyase reaction, trans-2-hexadecenal, induced apoptosis in several cell lines, and this indeed also by zosuquidar plus MK571 (Fig. 8C). Compared with doxorubicin, the effect of staurosporine was not significantly affected by the transporter inhibitors (Fig. 8B). Furthermore, the treatment with the inhibitors was not able to augment stauro-

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**Fig. 6.** Effect of various transporter inhibitors on compartmentalization of doxorubicin in Sgpl1−/−-MEFs and localization of Abcb1 at intracellular compartments in these cells. A: Sgpl1−/−-MEFs had been incubated for 30 min with vehicle, 15 µM MK571, 1 mM probenecid, or 100 µM verapamil, before addition of 1 µM doxorubicin and further cultivation in serum-free medium for 16 h. Shown are representative images of doxorubicin localization, analyzed by confocal microscopy. Two images of verapamil-treated cells were selected to show the range of the cellular response. B: Abcb1 was stained with a specific antibody in formaldehyde-fixed Sgpl1−/−-MEFs. Shown is a representative image.

**Fig. 7.** Inhibition of doxorubicin compartmentalization by combined treatment with inhibitors of Abcb1 and Abcc1. A: The cells had been incubated with vehicle or 1 µM zosuquidar plus 15 µM MK571 (ZO/MK), before addition of 1 µM doxorubicin (Doxo) and further cultivation in serum-free medium for 16 h. Shown are typical images of doxorubicin (red) and Hoechst 33342 (blue) fluorescence in Sgpl1+/+ and Sgpl1−/−-MEFs after treatment as indicated. Two images of Sgpl1−/−-cells treated with doxorubicin plus ZO/MK were selected to show the range of the cellular response. B: Hoechst 33342 staining in MEFs that had been incubated with ZO/MK in the absence of doxorubicin.
involved BAX activation, but it was dependent on upstream activation of c-Jun N-terminal kinase (JNK) and was prevented by the antioxidant N-acetylcysteine (32). The chemoresistance of MEFs isolated from S1P lyase knockout mice, however, has been traced back to upregulation of Bcl-2 and Bcl-xL (19). Here, we add another facet to the picture by demonstrating a link between S1P lyase and transcriptional regulation of multidrug transporters, which we demonstrate to be functionally relevant for the well-known chemoresistance of S1P lyase-deficient MEFs.

Following the hypothesis that accumulation of S1P in S1P lyase-deficient MEFs might induce a counterregulatory upregulation of S1P secretion, in our investigation we focused on ABC transporters that have been associated with transport of S1P (Abcc1, Abca1, and Abcg2) or regulation by S1P (Abcb1). We observed that Abcc1 mRNA and protein, and Abca1 as well as Abcb1 mRNA, were upregulated in Sgpl1−/− MEFs, while Abcg2 mRNA expression was not significantly altered. Although, with the exception of the Abcb1a transcript, which was strongly induced, the upregulation on the mRNA level was only ~2- to 3-fold, the microscopic images displaying the compartmentalization of the fluo-4 and doxorubicin in Sgpl1+/+ but not wild-type MEFs clearly demonstrate the functional effectiveness of the transporters in the knockout cells.

Not much is known so far about the link between multidrug transporters and S1P metabolism. In a pioneering study, Pilorget et al. (27) have shown that overexpression of SphK1 in a cerebral endothelial cell line induced upregulation of Abcb1 protein and Abcb1b, but not Abcb1a, mRNA expression. Furthermore, extracellular S1P stimulated Abcb1 transport activity via S1P1 and S1P3 receptors, and as data not shown, it was mentioned that exogenous S1P did not modulate Abcb1 expression, suggesting differential activities of intracellular SphK1 and extracellular S1P (27). On the other hand, Cannon et al. (33) and Miller (34) showed recently that exogenous S1P decreased Abcb1 activity via S1P1 in isolated mouse brain and spinal cord capillaries, as well as in isolated killifish renal proximal tubules, respectively. Because this effect was rapid and reversible, it did not rely on transcriptional regulation, but rather on alteration of the transporter turnover number or on trafficking of the transport protein away from the exterior surface of the luminal plasma membrane (33).

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In line with this activity, exogenous cell permeable C2-ceramide was able to increase cholesterol efflux to apolipoprotein A-I by increasing the cell surface presence of Abca1 (35). However, these data provide no possible explanation for why several diverse ABC transporters were upregulated in S1P lyase-deficient MEFs.

It is known that inhibition of HDACs can lead to the induction of a broad range of multidrug transporters, which constitutes a major problem in the development of HDAC inhibitors for cancer treatment [see, e.g., Hauswald et al. (36)]. We had observed a reduced expression of class I HDACs and a decreased HDAC activity in Sgpl1−/−MEFs (26), in line with the role of nuclear S1P in regulating HDAC activity reported previously (37). Therefore, it was reasonable to assume that upregulation of the transporters could be a result of HDAC inhibition in these cells. However, the
was redistributed into intracellular compartments in localized in the nuclei of either cell type, and only with time et al. (19). However, in our study, doxorubicin was initially explained why Colié et al. (19) did not observe a difference in ER and lysosomes. Although we did not further analyze the localization of Abca1 and spinster-2, it is obvious that a dysfunctional insertion of the transporters into the plasma membrane would explain why S1P fluo-4, which is a substrate of Abcc1, strongly suggests that in heterogeneity of the MEFs (25) and suggesting that other multidrug transporters, which were not analyzed in the present study, contributed to the effect. Finally, the combined treatment with zosuquidar plus MK571 rendered the Sgpl1+/−-MEFs sensitive to doxorubicin-induced cytotoxicity and apoptosis. Interestingly, the influence of the inhibitors on staurosporine-induced cytotoxicity was not significant, and staurosporine-induced caspase-3 cleavage was not enhanced by zosuquidar plus MK571. These data also suggest that other antiapoptotic mechanisms are active in these cells, which could be other not yet considered ABC transporters or Bcl-2 and Bcl-xL upregulation as described (19).

Taken together, our study presents the multidrug transporters as important targets of S1P metabolism and components of the chemoresistance of S1P lyase-deficient cells. Because S1P lyase downregulation has been associated with cancer cell survival and malignancy (see the introduction), S1P lyase stimulation has been suggested as a strategy to sensitize cancer cells to anticancer treatment (21). Therefore, the influence of S1P lyase overexpression, and of yet to be identified activators or inducers of this enzyme, on multidrug transporter expression deserves comprehensive investigation. Further studies are required to analyze in greater detail the influence of SphKs, S1P phosphatases, and their respective inhibitors on multidrug transporter expression, subcellular localization, and activity.

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