Metabolism of the unnatural anticancer lipid safingol, L-*threo*-dihydrosphingosine, in cultured cells

Mihaela Dragusin, Cristian Gurgui,[1] Günter Schwarzmann, Joerg Hoernschemeyer, and Gerhild van Echten-Deckert[2]

Kekulé-Institut für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Strasse 1, 53121 Bonn, Germany

Abstract We studied the metabolism of radioactively labeled safingol (L-*threo*-dihydrosphingosine) in primary cultured neurons, B104 neuroblastoma cells, and Swiss 3T3 fibroblasts, and compared it to that of its natural stereoisomer D-*erythro*-dihydrosphingosine. Both sphingoid bases are used as biosynthetic precursors for complex sphingolipids, albeit to different rates. Whereas a considerable amount of the natural sphingoid base is also directed to the catabolic pathway (20–66%, cell type dependent), only a minor amount of the nonnatural safingol is subjected to catabolic cleavage, most of it being *N*-acylated to the respective stereochemical variant of dihydroceramide. Interestingly, *N*-acylation of safingol to L-*threo*-dihydroceramide is less sensitive to fumonisin B1 than the formation of the natural D-*erythro*-dihydroceramide. In addition, safingol-derived L-*threo*-dihydroceramide, unlike its physiologic counterpart, is not desaturated. Most of it either accumulates in the cells (up to 50%) or is used as a biosynthetic precursor of the respective dihydrosphingomyelin (up to 45%). About 5% is, however, glucosylated and channeled into the glycosphingolipid biosynthetic pathway. Our results demonstrate that, despite its nonnatural stereochemistry, safingol is recognized and metabolized preferentially by enzymes of the sphingolipid biosynthetic pathway. Furthermore, our data suggest that the cytotoxic potential of safingol is reduced rather than enhanced via its metabolic conversion.—Dragusin, M., C. Gurgui, G. Schwarzmann, J. Hoernschemeyer, and G. van Echten-Deckert. **Metabolism of the unnatural anticancer lipid safingol, L-*threo*-dihydrosphingosine, in cultured cells. *J. Lipid Res.* 2003.** 44: **1772–1779.**

Supplementary key words primary cultured neurons • neuroblastoma cells B104 • Swiss 3T3 cells • L-*threo*-sphinganine • ceramide

Sphingolipids occur in all eukaryotic cells, where they are primarily components of the plasma membrane. Their ceramide backbone anchors them in the outer leaflet of the lipid bilayer. Their hydrophilic moiety, composed of carbohydrate chains or phosphorylcholine in the case of glycosphingolipids (GSLs) and sphingomyelin (SM), respectively, faces the extracellular space. Gangliosides are sialic acid-containing GSLs and are specifically abundant in the central nervous system, where they have been associated with development and maturation of the brain, neuritogenesis, synaptic transmission, memory formation, and synaptic aging (1). Ceramide is not only a key intermediate in the synthetic and degradative pathway of sphingolipid metabolism but also a key player in the antiproliferative cellular responses, including apoptosis, cell-cycle arrest, differentiation, and senescence (2). In contrast, its catabolic intermediate sphingosine 1-phosphate (S1P) has been implicated as a second messenger in cellular proliferation and survival (3), and also in protection against ceramide-mediated apoptosis (4). Thus, the dynamic balance between intracellular S1P and ceramide, also known as sphingolipid rheostat, appears to be essential for the determination of whether cells survive or die (5).

Safingol (L-*threo*-dihydrosphingosine) is a nonnatural isomer of dihydrosphingosine (sphinganine). Usually sphingoid bases contain two chiral centers, namely at carbon atoms 2 and 3. Natural sphingoid bases occur in the D-*erythro* (2S, 3R) configuration, but three additional nonnatural stereoisomers exist. Among the unnatural sphingoid bases, L-*threo*(2*S*, 3*S*)-dihydrosphingosine (safingol) is of particular interest due to its anticancer activity. It was shown to synergistically increase the toxicity of established chemotherapeutic agents in several cancer cells in vitro (6), as well as in preclinical animal studies (7) and in a phase I clinical trial (8). The anticancer properties of safingol can be explained by its inhibitory effect on the activity of either protein kinase C (PKC) or sphingosine kinase. The competitive interaction of safingol with the

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Abbreviations: FB1, fumonisin B1; GSL, glycosphingolipid; S1P, sphingosine 1-phosphate; SM, sphingomyelin.

[1] Present address of C. Gurgui: Institut für Physiologie II, Wilhelmstrasse 31, 53111 Bonn, Germany.

[2] To whom correspondence should be addressed.
e-mail: g.echten.deckert@uni-bonn.de

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regulatory phorbol binding domain of PKC could be correlated with partial inhibition of the multidrug resistance phenotype of certain tumor cells (9).

As a competitive inhibitor of sphingosine kinase (10), safingol could prevent the formation of S1P from sphingosine, which in turn is exclusively formed from ceramide (11). According to the sphingolipid rheostat concept, the imbalance in favor of ceramide at the expense of S1P should direct the cells into apoptosis.

Considering the fact that safingol is a stereoisomer of an essential precursor of the sphingolipid biosynthetic pathway, the question concerning its metabolic fate arises.

We have recently shown that in neuroblastoma cells (12) as well as in primary cultured neurons (unpublished observations), the inhibitory effect of safingol (20 μM) on sphingosine kinase catalyzed phosphorylation of the synthetic sphingosine analog *cis*-4-methylsphingosine decreases over time. These findings indicate that, although safingol is a nonnatural stereoisomer, it appears to be efficiently metabolized by neuronal cells. Reports concerning the stereospecificity of enzymes involved in sphingolipid biosynthesis are, however, quite confusing. Stoffel and Bister (13) showed 30 years ago that in rat liver, L-*threo*-sphinganine is used like its natural analog as a precursor for the biosynthesis of SM and of cerebrosides. In contrast, Kok et al. (14), who studied the metabolism of dihydroceramide in three different cell lines, reported that only the D-*erythro*-isomer is converted to more complex sphingolipids, the enzymes involved being highly stereoselective. In a very recent study, Venkataraman and Futerman (15) showed that, whereas both L-*threo*- and D-*erythro*-sphinganine are metabolized via the respective ceramides to SM, only the natural D-*erythro*-isomer is converted to glucosylceramide in rat liver microsomes as well as in cultured baby hamster kidney (BHK) cells. As safingol has already been used in phase 1 clinical trials (8), knowledge of its metabolism is essential. Therefore, we prepared radioactively labeled safingol and followed its metabolism in three different cell types in comparison with that of its natural stereochemical variant D-*erythro*-sphinganine. Our data demonstrate for the first time that safingol, like sphinganine, is used as a biosynthetic precursor for all complex sphingolipids by cultured cells. However, there are essential differences between the metabolism of the natural and the nonnatural compounds, which in the end appear to define a "strategy" that finally helps at least the cells investigated here to escape the acute intrinsic toxicity of the drug.

MATERIALS AND METHODS

Materials

Six-day-old NMRI (Navy Marine Research Institute) mice were bred in the animal house of the University in Bonn (Germany).

L-[3-^{14}C]serine (54 mCi/mmol) was purchased from Amersham-Buchler (Braunschweig, Germany). D-*erythro*- and L-*threo*-[4,5-^{3}H]sphinganine (250 Ci/mol) were obtained according to Schwarzmann (16). Recombinant human acid sphingomyelinase was a kind gift from R. J. Desnick and E. H. Schuchman, Mount Sinai School of Medicine, New York. *Vibrio cholerae* and *Clostridium perfringens* sialidase and fumonisin B1 (FB1) were from Sigma (Taufkirchen, Germany). Culture media (Dulbecco's modified Eagle's medium, DMEM; and minimal essential medium, MEM) containing Glutamax® were obtained from Life Technologies, Inc. (Karlsruhe, Germany). DNase was from Roche (Mannheim, Germany). Fetal calf serum, horse serum, and trypsin were supplied by Cytogen (Berlin, Germany). The plastic culture dishes were from Falcon (Heidelberg, Germany). LiChroprep® RP-18 and thin-layer silica gel 60 plates were purchased from Merck (Darmstadt, Germany). DEAE-Sephadex A-25 was from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other chemicals were of analytical grade and obtained from Sigma (Taufkirchen, Germany) or Merck.

Cell culture

Granule cells were cultured from cerebella of 6-day-old mice as described before (17). Briefly, cells were isolated by mild trypsinization (0.05%, w/v) and dissociated by repeated passage through a constricted Pasteur pipette in a DNase solution (0.1%, w/v). The cells were then suspended in DMEM containing 10% heat-inactivated horse serum and plated onto poly-L-lysine-coated 8 cm^2 Petri dishes (6×10^6 cells/dish). Twenty-four hours after plating, cytosine arabinoside was added to the medium (4×10^{-5} M) to arrest the division of nonneuronal cells. After 5 or 6 days in culture, cells were used for metabolic studies.

The rat neuroblastoma B104 cell line (ICLCATL99008) that originates in the central nervous system (18), as well as Swiss 3T3 fibroblasts (CCL92), were routinely cultured in DMEM, supplemented with 2 mM glutamine, 10% heat-inactivated fetal calf serum, and antibiotics (penicillin 100 U/l and streptomycin 100 mg/l). For experiments, cells were subcultured in 8 cm^2 Petri dishes. Medium was renewed every 48 h until confluency was reached. Swiss 3T3 fibroblasts were kept confluent and quiescent for 5 days before use. Experiments were performed in DMEM supplemented with 3% heat-inactivated fetal calf serum (neuroblastoma cells) or in DMEM-Waymouth medium (1:1; v/v) supplemented with 20 μg/ml BSA and 5 μg/ml transferrin (Swiss 3T3 cells).

Sphingolipid labeling, extraction, and analysis

From the cells cultured in 8 cm^2 plastic dishes, medium was removed and the cells were rinsed two times with MEM. The cells were metabolically labeled in MEM containing 0.3% horse serum and 1% cytosine arabinoside (cerebellar neurons), or 0.3% fetal calf serum (neuroblastoma B104 cells and Swiss 3T3 fibroblasts) by addition of 1 μCi/ml of either [^{14}C]serine or [^{3}H]sphinganine. After 24 h, cells were washed three times with phosphate-buffered saline, harvested, and centrifuged at 3,000 *g* for 10 min. Total lipids were extracted from cell pellets with 6 ml of chloroform-methanol-water-pyridine (10:5:1:0.1; v/v/v/v) for 24 h at 50°C. Phospholipids were degraded by mild alkaline hydrolysis with methanolic NaOH (100 mM) for 2 h at 37°C. The lipid extracts were desalted by reversed-phase chromatography on LiChroprep RP18, applied to TLC plates, and developed with the indicated solvents. Sphingolipids were visualized by autoradiography using the bio-imaging analyser Fujix Bas1000 software, TINA 2.09, (Raytest, Straubenhardt, Germany) and identified by their R_f values. In some experiments, prior to TLC, lipid extracts were additionally subjected to anion-exchange chromatography using DEAE-Sephadex A-25 as resin (19).

Identification of SM by sphingomyelinase digestion

The lipid substrate was either the neutral sphingolipid fraction obtained by anion-exchange chromatography as described

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above or the scraped and reextracted TLC band designated to be SM. The reaction mixture in a final volume of 60 μl contained 250 mM acetate buffer (pH 4.5), 0.1% Nonidet P40, and 0.5 μg/μl recombinant human acid sphingomyelinase. After incubation overnight at 37°C, the reaction was stopped by the addition of 800 μl of chloroform-methanol (2:1; v/v). Then 250 μl of water was added, and lipids were extracted by phase separation. The organic (lower) phase, after concentration, was applied to a TLC plate that was developed with chloroform-methanol-2 *N*-ammonia (65:25:4; v/v/v). Enzymatic digestion of authentic SM was run in parallel.

Identification of gangliosides by sialidase digestion

The anionic fraction obtained by anion-exchange chromatography as described above was used as substrate. The reaction mixture, in a final volume of 50 μl, contained 100 mM acetate buffer (pH 5), 0.5% Nonidet P40, and sialidase either from *V. cholerae* (0.16 U) or from *C. perfringens* (0.25 U). After incubation at 37°C overnight, the reaction was stopped by adding 1 ml of chloroform-methanol (2:1, v/v). The samples were desalted by reversed-phase chromatography on silica gel LiChroprep RP18, and separated by TLC using chloroform-methanol-0.22% aqueous $CaCl_2$ (60:35:8; v/v/v) as a solvent system.

Analysis of saturation of safingol-derived ceramide

Regions containing radioactively labeled (dihydro)ceramide were scraped, reextracted from the TLC plates, and subjected to acid hydrolysis in 1 ml of anhydrous methanolic hydrogen chloride (0.5 M) at 63°C overnight. The released radioactively labeled sphingoid bases were monitored by TLC using chloroform-methanol-2 *N*-ammonia (65:25:4; v/v/v) as solvent system.

Electrospray mass spectrometry

Mass spectra were recorded in positive ion mode on a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray source. Lipid samples to be analyzed were dissolved in chloroform-methanol (1:1; v/v). Solutions were injected into the mass spectrometer by glass capillaries (long type; Protana, Odense, Denmark) using a capillary voltage of 1,000 V and a cone voltage of 50 V at 70°C. Instrument calibration was done with a mixture of sodium iodide and cesium iodide in 50% aqueous acetonitrile with 0.1% formic acid. For tandem mass spectrometry experiments, argon was used as a collision gas and fragmentation was observed at energy values from 20 to 50 eV.

Protein determination

Cell protein was quantified as described by Bradford (20) using BSA as a standard. Prior to lipid extraction, cell pellets were homogenized in 400 μl of water and aliquots were used for protein determination.

RESULTS

Safingol is preferentially metabolized to (dihydro)ceramide and (dihydro)SM, whereas most of its physiological counterpart D-*erythro*-sphinganine is transformed into fatty acids, SM, and complex gangliosides in cultured cells.

To analyze the metabolism of safingol, we have performed several metabolic labeling studies in primary cultured cerebellar neurons. The lipid profiles derived from tritium-labeled D-*erythro*- and L-*threo*-sphinganine, as well as from [^{14}C]serine, were studied in parallel. As illustrated in **Fig. 1**, similar metabolic products can be detected from both sphinganine stereoisomers, although the rates of certain products differ significantly. Thus, the naturally occurring D-*erythro*-sphinganine is used primarily as a biosynthetic precursor for (dihydro)ceramide that is further metabolized to (dihydro)SM and mostly to complex gangliosides (Fig. 1A and **Table 1**). In contrast, (dihydro)ceramide formed from safingol was channeled primarily into the formation of (dihydro)SM, and to a much lesser extent to complex gangliosides. Moreover, L-*threo*-(dihydro)-ceramide derived from safingol appeared to be metabolically more stable than its D-*erythro*-stereoisomer, thus accumulating in the cells (Fig. 1B, Table 1). Furthermore, about one-third of the lipid-associated radioactivity derived from D-*erythro*-sphinganine was found in the fatty acid fraction released from phospholipids during alkaline treatment (Fig. 1B, Table 1), indicating that this sphinganine stereoisomer is also directed toward the degradation pathway (21, 22). In contrast, the amount of safingol channeled to the catabolic pathway averaged only less than 5% of the lipid-associated radioactivity (Fig. 1B, Ta-

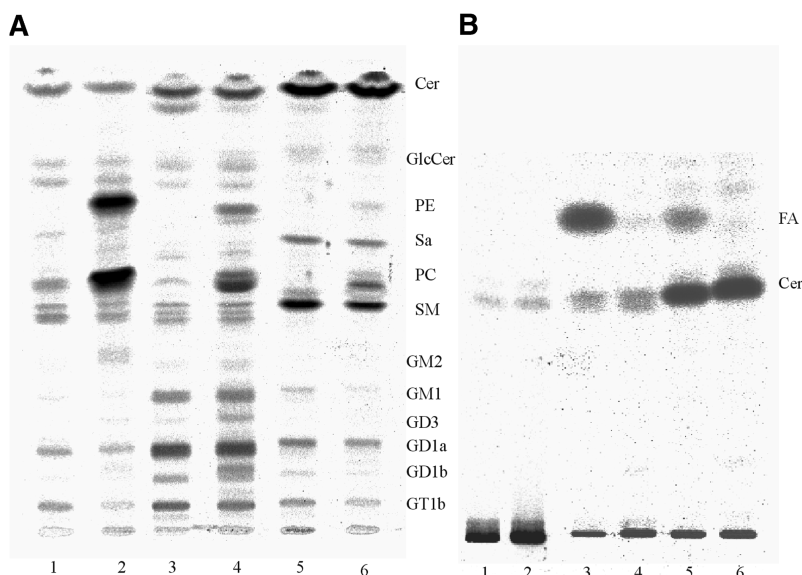

Fig. 1. Incorporation of tritiated safingol into sphingolipids of primary cultured cerebellar neurons. Cells were metabolically labeled for 24 h with [^{14}C]serine (Lanes 1 and 2), D-[^{3}H]*erythro*-sphinganine (Lanes 3 and 4), or L-[^{3}H]*threo*-sphinganine (Lanes 5 and 6). Cells were then harvested, and sphingolipids were extracted, isolated, separated by TLC, and detected, as described in Materials and Methods. In some samples, phospholipids were removed by alkaline treatment (lanes 1, 3, and 5). The TLC plate was developed in (A) chloroform-methanol-0.22% aqueous $CaCl_2$ (60:35:8; v/v/v) or (B) in chloroform-methanol-acetic acid (190:9:1; v/v/v). The mobility of standard sphingolipids is indicated. The terminology of gangliosides (GT1b, GD1b, GD1a, GD3, GM1, GM2) is according to Svennerholm (41). Cer, ceramide; FA, fatty acid; GlcCer, glucosylceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Sa, sphinganine; SM, sphingomyelin.

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TABLE 1. Formation of sphingolipids and fatty acids from L-*threo* (L-t)- and D-*erythro* (D-e)-sphinganine in primary cultured cerebellar neurons, Swiss 3T3 fibroblasts, and B104 neuroblastoma cells

	D-e-Sa			L-t-Sa		
Lipid Fraction	PCN	S3T3	B104	PCN	S3T3	B104
FA[b]	34.37	21.92	66.83	4.47	0.37	22.47
(DH)Cer	7.32	1.77	1.82	52.35	8.98	12.59
Neutral GSL[a]	7.82	2.38	nd	3.46	0.80	nd
(DH)SM	10.51	18.46	13.76	29.33	34.23	45.60
Gangliosides[a]	39.49	7.80	13.68	5.74	0.80	3.44
Sa	1.83	1.42	nd	3.66	3.28	nd

B104, B104 neuroblastoma cells; (DH)Cer, (dihydro)ceramide; (DH)SM, (dihydro)sphingomyelin; FA, fatty acid; GSL, glycosphingolipid; nd, not determined; PCN, primary cultured cerebellar neuron; Sa, sphinganine; S3T3, Swiss 3T3 fibroblasts. Cells were cultured in the presence of [^{3}H]safingol (L-t-Sa) or D-[^{3}H]*erythro*-sphinganine (D-e-Sa). After 24 h, cells were harvested and lipids analyzed as described in Materials and Methods. Each lipid fraction is expressed as the percentage of total lipid-associated radioactivity, which amounted in the case of D-e-Sa to 2,530,000, 2,100,000, and 1,900,000 cpm/mg of protein in PCN, S3T3, and B104, respectively, and in the case of L-t-Sa to 3,300,000, 2,600,000, and 2,400,000 cpm/mg of protein in PCN, S3T3, and B104, respectively. Data are from one representative out of three independent experiments that gave similar results. Note that for the sake of comparability, only glucosylceramide and lactosylceramide were included in the neutral GSL fraction, whereas other lipid species (e.g., globosides that are enriched especially in fibroblasts) were omitted.

[a] Both saturated and unsaturated forms.

[b] FA levels were measured after alkaline methanolysis.

ble 1). As shown in Table 1, in Swiss 3T3 fibroblasts, like in primary cultured cerebellar neurons, safingol is primarily *N*-acylated to (dihydro)ceramide that is then mainly used for the formation of (dihydro)SM. Also, a small but significant amount is converted into gangliosides (Fig. 1A, Table 1). A similar metabolism of safingol was found in neuroblastoma B104 cells (Table 1). However, in these tumor cells, unlike in the postmitotic neurons or in the contact inhibitory Swiss 3T3 cells, a respectable amount of safingol is channeled into the catabolic pathway, as indicated by the relatively high amount of fatty acid-associated radioactivity obtained (~30% of the radioactivity obtained in the fatty acid fraction from the D-*erythro*-diastereoisomer). Thus, when compared with its natural stereoisomer, the metabolism of safingol appears to be similar in all three cell types investigated, except for its conversion into fatty acids. The latter is much higher in the neuroblastoma cells (see above) than in primary cultured neurons and in Swiss 3T3 fibroblasts (13% and 1.7%, respectively, of the radioactivity obtained in the fatty acid fraction from the D-*erythro*-diastereoisomer).

The time dependence of the incorporation of label into fatty acids as well as into the main sphingolipid fractions analyzed in primary cultured cerebellar neurons is depicted in **Fig. 2**. The safingol-derived radioactivity recovered in the (dihydro)ceramide fraction reached its maximum after 16 h and then decreased, probably due to the complete consumption of safingol. The amount of radioactive SM derived from safingol still continued to increase even after 24 h due the huge pool of (dihydro)ceramide that was almost exclusively channeled into the (dihydro)SM pathway. However, after 8 h, about 5% of the lipid-associated radioactivity was recovered from the ganglioside fraction, and it was found to slightly increase with ongoing time.

Conversion of safingol to dihydroceramide is catalized in part by an FB1-insensitive enzyme in primary cultured cerebellar neurons

It is known that in the presence of FB1, an inhibitor of sphinganine-*N*-acyltransferase (dihydroceramide synthase), the accumulated long-chain bases sphinganine and sphingosine are directed toward the degradation pathway (23). To find out to what extent we can increase the amount of safingol that enters the catabolic pathway, we treated the cells with 25 μM of FB1 for 24 h prior to addition of the radioactively labeled sphinganines. As expected, in the presence of FB1, a 3-fold increase of fatty acids derived from safingol was observed (**Fig. 3A**). However, the amount of nonmetabolized safingol was much higher (up to 10 times over the untreated controls) than that of its natural D-*erythro* isomer (Fig. 3B). Whereas the formation of D-*erythro*-cer-

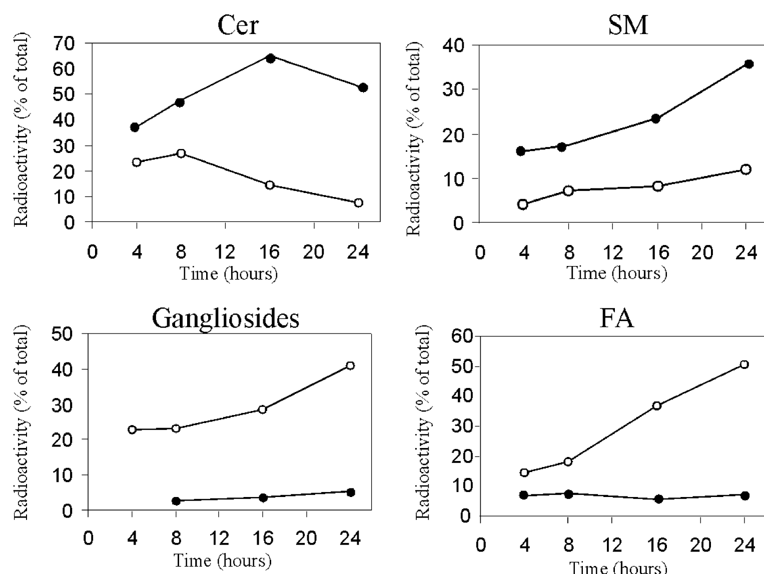

Fig. 2. Time course of the incorporation of tritiated safingol into different lipid species in primary cultured cerebellar neurons. Cells were cultured in the presence of [^{3}H]safingol (closed circle) or D-[^{3}H]*erythro*-sphinganine (open circle). After the indicated times, cells were harvested and lipids analyzed as described in Materials and Methods. Results are expressed as radioactivity incorporated into the indicated lipid species relative to the radioactivity associated with the whole lipid fraction (total). Data are from one representative experiment out of at least three different experiments yielding similar results. Note that FA levels were measured after alkaline methanolysis. SM, (dihydro)sphingomyelin.

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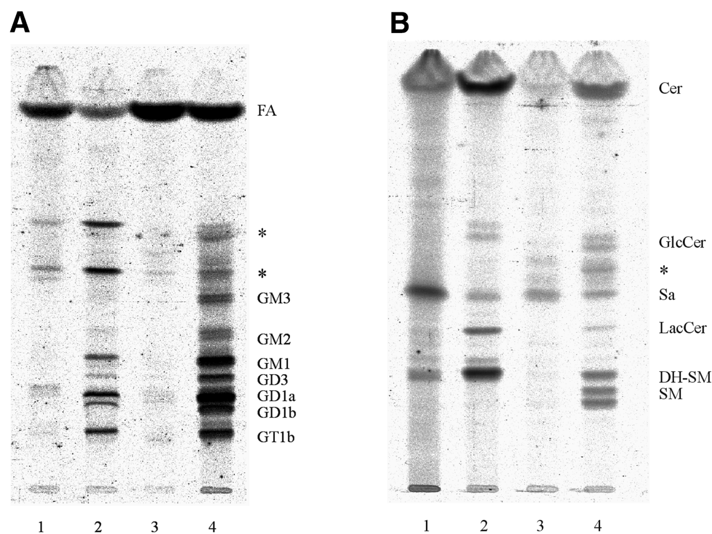

Fig. 3. The effect of fumonisin B1 (FB1) on the metabolism of safingol in cultured cerebellar neurons. Cells were cultured in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of FB1 (25 μM) for 24 h. Respective media were then renewed and cell cultures continued in the presence of [^{3}H]safingol (lanes 1 and 2) and D-[^{3}H]*erythro*-sphinganine (lanes 3 and 4), respectively. After 24 h, cells were harvested and lipids extracted as described in Materials and Methods. Phospholipids were removed by mild alkaline treatment. The desalted lipid extract was subjected to anion-exchange chromatography. A: The anionic fraction was separated by TLC using chloroform-methanol-0.22% aqueous $CaCl_2$ (60:35:8; v/v/v) as solvent system. B: The neutral fraction was separated by TLC using chloroform-methanol-2 *N*-ammonia (65:25:4; v/v/v) as solvent system. The mobility of standard lipids is indicated. The bands corresponding to (dihydro)sphingomyelin (DH-SM and SM) were sensitive to acid sphingomyelinase digestion (not shown). LacCer, lactosylceramide; Sa (both isomers). * Unidentified bands, but probably artifacts of the anion-exchange chromatography.

amide and hence of SM and complex gangliosides was almost completely repressed by the mycotoxin (down to 5–10% when compared with untreated controls), to our surprise L-*threo*-(dihydro)ceramide was further synthesized (25–30% relative to untreated controls) and partially directed toward the formation of the respective (dihydro)SMs (25–30% relative to controls (Fig. 3B). This result indicates that a different enzyme, namely an FB1-insensitive one, is also involved in the formation of this unnatural (dihydro)ceramide.

Safingol-derived dihydroceramide is not subjected to desaturation in cultured cells

It is well known that the sphingolipid biosynthetic pathway contains dangerous intermediates, ceramide, for example (24). The direct biosynthetic precursor of ceramide is the relatively inactive dihydroceramide. Because safingol is converted to a quite stable unnatural (dihydro)ceramide stereoisomer, it was important to find out whether or not this compound represents the biologically active desaturated or the less-active saturated species. In a first approach, the TLC bands corresponding to (dihydro)ceramide were scraped from the TLC plate and the lipids reextracted and subjected to acid hydrolysis to release the sphinganine and sphingosine backbones. As shown in **Fig. 4**, after acid hydrolysis, L-*threo*-(dihydro)ceramide releases only sphinganine, whereas D-*erythro*-(dihydro)ceramide leads to almost equal amounts of both sphingosine and sphinganine. Considering the loss of tritium label (about 50%) in consequence of the introduction of the 4,5-*trans* double bond, we conclude that about two-thirds of the D-*erythro*-sphinganine-derived dihydroceramide was desaturated, whereas one-third remained saturated. In a second approach, we then analyzed the sphingoid backbones of all sphingolipids synthesized from tritium-labeled safingol and D-*erythro*-sphinganine, respectively. As in the former approach, safingol-derived sphingolipids were found to exclusively contain the saturated sphingoid base L-*threo*-sphinganine in their backbones. Similar results were obtained for neuroblastoma B104 cells and also Swiss 3T3 fibroblasts (not shown). These findings demonstrate that the unnatural L-*threo*-dihydroceramide, in contrast to its natural diastereoisomer, is not subjected to desaturation in cultured cells. Furthermore, as shown before (14), the results indicate that introduction of the 4,5-*trans* double bond into the sphingoid backbone is not crucial for further

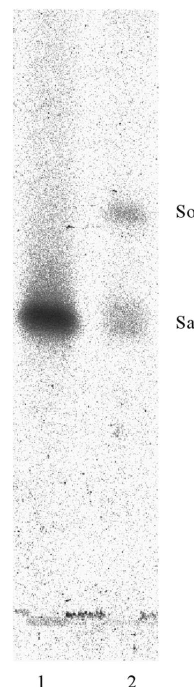

Fig. 4. Analysis of radiolabeled sphingoid bases released from (dihydro)ceramide derived from primary cultured cerebellar neurons. Regions containing L-*threo*-[^{3}H](dihydro)ceramide and D-*erythro*-[^{3}H](dihydro)ceramide, respectively, were scraped, reextracted from the TLC plates, and subjected to acid hydrolysis as described in Materials and Methods. The released radioactively labeled sphingoid bases were separated by TLC using chloroform-methanol-2 *N*-ammonia (65:25:4; v/v/v) as solvent system and identified by their R_f values. Lane 1: hydrolyzed L-*threo*-[^{3}H](dihydro)ceramide; lane 2: hydrolyzed D-*erythro*-[^{3}H](dihydro) ceramide. The mobility of standard sphingoid bases is indicated. Sa, both isomers; So, sphingosine (both isomers).

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metabolic conversion of dihydroceramide to more complex sphingolipids.

In addition, ESI-tandem mass spectrometry analysis revealed that the lipid bands corresponding to safingol-derived (dihydro)ceramide and (dihydro)SM amount $[M+Na]^+ = 590.5$ and $[M+Na]^+ = 755.6$, respectively. These values match the masses of the respective saturated (dihydro) counterparts containing C18-(stearoyl) fatty acid.

DISCUSSION

In the present study, we show that the anticancer lipid safingol is metabolized by cultured cells, despite its unnatural stereochemical structure. We have shown earlier that natural sphingoid bases added to the culture medium are taken up by the cells and are efficiently incorporated into cellular sphingolipids (25). Alternatively, sphingoid bases can be phosphorylated and channeled to catabolic breakdown yielding fatty acid aldehydes (26).

In all three cell types investigated, safingol, like its natural diastereoisomer, was mainly *N*-acylated to the respective dihydroceramides (**Fig. 5**). This was not surprising, in as much as it has been demonstrated previously in vitro and in cultured BHK cells that dihydroceramide synthase can metabolize L-*threo*-sphinganine (15). However, the fate of the formed L-*threo*-dihydroceramide was unexpected, because a considerable amount was directly converted to L-*threo*-dihydrosphingomyelin. In contrast to previous findings in BHK cells (15), our results indicate that none of the safingol-derived L-*threo*-dihydroceramide was desaturated. We have shown previously that the stereochemistry of the sphingoid base strongly affects the desaturase activity in vitro; the desaturation of L-*threo*-octanoylsphinganine is 10-fold lower than that of D-*erythro*-octanoylsphinganine (27). Apparently, either the stereo-specificity of dihydroceramide desaturase is tissue dependent or safingol-derived dihydroceramide bypasses the desaturation site. The fact is, the relatively inactive L-*threo*-dihydroceramide was not converted into its bioactive desaturated form. This is an important finding, in so far as it rules out the possibility that the drug itself is converted into a bioactive intermediate. The conversion of safingol-derived L-*threo*-dihydroceramide to L-*threo*-ceramide could dramatically affect cell behavior, because it has been shown that this ceramide stereoisomer is severalfold more potent in inducing nucleosomal fragmentation than its D-*erythro*-diastereoisomer (28). In addition, the formation of the respective L-*threo*-S1Ps can be excluded, because this intermediate can be formed exclusively via the respective ceramides (11, 27).

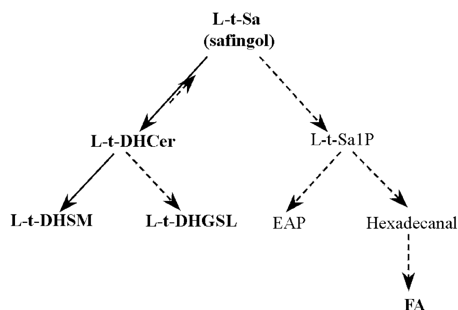

Fig. 5. Scheme of safingol metabolism. Possible metabolic routes of sphingoid bases are shown. Solid arrows: major metabolic pathway of safingol. Dotted arrows: minor metabolic route of safingol. L-t-Sa, L-*threo*-sphinganine; L-t-DHCer, L-*threo*-dihydroceramide; L-t-DHSM, L-*threo*-dihydrosphingomyelin; L-t-DHGSL, L-*threo*-dihydroglycosphingolipids; Sa1P, sphinganine-1-phosphate; EAP, ethanolamine-1-phosphate. Bold, quantitatively evaluated lipid fractions. Note that the desaturation step was omitted in this scheme, because we have shown that this stereoisomer, in contrast to the natural one, is not desaturated.

Interestingly, *N*-acylation of safingol, in contrast to that of its natural counterpart, was found to be at least in part FB1 resistant. This result indicates that either the reverse activity of brain ceramidase (29) or the recently described upstream of growth and differentiation factor 1 (UOG1)-regulated ceramide synthase (30) could be responsible for the conversion of safingol into L-*threo*-dihydroceramide. In as much as the former enzyme was shown to exert poor activity toward the unnatural stereoisomers of sphingosine, it appears more likely that the latter one catalyzes *N*-acylation of safingol. Moreover, the UOG1-regulated enzyme was reported to use preferentially stearoyl-CoA, thus significantly producing *N*-stearoyl-sphinganine. Accordingly, the bulk of safingol-derived dihydroceramide and dihydrosphingomyelin contains stearic acid in the fatty acid moiety. In addition, *uog1* is expressed at high levels in brain (31). However, further experiments are necessary to verify these assumptions.

The natural D-*erythro*-C18-(dihydro)ceramide synthesized in UOG1-overexpressing cells was shown to be preferentially channeled into the pathway of neutral GSL but not gangliosides (30). In contrast, safingol-derived L-*threo*-C18-dihydroceramide, unlike its D-*erythro* stereoisomer, accumulates mainly in the cells, suggesting that it is either metabolically quite stable or selectively retained in the endoplasmic reticulum, as suggested previously (14). The latter possibility seems rather unlikely, in so far as a considerable amount of safingol-derived dihydroceramide is converted into the respective dihydrosphingomyelins, and a small but significant part is channeled into the ganglioside biosynthetic pathway (Fig. 5). The second observation is of some surprise, because glycosylation of this unnatural dihydroceramide isomer has not been reported previously (15, 32). The metabolic stability of safingol-derived L-*threo*-dihydroceramide is consistent, with ceramidase preferentially hydrolyzing D-*erythro*-ceramide. Of the four possible stereoisomers of ceramide, rat brain ceramidase was shown to use only the natural D-*erythro*-isomer as substrate (33). Moreover, removal of the 4,5-*trans* double bond decreased the affinity of the enzyme toward its substrate by 90% (33). Thus, conversion of safingol to L-*threo*-dihydroceramide appears to be a one-way street.

An alternative metabolic fate of exogenous safingol is its direct phosphorylation and subsequent cleavage yielding phosphorylethanolamine and palmitoyl aldehyde (Fig. 5). Essentially, two enzymatic activities, namely a kinase and a lyase, are needed for this degradative pathway. The amount

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of 4,5-[^{3}H]safingol following this catabolic route should therefore be reproduced by the radioactivity recovered in the fatty acid fraction. Our results strongly suggest that, except for neuroblastoma cells, only a minor fraction of safingol is directed toward this catabolic pathway. This is not surprising, because lyase activity was found to be stereospecific in all tissues investigated so far (13, 34). In the brain, sphingosine kinase was also reported to act specifically on the D-*erythro*-isomer (10), whereas in Swiss 3T3 fibroblasts, L-*threo*-(dihydro)sphingosines were found to be readily phosphorylated (35). However, the formed sphingoid phosphates did not show any of the biological effects described for D-*erythro*- S1P (36). The relatively high amount of safingol-derived radioactivity recovered in the fatty acid fraction of neuroblastoma cells strongly suggests that in both these cells, the kinase and the lyase are not stereoselective, thus actively catalyzing safingol breakdown.

Taken together, our studies clearly show that safingol is actively metabolized in cultured cells being used as a biosynthetic precursor for all sphingolipid species. In contrast to predictions from other studies (13, 15, 37), our data clearly demonstrate that the formation of the dangerous intermediate L-*threo*-ceramide can be excluded in all three cell types investigated. Interestingly, the enzyme catalyzing *N*-acylation of safingol is apparently different from the FB1-sensitive ceramide synthase that converts its natural sphinganine stereoisomer to dihydroceramide, unlike previously reported in neuroblastoma cells (37).

Despite an active metabolic consumption, safingol was shown to affect cell behavior when added exogenously, and the consequence was growth arrest and cytotoxicity (37, 38). Because our data clearly show that safingol is only converted into less-bioactive compounds like dihydroceramide and more complex sphingolipids, its antineoplastic activity most probably cannot be explained by the formation of L-*threo*-ceramide, as suggested before (37). From our results, it appears more likely that the cytotoxic effect of safingol is a result of blocking sphingosine kinase on the one hand (12, 39, 40) and PKC on the other hand (9, 38). However, we cannot exclude that the different cell systems used in different studies might explain the different results.

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