Molecular Pharmacology Fast Forward. Published on September 15, 2004 as doi:10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

Specificity of the dihydroceramide desaturase inhibitor GT11 in primary cultured cerebellar neurons

Gemma Triola, Gemma Fabrias, Mihaela Dragusin, Lars Niederhausen, Roland Broere, Amadeu Llebaria, and Gerhild van Echten-Deckert

From the Research Unit on Bioactive Molecules (RUBAM), Dept. of Biological Organic Chemistry, IIQAB, CSIC, Jordi Girona 18, 08034 Barcelona, Spain (G.T., G.F., A.L.) and the [§]Kekulé-Institut für Organische Chemie und Biochemie der Universität Bonn, Gerhard-Domagk-Strasse 1, 53121 Bonn, Germany (M.D., L.N., R.B., G.vE-D.)

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: GT11 inhibits dihydroceramide desaturase in neurons

Corresponding author

Gerhild van Echten-Deckert

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

phone 0049-228-732703; fax 0049-228-737778; e-mail: g.echten.deckert@uni-bonn.de

Text pages:

Figures: 9

References: 30

Abstract: 204 words

Introduction: 663 words

Discussion: 1242 words

Non-standard abbreviations used are:

GT11, *N*-[(1*R*,2*S*)-2-hydroxy-1-hydroxymethyl-2-(2-tridecyl-1-cyclopropenyl)ethyl]octanamide; GT17, *N*-[(1*R*,2*S*)-2-hydroxy-1-hydroxymethyl-2-(2-tridecyl-1-cyclopropenyl)ethyl]amine; DHS, sphinganine (dihydrosphingosine); DHS1P, sphinganine-1-phosphate; ER, endoplasmic reticulum; PE, phosphatidylethanolamine; SL(s), sphingolipid(s); S1P, sphingosine-1-phosphate; SPT, serine palmitoyltransferase; TLC, thin layer chromatography.

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Dihydroceramide desaturase catalyses the conversion of the innocuous precursor (dihydroceramide) into a highly bioactive product (ceramide). We studied the effect of GT11, the first inhibitor of this enzyme, in primary cultured cerebellar neurons. Although desaturase was efficiently inhibited (IC_{50} of 23 nM) the compound lost its specificity at higher concentrations. From 5 µM upwards GT11 also decreased *de novo* sphingolipid biosynthesis. Studies with two differentially labeled radioactive analogs of GT11 support that the inhibitor itself and not a downstream metabolic product, interferes with sphingolipid biosynthesis. Interestingly, serine palmitoyltransferase activity decreased in the presence of high concentrations of GT11 in intact cells, but not when added directly into cell homogenates. However, suppression of enzyme transcription could not be detected. But at high concentrations GT11 provoked an accumulation of sphingosine-1-phosphate and especially of dihydrosphingosine-1-phosphate, suggesting a decreased activity of sphingosine-1-phosphate lyase. Enzyme activity measurements indeed supported this assumption. Thus, at higher concentrations, GT11 interferes with lyase activity inducing an accumulation of sphingoid base phosphates that, in turn down-regulate serine palmitoyltransferase activity.

At low concentrations GT11 is, however, the first specific inhibitor of dihydroceramide desaturase described so far. Considering the pro-apoptotic and pro-inflammatory effect of ceramide, GT11 could also turn out to be a novel cell-protective agent.

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

Ceramide the hydrophobic membrane anchor of sphingolipids (SLs) has been implicated as a bioactive molecule in cell growth regulation, apoptosis, senescence and diverse cell responses, particularly linked to stress situations (Hannun and Luberto, 2000; Hannun and Obeid, 2002). In addition ceramide is a key intermediate in the metabolism of all SLs. Among the latter are several bioactive molecules including sphingomyelin (Marchesini and Hannun, 2004), glucosylceramide (Bleicher and Cabot, 2002), and last but not least complex glycosphingolipids (Huwiler et al., 2000) with diverse functions in the control of cell fate. Interestingly, ceramide is also the only metabolic precursor for sphingosine-1-phosphate (S1P), which among a multitude of specific cellular responses is known to counteract the proapoptotic action of ceramide (Spiegel and Milstien, 2003). Moreover, several studies report on the therapeutic potential of modulating intracellular ceramide levels (Kolesnick, 2002).

The *de novo* biosynthesis of ceramide requires four sequential steps catalyzed by membrane-bound enzymes active at the cytosolic face of the ER (van Echten-Deckert and Sandhoff, 1999). The first and rate-limiting step is catalyzed by serine palmitoyltransferase (SPT) and yields 3-ketosphinganine, which is rapidly reduced to D-*erythro*-sphinganine. N-Acylation of sphinganine leads to dihydroceramide, which is then converted to ceramide by the introduction of a 4,5-*trans*-double bond (Michel et al. 1997). Since dihydroceramide is usually much less effective or fails to show the bioactivity of ceramide (Bielawska et al., 1993) this step catalyzed by dihydroceramide desaturase appears to be of particular importance. Also, induction of ceramide but not of dihydroceramide formation by some chemotherapeutic agents (such as daunorubicin) has been proposed to mediate their toxicity (Hannun and Luberto, 2000). It should be noted that the 4,5 double bond of ceramide is not essential for anabolic enzymes that catalyze the formation of sphingomyelin or glycosphingolipids. Thus inhibition of the desaturation step does not end up in complete SL depletion of cells. However, Δ^4desaturated sphingolipids were proposed to be involved in cell

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

cycle control during *Drosophila* spermatogenesis (Ternes et al., 2002). Taken together these findings indicate that the 4,5-*trans*-double bond contributes to the signaling function of ceramide and possibly of its metabolites. The enzyme catalyzing the desaturation of dihydroceramide in mammals is localized in the cytosolic leaflet of ER membranes (Michel and van Echten-Deckert, 1997) and is highly specific for D-*erythro*-dihydroceramide (Michel et al., 1997). Based on a bioinformatic strategy, a putative SL Δ^4desaturase family with members from animals, plants and fungi has been identified and characterized (Ternes et al., 2002). Although molecular and genetic tools are now available to study Δ^4desaturated SLs, it is still not clear whether this SL desaturase family also includes the dihydroceramide desaturase described previously (Michel and van Echten-Deckert, 1997; Michel et al, 1997). The synthesis of GT11, a cyclopropene-containing ceramide analog and its inhibitory effect on the activity of dihydroceramide desaturase in rat liver microsomes has been reported recently (Triola et al., 2001). Kinetic studies performed in rat liver microsomes using different concentrations of substrate and GT11 have evidenced that this ceramide analog is a competitive inhibitor (K_i = 6 μM) of dihydroceramide desaturase (Triola et al., 2003). Structure-function studies performed with GT11 related synthetic analogs have revealed that the cyclopropene ring instead of the 4,5-*trans*-double bond, the natural 2*S*,3*R* (D-*erythro*) stereochemistry as well as the presence of a free hydroxyl group at C1 are crucial for inhibition of dihydroceramide desaturase (Triola et al., 2003). The idea that an inhibitor of dihydroceramide desaturation and hence of *de novo* ceramide formation might possibly be a useful cell protective agent, prompted us to investigate the effects of GT11 in primary cultured cerebellar neurons. In addition to being a validated model for studies concerning synthetic analogs that interfere with SL metabolism (van Echten-Deckert et al., 1997 and 1998), these cells could also provide data concerning a potential pharmacological utility of GT11 as a neuroprotective drug. We report here that low concentrations (up to 1 μM) of

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

GT11 specifically and effectively inhibit dihydroceramide desaturase in primary cultured cerebellar neurons. However, the inhibitor looses specificity at higher concentrations.

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

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). GT11 (Fig. 1) was synthesized as described (Triola et al., 2001). [^{14}C]GT11 (5 Ci/mol) was obtained accordingly using [1-^{14}C]octanoic acid (5 Ci/mol) in the final acylation step. [1-^{3}H]GT11 (3.75 Ci/mmol) was prepared as illustrated in Fig. 1. *N*-[1-^{14}C]octanoyl D-*erythro*-sphinganine was synthesized as described before (Michel et al., 1997). L-[3-^{14}C]serine (54 mCi/mmol) was purchased from Amersham-Buchler (Braunschweig, Germany). D-*erythro*-[4,5-^{3}H]sphinganine (250 Ci/mol) was obtained according to Schwarzmann (1978). D-*erythro*[4,5-^{3}H]DHS1P (50-60Ci/mmol) and DHS1P were both from BioTrend (Koeln, Germany). Culture media (Dulbecco's modified Eagle's medium, DMEM and minimal essential medium, MEM) containing Glutamax^{R} were obtained from Life Technologies, Inc. (Karlsruhe, Germany). DNase was from Roche (Mannheim, Germany). Horse serum and trypsin were supplied from Cytogen (Berlin, Germany). Bovine serum albumin (fatty acid free) was from Sigma (Taufkirchen, Germany). The plastic culture dishes were from Falcon (Heidelberg, Germany). LiChroprep^{R} 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 (Darmstadt, Germany).

Cell Culture. Granule cells were cultured from cerebella of 6-day-old mice as described before (van Echten-Deckert et al., 1998). 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 x 10^{6} cells/dish). 24 h after plating, cytosine arabinoside was added to the medium (4 x 10^{-5} M) to

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

arrest the division of non-neuronal cells. After 5-6 days in culture, cells were used for metabolic studies.

Sphingolipid labeling, extraction and analysis. Medium was removed from the culture dishes 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 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 3000 x *g* for 10 min. Total lipids were extracted from cell pellets with 6 ml of chloroform/methanol/water/pyridine (10:5:1:0.1, by volume) for 24 h at 50^{o}C (van Echten-Deckert, 2000). Phospholipids were degraded by mild alkaline hydrolysis with methanolic NaOH (100 mM) for 2 h at 37^{o}C. The lipid extracts were desalted by reversed-phase chromatography on LiChroprep RP18, applied to thin layer chromatography (TLC) plates, and developed with the indicated solvents. SLs 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.

Dihydroceramide desaturase activity. Enzymatic activity of dihydroceramide desaturase was measured in cell homogenate with *N*-[1-^{14}C]octanoyl D-*erythro*-sphinganine as substrate, essentially as described previously (Schulze et al., 2000). In a final volume of 300 µl, the assay mixture contained phosphate buffer (100 mM, pH 7.4), substrate (12.5 µM, prepared as 1:2 complexes with fatty acid free bovine serum albumin) and 300 µg of cell protein. After a pre-incubation time of 5 min at 37°C, the reaction was started by addition of 1 µmol NADH in 30 µl phosphate buffer. After 60 min at 37°C the reaction was terminated by addition of 200 µl of $CHCl_3$/CH_3OH (83:17, by volume) on ice. Lipid extraction was achieved by addition of 343 µl CH_3OH and 22 µl $CHCl_3$, and vigorously mixing for 20 min. Phases were separated by centrifugation and the lower phase was collected. The extraction procedure was repeated twice with 200 µl of $CHCl_3$/CH_3OH (83:17, by volume). The combined organic

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

phases were evaporated under a stream of N_2 and dissolved in 30 µl of $CHCl_3/CH_3OH$ (1:1, by volume). Lipids were separated by TLC on silica gel/sodium borate plates developed with $CHCl_3/CH_3OH$ (9:1, by volume). The radioactively labeled desaturated product N-[1-^{14}C] octanoyl D-*erythro*-sphingosine was detected and quantified by phosphoimaging.

SPT activity. Enzymatic activity of SPT was measured using [^{14}C]serine and palmitoyl-CoA as substrates (Merrill and Wang, 1992). The assay mixture contained 0.1 M Hepes (pH 7.4), 5 mM dithiothreitol, 10 mM EDTA, 50 µM pyridoxal 5'phosphate, 1.2 mM L-[^{14}C]serine (1.6 µCi), 0.15 mM palmitoyl-CoA and 100 - 150 µg of cell protein, in a total volume of 100 µl. After incubation for 10 min at 37 ^{o}C, reactions were terminated by addition of chloroform/methanol (5:3, by volume). The lipids were extracted by phase separation and applied to a TLC plate, which was developed with chloroform/methanol/2 M NH_4OH (40:10:1; by volume). Radioactively labeled 3-ketosphinganine was detected and quantified by phosphoimaging.

S1P lyase activity. Enzymatic activity was determined in cell homogenates using [4,5-^{3}H]sphinganine-1-phosphate as substrate, essentially as described by van Veldhoven (2000). In a final volume of 200 µl, the assay mixture contained phosphate buffer (100 mM, pH 7.4), 25 mM NaF, 0.1 % Triton X-100, 0.5 mM EDTA, 2 mM dithiothreitol, 0.25 mM pyridoxal 5'phosphate, 40 µM substrate (0.5 µCi) and 100 µg cell protein. The reaction was performed for 60 min at 37 °C and terminated by addition of 0.2 ml of $HClO_4$ (1 %), and of 1.5 ml of chloroform/methanol (1:2, by volume). Phase separation was induced by adding 0.5 ml of chloroform and 0.5 ml of $HClO_4$. After thorough vortexing and centrifugation, lower phase was washed twice with 1 ml of $HClO_4$/chloroform (8:2, by volume). Then a known volume of the lower phase was transferred to new tubes, dried under nitrogen, and the residue dissolved in chloroform/methanol (8:2, by volume) containing 5 mM of palmitic acid and hexadecanol as carriers. Labeled lipids were resolved by TLC with hexane/diethylether/ acetic acid

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

(70:30:1, by volume). Radioactively labeled hexadecanal as well as palmitic acid and hexadecanol derived thereof were quantified by phosphoimaging.

Labeling of cells with $^{32}P_i$. Cells were washed with phosphate-free MEM and subsequently incubated with this medium containing $^{32}P_i$ (40 µCi/ml) for 24 h as described (Zhang et al., 1991). The cells were then treated with the indicated concentration of GT11 or GT17 for the indicated times. Sphingoid base phosphates were extracted following the method of Yatomi et al. (1995), as described in detail previously (van Echten-Deckert et al., 1997). Phosphorylated sphingoid bases were resolved by TLC with 1-butanol/methanol/acetic acid/water (80:20:10:20, by volume), visualized by autoradiography, identified by their R_f value and quantitatively evaluated using the bio-imaging analyzer Fujix Bas 1000 using software TINA 2.09 (Raytest, Straubenhardt, Germany).

Reverse-transcription polymerase chain reaction (RT-PCR). Total cellular RNA was prepared using the RNeasy Mini Kit and the RNaseFreeDNaseI-set (QIAGEN, Hilden, Germany) following provider's instructions. For each reverse-transcription reaction, 0.4 µg of total RNA was reversely transcribed using SuperScript II First-Strand Synthesis System for RT-PCR with Random Primers (Invitrogen, Karlruhe, Germany) according to manufacturer's instructions. Semi-quantitative PCR was performed using gene-specific primers: S1P lyase 5`-ATGGATCCTGTCCCCGAAGT-3`(forward), 5`-CACCTTTCACCCGGAAATCA-3` (reverse), 27 cycles; SPT-LCB1 5`-CGAGGCTCCAGCATACCATC-3` (forward), 5`-TGGCTGCCACTCTTCAATCA-3` (reverse), 30 cycles; SPT-LCB2 5`-AGCTGCTGAAGTCCTCAAGGA-3` (forward), 5`-GGTCATAGCAGCTTCCACACC-3` (reverse), 29 cycles. Results were normalised using the housekeeping genes: 18S rRNA 5`-GCATGGCCGTTCTTAGTTGG-3` (forward), 5`-TGAACGCCACTTGTCCCTCT-3` (reverse), 16 cycles; Synaptophysin 5`-CGATGTGAAGATGGCCACTGA-3` (forward), 5`-

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

CCGAGGTGTTGAGTCCTGAAGTC-3` (reverse), 25 cycles and hypoxanthinphosphoribosyltransferase (HPRT) 5`-TCCTGTGGCCATCTGCCTAGTA-3` (forward), 5`-GGACGCAGCAACTGACATTTCTA-3` (reverse), 29 cycles. All reactions were carried out with taq DNA polymerase (Amersham, Freiburg, Germany) in a MJ PTC 200 thermal cycler (Biozym, Hess. Oldendorf, Germany). Annealing was at 58°C and product size was between 100 – 150 base pairs.

Quantitative real time PCR was performed using the Quantitect SYBR Green PCR Kit (QIAGEN, Hilden, Germany), 35 cycles, annealing at 58°C and an ABI 7700 Sequence Detector with software version 1.7 (Applied Biosystems, Darmstadt, Germany). The same primer sets described above were used. Results were normalised using the same housekeeping genes as mentioned above. Melting curve analysis was performed to confirm production of a single product in these reactions.

Protein determination. Cell protein was quantified as described by Bradford (1976) using bovine serum albumin as a standard. Prior to lipid extraction, cell pellets were homogenized in 400 µl of water and aliquots were used for protein determination.

Cell viability assay. The effect of GT11 on cell viability was determined using the Cell Titer-Blue™ Cell Viability Assay from PROMEGA (Mannheim, Germany). The assay was performed in 96-well microtiter plates (Falcon, Heidelberg, Germany) according to manufacturer's instructions.

Presentation of data. Results presented as TLC images correspond to data obtained with at least two different cell preparations.

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

Results

GT11 is a potent inhibitor of dihydroceramide desaturase in primary cultured cerebellar neurons. The effect of GT11 (Fig. 1) on the activity of dihydroceramide desaturase in primary cultured cerebellar neurons was studied by following the incorporation of L-[3-^{14}C]serine into cellular ceramide. As illustrated in Fig. 2, a dose-dependent decrease of dihydroceramide desaturation and hence of *de novo* ceramide formation was observed in the presence of 0.001 to 1 µM GT11. On the bases of these data an IC_{50} of 23 nM was calculated. Furthermore, the effect of GT11 on neuronal desaturase activity was measured *in vitro*, using cell homogenates as an enzyme source. Activity of dihydroceramide desaturase was inhibited by 83% and 55% when 12.5 µM and 1.25 µM GT11 were directly added to the enzyme assay, respectively.

High concentrations of GT11 decrease the incorporation of [^{14}C]serine, but not that of [^{3}H]sphinganine, into cellular sphingolipids in primary cultured cerebellar neurons. Interestingly, when higher amounts (≥ 5µM) of the inhibitor were added to the culture medium, *de novo* SL biosynthesis monitored by incorporation of [^{14}C]serine into cellular SLs was strongly reduced (Fig. 3A). Thus, the incorporation of [^{14}C]serine into cellular SLs amounted only 10%, 30% and 50% in the presence of 20 µM, 10µM and 5 µM of GT11, respectively, relative to untreated controls. However, this reduction did not occur when instead of [^{14}C]serine, [^{3}H]sphinganine was used as a biosynthetic precursor (Fig. 3B). These results strongly indicate that concentrations of GT11 from 5 µM upwards affect an enzymatic step upstream of sphinganine formation. The increased radioactive labeling of some SL species in the presence of the desaturase inhibitor GT11 (1-20 µM) when compared with that of SLs in control cells might be explained by the loss of tritium label in the latter as a consequence of desaturation. In addition, the rate of metabolism of saturated SLs formed in the presence of GT11 might differ from that of their desaturated counterparts formed in

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

control cells. Note that the amount of radioactively labeled fatty acids recovered in the lipid fraction decreases with increasing concentrations of GT11 (Fig. 3C), indicating that this compound might interfere with the conversion of [^{3}H]sphinganine, via phosphorylation and lyase cleavage, into phosphoethanolamine and the fatty acid precursor, palmitaldehyde (see also Fig. 9). Worth mentioning is the fact that cell viability was not significantly affected by GT11 up to a concentration of 10 µM for 48 h.

High concentrations of GT11 decrease SPT activity in primary cultured cerebellar neurons. To find out if GT11 affects the activity of SPT, the rate-limiting enzyme of *de novo* SL biosynthesis, neurons were cultured for 24 h in the presence of different concentrations of GT11 prior to the enzyme assay performed in cell homogenates. As shown in Fig 4, in contrast to low concentrations (1µM), which did not affect SPT activity significantly, high concentrations (10 µM) of GT11 reduced the enzyme activity by about 90 %**.** However, when the same concentrations of inhibitor were directly added to the cell homogenate in the *in vitro* enzyme assay, no effect on SPT activity was observed (not shown). These results indicate that cell integrity is essential for the effect of GT11 on SPT activity. Cell integrity could be crucial either for metabolism of GT11, or for the regulation mechanism induced by higher doses of the inhibitor.

Metabolism of radioactively labeled GT11 in primary cultured cerebellar neurons. To find out, if GT11 itself or a metabolic product derived from GT11 is responsible for the decrease of *de novo* SL biosynthesis, the metabolic fate of two different radioactively labeled inhibitors was investigated in primary cultured cerebellar neurons. [^{14}C]GT11 (see materials) is labeled in its fatty acid moiety, whereas [^{3}H]GT11 is labeled in the sphingoid part of the molecule (Fig. 1). Both, differentially labeled radioactive analogs of GT11 were rapidly internalized in the cells. After one hour only about 30 % of the added radioactivity was detectable in the culture medium. This value remained constant up to 24 hours. About 75 % of

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

lipid associated radioactivity migrated with [^{14}C]GT11 (Fig. 5A). In case of [^{3}H]GT11 most of the metabolized fraction of the lipid (about 25%) were recovered in the phosphatidylethanolamine (PE) fraction (Fig. 5B), indicating that the compound was degraded down to phosphorylethanolamine by the stepwise action of ceramidase, sphinganine kinase and S1P lyase (see also Fig. 9). Interestingly, the amount of labeled PE formed from [^{3}H]GT11 decreased with increasing concentrations of added GT11. This result indicates that GT11 might interfere in a concentration dependent manner with at least one of the catabolic enzymes mentioned above.

High concentrations of GT11 induce an accumulation of phosphorylated long chain bases. Although GT11 appears to be metabolically quite stable, it became clear from the studies described above that the compound is partially directed into the catabolic pathway. Since phosphorylation is a key-step in this pathway, the cells were labeled with $^{32}P_i$ prior to treatment with different concentrations of either GT11 or its deacylated derivative GT17 (Fig. 1), in order to get a better insight into the fate of GT11 derived phophorylated metabolites. After 1 h levels of phosphorylated sphingoid bases were comparable in controls and GT11 (both, low and high concentration) as well as in GT17 treated cells (not shown). In contrast, after 24 h a clear cut accumulation of both, S1P and DHS1P was observed in the presence of the high concentration of GT11, while no changes of the two phosphorylated sphingoid bases were detected in the other samples (Fig. 6). The accumulation of DHS1P was especially surprising since SPT activity was found to be decreased after GT11 (10 μM) treatment. Note that phosphorylated GT17 was equally abundant in cells treated with GT17 or with the high concentration of GT11 (10 μM). In light of these findings, it appears likely that high concentrations of GT11 affect the S1P lyase activity prior to SPT down-regulation.

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

High concentrations of GT11 decrease sphinsosine-1-phosphate lyase activity in primary cultured cerebellar neurons. As shown above, high concentrations of GT11 induce an accumulation of phosphorylated long chain bases in cultured neurons after 24 h of incubation. In addition, the formation of PE from [^{3}H]GT11 is reduced by high concentrations of GT11 (see above). Finally, the formation of fatty acids from [4,5-^{3}H]sphinganine is strongly reduced in the presence of 10 µM of GT11 (see Fig 3C). Taken together these findings strongly suggest that GT11 at high concentrations interferes with the activity of S1P lyase. Therefore, lyase activity was measured after incubating the cells for 24 h with 1 and 10 µM GT11, respectively. As shown in Fig 7, lyase activity was strongly reduced after pre-incubation of cells in the presence of 10 µM of GT11, whereas 1 µM of GT11 had no effect. However, when GT11 (10 µM) was added directly to the cell homogenate in the *in vitro* lyase assay it had no effect on enzyme activity (not shown), suggesting that cell integrity is crucial for its inhibitory effect.

Deacylated GT11 (GT17) does not decrease neither [^{14}C]serine incorporation into cellular sphingolipids nor [^{3}H]sphinganine incorporation into cellular fatty acids. As shown above, both GT11 (10 µM) and GT17 (10 µM) induce the formation of phosphorylated GT17. To analyze whether this metabolite of GT11 mimics the effects on SL metabolism observed at high concentrations of GT11, *de novo* SL biosynthesis as well as [^{3}H]sphinganine catabolism, were studied in the presence of GT17. The deacylated derivative of GT11 had no effect, neither on *de novo* SL biosynthesis nor on the incorporation of [^{3}H]sphinganine into cellular fatty acids (not shown).

High concentrations of GT11 do not affect expression of SPT and of sphingosine-1-phosphate lyase in primary cultured cerebellar neurons. To find out if reduction of enzymatic activity of S1P lyase and also of SPT in neurons treated with high concentrations

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

of GT11 occurs on transcriptional level, mRNA amounts of SPT including the regulatory (LCB1) and the catalytically active subunit (LCB2) as well as of S1P lyase were assessed by PCR methods. As illustrated in Fig. 8 no significant changes in their expression could be observed upon treatment with 10 μM of GT11 for up to 48 h.

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Previous results from our laboratories have shown that the cyclopropene-containing ceramide analog GT11 is a competitive inhibitor of dihydroceramide desaturase activity in rat liver microsomes (Triola et al., 2001 and 2003). In the present study, GT11 was found to exhibit a much more pronounced inhibitory effect on dihydroceramide desaturase activity in primary cultured cerebellar neurons. The calculated IC_{50} of 23 nM is by about three and two orders of magnitude lower than that found in rat liver microsomes and in neuronal cell homogenates, respectively. Thus the inhibitory property of GT11 is much more pronounced *in vivo* than *in vitro*. This could be explained by the *in vivo* situation that might allow for a local sub-cellular enrichment of the inhibitor at its site of action, the ER. This assumption is supported by earlier observations that metabolically stable ceramide analogues become stuck to intracellular membranes especially the ER whereas Golgi staining requires metabolic processing of ceramide to glucosylceramide and/or sphingomyelin (Putz and Schwarzmann 1995). Since we have shown that GT11 is not metabolized to either of the two SLs, it appears very likely that it becomes stuck to ER membranes. Preliminary experiments with the fluorescent analog NBD-GT11 (N-6(7-nitro-2,1,3-benzoxadiazol-4-yl)aminododecanoyl-D-*erythro*-GT11) confirm this assumption. However, tissue and species specificity cannot be excluded either at present time.

Besides a high inhibitory potency, the quality of an inhibitor is also dependent on its specificity towards a certain enzymatic activity. In case of GT11, no collateral effects on SL metabolism in the concentration range of 0.001-1 µM could be observed. However, doses of GT11 ≥ 5 µM markedly blocked *de novo* SL biosynthesis in primary cultured neurons when radioactively labeled serine was used as a biosynthetic precursor. Surprisingly, SL biosynthesis was not affected at similar concentrations of GT11 when tritiated sphinganine was used as a biosynthetic precursor instead of serine. This result indicates that at higher doses (from 5 µM upwards), besides inhibiting dihydroceramide desaturase activity, GT11

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

interferes with a step upstream of *de novo* sphinganine formation. We could indeed show that the activity of SPT, the rate-limiting enzyme of SL biosynthesis, was markedly decreased when cells were cultured for 24 h with doses of the inhibitor as high as 5 µM and upwards. However, expression of SPT was not significantly affected. Also, the same high concentrations of GT11 did not affect SPT activity in cell homogenates *in vitro*, thus indicating that the integrity of the cell was required for this effect. Two possible explanations appeared most likely. i) GT11 rather down-regulates than inhibits SPT as shown before for long chain bases (Mandon et al., 1991) and also for the synthetic sphingosine analog *cis*-4-methylsphingosine (van Echten-Deckert et al., 1997). ii) Not GT11 itself, but a metabolic product derived from GT11 is responsible for the decreased SPT activity. The latter possibility is, however, widely excluded by our experimental data. Thus, metabolic studies with radioactively labeled GT11 analogs indicated that only a low percentage of the inhibitor is subjected to metabolism and directed rather to the catabolic than to the anabolic route. However, GT17 the sphingoid base that would derive from enzymatic deacylation of GT11 did not affect incorporation of radioactively labeled serine into cellular SLs when added to the culture medium. And finally, labeling experiments with inorganic phosphate demonstrated that only high concentrations of GT11 induce the accumulation of S1P and especially that of DHS1P after 24 h. In analogy with previous results showing that phosphorylated sphingoid bases play a critical role in down-regulation of the *de novo* pathway by affecting the rate-limiting biosynthetic enzyme, SPT (van Echten-Deckert et al., 1997, Le Stunff et al., 2002), we believe that those two compounds, S1P and DHS1P, are responsible for the decreased SPT activity induced by high concentrations of GT11. Since, as mentioned above, only a low percentage of GT11 is catabolyzed and GT17 does not mimic any of the effects of GT11, we concluded that GT11 itself, in addition to being a very efficient inhibitor of dihydroceramide desaturase activity, also induces accumulation of phosphorylated sphingoid bases when applied in high concentrations (≥ 5µM) to cultured neurons.

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

The enzyme responsible for a rapid breakdown of sphingoid phosphates is S1P lyase (van Veldhoven and Mannaerts, 1993; van Veldhoven, 2000). It catalyzes the cleavage of S1P and/or DHS1P into phosphoethanolamine and hexadecenal and/or hexadecanal (Fig. 9). Both breakdown products are then mainly incorporated into glycerophospholipids. After its activation with CTP, phosphoethanolamine is primarily used in the PE biosynthetic pathway, whereas the fatty aldehyde is oxidized to the respective fatty acid, which can be linked to glycerol via an alkali-labile ester bond. All our experimental data point to an interference of GT11 with S1P lyase activity. i) Culturing of neurons for 24 h in the presence of 10 µM of GT11 almost completely abolished lyase activity, whereas 1 µM GT11 did not affect this enzyme at all. ii) When [^{3}H]sphinganine was used in metabolic labeling experiments, incorporation of label into fatty acids (derived from the lyase-born aldehyde), was inhibited by GT11 in a concentration dependent manner (lowest effective concentration tested being 5 µM). iii) The amount of radioactive PE derived from [^{3}H]GT11 (tritiated at carbon atom C1 of the sphingoid base) decreased with increasing concentrations of GT11 applied. The mechanism by which GT11 affects lyase activity is still unknown at present. Although we did not find any regulation of lyase on transcriptional level, our results indicate that cell integrity is crucial for decrease of lyase activity by high concentrations of GT11 since its direct addition to the *in vitro* assay did not affect enzymatic activity. The latter finding argues against the possibility that GT11 interferes with lyase activity on molecular level by the reaction of its cyclopropene ring with the catalytically essential cysteine residue(s) (van Veldhoven et al., 2000). In addition, lyase is a pyridoxal phosphate-dependent enzyme (van Veldhoven and Mannaerts, 1993). Although GT11 is metabolized at a low rate, it is possible that its long-chain base phosphate interacts with the lyase-pyridoxal phosphate complex to form the substrate-enzyme imino-intermediate, thus competing with the natural substrate. However, since deacylated GT11 (GT17) did not mimic any of the effects of GT11 in cultured neurons, we also excluded the competitive inhibition of lyase by this metabolic

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

product of GT11. Thus, in contrast to the inhibitory effect of GT11 on lyase activity, which requires cell integrity, inhibition of dihydroceramide desaturase is independent of such requirements and it occurs at much lower concentrations.

In conclusion, the data presented in this study show that GT11 has a dual, dose-dependent effect on sphingolipid metabolism in cultured cerebellar neurons (Fig.9). At low concentrations (1-1000 nM) it specifically inhibits dihydroceramide desaturase, thus preventing the formation of ceramide and leading to the formation of saturated complex sphingolipids from dihydroceramide. The latter is, alternatively, subjected to catabolic breakdown by hydrolysis (ceramidase), phosphorylation (S1P kinase) and retroaldolic cleavage (S1P lyase) to phosphoethanolamine and palmitaldehyde. At high concentrations (from 5 µM upwards) GT11 induces in addition accumulation of S1P and DHS1P, most probably by interfering with S1P lyase activity. Accumulated sphingoid phosphates down-regulate SPT activity and hence *de novo* SL biosynthesis, most probably via a feedback mechanism.

Due to its highly specific inhibitory potency, we believe that GT11, despite its collateral effects at concentrations as high as 5 µM and upwards, represents an indispensable tool to study the physiological function of dihydroceramide desaturation. In addition, its lack of cytotoxicity in primary cultured neurons is a prerequisite for future studies concerning its potential neuroprotective properties against ceramide mediated stress responses.

Acknowledgements

We thank Andrea Raths for excellent technical assistance, Dr. J. Casas for helpful discussions and Prof. K. Sandhoff for the possibility to work in his department. G. Triola would like to thank Almirall Prodesfarma S.A.for awarding her The Young Researchers' Prize.

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

References

Bielawska A, Crane HM, Liotta D, Obeid LM, and Hannun YA (1993) Selectivity of ceramide-mediated biology. Lack of activity of erythro-dihydroceramide. *J Biol Chem* **268:**26226-26232.

Bleicher RJ and Cabot MC (2002) Glucosylceramide synthase and apoptosis. *Biochim Biophys Acta* **1585:**172-178.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248-254.

Hannun YA and Obeid LM (2002) The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind. *J Biol Chem* **277:**25847-25850.

Hannun YA, and Luberto C (2000) Ceramide in the eukaryotic stress response. *Trends Cell Biol* **10:**73-80.

Huwiler A, Kolter T, Pfeilschifter J, and Sandhoff K (2000) Physiology and pathophysiology of sphingolipid metabolism and signaling. *Biochim Biophys Acta* **1485:**63-99.

Kolesnick R (2002) The therapeutic potential of modulating the ceramide/sphingomyelin pathway. *J Clin Invest* **110:**3-8.

Le Stunff H, Galve-Roperh I, Peterson C, Milstien S, and Spiegel S (2002) Sphingosine-1-phosphate phosphohydrolase in regulation of sphingolipid metabolism and apoptosis. *J Cell Biol* **158:**1039-1049.

Mandon EC, van Echten G, Birk R, Schmidt RR, and Sandhoff K (1991) Sphingolipid biosynthesis in cultured neurons. Down-regulation of serine palmitoyltransferase by sphingoid bases. *Eur J Biochem* **198:**667–674.

Marchesini N and Hannun YA (2004) Acid and neutral sphingomyelinases: roles and mechanisms of regulation. *Biochem Cell Biol* **82:**27-44.

Merril AH Jr and Wang E (1992) Ceramide synthase. *Methods Enzymol* **209:**427-437.

Michel C and van Echten-Deckert G (1997) Conversion of dihydroceramide to ceramide occurs at the cytosolic face of the endoplasmic reticulum. *FEBS Lett* **416:** 153-155.

Michel C, van Echten-Deckert G, Rother J, Sandhoff K, Wang E, Merrill AHJr (1997) Characterization of ceramide synthesis. A dihydroceramide desaturase introduces the 4,5-trans-double bond of sphingosine at the level of dihydroceramide. *J Biol Chem* **272:** 22432-22437.

Putz U, Schwarzmann G (1995) Golgi staining by two fluorescent ceramide analogues in cultured fibroblasts requires metabolism. *Eur J Cell Biol* **68:** 113-121.

Schulze H, Michel C, and van Echten-Deckert G (2000) Dihydroceramide desaturase. *Methods Enzymol* **311:**22-30.

Schwarzmann G (1978) A simple and novel method for tritium labeling of gangliosides and other sphingolipids. *Biochim Biophys Acta* **529:**106-114.

Spiegel S and Milstien S (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nature Reviews* **4:**397- 407.

Svennerholm L (1963) Chromatographic separation of human brain gangliosides. *J Neurochem* **10:**613-623.

Ternes P, Franke S, Zähringer U, Sperling P, and Heinze E (2002) Identification and characterization of a sphingolipid delta 4-desaturase family. *J Biol Chem* **277:**25512-25518.

Triola G, Fabriàs G, and Llebaria A (2001) Synthesis of a cyclopropene analogue of ceramide, a potent inhibitor of dihydroceramide desaturase. *Angew Chem Int Ed Engl* **40:**1960-1962.

Triola G, Fabriàs G, Casas J, and Llebaria A (2003) Synthesis of cyclopropene analogues of ceramide and their effect on dihydroceramide desaturase. *J Org Chem* **68:**9924-9932.

van Echten-Deckert G (2000) Sphingolipid extraction and analysis by thin-layer chromatography. *Methods Enzymol* **312:**64-79.

van Echten-Deckert G and Sandhoff K (1999) Organization and topology of sphingolipid metabolism, in *Comprehensive Natural Products Chemistry* (Pinto BM ed) pp 87-106, Elsevier Science Amsterdam-Oxford.

van Echten-Deckert G, Giannis A, Schwarz A, Futerman A, and Sandhoff K (1998) 1-Methylthiodihydroceramide, a novel analog of dihydroceramide, stimulates sphinganine degradation resulting in decreased de novo sphingolipid biosynthesis. *J Biol Chem* **273:**1184-1191.

van Echten-Deckert G, Zschoche A, Bär T, Schmidt RR, Raths A, Heinemann T, and Sandhoff K (1997) cis-4-Methylsphingosine decreases sphingolipid biosynthesis by specifically interfering with serine palmitoyltransferase activity in primary cultured neurons. *J Biol Chem* **272:**15825-15833.

van Veldhoven PP (2000) Sphingosine-1-phosphate lyase. *Methods Enzymol* **311:** 244-254.

van Veldhoven PP, and Mannaerts GP (1993) Sphingosine-phosphate lyase. *Adv Lipid Res* **26:**69–98.

van Veldhoven PP, Gijsbers S, Mannaerts GP, Vermeesch JR, Brys V (2000) Human sphingosine-1-phosphate lyase: cDNA cloning, functional expression studies and mapping to chromosome 10q22(1). *Biochim Biophys Acta* **1487**: 128-134.

Yatomi Y, Ruan F, Ohta H, Welch RJ, Hakomori S, and Igarashi Y (1995) Quantitative measurement of sphingosine 1-phosphate in biological samples by acylation with radioactive acetic anhydride. *Anal Biochem* **230:**315-320.

Zhang H, Desai NN, Olivera A, Seki T, Brooker G, and Spiegel S (1991) Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J Cell Biol* **114:**155-167.

Footnotes

This study was supported by the Deutsche Forschungsgemeinschaft and the Spanish Ministry of Science and Technology (Grants BQU2002-00786 and BQU2002-03737). GT was financially supported by the Generalitat de Catalunya with a pre-doctoral fellowship and a 6-month sojourn in Bonn.

PD Dr. Gerhild van Echten-Deckert, Kekulé-Institut für Organische Chemie und Biochemie, Gerhard-Domagk-Str. 1, 53121 Bonn, Germany

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for figures

Fig. 1. Chemical Structure (A) and synthesis of [1-^{3}H]GT11 (B). The tritiated GT11 analog was prepared from the cyclic carbamate **(i)**. Oxidation (b, Dess-Martin Periodinane, CH_2Cl_2) to the aldehyde **(ii)** and its further reduction (c, NaB^3H_4/methanol) yielded the tritiated alcohol **(iii)**, which, upon deprotection and acylation afforded [1-^{3}H]GT11. Reagents and conditions of the reactions a and d are as described by Triola et al. (2001 and 2003)

Fig. 2. The effect of GT11 on dihydroceramide desaturation in primary cultured neurons. Cells were incubated in the absence (C, control) or presence of the indicated concentrations of GT11. After 24 h respective media were renewed and [^{14}C]serine added for additional 24 h. Then cells were harvested and lipids extracted and isolated as described in *Materials and Methods*. Ceramide (Cer) was separated from dihydroceramide (DHCer) by TLC on borate treated silica gel plates with chloroform/methanol (9:1, by volume) as solvent system. The radioactively labeled ceramide and dihydroceramide were quantitatively evaluated as described in *Materials and Methods.* Inhibition of desaturation of DHCer to Cer was calculated relative to controls. The results are given as the mean of double determinations from one representative experiment that was repeated twice with similar results. The standard deviation values were always in the range of ± 5 % of the mean.

Fig. 3. The effect of GT11 on [^{14}C]serine and [^{3}H]sphinganine incorporation into cellular sphingolipids. Primary cultured neurons were incubated in the absence (C, control) or presence of the indicated concentrations of GT11. After 24 h 1 µCi of either [^{14}C]serine (A) or of [^{3}H]sphinganine (B and C) were added to the renewed medium. 24 h later cells were harvested and lipids extracted, isolated, separated by TLC and detected as described in *Materials and Methods*. The solvent system used was chloroform/methanol/ 0.22% aqueous $CaCl_2$, 60:35:8, by volume (A and B) or chloroform/methanol/ acetic acid, 190:9:1, by

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

volume (C). The terminology of gangliosides (GT1b, GD1b, GD1a, GD3, GM1, GM2, GM3) is according to Svennerholm (1963). Other abbreviations are as follows: SM, sphingomyelin; LacCer, lactosylceramide; GlcCer, glucosylceramide; * Unidentified bands

Fig. 4. The effect of GT11 on SPT activity in primary cultured neurons. Cells were incubated in the absence (C, control) or presence of the indicated concentrations of GT11. After 24 h cells were harvested, homogenized and enzyme activity determined as described in *Materials and Methods*. B, blank with boiled homogenate. Data are from three separate experiments, each with double determinations.

Fig. 5. Metabolism of radioactively labeled GT11 in primary cultured neurons. Cells were incubated in the presence of the indicated concentrations of GT11 including 5 nCi of [^{14}C]GT11 (A) or 0.72 µCi of [^{3}H]GT11 (B), respectively. After 24 h cells were harvested and lipids extracted, isolated, separated by TLC (A: chloroform/methanol/ acetic acid, 190:9:1, by volume; B: chloroform/methanol/ 0.22% aqueous $CaCl_2$, 60:35:8, by volume) and detected as described in *Materials and Methods*. Note that alkaline treatment was omitted to keep phospholipids intact. PE, phosphatidylethanolamine.

Fig. 6. Determination of $^{32}P_i$-labeled sphingoid phosphates in primary cultured cerebellar neurons. Cells were washed with phosphate-free MEM and incubated with the same medium containing $^{32}P_i$ (40 µCi/ml) for 24 h. Then vehicle (lane C), or the indicated concentration of GT11 or GT17 was added for 24 h. Cells were harvested and sphingosine phosphates extracted, resolved by TLC and determined as described in *Materials and Methods*. The R_F value of authentic sphingoid phosphates is indicated. Similar data were obtained in two separate experiments, each with double determinations.

Molecular Pharmacology Fast Forward. Published on September 15, 2004 as DOI: 10.1124/mol.104.003681
This article has not been copyedited and formatted. The final version may differ from this version.

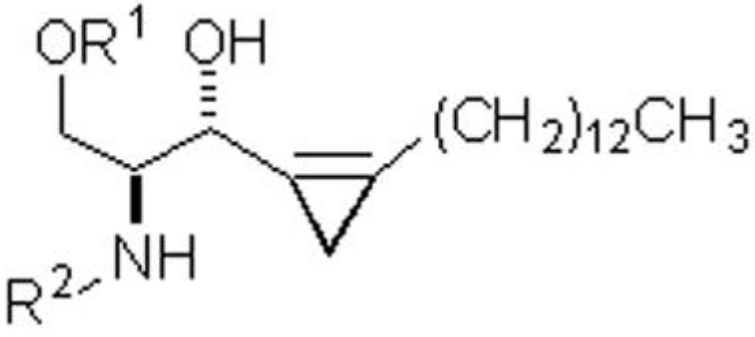
Fig. 7. The effect of GT11 on S1P lyase activity in primary cultured neurons. Cells were incubated in the absence (C, control) or presence of the indicated concentrations of GT11. After 24 h cells were harvested, homogenized and S1P lyase activity determined as described in *Materials and Methods*. B, blank with boiled homogenate. Data are from two separate experiments, each with double determinations.

Fig. 8. The effect of GT11 on mRNA levels of SPT and S1P lyase in primary cultured neurons. Cells were incubated for the indicated times (*black bars* 24 h, *gray bars* 48 h) in the absence (control) or presence of 10 µM of GT11. After 24 h respective media were renewed. Levels of SPT (LCB1 and LCB2) and of S1P lyase mRNA were determined by quantitative real time PCR (A) and by semi-quantitative RT-PCR (B) as described in *Materials and Methods*. (A) Data are means from three different experiments, each with triple determinations. (B) One representative out of three experiments with similar results from different cell preparations. C, control; HPRT, hypoxanthinephosphoribosyltransferase; LCB, long chain base; Synapto, synaptophysine; 18S, 18S rRNA.

Fig. 9. Proposed mechanism for the effect of GT11 on sphingolipid metabolism in primary cultured cerebellar neurons. At sub-micromolar concentrations (1 – 1000 nM) GT11 specifically inhibits dihydroceramide desaturase. At concentrations above 5 µM, GT11 also decreases lyase activity. This effect results in the accumulation of sphingoid-1-phosphates, which down-regulate SPT. Note that an accumulation of S1P is possible despite a complete inhibition of dihydroceramide desaturase from catabolic ceramide pools formed by breakdown of sphingomyelin (SM) and of glycosphingolipids (GSL). AL, aldehyde; EAP, ethanolaminephosphate; (DH)Cer, (dihydro)ceramide; (DH)GSL, (dihydro)glycosphingolipids; (DH)SM, (dihydro)sphingomyelin; Sa, sphinganine; Sa1P, sphinganine-1-phosphate; So, sphingosine; S1P, sphingosine-1-phosphate.

Fig. 1

A



GT11: R^1=H, R^2=CO(CH_2)$_6CH_3$

[^{14}C]GT11: R^1=H, R^2=^{14}CO(CH_2)$_6CH_3$

GT17: R^1=R^2=H

B

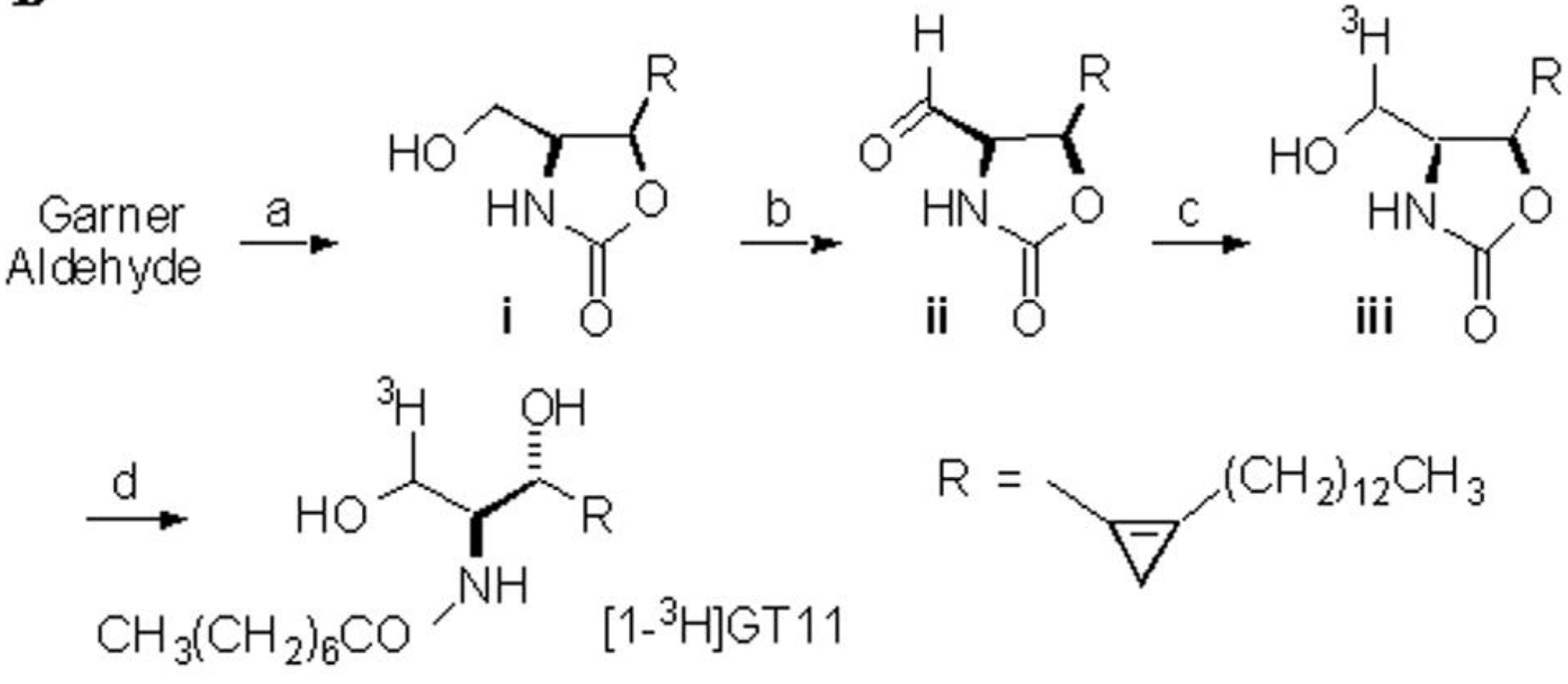
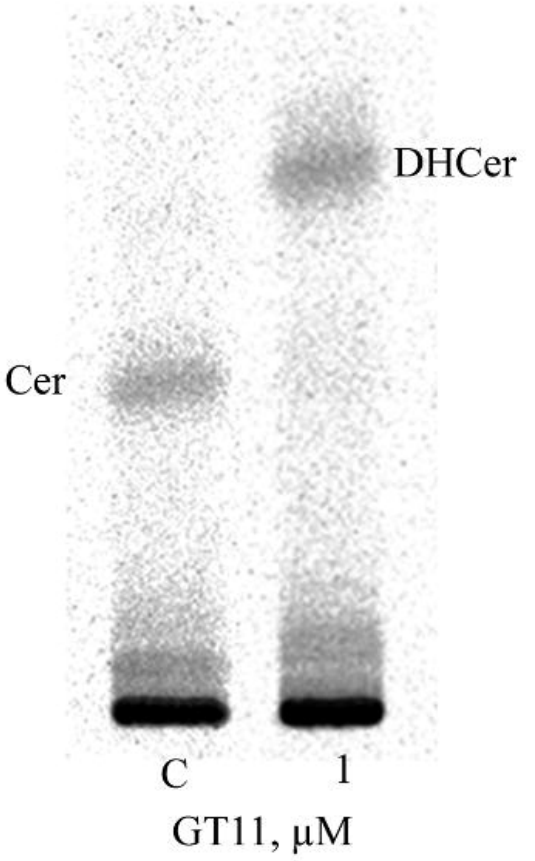


Fig 2

A


DHCer
Cer
C
1
GT11, μM

B

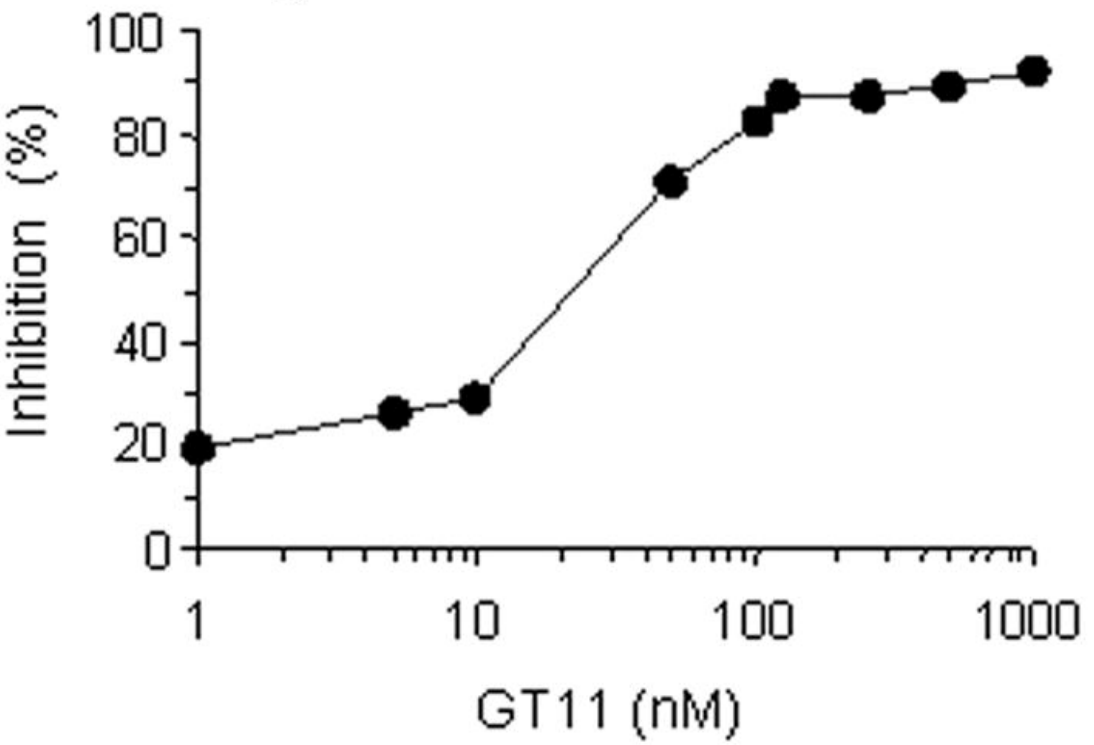

100
80
60
40
20
0
Inhibition (%)
1
10
100
1000
GT11 (nM)

Fig. 3

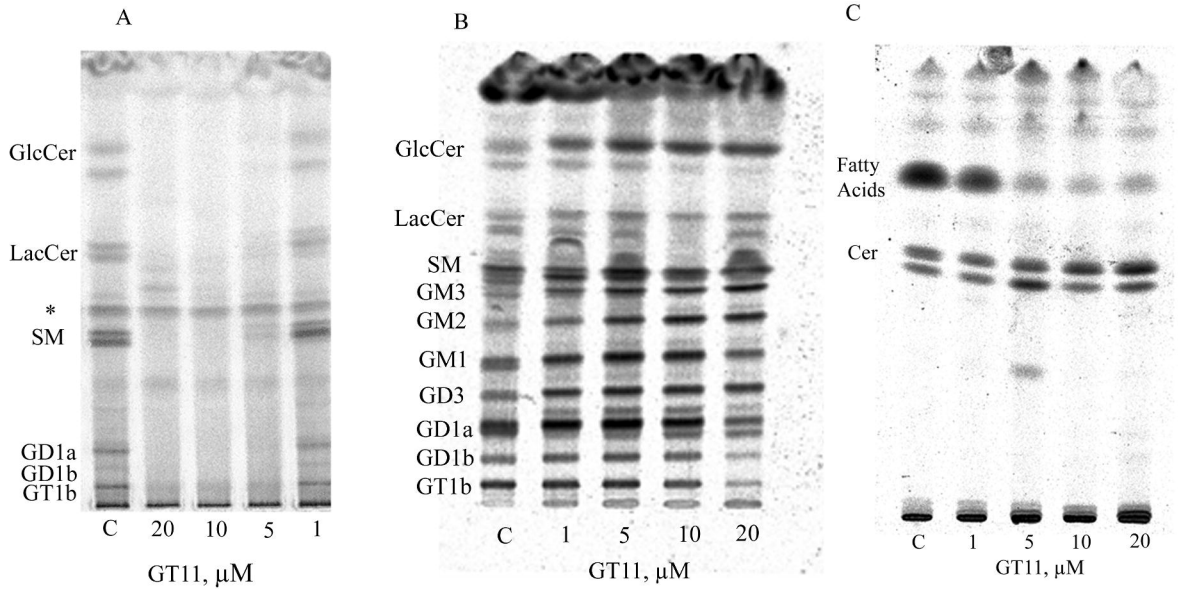

A
GlcCer
LacCer
*
SM
GD1a
GD1b
GT1b
C 20 10 5 1
GT11, μM
B
GlcCer
LacCer
SM
GM3
GM2
GM1
GD3
GD1a
GD1b
GT1b
C 1 5 10 20
GT11, μM
C
Fatty Acids
Cer
C 1 5 10 20
GT11, μM

Fig. 4

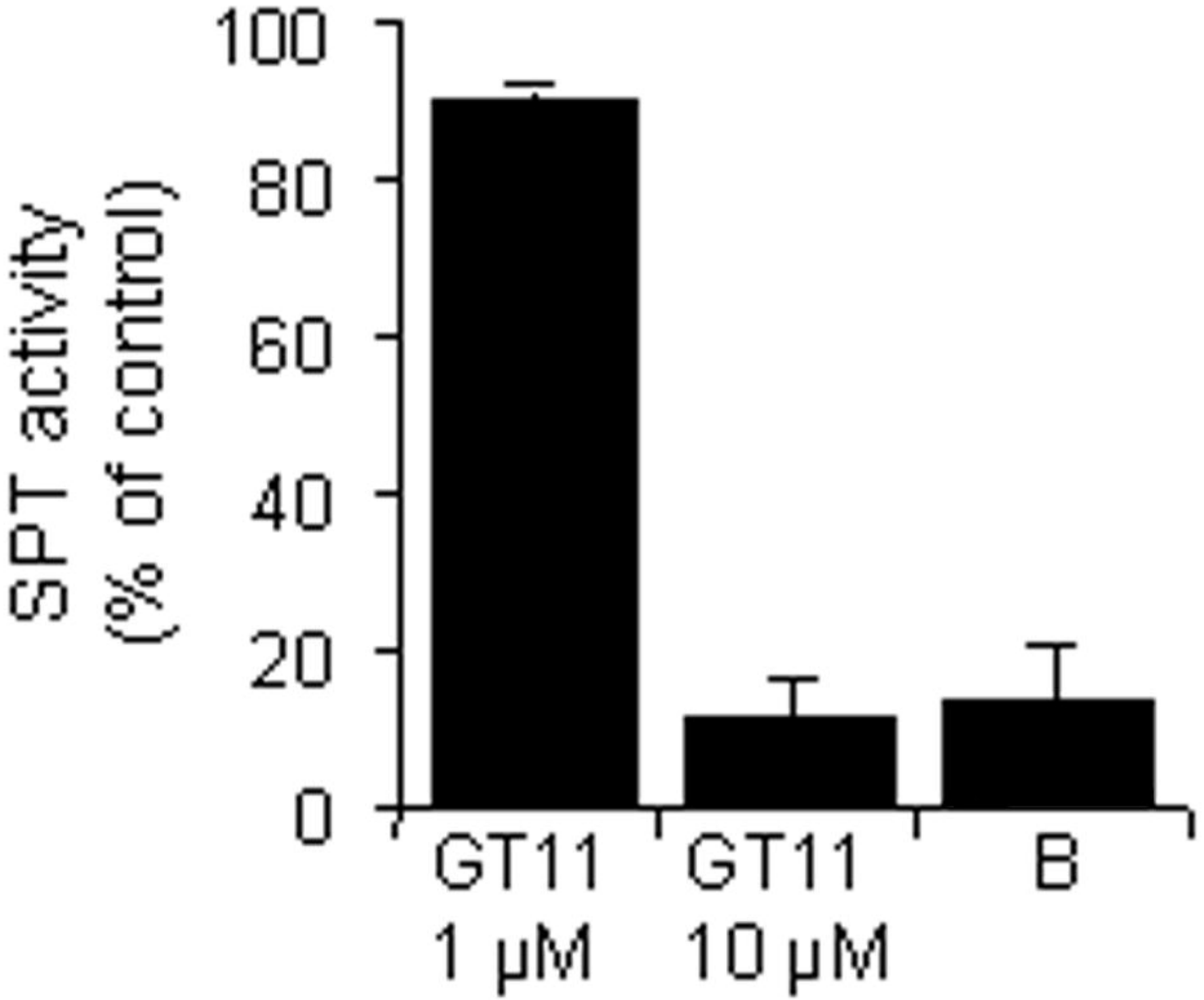


Fig.5

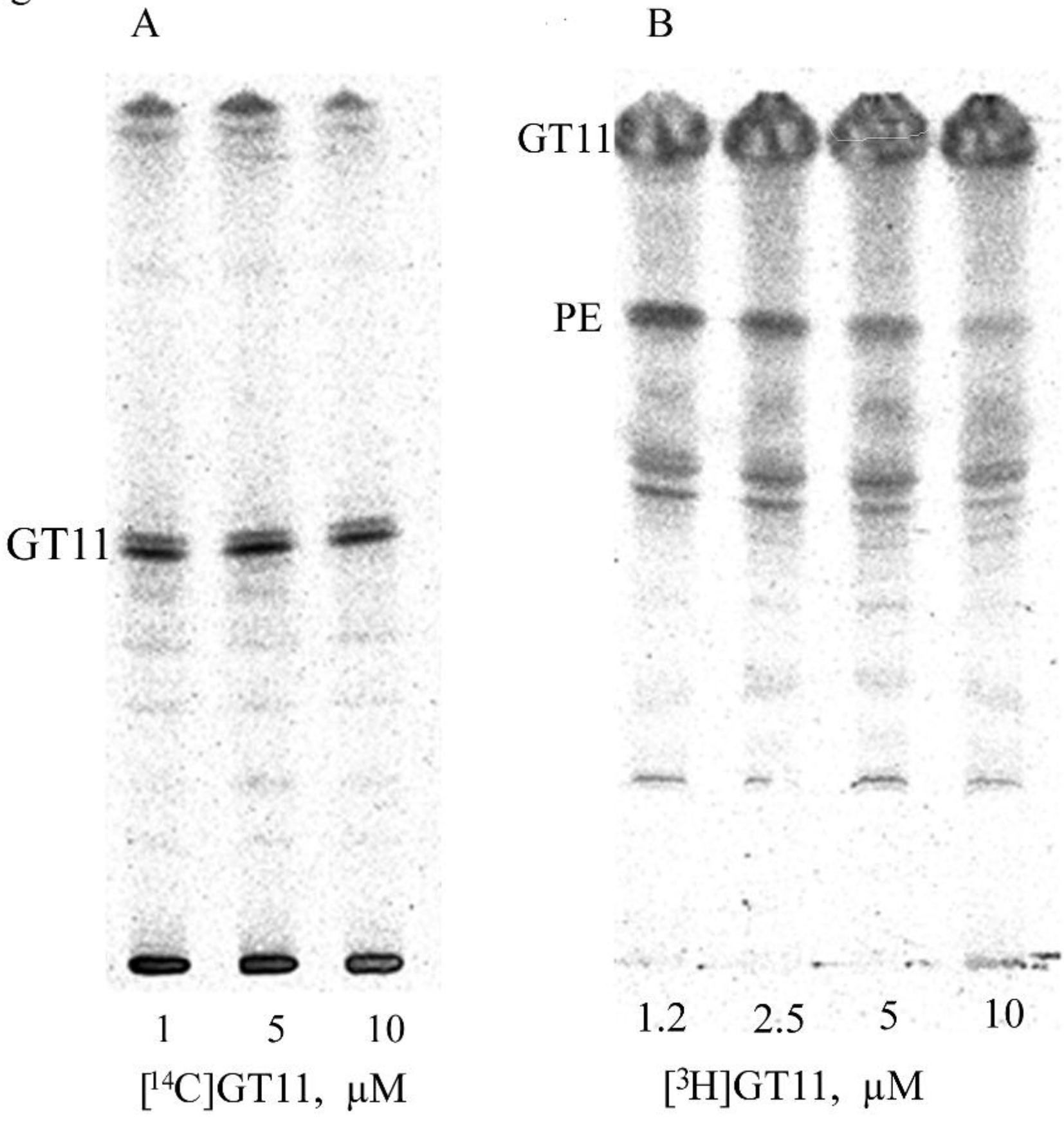


Fig. 6

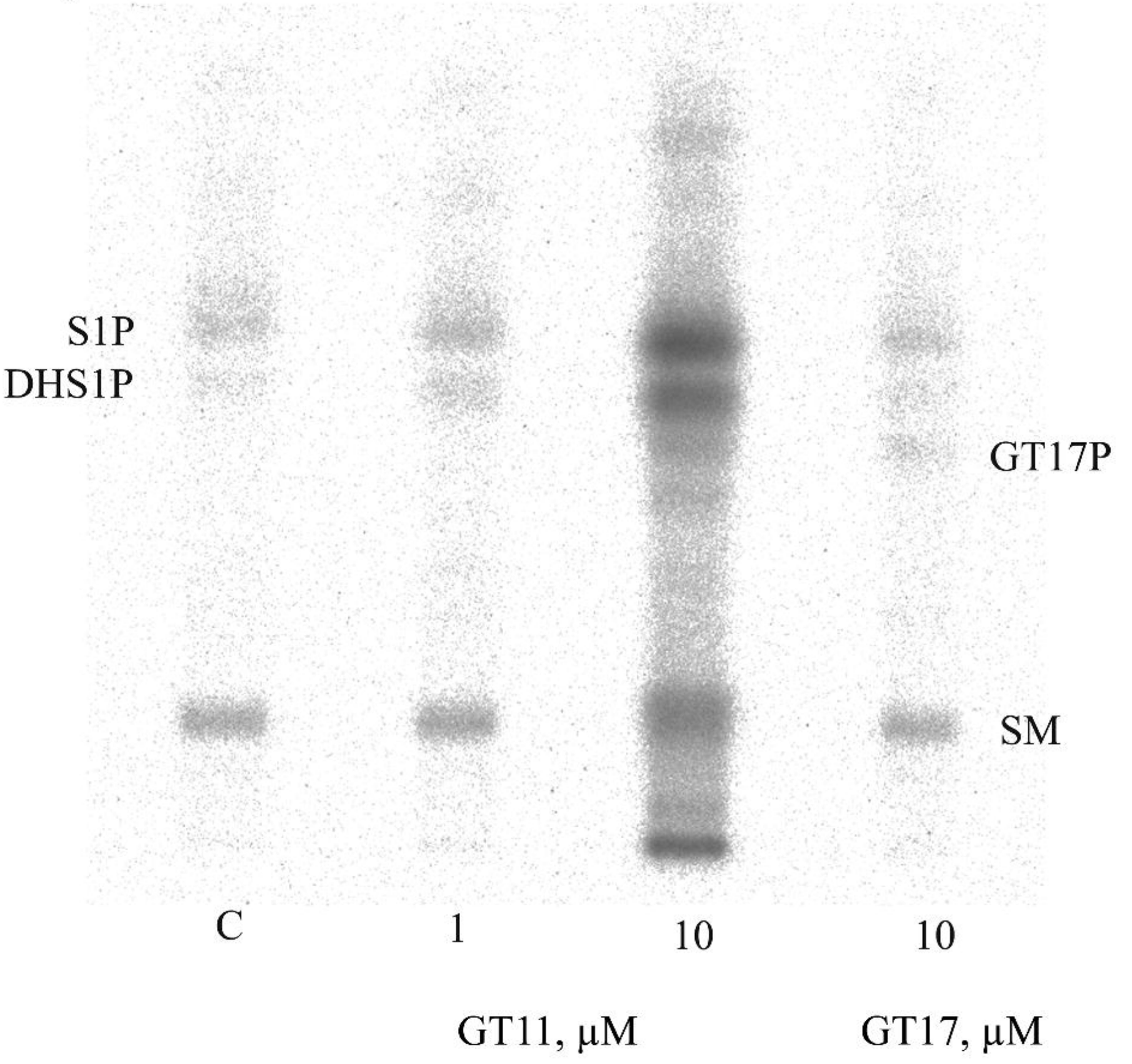


Fig. 7

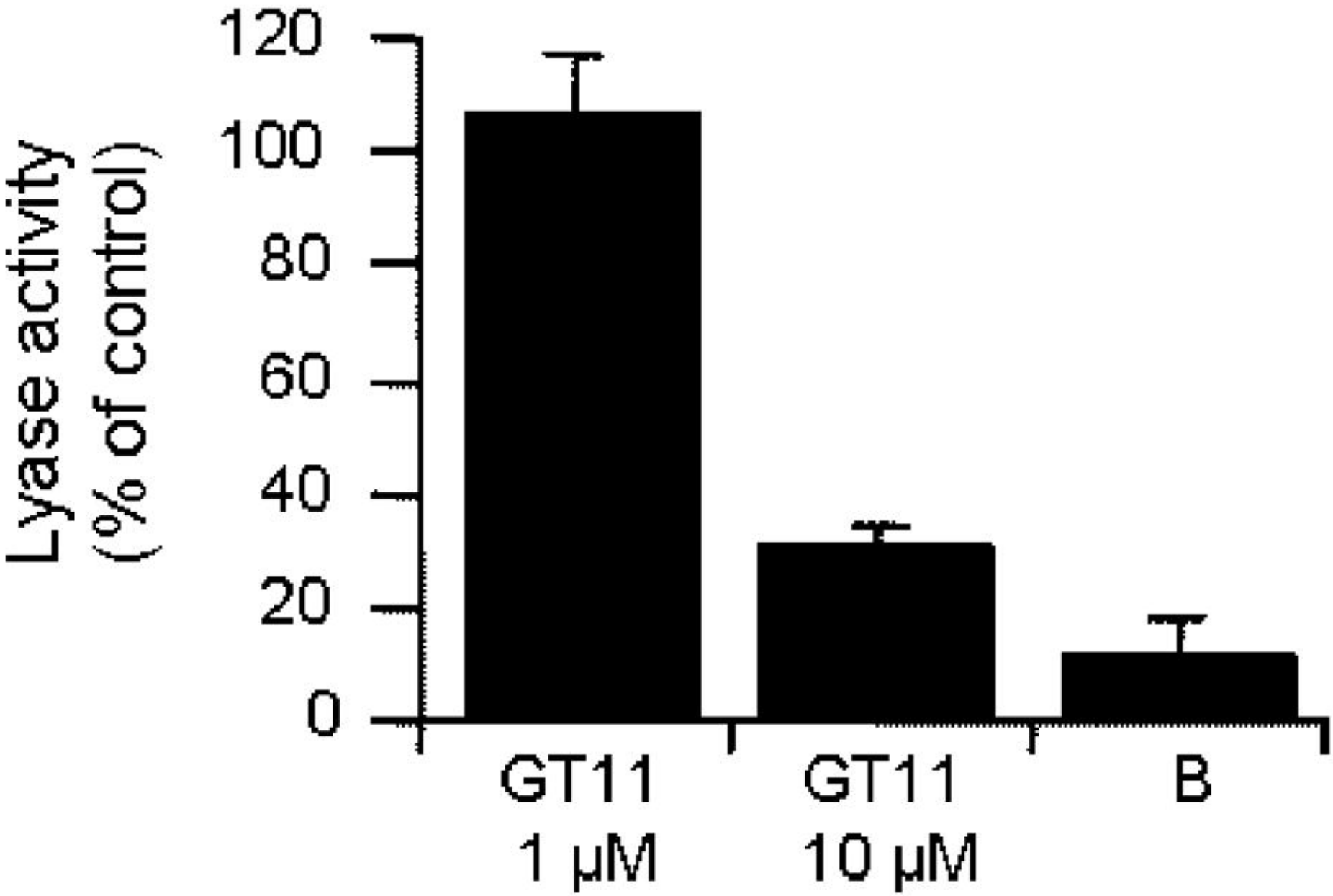

Lyase activity
(% of control)
120
100
80
60
40
20
0
GT11
1 μM
GT11
10 μM
B

Fig. 8

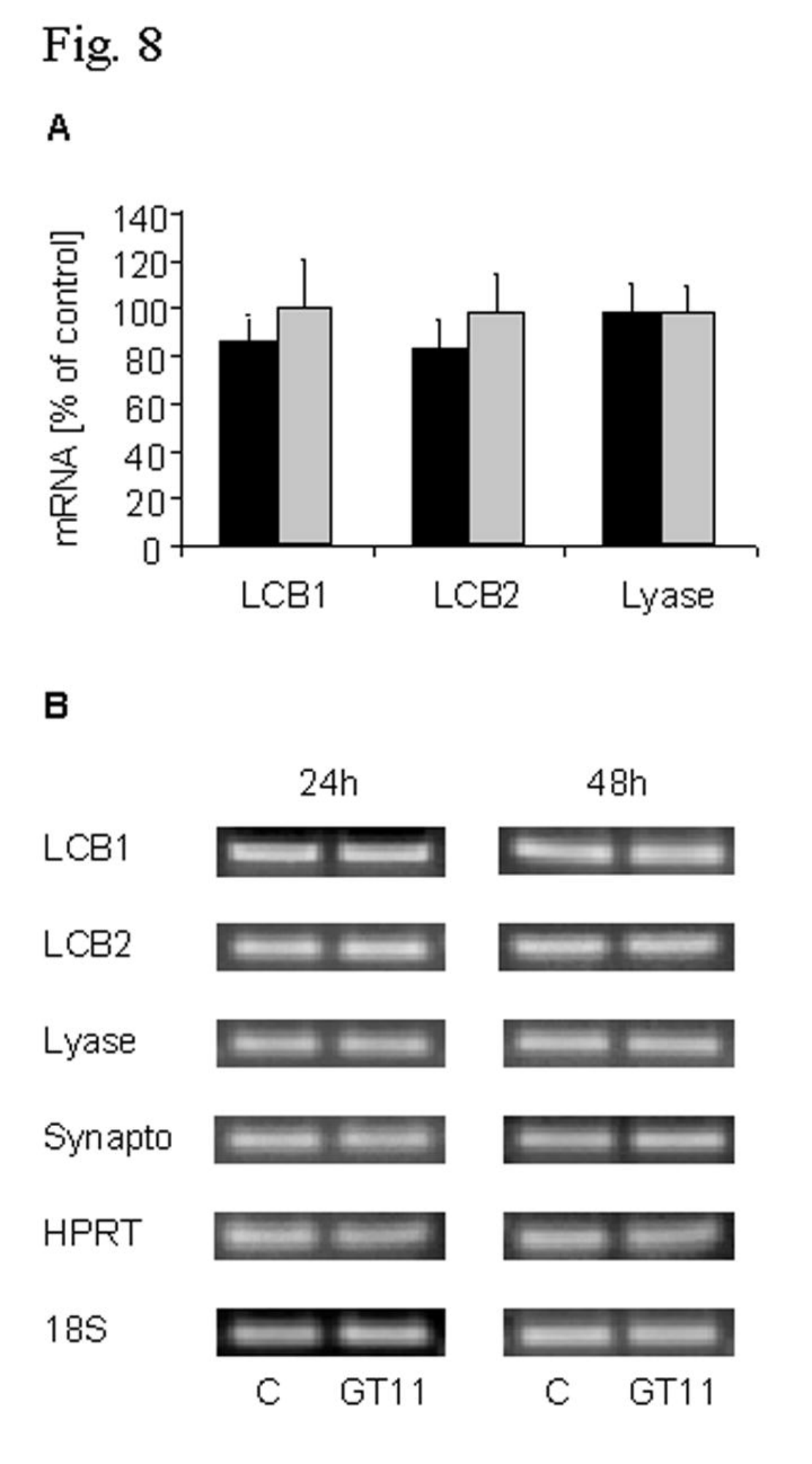
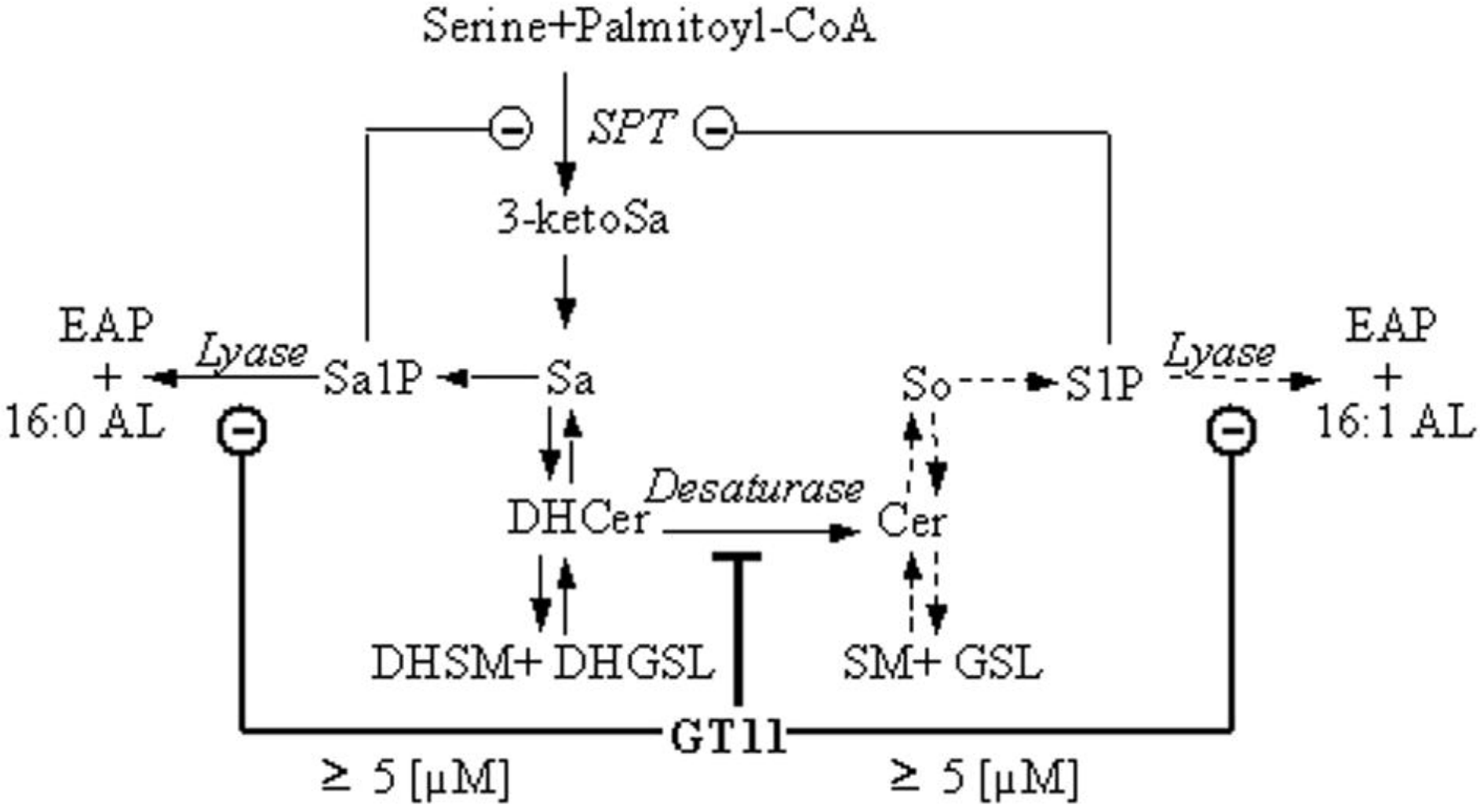

A
mRNA [% of control]
140
120
100
80
60
40
20
0
LCB1
LCB2
Lyase
B
24h
48h
LCB1
LCB2
Lyase
Synapto
HPRT
18S
C
GT11
C
GT11

Fig. 9


Serine+Palmitoyl-CoA
SPT
3-ketoSa
EAP
+
16:0 AL
Lyase
Sa1P
Sa
So
S1P
Lyase
EAP
+
16:1 AL
Desaturase
DHCer
Cer
DHSM+ DHGSL
SM+ GSL
GT11
≥ 5 [μM]
≥ 5 [μM]