ALTERED SPHINGOLIPID METABOLISM IN N-(4-HYDROXYPHENYL)RETINAMIDE-RESISTANT A2780 HUMAN OVARIAN CARCINOMA CELLS

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Running title: Sphingolipids in retinoid-resistant cells

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ABSTRACT

In the present work, we studied the effects of fenretinide (HPR), a hydroxyphenyl derivative of all-trans-retinoic acid, on sphingolipid metabolism and expression in human ovarian carcinoma A2780 cells. A2780 cells, which are sensitive to a pharmacologically achievable HPR concentration, become 10-fold more resistant after exposure to increasing HPR concentrations. Our results showed that HPR was able to induce a dose- and time dependent increase in cellular ceramide levels in sensitive, but not in resistant cells. This form of resistance in A2780 cells was not accompanied by the overexpression of multidrug resistance specific proteins MDR1 and MRP, whose mRNA levels did not differ in sensitive and resistant A2780 cells. HPR-resistant cells were characterized by an overall altered sphingolipid metabolism. The overall content in glycosphingolipids was similar in both cell types, but the expression of specific glycosphingolipids was different. More in detail, our findings indicated that glucosylceramide levels were similar in sensitive and resistant cells, but resistant cells were characterized by a 6-fold lower expression of lactosylceramide levels and by a 6-fold higher expression of ganglioside levels than sensitive cells. The main gangliosides from resistant A2780 cells were identified as GM3 and GM2. The possible metabolic mechanisms leading to this difference were investigated. Interestingly, the mRNA levels of glucosylceramide and lactosylceramide synthases were similar in sensitive and resistant cells, while GM3 synthase mRNA level and GM3 synthase activity were remarkably higher in resistant cells.
ABBREVIATIONS

Ganglioside and glycosphingolipid nomenclature is in accordance with Svennerholm (67), and the IUPAC-IUBMB recommendations (68). GlcCer, β-Glc-(1-1)-Cer; LacCer, β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM3, β²Neu5AcLacCer, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM2, β²Neu5AcGg₃Cer, β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM1, β²Neu5AcGg₄Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; Cer, ceramide, N-acyl-sphingosine; C2Cer, N-acetylsphingosine; C16Cer, N-palmitoylsphingosine; Sph, sphingosine, (2S,3R,4E)-2-amino-1,3-dihydroxy-octadecene; Chol, cholesterol; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; DMSO, dimethylsulfoxide; ESI-MS, electrospray ionization mass spectrometry; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GCS, glucosylceramide synthase; GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; GSL(s), glycosphingolipid(s); HPR, N-(4-hydroxyphenyl)retinamide; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography; MDR, multidrug resistance; MDR1, MDR1 P-glycoprotein; MRP, multidrug resistance-associated protein; PBS, phosphate buffered saline; RA, retinoic acid; RPMI 1640, Roswell Park Memorial Institute medium 1640; RT-PCR, reverse transcription polimerase chain reaction; SSC, standard sodium citrate; SDS, sodium dodecylsulfate;
INTRODUCTION

Sphingolipid metabolism plays a pivotal role in the mechanism of apoptosis induced in tumor cells. Ceramide, produced under physiological (tumor necrosis factor α, γ-interferon, interleukines) and pharmacological (anticancer drugs, including daunorubicin, vincristine, 1-α-D-arabinofuranosylcytosine, retinoids) stimuli by sphingomyelin hydrolysis or by de novo biosynthesis, is a mediator of apoptosis and an inhibitor of cell proliferation in a variety of tumor cell lines (reviewed in 1-2).

Interestingly, in tumor cell lines, resistance to chemotherapeutic treatments is often associated with an increased ability of the cell to glycosylate ceramide, as a consequence of a higher activity of glucosylceramide synthase (GCS) (3-12). High levels of GlcCer (6-9, 11-12) and of GCS activity (5, 8-9) and/or expression (8-9, 11-12) were detected in a number of drug-resistant cancer cell lines, and in specimens from cancer patients not responding to chemotherapy treatment (13). GlcCer accumulation results in the ability of drug-resistant cells to scavenge ceramide, thus preventing ceramide-induced apoptotic death. Multidrug resistance (MDR), a drug-resistant phenotype characterized by the ability of tumor cells to become insensitive to a variety of chemically unrelated chemotherapeutics after exposition to one single drug, is hallmarked by the overexpression of energy-dependent drug efflux pump proteins, such as MDR1 P-glycoprotein and multidrug-resistance associated protein, MRP (3, 14). Extensive studies by Cabot’s group lead to the conclusion that elevated GlcCer levels are a specific marker for MDR phenotype in cancer cells, and that modifying ceramide metabolism might represent a winning strategy to overcome drug resistance in tumor cells (4-11).

On the other hand, it has been shown (12, 15) that GlcCer accumulation is not the only consequence of an altered sphingolipid metabolism in MDR cancer cells. In MDR human ovarian carcinoma cells, SM and galactosylceramide levels are also higher respect to parental sensitive cells, while LacCer and all more complex GSLs are present in lower
amounts. These data can be at least in part interpreted in the scenario described above. But, in addition to this it is necessary to recall that sphingolipids and sphingolipid metabolites other than ceramide have biological activities that could be responsible for the acquisition of a drug-resistance phenotype (16-18).

Retinoids are natural and synthetic derivatives of vitamin A which play a critical role in different biological processes including morphogenesis in the embryo, cell proliferation, differentiation and apoptosis (19). Retinoids exert their effects by regulating gene expression through two classes of nuclear receptors: retinoic acid receptors (RARs) \(\alpha\), \(\beta\) and \(\gamma\) and retinoid X receptors (RXRs) \(\alpha\), \(\beta\) and \(\gamma\). RARs and RXRs activate gene transcription by binding as homo- or heterodimers to specific DNA sequences, the retinoic acid responsive elements (RARE) and the retinoid X responsive elements (RXRE), usually found in the 5'-flanking regions of responsive genes (20). Retinoids have been shown to have differentiating and antitumor activities in several experimental models and their effectiveness in the treatment and prevention of human cancer has already been established (21).

\(N\)-(4-hydroxyphenyl)retinamide (HPR), pharmacologically known as fenretinide, is a synthetic amide of all-trans retinoic acid (RA) which has shown reduced toxicity relative to RA, while maintaining a significant biological activity (22). HPR is under investigation in clinical trials as preventive and therapeutic agent, and has already shown a preventive effect for ovarian and breast cancers in premenopausal women (23-24) as well as chemopreventive and therapeutic efficacy against different tumors in animal models (22). In vitro studies have demonstrated that HPR has significant antiproliferative activity associated with induction of apoptosis in several tumor cell types (22). To date, the mechanism of action of HPR is poorly understood. Some studies suggest that the effects of this retinoid are mediated through RAR and RXR signaling (25-28). Other studies have shown that apoptosis in response to HPR primarily occurs by a receptor-independent
mechanism, which is accompanied by generation of reactive oxygen species (29-30) or increases in ceramide (31-32).

Discontinuation of retinoid treatment leads to recurrence of the lesion. However, as occurs with chemotherapeutic agents, continuous retinoid exposure might cause development of drug resistance. So far, only few in vitro models had been developed to investigate mechanisms and molecular characteristics associated with retinoid resistance (33-34). In a recent study, we hypothesized that continuous HPR treatment might lead to resistance to the retinoid. We showed that when A2780 human ovarian carcinoma cells, that are very sensitive to HPR (35), were continuously exposed to the drug, they developed a 10-fold resistance to the drug (34). Differences in HPR uptake and metabolism were observed between sensitive and resistant cells (34). HPR intracellular peak levels were two times lower and a polar metabolite, not detected in sensitive cells, was found in cell extracts from resistant cells. Moreover the development of HPR resistance was associated with changes in marker expression, suggestive of a more differentiated status (34). The expression of retinoic acid receptor β (RARβ), a putative tumor suppressor (36), was markedly increased whereas the expression of cell surface molecules associated with tumor progression including HER-2 laminin receptor and β1 integrin was markedly reduced.

An increase in the cellular levels of ceramide upon HPR treatment was reported in neuroblastoma (37-38) and breast cancer cell lines (32). In the present work, we studied the possible involvement of ceramide in HPR-induced apoptosis in A2780 human ovarian carcinoma cells. Moreover, we assessed whether resistance to HPR was associated with modifications of sphingolipid patterns and metabolism in these cells.
EXPERIMENTAL PROCEDURES

Chemicals. Commercial chemicals were the purest available, common solvents were distilled before use and water was doubly distilled in a glass apparatus. 4-[3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenamido]-1-hydroxybenzene, known as N-(4-hydroxyphenyl)retinamide (HPR), obtained from J. A. Crowell Division of Cancer Prevention, National Cancer Institute, (Bethesda, MD, USA) was dissolved in DMSO at a concentration of 10 mM and stored at –80°C under N₂ in the dark. Sodium boro[³H]hydride was from Amersham Biosciences (specific radioactivity 12.0 Ci/mmol). Clostridium perfringens sialidase was from Boehringer, Vibrio cholerae sialidase from Sigma.

Lipids and radioactive lipids. Sphingolipids and glycerolipids to be used as standards were extracted from rat brain, purified and characterized (39). Gangliosides were extracted from bovine brain and purified by partitioning (39). GM1 was prepared from the bovine brain ganglioside mixture by enzymatic treatment with Clostridium perfringens sialidase (40). GM3 was prepared from GM1 using the GM1-lactone hydrolysis procedure (41). Lactosylceramide was prepared from GM3 by enzymatic treatment with Vibrio cholerae sialidase (42). Sphingosine was prepared from cerebroside (43). [1-³H]sphingosine was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium boro[³H]hydride (44) (radiochemical purity over 98%; specific radioactivity 2.2 Ci/mmol). N-acetylsphingosine (C2Cer) and N-palmitoylsphingosine (C16Cer) were prepared by N-acylation of sphingosine using acetic anhydride and hexadecanoic anhydride, respectively (45). The same procedure applied to [1-³H]sphingosine was used to synthesize tritium-labeled N-palmitoylsphingosine (radiochemical purity over 99%; specific radioactivity 2.2 Ci/mmol). GM3 tritium labeled at the position 3 of sphingosine, [3-³H(sphingosine)]GM3, was prepared by the dichlorodicyanobenzoquinone-sodium borohydride method (46), and the natural erythro diastereoisomer was purified by reverse phase HPLC as described (47) (radiochemical
purity 98%, specific radioactivity 1.2 Ci/mmol). [3-\textsuperscript{3}H(\textit{sphingosine})]LacCer was prepared from [3-\textsuperscript{3}H(\textit{sphingosine})]GM3 by enzymatic treatment with \textit{Vibrio cholerae} sialidase and purified by silica gel column chromatography (radiochemical purity over 99%, specific radioactivity 1.2 Ci/mmol). \([\text{H}]\)lipids used as chromatographic standards were prepared from [1-\textsuperscript{3}H]sphingosine-fed cell cultures as previously described (48).

**Cell cultures.** An HPR-resistant cell line, A2780/HPR, was developed from parental A2780 cells by in vitro incubation of A2780 cells with increasing concentrations of HPR as previously described (34). Briefly, cells surviving 60 rounds of selections in HPR containing medium (3 rounds at 1 µM, 11 rounds at 2 µM, 8 rounds at 3 µM and 38 rounds at 5 µM) were cloned by limiting dilution. One clone (A2780/HPR) was expanded and when tested for HPR sensitivity demonstrated a 10 fold resistance to HPR, which was slightly reversible upon drug removal for 5 rounds (34). Therefore A2780/HPR cells were continuously maintained in 5 µM HPR and seeded without the drug in all the experiments. A2780 and A2780/HPR cells were grown in monolayer in RPMI 1640 medium (Biowhittaker, Belgium) containing 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) in 5% CO\textsubscript{2} at 37 °C.

**Effects of C2Cer on A2780 and A2780/HPR cells.** Cells were seeded at density of 10,000 cells per well in 96-well tissue culture plates, treated on the next day with different concentrations of C2Cer in absence of FBS and incubated for one additional day. Control cultures received the same amount of ethanol as the treated cultures (0.1%). Cell number was estimated by using the sulforhodamine B (SRB) assay (49). Two analyses were performed and four replicate wells were used for each analysis. Apoptotic cells were identified by the Terminal dUTP nick-end labeling (TUNEL) method using an \textit{in situ} cell death detection kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, A2780 and A2780/HPR cells were treated 24 hr after seeding with 2.5 µM C2Cer in absence of FBS, incubated for one additional day, harvested and washed twice in PBS. After centrifugation, cells were fixed in 4% paraformaldehyde for 1 hour at
26°C, and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate in PBS. After washing, the cells were resuspended in TUNEL reaction mixture containing FITC-dUTP and terminal-deoxy-nucleotidyl- transferase (TdT). Control cells were suspended in the TUNEL reaction mixture containing FITC-dUTP without TdT and incubations were performed for 1 hour at 37°C before washing the cells twice. The number of TUNEL-positive cells, as detected by fluorescent microscopy, was assessed on at least 100 cells in two different smears and referred to the whole cell population.

Northern analysis. Total cellular RNA was isolated using the TriReagent method (Molecular Research Center, Cincinnati). Twenty µg of total RNA were fractionated in formaldehyde/Mops agarose gel and blotted on Hybond N+ nylon membranes (Amersham Corp., Arlington Heights, IL). Filters were hybridized with cDNA probes for multidrug resistance (MDR1), multidrug resistance-associated protein (MRP), GlcCer synthase, LacCer synthase, and GM3 synthase, obtained by RT-PCR using A2780/HPR cDNA as template. Briefly, single-stranded cDNA was synthesized from 2 µg RNA by reverse transcription, then amplified by PCR. The primer pairs used for PCR amplification are the following: MDR1: sense, 5’-AAAAAGATCAACTCGTAGGAGTG-3’, and antisense, 5’-GCACAAAATACACCAAACAA-3’ (161 pb amplified product). MRP: sense, 5’-CCACCTCCTCATTGCATCCACCTTG-3’, and antisense, 5’-GGAAACCATCCACGACCCTAATCCCT-3’ (296 pb amplified product). GlcCer synthase: sense, 5’-TCTACACCGATTACACCTC-3’, and antisense, 5’-TGGGATAATCCAATTCAAAGAAT-3’ (147 pb amplified product). LacCer synthase: sense, 5’-AAAAACAGTACGCTCAACGG-3’, and antisense, 5’-ATCATCTTCTCTCCTCCCCATC-3’ (676 pb amplified product). GM3 synthase: sense, 5’-AAAAATGCATTATGTGGACCC-3’, and antisense, 5’-GTCTTGGCTTTCAAGTGTTCA-3’ (303 pb amplified product). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense, 5’-CCATGGAGAAGGCTGCGC-3’, and antisense 5’-
CAAAGTTGTCATGGATGACC-3' (195 pb amplified product). The cycling program was
94°C for 30s, 53 °C for 30 s, and 72 °C for 60 s for 35 cycles. PCR fragments were labeled
with [α-32P] dCTP (3,000Ci/nmol, ICN, Milan, Italy) using multiprime DNA labeling system
(Amersham) to a specific activity of approximately 2 x 10^9 cpm/µg cDNA. Following
hybridization for 3-12 h at 66 °C in Rapid-Hyb Buffer (Amersham) the blots were washed
twice with 2X SSC/ 0.1 % SDS at room temperature for 10 min. The final washes were
done with 0.1 X SSC/ 0.1 % SDS at 65 °C for 20 min for at least two times. Membranes
were exposed to X-ray film (Hyperfilm-MP, Amersham) using double intensifying screens.
RNA from human colon cancer LOVO-DX cells (kindly provided by Monica Binaschi) was
used as positive control for MDR1 (50)

Treatment of cell cultures with [3H]sphingosine. 24 h after seeding, cells were
incubated in the presence of 3x10^-8 M [1-3H]sphingosine (5 ml/dish) in culture medium for
2 h (pulse). After the pulse, the medium was replaced with fresh medium without
radioactive sphingosine and cells were further incubated for up to four days (chase). Under
these conditions all sphingolipids (including ceramide, SM, neutral glycolipids and
gangliosides), and PE (obtained by recycling of radioactive ethanolamine formed in the
catabolism of [1-3H]sphingosine) were metabolically radiolabeled (32, 48, 51).

Treatment of [3H]sphingosine-labeled cell cultures with HPR. After metabolic labeling
of cell lipids with [1-3H]sphingosine, at the end of a 24 h chase, the medium was replaced
with medium containing HPR at final concentrations of 5 and 10 µM for up to 72 h. Time-
matched control cells received the same amount of DMSO as HPR treated cells (0.1%).

Lipid extraction and determination. In the experiments described above, at the end of
the treatment periods, cells adherent to the dishes were harvested in ice-cold water (2 mL)
by scraping with a rubber policeman. Cells floating in the culture medium were collected by
centrifugation. Adherent and floating cells were analyzed to determine the content of
radioactivity associated with lipids. Samples were lyophilized and lipids were extracted
twice with chloroform/methanol 2:1 by vol (1st extraction 1.5 ml, 2nd extraction 0.25 ml) (51). The total lipid extracts were subjected to a two-phase partitioning as previously described (52), resulting in the separation of an aqueous phase containing gangliosides and in an organic phase containing all other lipids. Aliquots of total lipid extracts, aqueous and organic phases were analyzed by HPTLC as described below, followed by radioactivity imaging for quantification of radioactivity.

Identity of radioactive lipids separated by HPTLC (using HPTLC Silica Gel 60 plates from Merck, Germany) was assessed by comigration with standard lipids and confirmed by susceptibility of compounds to the following enzymatic and chemical treatments (51). A sample of the aqueous phase was treated at 37°C for 2 h, in 50 µL of water, in the presence of 1 mU of *Vibrio cholerae* sialidase, to yield GM1. For the identification of SM, a sample of the organic phase was treated at 37°C overnight in 30 µL of 100 mM Tris-HCl, pH 7.4, 0.5 mM MgCl2, 0.05% sodium deoxycholate, in the presence of 11 mU of *Staphylococcus aureus* sphingomyelinase, to yield ceramide; PE contained in the organic phase was characterized following its degradation under alkaline conditions. The enzymatic or chemical reaction mixtures were separated by HPTLC and the reaction products identified by chromatographic comparison with standard lipids.

The total lipid extract from about 50 mg of cell proteins was analysed for mass lipid content. The phospholipid content was determined in the organic phase as phosphate after perchloric acid digestion by the method of Bartlett (53). Gangliosides from the aqueous phase and phospholipids, and cholesterol from the organic phase were separated by HPTLC as described below. Identification of lipids after separation was assessed by comigration with standard lipids and confirmed by their susceptibility to enzymatic and chemical treatments as previously described (51). After chromatographic separation, compounds were chemically detected and their amount was determined by
densitometry as described below. Gangliosides from the aqueous phase were characterized by HPLC-ESI-MS (46) (see below).

**GM3 synthase assay.** Cells cultured in 100-mm dishes as described above were harvested using a plastic scraper and washed two times with phosphate–buffered saline. GM3 synthase activity was assayed as previously described (54-55) with some modifications. Cells were resuspended in 150 mM sodium cacodylate-HCl buffer, pH 6.6 (20 mg cell protein/ml) with protease inhibitors (2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.0016 mM aprotinin, 0.044 mM leupeptin, 0.08 mM bestatin, 0.03 mM pepstatin A, 0.028 mM E-64) (Sigma) and homogenized with a Dounce homogenizer (10 strokes, tight). In each reaction tube, 10 µl Triton CF-54 1.5% (v/v) in chloroform/methanol 2/1 were mixed with 0.5-50 nmoles [3-$^3$H(sphingosine)]LacCer (corresponding to 45 nCi) from a stock solution in chloroform/methanol 2/1, and dried under $\text{N}_2$. To this mixture, 8 µl 750 mM sodium cacodylate-HCl buffer, pH 6.6, 4 µl 125 mM MgCl$_2$, 4 µl 125 mM 2-mercaptoethanol, 10 µl 5 mM CMP-NeuAc, and 10 µl cell homogenate (containing 200 µg of protein) were added in a total reaction volume of 50 µl. Negative controls were performed using heat-inactivated cell homogenates (100°C for 3 min). The incubation was performed at 37°C for 3 hours with continuous shaking. Reaction was stopped by adding 1.5 ml of chloroform/methanol 2/1. The reaction mixture was analyzed by HPTLC using the solvent system chloroform/methanol/water 55:20:3 by vol. Radioactive lipids were detected and quantified by radioactivity imaging as described below.

**Ceramidase assay.** Cells homogenates were prepared as described for the GM3 synthase assay, using 20 mM Tris-HCl, pH 7.4 as homogenization buffer. The activity of acidic, neutral and alkaline ceramidase was assayed as previously described (56-57) with some modifications. In each reaction tube, 25 µl 0.1% Triton-X 100 (v/v) in chloroform/methanol 2/1 and 25 µl 0.2 % sodium cholate (w/v) in chloroform/methanol 2/1
were mixed with 2-100 pmoles [1-3H(sphingosine)]C16Cer (corresponding to 4.5 nCi) from a stock solution in chloroform/methanol 2/1, and dried under N2. To this mixture, 7.5 µl of water was added and the tubes were sonicated for 3 minutes, heated for 5 sec at 80° C and then put on ice. Ceramidase activities were assayed by adding 12.5 µl 400 mM sodium acetate buffer, pH 4.5 (for the acidic enzyme), or 12.5 µl 400 mM Tris-HCl, pH 7.4 (for the neutral enzyme) or 12.5 µl 400 mM glycine–NaOH, pH 9.0 (for the alkaline enzyme), 5 µl 50 mM MgCl2 and 10 µl cell homogenate (containing 200 µg of protein) were added in a total reaction volume of 50 µl. Negative controls were performed using heat-inactivated cell homogenates (100°C for 3 min). The incubation was performed at 37°C for 1 hours with continuous shaking. Reaction was stopped by adding 1.5 ml of chloroform/methanol 2/1. The reaction mixture was analyzed as described for GM3 synthase assay.

Analytical procedures. Lipids were separated by monodimensional and two-dimensional HPTLC carried out with the following solvent systems: chloroform/methanol/0.2% aqueous CaCl2, 55:45:10 by vol, to separate ceramide from sphingosine; chloroform/methanol/0.2% aqueous CaCl2, 50:42:11 or 55:45:10 by vol and chloroform/methanol/0.2% aqueous CaCl2/32% NH3, 60:50:9:1 by vol, to analyze total lipids and gangliosides; chloroform/methanol/water 55:20:3 by vol, to analyze total lipids, lipids from the organic phase and to separate GM3 from LacCer; n-butanol/acetic acid/water 3:1:1 by vol and hexane/chloroform/acetone/acetic acid 10:35:10:1, by vol., to analyze ceramide. Endogenous phospholipids from organic phase were separated by mono- or bidimensional HPTLC using the following solvent systems and conditions (51): chloroform/methanol/acetic acid/water, 30:20:2:1 by vol, to analyze glycerophospholipids and SM; chloroform/methanol/acetic acid/water, 30:20:2:1 by vol, 1st run and 2nd run with intermediate exposure to HCl vapours for 15 min, to analyze plasmalogens of
glycerophospholipids. The solvent system chloroform/methanol/water, 55:40:3 by vol was used to analyze neutral GSLs and SM from organic phase after alkaline treatment. Hexane/diethylether/acetic acid, 80:20:1, by vol and hexane/ethylacetate, 3:2 by vol were used to analyze cholesterol.

Ganglioside, neutral GSL and phospholipid species were quantified after separation on HPTLC followed by specific detection with a \( p \)-dimethylaminobenzaldehyde reagent (58), an aniline/diphenylamine reagent (59) or a molybdate reagent (60), respectively. The relative amounts of each ganglioside, neutral GSL or phospholipid were determined by densitometry. The mass content of each phospholipid was calculated on the basis of the percent distribution and total phospholipid content, determined as described above. The quantity of each ganglioside, neutral GSL or of SM was determined by densitometry and comparison with known amounts of standard compounds using the Molecular Analyst program (Bio-Rad Laboratories). Cholesterol was quantified by visualization with 15% concentrated sulfuric acid in 1-butanol (48). The quantity of cholesterol was determined by densitometry and comparison with 0.1-2 \( \mu g \) of standard compounds.

Radioactive lipids were detected and quantified by radioactivity imaging performed with a Beta-Imager 2000 instrument (Biospace, Paris) using an acquisition time of about 48 h. The radioactivity associated with individual lipids was determined with the specific \( \beta \)-Vision software provided by Biospace.

The radioactivity associated with cells, lipids, lipid extracts and aqueous or organic phases was determined by liquid scintillation counting.

Structural characterization of gangliosides has been carried out using a ThermoQuest Finningan LCQDeca mass spectrometer (FINNIGAN MAT, San Jose,CA, USA) equipped with an ESI ion source and a Xcalibur data system and connected to a HPLC TSP P4000 quaternary pump and an automatic solvent degasser. Optimum conditions included: sheath gas flow of 70 arbitrary units, auxiliary gas flow of 10 arbitrary
units, spray voltage of 4 Kv, capillary voltage of -42 v, capillary temperature of 260°C, fragmentor voltage (used for insurge collision induced dissociation) of 40-80%. Mass spectra were acquired over a m/z range 200-2000.

HPLC separation of gangliosides dissolved in CH$_3$CN/5mM ammonium acetate buffer pH 7, 64:36 by vol, was carried out on a 5 µm LiChrospher C8 reverse-phase column (250x4mm) (Merck) at a flow rate of 0.5 ml/min using a gradient formed by the solvent system A, consisting of CH$_3$CN/5 mM ammonium acetate buffer, pH 7, 15:85 by vol, and solvent system B containing CH$_3$CN/H$_2$O, 85:15 by vol. The gradient was linear from 30:70 to 20:80, by vol, of A:B over 25 min, then to100 of B in 5 min.

The protein content was determined according to Lowry (61), using bovine serum albumin as the reference standard.
RESULTS AND DISCUSSION

Effect of HPR on ceramide levels in A2780 cells. The possible involvement of ceramide in the effect of HPR in A2780 human ovarian carcinoma cells was investigated. To this purpose, A2780 and A2780/HPR cells were incubated in the presence of $[1^{-3}{\text{H}}]$sphingosine for 2 h, followed by 24 h chase. $[1^{-3}{\text{H}}]$sphingosine is efficiently incorporated by cultured cells and converted into ceramide, that is further utilized for the synthesis of more complex sphingolipids. This metabolic radiolabeling approach was successfully used in the past as a valuable tool to quantitatively and sensitively determine changes in cellular ceramide levels elicited by different treatments, including RA (62) and HPR (32). Cells were then treated with 5 and 10 $\mu$M HPR for 48 and 72 h. As previously reported (35), treatment of A2780 cells with HPR under these experimental conditions was able to induce apoptosis, and a relevant number of cells (25.0 and 60.6% of total cells after treatment with 5 $\mu$M HPR for 48 and 72 h, respectively) were detached from the culture dishes and floating in the culture medium. The same treatment had only weak effect (3.8 and 5.5% of total cells were floating after treatment with 5 $\mu$M HPR for 48 and 72 h, respectively) on A2780/HPR cells (34). Cell lipids were extracted from both cell types, separated by HPTLC and analyzed by digital autoradiography. The values for radioactivity incorporation into ceramide in control and HPR treated cells are reported in Figure 1. Under these experimental conditions, treatment of A2780 cells with HPR increased the incorporation of radioactivity from $[1^{-3}{\text{H}}]$sphingosine into ceramide in floating cells respect to untreated cells or adherent cells (Figure 1, Panels A and B). On the other hand, in the case of A2780/HPR cells, the incorporation of $[1^{-3}{\text{H}}]$sphingosine into ceramide was very similar in adherent and floating cells, treated with HPR or not (Figure 1, Panels C and D). HPR treatment of A2780/HPR cells is thus not able to determine a raise in the production of radioactive ceramide.
Effects of C2Cer on A2780 and A2780/HPR cells. To further examine the involvement of ceramide in the HPR effect in A2780 cells, we investigated whether cells resistant to HPR were also resistant to ceramide. To this aim, the antiproliferative and apoptotic effect of C2Cer was evaluated in parental and HPR-resistant cells. After 24 hr of exposure to the drug, the sensitivity of the two cell lines to C2Cer was different (Figure 2, Panel A): doses of C2Cer ranging from 1 µM to 5 µM caused approximately 60% growth inhibition in A2780 whereas the same doses were ineffective in A2780/HPR cells. No difference in sensitivity to C2Cer between A2780 and A2780/HPR cells was observed at doses higher than 7.5 µM, that caused a strong dose-dependent growth inhibition in both cell lines. TUNEL analysis performed in cells treated with 2.5 µM C2Cer showed an increase in the percentage of apoptotic cells, compared to untreated cells, in A2780 cells, whereas no increase was observed in A2780/HPR cells (Figure 2, Panel B).

Expression levels of MDR1 and MRP. In several tumor cell lines, the loss of ability to respond to anticancer drugs with the raise of cellular ceramide levels hallmark the acquisition of the MDR phenotype. To understand whether HPR-resistance in A2780 cells is accompanied by the overexpression of multidrug resistance specific proteins, MDR1 and MRP mRNA expression levels were analyzed in parental and resistant cells (Figure 3). No differences were observed in gene expression between sensitive and resistant cells: MDR1 mRNA was not detected in A2780 and A2780/HPR cells, whereas MRP1 was expressed in both cell lines at similar levels.

Sphingolipid metabolism in A2780 and A2780/HPR cells. To study possible differences in sphingolipid patterns and metabolism, A2780 cells and A2780/HPR cells were incubated in the presence of [1-3H]sphingosine for 2 h, followed by different chase times (up to 4 days). As reported for other cell types (48, 51), under these conditions all sphingolipids (including ceramide, SM, neutral glycolipids and gangliosides), were efficiently metabolically radiolabeled, reaching a steady-state distribution of radioactivity.
after 48 h (data not shown). The use of \([1-^3H]\)sphingosine as metabolic precursor allows the quantitative analysis of cell sphingolipids with very high sensitivity. Moreover, during the degradation of the exogenous \([1-^3H]\)sphingosine taken up by the cells or of \([1-^3H]\)sphingosine-containing sphingolipids, radioactive ethanolamine is formed, that is recycled for the synthesis of phosphatidylethanolamine (PE). The formation of \(\[^3H\]PE\) is thus a very useful and sensitive tool to quantify sphingolipid catabolism.

Under these experimental conditions, at all investigated chase times A2780 and A2780/HPR cells incorporated comparable amounts of radioactivity (Table 1). Cell lipids were extracted, separated by HPTLC and analyzed by digital autoradiography. Figure 4 shows the patterns of radioactive lipids extracted from A2780 and A2780/HPR cells after HPTLC separation. In both cell lines, radioactive bands co-migrating with standard ceramide, glucosylceramide, PE, lactosylceramide and SM were detectable. In the case of A2780/HPR cells, at least two intense radioactive bands migrating below SM were also present. To allow a better resolution of these lipid profiles, the total lipid extracts were subjected to a two-phase partitioning, resulting in the separation of an aqueous phase containing gangliosides and an organic phase containing all less polar lipids (including radioactive ceramide, PE, neutral glycosphingolipids and SM), that were further analyzed. As reported in Table 1, most radioactive lipids were associated with the organic phase in both A2780 and A2780/HPR cells. Remarkably, independently from the chase time, the radioactivity associated with the aqueous phase in the case of resistant A2780/HPR cells was 3 times higher than in sensitive A2780 cells.

Figure 5 shows the patterns of radioactive lipids present in aqueous (Panel A) and organic phases (Panel B) obtained from A2780 and A2780/HPR cell lipid extracts after HPTLC separation. The major radioactive bands present in the aqueous phase from both cell types showed a similar chromatographic behavior. On the basis of their chromatographic migration and sensitivity to \(Vibrio cholerae\) sialidase treatment, the two
upper bands were identified as GM3, and the two lower bands, accounting for about 60% of the aqueous phase radioactivity in the case of A2780/HPR cells, as GM2. In the organic phase from both cell types (Figure 5, Panel B), ceramide, glucosylceramide, lactosylceramide, PE and SM were identified.

The quantitative data, summarized in Table 2 as radioactivity incorporation into different lipids, indicate that the metabolic use of [1-3H]sphingosine is different A2780 and A2780/HPR cells, thus resulting in quantitatively different [3H]lipids patterns. In both cell types, a significant portion of [1-3H]sphingosine-containing lipids underwent complete degradation, as indicated by the formation of high levels of radioactive PE (particularly in the case of A2780/HPR cells). In both cell types, more than 50% of radioactivity from [1-3H]sphingosine was associated with complex sphingolipids, including SM and glycosphingolipids. Albeit the total radioactivity associated with glycosphingolipids was similar in A2780 and A2780/HPR cells, its distribution among different glycosphingolipids was radically different in these two cell lines. In particular, A2780 cells were characterized by high levels of LacCer (about 6-fold higher than in A2780/HPR), while A2780/HPR express remarkably more elevated (6-fold higher) ganglioside levels than the parental cell line.

**Lipid composition of A2780 and A2780/HPR cells.** The data obtained using metabolic labeling with [1-3H]sphingosine strongly indicate that, in A2780 cells, resistance to HPR is linked to an alteration in sphingolipid metabolism leading to an increased expression of gangliosides. To evaluate possible alterations in the metabolism of other lipid classes, we analyzed the mass content of cholesterol and phospholipids in A2780 and A2780/HPR cells. Cholesterol content was 45.20±3.12 and 45.20±2.97 nmoles/mg of cell protein in A2780 and A2780/HPR cells, respectively (Figure 6, Panel A). Phospholipid phosphorous content was 130.60± 5.21 and 118.30± 6.13 nmoles/mg of cell protein in A2780 and A2780/HPR cells, respectively, and the phospholipid pattern was very similar in both cell
types (Figure 6, Panel B). Thus, A2780 and A2780/HPR cells were almost identical in their bulk lipid composition.

In the case of sphingolipids, colorimetric chemical detection did not allow an accurate quantification, due to their low cellular content. However, the qualitative comparison of endogenous neutral glycosphingolipid and ganglioside patterns confirmed the radiolabeling data. Mass spectrometry analyses showed that GM2 and GM3 were the main components of the ganglioside mixture extracted from A2780/HPR cells. Both gangliosides were present as molecular species containing d18:1-16:0, d18:1-18:0, d18:1-24:1 and d18:1-24:0 ceramide. As shown in Figure 7, the GM2 and GM3 species were characterized by the deprotonated molecular ions [M–H]$^-$ at m/z 1354, 1382, 1464, 1466 and 1151, 1179, 1261, 1263, respectively. Figure 7 shows as example the characterization of GM2(d18:1-16:0) and GM3(d18:1-16:0). MS2 of molecular ion [M–H]$^-$ gave typical fragmentation pattern due to the sequential loss of sugar units, providing information about the carbohydrate structure and the total ceramide mass; GM3 and GM2 molecular species contained N-acetylneuraminic acid, while species containing N-glycolyneuraminic acid were undetectable.
Expression levels of GlcCer synthase, LacCer synthase and GM3 synthase in A2780 and A2780/HPR cells. To investigate potential mechanisms underlying the observed differences in sphingolipid levels in A2780 and A2780/HPR cells, the mRNA expression of the enzymes GlcCer synthase, LacCer synthase, and GM3 synthase was evaluated in parental and resistant cells. A2780/HPR cells showed GlcCer synthase and LacCer synthase mRNA levels similar to those of A2780 cells, whereas GM3 synthase mRNA was markedly increased (Figure 8). In A2780 cells, the mRNA levels of GM3 synthase were below the detection limit of our Northern blot analysis. Using RT-PCR, we were able to detect in A2780 cells a faint band relative to GM3 synthase.

To assess if observed changes in the mRNA levels of GM3 synthase were paralleled by differences in the enzyme levels, we measured the activity of GM3 synthase in A2780 and A2780/HPR cells by the mean of a cell-free assay using radioactive LacCer as substrate. As indicated in Figure 9, the *in vitro* activity of GM3 synthase was 20-fold higher in resistant A2780/HPR cells than in sensitive A2780 cells.

Ceramidase activities in A2780 and A2780/HPR cells. Among the metabolic mechanisms that contribute to the regulation of cellular ceramide levels, a possible role of ceramidases (enzymes that cleave the *N*-acyl bond of ceramide) was recently highlighted (reviewed in 63). To determine if differences in the activity of ceramidases were associated with the resistance to HPR in human ovarian carcinoma cells, we measured the *in vitro* activity of ceramidase at acidic, neutral and alkaline pH in A2780 and A2780/HPR cells using [1-\(^{3}\text{H}\)(sphingosine)]C16Cer as substrate. As reported in Table 3, the *in vitro* activities of acid, neutral and alkaline ceramidases were similar in sensitive and resistant cells.
CONCLUSIONS

The synthetic retinoid HPR exerts an apoptotic effect in several tumor cell lines, including A2780 (28). In the case of neuroblastoma (36-37), breast carcinoma (32) and acute lymphoblastic leukaemia cell lines (64), HPR-induced apoptosis is accompanied by the elevation of ceramide cellular levels. In the present paper, we show that this is the case also for human ovarian carcinoma A2780 cells. The administration of pro-apoptotic concentrations of HPR to A2780 cells resulted in an up to 10-fold increase of cellular ceramide levels. The raise of cellular ceramide levels was not observed when A2780/HPR cells, resistant to HPR-induced apoptosis (34), were treated with HPR under the same experimental conditions. These data indicates that: a) ceramide is a possible mediator in HPR-induced apoptosis in parental A2780 cells and b) the different sensitivity of parental A2780 and A2780/HPR cells to HPR might be linked to their different ability to respond with an increase in the cellular levels of the pro-apoptotic sphingolipid ceramide. Interestingly, a parallel between ceramide generation in response to HPR treatment and sensitivity to HPR was observed in cells of lymphoid origin (64). Different acute lymphoblastic leukemia cell lines respond to cytotoxic HPR treatment with an increase in ceramide. On the other hand, HPR is not cytotoxic and has no effect on ceramide levels in non-malignant lymphoid cell types (64).

In the well studied case of multidrug resistance (3-12, 14), MDR cells are able to escape the drug-induced apoptotic death elicited in their sensitive counterparts by a raise in the cellular ceramide levels through two distinctive features. MDR cells express high levels of efflux pump proteins belonging to the ATP-binding cassette superfamily of membrane transport proteins, such as MDR1 and MRP. The overexpression of energy-dependent drug efflux pump proteins might lead to lowering intracellular drug levels to sublethal concentrations. MDR cells show increased levels of GlcCer and GlcCer synthase expression and/or activity respect to their sensitive counterparts. The ability to scavenge
ceramide by converting it to GlcCer could represent an efficient way to reduce the drug effects at the intracellular level. In addition, in 2780AD cells, a MDR cell line deriving from the parental A2780 line, besides enhanced levels of glucosylceramide, more complex alteration of sphingolipid metabolism respect to the parental A2780 cells were reported (12). In these cells, a reduced synthesis of LacCer was observed, that resulted in lower levels of LacCer, GM3 and GM2 and higher levels of galactosylceramide and SM.

According to this knowledge, the resistance to a retinoid in A2780 cells herein reported, does not seems to be related to MDR. In fact, our data indicate that levels of MDR1 mRNA and MRP mRNA, as well as those of GlcCer and GlcCer synthase mRNA were substantially unchanged in A2780/HPR cells respect to parental A2780 cells.

Instead striking differences were observed between A2780 and A2780/HPR cells in the metabolism of more complex glycosphingolipids. In A2780/HPR cells, the sphingolipid metabolism is markedly oriented toward the synthesis of gangliosides. We characterized the main gangliosides of A2780/HPR cells as GM3 and GM2, and their levels were 6-fold higher than in parental cells, while LacCer, the direct neutral glycolipid precursor of gangliosides, was proportionally lower. Finally, differences were observed in the mRNA levels of the enzyme GM3 synthase, that is more expressed in A2780/HPR than in sensitive A2780 cells. This difference was reflected by the in vitro activity of GM3 synthase, that was 20-fold higher in resistant A2780/HPR cells than in sensitive A2780 cells.

Thus, the data presented in this paper indicate that all the biosynthetic pathway downstream of ceramide is more active in A2780/HPR cells than in the parental sensitive cell line, leading to the expression of higher levels of GM3 and GM2 gangliosides. This particular alteration in sphingolipid metabolism associated with the development of resistance to an apoptosis inducing drug is reported here for the first time.
It could be argued that an overall increased flow through the ganglioside biosynthetic pathway is also a mechanism, even if quite elaborate, to scavenge ceramide thus counteracting the apoptotic effect of HPR, in analogy with what observed in MDR cells synthesizing high GlcCer levels. However, the role acknowledged to complex sphingolipids in modulating the properties of tumor cells (such as adhesion, motility, invasiveness and proliferation) is well documented (reviewed in 16), even if less codified that the one of ceramide in tumor cell apoptosis. In bladder cancer, a specific role for GM3 ganglioside as a negative modulator of malignant potential of this tumor was hypothesized. In fact, superficial bladder tumors express higher levels of GM3 and GM3 synthase compared with invasive tumors (65), and exogenous GM3 treatment (65) or GM3 synthase overexpression (66) reduced invasion potential and induced apoptosis in bladder tumor cell lines. Even in the case of MDR cells (where the link between sensitivity to drug and generation of ceramide as described above seems extremely well established), a possible role for GlcCer as substrate or modulator of the pump activity of MDR related proteins (that are interestingly localized at the level of plasma membrane in sphingolipid-rich environments) was suggested (3). As presented in this paper, the evidence we obtained using A2780/HPR cells suggest that, also in the case of retinoids, differences in sphingolipid metabolism are related to differences in the sensitivity of these cells to the drug, as previously reported for chemoterapeutic drugs. However, in our case these differences might be not simply related to the ability of cells to neutralize the proapoptotic action of ceramide. In particular, our data suggest that changes in the expression of gangliosides could contribute to the onset of a resistant phenotype in tumor cells.
REFERENCES


FIGURE LEGENDS

FIGURE 1.

Ceramide detection in A2780 and A2780/HPR cells. After cell lipid metabolic labeling with [1-3H]sphingosine as described under “Materials and Methods”, A2780 (Panels A and B) and A2780/HPR (Panels C and D) cells were treated with vehicle (white) or 5 µM (grey) and 10 µM HPR (black) for 48 or 72 h. Panel A: A2780, adherent cells; Panel B: A2780, floating cells; Panel C: A2780/HPR, adherent cells; Panel D: A2780/HPR, floating cells. Radioactive lipids were extracted, separated by HPTLC detected by digital autoradiography (250 dpm applied on a 3 mm line. Time of acquisition: 48 h) and radioactivity associated with ceramide was quantitatively determined. Data are expressed as nCi/mg of cell protein and are the means of three different experiments, with the S.D. never exceeding 10% of the mean values.

FIGURE 2.

C2Cer effects on A2780 and A2780/HPR cells. Panel A: C2Cer antiproliferative activity on A2780 (open circle) and A2780/HPR (solid circle) cells. Cells were treated 24 hr after seeding, and surviving cell number was evaluated 1 day later. Data are expressed as percentage of control untreated cells. Results are the mean ± SD of two independent experiments. Panel B: Detection of DNA fragmentation induced by C2Cer. A2780 (Lane 1) and A2780/HPR (Lane 2) cells were treated with vehicle (white) or 2.5 µM C2Cer (grey) for 24 hr one day after seeding. Cells were then harvested, processed for TUNEL reaction and observed using a fluorescence microscope. The results are referred to the whole population and represent the mean ± SD of two independent experiments.
FIGURE 3.

Constitutive expression of MDR1 and MRP mRNA in A2780 and A2780/HPR cells. Northern blot analysis was performed as described under “Material and Methods”. Each lane contains 20 µg of total RNA. Filters were hybridized with cDNA probes for multidrug resistance (MDR1) (Panel A), and multidrug resistance-associated protein (MRP) (Panel B), and as a control for loading, the filters were stripped and rehybridized with GAPDH (lower part of each panel). Lane 1: A2780 cells; Lane 2: A2780/HPR cells; Lane 3: RNA from LOVO-DX cells was used as a positive control for MDR1.

FIGURE 4.

Radioactive lipid patterns in total lipid extracts from A2780 and A2780/HPR cells. After metabolic labeling with [1-3H]sphingosine for 2 h pulse followed by 48 h chase, lipids were extracted from A2780 (Lane 1) and A2780/HPR (Lane 2) cells and separated by HPTLC in solvent system chloroform/methanol/water 55:20:3 by vol. Radioactive lipids were detected by digital autoradiography (250 dpm applied on a 3 mm line. Time of acquisition: 48 hr). The position of pure standard lipids is indicated on the left margin of the panel. Pattern is representative of that obtained in three different experiments.

FIGURE 5.

Radioactive lipid patterns in aqueous and organic phases obtained after phase separation of total lipid extracts from A2780 and A2780/HPR cells. After metabolic labeling with [1-3H]sphingosine for 2 h pulse followed by 48 h chase, lipids were extracted from A2780 (Lanes 1 and 3) and A2780/HPR (Lanes 2 and 4) cells. Total lipid extracts were subjected to phase separation as described under “Materials and Methods”. Lipids from the aqueous (Panel A) and organic (Panel B) phases were separated by HPTLC using solvent systems chloroform/methanol/0.2% aqueous CaCl₂, 50:42:11, by vol. and
chloroform/methanol/water 55:20:3 by vol., respectively. To selectively remove PE and allow the quantitative detection of LacCer, aliquots of the organic phases were subjected to chemical treatment under alkaline conditions (Lanes 3 and 4). Aliquots of aqueous and organic phases corresponding to identical amounts of cell proteins were analyzed. Radioactive lipids were detected by digital autoradiography (250-1000 dpm applied on a 3 mm line. Time of acquisition: 48 h). The position of pure standard lipids is indicated on the left margin of the panel. Pattern is representative of that obtained in three different experiments.

FIGURE 6.

Patterns of endogenous lipids from A2780 and A2780/HPR cells. Cholesterol and phospholipids from A2780 (Lane 1) and A2780/HPR cells (Lane 2) were analyzed by HPTLC followed by chemical detection as described under “Materials and Methods”. Aliquots of samples corresponding to identical amounts of cell proteins were analyzed for both cell types. Panel A: Cholesterol was separated from organic phases (corresponding to 0.4 mg of cell proteins). Solvent system hexane/ethylacetate, 3:2 by vol., visualization by charring with 15% concentrated sulfuric acid in 1-butanol. Panel B: Phospholipids were separated from organic phases (corresponding to 0.6 mg of cell proteins). Solvent system chloroform/methanol/acetic acid/water, 30:20:2:1 by vol., visualization by detection with a molybdate reagent.

FIGURE 7.

Mass spectra of main gangliosides from A2780 and A2780/HPR cells. Lipids were extracted from A2780 and A2780/HPR cells, total lipid extracts were subjected to phase separation as described under “Materials and Methods” and gangliosides present in the aqueous phases were analyzed by HPLC-ESI-MS. Panel A, total negative ESI-MS
spectrum of the gangliosides from HPR resistant cells, with indication of the main GM3 and GM2 molecular species differing in the acyl structure. Panel B, HPLC-ESI-MS mass spectrum of the GM3(d18:1-16:0) molecular species together with the MS2 spectrum derived from ion at m/z 1151 and the MS3 spectrum derived from the ion at m/z 536. Panel C, HPLC-ESI-MS mass spectrum of the GM2(d18:1-16:0) molecular species together with the MS2 spectrum derived from ion at m/z 1354 and the MS3 spectrum derived from the ion at m/z 536.

FIGURE 8.

**Constitutive expression for GlcCer synthase, LacCer synthase, and GM3 synthase mRNA in A2780 and A2780/HPR cells.** Northern blot analysis was performed as described under “Material and Methods”. Each lane contains 20 µg of total RNA. Filters were hybridized with cDNA probes for GlcCer synthase (Panel A), LacCer synthase (Panel B), and GM3 synthase (Panel C), and as a control for loading, the filters were stripped and rehybridized with GAPDH (lower part of each panel). Lane 1: A2780 cells; Lane 2: A2780/HPR cells.

FIGURE 9.

**GM3 synthase activity in A2780 and A2780/HPR cells.** GM3 synthase activity was assayed on cell homogenates from A2780 (1) and A2780/HPR cells (2) as described under “Materials and Methods” in the presence of 10 µM (dark grey) or 100 µM (light grey) [3-3H(sphingosine)]LacCer (corresponding to 45 nCi/assay). Aliquots of cell homogenates containing the same amount of proteins (200 µg) were added in a total reaction volume of 50 µl. The incubation was performed at 37°C for 3 hours. Negative controls were performed using heat-inactivated cell homogenates. The reaction mixture was analyzed by HPTLC using the solvent system chloroform/methanol/water 55:20:3 by vol.
Radioactive lipids were detected and quantified by radioactivity imaging as described in the legend of Figure 1. Data are expressed as pmoles of formed GM3/hr/mg of cell protein and are the means of three different experiments, with the S.D. never exceeding 10% of the mean values.
Table 1. Radioactivity incorporation into total lipid extracts, aqueous and organic phases in A2780 and A2780/HPR cells after 2 hour pulse with \([1-^3H]\)sphingosine followed by 48, 72 and 96 h chase.

<table>
<thead>
<tr>
<th></th>
<th>Total lipid extract</th>
<th>Organic phase</th>
<th>Aqueous phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nCi/mg of cell protein</td>
<td>nCi/mg of cell protein</td>
<td>% of radioactivity in total lipid extract</td>
</tr>
<tr>
<td>A2780</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>312.17</td>
<td>295.25</td>
<td>94.6</td>
</tr>
<tr>
<td>72 h</td>
<td>170.25</td>
<td>159.91</td>
<td>93.9</td>
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<tr>
<td>96 h</td>
<td>55.28</td>
<td>51.67</td>
<td>93.5</td>
</tr>
<tr>
<td>A2780/HPR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>242.37</td>
<td>192.21</td>
<td>79.3</td>
</tr>
<tr>
<td>72 h</td>
<td>109.06</td>
<td>86.81</td>
<td>79.6</td>
</tr>
<tr>
<td>96 h</td>
<td>48.56</td>
<td>39.73</td>
<td>81.8</td>
</tr>
</tbody>
</table>
Table 2. Radioactivity incorporation into different lipids after metabolic labeling of A2780 and A2780/HPR cells with [1-$^3$H]sphingosine (2 hour pulse followed by 48 h chase).

<table>
<thead>
<tr>
<th></th>
<th>A2780</th>
<th>A2780/HPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>nCi/mg protein</td>
<td>% of lipid radioactivity</td>
<td>nCi/mg protein</td>
</tr>
<tr>
<td>Total</td>
<td>271.59 100</td>
<td>222.18 100</td>
</tr>
<tr>
<td>PE</td>
<td>63.71 23.46</td>
<td>85.81 38.62</td>
</tr>
<tr>
<td>SPHINGOLIPIDS</td>
<td>207.88 76.54</td>
<td>136.37 61.38</td>
</tr>
<tr>
<td>SM</td>
<td>118.30 43.56</td>
<td>55.84 25.13</td>
</tr>
<tr>
<td>Cer</td>
<td>8.32 3.06</td>
<td>9.91 4.46</td>
</tr>
<tr>
<td>GSLs</td>
<td>81.26 29.92</td>
<td>70.62 31.78</td>
</tr>
<tr>
<td>GlcCer</td>
<td>18.48 6.80</td>
<td>13.74 6.18</td>
</tr>
<tr>
<td>LacCer</td>
<td>53.09 19.55</td>
<td>6.72 3.02</td>
</tr>
<tr>
<td>GM3</td>
<td>3.23 1.19</td>
<td>15.27 6.87</td>
</tr>
<tr>
<td>GM2</td>
<td>5.91 2.18</td>
<td>31.50 14.18</td>
</tr>
<tr>
<td>Other gangliosides</td>
<td>0.55 0.20</td>
<td>3.39 1.53</td>
</tr>
</tbody>
</table>
Table 3. Activity of acid, alkaline and neutral ceramidase in A2780 and A2780/HPR cells.
Ceramidase activity was measured by the mean of a cell-free assay at pH 4.5, 9.0 and 7.4 using radioactive ceramide as substrate at three different concentrations. The activities are expressed as pmol of hydrolized ceramide/hr/mg of cell protein and are the means of three different experiments, with the S.D. never exceeding 15% of the mean values.

<table>
<thead>
<tr>
<th></th>
<th>0.043 µM Ceramide</th>
<th>0.43 µM Ceramide</th>
<th>2 µM Ceramide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A2780</td>
<td>A2780/HPR</td>
<td>A2780</td>
</tr>
<tr>
<td>Acid Ceramidase</td>
<td>1.159</td>
<td>0.928</td>
<td>7.912</td>
</tr>
<tr>
<td>Alkaline Ceramidase</td>
<td>0.272</td>
<td>0.225</td>
<td>2.171</td>
</tr>
<tr>
<td>Neutral Ceramidase</td>
<td>0.173</td>
<td>0.144</td>
<td>1.944</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

A

Live cells (% of control)

C2Cer (μM)

B

% of TUNEL positive cells

C2Cer 2.5 μM

1 2
Figure 3

A

MDR1

GAPDH

1 2 3

B

MRP

GAPDH

1 2
Figure 5

A

GM3
GM2

1
2

B

Cer
GlcCer
PE
LacCer
SM

1
2
3
4
Figure 6

A

Chol

B

PE

PI

PS

PC

SM

1 2

1 2
A

Relative Abundance

MS1

[\text{GM3(d18:1-16:0)}]

[\text{GM3(d18:1-18:0)}]

[\text{GM3(d18:1-24:1)}]

[\text{GM3(d18:1-24:0)}]

GM2(d18:1-16:0)

GM2(d18:1-24:0)

GM2(d18:1-24:1)

GM2(d18:1-18:0)

GM2(d18:1-24:1)

B

GM3(d18:1-16:0)

MS1

[M - H]⁻

1151

1233

C

GM2(d18:1-16:0)

MS1

[M - H]⁻

1354

1436

GM2(d18:1-16:0)

GM2(d18:1-24:0)

GM2(d18:1-24:1)

GM2(d18:1-18:0)

GM2(d18:1-24:1)

Relative Abundance

MS2

[Cer - H]⁻ [GlcCer - H]⁻

536 698

[LacCer - H]⁻

860

[\text{GM2(d18:1-16:0)}]

[\text{GM2(d18:1-24:0)}]

[\text{GM2(d18:1-24:1)}]

[\text{GM2(d18:1-18:0)}]

[\text{GM2(d18:1-24:1)}]

Relative Abundance

MS3

[Cer - H]⁻ [GlcCer - H]⁻ [LacCer - H]⁻

1133 536 698

280 506 1336

263

[\text{GM2(d18:1-16:0)}]

[\text{GM2(d18:1-24:0)}]

[\text{GM2(d18:1-24:1)}]

[\text{GM2(d18:1-18:0)}]

[\text{GM2(d18:1-24:1)}]
Figure 8

A
GlcCer synthase
GAPDH

B
LacCer synthase
GAPDH

C
GM3 synthase
GAPDH
Figure 9

pmol/hr/mg protein

1  2

0  400  800  1200  1600

1  2

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