Glycoconj J (2009) 26:647–661
DOI 10.1007/s10719-008-9219-4

Post-translational and transcriptional regulation of glycolipid glycosyltransferase genes in apoptotic breast carcinoma cells: VII. Studied by DNA-microarray after treatment with L-PPMP

Rui Ma · N. Matthew Decker · Vesta Anilus · Joseph R. Moskal · Joseph Burgdorf · James R. Johnson · Manju Basu · Sipra Banerjee · Subhash Basu

Received: 15 July 2008 / Revised: 4 November 2008 / Accepted: 25 November 2008 / Published online: 29 January 2009

Abstract Functions of glycosphingolipids on the eukaryotic cell membranes during the onset of oncogenic processes and cell death are not well understood. Inhibitors of glycosphingolipid biosynthesis were recently found to trigger apoptosis in many carcinoma including breast cancer SKBR-3, MCF-7, and MDA-468 cells through either intrinsic or extrinsic apoptotic pathways as we previously reported. These anti-cancer inhibitors could increase ceramide concentration by blocking functions of glycolipid glycosyltransferases (GLTs). In this study, using a novel fluorescent dye (ASK-0) revealed the damage of cell organelle membranes by an inhibitor of glucosylceramide biosynthesis (L-PPMP). A highly drug- and cell-dependent regulation of MAPKs was also found by *cis*-platin and L-PPMP when inducing apoptosis in SKBR-3, MCF-7, and MDA-468 cells. A dose and time-dependent regulation of GLTs were investigated by enzymatic assay and DNA microarray analyses. These GLTs are involved in biosynthesis of Le^X and sialosyl-Le^X (neolactosyl-ceramide series) such as GalT-4 (UDP-Gal: LcOse3cer β−galactosyltransferase, GalT-5 (UDP-Gal: nLcOse4Cer α1, 3galactosyltransferase, FucT-3 (GDP-Fucose: LM1 α1, 4fucosyltransferase). A similar effect was observed with the GLTs involved in the biosyntheses of Gg-series gangliosides, such as SAT-4 (CMP-NeuAc: GgOse4Cer α2, 3sialyltransferase, and SAT-2 (CMP-NeuAc: GM3 α2, 8sialyltransferase). The glycol-related gene DNA-microarrays also suggested the transcriptional regulation of several GLTs involved in the biosynthesis of neolactosylceramide containing cell-surface antigens in these apoptotic breast carcinoma cells. In the early apoptotic stages (2 to 6 h after L-PPMP treatment) in addition to GlcT-1 gene, several genes (βGalTs and βGlcNAcTs) in the SA-Le^a pathway were stimulated.

R. Ma · N. Matthew Decker · V. Anilus · J. R. Johnson · M. Basu · S. Basu (✉)
Department of Chemistry and Biochemistry,
The University of Notre Dame,
Notre Dame, IN 46556, USA
e-mail: sbasu@nd.edu

J. R. Moskal · J. Burgdorf
The Falk Center for Molecular Therapeutics,
Department of Biomedical Engineering,
McCormick School of Engineering and Applied Sciences,
Northwestern University,
Evanston, IL 60201, USA

S. Banerjee
Department of Cancer Biology, Cleveland Clinic Foundation,
Cleveland, OH 44129, USA

Keywords Apoptosis · AKS-0 · Breast carcinoma cells · Carpases · *cis*-platin · DNA-microarray · GSL · Gangliosides · Glycosyltransferases · FucT · GlcT · GD3 · GD1b · D-PDMP · L-PPMP · LM1 · Le^X · MAPKinases · SA-Le^a · SA-Le^X · PSS-380 · Phosphatidylserine

Abbreviation

GSLs	Glycosphingolipids
GLTs	glycolipid glycosyltransferases
GalT	galactosyltransferases
SAT	sialyltransferases
FucT	fucosyltransferases
GlcT	glycosyltransferase

L-PPMP	L-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol
D-PDMP	D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
SA-Le^X	sialyl-fucosyl-neolactotetraosyl-ceramide
cis-Platin	*cis*-diamino-dichloro platinum

Introduction

Studies on initiation and regulation of the apoptotic cascades are of great interest in oncogenic drug discovery. Glycosphingolipids (GSLs) comprise an important outer-leaflet structural and signaling lipid in the higher eukaryotic cells. Importantly, the glycolipid-enriched membrane domain (Rafts) also includes proteins related to signal transduction including the apoptotic signal. GSLs are synthesized from ceramide in the Golgi bodies [1–4], and subsequently distributed to different areas of the cell. The glycosphingolipids are derived from the sphingolipid ceramide by the action of a glucosyltransferase, GlcT [1–5], to generate glucosylceramide, a precursor of other long chain glycolipids [6–9] (Fig. 1). However, ceramide is widely agreed to be a pro-apoptotic messenger in eukaryotic cells [10]. If the GSLs biosynthesis is blocked [11–13] in the first committed step of glucosyltransferase (GlcT), the ceramide will accumulate in the cells, which may be the cause for apoptosis. At present it is not known why ablation of the GlcT gene causes the enhancement of apoptotic cell death because GlcT has at least two distinct functions, one of which is a negative regulatory factor for intracellular ceramide content through ceramide glycosylation. The other is a positive regulatory role of the glycolipid biosynthesis. The action of ceramide-like substances (D- and L-PDMP) on sphingolipid glycosyltransferases, purified lactosylceramide synthase, has also been studied [14]. Using the GlcT-specific inhibitor L-*threo*-PPMP [11-13] in human breast carcinoma cells [14-19], it is suggested that a systematic study of different stereo isomers of these inhibitors of GSL biosynthesis is needed to establish a relation of these GSL biosynthesis inhibitors to the apoptotic processes in the level of other glycosyltransferase activities [20, 21]. Such studies would be important in the search for chemotherapeutic drugs for treatment of breast and other highly metastatic cancer cancers.

Abnormal expression of any particular glycosyltransferase may lead to a distorted sequence and may give rise to aberrant oligosaccharides in tumor and metastatic cancer cells [14, 22]. Many tumor-associated antigens are characterized as glycolipids and glycoproteins [23–25]. Comparative studies between normal and tumor cells showed changes in glycan structures during oncogenic transformation. Complete biosynthetic steps of these GSLs have been established [6–8]. SA-Le^x(SAα2,3Galβ1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glc-Ceramide) and SA-Lea(SAα2,3Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4 Glc-Ceramide) have been suggested as markers for metastatic breast carcinoma cells [26]. Exact abnormalities of these oligosaccharide-antigens (glycolipid or glycoproteins) and the regulation of these GSLs during apoptosis in tumor or cancer cells is not known

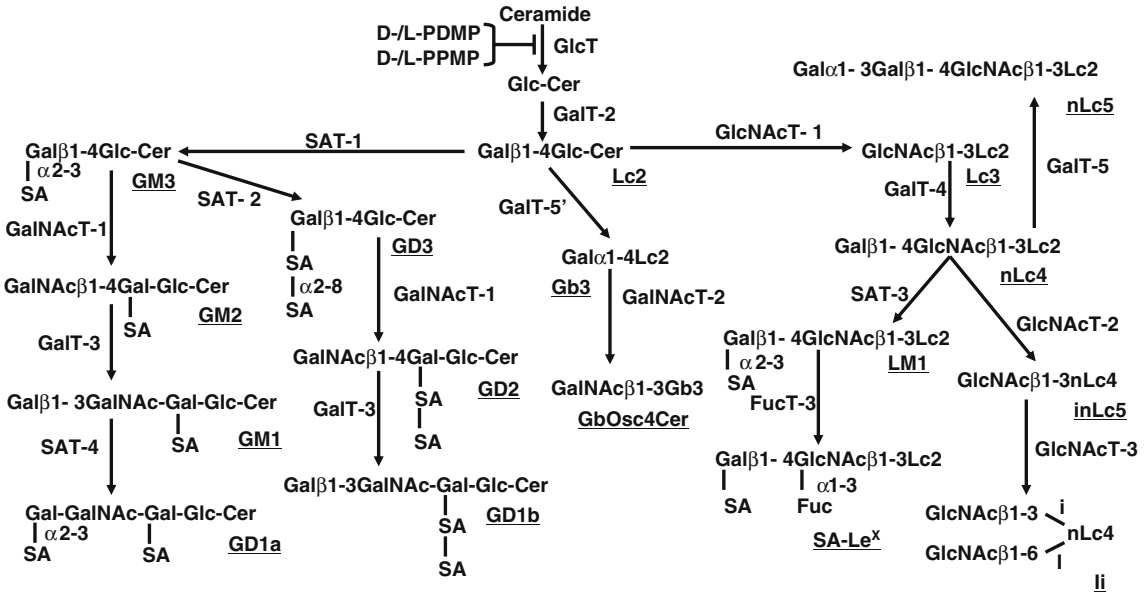


Fig. 1 Proposed pathways for biosynthesis of sialosyl glycosphingolipids in animal cells

or has been poorly understood until now. Our present goal is to correlate the changes in the expression of cancer cell surface glycosphingolipids (Glyco-biomarkers) and the signal transduction with apoptosis induced by anti-cancer drugs, inhibitors of glycosphingolipid biosynthesis such as L-PPMP.

Microarray-based assays are rapidly becoming one of the powerful technologies in high-throughput-gene-expression analyses. In order to focus on intrinsically complex regulation of synthesis and degradation of the specific glycoconjugates that are essential for breast cancer, a total of 359 genes comprising the human glycobiology microarray are complied from currently available NCBI/EMBL/TIGR human sequence database and the Consortium for Functional Glycomics-CAZY databases [27]. A unique sense 45-mer oligonucleotides corresponding to mRNAs of each gene are individually synthesized, purified, and immobilized via a 5′ amino linker onto aldehyde-coated microarrays. The labeled apoptotic cell aRNA from L-PPMP-treated cells were hybridized with the array to study the change of GSL-GLT expression.

Material and methods

Cells MCF-7, MDA-468, and SKBR-3 human breast adenocarcinoma cell lines were the kind gifts of Dr. S. Banerjee (Cleveland Clinic Foundation, Cleveland, OH). RPMI media 1640, fetal bovine serum, Trypsin-EDTA, Penicillin-Streptomycin, L-Glutamine were purchased from Invitrogen, CA.

Apoptotic agents D/L-*threo*-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (D/L-*threo*-PPMP) and D/L-*threo*-1-Phenyl-2-decanoylamino-3-morpholino-1-propanol (D/L-*threo*-PDMP) were gifts from Dr. J-i. Inokuchi of Hokkaido University, Japan; *Cis*-Diammineplatinum (II) dichloride (*cis*-Platin) were purchased from Sigma Chemical Co. (St. Louis, MO) (Fig. 2).

Antibodies Rabbit anti-human-phos-p38 MAb (3D7), rabbit anti-human-phos-ERK PAb, rabbit anti-human-phos-ERK MAb (20G11), rabbit anti-human-phos-SAPK/JNK MAb (98F2) were from Cell Signaling Technology, Inc. (Danvers, MA); Rabbit anti-human-actin, Goat anti-mouse-IgG-ALP conjugate, Goat anti-rabbit-IgG-ALP conjugate were from Sigma Chemical Co. (St. Louis, MO).

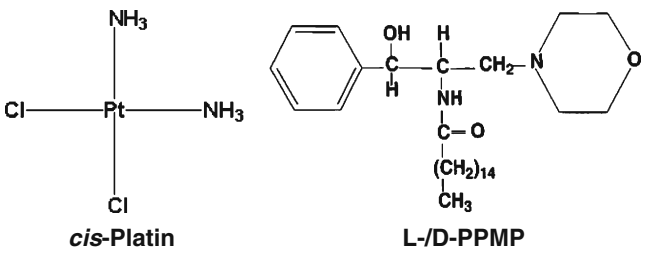


Fig. 2 Structures of anti-cancer apoptotic agents

Staining agents Trypan blue, Propidium iodide were from Sigma Chemical Co. (St. Louis, MO); PSS-380 and Squaraine-derived Rotaxane AKS-0, and MitoTracker Red 580 were kind gifts of Dr. Smith and Dr. Goodson (Department of Chemistry and Biochemistry, University of Notre Dame, IN).

Substrates for glycosyltransferases: donors UDP-[^{3}H]-Galactose, [^{3}H]-L-fucose were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). GDP-[^{3}H]-L-Fucose was prepared in our laboratory [21] and all other glycosphingolipids were the stock prepared and identified in our laboratory previously [28, 29].

DNA microarray agents RNeasy Mini Kit was purchased from Qiagen (Germany); Amino Allyl MessageAmp™ II aRNA Amplification Kit, Ambion (Austin, TX); Micro Bio-Spin Chromatography Columns were purchased from Bio-Rad Laboratories (Hercules, CA), Glycobiology microarray [30] containing all human 359 glyco-related genes cloned to date was the kind gift from Dr. Moskal (Department of Biomedical Engineering, Northwestern University, IL).

Cell culture All the cells were maintained in RPMI media 1640, supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 4 mM L-glutamine at 5% CO_2, 37°C. The cells were synchronized in 0.5% FBS before drug treatment.

AKS-0/PSS-380 staining Cells were cultured and synchronized on the Nunc Lab-Tek 8-well Chambered #1.0 Borosilicate Cover glass system at the starting concentration of 1×10^4 cells/200 μl per well. After synchronization and drug treatment, 10 μl of 1 mM AKS-0 stock in DMSO was added to the medium (~200 μl) and incubated at 37°C for 30 min. Then each well was washed with 200 μl TES buffer mixture and stained with 25 μM PSS-380 as described above. The cells were washed with 200 μl TES buffer mixture once and soaked in 100 μl of fresh TES buffer mixture for fluorescence observation as described previously [21, 30–32].

Western blot Cell homogenate was used to blot with anti-human-phos-p38 MAb, anti-human-phos-ERK PAb, and human-phos-SAPK/JNK MAb as previously described [20, 21].

Enzymatic assay of glycosyltransferases

Preparation of enzyme All the steps of enzyme preparation were carried out at 4°C. The cells were homogenized in 500 μl HEMPIS buffer (50 mM HEPES pH 7.4, 10 mM EDTA, 0.1% β-mercaptoethanol, 5 mM proteinase inhibitor AANB, 0.32 M sucrose), followed by sonication for 3×10 s [15–21].

GalT-4 The complete incubation mixture for GalT-4 assay [1, 2] contained the following components (in μmol unless otherwise stated): substrate LcOsc3Cer 0.02; Triton-X100 detergent 25–50 μg (1/4 weight of protein amount); HEPES pH 6.8; $MnCl_2$ 0.5; UDP-[^{3}H]-galactose 0.01 (5×10^6 cpm/μmol on Whatman 1mm paper); enzyme protein 100–200 μg.

GalT-5 The complete incubation mixture for GalT-5 [1, 3] assay contained the following components (in μmol unless otherwise stated): substrate nLcOsc4Cer 0.02; Triton-X100 detergent 25–50 μg (1/4 weight of protein amount); HEPES pH 6.8; MnCl2 0.5; UDP-[^{3}H]-galactose 0.01 (5×10^6 cpm/μmol on Whatman 1 mm paper); enzyme protein 100–200 μg.

FucT-3 The complete incubation mixture for FucT-3 assay [1, 4] contained the following components (in μmol unless otherwise stated): substrate LM1 0.01; sodium taurodeoxycholate (Na-TDC) detergent 200–400 μg (same weight as protein amount); Carcodylate–HCl 5 pH 6.5; $MnCl_2$ 0.25; GDP-[^{3}H]-fucose 0.01 (8.5×10^6 cpm/μmol on Whatman 1 mm paper); enzyme protein 200–400 μg.

DNA microarray Total RNA Isolation, cDNA synthesis, *in vitro* transcription of antisense RNA (aRNA), aRNA labeling and purification, and chip hybridization were followed with a modified protocols given below and were based on the previous methods [22, 33, 34]. Then the chips were scanned. The scanned files in BlueFuse format were analyzed with GeneTraffic (DUO) v3.2-11.

Total RNA isolation The total RNA from a cell sample was prepared [21, 22] with Qiagen RNeasy Mini Kit. After the drug treatment, the cells in a T25 culture flask (~5×10^6 cells) were collected with trypsin digestion and spun in a 15-ml conical tube. The cells were washed with PBS once, and tapping the tube loosened the pellet. The lysate was prepared with 350 μl buffer RLT and vortexed. The cell lysate was then passed through a blunt 21-gauge needle fitted to an RNase-free syringe. Then 350 μl ethanol was added and mixed well by pipetting.The sample was transferred to an RNeasy spin column placed in a 2 ml collection tube, and centrifuged for 15 s at 10,000xg. The flow-through was discarded, and the column was washed with 700 μl Buffer RW1 followed by centrifuge for 15 s at 10,000×*g*. The flow-through was discarded again and 500 μl buffer RPE was added followed by centrifuge for 15 s at 10,000×*g*. This step was repeated with another 500 μl buffer RPE followed by centrifuge for 2 min at 10,000×*g*. The flow-through was discarded, and the empty column was spun for two more minutes at 10,000×*g*. The RNeasy spin column was filled with 25 μl RNase-free water and placed in a new 1.5 ml collection tube. The column was centrifuged for 2 min at 10,000×*g* to elute the RNA. This step was repeated once with 25 μl more RNase-free water. The RNA sample was then stored at −70°C for further analysis.

RNA integrity examination From the frozen RNA, a 5 μl sample was added to 1 ml 10 mM Tris–HCl pH 7.0 to be measured at OD_{260} and OD_{280}. The ratio need to be greater than 1.8, and the amount of RNA in 5 μl sample is equal to 44 μg×OD_{260}. Then 1–2 μg of RNA was loaded to 1% agarose gel and run in TAE buffer to check the integrity [21, 22, 33, 34].

Preparation of Labeled aRNA The indirect aminoallyl RNA labeling was carried out with Ambion Amino Allyl MessageAmp™ II aRNA Amplification Kit based on published protocol [33, 34]. Details are given in the following steps.

First and second strand cDNA synthesis and purification of cDNA From total RNA in the amount of 5 μg purified cDNA was prepared by the methods published before [21, 22, 33, 34].

In vitro transcription of antisense RNA (aRNA) In a nuclease-free microcentrifuge tube, the following mixture of IVT Master Mix was added to 14 μl double-stranded cDNA: 3 μl a UTP Solution (50 mM), 12 μl ATP, CTP, GTP Mix (25 mM), 3 μl UTP solution (50 mM), 4 μl T7 10× reaction buffer, 4 μl T7 enzyme mix. The mixture was pipetted up and down a few times followed by a quick centrifuge. The incubation was carried out at 37°C overnight (6 to 14 h) in a hybridization oven. The reaction was stopped by adding nuclease-free water to each aRNA sample.

aRNA Purification All centrifugations in this purification procedure were performed at 10,000xg (~8,000 rpm) at room temperature. To each aRNA sample, 350 μl of aRNA Binding Buffer was added and mixed thoroughly by gentle vortexing. Then 250 μl of ACS grade 100% ethanol was added and mixed by pipetting. This sample mixture was transferred immediately to the center of an aRNA Filter

Cartridge. The filter cartridge was centrifuged for 1 min and the flow-through was discarded. Then the filter was washed with 650 μl aRNA Wash Buffer and spun for 1 min. The flow-through was discarded and the filter cartridge was centrifuged for an additional minute. The filter cartridge was then transferred to a fresh aRNA collection Tube. The 100 μl preheated 50–55°C Nuclease-free water was added to the center of the filter and left at room temperature for 2 min. The eluate was collected by centrifuge for 1.5 min. The concentration of purified aRNA was determined by using NanoDrop ND-1000 spectrophotometer [22, 33, 34].

Dye coupling of aRNA Each tube from Cy3 or Cy5 mono-Reactive Dye Pack (Amersham) was resuspended with 95 μl DMSO. To precipitate 5 mg human Cot-1 DNA (10 mg/ml), 925 μl 100% ethanol and 75 μl 3 M sodium acetate (pH 5.2) were added, and the mixture was centrifuged at 14,000×*g* for 30 min. The supernatant was removed and the pellet was air dried for 5 min. The pellet was dissolved in 50 μl Nuclease-free Water. The 8 mg/ml Poly d(A)40-60 was prepared by adding 32 μl Nuclease-free water to the pellet. To label the aRNA, 20 μg aRNA aliquot was dried in speedvac for 1 h. The aRNA pellet was resuspended in 5 μl Coupling Buffer from the Ambion kit. And 5 μl Cy-ester dye was added to sample (Cy5) or reference human RNA (Cy3), and mixed well. The reaction was incubated at room temperature in a dark room for 1 h. Then 10 μl nuclease-free water was added to stop the reaction.

Dye-labeled aRNA purification The labeled aRNA was purified with Bio-Rad Micro Bio-Spin Chromatography Columns. The new columns were inverted sharply several times to resuspend the settled gel and remove any bubbles. Then the tip of the column was snapped off. The column was placed in a 2.0 ml microcentrifuge tube and the top cap was removed to allow the excess packing buffer to drain by gravity to the top of the gel bed (about 2 min). The drained buffer was then discarded. The column was centrifuged for 2 min at 1,000×*g*, and the buffer was discarded again. Then the column was placed in a clean 1.5 ml microcentrifuge tube, and the labeled aRNA sample was carefully applied to the center of the gel matrix. The column was spun at 1,200×*g* for 2 min to elute a clean labeled sample. The concentration of purified, labeled aRNA was determined by using NanoDrop ND-1000 spectrophotometer.

Chip hybridization The volume of the hybridization solution was determined as an array covered by 24×50 mm m-Series Lifterslip 50 μl. Then 10 μl of 3× SSC buffer was added to each hybridization chamber to keep moisture [22]. The m-Series Lifterslips were washed with 0.1% SDS and rinsed with diH_2O followed by spin drying in 50 ml tubes. For 50 μl of hybridization, 2 μg of each Cy3 and Cy5 labeled aRNAs were pooled into a single PCR tube and adjusted the volume to 40 μl with nuclease-free water. Then 4 μl of Amersham Liquid Block, 1 μl of Poly d(A)(8 μg/μl), 1 μl Human Cot-1 DNA (10 μg/μl), and 6 μl of 20× SSC were added to the PCR tube. The hybridization mixture was heated to 98°C for 2.5 min and cooled to room temperature. Then 1.25 μl 10% SDS was added to each reaction and mixed well. The m-Series Lifterslips was placed over each array. Then 80% of the hybridization mixture was carefully transferred to the right side. The flow crossed the array by capillary action. The final 20% of the mix was pipetted to the left side of the array. The lid of the hybridization chamber was screwed on and the chamber was placed in the 46°C hybridization oven overnight. To wash the array, four solutions in 500 ml glass jars were made as follows: Wash 1: 500 ml 0.5× SSC + 500 μl 10% SDS + 5 μl 1 M DTT; Wash 2: 500 ml Buffer 0.2× SSC + 5 μl 1 M DTT; Wash 3: 500 ml 0.1× SSC + 5 μl 1 M DTT; Wash 4: 250 ml 0.1× SSC + 250 ml dH_2O + 5 μl 1 M DTT. The chip with coverslip affixed was placed into Wash 1. The coverslip was slided off once submerged and the chip was washed for 4 min at room temperature covered with tinfoil. The slide rack was transferred to Wash 2 and gently ducked for few seconds followed by washing 4 min at room temperature covered with tinfoil. The same step was repeated with Wash 3. Then the slide rack was transferred to Wash 4 and washed 4 min at room temperature covered with tinfoil. Then each slide was transferred to a 50 ml conical tube with a scrunched up Kimwipe in the bottom. The tubes were spun immediately in swinging bucker centrifuge at 1,200 rpm for 2.5 min. Then the chips were scanned. The scanned files in BlueFuse format were analyzed with GeneTraffic (DUO) v3.2-11 [22].

Results

Cell growth inhibition and visible damage

MCF-7, MDA-468, and SKBR-3 cells were synchronized with 0.5% FBS-RPMI 1640 medium for 24 h twice followed by the treatment with *cis*-platin (Fig. 3) or L-PPMP (Fig. 4) separately**.** After 24 h of treatment, the viability of cells was examined with Trypan blue. Dead blue-stained cells were counted along with the total cells. Then the percentage of live cells in total cells was plotted at increasing concentrations of *cis*-platin (Fig. 3) between 0 to 75 μM with MCF-7, 0 to 160 μM with MDA-468, and 0 to 160 μM with SKBR-3; or L-PPMP (Fig. 4) between 0 to 50 μM with MCF-7, 0 to 16 μM with MDA-468, and 0 to 32 μM with SKBR-3. With these curves, the 24-h ED_{50} values of cell growth inhibition by *cis*-platin [35-37] for

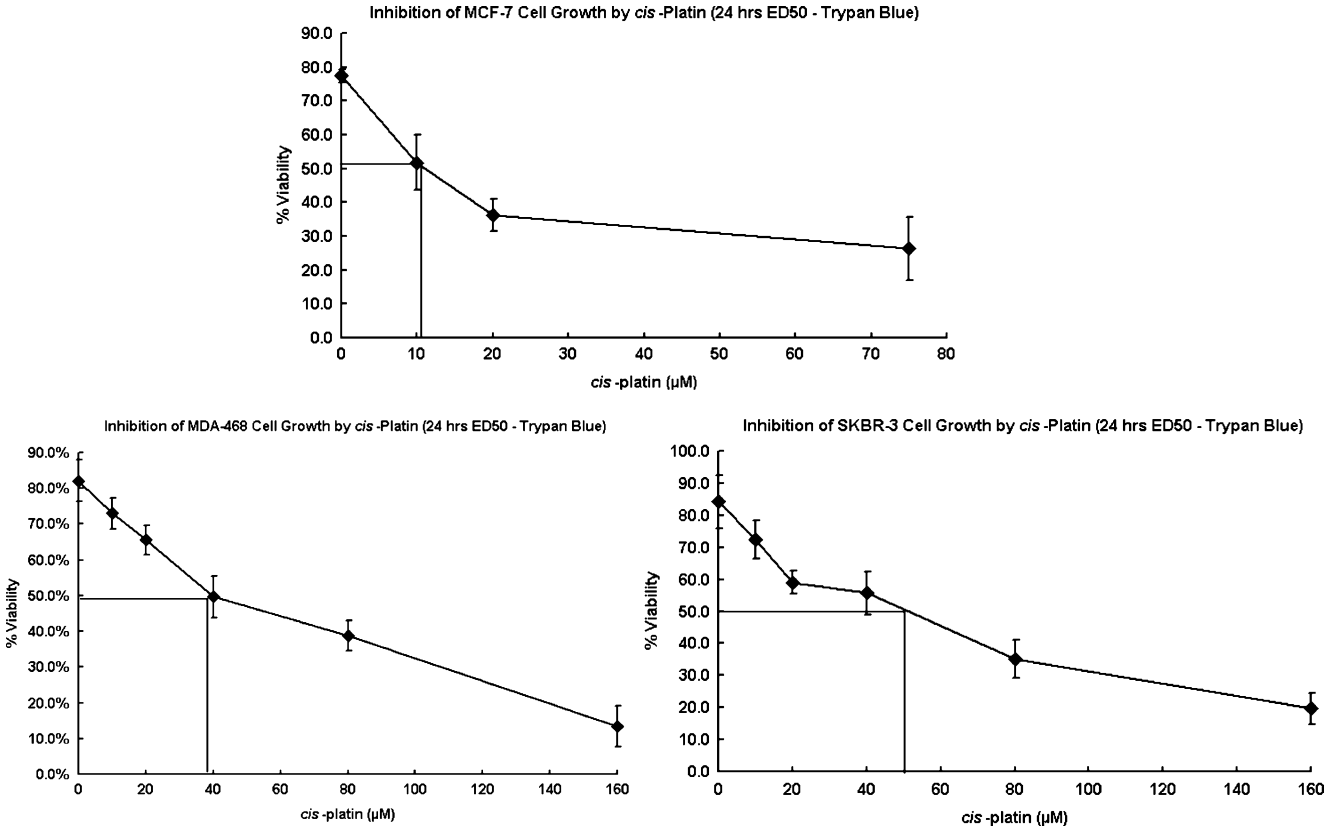


Fig. 3 Identification half effective dose (ED50) of cis-Platin on breast cancer cells(standard deviations were obtained from at least three experiments performed under same experimental conditions)

MCF-7, MDA-468, and SKBR-3 were determined at about 10 μM, 40 μM, and 50 μM. The 24-h ED_{50} values of cell-growth inhibition by L-PPMP for MCF-7, MDA-468, and SKBR-3 were at 4 μM, 7 μM, and 6 μM, respectively. The L-PPMP was found to trigger cell death with lower concentrations compared with *cis*-platin under the same growth condition.

Two novel synthetic organic fluorescent dyes [38, 39] from Dr. Smith's group at Notre Dame and a DNA-binding dye, propidium iodide (Fig. 5), were used to monitor the morphological changes of SKBR-3 cells treated with L-PPMP. Similar experiment results on other cells with L-PPMP or *cis*-platin were reported previously in our other publications [15–21]. PSS-380 is one of such fluorescent dyes with Zn^{2+}-2,2′-dipicolylamine complex which can efficiently label apoptotic cells and has been used in our group for the last few years [15–21]. In this study we used AKS-0, a permanently interlocked Leigh-type rotaxane, and an endoplasmic reticulum membrane-binding dye [31, 32]. AKS-0 was found to bind many cell organelles with membrane compartment structure, such as mitochondria, endoplasmic reticulum (ER), and Golgi apparatus (Fig. 5). SKBR-3 cells were treated with 10 μM L-PPMP for 24 h. Then the cells were stained with PSS-380 and AKS-0 together (Fig. 6). As it shown, PSS-380 detected the apoptotic phosphatidylserine externalization, and AKS-0 stained the organelle membrane system. In the control cells, the details of intracellular membrane such as the shape of mitochondria as little particles can be visualized. However, in the L-PPMP-treated cell, the mitochondria and ER membrane fused, gathered together on one side of the plasma membranes. This reveals the changes of cell membrane system during apoptosis and indicates that during apoptosis, the nucleus expanded, forcing the cytosol plasma to shrink. Thus, AKS-0 is shown to be a very useful tool to observe changes in intracellular membranes during apoptosis.

MAPKs signaling

The process of apoptosis is usually triggered by various signals which could be originated extracellular through caspase-8 or intracellular through caspase-9 [40, 41]. The mitogen activated protein kinases (MAPKs) and NF-κB pathways can also play important roles in apoptosis [42] (Fig. 7). To identify the involvement of MAPKs in the L-PPMP- and *cis*-platin-induced human breast cancer cell apoptosis, the three cell lines, MCF-7, MDA-468, and SKBR-3, were treated with 10 μM L-PPMP or 100 μM *cis*-platin individually for 2 h. The samples of each cell

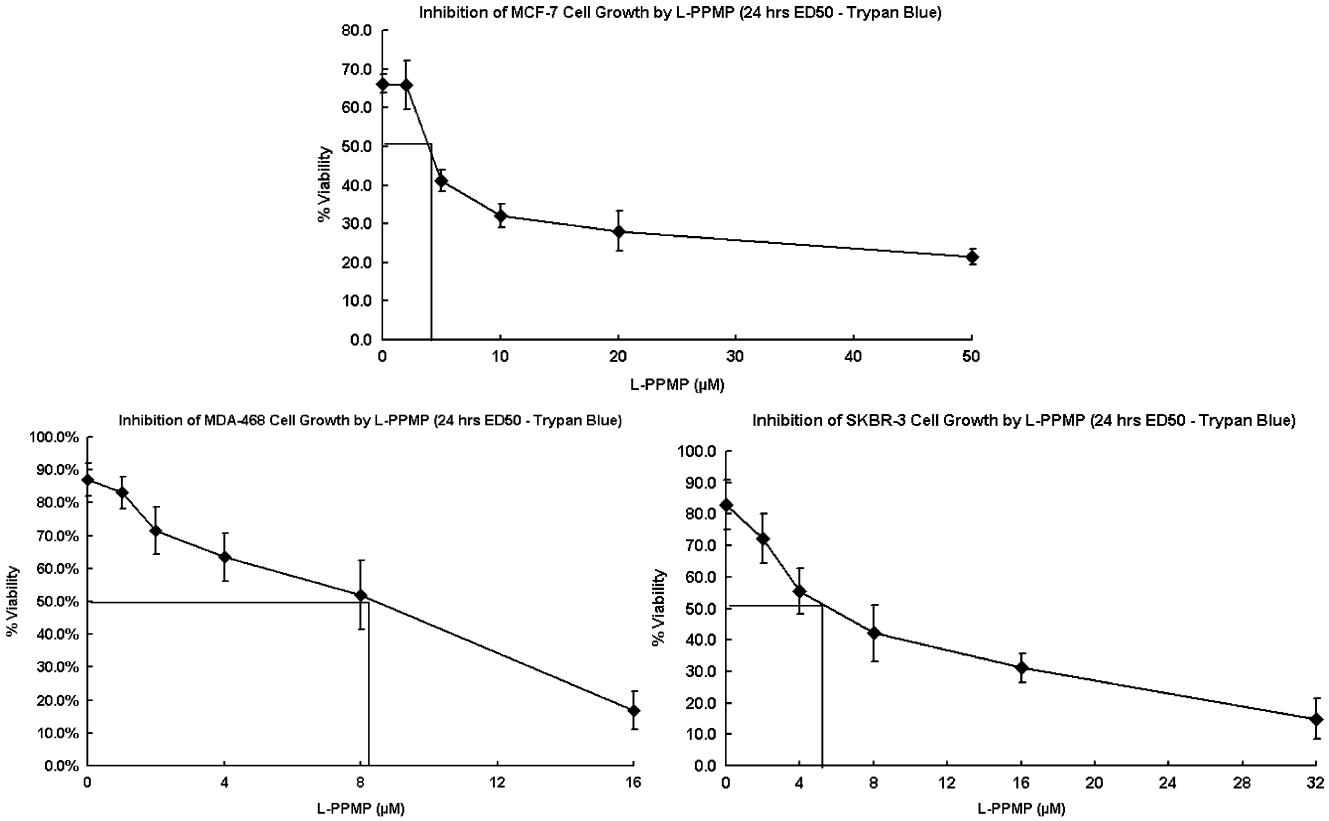
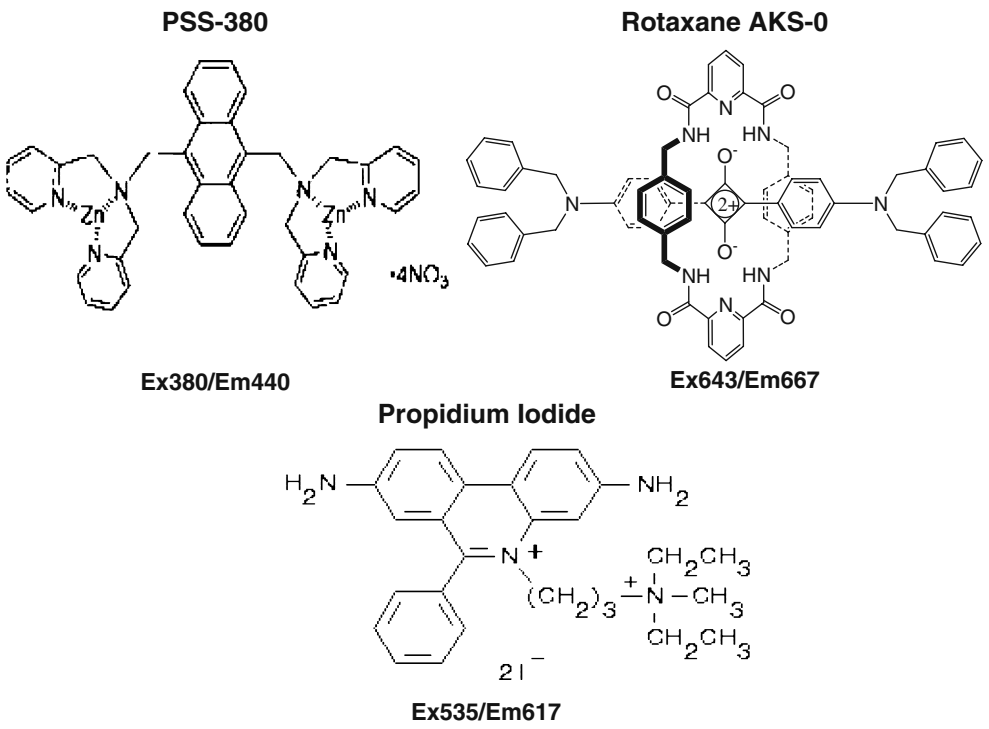


Fig. 4 Identification half effective dose (ED50) of L-PPMP on breast cancer cells (standard deviations were obtained from at least three experiments performed under same experimental conditions)

Fig. 5 Structures of fluorescent dyes



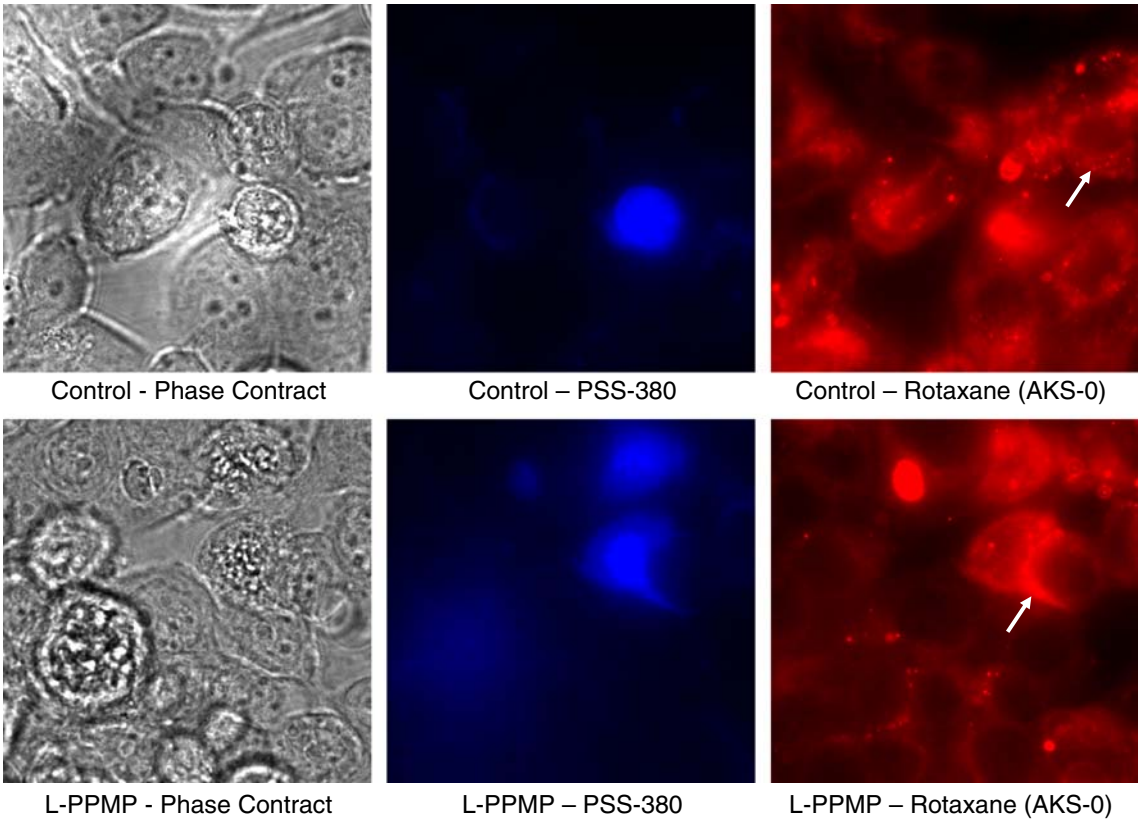


Fig. 6 SKBR-3 cells treated with 10 μM L-PPMP and incubated for 24 h in the presence of PSS-380 and AKS-0

homogenate were characterized with western blot using anti-human-phospho-MAPK-specific MAbs from Cell Signaling Technology, Inc. (Danvers, MA): anti-phos-ERK (T202/Y204) (Clone# 20G11), anti-phos-JNK (T183/Y185)(Clone# 98F2), and anti-phos-p38 (T180/Y182) (Clone# 3D7) as shown in Fig. 8. The anti-human-actin antibody was used for each western blot as an internal control of total protein loading. A sample of anti-actin blotting was shown in the lowest panel of Fig. 8. As the blot shows, the activation pattern of MAPKs was complicated depending on cell type and drugs. L-PPMP with 10 μM/2-h treatment was observed to enhance ERK phosphorylation, especially the p44 protein, in both MDA-468 and MCF-7 cells. However, under the same treatment condition, L-PPMP inhibited the phosphorylation of ERK in SKBR-3 cells. This inhibition turned out to be greater in the p42 protein than the p44 protein. In contrast, the *cis*-platin (100 μM/2-h) was shown not to influence the status of ERK phosphorylation in any of the three cell lines (or slight inhibition in MDA-468 cells). No notable changes of ERK phosphorylation by *cis*-platin were identified compared with each control.

The changes of JNK phosphorylation by L-PPMP varied also in different cell lines. L-PPMP induced the JNK, especially the p54, phosphorylation in MDA-468 cells. However, L-PPMP suppressed both of JNK p54 and p46 phosphorylation in MCF-7 and SKBR-3 cells. *cis*-platin was shown to slightly decrease the activation of JNK in MDA-468 and MCF-7 cells (or showed no change), but to enhance it in SKBR-3 cells. The phosphorylation status of p38 by L-PPMP and *cis*-platin was shown not to be the same either. The p38 activation by L-PPMP was observed to increase in MDA-468 cells but decrease in MCF-7 and SKBR-3 cells. Similar to ERK, *cis*-platin did not show much ability to disturb the p38 activation in all of the three cell lines.

Regulation of GSL GLT enzymatic activities by L-PPMP

The microdomains, also known as rafts, are the certain lipid aggregations on the cell surfaces containing specific glycosphingolipids (GSLs). Now Individual steps for its biosynthesis from ceramide [6–8] and the enzymes catalyzing the reactions have been characterized in our laboratory and other laboratories as shown in Fig. 1. Activities of different GSL glycosyltransferases were characterized in human breast cancer MCF-7, MDA-468, and SKBR-3 cells as listed in Table 1. The cells were harvested when reaching confluence and homogenized in HEMPIS (pH=7.4) buffer. The unit of enzyme level was defined as "cpm per unit time", which represents incorporation of 10^{-9} μmol nucleotide sugar per microgram protein

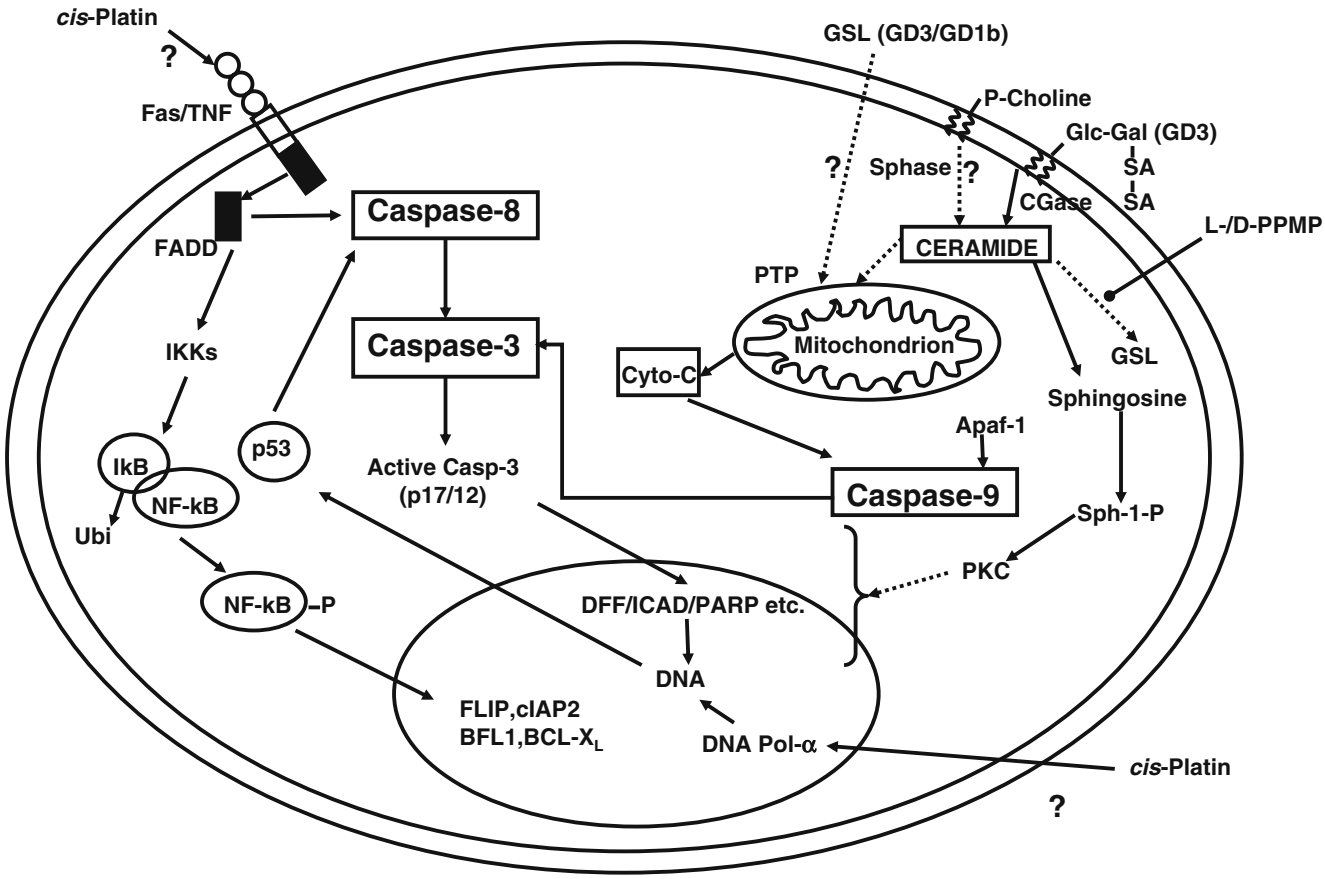


Fig. 7 Schematic diagram of extrinsic and intrinsic pathways in apoptosis

of cell lysate. No activity or "–" indicates the enzyme level is less than 200 cpm; Low activity or "+" represents the enzyme level is between 200 cpm and 1,000 cpm; medium activity or "++" indicates the enzyme level is between 1,000 and 5,000 cpm; high activity or "+++" indicates the enzyme level is above 5,000 cpm; "N.T." indicates the cells were not tested for this enzyme. In SKBR-3 Cells, GalT-2, GalT-3, SAT-1, and SAT-2 activities were not detected. All activities were compared as per milligram protein. The GalT-4 level was high; GalT-5 and SAT-4′ levels were

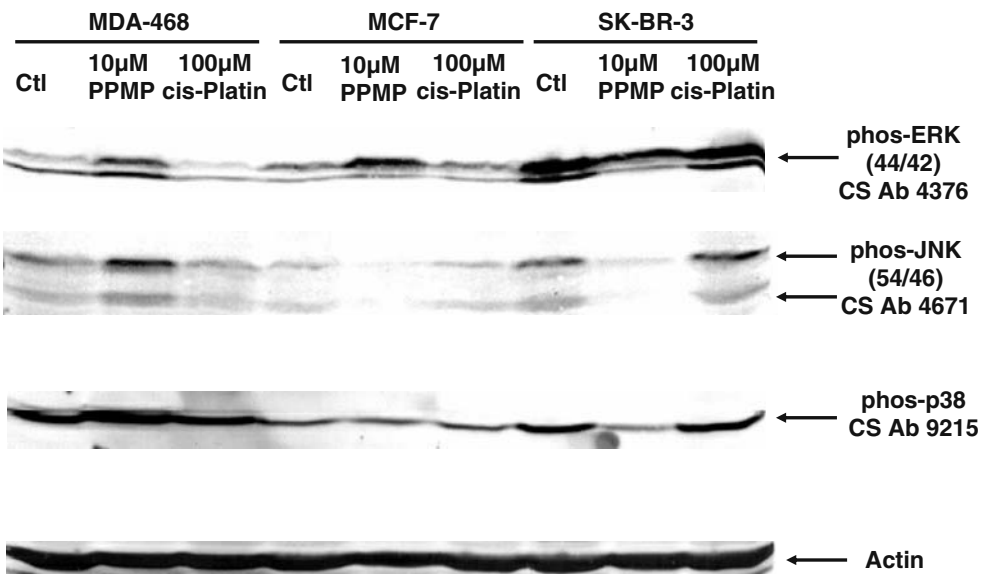


Fig. 8 Identification of changes of MAPKs in apoptotic cells by Western blot

Table 1 Activities of GSL: glycosyltransferases in non-apoptotic human breast carcinoma cells (conclusions were obtained from at least three experiments performed at different times under same experimental conditions)

Enzyme(Substrate)	SK-BR-3	MDA-468	MCF-7
GlcT(Ceramide)	N.T.	N.T.	–
GalT-2(Glc-Cer)	–	N.T.	N.T.
GalT-3(GM2)	–	N.T.	N.T.
GalT-4(Lc3)	+++	+++	+
GalT-5(nLc4)	++	+	+
SAT-1 (Lc2)	–	–	–
SAT-2 (GM3)	–	–	+
SAT-3 (nLc4)	+	–	–
SAT-4 (GM1)	+	+	+
SAT-4′ (Gg4)	++	++	++

(–) <200 cpm/"+" 200–1,000 cpm/"++" 1,000–5,000 cpm/"+++" >5,000 cpm/"N.T." not tested
1 cpm count=10^{-9} μmol nucleotide sugar incorporated per μg protein of cell lysate in 4 h

medium; and SAT-3 and SAT-4 levels were low in SKBR-3. In MDA-468 cells, SAT-1, SAT-2, and SAT-3 activities were not detected. The GalT-4 level is high; SAT-4′ level is medium; and GalT-5 and SAT-4 level is low in MDA-468 cells. In MCF-7 cells, GlcT, SAT-1, and SAT-3 activities were not detected. The SAT-4′ level is medium, and GalT-4, GalT-5, SAT-2, and SAT-4 levels were low in MCF-7 cells. The fucosyltransferase activities in SKBR-3 cells were also determined with LM1 and nLc4 as substrates. The FucT-3 activity with LM1 was found to be 147 pmol [^{3}H]-fucose incorporated/mg protein/2 h from GDP-[^{3}H]-fucose, and activity of nLc4 was to be found only 34 pmol [^{3}H]-fucose incorporated/mg protein/2h.GalT-4 in all three breast cancer cell lines. This information was used in the following experiments to characterize the regulation of GSL glycosyltransferase by anti-cancer agents.

To study the changes of GalT-4 and GalT-5 in MCF-7, MDA-468, and SKBR-3, the three cell lines were treated with 2 μM L-PPMP for 2 and 6 h, and the activities of GalT-4 and GalT-5 were tested with the protocol stated before [1–3]. The results were summarized in Figs. 9 and 10. GalT-4 activity was found to be inhibited by L-PPMP in both time points of 2 and 6 h incubation (Fig. 9). In MCF-7 cell, the inhibition in the 2-h sample was higher than in the 6-h sample. In two other cell lines, the level of GalT-4 inhibition by L-PPMP was increased as treatment time increased from 2 h to 6 h. All the inhibition levels were between 10% and 40%. As shown in Fig. 10, GalT-5 activity was found to be stimulated by L-PPMP in MCF-7 and MDA-468 cells at 2 h of treatment. However, when the treatment time extended to 6 h, the GalT-5 was inhibited by L-PPMP. In SKBR-3 cells, L-PPMP inhibited GalT-5 activity in both 2-h and 6-h treatments with about 60% decrease. The overall regulation of Glycosphingolipid:GLTs enzymatic activities during apoptosis triggered by *cis*-platin or L-PPMP is listed in Table 2. A major trend of Glycosphingolipid:GLTs inhibition was observed with few exceptions.

To study the changes of fucose-containing glycolipid biosynthesis by L-PPMP, MCF-7, MDA-468 and SKBR-3, cells were treated with 1 to 10 μM L-PPMP for 48 h. The cell samples were analyzed with GF/A filtering assays (Fig. 11) as the methods previously described [17]. The data points in diamonds represent the average counts without chloroform/methanol wash (radioactive fucose in both glycoprotein and glycolipid). The data points in squares represent the count difference in the sample with or without chloroform/methanol wash. This is the radio-

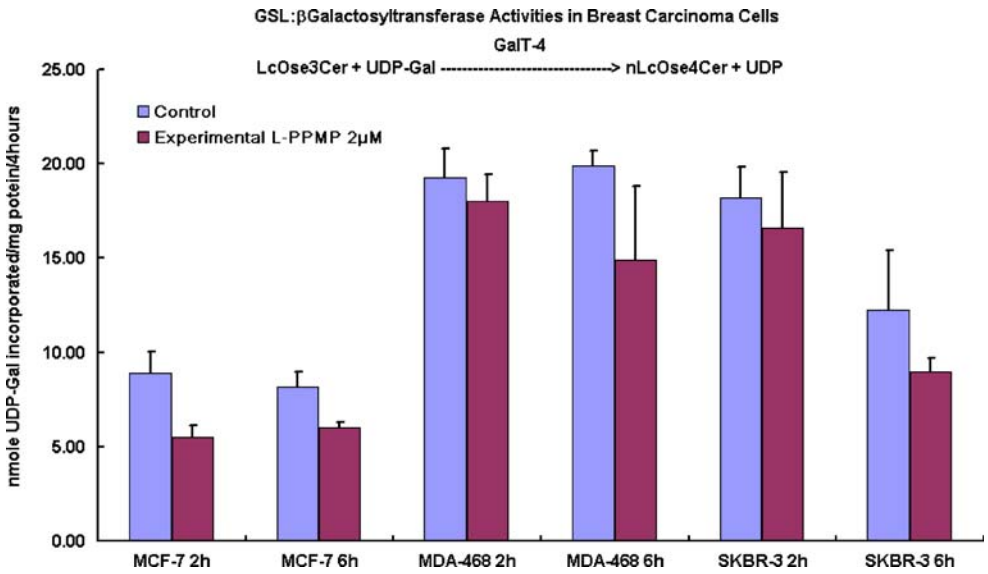


Fig. 9 Post-translational activity of GalT-4 in apoptotic breast carcinoma cells (standard deviations were obtained from at least three experiments performed under same experimental conditions)

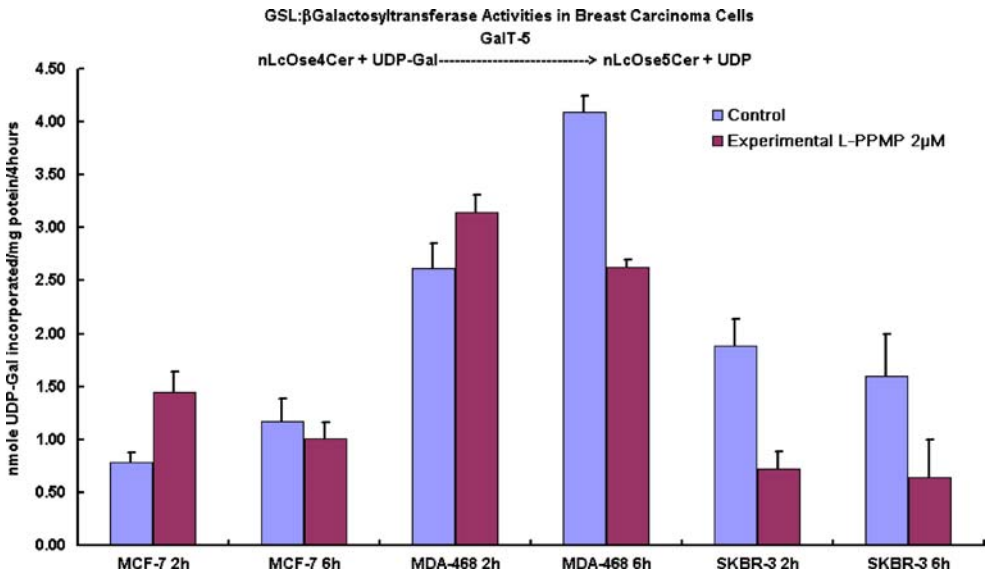


Fig. 10 Post-translational activity of GalT-5 in apoptotic breast carcinoma cells

activity of fucose in glycolipids only. In each cell line, L-PPMP inhibited glycoprotein and glycolipid biosynthesis. For the glycolipid biosynthesis, in MCF-7 cells L-PPMP did not show the ability of inhibition except at 1 μM. However, in MDA-468 cells L-PPMP could increase the formation of fucose-containing glycolipid at 2 μM, but it inhibited glycolipid at level 10 μM. In SKBR-3 cells, L-PPMP was shown to inhibit both the fucose-containing glycoprotein and glycolipid biosynthesis.

The FucT-3 activities in SKBR-3 by L-PPMP treatment for 48 h were tested (Fig. 12). The L-PPMP was found to obviously decrease the FucT-3 activity from 1 to 10 μM treatment conditions in a dose-dependent manner. By giving 10 μM L-PPMP at the highest level, the FucT-3 activity was inhibited at about 30% compared with the control cells. This suggests the decrease levels of SA-Le^X biosynthesis during apoptosis were induced by L-PPMP.

Table 2 Overall conclusion of post-translational activities of several glycosphingolipid: GLTs (cell, agents, and time mentioned in the table)

GSLGLT catalyzed	Reaction enzymatic	Activity
GalT-4	Lc3 (GlcNAc-Gal-Glc-Cer)→ Galβ-Lc3	Decrease (MCF-7/L-PPMP-2 h, 6 h; SKBR-3/*cis*P, L-PPMP and MDA-468/L-PPMP, 48 h)
GalT-5	Lc4) (Gal-GlcNAc-Gal-Glc-Cer)→Galα-Lc4	Decrease (SKBR-3/L-PPMP-2 h, 6 h; MCF-7,MDA-468/L-PPMP-6 h; SKBR-3/*cis*P and MDA-468/L-PPMP-48 h)
SAT-2	GM3→GD3	Decrease (MCF-7/*cis*P,L-PPMP-48 h)
SAT-4	GM1→GD1a	Decrease (MCF-7/L-PPMP, SKBR-3/*cis*P and MDA-468/LPPMP-48 h)
SAT-4′	Gg4 (Gal-GalNAc-Gal-Glc-Cer)→GM1b	Decrease (SKBR-3/*cis*P and MDA-468/L-PPMP-48 h)
FucT-3	LM1→SA-LeX	Decrease (SKBR-3/L-PPMP-48 h)

Regulation of GSL GLT mRNA expression by L-PPMP

Table 3 summarizes the change of genes that are involved in GSL biosynthesis with 2-h L-PPMP treatment. In MCF-7 cells, as mentioned above, B3GALT5, which makes the Galβ1-3GlcNAc bond, was increased at the mRNA level as well as in the 24-h treatment. This could reflect the feedback mechanism when the cells detect the decreased enzyme level led by L-PPMP. In addition, the two variants of GlcNAcT-2 (B3GNT1 and B3GNT3) were found to change significantly. Interestingly, the B3GNT1 was decreased and B3GNT3 was increased by L-PPMP. This suggests different regulatory elements among the DNA of these two genes upon the signal from L-PPMP. In the MDA-468 cells treated with L-PPMP for 2 h, the variant of GlcT gene (UGCGL2) was up-regulated. Similar to MCF-7 cells, the two variants of GlcNAcT-2 (B3GNT1 and B3GNT4) are found to change too. The B3GNT1 was decreased and B3GNT4 was increased. This conforms to the different regulations of GlcNAcT-2 by L-PPMP in the SKBR-3 cells treated with L-PPMP for 2 h, only one variant of GlcNAcT-2 (B3GNT4) was elevated by L-PPMP. The ST6GAL1 gene is in the N-Glycan biosynthesis making NeuAcα2-6Gal linkage inhibited by L-PPMP.

Discussion

Glycolipids, glycoproteins, and proteoglycans are ubiquitous on eukaryotic cell surfaces. Isolation and structural characterization of some of these compounds from normal

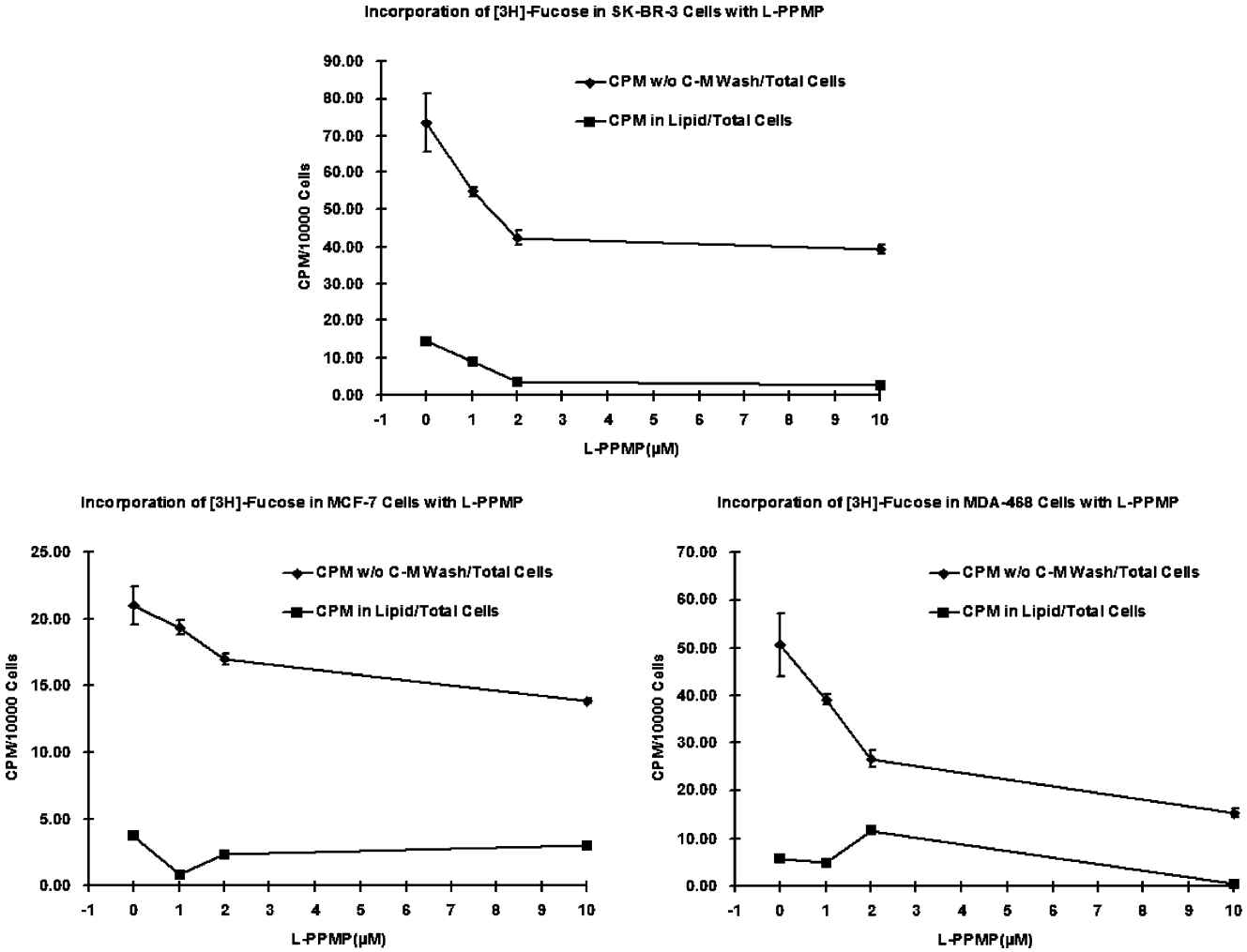


Fig. 11 Incorporation of L-[^{3}H]-Fucose in the total GSLs in breast cancer cells treated with L-PPMP (standard deviations were obtained from at least three experiments performed under same experimental conditions)

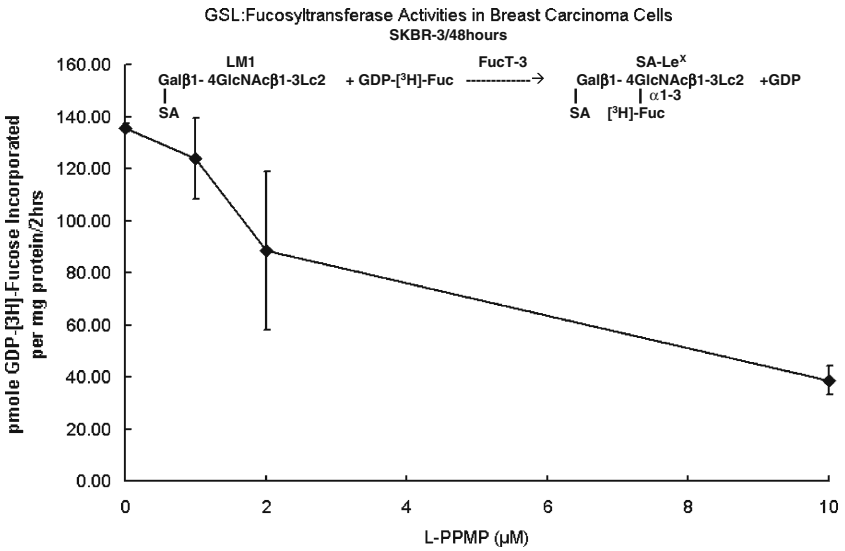


Fig. 12 Post-translational activity of FucT-3 in apoptotic breast carcinoma cells (standard deviations were obtained from at least three experiments performed under same experimental conditions)

Table 3 Changes of GLT gene expressions in apoptotic breast cancer cells studied by DNA-microarray after treatment with 2 μM L-PPMP for 2 h (p-value≤0.05)

Cell line	GLT gene name	Linkage formed	Fold change*
MCF-7	B3GALT5	Galβ1-3GlcNAc-R1	1.19–1.33
	B3GNT3	GlcNAcβ1-3 Gal-R2	1.20
	B3GNT1	GlcNAcβ1-3 Gal-R2	−1.19
MDA-468	UGCGL2	Glcβ1-1 Cer	1.28
	B3GNT1	GlcNAcβ1-3 Gal-R2	−1.34
SKBR-3	ST6GAL1	NeuAcα2-6Galβ1-3GlcNAc-R3	−1.55

Core Blood Type: Galβ1-3/4GlcNAcβ1-3Galβ1-4-R1/R2
R1=Glcβ1-1 Cer/R2=OligoN-Protein
*$p \leq 0.05$

and cancer cells clearly show changes during embryogenesis, differentiation, and onset of oncogenic processes. Isolation and characterization of individual glycosyltransferases which catalyze the each step for the biosyntheses of different glycolipids (Fig. 1) have been established in the last four decades [6-8]. In the last two and a half decades the putative structures of almost 300 gene sequences of glycosyltransferases have been established. Each Glycoconjugate is characterized by its unique structure synthesized by the activities of a set of specific glycosyltransferases. It is also established that a specific linkage between two sugars is established by the catalysis of a specific gene product (or a glycosyltransferase) as proposed by Professor Saul Roseman [9]. Sometimes with little variation of a gene sequence for different linkage formation is also catalyzed by the original sequence of the protein as observed recently [43]. However, many questions remain unanswered: (1) what are the active sites of these enzymes in their protein sequences? (2) How are these enzymes transcriptionally regulated? (3) How are these glycosyltransferases post-translationally modified and regulated? (4) How are these glycosyltransferases regulated in apoptotic, or metastatic cells?

To understand the regulation or the signaling mechanism for any agent, the interactions of any network should be defined within the cellular context [44]. This includes the activation of protein kinases, and the subcellular distribution of these transducers to bring them into contact with appropriate targets in the late phase of apoptosis. After the reorganization and modification of the Golgi bodies, which contain several of these glycosyltransferases. In the present study we checked activities of several glycolipid: glcosyltransferases and they were routinely inactivated (Table 2) after 24 h treatment with the apoptotic reagents. However, in the early stages (between 2 to 6 h of induction) several genes of glycosyltransferases (in the glycolipids or glycoprotein biosynthetic pathways) are transcriptionally regulated (Table 3).

In the presence of L-PPMP (the inhibitor of GlcT-1 (UDP-Glc: ceramide1,1 glucosyltransferase; Fig. 1; Table 3) is transcriptionally induced. It is suggested from our present studies that induction of apoptosis is a time dependent process in the breast carcinoma cells (+/− p53 gene). In addition to L-PPMP some of these other apoptotic agents such as *cis*-platin (Fig. 2) is under study in our laboratory. If these potential agents are considered as future drugs, then our present investigation would suggest that these drugs be applied in sub-toxic level because the killing mechanism is through apoptotic induction. However, our present studies also indicate that several kinases are activated during apoptotic processes initiated by D-PDMP (data will be presented elsewhere), the inhibitors of the glycolipid biosynthetic pathways. These inhibitors are increasing intrinsic concentrations of ceramide [15, 16] but how they are affecting the MAPkinase signaling pathway is not known at present.. The p38 MAPKs are key regulators of inflammatory cytokine expression and are involved in the disease processes. The ERK, JNK, and p38 pathways are all molecular targets for drug development. Activators or inhibitors of MAPKs, the regulators of gene expression, will be a family of drugs for treatment of human cancers by initiating apoptotic pathways [45, 46]. Our present studies with three breast carcinoma cell lines under apoptosis (induced by *cis*-platin or L-PPMP) suggest that *cis*-platin did not stimulate p38 phosphorylation. *cis*-platin is proved to be inhibiting DNA polymerase-alpha [47] and the Helicase III [18, 48] in these breast carcinoma cells. It is also established that *cis*-platin binds to the Zn-binding domain in the active site of DNA polymerase-alpha [49, 50]. The exact relation of the inactivation of the replication complex and initiation of apoptotic process is not known [17, 48]. However, activation of p38 in all three-cell lines was also different when induced with L-PPMP. This suggests the regulation of glycosyltransferase genes during apoptosis could occur by two different pathways in the early stages of apoptosis (between 2 to 6 h). Inactivation of several glycosyltransferase activities during a 24-h treatment may cause damage to the Golgi-bodies (as evidenced by AKS-0 fluorescence studies) which could be due to activation of several unknown proteases in addition to the activation of several Caspases as studied earlier [15–21]. Present study shows changes in the expressions of a few glycosyltransferase genes expressions during apoptosis in the breast cancer cells. Damages of Golgi-bodies in late stages of apoptosis could cause the inactivation of several glycolipid glycosyltransferase activities post-translationally.

Acknowledgments This article was written based on research supported by grants NS-18005 (NIMDS), CA-14764 (NCI) and grants-in-aid from the Bayer Corporation Elkhart, Indiana, and Coleman Foundation, Chicago, IL. to S. Basu. We are grateful to Dr. Surendra Gupta of American Radiolabeled Corporation, St. Louis

and Sudip Basu of Moraveck Chemicals Corporation, Brea, CA, for providing us radioactive precursor chemicals need for this project. We are thankful to Professor Bradley Smith of the University of Notre Dame for his newly synthesized PSS-380 and AKS-0 dyes used for phosphatidylserine-binding and mitochondrial and Golgi-membrane scrambling studies. Our special thanks to Professor Jen-ichi Inokuchi of Hokkaido. University, Hokkaido, Japan, for providing us gift samples of chemically synthesized L-/D-PPMP and L-/D-PDMP used as apoptotic agents. We gratefully acknowledge the editorial help of Ms. Dorisanne Nielsen, and Christopher Thomas during preparation of this manuscript.

References

1. Basu, M., De, T., Das, K.K., Kyle, J.W., Chon, H.C., Schaeper, R.J., Basu, S.: Glycosyltransferases involved in glycolipid biosynthesis. In: Ginsburg, V. (ed.) Methods in Enzymology, pp. 575–607. Academic, New York (1987)
2. Basu, M., Basu, S.: Enzymatic synthesis of a tetraglycosylceramide by a galactosyltransferase from rabbit bone marrow. J. Biol. Chem. **247**(5), 1489–1495 (1972)
3. Basu, M., Basu, S.: Enzymatic synthesis of a blood group B-related pentaglycosylceramide by an alpha-galactosyltransferase from rabbit bone marrow. J. Biol. Chem. **248**(5), 1700–1706 (1973)
4. Basu, M., Hawes, J.W., Li, Z., Ghosh, S., Khan, F.A., Zhang, B.J., Basu, S.: Biosynthesis in vitro of SA-Lex and SA-diLex by alpha 1-3 fucosyltransferases from colon carcinoma cells and embryonic brain tissues. Glycobiology **1**(5), 527–535 (1991). doi:10.1093/glycob/1.5.527
5. Basu, S., Kaufman, B., Roseman, S.: Enzymatic synthesis of glucocerebroside by a glucosyltransferase from embryonic chicken brain. J. Biol. Chem. **248**(4), 1388–1394 (1973)
6. Basu, S.C.: The serendipity of ganglioside biosynthesis: pathway to CARS and HY-CARS glycosyltransferases. Glycobiology **1**(5), 469–475 (1991). doi:10.1093/glycob/1.5.469
7. Basu, S., Basu, M., Dastgheib, S., Hawes, J.W.: Biosynthesis and regulation of glycosphingolipids. In: Meth-Cohn, O., Pinto, B.M., Barton, D.H.R., Nakanishi, K. (eds.) Comprehensive Natural Products Chemistry, pp. 107–128. Pergamon, New York (1999)
8. Basu, S., Das, K., Basu, M.: Glycosyltransferase in glycosphingolipid biosynthesis. In: Ernst, B., Sinay, P., Hart, G. (eds.) Oligosaccharides in Chemistry and Biology—A Comprehensive Handbook, pp. 329–347. Wiley-VCH Verlag Gmbh, Germany (2000)
9. Roseman, S.: The synthesis of complex carbohydrates by multiglycosyltransferase systems and their potential function in intercellular adhesion. Chem. Phys. Lipids **5**(1), 270–297 (1970). doi:10.1016/0009-3084(70)90024-1
10. Siskind, L.J.: Mitochondrial ceramide and the induction of apoptosis. J. Bioenerg. Biomembr. **37**(3), 143–153 (2005). doi:10.1007/s10863-005-6567-7
11. Inokuchi, J., Radin, N.S.: Preparation of the active isomer of 1-phenyl-2-decanoylamino-3-morpholino-1-propanol, inhibitor of murine glucocerebroside synthetase. J. Lipid Res. **28**(5), 565–571 (1987)
12. Radin, N.S.: Chemotherapy by slowing glucosphingolipid synthesis. Biochem. Pharmacol. **57**(6), 589–595 (1999). doi:10.1016/S0006-2952(98)00274-3
13. Manning, L.S., Radin, N.S.: Effects of the glucolipid synthase inhibitor, P4, on functional and phenotypic parameters of murine myeloma cells. Br. J. Cancer **81**(6), 952–958 (1999). doi:10.1038/sj.bjc.6690792
14. Chatterjee, S., Cleveland, T., Shi, W.Y., Inokuchi, J., Radin, N.S.: Studies of the action of ceramide-like substances (D- and L-PDMP) on sphingolipid glycosyltransferases and purified lactosylceramide synthase. Glycoconj. J. **13**(3), 481–486 (1996). doi:10.1007/BF00731481
15. Basu, S., Ma, R., Mikulla, B., Bradley, M., Moulton, C., Basu, M., Banerjee, S., Inokuchi, J.: Apoptosis of human carcinoma cells in the presence of inhibitors of glycosphingolipid biosynthesis: I. Treatment of Colo-205 and SKBR3 cells with isomers of PDMP and PPMP. Glycoconj. J. **20**(3), 157–168 (2004). doi:10.1023/B:GLYC.0000024254.64450.8b
16. Ma, R., Koulov, A., Moulton, C., Basu, M., Banerjee, S., Goodson, H., Basu, S.: Apoptosis of human breast carcinoma cells in the presence of disialosyl gangliosides: II. Treatment of SKBR3 cells with GD3 and GD1b gangliosides. Glycoconj. J. **20**(5), 319–330 (2004). doi:10.1023/B:GLYC.0000033628.39302.ae
17. Basu, S., Ma, R., Boyle, P.J., Mikulla, B., Bradley, M., Smith, B., Basu, M., Banerjee, S.: Apoptosis of human carcinoma cells in the presence of potential anti-cancer drugs: III. Treatment of Colo-205 and SKBR3 cells with: cis -platin, Tamoxifen, Melphalan, Betulinic acid, L-PDMP, L-PPMP, and GD3 ganglioside. Glycoconj. J. **20**(9), 563–577 (2004). doi:10.1023/B:GLYC.0000043293.46845.07
18. Boyle, P.J., Ma, R., Tuteja, N., Banerjee, S., Basu, S.: Apoptosis of human breast carcinoma cells in the presence of cis-platin and L-/D-PPMP: IV. Modulation of replication complexes and glycolipid: glycosyltransferases. Glycoconj. J. **23**(3–4), 175–187 (2006). doi:10.1007/s10719-006-7923-5
19. Basu, S., Ma, R., Basu, M., Goodson, H., Smith, B., Banerjee, S.: Glycosphingolipid metabolism and signaling in apoptotic cancer cells, lipids: sphingolipid metabolizing enzymes, In: Haldar, D., Das, S.K. (eds.) Research Signpost, p. 81–100. India (2004)
20. Ma, R., Hopp, E.A., Decker, M.N., Loucks, A.J., Johnson, J.R., Moskal, J.R., Basu, M., Banerjee, S., Basu, S.: Regulation of glycosyltransferase genes in apoptotic breast cancer cells induced by L-PPMP and *cis*-Platin. In Proceedings of the Molecular Immunology Cancer Congress. Kluwer, Boston (in press) (2009)
21. Ma, R.: Apoptosis of breast and colon carcinoma cells by inhibitors of glycolipid and DNA biosynthesis. In Chemistry and Biochemistry, p. 272. University of Notre Dame, Notre Dame (2007)
22. Kroes, R.A., Panksepp, J., Burgdorf, J., Otto, N.J., Moskal, J.R.: Modeling depression: social dominance-submission gene expression patterns in rat neocortex. Neuroscience **137**(1), 37–49 (2006). doi:10.1016/j.neuroscience.2005.08.076
23. Ritter, G., Livingston, P.O.: Ganglioside antigens expressed by human cancer cells. Semin. Cancer Biol. **2**(6), 401–409 (1991)
24. Hakomori, S.: Tumor-associated carbohydrate antigens defining tumor malignancy: basis for development of anti-cancer vaccines. Adv. Exp. Med. Biol. **491**, 369–402 (2001)
25. Nohara, K., Wang, F., Spiegel, S.: Glycosphingolipid composition of MDA-MB-231 and MCF-7 human breast cancer cell lines. Breast Cancer Res. Treat. **48**(2), 149–157 (1998). doi:10.1023/A:1005986606010
26. Ugorski, M., Laskowska, A.: Sialyl Lewis(a): a tumor-associated carbohydrate antigen involved in adhesion and metastatic potential of cancer cells. Acta Biochim. Pol. **49**(2), 303–311 (2002)
27. Kikuchi, N., Narimatsu, H.: Bioinformatics for comprehensive finding and analysis of glycosyltransferases. Biochim. Biophys. Acta **1760**(4), 578–583 (2006)
28. Higashi, H., Basu, M., Basu, S.: Biosynthesis in vitro of disialosylneolactotetraosylceramide by a solubilized sialyltransferase from embryonic chicken brain. J. Biol. Chem. **260**(2), 824–828 (1985)
29. Das, K.K., Basu, M., Basu, S.: A rapid preparative method for isolation of neutral and acidic glycosphingolipids by radial thin-layer chromatography. Anal. Biochem. **143**(1), 125–134 (1984). doi:10.1016/0003-2697(84)90566-9

30. Tusher, V.G., Tibshirani, R., Chu, G.: Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U.S.A. **98**(9), 5116–5121 (2001). doi:10.1073/pnas.091062498
31. Johnson, J.R.: Targeted delivery of molecular cargo and fluorescent bioimaging agents. In Chemistry and Biochemistry. University of Notre Dame: Notre Dame (2008).
32. Arunkumar, E., Fu, N., Smith, B.D.: Squaraine-derived rotaxanes: highly stable, fluorescent near-IR dyes. Chemistry (Easton) **12** (17), 4684–4690 (2006)
33. Yamamoto, H., Oviedo, A., Sweeley, C., Saito, T., Moskal, J.R.: Alpha2,6-sialylation of cell-surface N-glycans inhibits glioma formation in vivo. Cancer Res. **61**(18), 6822–6829 (2001)
34. Moskal, J.R., Kroes, R.A.: The development of glycobiology-based therapeutics for the treatment of brain tumors. Curr. Drug Targets (in press)
35. Siddik, Z.H.: Cisplatin: mode of cytotoxic action and molecular basis of resistance. Oncogene **22**(47), 7265–7279 (2003). doi:10.1038/sj.onc.1206933
36. Rosenberg, B., VanCamp, L., Trosko, J.E., Mansour, V.H.: Platinum compounds: a new class of potent antitumour agents. Nature **222**(5191), 385–386 (1969). doi:10.1038/222385a0
37. Jamieson, E.R., Lippard, S.J.: Structure, Recognition, and Processing of Cisplatin-DNA Adducts. Chem. Rev. **99**(9), 2467–2498 (1999). doi:10.1021/cr980421n
38. Koulov, A.V., Stucker, K.A., Lakshmi, C., Robinson, J.P., Smith, B.D.: Detection of apoptotic cells using a synthetic fluorescent sensor for membrane surfaces that contain phosphatidylserine. Cell Death Differ. **10**(12), 1357–1359 (2003). doi:10.1038/sj.cdd.4401315
39. Hanshaw, R.G., Smith, B.D.: New reagents for phosphatidylserine recognition and detection of apoptosis. Bioorg. Med. Chem. **13** (17), 5035–5042 (2005). doi:10.1016/j.bmc.2005.04.071
40. Strasser, A., O'Connor, L., Dixit, V.M.: Apoptosis signaling. Annu. Rev. Biochem. **69**, 217–245 (2000). doi:10.1146/annurev.biochem.69.1.217
41. Fulda, S., Debatin, K.M.: Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene **25**(34), 4798–4811 (2006). doi:10.1038/sj.onc.1209608
42. Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., Greenberg, M.E.: Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270**(5240), 1326–1331 (1995). doi:10.1126/science.270.5240.1326
43. Qasba, P.K., Ramakrishnan, B., Boeggeman, E.: Structure and function of beta -1,4-galactosyltransferase. Curr. Drug Targets **9** (4), 292–309 (2008). doi:10.2174/138945008783954943
44. Sen, R., Baltimore, D.: Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46**(5), 705–716 (1986). doi:10.1016/0092-8674(86)90346-6
45. Lewis, T.S., Shapiro, P.S., Ahn, N.G.: Signal transduction through MAP kinase cascades. Adv. Cancer Res. **74**, 49–139 (1998). doi:10.1016/S0065-230X(08)60765-4
46. Zarubin, T., Han, J.: Activation and signaling of the p38 MAP kinase pathway. Cell Res. **15**(1), 11–18 (2005). doi:10.1038/sj.cr.7290257
47. Kelley, T.J., Moghaddas, S., Bose, R., Basu, S.: Inhibition of immunopurified DNA polymerase-alpha from PA-3 prostate tumor cells by platinum (II) antitumor drugs. Cancer Biochem. Biophys. **13**(3), 135–146 (1993)
48. Boyle, P.J.: Characterization of DNA helicase-III in replication complexes isolated from embryonic chicken brains and breast carcinoma cells. In Chemistry and Biochemistry, p. 188. University of Notre Dame: Notre Dame. (2005)
49. Bose, R.N., Li, D., Kennedy, M., Basu, S. (1995) Facile Formation of cis-platin nanopeptide complex of human DNA polymerase-a origin. J. Chem. Soc. Commun. Royal Soc. Chem., 1731–1732.
50. Bose, R.N., Li, D., Yang, W.W., Basu, S.: NMR structures of a nonapeptide from DNA binding domain of human polymerase-alpha determined by iterative complete-relaxation-matrix approach. J. Biomol. Struct. Dyn. **16**(5), 1075–1085 (1999)