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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

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Breast cancer is the most common type of cancer, predominantly among women over 20, whereas colo-rectal cancer occurs in both men and women over the age of 50. Chemotherapy of both cancers affect rapidly growing normal as well as cancer cells. Cancer cells are non-apoptotic. Seven anti-cancer agents (*cis*-platin, Tamoxifen, Melphalan, Betulinic acid, D-PDMP, L-PPMP, and GD3) have been tested with human breast (SKBR3) and colon (Colo-205) carcinoma cells for their apoptotic effect and found to be positive by several assay systems. Colo-205 cells were obtained from ATCC, and the SKBR3 cells were a gift from the Cleveland Clinic. All of these six agents killed those two cell lines in a dose-dependent manner. In the early apoptotic stage (6 h), these cells showed only a flopping of phosphatidylserine on the outer lamella of the plasma membranes as evidenced by the binding of a novel fluorescent dye PSS-380. After 24 h of the treatment, those apoptotic cells showed damage of the plasma as well as the nuclear membrane as evidenced by binding of propidium iodide to the nuclear DNA. DNA laddering assay viewed further breakdown of DNA by 1% agarose gel electrophoresis analysis. It is concluded that during apoptosis the signaling by Mitochondrial Signaling Pathway (MSP) is stimulated by some of these agents. Caspase 3 was activated with the concomitant appearance of its p17 polypeptide as viewed by Westernblot analyses. Incorporation of radioactivity from [U-^{14}C]-L-serine in total sphingolipid mixture was observed between 2 and 4 micromolar concentrations of most of the agents except *cis*-platin. However, apoptosis in carcinoma cells in the presence of *cis*-platin is induced by a caspase 3 activation pathway without any increase in synthesis of ceramide.
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Keywords: **apoptosis, anti-cancer drugs, betulinic acid, carcinoma cells, *cis*-platin, DNA polymerase-α, helicase-III, GD3, Gangliosides, D-PDMP, L-PPMP, Melphalan, Tamoxifen, replisome complex**

Introduction

Apoptosis, or programmed cell death [1,2] plays an important role in normal cell development and in cells under diseased conditions as well. Tumor or cancer cell death can be triggered by necrosis or apoptosis induced by anti-cancer drugs or agents [3–7]. However, during chemotherapy [8,9] how these agents induce apoptosis in a specific cancer cell is little known and needs to be explored carefully [10,11]. During apoptosis there are morphological and molecular changes. Morphological change includes: chromatin condensation, cytoplasmic shrinkage, and plasma membrane blebbing. Swelling and permeability change of mitochondrial membrane occurs with the release of cytochrome c from the mitochondrial inner membrane. During induction of apoptosis, randomized distribution of phosphatidylserine occurs on the plasma membrane (from the inside to the outer leaflet). There are some major proteins playing important roles in apoptotic processes that initiate the activation of Caspases, Adaptor Proteins, and TNF Receptor Super Family with concomitant degradation of chromosomal DNA. However,

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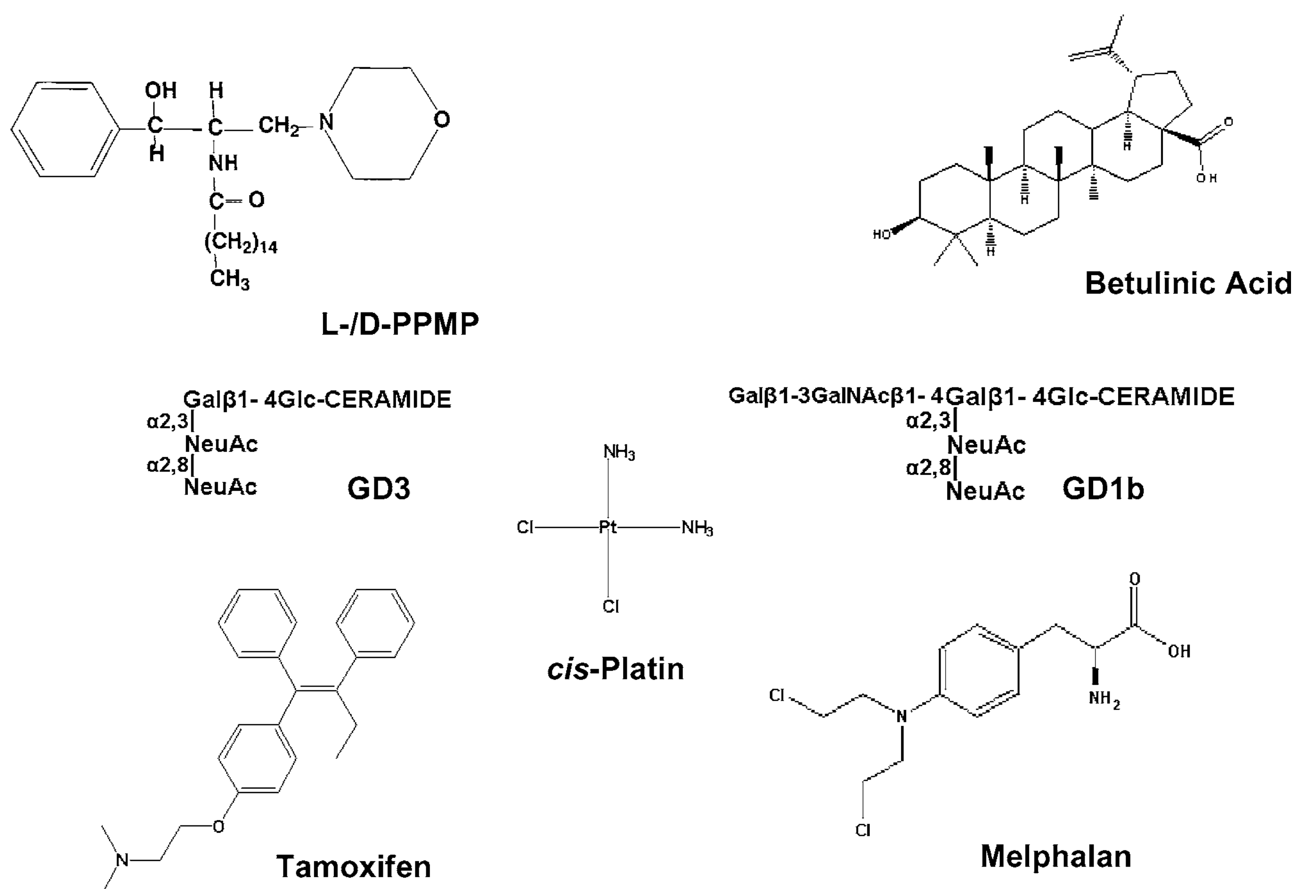


Figure 1. Structure of apoptotic drugs.

very little is known about the discontinuity of DNA synthesis or stability of the Replication Complex [12,13] (containing DNA Polymerases, Primase and Helicases) during apoptotic processes. Our present studies indicated that these anti-cancer agents (Figure 1) could be effective drug or apoptotic agents causing increased degradation of DNA and inhibition of DNA synthesis also. The effectiveness of their antitumor activities in the body depends on the successful delivery of the drugs to the specific tumor area in the body. In the following section we would like to discuss about some background work done with each of the anti-cancer agents used in our present studies.

Tamoxifen (TMF)

Inflammatory breast cancer (IBC) is the most aggressive form of breast cancer disease. A nationwide (US) survey [14,15] conducted among 40 to 59 years old women from 1994 through 1998 showed a rate of 1.3 per 100,000 for all races combined. African-American women had the highest risk of IBC (1.6) and Asian and Pacific Islander women the lowest (0.7). Despite decades of use and considerable research, the role of estrogen alone in preventing chronic breast cancer disease in postmenopausal women remains uncertain. In addition to breast cancer [16–18], Tamoxifen (TMF) has been shown to cause apoptosis in various cancer cells [19–25] including human cervical carcinoma (HeLa), murine erythroleukemic (MEL) cells, AIDS-related Kaposi Sarcoma cells, head and neck cancer cells, pituitary tumor cells, human hepatoblastoma (hepG2) cells, human glioblastoma cells, rat glioma cells, and in multiple myeloma cell lines. Apoptosis could be a major mechanism of the antitumor effect of many anti-cancer agents. Normal human mammary epithelial cells (HMECs), unlike estrogen receptor-positive (ER+) breast cancer cells, typically express low nuclear levels of ER (ER-poor). It has been demonstrated recently that ER-poor HMECs acutely transduced with human papillomavirus-16E6 (HMEC-E6) also went through apoptosis (induction of rapid MSP, mitochondrial-signaling pathway) in the presence of 1 μM Tamoxifen. TMF could be more effective at low doses if it is targeted properly.

Cis-platin (*cis*-DDP)

Pre-operative chemotherapy is most important in clinical treatment in testicular, ovarian, or breast cancer patients. In case of testicular cancer treatment, multiple anti-cancer drugs such as Adriamycin, *cis*-platin (*cis*-DDP), cyclophosphamide (CPM), methotrexate (MTX), and Vincristine (VCR) are commonly used in combination. *Cis*-DDP (*cis*-platin) is effective as an

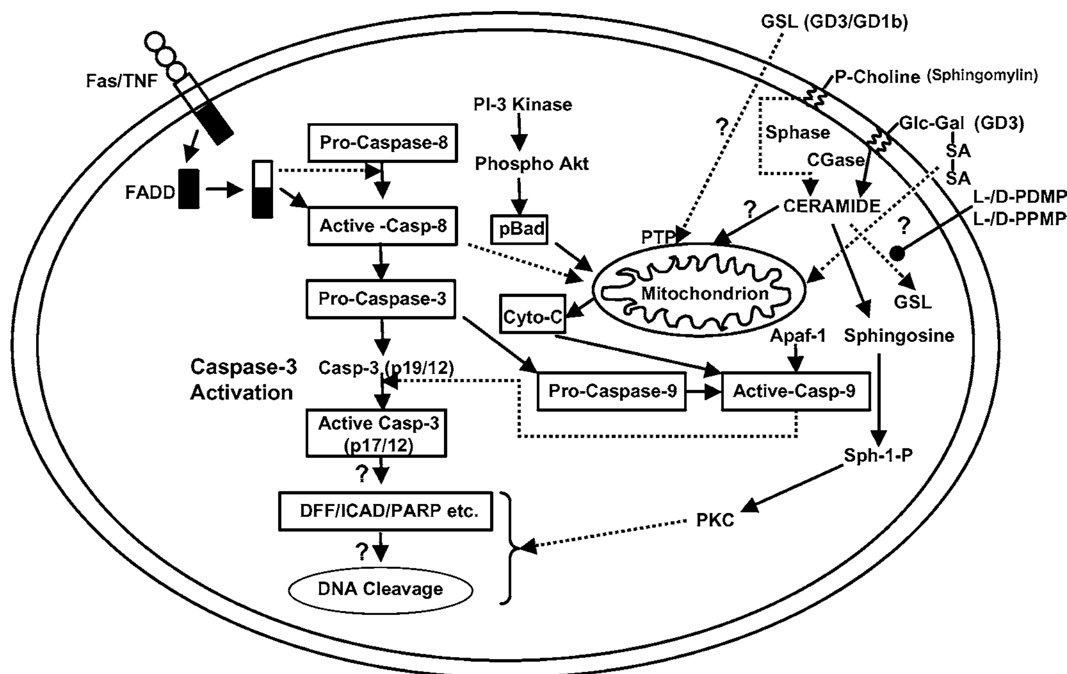


Figure 2. Mitochondrial signaling pathway (MSP) scheme.

anticancer agent in the testicular cancer, but it is rarely used for breast cancer patients. Recently, it has been demonstrated that CDDP specifically induces apoptosis via activation of Caspases (−3, −6, and −8) in osteosarcoma [27] and ovarian cancer cells [28]. Only recently it has been found that pretreatment with *cis*-platin increases the effectiveness of Tamoxifen treatment in breast cancer patients [20].

Recently, we reported that the mechanism of action of *cis*-platin inhibition (Figure 3) of replication is mediated by binding to DNA as well as polymerase α enzyme [29].*Cis*-platin binds to the Zn-binding domain of the DNA Polymerase-α much tighter (1000 times stronger) than the binding with the N-7 position guanine groups in DNA [30]. From our 2D-NMR studies we concluded that the divalent Pt (II) binds with two vicinal Cysteine groups at the Zn-binding domain of the enzyme (Figure 3) [31]. It is believed that in some cancer cells, the components of the apoptotic signaling pathways are expressed but remain inhibited until proper signaling is triggered. Whether *cis*-platin inhibits any inhibitory action of apoptosis is not known yet (Figure 4).

This information prompted us to study the apoptotic effect of *cis*-platin on colon carcinoma cells Colo-205 and human breast cancer SKBR3 cells. From our present studies it is evident that induction of apoptosis by *cis*-Platin perhaps occurs by a non-MSP (mitochondrial signal transduction pathway) by activation of Caspase-3 (Figure 2). Both testicular and breast cancer patients are treated with high doses of *cis*-platin. Liposomes [32] encapsulated *cis*-platin is expected to reduce the effective concentration of *cis*-platin from 100 micromolar to 5 to 10 micromolar range (below toxic level) for induction of apoptosis.

Betulinic acid (BetA)

Betulinic acid is a naturally occurring pentacyclic triterpenoid (Figure 1), which has demonstrated selective toxicity against a number of specific tumor types and a variety of infectious agents such as HIV [33], the Malaria parasite, and bacteria [34]. Biological activity was demonstrated in melanoma cell lines [35] and was confirmed in mice bearing human melanoma xenografts [36]. BetA exhibited potent antitumor activity on

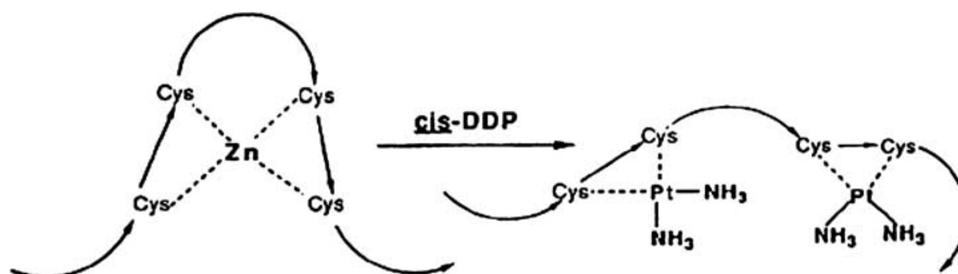


Figure 3. Possible mechanism of *cis*-platin binding to the zinc-finger domain of DNA Polymerase-α.

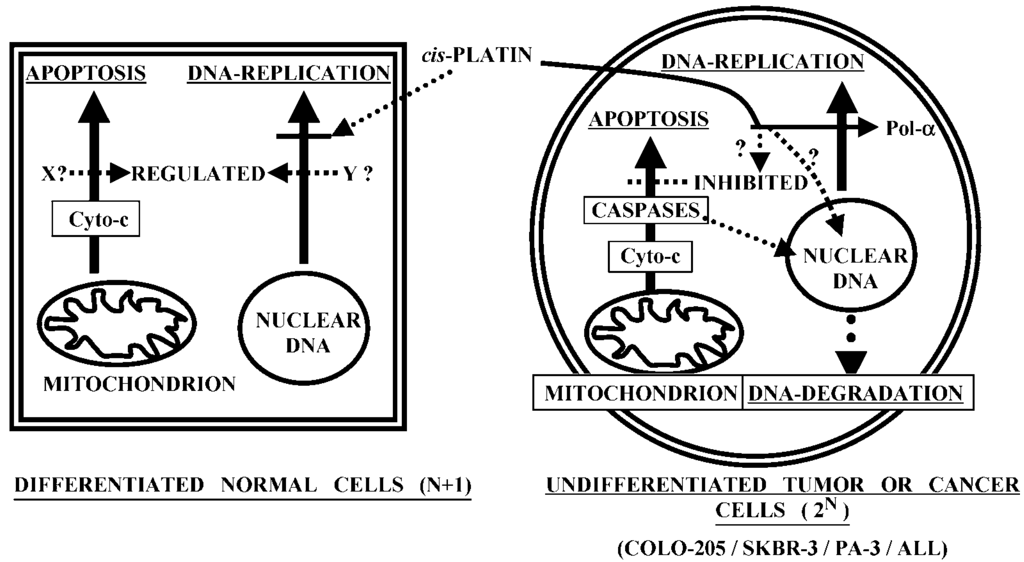


Figure 4. A scheme of probable role of *cis*-platin in the apoptotic pathway.

neuroblastoma cells resistant to CD95 or doxorubicin-triggered apoptosis and on primary tumor cells from patients with neuroblastodermal tumors [37]. BetA treatment results in the release of soluble apoptogenic factors such as a cytochrome *c* or Apaf-1 from mitochondria into the cytosol. Detailed study on the activation of Caspase pathway in colon and breast carcinoma cell lines has not been established yet.

L-PPMP and D-PDMP

The microdomains (rafts) on the cell surfaces contain aggregates of glycosphingolipids (GSLs), sphingomyelin (SM), and cholesterol. These lipid-islands while floating on the phospholipids bilayers do also contain various signaling molecules such as Src family kinases and transmembrane receptors. Microdomains are believed to mediate extracellular signals. GSLs are assumed to function as a regulator of various proteins in the microdomains. Until now very little information is available on the direct role of GSLs in the regulation of the GSL or other gene-function. In recent years, several reports are available on the induction of MSP (Mitochondrial Signaling Pathway) by Ceramide and GD3 [38–46]. We have reported recently the induction of MSP in both Colo-205 and SKBR3 by exogenously added GD3 or GD1b [11,26]. Introducing SAT-1 gene (Figure 5) in Lactosyl-Ceramide-expressing cells (cloned from wild type 3LL Lewis lung carcinoma cells) [47], it was observed that

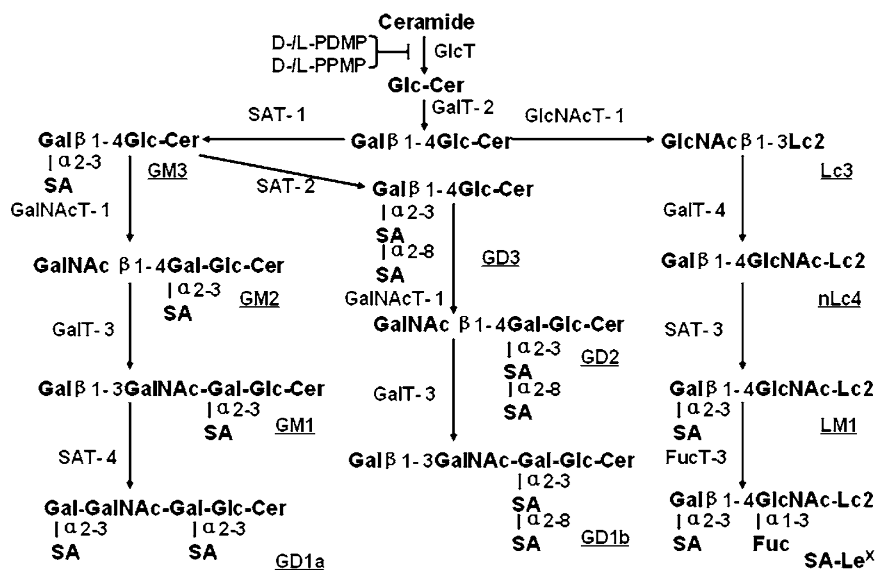


Figure 5. Inhibition of GlcT in the proposed pathway for biosynthesis of gangliosides and SA-Le^X by PPMP and PDMP.

anchorage-independent growth was promoted, and expression of PDGFα-receptor mRNA was specifically reduced. We have also observed recently [10] that during apoptosis of breast cancer cells by L-PPMP, the crucial GalT-4 activity (Figure 5) that catalyzes synthesis of the intermediate nLcOse4Cer, a precursor of SA-Le^X (carcinoma cell surface antigen), is also modulated (decreased).

Ceramide is a transmembrane sphingolipid composed of an *N*-acylated (C_{14} to C-fatty acids) sphingosine (C18-erythro-4, 5-trans unsaturated amino alcohol] in most of the eukaryotic animal cells. In yeast cells [48,49] and squid cells [50], polyunsaturated sphingosine has been reported. Ceramides containing phyto-sphingosines are quite common in plant cells [51–53]. Hydrolysis of sphingomyelin (SM) to generate ceramide in HL-60 human leukemia cells in response to the action of 1,25-dihydroxyvitamin D3 led to the suggestion that sphingolipid metabolism is regulated in response to extracellular agents [54,55]. Ceramide-induced cell death is also correlated with DNA-fragmentation in the apoptotic programmed cell death [46]. Several comprehensive reviews have been published recently on the role of exogenously added ceramide in the apoptosis of cultured tumor cells [56–60].

In spite of many papers published on this subject, very little is known about generation of ceramide during apoptosis by anti-cancer drugs in the cancer cells. Our present goal is to correlate the changes in the expression of cancer cell surface glycosphingolipids with apoptosis induced by anti-cancer drugs, inhibitors of glycosphingolipid biosynthesis (L-/D-PPMP; L-/D-PDMP), and disialogangliosides (GD3 and GD1b) (Figure 1). The instant production of ceramide under stress could occur by the action of sphingomyelinase (SMase) as well as by the action of the novel ceramide glycanase (CGase; Figures 6 and 7) that cleaves between ceramide and the oligosaccharides [61–63]. Activity of CGase is widely distributed in nature: (bacteria [64], leech [65], clam [62], rat mammary, and other tissues [61] as well as in human colon carcinoma cells [63]). Regulation of CGase activity during apoptosis or metastasis is under study in our laboratory. It appears that in addition to SMase (sphingomyelinase), CGase (Figures 6 and 7) may have had an important role in cell signaling. Ceramide produced by the action of these two enzymes could lead to the production of sphingosine or sphingosine-1-PO_4^{-2}, (Figure 7) which have been recognized in recent years as bio-regulators (66.67).

GSLs are synthesized from ceramide (Figure 5) in the Golgi [68–70], and are subsequently distributed to different compartments of a cell, most predominantly in the plasma membrane where they are recruited by signaling microdomains (rafts). A recently characterized trafficking of ceramide [59,60,71] and GD3 ganglioside to mitochondria [38–41,47] has revealed a novel function of this GSL as a death effector. These observations initiated the idea that some of the GSL-GLT (glycolipid glycosyltransferase) activities could be regulated during metastasis or apoptosis induced by anti-cancer agents. However, SA-Le^x (SAα2,3Gal β1,4(Fucα1,3)GlcNAcβ1,3Galβ1,4Glc-Ceramide), and SA Le^a(SAα2,3Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4Glc-Ceramide) have been suggested as markers for metastatic breast carcinoma cells [72–74].

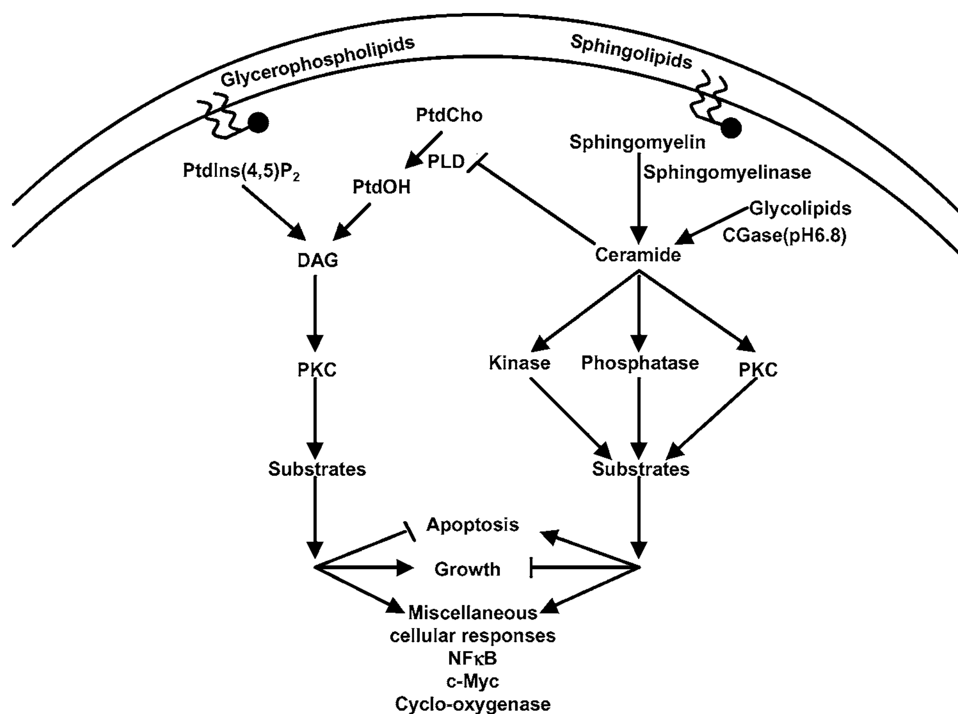


Figure 6. Signal transduction by intracellular ceramide.

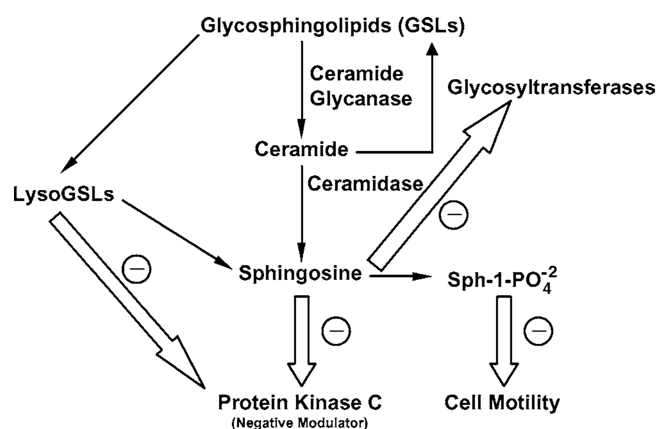


Figure 7. Modulation of cell signaling by ceramide and sphingosine.

Biosyntheses *in vitro* of GD3 and SA-Le^x from ceramide and related GSLs in embryonic brains and in metastatic breast and colon carcinoma cells (Figure 5) have been studied in our laboratory in recent years [75–87]. The glycolipid-glycosyltransferases (GSL-GLTs) that catalyze the synthesis of GD3 from ceramide are GlcT-1 (UDP-Glc: Ceramideβ1,1glucosyl transferase [69,70,76,83]; GalT-4 (UDP-Gal: LcOse3Cerβ1,4galactosyltransferase [70,76,83,85]; SAT-1 (CMP-NeuAc: LacCer α2,3Sialyl transferase [70,75,76,83,87] and SAT-2 (CMP-NeuAc:GM3gα2,8 Sialyltransferase [68,70,75–77,84] (Figure 5). All of those GSL-GLT activities were initially characterized in developing chicken brains [69,70,75–77,81,83,84–86], and carcinoma cells [78–80,82,83] in this laboratory. However, transcriptional or posttranslational regulation of these transferases during apoptosis initiated by anti-cancer drugs and agents mentioned above are not known yet and will be the future field to be explored.

Materials and methods

Cell culture

The human colonic cancer cell line (Colo-205) and the breast cancer cell line (SKBR3) were grown in RPMI1640 and DMEM media, respectively, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 mM L-glutamine. Human breast carcinoma cell lines SKBR3 were obtained from the Cleveland Clinic, Cleveland, OH, and have been maintained in our Notre Dame laboratory for the past two years. Colo-205 cells were purchased from ATCC, have been maintained in our laboratory for several years, and have been used to elucidate the SA-Le^X biosynthetic pathway and the identification of all the GSL-glycosyltransferases involved in that pathway [79–84]. When cells were 90% confluent (5×10^6 per 25 cm^2 T flask, they were passaged or harvested for biochemical work. The semi-confluent cells were treated twice with 0.5 mM hydroxyurea for 24 h under the same culturing conditions to obtain synchronized cultures before apoptotic reagents were used. Hydroxyurea was removed, and cells were treated with different apoptotic reagents, Tamoxifen, *cis*-platin, D-PDMP, L-PPMP, Melphalan, and GD3 (Colo-205/ Figure 8; SKBR3/ Figure 9), and 0.5 μCi ^{14}C-Serine per T-flask to study *in vivo* (in whole cells) biosynthesis of sphingolipids for 24 h.

Analysis of radioactive sphingolipids synthesized from ^{14}*C-L-Serine*

The C-2 and C-3 Carbons of L-serine are incorporated into C-1 and C-2 of the Sphingosine, respectively, when added to media containing non-apoptotic or apoptotic cells. After incubation with 0.5 to 1.0 μCi/T-flask, cells were scraped off and transferred into 12-ml graduated conical tubes followed by 2 times of phosphate-buffered saline (PBS) wash. Finally all the cells were suspended in 5 ml PBS, and cell counts are performed. These cells are used for further analysis of incorporation of radioactivity of [^{14}C] L-serine in radioactive total sphingolipid, glycosphingolipids, and ceramide following protocol as indicated in our recent publications [10,11,26].

GF/A Filtering Assay-0.5 ml suspension of cells is loaded onto a GF/A glass filter, which had been treated with 50 mM sodium pyrophosphate. Then the samples (in duplicates) on each GF/A disc were washed twice with 5% TCA followed by 2 times chloroform/methanol (2:1) wash and acetone wash; GF/A discs were then fully dried and counted in toluene scintillation system. Incorporation of radioactivity was determined in protein as well as total sphingolipids [10,11,26]. Sphingolipids were further analyzed as follows.

Extraction and analysis of radioactive of *glycosphingolipids*

Labeled cells (0.5 ml) were centrifuged and resuspended with 0.2 ml 0.1 M NaOH and 0.5 ml chloroform/methanol (2:1) and incubated at 37°C for 1 h. Fifty microliters upper layer or lower layer was then spotted onto a 4 cm^2 Whatman 3MM paper and quantitated by toluene scintillation system. The extraction procedure has been published previously [11] and is described in the previous section also. The radioactivities on these dried discs were quantitated with a toluene scintillation system. The rest of the harvested cells were then used for extraction of [^{14}C]-GSLs using 0.05 to 0. 1 ml of $CHCl_3$: Methanol (2:1) and 0.2 volume of 0.1 N NaOH. The upper layer and the lower layer were separated and dried before further analysis by TLC using specific solvent systems. Incorporation of radioactivity in ceramide was quantitated by TLC, using $CHCl_3$-Methanol-water (80:18:2) solvent system. Migration of radioactivity in standard ceramide or GSLs areas were scraped and quantitated by the toluene liquid scintillation system. For further analysis of radioactive glycosphingolipids and ceramide, larger quantities of cells were extracted as above, concentrated, and purified by silicic acid columns, and further analyzed by TLC plates as published previously [83–88].

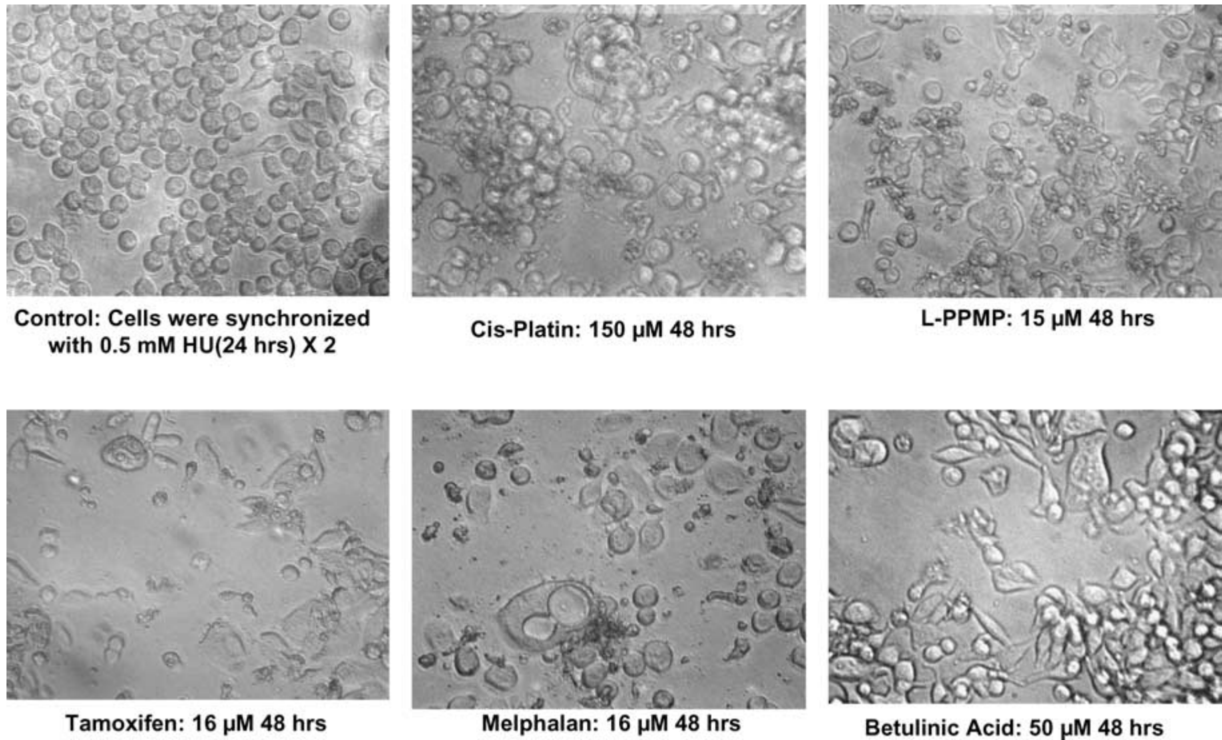


Figure 8. Apoptosis of human colon carcinoma colo-205 cells by anti-cancer agents (*cis*-platin, L-PPMP, tamoxifen, melphalan, and betulinic acid). [Phase contrast microscopic studies]

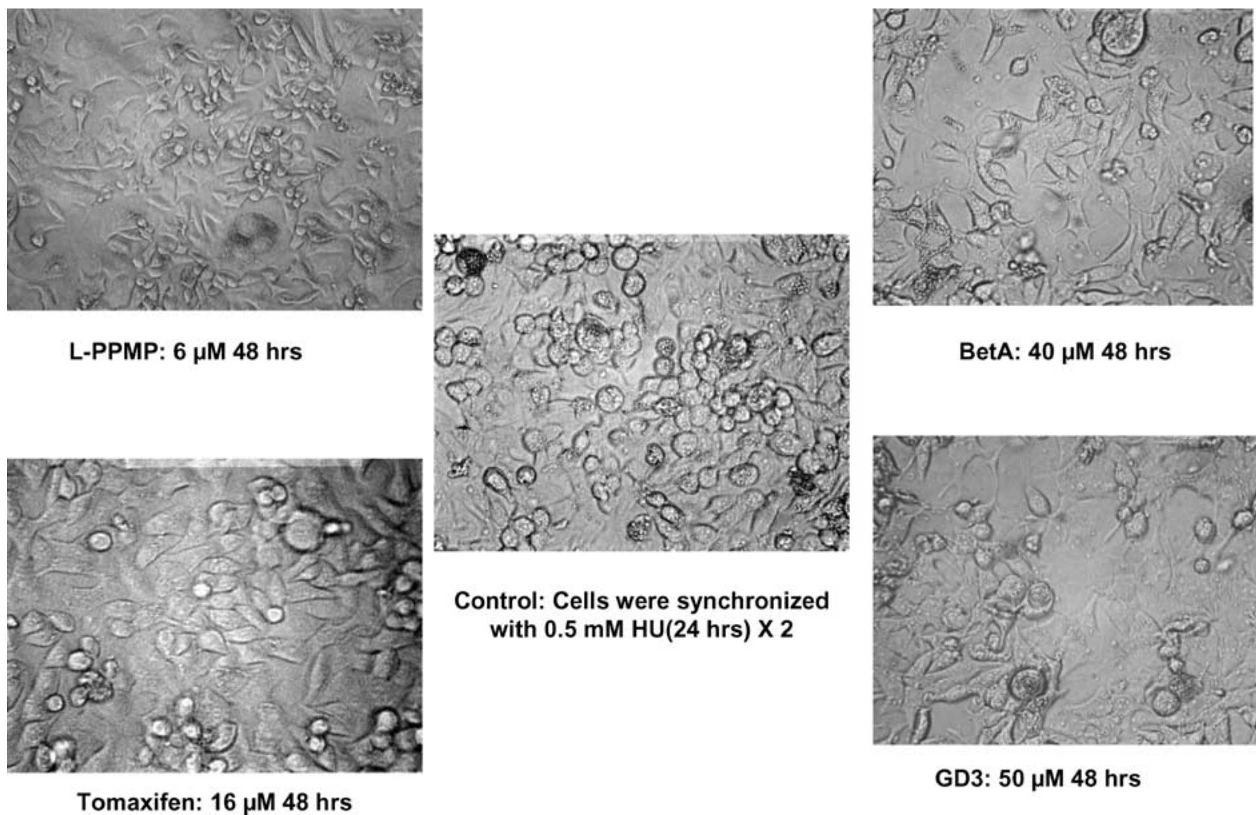


Figure 9. Apoptosis of human breast carcinoma cells by anti-cancer agents (L-PPMP, betulinic acid, tamoxifen, and GD3 ganglioside). [Phase contrast microscopic studies].

Western blot for identification of activation of caspases

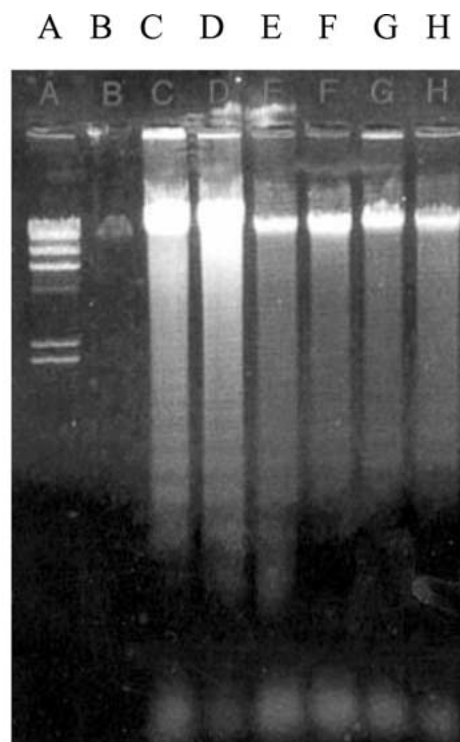
Drug treated cells (0.5 ml aliquots) were pelleted and resuspended with 0.1 μl lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, and 50 mM DTT) followed by homogenization with 3×10 s sonication. The homogenized samples were then incubated at 37°C for 1 h before 5 min of denaturation at 95°C and loaded onto SDS-PAGE gel. The protein mixture in the amount of 20 to 25 micrograms was loaded for each sample and blotted to nitrocellulose membranes; further methodology was published in our recent publications [10,11,26]. Nonspecific binding was blocked by incubation in Tris-buffered saline containing 5% bovine serum albumin [89] and 0.1% Tween-20 for 1 h at room temperature. The blots were then incubated overnight at 4°C in blocking buffer containing the primary antibody. Antibodies used were a rabbit polyclonal anti-Caspase-3 antibody raised against full-length human Caspase-3 diluted 1:1,000. Afterward, membranes were washed and incubated with anti-rabbit IgG-Alkaline phosphatase conjugate (1:3,000; Sigma). Antibody-conjugated alkaline phosphatase activity was visualized using the NBT-BCIP reagent in the AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$). Activation of Caspase-3 in apoptotic Colo-205 cells in the presence *of cis*-platin (Figure 12) and SKBR3 cells in the presence of GD3 ganglioside [10] was observed.

Fluorescence staining of PSS-380 and Propidium Iodine

Cells cultured on Falcon Microslide System (Fisher) were synchronized 2 times (24 h each) with 0.5 mM hydroxyurea before treatment with apoptotic reagents under different conditions. The cells were then washed 2 times with TES buffer (5 mM *N*-tris [Hydroxymethyl]-2-aminoethane-sulfonic acid: TES, 150 mM NaCl, pH7.4), followed by incubation with 200 μl new TES buffer containing 25 μM PSS-380 [90] and 0.25 μg/ml Propidium Iodide at 37°C for 10 min. The buffer was removed after staining, and the cells were washed with TES buffer once before observation for fluorescence [11]. The PSS-380 dye was used instead of annexin-V [91] to recognize phosphatidyl serine on the outer leaflet of the apoptotic cells.

DNA laddering analysis

Colo-205 cells (0.5 ml) are pelleted and resuspended with 0.1 ml lysis buffer (20 mM Tris-HCl pH 8.0, 20 mM NaCl, 20 mM EDTA, and 10% w/v SDS) followed by sonication for 3×10 s. The homogenized samples are incubated at 37°C for 1 h before adding 0.4 ml deionized water and 100 microgram/ml Proteinase K. After that Phenol/chloroform (1:1) 0.5 ml) was added to the homogenate, vortexed, and placed in ice for 15 min. The samples were centrifuged at 4°C (5,000X g) for 20 min. The upper layer was then transferred to a new tube and 0.5 ml of chloroform was added to it. Further analysis of the degraded DNA (Figure 10) was performed by the method published recently [10,11].



Gel electrophoresis showing DNA laddering. Lanes A-G go as follows: lambda standard (A), control (B), 132 μM cisplatin (C-D), 66 μM cisplatin (E-F), and 33 μM cisplatin (G-H).

Figure 10. DNA laddering examination with apoptotic human colon carcinoma Colo-205 cells treated with *cis*-platin.

Results and discussion

DNA laddering analysis with the apoptotic carcinoma cells initiated by L-PPMP

Treatment of human carcinoma cells (phase contrast picture of colon, Colo-205 cells; Figure 8) and (phase contrast pictures of breast, SKBR3 cells; Figure 9) with varying concentrations of *cis*-platin, L-PPMP, Tamoxifen, GD3, Melphalan or Betulinic acid led to observed cell blebbing and DNA condensation, while the control cells remained uniformly round and smooth on the outer surfaces with no damaged DNA as evidenced from negative staining by Propidium Iodide. The DNA laddering [92] resulted in altering UV Fluorescent bright and dark bands, and increased concentration of *cis*-platin (33 to 132 μM) led to brighter banding, indicating a greater degree of systematic DNA fragmentation (Figure 10). Similar results were observed with the isomers of L-PPMP or L-/D-PDMP [10]. The observed blebbing of both the Colo-205 and the SKBR3 cells with *cis*-platin and isomers of GSL: GlcT (glucosyltransferase) inhibitors (L-/D-PPMP or L-/D-PDMP) suggested that these agents were inducing apoptosis in these carcinoma cells after 6 h and damaging DNA between 24 and 48 h after treatment. Recent report shows [93] treatment of multidrug

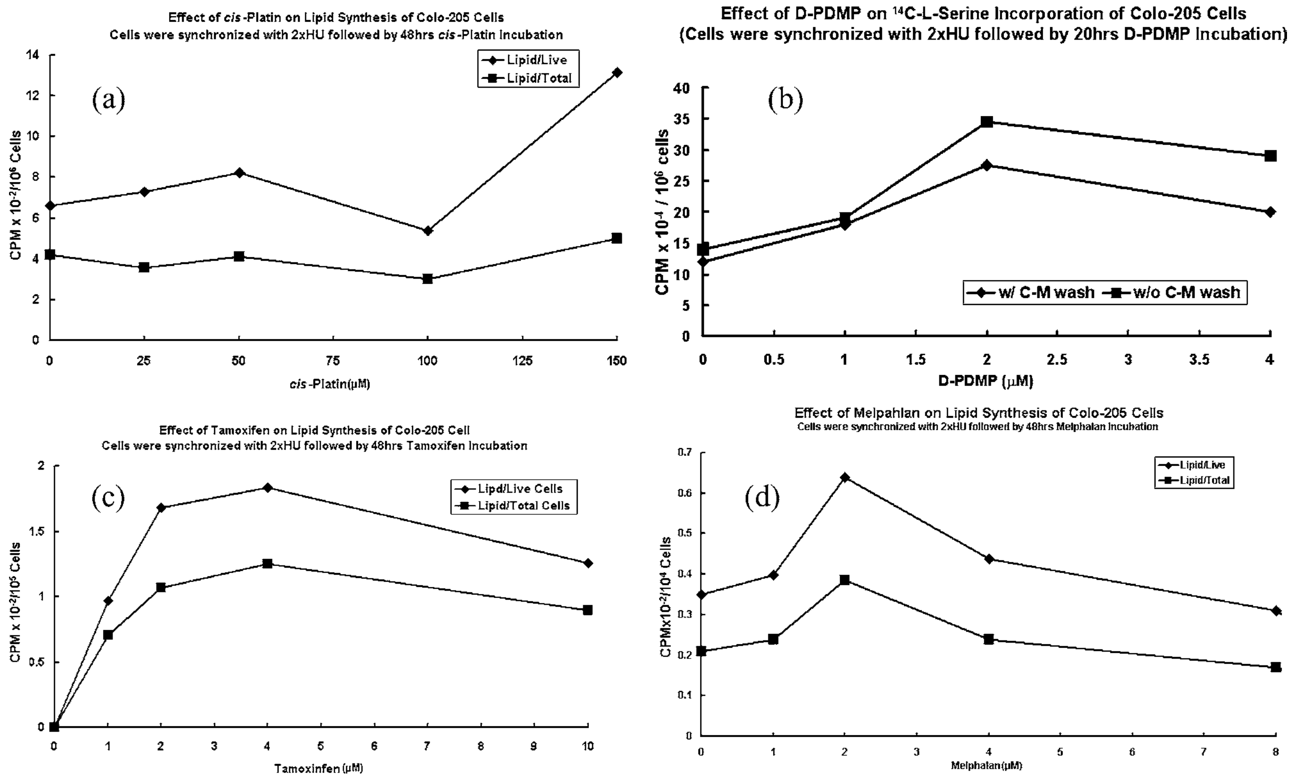


Figure 11. Effect of anti-cancer agents (cis-platin, D-PDMP, tamoxifen and melphalan) on the synthesis of sphingollipids in apoptotic humna colon carcinoma Colo-205 cells.

resistant P-glycoprotein (P-gp) expressing cancer cells (MCF-7-ADR) (Adriamycin resistant breast cancer) with L-PPMP diminishes expression of MDR1 and enhances responses of chemotherapy.

Incorporation of ^{14}C-Serine in ceramide in apoptotic carcinoma cells

Synchronized Colo-205 cells were treated with *cis*-platin (Figure 11a), D-PDMP (Figure 11b), Tamoxifen (Figure 11c) and Melphalan (Figure 11d), in the presence of uniformly labeled L-^{14}C-Serine. Incorporation of radioactivity was determined in total sphingolipids in Figure 11a, c, and d whereas the panel b represents the incorporation of radioactivity in live Colo-205 cells per 10^6 cells before and after chloroform-methanol wash only. Maximum incorporation of ^{14}C-serine into the total sphingolipid was observed in Colo-205 with Tamoxifen (Figure 11c) and Melphalan (Figure 11d) at concentrations between 2 and 4 μM. Inhibition of incorporation was observed in higher concentrations of the anti-cancer agents. The reason for increase in total sphingolipids in the presence of 2 to 4 micromolar D-PDMP (Figure 11b) and other anti-cancer agents in Colo-205 apoptotic live cells could be due to transient increase in the concentration of ceramide in those apoptotic cells. In case of *cis*-platin (Figure 11a) the total sphingolipid synthesized remained almost constant over a range of 20 to 150 micromolar

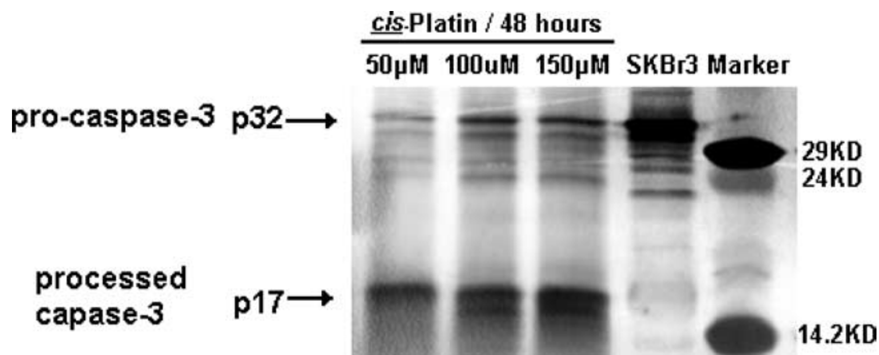


Figure 12. Activation of caspase-3 in human breast carcinoma cells treated with *cis*-platin.

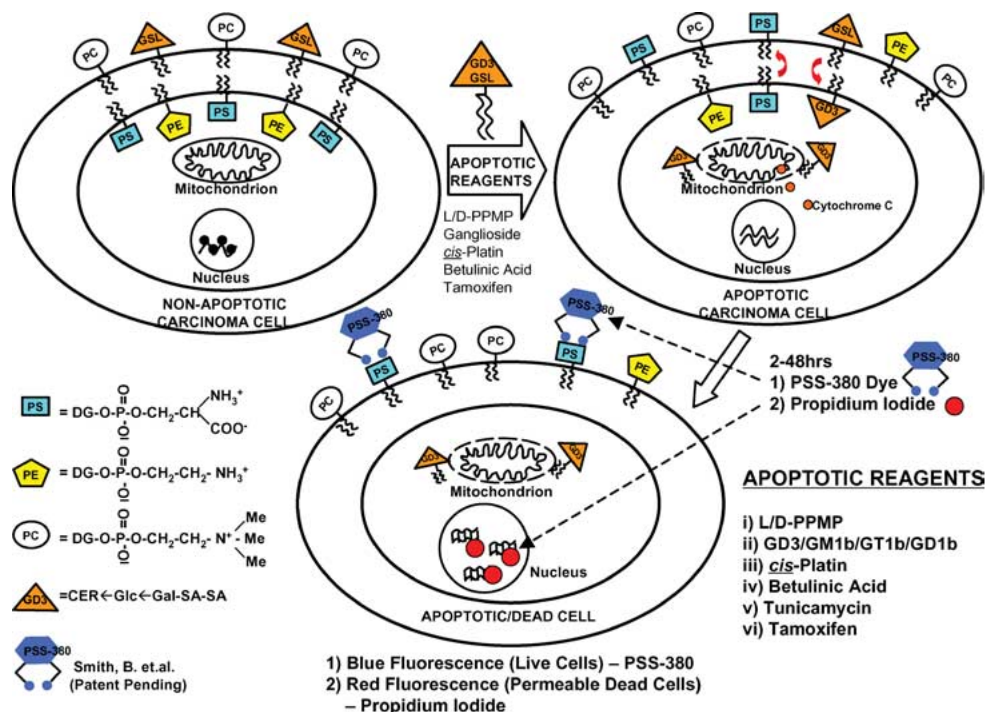


Figure 13. Identification of apoptotic carcinoma cells by fluorescent dyes (PSS-380 and propidium iodide), a scheme.

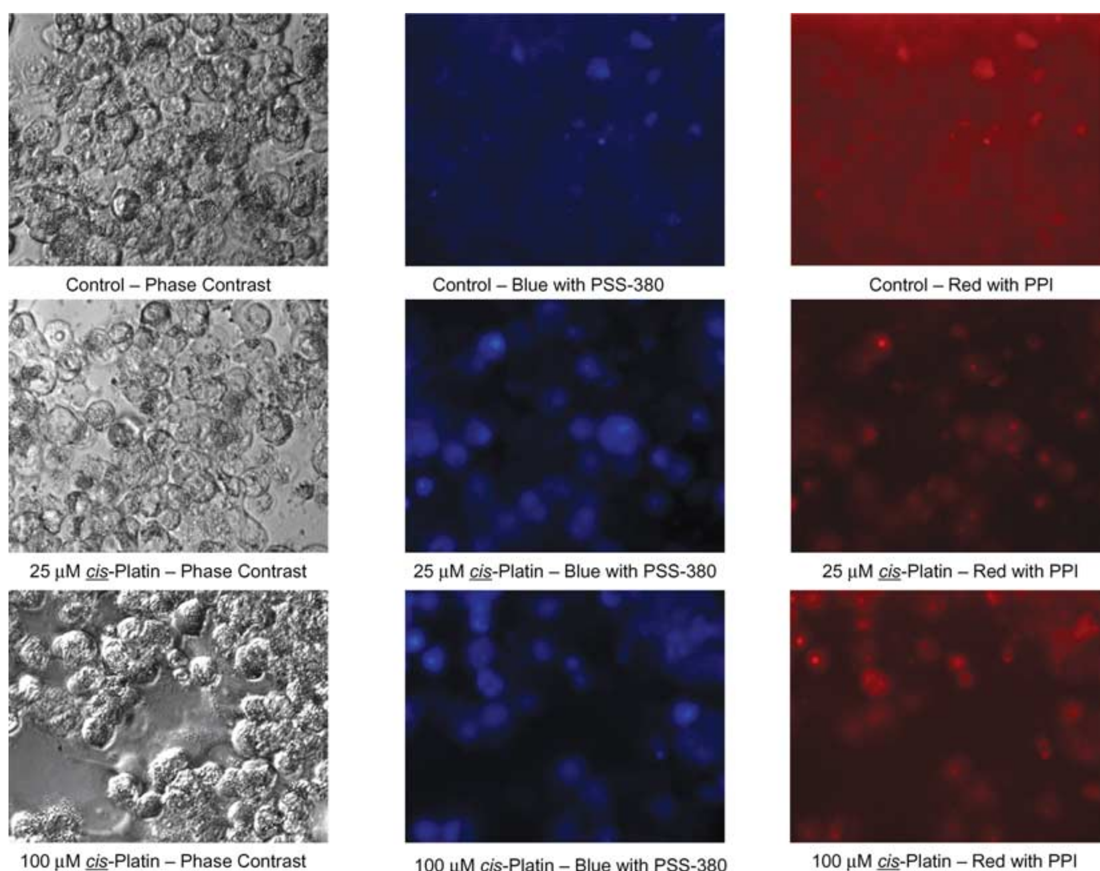


Figure 14. Fluorescent microscopy of the apoptotic human breast carcinoma SKBR3 cells induced by cis-platin (25 and 100 μM after 24 h.

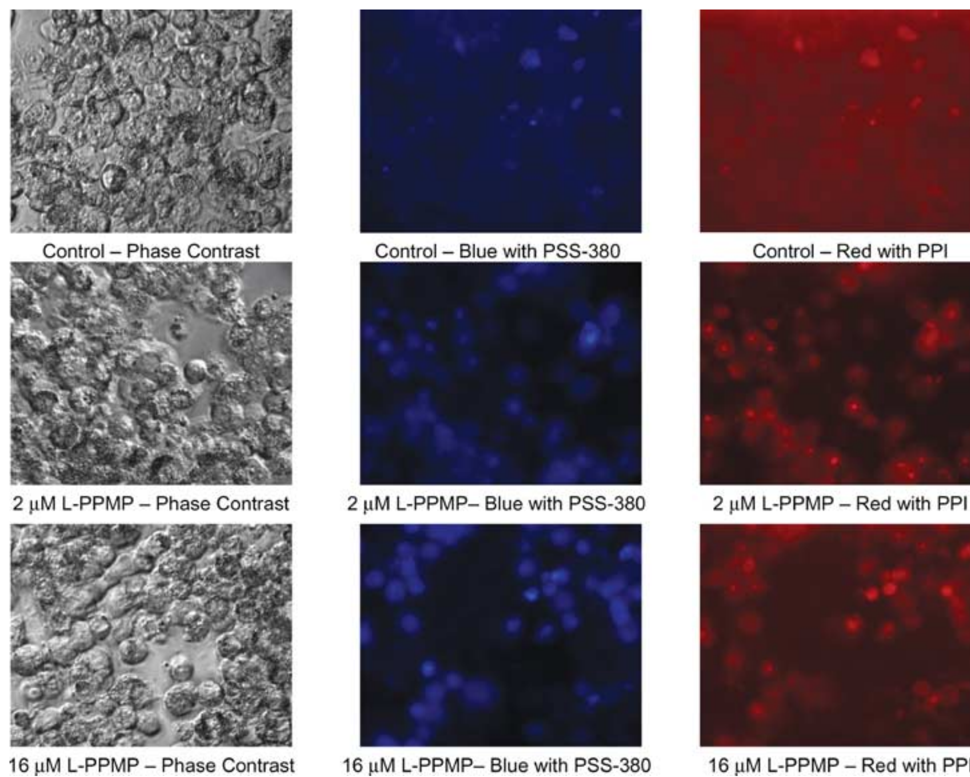


Figure 15. Fluorescent microscopy of the apoptotic human breast carcinoma SKBR3 cells induced by L-PPMP (2 and 16 μM) after 24 h.

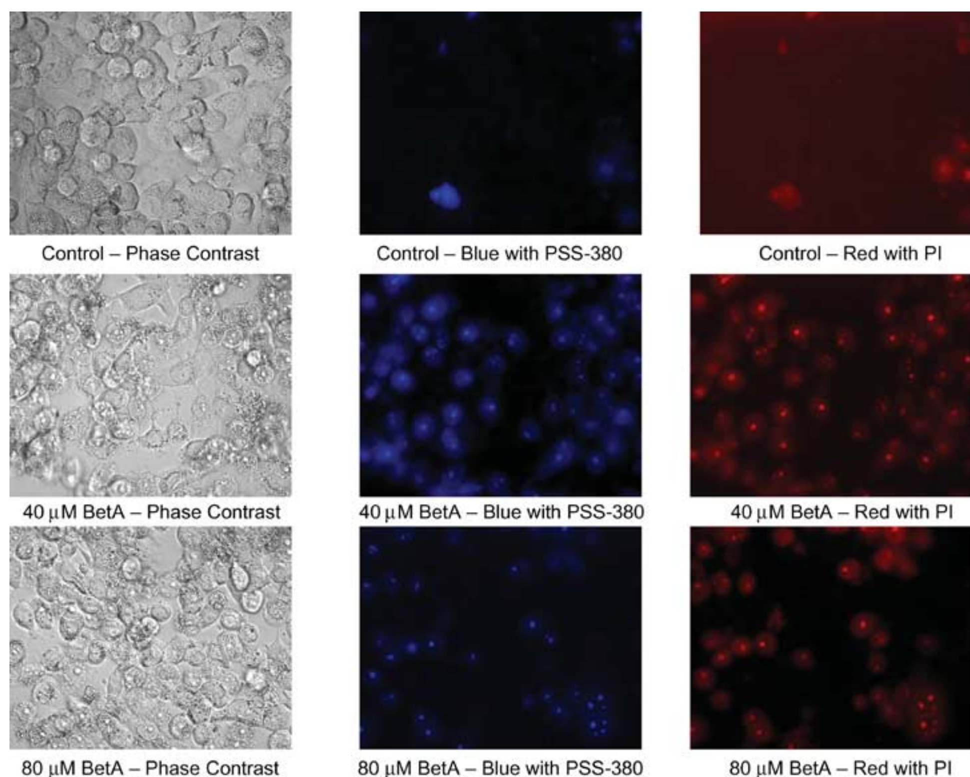


Figure 16. Fluorescent microscopy of the apoptotic human breast carcinoma SKBR3 cells induced by betulinic aid (40 and 80 μM) after 24 h.

range. The increase in radioactive sphingolipids in live cells could be due to the presence of *cis*-platin resistant live cells.

Westernblot analysis of Caspase-3 activation in apoptotic carcinoma cells by GSL biosynthesis inhibitors (L-/D-PPMP)

Caspases make up part of the cascade a cell follows in order to undergo apoptosis. Caspase-3 is one of the effector Caspases [10]. When activated, it cleaves proteins by recognizing the amino acid sequence DEVD. Upon recognition of the target proteins, the nucleus is broken down, starting with the disassembly of the chromosomes [10]. We have studied extensively the Caspase-3 activation in the presence of all the isomers of the inhibitor of GlcT (UDP-Glc: Ceramide β1,1 glucosyltransferase) [10,11,26]. A typical activation profile of Caspase-3 as evidenced in SKBR3 cells in the presence of cis-platin (50 to 150 micromolar) is shown in Figure 12. Appearance of p 17 peptide fragment from the pro-Caspase-3 p 32 was observed when tested by Western blot analysis (see method section). These results suggested the involvement of Caspase-3 activation during apoptosis of the human breast carcinoma cells (SKBR3) occurred only at a high concentrations of *cis*-platin. At present it is not known why this drug is effective mostly with the testicular cancers and not in other cancers. Perhaps the transport of this inorganic drug is limited from cell to cell. A similar study is needed with cultured testicular cancer cells to test the comparison of effective concentrations needed for caspase-3 activation.

Detection of translocation of membrane phosphatidylserine using a novel fluorescent dye

One important phenomenon of apoptotic cells is the randomized distribution of phosphatidylserine (PS) between the inner and outer leaflets of the plasma membrane. In normal cells, the phosphatidylserine is present in the inner leaflet of the cell membrane [90]. During apoptosis the flopping of phosphatidylserine from inside to the outer leaflet of the cell membrane can be detected (Figure 13). Recently, at Notre Dame a novel dye, PSS-380, has been synthesized that can bind phosphate derivatives with negative charges (*e.g.* phosphatidylserine or DNA at physiological pH) [90]. However, the dye PSS-380 does not bind to the nonapoptotic or undamaged cancer cells [10,11,26,90] The dye, PSS-380, could be used as a membrane phosphatidylserine detector in the early as well as late stages of apoptotic processes instead of annexin V [91]. In the later stage of apoptosis, as it is shown here the cell membrane permeability changes; then both PSS-380 and Propidium Iodide (a DNA binding dye (11)) can enter into the cell nucleus. In this experiment, synchronized SKBR3 cells were treated at first with *cis*-platin (Figure 14), L-PPMP (Figure 15) or Betulinic acid (Figure 16), washed twice with TES buffer (5 mM TES, 150 mM NaCl, pH7.4), and then the dyes PSS-380 (25 μM) and Propidium Iodide (0.25 μg/ml) were added. Within 24 h of treatment of SKBR3 cells with the anti-cancer agents, the dye PSS-380 [90] binds to both outer and inner leaflet phosphatidylserine (PS) with apparent damage of the outer plasma membrane also. However, in our previous papers we published no apparent damage of the outer membranes after 6 h of treatment with those ant-cancer agents (L-PPMP) [10,26] and GD3 (11,236). During 6 h of apoptosis the dye PSS-380 bound to the phophatidylserine of the outer leaflet only. The degree of apoptosis increased with increasing concentration of the agents. The nuclear membrane and DNA damage were evidenced by the appearance of bright red fluorescence (right panels of Figures 14 to 16). The Propidium Iodide dye bound to nuclear DNA with red fluorescence.

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