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Apoptosis of human breast carcinoma cells in the presence of *cis*-platin and L-/D-PPMP: IV. Modulation of replication complexes and glycolipid: Glycosyltransferases

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Abstract Apoptosis of human breast carcinoma cells (SKBR-3, MCF-7, and MDA-468) has been observed after treatment of these cells with anti-cancer drug cis-platin and glycosphingolipid biosynthesis inhibitor L- and D-PPMP, respectively. These drugs initiated apoptosis in a dose-dependent manner as measured by phenotypic morphological changes, by binding of a fluorescent phophatidyl serine-specific dye (PSS-380) onto the outer leaflet of the cell membranes, and by activation of caspases, −3, −8, and −9. It was observed that in two hours very little apoptotic process had started but predominant biochemical changes occurred after 6 h. DNA degradation started after 24 hours of drug treatment. However, very little is known about the stability of the "Replication Complexes" during the apoptotic process. DNA helicases are motor proteins that catalyze the melting of genomic DNA during its replication, repair, and recombination processes. Previously, DNA helicase-III was characterized as a component of the replication complexes isolated from embryonic chicken brains as well as breast and colon carcinoma cells. Helicase activities were measured by a novel method (ROME assay), and DNA polymerase-α activities were determined by regular chain extension of the nicked ACT-DNA, by determining values obtained from +/− aphidicolin-treated incubation mixtures. In all three breast carcinoma cell lines, a common trend was observed: a decrease of activities of DNA polymerase-α and Helicase III. A sharp decrease of activities of the glycolipid sialyltransferases: SAT-2 (CMP-NeuAc; GD3 α2-8 sialyltransferase) and SAT-4 (CMP-NeuAc: GM1a α2-3 sialyltransferase) was observed in the apoptotic carcinoma cells treated with L-PPMP compared with *cis*-platin.

Keywords Apoptosis · Anti-cancer drugs · Carcinoma cells · *cis*-platin · Caspase-3 · Caspase-9 · DNA polymerase-α · Helicase-III · GD3 ganglioside · GD1a ganglioside · D-PPMP · L-PPMP · SAT-2 · SAT-3 · SAT-4

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Introduction

Apoptosis, or programmed cell death, is a cellular phenomena that was first recognized over one hundred years ago [1]. In non-diseased tissues, apoptosis is a precisely regulated process which plays roles in embryological molding, normal cell turnover, immune regulation, and hormone-dependent atrophy [2]. Apoptosis maintains homeostasis by inducing cell death in senescent cells. Precise regulation of apoptosis is required to maintain homeostasis with the organism. Diseased states can arise from both over-active and under-active apoptotic machinery. Failure in apoptosis leads to accumulation of abnormal cells which can lead to cancer and autoimmune diseases. On the other hand, overactive apoptosis causes tissue destruction as seen in hepatitis, AIDS, and neurodegenerative diseases. The pathways by which apoptosis is initiated have been an area of intense study recently [3, 4]. Several apoptotic signaling pathways and their machinery have been identified. The goal of cancer chemotherapy is to induce cell death

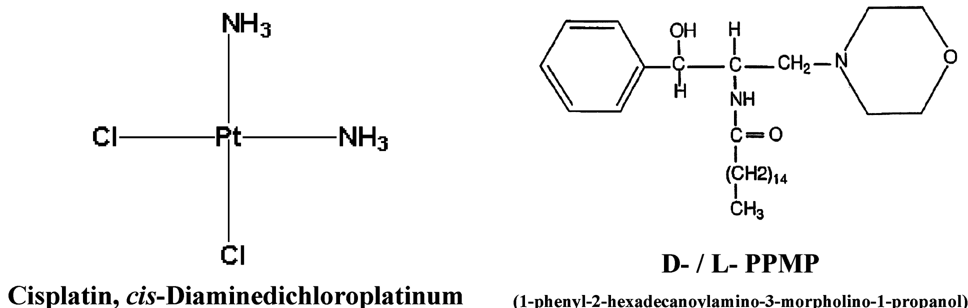


Fig. 1 Structures of anti-cancer agents: cis-platin (*cis*-Diaminedichloro-platinum) and D/ L-PPMP (1-phenyl-2hexadecanoyl-/palmitoylamino-3-morpholino-1-propanol)

in tumor cells while leaving non-transformed cells unharmed. During chemotherapy, anti-cancer agents can induce cell death through both necrotic and apoptotic pathways [5–9].

The role of lipids as signaling molecules has been established in the stress response of many cells. A relationship between lipid mediators and apoptosis has been established [10]. Glycosphingolipids (GSLs) (ganglio-, globo-, and lacto-) have been shown to play a role in the apoptotic signaling process [11]. In particular, disialosylgangliosides have been shown to play a significant role in apoptosis [12–21]. Lipid rafts on cell surfaces are aggregates of GSLs, sphingomyelin, and cholesterol. These rafts contain signaling proteins, such as transmembrane receptors and Src family kinases, and are believed to mediate extracellular signals. In addition to membrane signaling, a recent study has discovered trafficking of GD3 to the mitochondrial membrane [22], suggesting a role in initiation of the mitochondrial signaling pathway for apoptosis. In a recent study, we have reported induction of apoptosis upon addition of exogenous disialosylgangliosides GD3 and GD1b to breast (SKBR3) cells [23].

Human Breast Carcinoma Cells

SK-BR-3, MCF-7, and MDA-468

Derived from pleural effusion of breast carcinoma

Cells	HER2 Status	ER	PgR	p53
SK-BR-3	High	-	-	Mutant
MCF-7	Normal	+	+	Normal
MDA-468	Normal	-	-	Mutant

•HER2 (c-erbB-2/neu) protein

HER2 comes from a proto-oncogene encoding a transmembrane glycoprotein of 185 kDa (p185(HER2)) with intrinsic tyrosine kinase activity. HER2 gives the cells different responsiveness to anti-cancer drugs versus HER2 negative breast cancers cells

•Estrogen receptor (ER) and progesterone receptor (PgR)

Fig. 2 Receptors and p53 genes in expression in different breast carcinoma cells

In addition to GSLs, ceramide has been implicated in apoptosis [24–29]. Ceramide is a transmembrane sphingolipid composed of an *N*-acylated (C_{14}-C_{26}-fatty acids) sphingosine (C_{18}-erythro-4, 5-trans unsaturated amino alcohol) in most eukaryotic cells. Generation of ceramide from sphingomyelin has been reported in HL-60 human leukemia cells in response to treatment with 1, 25-dihydroxy vitamin D3 [30,31]. Also, we have previously reported the generation of ceramide upon treatment of colon (colo-205) and breast (SKBR3) cells with inhibitors of GlcT (isomers of PPMP and PDMP) (Fig. 1, Fig. 3), which is the first committed step of GSL biosynthesis [32]. In addition to the increase in ceramide concentration, we have also reported the decrease in GalT-4 activity [(UDP-Gal: LcOse3Cer β1-4 galactosyltransferase] in apoptotic breast carcinoma cells [32]. Production of ceramide under stress conditions could result from the action of either sphingomyelinase (SMase) [33] or the novel ceramide glycanase (CGase) [34,35], which cleaves between the ceramide and oligosaccharide moieties of GSLs [34, 36]. Ceramide produced under these conditions may lead to the synthesis of the signaling molecules sphingosine and sphingosine-1-PO_4 [37,38].

A series of characteristic molecular and morphological changes occurs as a result of apoptosis. Morphological changes include chromatin condensation, plasma membrane blebbing, and cytoplasmic shrinkage [39]. Biochemical characteristics of apoptosis include internucleosomal DNA cleavage [40], phosphatidylserine externalization [41], and proteolytic cleavage of a number of target proteins [42]. Apoptotic DNA cleavage was first reported in mouse thymocytes treated with glucocorticoid [43]. It was observed that treatment resulted in the release of nucleosome multimers from the nucleus. From these results, it was hypothesized that a double-strand specific endonuclease is activated upon apoptosis which cleaves the internucleosomal DNA. The factor responsible for apoptotic DNA fragmentation was first purified from HeLa cells as a heterodimeric protein called DNA fragmentation factor (DFF). It consists of 45- and 40-kDa subunits (DFF 40 and 45) [44]. When treated with Caspase-3, DFF 45 is cleaved and DFF 40 was then active to catalyze

Fig. 3 Pathways of GalNAc- and GlcNAc-containing gangliosides in neuronal and cancer cell surfaces as proposed by Basu and Roseman [55,58, and 85–87]

DNA degradation. Later studies purified a 40-kDa DNase from mouse lymphoma WR19L [45] and human Jurkat cells [46]. Similarly, the proteins isolated from the other sources were inhibited by DFF 45, and the inhibition was reversed by treatment with Caspase-3.

Pre-operative chemotherapy is of utmost importance in many forms of cancer, In the case of testicular cancer, one drug commonly used is *cis*-platin (*cis*-DDP) (Fig. 1), in combination with several other drugs including adriamycin, cyclophosphamide, methotrexate, and vincristine. Although *cis*-platin is extremely effective against testicular cancer, it is rarely used to treat other forms of cancer. Recent studies, however, have shown that *cis*-platin is able to induce apoptosis in osteocarcinoma [47] and ovarian cancer cells [48]. A study has also shown that pre-treatment with *cis*-platin increases the efficacy of tamoxifen treatment in breast cancer patients [49].

It is commonly thought that the main target of many anti-cancer drugs, including *cis*-platin, is thought to be DNA. *Cis*-platin has been shown to damage DNA by forming intra- and inter-strand crosslinks by binding to the N7 of adjacent guanines [50]. *Cis*-platin, however, has also been shown to bind strongly to cysteine residues. In fact our studies have shown that *cis*-platin is able to bind to, and inhibit, DNA polymerase-α suggesting another method for the cytotoxicity of *cis*-platin [51]. We have also shown that *cis*-platin binds to the Zn-binding domain of DNA polymerase-α approximately 1000 times stronger than binding to guanine [52]. Structural studies of the Zn-binding domain of DNA polymerase-α have shown that divalent Pt (II) binds, to vicinal cysteine residues within the protein [53]. These studies have led to the hypothesis that during *cis*-platin induced apoptosis, in addition to DNA degradation by DFF, synthesis of DNA may also be inhibited.

In the present study, we report the induction of apoptosis in breast cancer cells (MDA-468 and MCF-7) (Fig. 2) upon treatment with L-PPMP and *cis*-platin. Induction of apoptosis was observed through the binding of the fluorescent dye PSS-380, which binds to cell surface-exposed phosphatidyl serine, and by caspase activation. Upon induction of apoptosis, we observed inhibition of DNA replication enzyme (DNA polymerase-α and DNA helicase-III) activities.

Apoptosis is associated with Le^X expression in various types of cancer cells [54]. It is also observed that GD3 (disialosyl-lactosyl-ceramide; NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer) may additionally affect some yet unknown process to enhance apoptosis [22, 23]. The addition of GD3 or GD1b to both Colo-205 (human colon carcinoma) and SKBR-3 (human breast carcinoma) cells caused apoptosis within 6 h and initiated activation of Caspases (-3 and -9) within 16 to 24 h [23,55]. Our recent observation on the decrease of β1-4GalT-4 expression in Colo-205 after apoptotic activation with L-PPMP [32, 56] may suggest a regulation induced by anti-cancer drugs for metastasis or apoptosis [32]. In a mixture of Le^X-positive and Le^X-negative rat basophilic leukemia (RBL) cells most of the aggregates were composed exclusively of Le^X-positive cells. These data suggest that interactions between Le^X GSLs in opposite cell surfaces are strong enough to allow formation of stable cell to cell contacts [56] which perhaps also occur during metastasis of LeX-positive cancer cells.

However, occurrences of the inhibition of the sialyltransferases and the reduction of cell surface SA-Le^X or the increase of Le^X GSLs during metastasis or apoptosis are not yet known and are being studied now.

Materials and methods

SKBR3 and MCF-7 (Fig. 2) breast cancer cell lines were obtained from Dr. Sipra Banerjee of the Cleveland Clinic, Cleveland, OH. DMEM cell culture medium powder was from Gibco/Invitrogen Corporation (Carlsbad, CA). Penicillin, streptomycin, and L-glutamine were from Gibco. Fetal bovine serum was purchased from Intergen (Purchase, NY) and Gibco. L-PPMP was a gift from Dr. J-I Inokuchi of Hokkaido University, Japan. GF/A glass microfiber filters and Millipore Microcon-30 centrifugal filtration devices were from Fisher Scientific (Pittsburgh, PA). Pierce BCA Micro Protein Assay kit is from Pierce Biotechnology, Inc. (Rockford, IL). Rabbit anti-caspase-8 and -9 polyclonal antibodies were from Cell Signaling Tech., Inc. (Danver, MA). [^{3}H]-dTTP was obtained from Moravek Biochemicals (Brea, CA). Goat anti-rabbit IgG antibody alkaline phosphatase conjugate, NBT (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indoyl-phosphate) alkaline phosphatase developing dye, and all other reagents were from Sigma (St. Louis, MO).

Cell culture

Human breast cancer cell lines (SKBR3 and MCF-7) were grown in DMEM and RPMI-1640 media, respectively, Both were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 mM L-glutamine. When cells were 90% confluent, they were used for passage or harvested for biochemical work. Cell synchronization was performed by treating the semi-confluent cells twice with 0.5 mM hydroxyurea for 24 h under the same culturing conditions. Hydroxyurea was then removed and the cells were treated with the apoptotic reagents, L-PPMP and *cis*-platin, under the indicated conditions.

Western blot for identification of activation of caspases

Drug-treated cells were pelleted and resuspended in 0.1 ml lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, and 50 mM DTT) and homogenized by sonication (3 × 10 s). The homogenized samples were then denatured for 5 min at 95°C and were loaded onto an SDS-PAGE gel. Protein samples (20–25 μg) were loaded onto the gel and blotted to nitrocellulose membranes. Non-specific binding was blocked by incubation in Tris-buffered saline containing 5% bovine serum albumin and 0.1% Tween-20 for one hour at room temperature. The blots were then incubated overnight at 4°C in blocking buffer containing the primary antibody. Antibodies used were rabbit polyclonal antibodies raised against full length human Caspase-8 or -9 diluted 1:500. Afterward, membranes were washed and incubated with anti-rabbit IgG-alkaline phosphatase conjugated antibodies (1:3,000; Sigma). Antibody-conjugated alkaline phosphatase-activity was visualized using the NBT-BCIP reagent in AP buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) [23, 32].

Fluorescence staining with PSS-380 and propidium iodide

Cells cultured on Falcon Microslide System (Fisher) were synchronized two times (24 h each) with 0.5 mM hydroxyurea before treatment with different apoptotic reagents under different conditions. The cells were then washed two times with TES buffer (5 mM *N-tris* [hydroxymethyl]-2-aminoethane sulfonic acid (TES), 150 mM NaCl, pH 7.4) followed by incubation with 0.2 ml new TES buffer containing 25 μM PSS-380 [57] and 0.25 μg/ml propidium iodide for ten minutes at 37°C. The PSS-380 dye was used instead of annexin-V to recognize phosphatidyl serine on the outer leaflet of the plasma membrane [57].

Assay of Sialyltransferases [SAT-2 (CMP-NeuAc; GD3 α2-8 sialyltransferase and SAT-4 (CMP-NeuAc: GM1a α2-3 sialyltransferase)]

Enzymatic assays for SATs were carried out as described previously [58], with some modifications to provide optimum conditions with activities present in the Golgi membrane fractions isolated from MCF-7 cells. The incubation mixtures contained the following components in a final incubation volume of 35 μl:0.5 mM acceptor glycolipids, 300 mM Cacodylate buffer pH 6.5, 30 mM $MgCl_2$, Golgi membrane fraction (20 μl containing 200–400 μg protein), CMP- [^{3}H]-NeuAc (70,000–80,000 CPM, specific activity 3 to 4 × 10^6 CPM/μmole). Triton CF-54 was used at a ratio of protein:detergent (1 to 1) and was used in mixed micelle form during drying of the acceptor glycolipid dissolved in chloroform-methanol (2:1) followed by vacuum drying in a rotary dessicator (Speedvac's original prototype). Incubations were carried out for 2 to 4 h at 37°C, and final results were obtained in the range where the reaction rate remained constant within the incubation time and proportional to the added protein concentrations. The reactions were stopped with the addition of 25 mM EDTA and 20 μl methanol. The mixture was quantitatively transferred to Whatman 1 MM chromatographic paper followed by descending chromatography with 1% sodium tetraborate

(pH 9.1). The appropriate areas of the chromatogram (origin and next inch) were cut and the amounts [^{3}H]-NeuAc transferred to glycolipids was quantitated by a toluene liquid scintillation system.

Assay of DNA polymerase-α

DNA polymerase-α activity was assayed by the extent of incorporation of [^{3}H] dTMP into acid-insoluble activated calf thymus DNA in the presence and absence of aphidicolin as previously described [59]. The components of the assay consisted of 20 μg activated calf thymus DNA, 50 mM Tris-HCl (pH 8.0), 15 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 25 μg BSA, 50 μM unlabeled dNTP, and 50 μM [^{3}H]-dTTP (specific activity 150 to 200 CPM/pmole), and enzyme in a total volume of 0.1 ml. Incubation was carried out for 1 h at 37°C. The reaction was stopped by the addition of 50 μg BSA and 1 ml 10% TCA containing 100 mM sodium pyrophosphate. Reactions were incubated on ice for 30 min to allow complete precipitation to occur. The precipitate was then collected on a GF/A glass filter and washed with 15 ml 5% TCA, 50 mM sodium pyrophosphate, and 3 ml each of 95% ethanol and acetone. The filters were then dried, and the radioactivity incorporated into acid-insoluble material was quantitated by liquid scintillation counting in a toluene-based counting system.

Radioactive oligonucleotide in membrane filtration effluent (ROME) assay for helicase activity

Helicase activity was routinely measured by the extent of [^{3}H]-oligonucleotide (>60 nt) released from a [^{3}H]-calf thymus DNA ([^{3}H]-CT-DNA) substrate [59]. Helicase activity was measured by incubating the enzyme with 1 to 2 μg (800,000 CPM) [^{3}H]-CT-DNA in 20 mM Tris-HCl pH 8.0, 1 mM $MgCl_2$, 4 mM ATP, 150 mM KCl, 8 mM DTT, 4% sucrose, and 100 μg/ml BSA for one hour at 37°C. The assay mixture was then diluted to 150 μl with water and filtered through a Microcon-30 centrifugal filtration device. The filtrate was then separated by descending paper chromatography using 100 ml K-PO_4 pH 6.8, 60 g ammonium sulfate, 2ml n-propanol as the solvent. The origin was counted by liquid scintillation to quantify the amount of released oligonucleotide in the Microcon filtrate.

Results

Dose-response curve after treatment with *cis*-platin and L-PPMP

Synchronized breast carcinoma (MCF-7) cells were treated with varying concentrations of either *cis*-platin (DNA crosslinker / DNA polymerase-α inhibitor) or L-PPMP (GlcT inhibitor for 48 h). Viability was measured by the exclusion of the dye trypan blue [60]. Cells were stained and subsequently counted. The cells that were able to exclude the dye were counted as live cells while cells stained by the dye were counted as dead. Upon treatment with both *cis*-platin and L-PPMP, cell death occurred in a dose-dependent manner. Treatment with *cis*-platin resulted in significant cell death with 10 μM concentration and death of half of the viable cell population with approximately 20 μM treatment (Fig. 4, panel a). MCF-7 cells were more sensitive to treatment with L-PPMP with significant cell death evident upon treatment with 5 μM L-PPMP and death of half of the viable cells upon treatment with 10 μM L-PPMP (Fig. 4, panel b).

Detection of phosphatidylserine translocation after treatment with L-PPMP

Synchronized MCF-7 cells were treated with the GlcT inhibitor L-PPMP. Phosphatidylserine (PS) flop was investigated by staining the treated cells with the novel PS specific dye PSS-380 [57]. In addition, the cells were stained with the DNA binding dye propidium iodide PI to check for membrane permeability. At the beginning of treatment, the majority of the cells are not stained with PSS-380 or PI indicating that the cells are not yet undergoing apoptosis (Fig. 5-Control). After 6 h of treatment, however, the cells are stained heavily with PSS-380 but not stained with PI (Fig. 5, 6 h). This indicates the induction of apoptosis has begun in these cells. PS flop is one of the early phenomena of apoptosis. At this point in the treatment, however, the cell membrane remains intact, indicating the cells are not in the late stages of apoptosis. Twenty-four h of treatment with L-PPMP induced apoptosis in the cells (Fig. 5, 24 h). After 24 h treatment, the cells are heavily stained with both PSS-380 and PI. This indicates the cells are permeabilized and most likely dead at this point.

Activation of caspases by treatment with *cis*-platin and L-PPMP

Caspases are a family of proteases activated upon induction of apoptosis and part of the apoptosis signal transduction pathway [61–67]. Upon activation, caspases cleave a number of intracellular targets including other downstream caspases. Activation of caspases has been shown to result in both nuclear envelope breakdown and chromosomal fragmentation. In the current study, the activation of caspases upon treatment with both *cis*-platin and L-PPMP was investigated. Caspase-3 is a downstream effector caspase. Upon

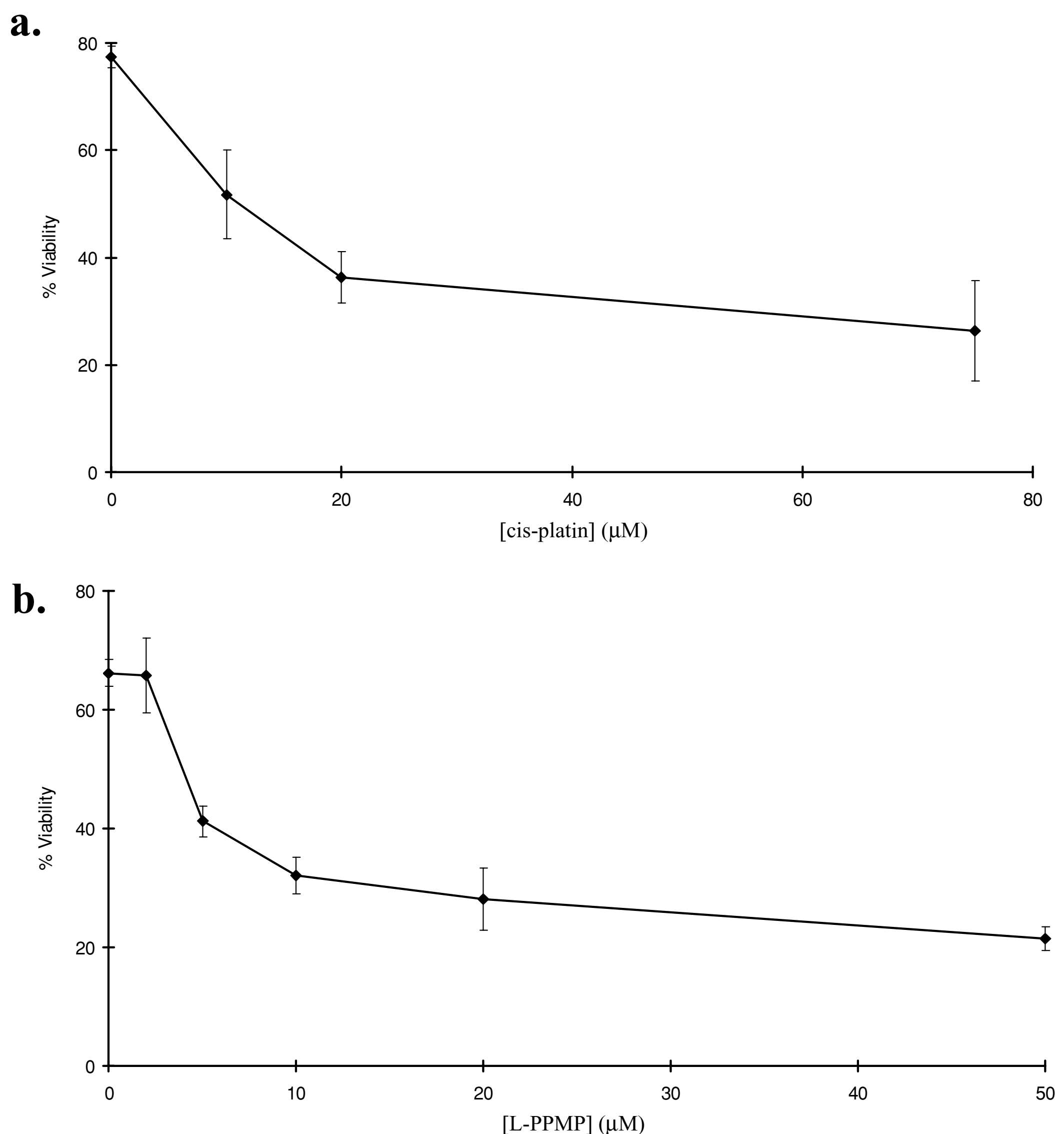


Fig. 4 Viability of human breast carcinoma MCF-7 cells in the presence of *cis*-platin and L-PPMP

activation, Caspase-3 cleaves a number of targets responsible for the effects of apoptosis. We have previously reported the activation of Caspase-3 by treatment of SKBR3 cells with both *cis*-platin and L-PPMP. Activation of Caspase-3 is detected by processing of the pro-form (32 kDa) to the active form (17 kDa). Activation of Caspase-3 was not established at present upon treatment of MCF-7 with *cis*-platin. However, activation of Caspase-9 was observed in both SKBR-3 (Fig. 6, panel a) and MCF-7 (Fig. 6, panel b) after teatment with *cis*-platin.

Apoptosis can be initiated through two convergent pathways: the extrinsic (receptor-mediated) and intrinsic (mitochondrial-mediated) pathways. Initiation of the extrinsic pathway begins with activation of Caspase-8 while initiation of the intrinsic pathway is mediated by activation of Caspase-9 [64–66]. The two pathways then converge through the activation of one of the effector caspases, such as Caspase-3. Since activation of Caspase-3 has already been established, it should be investigated whether this occurs due to the activation of the intrinsic or the extrinsic signaling pathways. Upon treatment with *cis*-platin, activation of Caspase-9 was observed in both MCF-7 (Fig. 6, panel b) and SKBR3 (Fig. 6, panel a) cells. In addition, activation of Caspase-8 was observed in *cis*-platin-treated SKBR3 cells (Fig. 7). These results suggest the induction of both the intrinsic and the extrinsic apoptotic signaling pathways occurred upon *cis*-platin treatment of some breast carcinoma cells [55,67,68]. However, it is reported Caspase-3 is absent in MCF-7 cells [65]. Perhaps the signal is stopped at the Caspase-9 activation level in these cells. Whether any non-caspase pathway is responsible for DNA degradation in MCF-7 cells is under investigation in our laboratory.

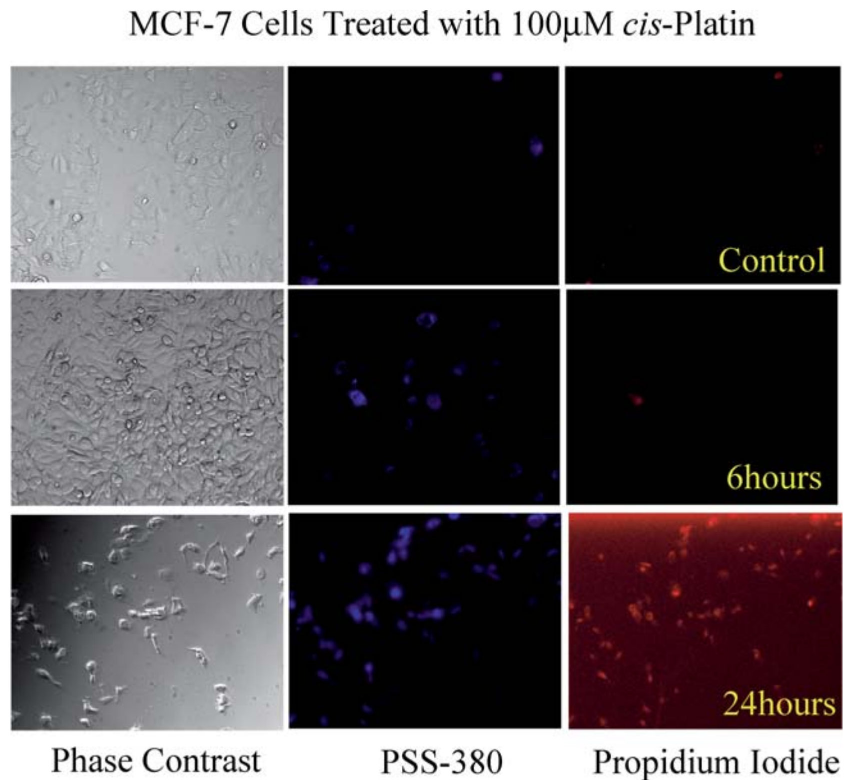


Fig. 5 PSS-380 and Propidium iodide binding to MCF-7 cells during 6 h and 24 h after treatment with 100 μm *cis*-platin

Modulation of SAT activities (SAT-2 and SAT-4) in apoptotic carcinoma cells induced by *cis*-Platin and L-PPMP

A sharp decrease of activity of the glycolipid sialyltransferase: SAT-2 (CMP-NeuAc; GD3 α2-8 sialyltransferase (Fig. 8a) was observed in MCF-7 cells treated with 80 μM cis-platin (70%) or 1 to 8 μM L-PPMP (80%). Under the same conditions the SAT-4 activity (CMP-NeuAc: GM1a α2-3 sialyltransferase) (Fig. 8b) [55,58, 85–87] remained unchanged when treated with *cis*-platin between 10 to 80 μM *cis*-platin. However, a 70 to 80% decrease in both SAT-2 and SAT-4 activities resulted after the treatment with 10 to 80 μM L-PPMP (Fig. 8b). These results suggest different modes of regulations in the apoptotic MCF-7 cells induced by *cis*-platin (thiol binder reagent) or L-PPMP (an inhibitor of GlcT; Fig. 3).

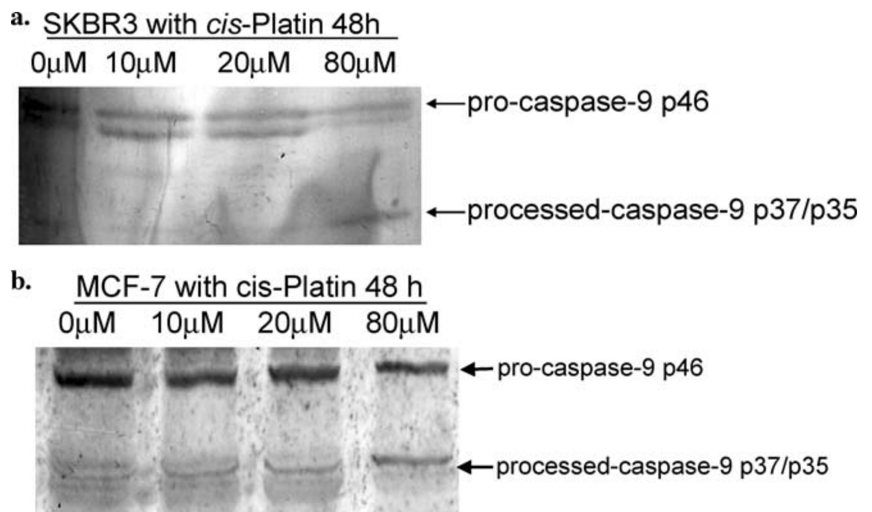


Fig. 6 Western Blot analyses: (a) Activation of Caspase-9 in SKBR-3 cells after 48 h treatment with *cis*-platin (10–80 μM). (b) Activation of Caspase-9 in MCF-7 after 48 h treatment with *cis*-platin (10–80 μM)

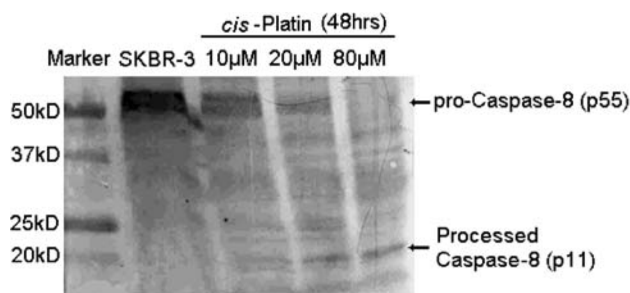


Fig. 7 Western Blot analysis: Activation of Caspase-8 in SKBR-3 cells after 48 h treatment with *cis*-platin (10–80 μM)

Modulation of DNA biosynthetic enzyme activities upon induction of apoptosis by *cis*-platin

Thiol groups are an attractive target for *cis*-platin binding within the cell due to the high intracellular concentration (up to 10 mM) of thiols and the high affinity of *cis*-platin for thiols. Studies have shown that in the presence of physiological thiol concentrations, the amount of *cis*-platin bound to DNA is less than 2% of the total concentration [68]. One potential target of thiol-*cis*-platin binding is DNA polymerase-α. DNA polymerase-α is known to be directly inhibited by *cis*-platin. Inhibition of enzymatic activity was observed when the enzyme was pretreated with *cis*-platin. Alternatively, treatment of the DNA substrate with *cis*-platin previous to the enzymatic assay showed no inhibition of the enzyme [51]. This discounts the presence of platinum-DNA adducts as the method of inhibition. Although the structure of pol-α is still unknown, based on amino acid homology, it is hypothesized that the C-terminal region of the protein contains a four cysteine-zinc binding domain. It is thought that this zinc-binding domain is crucial for enzymatic recognition of the DNA substrate. The proposed model for *cis*-platin inhibition of pol-α envisions *cis*-platin forming covalent bonds with the cysteines, thereby displacing the zinc. The displacement of zinc results in structural perturbation of the zinc-binding domain which renders pol-α unable to bind to the DNA substrate.

Synchronized MCF-7 cells were treated with varying concentrations of *cis*-platin for 48 h and then harvested and homogenized. The resulting soluble protein fraction was used as the enzyme source for the enzymatic activity assays. At low concentrations (10 μM) there was an increase in DNA polymerase-α activity in the MCF-7 cells. At concentrations above 10 μM, however, activity began to be inhibited. The highest concentration of *cis*-platin investigated (80 μM) resulted in an 80% decrease in DNA polymerase-α activity compared with the untreated cells (Fig. 9, panel a). In a similar fashion to DNA polymerase-α, DNA helicase activities were observed to decrease in MCF-7 cells. Like DNA polymerase-α, the DNA helicase-III activity increased slightly upon treatment with 10 μM *cis*-platin. At the higher concentrations, however, activity decreased. After treatment with 80 μM *cis*-platin, DNA helicase-III [69] activity was decreased by 70% compared with the control (Fig. 9, panel b).

In a separate experiment, synchronized MDA-468 cells were treated with varying concentrations of L-PPMP. DNA helicase activity was measured using the soluble protein fraction as the enzyme source. At concentrations as low as 2 μM, a significant decrease in DNA helicase activity was observed. With increasing concentrations of L-PPMP treatment, approximately 50% inhibition was observed.

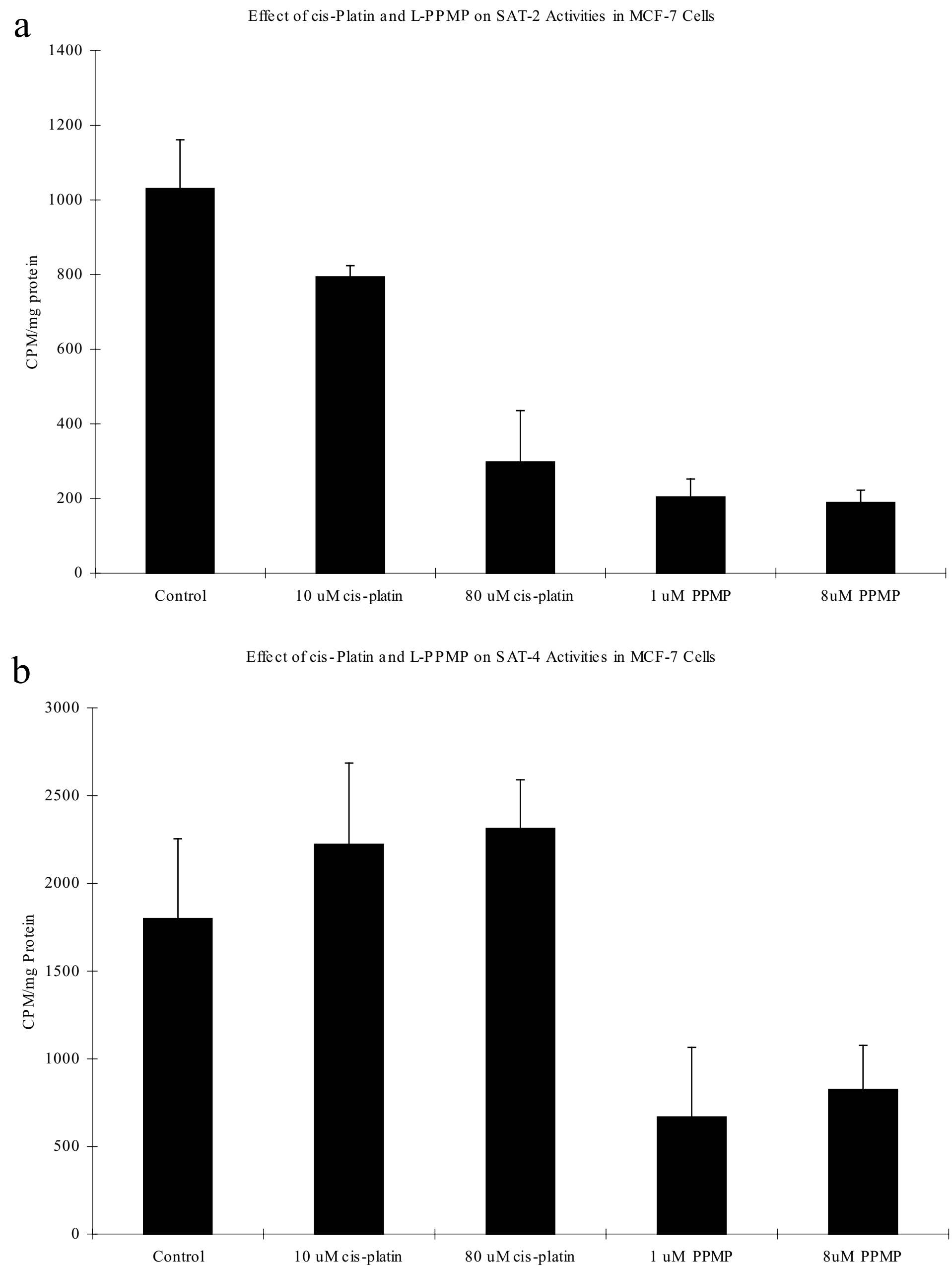
Discussion

Cis-platin was the first in a series of platinum-based compounds demonstrating significant and clinically relevant anti-tumor activity. *Cis*-platin was first synthesized and characterized well over one-hundred years before its anti-tumor activity was recognized [70]. The anti-tumor activity of *cis*-platin was discovered after it was observed by Rosenberg and co-workers that *E. coli* grown in an electric field exhibited filamentous DNA growth indicative of DNA damaging agents and inhibition of cell division. In 1965, it was reported that the cytostatic effect was caused by electrochemical reactions taking place at the platinum electrode forming *cis*-platin [71]. Further studies then revealed that *cis*-platin had potent anti-tumor activity [72].

It is commonly believed that the *cis*-platin binds to and damages DNA and the observed cytotoxic effects of *cis*-platin are due to DNA damage. It has been shown that *cis*-platin cause inter- and intra-strand lesions by binding to the N7 atom of adjacent guanines [73]. *Cis*-platin has been shown to induce apoptosis in osteocavcinoma cells through activation of caspases −3, −6, and −9 [47].

Reports from our laboratory, however, have suggested an alternative mechanism of *cis*-platin-induced cytotoxicity. We have reported that DNA polymerase-α from PA-3 (rat prostate) is inhibited through direct interaction with the enzyme. It was also observed that, upon treatment with *cis*-platin, there was a concomitant release of zinc. Finally, it was shown that *cis*-platin binds to thiols much stronger than it binds with guanine [51]. This led to the hypothesis that *cis*-platin inhibits DNA polymerase-α by binding to the zinc-binding domain, displacing the zinc, and perturbing the structure of the domain [52,53]. It is thought the enzyme recognizes the DNA substrate through the zinc-binding domain, so structural perturbation may interfere with the ability of the enzyme to recognize its substrate. This hypothesis was investigated through a series structural studies looking at the ability of the enzyme to bind *cis*-platin. First, it was shown that *cis*-platin is able to bind to a nonapeptide from near the

Fig. 8 Decrease in DNA Polymerase-α and Helicase activities in synchronized MCF-7 cells treated with *cis*-platin (10–80 μM) and L-PPMP (2–16 μM)



zinc-binding domain from DNA polymerase-α through two cysteines separated by a proline [74]. It was later shown that *cis*-platin binds to a 38-mer from the zinc-binding domain of DNA polymerase-α and causes severe structural changes in the peptide [75].

All of the previously mentioned studies regarding *cis*-platin and DNA polymerase-α interaction have involved *in vitro* studies. In the current studies, we sought to determine whether *cis*-platin inhibition of DNA polymerase-α occurs in cultured cells. Breast cancer cells (MCF-7, MDA-468, and SKBR3) were treated with *cis*-platin under various conditions (concentration and time). Through several biochemical methods, it was shown that *cis*-platin induced apoptosis in these cell lines. Phosphatidyl serine randomization was observed in a time-dependent manner through the binding of the novel fluorescent dye PSS-380 to plasma membrane exposed phosphatidyl serine. This was shown to occur within 6 h of *cis*-platin treatment indicating that phosphatidyl serine randomization is one of the early processes of apoptosis. Also, it was shown that Caspase-3 was activated by *cis*-platin in a dose-dependent manner. Activation of Caspase-3 is one of the downstream steps of both extrinsic (receptor-mediated) and intrinsic (mitochondria-mediated) pathways of apoptosis [76]. Therefore, activation of Caspase-3 indicates that apoptosis is occurring through one of these pathways. Finally, it was shown that treatment with *cis*-platin causes genomic

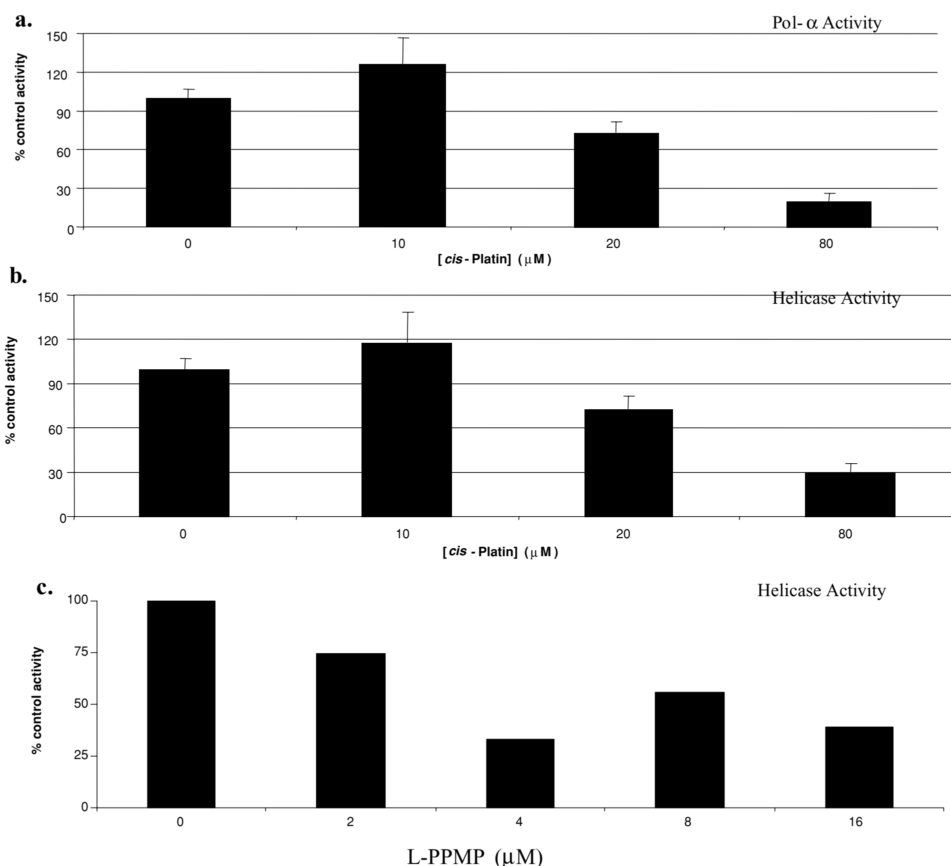


Fig. 9 Decrease in sialyltransferase activities (SAT-2, CMP-NeuAc: GM3 a2-8 sialyltransferase; SAT-4, CMP = NeuAc: nLcose4-cer a2-3sialyltransferase) in human breast carcinoma MCF-7 cells after treatment with *cis*-platin (10–80 μM) and L-PPMP (2–8 μM)

DNA degradation in a dose-dependent manner after forty-eight hours of treatment. Genomic DNA degradation is one of the late steps of apoptosis. Taken together, these three pieces of data indicate that *cis*-platin is initiating apoptosis in these cell lines and initiation of apoptosis is occurring in a dose-dependent and time-dependent manner.

As was reported previously, *cis*-platin is known to inhibit DNA polymerase-α *in vitro*. In the current study, we investigate the inhibition of DNA polymerase-α by *cis*-platin treatment of cultured cells. Breast cancer cells were treated with *cis*-platin under the indicated conditions. After treatment, the cells were harvested, lysed, and assayed for DNA polymerase-α and DNA helicase activity.

In all three cell lines tested, treatment with *cis*-platin inhibited both DNA polymerase-α and DNA helicase activities. Interestingly, the cell lines exhibited different levels of inhibition holding true for both cell lines. In both cases, enzymes from MCF-7 cells were most resistant to inhibition, followed by MDA-468, and finally SKBR3 was least resistant to inhibition. There can be several explanations for this observation. The cell lines may differ in the uptake of *cis*-platin. SKBR3 cells may be more efficient in uptake of *cis*-platin than MCF-7 cells. Alternatively, the cells may take up the drug with equal efficiency but the efflux of *cis*-platin may not be equal among the cell lines. Also, since it was shown that *cis*-platin binds strongly to thiols, perhaps the cell lines express differing amounts of thiol-rich proteins, or they may up-regulate expression of thiol-rich proteins in response to *cis*-platin treatment, as seen in *cis*-platin-resistant cell lines. As is apparent in the western blots of the enzymes decreased enzymatic activity at higher concentration *cis*-platin may result from either apoptosis-related proteolytic cleavage of the enzyme or loss of the intracellular concentration of the enzyme due to increased membrane permeability. At lower concentrations of *cis*-platin treatment, however, inhibition of enzymatic activity and concomitant decrease of the stability of the replication complex (Fig. 10) was observed (data not shown and to be published elsewhere) while the amount of the enzyme remains relatively constant as determined by western blot analyses [59].

The acidic glycosphingolipids such as GD3, sialyl-Le^a and sialyl-Le^X were demonstrated to be highly expressed in many malignant cancers. The levels of expression of these carbohydrate tumor markers are also positively correlated with patient survival and are an indicator of the metastatic behavior of invasive cancer cells [77–84]. These glycosphingolipids of the ganglioside family (GD3; NeuAcα2-8NeuAcα2-3Galβ1-4Glc-ceramide [77–80] and Lewis family (having a core tetraglycosylceramide; Galβ 1-3/or 4(Fuc α1,4) GlcNAcβ1-3Galβ1-4Glc-ceramide)-containing sialic acid, bind with selectins [81–84] expressed on the cell surfaces. Inhibition of synthesis of these GSLs might prevent metastasis

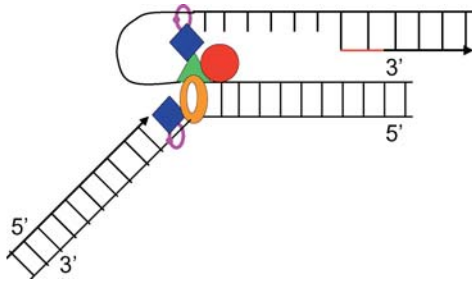


Fig. 10 A model for helicase activity in a multienzyme replication complex. Red circle—DNA Poymerase-α; blue square, DNA Polymerase-δ; green triangle, Primase; yellow circle, Helicases; pink circle, PCNA; Upper DNA strand is the lagging strand and the lower DNA strand is the leading strand

of breast cancer cells. Our present observation about regulation (sharp reduction) of both SAT-2 (GD3 synthase) and SAT-4 (iso-enzyme of SAT-3 responsible for biosynthesis of SA-Le^X) during apoptosis of breast carcinoma cells (MCF-7) could be an added feature for the increased potency and specificity of the glycolipid:glycosyltransferases required for therapeutic purposes. Biosynthetic pathways (Fig. 3) [55,58, 85–88] for GD3 ganglioside and sialyl-Le^X in embryonic brain cells [58,85–88] and non-apoptotic carcinoma [84–87] cells have been proposed by Basu *et al.* and Roseman and his coworkers in last three decades. Regulation of expression of these glycolipid: glycosyltransferases during apoptosis induced by anti-cancer drugs or agents are not known until now.

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