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3′-Azidothymidine significantly alters glycosphingolipid synthesis in melanoma cells and decreases the shedding of gangliosides

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In this report, we establish that 3′-azido-3′-deoxythymidine (AZT) treatment of melanoma cells greatly alters the pattern of glycosphingolipid biosynthesis. In SK-MEL-30 cells, synthesis of the gangliosides GM3 and GD3 was significantly inhibited (60% and 50% of control, respectively) and the production of their precursor, lactosylceramide, was stimulated by 2.5-fold. Control experiments established that phospholipid synthesis was not affected by AZT treatment, consistent with AZT treatment only affecting lipid biosynthetic reactions that involve glycosylation. Likely as a consequence of decreased rates of ganglioside synthesis, AZT treatment of SK-MEL-30 cells also significantly suppressed the amount of gangliosides shed from the membranes of these cells. Since shedding of gangliosides has been proposed to allow melanoma cells to avoid destruction by the immune system and alterations of glycosphingolipid levels are likely important for the malignant cell phenotype, these results may have important implications regarding the potential use of AZT or related glycosylation inhibitors as cancer chemotherapeutics.

Keywords: **melanoma, AZT, cancer, ganglioside**

Abbreviations: **AZT, 3′-azido-3′-deoxythymidine; Cer, ceramide; Crbr, cerebroside; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; GD3, disialyl lactosylceramide; GM3, sialyl lactosylceramide; HPTLC, high-performance thin layer chromatography; LacCer, lactosylceramide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NeuAc, N-acetylneuraminic acid; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.**

Introduction

Recent *in vitro* and whole cell work with human erythroleukemia cell lines has established that the anti-HIV chemotherapeutic 3′-azido-3′-deoxythymidine (AZT) potently inhibits protein and lipid glycosylation [1,2]. These effects of AZT most likely result from intracellular accumulation of its monophosphate form, AZTMP, which has been shown to competitively inhibit pyrimidine nucleotide-sugar import into the Golgi lumen and, consequently, modify the macromolecular glycosylation reactions that occur in this organelle. Although AZT treatment affects both protein and lipid glycosylation reactions, the synthesis of acidic glycosphingolipids shows the most severe alterations. In light of the involvement of these glycolipids in numerous cellular processes, it is predicted that this inhibition of their synthesis may lead to a number of biological effects including altered growth, adhesion, differentiation, and perhaps cytotoxicity [3–6].

Oncogenic transformation is often accompanied by drastic changes in glycosylation patterns that may be essential for the transformation process [7,8]. In melanoma cells, for example, the overexpression of the gangliosides GM3 and GD3 may help impart many of the oncogenic characteristics of these tumor cells [9]. Studies using monoclonal antibodies and classical glycosylation inhibitors have demonstrated a crucial role for gangliosides in the growth and adhesion of melanoma cells [3,10]. Furthermore, changes in ganglioside content of melanoma cells correlate with their metastatic potential [8].

The shedding of gangliosides from the cell membrane into the surrounding medium may be an important mechanism by which some tumor cells avoid destruction by the

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immune system [11–13]. For example, melanoma cells shed large amounts of gangliosides, both *in vitro* and in patients [14,15]. Shed glycosphingolipids potently modulate leukocyte receptor function, thereby preventing activation and proliferation of these cells and, consequently, abrogating the immune response in the tumor microenvironment [16].

Together, these data suggest that inhibition of ganglioside synthesis will impair the ability of melanoma cells to metastasize as well as make them more susceptible to destruction by the immune system due to reduced amounts of shed gangliosides. Consistent with this hypothesis, PDMP, a powerful inhibitor of glycosphingolipid synthesis, has been shown to inhibit metastasis and adhesion of various tumor cells in both animal and *in vitro* model systems [17].

To establish the potential of AZT as an anticancer chemotherapeutic via effects on glycosylation, we examined the impact of AZT on glycosphingolipid synthesis in cultured melanoma cells. AZT treatment potently inhibited the synthesis of gangliosides whereas the production of the neutral precursors to these glycolipids was stimulated. The amount of gangliosides shed was also significantly suppressed by AZT treatment. In contrast, the synthesis of phospholipids, a predominantly cytosolic process, was unaffected. The importance of these results with respect to AZT as a potential cancer chemotherapeutic is discussed.

Experimental Procedures

Materials

SK-MEL-30 cells were obtained from the American Type Culture Collection. Cells were maintained in and all experiments performed in RPMI 1640 media supplemented with 10% fetal calf serum, 100 mg/liter penicillin G, and 100 mg/liter streptomycin. Cell culture media and bovine fetal serum were obtained from Life Technologies, Inc. or Sigma. D-[1-^{14}C]Galactose was obtained from American Radiolabeled Chemical or DuPont NEN. L-[U-^{14}C]Serine and [^{32}P]orthophosphate were purchased from DuPont NEN. AZT, lipid and glycolipid carriers used in the extractions were from Sigma and HPTLC lipid standards were obtained from Matreya Inc.

Labeling and quantitative analysis of membrane glycosphingolipids

Unless otherwise indicated, glycosphingolipids were labeled by growing 1.5–2.5 $\times$ 10^5 cells in 2 mL of medium containing either [1-^{14}C]galactose (0.5 μCi/mL) or [U-^{14}C]serine (0.5 μCi/mL) for 24 h. Lipids were analyzed using the method described in Yan *et al.* [2]. Briefly, metabolically labeled cells were trypsinized for one minute and transferred to glass tubes, washed with PBS, and extracted consecutively with 5 ml each of 1:2, 1:1, and 2:1 $CHCl_3$:MeOH (v/v). Purified carrier disialogangliosides and ceramides (2.5 μg each) and 1.5 $\times$ 10^5 cpm of [^{3}H]N-acetylsphingosine were added during the first extraction to improve recovery and normalize the amount of lipids recovered, respectively. The pooled extracts were treated with methanolic NaOH (50 mM) for 2 h at 37°C to hydrolyze phospholipids. Following neutralization, lipids were desalted by chromatography over a Waters C-18 SepPak. Sphingolipids were separated by silica HPTLC (Whatman) developed using $CHCl_3$: MeOH: 0.22% aqueous CaCl2 (60:35:8, v/v/v) or $CHCl_3$: MeOH: 0.22% aqueous $CaCl_2$: CH_3COOH (60:35:7:1, v/v/v). Equal amounts of ^{3}H were loaded for each sample so as to normalize for total recovery in each sample. [^{14}C]-Labeled compounds were visualized and quantified using a Phosphorimager (Molecular Dynamics). Lipid standards were detected with orcinol/H_2SO_4 reagent.

Purification of shed glycosphingolipids

Analysis of shed lipids was performed using a slight modification of the procedure described above. SK-MEL-30 cells were labeled with [^{14}C]galactose for 24 h as before. The medium in which the cells grew was transferred into a glass tube, and the cells remaining in the plate were washed twice with PBS that was then combined with the medium. After brief centrifugation to remove any whole cells (100 $\times$ g $\times$ 10 min.), the supernatant was centrifuged for 25 min at 70000 $\times$ g in order to remove microsomes and other cellular debris. The shed lipids were then purified using the procedure described above, separated by HPTLC developed using $CHCl_3$: MeOH: 0.22% aqueous $CaCl_2$ (65:45:8), and quantified by Phosphorimager analysis.

Labeling and quantitative analysis of phospholipids

Phospholipid synthesis was analyzed by first incubating SK-MEL-30 cells (2.0 $\times$ 10^5) in 2 mL of medium containing [^{32}P] orthophosphate (50 μCi) for 24 h. At the end of the incubation, the cells were washed three times with PBS and harvested in 1.8 mL of PBS using a cell scraper (Costar). Following transfer to glass tubes, 4 mL of MeOH and 2 mL of $CHCl_3$ were added and the tubes vortexed to create a Bligh and Dyer monophase [18]. Sphingomyelin, PC, PS, PI, PE, and PA (50 μg each) were added to serve as TLC standards, followed by 1 mL of $CHCl_3$ and 0.9 mL of PBS. After rapid mixing, the samples were centrifuged at 1000 $\times$ g for 5 min. to form a Bligh and Dyer biphase. Upon removing the upper aqueous phase and washing the lower organic phase three times with practical aqueous phase, the organic phase was dried under N_2 and the residue resuspended in $CHCl_3$:MeOH (9:1). Phospholipids were separated using 2D silica gel TLC (Merck) developed using $CHCl_3$:MeOH:HOAc (65:25:10) in the first dimension and $CHCl_3$:MeOH:formic acid (65:25:10) in the second dimension. [^{32}P]-Labeled compounds were visualized and quanti-

fied using a Phosphorimager. Lipid standards were detected with iodine vapors.

Determination of cell growth rate

Cells (1.0×10^5) were plated in 60 mm dishes (Corning) and grown for 24 h in 8 mL of RPMI 1640 before treatment with various concentrations of AZT. Cell proliferation was assessed using the colorimetric MTT assay [19]. After incubation of the cells for various amounts of time, the medium was replaced with 7 mL of 1 mg/mL MTT in RPMI 1640 without phenol red (Sigma). Cells were incubated for 30 min at 37°C, at which time the medium was removed and replaced with 10 mL DMSO to solubilize the product formazan. Quantitation was performed at 562 nm using an ELISA plate reader (Molecular Devices). All time points were measured in triplicate.

Synthesis of [^{3}H] N-acetylsphingosine

[^{3}H]-Acetic anhydride (0.096 mmol, 52 mCi mmol^{-1}) was added to 3 mg (0.01 mmol) of sphingosine dissolved in 0.3 mL of iPrOH. After incubating the reaction for 1.5 h at room temperature, 0.31 mL of 0.1 M KCl, 33 mM Tris, pH 7.4 were added to quench any remaining acetic anhydride. The reaction products were loaded onto a Waters C18 SepPak equilibrated with 1:1 MeOH: 0.1 M KCl, and the SepPak was then washed with 15 mL of ddH_2O. [^{3}H]-N-Acetylsphingosine was eluted by washing the SepPak sequentially with 1 mL of MeOH, 3 mL of 1:1 MeOH:$CHCl_3$, and 2 mL of 1:2 MeOH:$CHCl_3$. Products were analyzed for purity by HPTLC on silica gel developed using $CHCl_3$:MeOH:0.22% $CaCL_2$ (60:35:8). Detection with $KMnO_4$ revealed one product (R_f = 0.95) that comigrated with authentic ceramide (N-Acylsphingosine), and scintillation counting of the HPTLC showed that >99% of the [^{3}H] was present in this compound. The R_f of sphingosine under the described solvent conditions was 0.5. The final yield of [^{3}H]-N-acetylsphingosine was 0.006 mmol.

Results

We initially examined the effects of AZT on glycosphingolipid biosynthesis in the melanoma cell line SK-MEL-30. The plasma membrane of these cells contains high levels of acidic glycosphingolipids that may be important for their oncogenic phenotype. The effect of AZT treatment on glycosphingolipid synthesis was assessed by incubating cells with [^{14}C]galactose to label all newly synthesized glycosphingolipids, and analyzing the composition of these lipids by HPTLC (Fig. 1). Glycolipids were identified by comigration with standards under neutral and acidic solvent conditions. Major glycosphingolipids identified were the gangliosides GM3 and GD3, while two neutral species, lactosylceramide and glucosylceramide, constitute a smaller but significant fraction of the glycolipid membrane composition. GM3, GD3, and lactosylceramide were all present as doublets of equal intensity indicating significant modification of the fatty acid moieties of these glycolipids. This composition is similar to that previously reported for other melanoma cell lines [10,15].

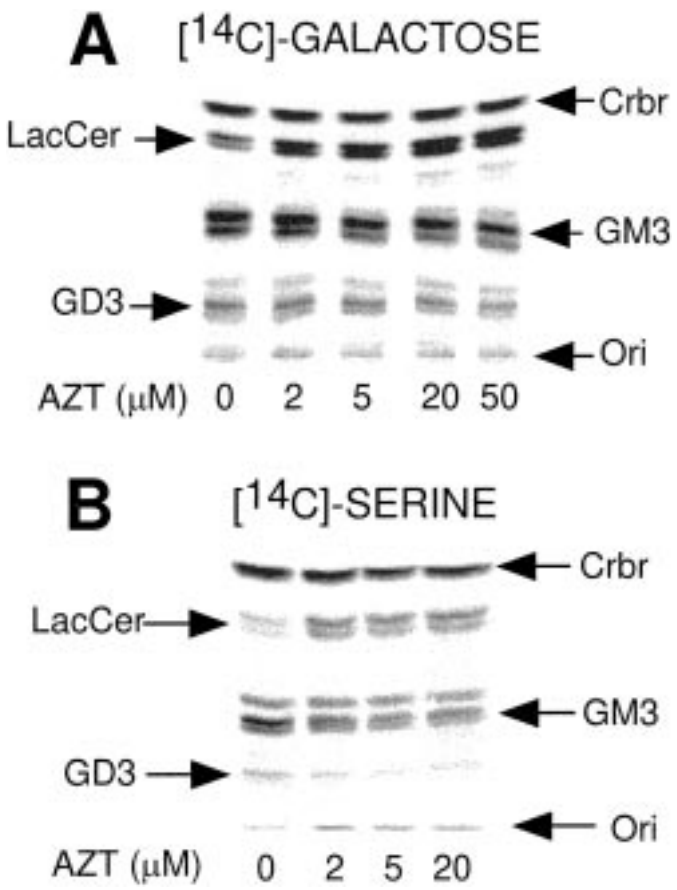

Figure 1. AZT treatment dramatically alters the synthesis of glycosphingolipids in SK-MEL-30 cells. Cells were labeled with [^{14}C]galactose (panel A) or [^{14}C]serine (panel B) for 24 h in the presence of the indicated AZT concentrations, and glycolipids were analyzed by HPTLC as described under Experimental Procedures. The phosphorimages of representative chromatograms obtained under neutral solvent conditions are shown. The mobilities of standards are indicated.

AZT treatment alters glycosphingolipid synthesis

Figure 1 shows that AZT treatment of SK-MEL-30 cells greatly altered the pattern of glycosphingolipid biosynthesis in a dose-dependent manner. Quantitative analysis of the data demonstrated that AZT treatment significantly inhibited synthesis of GM3 and GD3 while stimulating the production of their common precursor, lactosylceramide (Fig. 2). Metabolic labeling with the non-carbohydrate glycosphingolipid precursor, [^{14}C]serine, was performed to verify that the effects of AZT did not result from changes in sugar uptake or nucleotide sugar precursor pools. AZT

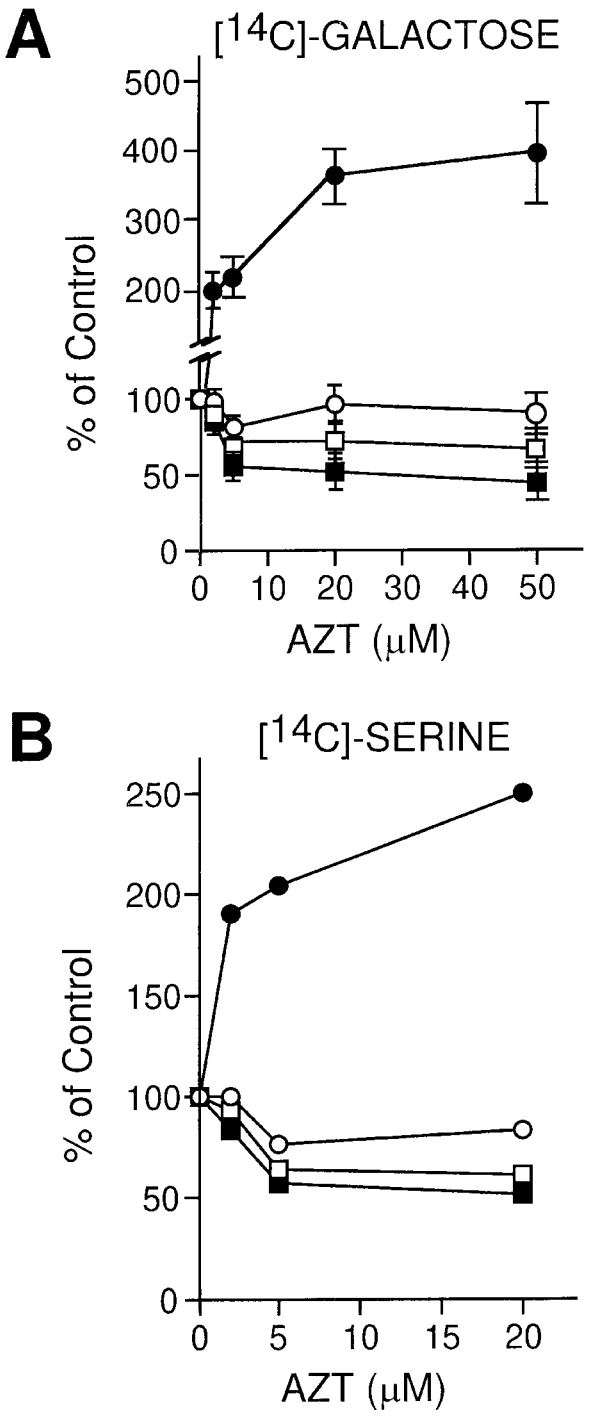

Figure 2. Quantitative analysis of the effects of AZT on glycosphingolipid synthesis. (Panel A) SK-MEL-30 cells were labeled with [^{14}C]galactose for 24 h in the presence of various AZT concentrations. For each sample of purified glycosphingolipids, equal amounts of total glycolipid originally extracted from the cells was loaded on the HPTLC. The amount of each species present at each AZT concentration is given relative to the amount present in untreated cells. The average of three independent experiments is shown. The lipids analyzed and their corresponding plot symbols are as follows: ○, cerebrosides; ●, lactosylceramide; □, GM3; ■, GD3. (Panel B) SK-MEL-30 cells were labeled with [^{14}C]serine for 24 h in the presence of various AZT concentrations. Glycolipids were analyzed and quantified as described for Panel A.

treatment again reduced ganglioside synthesis and caused accumulation of neutral glycolipids to levels very similar to those observed when using [^{14}C]galactose as a label (Figs. 1 and 2). Due to the similarity of effects observed using both metabolic labels, it is likely that the effects of AZT on glycosphingolipid synthesis do not result from changes in sugar metabolism. The small quantitative differences probably arise because only one [^{14}C]serine is incorporated per sphingolipid, whereas different numbers of [^{14}C]galactose may be incorporated into each lipid species due to the rapid equilibration of UDP-[^{14}C]glucose and UDP-[^{14}C]galactose by cellular epimerases (see Table 1).

The onset of AZT's effects is rapid

In the lipid-labeling experiments presented in Figure 1, cells were treated and labeled for 24 hours prior to analysis. Short treatment and labeling times were examined to test the possibility that the effects of AZT were due to alterations in the biosynthesis of the glycosylation machinery (sugar transferases, etc.), as opposed to direct effects on glycosylation. After treating cells with AZT for only 30 min., [^{14}C]galactose was added and newly synthesized glycolipids were analyzed after an additional 90 min incubation. This brief treatment and labeling time yielded results similar to those observed in the long term labeling and treatment experiments described above (Fig. 3). Quantitative analysis of the data in Figure 3 showed that 20 μM AZT increased lactosylceramide synthesis by 2.5-fold and inhibited GM3 and GD3 synthesis by 30 and 47%, respectively. These results indicate that changes in glycolipid synthesis are rapid and unlikely to result from AZT-induced alterations in the levels of proteins involved in glycosphingolipid synthesis.

The effects of AZT on glycosphingolipid synthesis were readily reversible. SK-MEL-30 cells were treated with 0 or 20 μM AZT for 24 h followed by removal of the drug, incubating in fresh medium for 5 h, and subsequent labeling with [^{14}C]galactose for 6 h. Cells were incubated in the fresh medium for 5 h before metabolic labeling in order to allow the cells to condition the medium and degrade most of the intracellular AZT metabolites. This incubation should be sufficient since previous studies with various human cell lines demonstrated that AZTMP is degraded with a half-life of 30–60 min [20–22]. The composition of newly synthesized glycosphingolipids recovered from AZT-treated and untreated samples following the labeling period was identical (±5%, data not shown). Thus, the effects of AZT are readily reversible, consistent with inhibition being due to the intracellular accumulation of AZTMP.

AZT treatment has no effect on phospholipid metabolism

AZT likely exerts its effect on glycosylation through its metabolite AZTMP, a competitive inhibitor of pyrimidine

Table 1. Structure of glycosphingolipids synthesized by SK-MEL-30 cells

Glycosphingolipid (abbreviation)	*Structure*
Glucosylceramide (GlcCer)	Glcβ1→1 Cer
Lactosylceramide (LacCer)	Galβ1→4Glcβ1→1 Cer
GM3	NeuAcα2→3Galβ1→4Glcβ1→1 Cer
GD3	NeuAcα2→3NeuAcα2→3Galβ1→4Glcβ1→1 Cer

nucleotide sugar import into the lumen of the Golgi complex. This mechanism of inhibition predicts that phospholipid synthesis, a process occurring predominantly on the cytosolic face of Endoplasmic Reticulum membranes and not involving sugar addition, will not be affected by AZT treatment. In order to test this hypothesis, SK-MEL-30 cells were metabolically labeled with [^{32}P]orthophosphate for 24 h in the presence of 0 or 50 μM AZT. Treatment with AZT had no significant effect on the amount (data not shown) and pattern of labeled phospholipids after 24 h (Table 2). These results demonstrate that AZT treatment does not affect all lipid biosynthesis, and suggest that the effects are limited to those reactions dependent on nucleotide-sugar import into the Golgi complex.[1]

AZT treatment reduces the amount of gangliosides shed from SK-MEL-30 cells

Melanoma cells shed large amounts of gangliosides, and the immunosuppressive properties of these gangliosides may be one way in which these cells avoid destruction by the immune system [12,14,16]. In order to test whether AZT treatment affects ganglioside shedding, cells were labeled with [^{14}C]galactose for 24 h in the presence or absence of AZT, and glycosphingolipids released into solution analyzed. Initially, we compared the composition of glycosphingolipids released into solution prior to any further processing. As shown in Figure 4, the shed fraction contained surprisingly large amounts of the neutral cerebrosides and lactosylceramide. Since Ladisch and coworkers recently showed that gangliosides can be released from cells either in a soluble form or as vesicles [23], we subjected the medium to ultracentrifugation in order to precipitate any vesicles, and then analyzed the soluble fraction as well as the pellet (Figs. 4 and 5). The pellet contained 13% of the total radioactivity incorporated into shed glycosphingolipids, and had a glycosphingolipid composition that was extremely similar to the cell membrane. The remaining 87% of the total radioactivity present in the soluble fraction, however, was greatly enriched in GM3 and GD3, and contained much less lactosylceramide and cerebrosides than did cell membranes. The reduced amount of neutral glycosphingolipids in the soluble shed fraction is consistent with their lower H_2O solubility as compared to that of gangliosides. Potentially, these glycosphingolipid-containing vesicles could have been released either from dying cells or as a normal metabolic process. In order to minimize the possibility that analysis of the shed glycosphingolipids might include those associated with vesicles and/or membrane fragments produced by any dying cells, the medium was cleared by ultracentrifugation for all further studies.

Quantitative analysis of the shed gangliosides reveals that treatment with low AZT concentrations inhibits the shedding of both GM3 and GD3 to an extent similar to that observed for the GM3 and GD3 remaining in the membrane (Figs. 5 and 2). As noted earlier, two forms of GM3 are present in the cell membrane. Figure 5A shows that both forms of GM3 are also shed into the medium. Further analysis of the GM3 doublet reveals that the majority of the shed glycolipids comigrate with the slower migrating, more hydrophilic component, whereas equal amounts of each species were present in the plasma membrane.

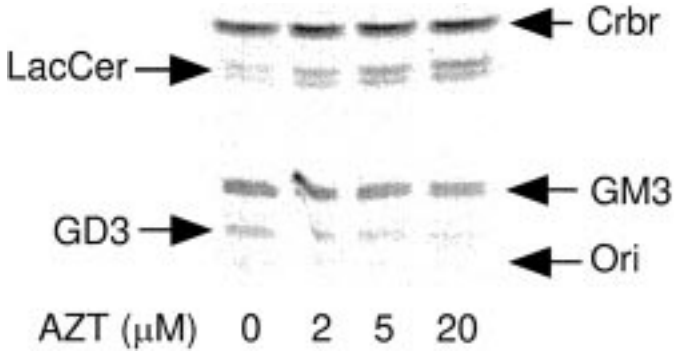

Figure 3. AZT treatment of SK-MEL-30 rapidly affects glycosphingolipid synthesis. Cells were pretreated with various AZT concentrations for 30 min followed by 90 min labeling with [^{14}C]galactose. The glycolipids were isolated and analyzed as described in Figure 1.

[1]Similar experiments performed using the erythroleukemia lines K562 and HEL revealed that AZT treatment did not alter phospholipid metabolism in these cell lines (data not shown).

Table 2. AZT treatment of SK-MEL-30 cells has no effect on phospholipid synthesis.

Phospholipid	*0 μM*	*50 μM*
PC	47 ± 3	46 ± 4
PE	36 ± 1	35 ± 1
PI	3.5 ± 1.3	3.3 ± 0.6
PS	12 ± 3	15 ± 2
PA	4.6 ± 1.4	4.3 ± 1.0
SM	1.6 ± 1.3	1.4 ± 1.1

Cells were labeled with [^{32}P]orthophosphate for 24 h in the presence or absence of 50 μM AZT. The phospholipids were isolated and analyzed by 2D-TLC as described under Experimental Procedures. AZT had no effect on the total amount of labeled phospholipids recovered. Equal amounts of [^{32}P]-labeled phospholipid were loaded from each sample and the amount of each phospholipid present were expressed as a fraction of the total present. The average of three independent experiments with standard deviation is reported here.

AZT treatment inhibits cell growth in culture

The profound effects of AZT on the synthesis of several classes of glycolipids reported above could easily interfere with the growth properties of treated cells. Indeed, AZT has been shown to be mildly cytostatic and/or cytotoxic in a variety of different cell lines [25,26]. To test this possibility, cells were grown in the presence of various concentrations of AZT and cell proliferation was measured at various times by means of the MTT assay. Similar to the effects of AZT on the growth of other cell lines, AZT treatment inhibited the growth of SK-MEL-30 cells in a dose-dependent manner (Fig. 6). After 12 h treatment with AZT, there was less than 5% growth inhibition at all the AZT concentrations tested, while 24 h treatment with 20 μM AZT decreased the cell number by only 10%. Importantly, these data indicate that the effects of AZT on glycosphingolipid synthesis cannot be accounted for by changes in the growth rate. The effect of 20 μM AZT on the growth rate also was measured at longer time points. Interestingly, AZT now exhibited much more significant inhibition of growth. Prior to AZT treatment, the doubling time of cells was 21 h, while after treatment for 48 h, the doubling time increased to 24 h, and after treatment for 96 h, the doubling time increased further to 55 h.

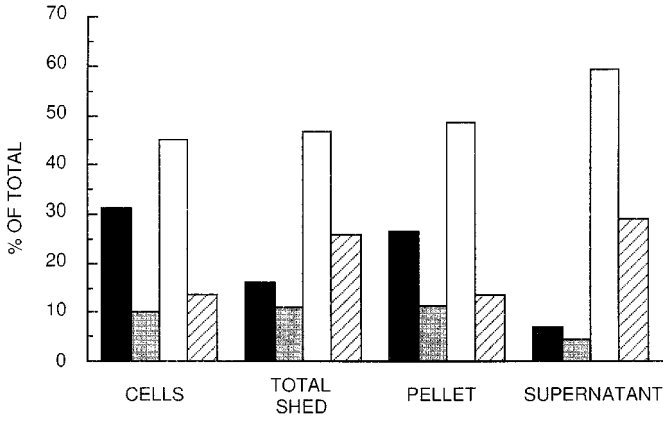

Figure 4. Analysis of relative amounts of glycolipids in cell membrane and shed fractions. Cell monolayers were labeled with [^{14}C]galactose for 24 h. Glycosphingolipids were recovered from both cell pellets and the medium before and after ultracentrifugation and analysis by HPTLC as described under Experimental Procedures. The fraction of total radioactivity in Crbr (solid bars), LacCer (gray bars), GM3 (open bars), and GD3 (hatched bars) is shown.

Discussion

The studies described above demonstrate that AZT treatment of cultured melanoma cells greatly alters glycosphingolipid synthesis. These data not only establish AZT as a general modulator of glycosylation reactions in human cell lines [2], but, in light of the importance of changes in glycosylation states to the phenotype and function of malignant cells, also raise the possibility that this nucleoside analog may prove to be a useful cancer chemotherapeutic.

AZT treatment inhibited the synthesis of acidic glycosphingolipids and caused accumulation of lactosylceramide. Several novel observations from this study support previous work showing that competitive inhibition of the import and accumulation of nucleotide-sugars in the Golgi apparatus by AZTMP is the most likely molecular mechanism for these effects of AZT [2]: (i) AZT treatment does not alter phospholipid synthesis, a predominantly cytosolic process that does not involve glycosylation reactions; (ii) the onset of AZT's effects is rapid and, therefore, likely does not involve alterations in protein synthesis; (iii) similar effects are observed whether using either galactose or serine as label, indicating that effects on glycosylation were not due to changes in sugar metabolism; and (iv) the effects are readily reversible upon removal of AZT. Importantly, since AZT treatment of SK-MEL-30 cells only slightly inhibits their growth at 24 h ($\leq$10%), the decreased production of gangliosides is not primarily due to an indirect effect on cell proliferation but rather a consequence of AZTMP's effect on nucleotide-sugar import.

The pattern of inhibition suggests that AZTMP affects most severely those reactions dependent on import of CMP-sialic acid into the Golgi complex. This observation cannot result from enhanced sensitivity of the CMP-sialic acid transporter to AZTMP since previous studies showed that it is ca. 6-fold less sensitive than the UDP-GlcNAc transporter [1]. Rather, the enhanced sensitivity indicates that import of CMP-sialic acid is more rate limiting for glycosylation than is import of other nucleotide sugars required for glycosphingolipid synthesis.

In addition to inhibiting the synthesis of gangliosides found in the cell membrane, AZT treatment of SK-MEL-30 cells also caused a severe reduction in the amount of

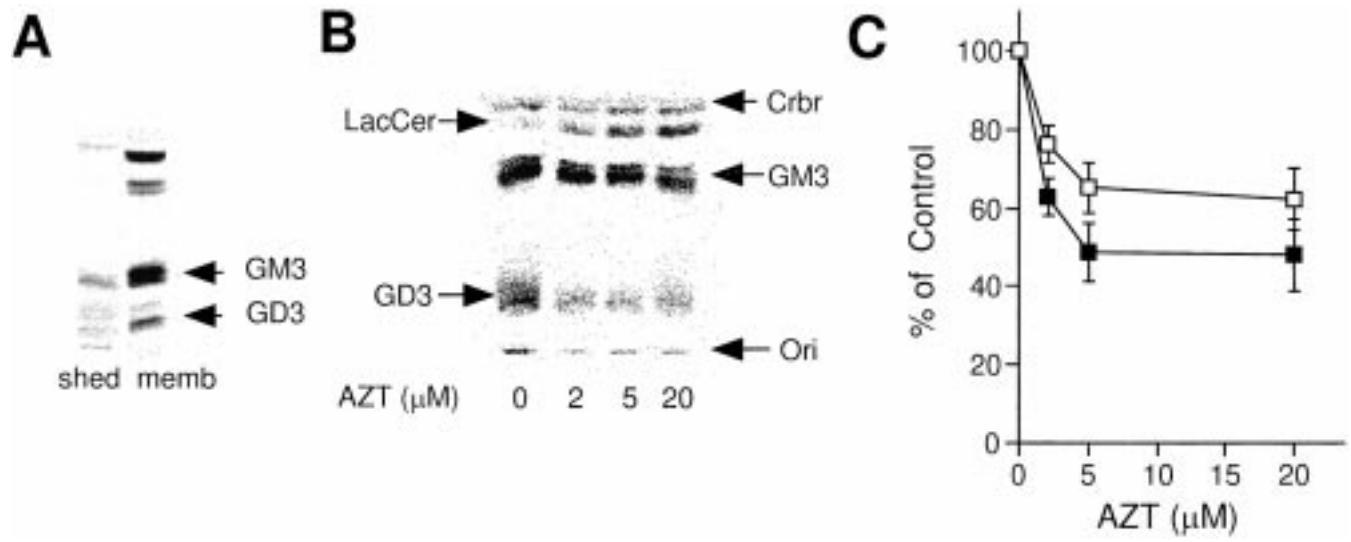

Figure 5. AZT treatment inhibits the amount of gangliosides shed from SK-MEL-30 cells. (Panel A) The phosphorimage of a representative chromatogram of shed lipids obtained under neutral solvent conditions optimized to increase resolution of acidic lipids ($CHCl^3$: MeOH: 0.22% aqueous $CaCl^2$ (47:47:8)) is shown. The mobility's of standards are indicated. (Panel B) Quantitative analysis of the effects of AZT on ganglioside shedding in SK-MEL-30 cells is shown. Similar results were obtained in two separate experiments and the average of these is presented here. The lipids analyzed and their corresponding plot symbols are as follows: □, GM3; ■, GD3.

gangliosides shed into the medium. Large amounts of gangliosides are known to be shed by melanoma cells, both in tissue culture as well as in patients [15]. It has been proposed that the resulting high concentrations of shed gangliosides in the tumor microenvironment may be a major mechanism by which melanoma cells avoid destruction by the immune system. This hypothesis is based on *in vivo* and *in vitro* studies which established that shed gangliosides can compromise immune responsiveness [11,27], although the precise mechanism(s) of this phenomenon has not been elucidated. The large diminution of gangliosides shed from the membranes of SK-MEL-30 cells in the presence of AZT (50% and 40% inhibition of GD3 and GM3 shedding, respectively, with 20 μM AZT) therefore raises the possibility that AZT treatment may enhance the ability of the immune system to detect and then eradicate melanoma cells.

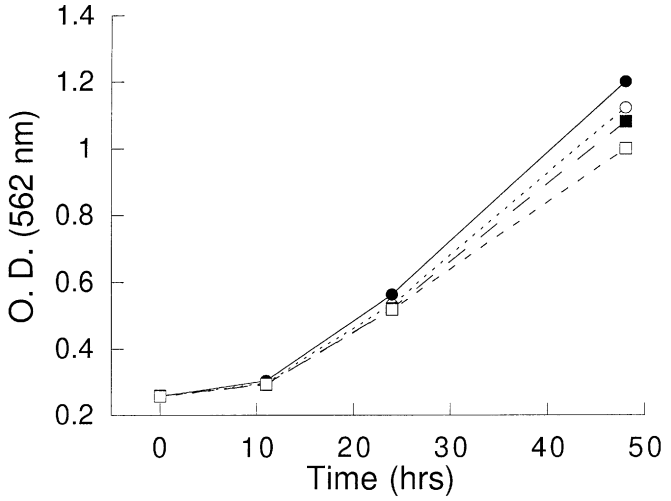

Figure 6. AZT treatment inhibits cell growth in SK-MEL-30 cells. The effect of AZT on the growth of SK-MEL-30 cells was determined at four different concentrations, 0 μM (closed circles), 5 μM (open circles), 10 μM (open diamonds), and 20 μM (closed diamonds) using the MTT colorimetric assay. The above experiment is a representative of several separate measurements.

Glycosphingolipids appear to be shed from melanoma cells either individually or in vesicles, similar to the results of Ladisch and coworkers for YAC-1 lymphoma cells [23]. In both cell lines, only a fraction of the shed glycosphingolipids are released in vesicles. While the glycosphingolipids released in vesicles closely mimicked the cell membrane composition, those released as individual molecules were specifically enriched for gangliosides. This latter result likely reflects the greater H_2O solubility of gangliosides compared to neutral species. Previous studies on ganglioside shedding have also demonstrated that the gangliosides containing shorter fatty acid tails are preferentially shed into the medium [24], while those containing longer, more hydrophilic fatty acid tails are preferentially retained in the membrane. Likewise, we found that SK-MEL-30 cells contain 2 forms of GM3, and the more hydrophilic species is preferentially shed.

The decrease in shed gangliosides upon treatment of cells with AZT likely reflects the decreased rate of ganglioside synthesis. Consistent with this idea, the extent to

which AZT inhibited ganglioside synthesis and release were remarkably similar. We cannot, however, exclude the possibility that AZT-treatment also affects the actual process of glycosphingolipid release from the membrane.

While the cytotoxic and cytostatic effects of AZT are often ascribed to effects on DNA replication in spite of AZT having minimal impact on this process [2], the ability of AZT to inhibit cell growth can readily be accounted for by its ability to modulate glycosphingolipid synthesis. Gangliosides such as GD3 and GM3 are known modulators of cell surface receptor function, and changes in the ganglioside composition of as little as 15% have been shown to dramatically affect growth receptor function [28]. Thus, in cells that are dependent on growth factors, AZT-induced changes in lipid glycosylation could inhibit receptor function and, therefore, block cell growth. Likewise, recent studies have shown that glycosphingolipids play a crucial role in the growth of cells in culture. For example, growth of the epidermoid carcinoma cell line A431 was inhibited after treatment with PDMP [3], and treatment of SK-MEL-28 cells with an anti-GD3 antibody greatly reduced their growth rate [10]. In addition, protein glycosylation occurs in the Golgi complex and this process will likely also be affected by AZT treatment [2]. Together, these data raise the possibility that the decreased growth of SK-MEL-30 cells in the presence of AZT was due to its effect on glycosphingolipid synthesis, as opposed to effects on DNA replication. Furthermore, the slow onset of AZT's growth inhibitory effects are consistent with an effect on glycosylation. Prior to AZT treatment, the cells contain the normal levels of glycosphingolipids. The large alterations in glycosphingolipid synthesis induced by AZT will not result in an immediate large change in membrane composition due to the dilutive effect of the normal glycosphingolipid pool with which the cells started, but rather will require several generations to be fully manifested, similar to the slow decrease in growth rate.

Inhibition of ganglioside synthesis via AZT treatment will likely alter multiple cellular functions, including adhesion and metastasis, and may provide an additional mechanism by which AZT could affect the growth of tumors *in vivo*. Recent work has demonstrated that inhibition of glycosphingolipid synthesis modulates the adhesive properties of cancer cells. For example, treatment of B16 melanoma cells with the glycosylation inhibitor PDMP was shown to inhibit attachment of these cells to the extracellular matrix proteins laminin and collagen and cell lines deficient in glycosphingolipid synthesis also lost the ability to bind to substratum [29]. Glycolipid depletion via treatment with PDMP also greatly inhibited the lung-colonizing capacity of lung carcinoma cells in inoculated mice, suggesting that glycosphingolipids are essential for the metastatic spread of tumor cells [17]. Furthermore, it has been demonstrated that glycosphingolipids directly modulate the function of integrins. Hakomori and co-workers showed that GM3 regulated the integrin receptor-mediated adhesion of a murine carcinoma cell line to fibronectin [30]. These data clearly demonstrate that the processes of cellular adhesion and metastasis are sensitive to changes in glycosphingolipid levels. We are presently examining if AZT treatment has similar effects on melanoma cells.

Notably, thymidine-based glycosylation inhibitors such as AZT provide a number of advantages over classical glycosylation inhibitors and may be better suited as cancer chemotherapeutic agents under some conditions. Classical glycosylation inhibitors (Tunicamycin, deoxynojirimycin, etc. [31]) suffer the severe drawback of being very nonspecific with respect to cell type affected and are thus quite toxic. Indeed, the aforementioned animal studies of the anti-cancer effects of PDMP revealed significant kidney and liver damage [6]. Likewise, many of the classical protein glycosylation inhibitors show severe toxicity due to their ability to inhibit glycosylation in multiple cell types. In contrast, thymidine-derived glycosylation inhibitors can, to some extent, be targeted to rapidly growing cells and may prevent some of these unwanted side-effects. The basis of this selectivity is that (i) the thymidine analog must be converted into the monophosphate by thymidine kinase in order to inhibit glycosylation; and (ii) high levels of thymidine kinase are only present in growing cells [32,33]. These observations predict that thymidine-based nucleosides should preferentially affect rapidly growing cells and could be especially useful for treating those types of cancer typified by rapid growth rates.

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