Tamoxifen retards glycosphingolipid metabolism in human cancer cells

Myles C. Cabot*, Armando E. Giuliano, Alon Volner, Tie-Yan Han

John Wayne Cancer Institute at Saint John's Hospital and Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404, USA

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Abstract In this study we provide evidence that tamoxifen, the widely used breast cancer drug, is a potent antagonist of glycolipid metabolism. When added to the medium of cultured multidrug resistant (MDR) KB-V-1 carcinoma cells, tamoxifen, at 5.0 μM, drastically lowered the levels of glucosylceramide (glc-cer), as evidenced by a reduction in glc-cer mass. In a similar fashion, in cultured human melanoma cells grown with [^{3}H]galactose, tamoxifen inhibited formation of glc-cer by 44%, and retarded lactosylceramide and ganglioside formation by 50 and 35%, respectively. When glc-cer synthase of melanoma was assayed in cell-free incubations, the inclusion of tamoxifen, at a 1:10 molar ratio with ceramide, inhibited glc-cer synthesis by 50%. These results clearly reveal a new action of tamoxifen and thereby pose intriguing questions regarding mechanisms of action in the realm of estrogen receptor-independent modalities, including effects on MDR.

Key words: Tamoxifen; Glycosphingolipids; Glucosylceramide; Multidrug resistance; Cancer

1. Introduction

The efficacy of tamoxifen, used for treatment of estrogen receptor-positive breast cancer, lies chiefly in its ability to antagonize the action of estrogen by binding competitively to the estrogen receptor [1,2]. It has, however, become increasingly apparent that many biological activities of tamoxifen are potentiated in a manner divorced from estrogen receptor machinery [3]. Examples of activities promoted by tamoxifen include protection against lipid peroxidation [4], induction of transforming growth factor-β [5], modification of protein kinase C activity [6–8], activation of lipid second messenger signaling [9], and antagonism of calmodulin [10]. Along these lines, the utility of tamoxifen in non-breast related disease, e.g. brain [11] and melanoma [12], has sparked interest and elicited a move to understand the multimodal mechanisms of action of this synthetic triphenylethylene [3]. Among the most intriguing properties of tamoxifen, independent of estrogen receptor status, is reversal of multidrug resistance (MDR) [3,13,14]. This has resulted in clinical studies[1] aimed at exploiting the chemosensitizing properties of tamoxifen [15,16].

We have recently demonstrated that specific glycosphingolipids, identified as glucosylceramides (glc-cer), accumulate in MDR cancer cells [17]. This finding poses new questions regarding the role of lipids in MDR neoplasms. As gleaned from the literature, it is now clear that tamoxifen impacts on cellular lipid metabolism [9,18–20] and on P-gp-dependent and -independent modes of MDR [21–23]. The association of tamoxifen with MDR reversal then becomes more interesting when considering the participation of lipids in drug transport. Reports have shown that P-gp ATPase activity is dependent on lipid environment [24], and that lipids interact with P-gp substrates [25].

Although tamoxifen has been shown to influence various aspects of lipid metabolism in intact cells and tissues, a direct impact on the enzymes of lipid metabolism has not been clearly evidenced. Herein we report that tamoxifen inhibits glucosylceramide synthase. As such, tamoxifen takes on a new role as a regulator of glycolipid metabolism. Because tamoxifen is used widely for treatment and for prevention of breast cancer and related compounds are making an appearance in the clinic, knowledge must be gained regarding the biological actions of these agents. Our results show that tamoxifen can exert considerable extra-nuclear influence. The impact of tamoxifen on ceramide glycosylation may be important in defining mechanisms underlying various aspects of tamoxifen action. Herein we offer some insight.

2. Materials and methods

2.1. Materials

Ceramide (brain) and phosphatidylcholine (dioleoyl) were from Avanti Polar Lipids (Alabaster, AL). Sulfatides (ceramide galactoside 3-sulfate, bovine) and glucosylceramide (Gaucher's spleen) were purchased from Matreya (Pleasant Gap, PA). D-[6-^{3}H]Galactose (29.5 Ci/mmol) was from DuPont NEN, and uridine diphosphate [6-^{3}H]glucose (15 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Dithiothreitol, β-NAD, tamoxifen (free base), estradiol and UDP-glucose (disodium salt) were from Sigma. 4-*hydroxy*-Tamoxifen was a gift from Dr. Dominique Salin-Drouin of Besins Iscovesco Laboratories (Paris, France). Silica gel G TLC plates were purchased from Analtech (Newark, DE), and solvents were from Fisher. RPMI-1640 medium was from Mediatech (Herndon, VA), and FBS was from Hyclone (Logan, UT). Tissue culture ware was purchased from Falcon.

2.2. Cell culture

The MDR cell line, KB-V-1, was kindly provided by Dr. Michael M. Gottesman, National Cancer Institute. The melanoma cell line, M-10, was established from human tumor [26]. Cells were grown in RPMI-1640 containing 10% FBS (v/v), 50 units/ml penicillin, 50 μg/ml streptomycin, and 584 mg/l L-glutamine, and subcultured as described [17].

Cells (at 60–70% confluence) were treated with tamoxifen (5.0 μM) for 24 h in medium containing 5% FBS. Tamoxifen, in acetone, was added from a 20 mM stock solution; acetone vehicle was present in controls. When utilized, [^{3}H]galactose was introduced at a concentration of 2.0 μCi/ml medium. Cells were harvested by removing medium, rinsing the monolayer twice with ice-cold phosphate-buffered saline, and scraping in ice-cold methanol containing 2% acetic acid. Lipid extraction [27] was continued in 1-dram glass vials with teflon-lined screw caps. The lower phase (biphasic extraction) containing

*Corresponding author. Fax: (1) (310) 998-3995.

Abbreviations: MDR, multidrug resistance; glc-cer, glucosylceramide; FBS, fetal bovine serum; TLC, thin-layer chromatography

(1) 'Phase I/II Trial of Concurrent ChemoBiologic Therapy with Escalating Doses of Tamoxifen for Metastatic Melanoma', S.J. O'Day, M.D. (Principal Investigator), P. Boasberg, M.D., D.S.B. Hoon, Ph.D., M.C. Cabot, Ph.D. and G. Gammon, M.D. (unpublished).

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total cell lipids was taken to dryness under a stream of nitrogen and analyzed.

2.3. Glucosylceramide synthase assay

Enzyme activity was assayed (M-10 cell homogenate) according to the method of Shukla and Radin [28], and tamoxifen was added to the lipoidal substrate. Components of the lipoidal substrate, after solvent evaporation under nitrogen, were immediately sonicated in water and used. Enzyme was prepared from M-10 cells by rinsing monolayers three times with ice-cold phosphate-buffered saline and scraping cells in ice-cold water (0.5 ml per 10 cm culture dish). The cellular preparation was sonicated, over ice, for 15 s. Protein was determined using the Pierce BCA Protein Assay Reagent. Enzyme incubations were carried out at 37°C in a shaking water bath, for 90 min.

2.4. Lipid analysis

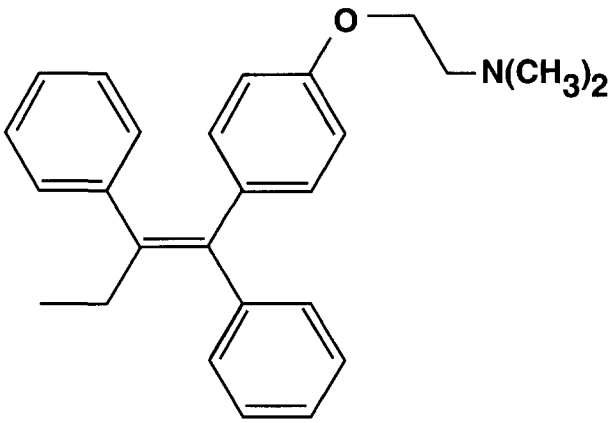
Glc-cer was resolved from other cell lipids by TLC in a solvent system containing chloroform/methanol/ammonium hydroxide (40:10:1, v/v). The radiolabeled glc-cer was scraped from the TLC plate, after visualization in iodine vapor, and analyzed in Ecolume fluid, by liquid scintillation spectrometry. Radiolabeled lactosylceramide and gangliosides were isolated and resolved by TLC as described [17]. To char lipids for mass quantitation, the TLC plates were sprayed with 30% sulfuric acid and heated in an oven at 180°C for 20 min.

Analysis of [^{3}H]glc-ser formed in cell-free assays was done by first terminating the reaction by addition of lipid extraction solvents [27]. After evaporating the lower phase (under nitrogen), 50 μl chloroform/methanol (1:1, v/v) was added to the sample and a 10 μl aliquot was applied to TLC. Commercial glc-cer (5–10 μg) was co-spotted. The [^{3}H]glc-cer was resolved in the above solvent system, and radioanalysis was by liquid scintillation spectrometry.

3. Results

The structure of tamoxifen, a synthetic nonsteroidal triphenylethylene derivative, is shown in Fig. 1. Although classified as an antiestrogen, this drug has complex actions [3]. In previous work [17], we learned that specific glycolipids (glc-cer) accumulate in MDR cancer cells. Among the cells evaluated were drug resistant breast cancer cells, and in these cells we noted that glc-cer levels were reduced upon exposure to tamoxifen. We sought to further explore this response in other MDR cells, most notably, melanoma, for which tamoxifen is a component of a combination chemotherapy regimen [12].

Fig. 2 demonstrates the glc-cer profile in KB-V-1 carcinoma cells that are grown in the absence and presence of tamoxifen. As shown in the right lane, tamoxifen, over a 24 h exposure



Tamoxifen

Fig. 1. The chemical structure of tamoxifen.

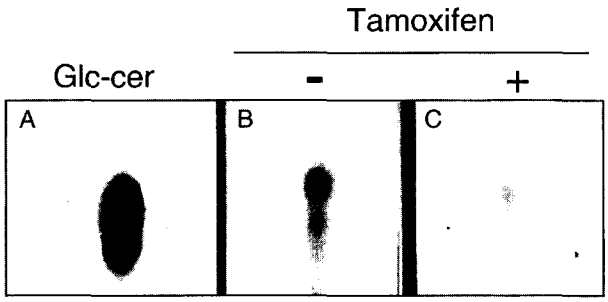


Fig. 2. Thin-layer chromatographic char of glucosylceramide levels in KB-V-1 cells grown in the absence and presence of tamoxifen. Cultures (10 cm dishes) were grown to 60–70% confluence and tamoxifen (5.0 μM) was added, where indicated, for 24 h. TLC chars were conducted as described in Section 2. A representative char from the cerebroside area of the TLC plate is shown. A: glc-cer commercial standard; B: M-10 cell levels of glc-cer (grown without tamoxifen); C: cell glc-cer levels grown in presence of tamoxifen.

period, drastically reduced the levels of cellular glc-cer. Over this same time period, the drug caused no significant change in cell number. These results show that tamoxifen can interfere with glycolipid metabolism in intact cells.

Using radiolabeled human melanoma (M-10 cells), we sought to confirm the influence of tamoxifen on glc-cer metabolism and, as well, assess cerebroside and ganglioside metabolism. As shown in Fig. 3, the addition of tamoxifen, to cultures of human melanoma, inhibited biosynthesis of glc-cer by 44%, based on tritium incorporated from [^{3}H]galactose. Tamoxifen also caused diminished lactosylceramide and ganglioside formation, 50 and 35%, respectively. The results from Figs. 2 and 3 strongly indicate that tamoxifen is an antagonist of glycolipid metabolism. Estradiol, tested at 5.0 nM and 5.0 μM, in M-10 cells under the conditions described in Fig. 3, was without influence.

To directly evaluate the influence of tamoxifen on glycolipid synthesis, we measured the effect of the drug on glucosylceramide synthase, the enzyme responsible for the first committed step of glycolipid synthesis. Table 1, using a crude enzyme preparation from melanoma cells, shows that tamoxifen inhibited ceramide glycosylation in the cell-free assay. Compared to control, tamoxifen, at a 1:20 molar ratio with ceramide, inhibited glc-cer synthesis by 32% (Table 1). The inhibition was dose-dependent, as shown by the near 50% reduction in glc-cer synthesis when tamoxifen was present at higher amounts (1:10 molar ration with ceramide). Interestingly, 4-*hydroxy*-tamoxifen, a major in vivo metabolite, of

Table 1
The influence of tamoxifen and 4-*hydroxy*-tamoxifen on cell-free glucosylceramide synthase activity in melanoma cell preparations

Addition	Glc-cer synthase activity (pmol glc-cer formed/mg protein)
Control	2967 ± 57
Tamoxifen (1:20)	2013 ± 167
Tamoxifen (1:10)	1604 ± 70
4-*hydroxy*-Tamoxifen (1:10)	2486 ± 118

Glc-cer synthase was assayed as described [28], using M-10 melanoma cell homogenate (130 μg protein/reaction) in a final volume of 0.2 ml. Incubations were for 90 min at 37°C. Numbers in parentheses denote the molar ratio of drug to ceramide in the reaction mixture. The mean ± S.D. of triplicate determinations is shown. Enzyme experiments were repeated many times with the same results.

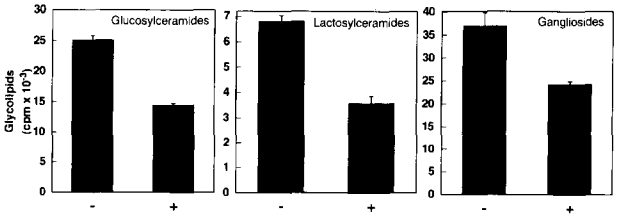


Fig. 3. Tamoxifen retards glycolipid synthesis in human melanoma cells. Cells (M-10), grown to 60–70% confluence, were switched to medium containing 5% FBS and cultured for 24 h, without or with tamoxifen (5.0 μM), in medium containing [^{3}H]galactose (2.0 μCi/ml). Cell harvest, extraction, and TLC analyses of radiolabeled glycolipids were conducted as described in Section 2. (−) indicates no tamoxifen; (+) indicates with tamoxifen.

rather minor chemical modification, was not nearly as effective as tamoxifen in blocking glc-cer synthesis (16% inhibition).

4. Discussion

Our results, presenting evidence that tamoxifen antagonizes glycolipid metabolism, are based upon (i) depletion of glc-cer levels in KB-V-1 cells grown in the presence of tamoxifen in the culture medium; (ii) reduced utilization of [^{3}H]galactose for synthesis of cerebrosides and gangliosides in cultured human melanoma cells; (iii) inhibition of glc-cer formation in cell-free assays of glc-cer synthase. In experiments with intact cells, drug had no influence on uptake of radio precursors. This study brings to light a new activity, one beyond the antiestrogen [3], for the popular chemotherapeutic agent tamoxifen.

Glycolipids exert major influence over cell growth, differentiation, and transformation [29,30], and as well play an intriguing role in tumor metastasis [31]. Therefore, agents that modify glycolipid metabolism would have wide biological application, especially in cell growth control. In our experiments, the levels of tamoxifen required to antagonize glycolipid biosynthesis are well within the clinical range for reversing MDR. In the cell-free assay, we were able, with crude enzyme, to achieve enzyme inhibition in the micromolar range, with ceramide, the cosubstrate, present at a 20-fold excess. Although we have as yet to approach the structural requirements, from experiments with 4-*hydroxy*-tamoxifen, it appears that slight chemical modification can greatly influence activity (Table 1).

A strong unifying aspect of this work and one that imports biological significance, is that of the relationship between human melanoma, gangliosides, and the Dartmouth regimen. The Dartmouth, used to treat advanced stage melanoma, contains tamoxifen [12]; however, the role of tamoxifen in estrogen receptor-independent cancers remains a curiosity. It is important to note that human melanoma express specific gangliosides, such as GD2, which increase as tumorigenesis progresses [32] and which may be associated with increased metastatic potential [31]. In this regard it would appear that tamoxifen imparts some therapeutic benefit via modification of glycolipid metabolism. This notion remains to be visited, but it is still tempting to throw out that agents regulating glycolipid metabolism, especially ceramide glycosylation, would be favorable models for drug design.

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