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Short communication

Targeting chemosensitizing doses of toremifene based on protein binding*

Gregory T. Wurz, Vernon D. Emshoff, Michael W. DeGregorio, and Valerie J. Wiebe

Department of Medicine, Division of Oncology, The University of Texas Health Science Center, San Antonio, Texas, USA

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Summary. Toremifene is currently being evaluated as a chemosensitizing agent in doxorubicin-resistant patients. Although concentrations of >2 μM reverse resistance in vitro, target concentrations required to reverse multidrug resistance (MDR) in vivo may be highly influenced by variables such as protein binding in serum. We examined the effects of high serum concentrations on the cellular accumulation of toremifene in an MDR MDA-MB-A-1 human breast-cancer cell line. We then examined the cellular accumulation of doxorubicin at various toremifene concentrations in 5% – 100% serum. We also measured the concentrations of toremifene and its major metabolites in plasma specimens obtained from two patients receiving 360 mg/day for 5 days in a phase I study. Our results show that (1) high serum concentrations decrease toremifene accumulation, (2) toremifene concentrations of ≥2.5 μM enhance doxorubicin accumulation, and (3) patients achieve plasma toremifene concentrations of 10–15 μM following doses of 360 mg/day × 5 days. Our findings suggest that in vivo toremifene concentrations well above those used to reverse resistance in vitro are required to overcome the effect of high serum-protein binding.

Introduction

Toremifene is a nonsteroidal triphenylethylene that is currently undergoing phase I evaluation for use as a chemosensitizing agent. Chemosensitizing agents are typically used to reverse multidrug resistance (MDR) to natural-product antineoplastics, particularly anthracyclines such as doxorubicin. When used prior to or in conjunction with anthracyclines, these agents enhance the cytotoxicity and correct the accumulation defect present in MDR cells [7]. MDR is believed to occur via the extrusion of drug from the cell by an active efflux pump termed P-glycoprotein [2, 3, 15] and is believed to result from the overexpression of an *mdr*-1 gene that encodes for P-glycoprotein [6].

Agents used as chemosensitizers are believed to compete with the cytotoxic drug for the active efflux pump. The correction of cellular cytotoxic drug accumulation is frequently used as a measure of an agent's ability to reverse MDR. Although many agents have been shown to correct the cellular accumulation defect in vitro and to enhance cytotoxicity in resistant cells, the concentrations of reversing agents required to achieve these effects in vivo cannot be achieved without the production of severe toxic effects in patients, including cardiotoxicity, neurologic complications, and immunosuppression [4, 9, 13, 15, 18]. In preliminary studies, we noted that toremifene and its major metabolites (*N*-desmethyltoremifene and 4-hydroxytoremifene) reversed MDR in vitro at concentrations that could be achieved in patients without resulting in significant toxicity [1]. However, concentrations that reverse resistance in vitro may not be sufficient to reverse resistance in vivo due to the reduced availability of toremifene from protein binding. Toremifene is reported to be extensively protein-bound (>95%) [14, 16]. In the present study, we assessed the effects of various serum concentrations on the cellular accumulation of toremifene in MDR breast-cancer cells.

Patients and methods

Methods

Cell culture. MDA-MB-A-1 doxorubicin-resistant cells were held under selective pressure (doxorubicin concentration, 1.0 μg/ml) prior to their use. Cells were grown in Iscove's modified Dulbecco's medium (IMEM) supplemented with 10% fetal bovine serum (FBS) in Corning T75 culture flasks and were maintained at 37° C in an atmosphere comprising 5% CO_2 and 95% air.

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Correspondence to: Valerie J. Wiebe, The University of Texas Health Science Center, Department of Medicine, Division of Oncology, 7703 Floyd Curl Drive San Antonio, Texas 78 284-7884, USA

Table 1. Toremifene accumulation in various FBS concentrations in MDR cells

% FBS	Toremifene concentration (μmol/10^6 cells)	% Change[a]
5	3.9 (3.5–4.2)	–
10	3.4 (3.1–3.7)	11.7 decrease
25	1.4 (1.3–1.5)	63.6 decrease
50	0.7 (0.7–0.7)	81.8 decrease
75	0.6 (0.4–0.8)	84.4 decrease
100	0.54 (0.53–0.55)	85.7 decrease

Data represent average values for duplicate samples. Numbers in parentheses represent ranges

[a] The percentage of change was calculated by subtracting the control toremifene concentration (5% FBS) from each sample toremifene concentration, dividing by the control concentration, and then multiplying by 100

Toremifene accumulation. Heat-inactivated FBS was diluted to various extents (0, 5%, 10%, 25%, 50%, 75%, and 100% serum) with IMEM. Serum was incubated at 37° C for 30 min with toremifene prior to cellular exposure. Cells were exposed to various concentrations of FBS containing a final toremifene concentration of 6.6 μM. Toremifene accumulation was evaluated by cell extraction followed by drug quantitation by high-performance liquid chromatography (HPLC) as previously described [16, 8].

Doxorubicin accumulation. To determine doxorubicin accumulation, we used an HPLC assay as previously described [5]. Cells were incubated for 72 h with toremifene-containing media prior to the addition of doxorubicin (2.0 μg/ml × 2 h). Cells were then processed and extracted, and the doxorubicin content was quantified by HPLC.

Patients

Plasma was obtained from two patients participating in a phase I study evaluating the efficacy of toremifene as a chemosensitizing agent, in which toremifene was given orally at 360 mg/day for 5 days. Plasma samples were drawn daily just before and at 2 and 4 h after dosing. Aliquots (1 ml) were placed in 16- × 125-mm glass extraction tubes, were spiked with an internal standard (nafoxidine hydrochloride) before being subjected to extraction, and were then assayed for toremifene and its metabolites [16].

Results

Table 1 shows that toremifene accumulation decreases with increasing FBS concentration. Cellular concentrations of toremifene following exposure to a concentration of 6.6 μM were 3.85 μM at 5% FBS and 0.55 μM at 100% FBS, a decrease of 85.7%. Clearly, increased protein binding in serum substantially decreases toremifene accumulation. Table 2 shows the effects of toremifene in 5% FBS on doxorubicin accumulation in MDA-MB-A-1 cells. At toremifene concentrations exceeding 2.0 μM, doxorubicin accumulation was substantially increased. Toremifene concentrations of >2 μM appear to be a threshold that corrects the accumulation defect in MDA-MB-A-1 doxorubicin-resistant cells.

Table 2. Doxorubicin accumulation in the presence and absence of toremifene in MDR cells

Toremifene concentration (μM)	Doxorubicin concentration (μmol/10^6 cells)	% Change
Control ($n = 4$)	1.09	
1.5 ($n = 3$)	1.56 (0.089)	43.8 increase
2.0 ($n = 3$)	1.41 (0.157)	29.5 increase
2.5 ($n = 3$)	2.11 (0.398)	93.9 increase
3.0 ($n = 3$)	2.08 (0.128)	91.1 increase

Numbers in parentheses represent SDs

[a] The percentage of change was calculated by subtracting the control doxorubicin concentration from the sample concentration, dividing by the control concentration, and then multiplying by 100

When the toremifene exposure concentration was raised to >10 μM in 100% FBS, the cellular accumulation of doxorubicin was increased by 145% relative to the control value, an increase similar to that seen in 5% FBS containing a toremifene concentration of 6.6 μM. Therefore, in patients receiving toremifene to reverse doxorubicin resistance, it must be assumed that toremifene is extensively protein-bound (>95%), and that toremifene concentrations on the order of >10 μM are required to overcome the effects of protein binding.

In the two patients studied, serum toremifene and its active metabolite *N*-desmethyltoremifene were shown to increase over the 5-day dosing period, achieving concentrations of 10.0–15.0 μM on day 5, when doxorubicin was given. Assuming that toremifene is approximately 95% protein-bound in human serum, these concentrations of toremifene appear to be sufficiently high to reverse doxorubicin resistance in these patients.

Discussion

The nonsteroidal triphenylethylene toremifene is an agent that has chemosensitizing activity in MDR cells at concentrations that can be achieved in humans without resulting in significant toxicity. The underlying mechanism by which toremifene reverses resistance has yet to be identified. Although it is true that several biochemical alterations have been observed, such as protein kinase C inhibition, calcium channel blockade, competition with cytotoxic drug at the energy-dependent efflux pump, and altered cell-membrane lipid integrity, there is little evidence linking these in vitro effects with the in vivo modulation of MDR [3, 10–13].

In a recent report, we presented evidence that the correction of the accumulation defect in MDA-MB-A-1 MDR cells by toremifene occurs at concentrations of >2.5 μM

[17]. The two patients participating in the toremifene phase I study who were selected for the present investigation were found to have between 10- and 15-μM concentrations of toremifene plus its major active metabolites (4-hydroxytoremifene and *N*-desmethyltoremifene) in their plasma.

The present study provides evidence that the cellular availability of toremifene is markedly influenced by high serum concentrations. Our results show that a marked decrease in toremifene accumulation occurs in MDR cells with increasing serum concentration (from 5% to 100% FBS). Due to the decreased availability of toremifene in serum and to the observation that 2.5 μM toremifene in 5% FBS can effectively increase doxorubicin accumulation in MDR cells, target concentrations of toremifene plus *N*-desmethyltoremifene must be above 10 μM to reverse MDR in vivo.

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