Effect of Tamoxifen on the Multidrug-resistant Phenotype in Human Breast Cancer Cells: Isobologram, Drug Accumulation, and Mr 170,000 Glycoprotein (gp170) Binding Studies

Fabio Leonessa, Mary Jacobson, Bryan Boyle, Jeremy Lippman, Michael McGarvey, and Robert Clarke

ABSTRACT

We have performed isobologram analyses of the ability of tamoxifen (TAM) to alter the response to Adriamycin (ADR) and vinblastine (VBL) in human breast cancer cells. MCF-7 cells express functional receptors for estrogen and progesterone but do not express detectable levels of Mr 170,000 glycoprotein (gp170). CL 10.3 and MCF-7ADR cells are MCF-7 variants which express gp170. CL 10.3 but not MCF-7ADR cells express functional steroid hormone receptors. Tamoxifen (1-2.5 #M) interacts synergistically with ADR and VBL in CL 10.3 and MCF-7ADR cells. TAM increases the cytotoxicity of VBL and ADR and the intracellular levels of [3H]VBL by approximately 2-3-fold. TAM also prevents the binding of [3H]azidopine to gp170. The ability of TAM to concurrently increase the cytotoxic effects of ADR and VBL, increase VBL accumulation, and inhibit the binding of azidopine to gp170 strongly implies that the synergistic effects of TAM are mediated through its effects on gp170. TAM produces an antagonistic to additive interaction with ADR and VBL in MCF-7 cells, and at high concentrations (5 #M) the synergy apparent in CL 10.3 and MCF-7ADR cells is lost. While TAM clearly has a significant potential for use as a chemosensitizing agent, the design of clinical trials may require careful consideration.

INTRODUCTION

The treatment of breast cancer is often confined to local modalities when there are no detectable metastases. However, a significant proportion of patients have already developed either clinical or occult metastatic disease by the time of diagnosis (1). These patients require systemic therapy. Many patients with metastatic breast cancer respond to a wide range of systemic treatments. Unfortunately, the majority of initially responsive tumors acquire a phenotype characterized by cross-resistance to multiple drugs, including agents that these patients have not previously received.

The development of a multiple drug-resistant phenotype in metastatic breast cancer is primarily responsible for the failure of current treatment regimens. The precise mechanisms responsible for conferring a multiple drug-resistant phenotype on breast cancer cells remain unclear. In some cells, this phenotype is accompanied by the expression of a membrane-bound glycoprotein (gp170), the product of the human MDR1 gene. In these cases resistance is the result of an energy-dependent increase in drug efflux (2). Many of the more widely used drugs in breast cancer treatment, including the Vinca alkaloids (e.g., vinblastine) and the anthracycline antibiotics (e.g., Adriamycin), are substrates for gp170. Expression of MDRI mRNA and/or gp170 has been widely reported in breast tumors (3-7). In some studies, detectable levels of expression in breast tumors correlate with failure of cytotoxic chemotherapy (3, 6), poor prognosis (7), and/or in vitro resistance to cytotoxic drugs (4, 6). Although gp170 is clearly expressed in a significant number of breast tumors, its precise functional and biological relevance remains to be determined.

Expression of MDRI has been associated with a loss of steroid hormone receptor expression and cross-resistance to antihormones (8). To determine the ability of MDRI expression to perturb hormone sensitivity, we previously introduced the human MDRI complementary DNA into MCF-7 human breast cancer cells using retroviral vector-mediated gene transfer (9). Transduced cells (CL 10.3) express gp170 and are resistant to drugs of the MDR1-mediated phenotype but retain expression of steroid hormone receptors. These cells retain sensitivity to the inhibitory effects of triphenylethylene antihormones (9). Thus, the loss of ER and progesterone receptor expression and the cross-resistance to TAM observed in some MDRI-expressing cells appear to arise independently of an overexpression of MDRI (9).

Some initial studies have suggested that hormonal agents may be substrates for gp170. Yang et al. (10) have shown that progesterone both can increase vinblastine accumulation in endometrial cells of mouse gravid uterus that express the mouse MDR1 genes and can prevent the binding of a photoactive substrate to the mouse MDR1 glycoprotein. The triphenylethylene antihormone TAM and its close structural analogue toremifene also appear to influence multidrug resistance in MDRI-expressing cells (11, 12). However, these studies were performed on cells selected in vitro for resistance to ADR (P388 drug-resistant subline; MCF7ADR). These cells may contain additional ADR resistance mechanisms unrelated to gp170. For example, we have recently demonstrated that MCF-7ADR but not CL 10.3 cells are cross-resistant to tumor necrosis factor. Because both ADR and tumor necrosis factor can inhibit cells by the generation of free radicals (13, 14), this cross-resistance in MCF7ADR cells strongly suggests the presence of ADR resistance mechanisms in addition to gp170, including altered expression of manganous superoxide dismutase. Steroids can alter resistance mediated through this pathway (14). Thus, effects on sensitivity to ADR mediated through resistance mechanisms that are independent of MDRI expression cannot be readily excluded when using MCF7ADR cells.

Despite the apparent monoclonal origin of breast tumors, they develop considerable subpopulation heterogeneity (15, 16). By the time a breast tumor mass is detected there is already marked heterogeneity of hormone receptor expression (17). Many of these populations respond differently to the effects of hormones/antihormones. There may also be interactions between cytotoxic drugs and hormonal agents (18, 19). For example, estrogens can increase or decrease the sensitivity to cytotoxic drugs, depending on the ER status of the target cell (20, 21). Some endocrine agents alone can induce significant inhibitory effects on responsive cells when administered at the concentrations required...
to influence multidrug resistance. TAM arrests cells that express functional ER in $G_0/G_1$ of the cell cycle (22). This could significantly increase the kinetic resistance of cells expressing ER but not gp170 and reduce the overall cytotoxic effects of cell cycle/phase-specific drugs in cells that concurrently express both ER and gp170. Thus, understanding the interactions between gp170, endocrine-reversing agents, and tumor subpopulations with differing levels of receptor and gp170 expression is critical for the development of novel chemotherapeutic therapies. Previous studies have not addressed whether endocrine-cytotoxic drug interactions in ER-positive gp170-expressing cells are synergistic, additive, or antagonistic.

The development of drugs that can reverse the MDR1 phenotype would be of significant clinical importance for breast cancer and other cancers associated with a MDR1-related multiple drug resistance phenotype. In this report we describe the ability of TAM to influence the MDR1 phenotype of human breast cancer cells. We demonstrate that the potential antagonistic effects of TAM on the response of ER-positive/gp170-negative MCF-7 cells to ADR and VBL are limited, relative to the synergistic effects on cells expressing gp170. We also demonstrate the ability of TAM to increase the intracellular accumulation of $[^3H]VBL$ in MCF-7 cells expressing gp170, but not in parental MCF-7 cells, and to prevent $[^3H]azidopine$ binding to gp170. Thus, triphenylethylenes interact synergistically with classical gp170 parenteral MCF-7 cells, and to prevent $[^3H]azidopine$ binding to gp170.

Determination of Cytotoxicity. Cytotoxicity was determined 72 h after removal of the drug, using a crystal violet dye uptake assay. At day 0 cells were plated into 96-well culture dishes (Costar, Cambridge, MA) in IMEM, at seeding densities appropriate for each cell line (MCF-7, 2 x 10^4 cells/well; CL 10.3, 5 x 10^4 cells/well; MCF-7ADR, 1 x 10^4 cells/well). Twenty-four h later (day 1) cells were exposed to TAM (1-5 μM) and an additional 24 h (day 2) they were re-fed with IMEM containing the appropriate concentrations of TAM (1-5 μM) and either ADR (5 nM to 3 μM) or VBL (0.5-100 nM). Control wells were treated with each drug alone and/or appropriate concentrations of the drug vehicles. A concurrent dose-response curve for TAM alone (1-10 μM) was also included in each experiment. Six or more wells were used for each treatment. Thus, the data for a typical experiment were obtained from approximately 900 individual measurements. All treatments were stopped 24 h after the beginning of the combined treatment, and the wells were rinsed, re-fed with fresh growth medium, and incubated for an additional 72 h.

Table 1 Phenotypes of the MCF-7, CL 10.3, and MCF-7ADR cells

<table>
<thead>
<tr>
<th>Sites/cell</th>
<th>ER</th>
<th>PGR</th>
<th>gp170</th>
<th>Tumorigenicity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>58,900</td>
<td>68,250</td>
<td>-</td>
<td>$+E2^a$</td>
<td>9,54</td>
</tr>
<tr>
<td>CL 10.3</td>
<td>78,547</td>
<td>89,995</td>
<td>+</td>
<td>$+E2$</td>
<td>9</td>
</tr>
<tr>
<td>MCF-7ADR</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>$-E2$</td>
<td>8, 9</td>
</tr>
</tbody>
</table>

$^a$E2, estradiol.
$^b$ND, not detected.

Cells were rinsed twice in phosphate buffer and stained with the crystal violet stain by incubation with staining solution (0.5%, w/v, crystal violet in 25%, v/v, methanol) for 5 min at 25°C. The stained cell monolayers were rinsed gently with distilled water and allowed to dry, and the dye was extracted by the addition of 0.1 M sodium citrate/50% (v/v) ethanol and incubation at room temperature for 10-15 min. Absorbance was read at 540 nm using a Dynatech MR700 enzyme-linked immunosorbent assay reader (Dynatech, Chantilly, VA). The degree of dye uptake, as measured by absorbance, is directly related to cell number (23, 24). The data are presented as the mean of the absorbance expressed as a percentage of untreated cell population values. The drug concentrations needed to achieve fixed levels of inhibition (ICn, where n varies from 25% to 80% inhibition) were estimated by interpolation using the appropriate dose-response curves. These data were then expressed as isofactor curves (isobios) and subjected to isobologram analysis for evaluation of the drug-drug interactions, as described below.

**Isobologram Analyses.** Isobolograms define the nature of the interaction between two or more drugs. A concave isobologram reflects antagonism, and a straight line joining each axis intercept represents zero interaction (additivity) between two drugs (25). A quantitative index of these interactions is provided by the isobologram equation (26), $(D/D_2 + T/T_2 - 1) = I_x$, where, for this study, $D$ and $T$ represent the respective concentrations of the cytotoxic drug (ADR or VBL) and antiestrogen (TAM) required to produce a fixed level of inhibition (ICx) when administered alone, and $D$ and $T$ represent the concentrations required for the same effect when the two drugs are administered in combination, and $I_x$ represents an index of drug interaction (interaction index). $I_x$ values of $<1$ indicate synergy, a value of 1 represents additivity, and values of $>1$ indicate antagonism. For all estimations of $I_x$, we used only isobios where intercept data for both axes were available.

**Effect of TAM on $[^3H]VBL$ Accumulation.** Cells (10^5) were seeded into 24-well dishes (Costar). Twenty-four h later, cells were incubated with 10 nM $[^3H]VBL$ (specific activity, 8.3 Ci/mmole; Amersham) for increasing periods from 60 sec to 3 h at 37°C, in the presence or absence of 1 μM TAM. Following incubation, uptake was stopped by rinsing the wells three times with 250 μl of buffer (IMEM with 0.1% bovine serum albumin and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4). Cells were removed by trypsinization and total cell-associated radioactivity was determined by liquid scintillation counting. Data points are presented as the mean ± SD of three or more determinations.

**Effect of TAM on $[^3H]Azidopine$ Binding to gp170.** To exclude the potentially confounding binding of TAM to additional sites (e.g., ER or estrogen binding sites) in the CL 10.3 cells, we used MCF-7ADR cells for the gp170 competitive binding analyses. Cells were grown to 90% confluence and incubated with 0.2 μM $[^3H]azidopine$ [2,6-dimethyl-4-(2’-trifluoromethylphenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid, ethyl, (N-4’-azido[3’,5’-3H]-benzoylaminohydroxy)diester; specific activity, 50 Ci/mmol; Amersham, Arlington Heights, IL] for 1 h, in the presence or absence of TAM citrate (1-100 μM) or the gp170 substrate VBL sulfate (11.0 μM). The treatment was followed by washing with phosphate-buffered saline and exposure of cells to UV light at a 15-cm distance for 20 min on ice. Cellular proteins were extracted in buffer containing 1% Nonident P-40, 150 mM NaCl, and 50 mM Tris. Following centrifugation for 5 min at 4°C in a benchtop microfuge (Brinkman Instruments Inc., Westbury, NY), the pellets were discarded, and the protein concentration in the supernatants was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Proteins were separated by molecular weight following electrophoresis in sodium dodecyl sulfate-Tris-glycine-polyacrylamide (4–20% gradient) gels. Electrophoresis was performed in a Novex X-cell Mini-Cell apparatus (Novex, San Diego, CA). The gels were treated with 20% (v/v) 2,5-diphenyloxazole in dimethylsulfoxide (27) and dried, and fluorography was performed at ~70°C.

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RESULTS

Differential Sensitivities of the Cell Lines to TAM, ADR, and VBL. Representative dose-response relationships for each drug alone and in combination are provided in Figs. 1 (left), 2 (left), and 3 (left). The dose-response relationships for the combination treatments are represented by a three-dimensional surface that connects the dose-response curves for each drug alone. The response of the respective cell line to each drug alone is represented by the interface between the edge of this surface and each of the vertical planes.

The ER-positive MCF-7 [Fig. 1 (left)] and CL 10.3 [Fig. 2 (left)] cells are clearly inhibited by TAM at all concentrations used in this study. This confirms our previous data showing that the expression of gp170 does not alter the response to TAM in ER-expressing cells (9). Concentrations of TAM of <5 μM do not consistently inhibit the ER-negative MCF-7ADR cells. However, at ≥5 μM TAM we observe a ≥25% inhibition of these cells (data not shown).

The relative differences in responsiveness to VBL and ADR among the cell lines are more readily appreciated at the lower levels of cell inhibition. We can define fold resistance at IC₅₀ as the ratio of the IC₅₀ values in the resistant and parental cells. Thus, the IC₅₀ values for CL 10.3 and MCF-7ADR cells indicate that these cells are 13-fold and 90-fold resistant, respectively, to ADR, relative to MCF-7 cells. This is in broad agreement with our previous data (9). For VBL, CL 10.3 and MCF-7ADR cells are 20-fold and 45-fold resistant, respectively, relative to MCF-7 cells. Clearly, sensitivity of the various cell lines is strongly associated with gp170 expression.

Effect of TAM on the Response of Cell Lines to ADR and VBL. The effects of TAM (1.0, 2.5, and 5.0 μM) on the response of the different cell lines (MCF-7, MCF-7ADR, and CL 10.3) to ADR and VBL are represented in Figs. 1 (left), 2 (left), and 3 (left), respectively. The magnitudes of the TAM/drug responses are readily apparent. TAM alone is inhibitory at many of the concentrations examined. This indicates the presence of a potentially significant additive/antagonist component. Thus, possible synergistic interactions between TAM and ADR/VBL cannot be accurately discriminated from additive or antagonistic effects on the basis of these representations alone.

To evaluate the interactions of TAM with ADR and VBL, we performed a series of isobologram transformations of multiple dose-response analyses. Representative transformations are presented graphically (isobolograms) in Figs. 1 (right), 2 (right), and 3 (right). The concavity of the isobolograms clearly indicates that TAM interacts synergistically with both ADR and VBL in the gp170-expressing MCF-7ADR and CL 10.3 cells. While Figs. 1–3 provide a graphical representation of representative experiments, Table 2 reports the means and 95% confidence intervals of the IC₅₀ values estimated at the IC₅₀ value [IC₅₀] for three or more experiments. The choice of IC₅₀ was determined by the individual characteristics of the dose-response curves obtained in multiple analyses. A significant sensitization to VBL and ADR is observed specifically in the CL 10.3 and MCF-7ADR cells and is already detected in the presence of 1 μM TAM. Increasing the concentration of TAM to 5 μM results in the loss of a significant synergistic interaction. This is evident for both ADR and VBL in CL 10.3 and MCF-7ADR cells (Table 2). In the parental MCF-7 cells, TAM/VBL and TAM/ADR interactions are approximately additive, although antagonism is evident in some isobols (e.g., Fig. 1 (right, bottom)).

TAM Effects on [³H]VBL Accumulation and [³H]Azidopine Binding to gp170. To avoid potential problems associated with additional ADR-resistance mechanisms in MCF-7ADR cells, we used VBL as the gp170 substrate for the competitive binding and drug accumulation studies. For the drug accumulation studies we used 1 μM TAM. This concentration is significantly higher than the Kᵣ of TAM for ER or antiestrogen binding sites (28) and ensures that there are significant levels of free TAM present in the ER-positive cells. Fur-
Fig. 2. Representative dose-response relationships (left) and isobologram transformations (right) for TAM/VBL (upper) and TAM/ADR (lower) in CL 10.3 cells. The data at left represent the mean of 6 or more determinations. The coefficient of variation among these mean values is approximately 15%. O.D., absorbance. The data at right represent the isobols estimated at the IC_{25} - IC_{50} levels.

Fig. 3. Representative dose-response relationships (left) and isobologram transformations (right) for TAM/VBL (upper) and TAM/ADR (lower) in MCF-7ADR cells. The data at left represent the mean of 6 or more determinations. The coefficient of variation among these mean values is approximately 15%. O.D., absorbance. The data at right represent the isobols estimated at the IC_{25} - IC_{50} levels.
thermore, this concentration of TAM produces clear synergistic effects on the cytotoxicity of VBL in both MCF-7ADR and CL 10.3 cells (Table 2). The data in Fig. 4 demonstrate the differential intracellular accumulation of [3H]VBL in each of the cell lines. TAM significantly increases the accumulation in MCF-7ADR and CL 10.3 cells, by approximately 3-fold. TAM has no effect in the parental MCF-7 cells.

The data in Fig. 5 demonstrate the ability of TAM to inhibit binding of the photoactive substrate [3H]azidopine to gp170. TAM (10–100 μM) inhibits azidopine binding in a dose-dependent manner. The lack of a significant effect on azidopine binding with 1 μM TAM probably reflects the relative affinities of TAM and azidopine for gp170.

**DISCUSSION**

Ford and Hait (29) have observed that many investigators estimate the magnitude of the effect of gp170-chemosensitizing agents by directly comparing the IC50 values for a cytotoxic drug in the presence and absence of a fixed concentration of a relatively nontoxic agent. This is often performed by estimating the IC50 ratios in the presence and absence of chemosensitizer. However, the validity of this approach decreases as the concentration of chemosensitizer approaches toxic doses, and it is inadequate in cases where the chemosensitizer exhibits inhibitory activity.

There are various potential methods that have been previously used to investigate drug-drug interactions (25, 30–32). Berenbaum (30, 33) has clearly described how the classical isobologram equation applies to drug combinations irrespective of either the shape of the constituent dose-response curves or the mechanism of action. In these analyses the interactions between the two drugs are represented graphically as isoeffect curves (isobolograms). TAM can inhibit all three cell lines (MCF-7, CL 10.3, and MCF-7ADR), and there are multiple potential mechanisms for TAM-induced toxicity, including ER interactions (34) and perturbations of either membrane fluidity (35) or calmodulin activity (36). Consequently, we applied classical isobologram analyses using the criteria described in detail by Berenbaum (26, 30).

In Figs. 1–3 and Table 2 we demonstrate that TAM (1 μM) interacts synergistically with VBL and ADR, decreasing the IC50 by approximately 2–3-fold in both CL 10.3 and MCF-7ADR cells. However, increasing the concentration of TAM from 2.5 μM to 5 μM increases the IC50 for TAM/ADR and TAM/VBL to nonsignificant levels in both CL 10.3 and MCF-7ADR cells.

The failure to consider adequately the toxicity of 5 μM TAM alone could lead to inappropriate mechanistic speculations regarding TAM/drug interactions. Using IC50 ratios (29), increasing the concentration of TAM from 2.5 μM to 5 μM would produce a misleading increase in the apparent degree of “sensitization” to ADR from 5.4 to 12.5-fold in MCF-7ADR cells. However, the isobologram analyses clearly demonstrate that this interaction is no longer synergistic.

Breast tumors are markedly heterogeneous with respect to the expression of both ER (37) and gp170 (38, 39). Consequently, we wished to determine the nature of TAM/drug interactions in ER-positive and -negative cells that concurrently express gp170. This is particularly important because TAM alone can arrest ER-positive cells in G0/G1 at the concentrations used in chemohormonal combinations (1–5 μM) (22, 35). A G0/G1 blockade could significantly increase the kinetic resistance to ADR and VBL in TAM-responsive cells (MCF-7 and CL 10.3). Furthermore, there have been some reports of interactions between TAM and cytotoxic drugs that are not substrates for gp170 (for recent reviews, see Refs. 40 and 41). For example, TAM reduces both the intracellular accumulation and cytotoxicity of Melphalan (42).

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**Table 2. Effect of TAM on VBL and ADR cytotoxicity**

Data represent the geometric mean and 95% confidence interval of 3 or more experiments, except where indicated. Ix(65) is the interaction index at the 65% cell growth inhibition level, as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Cell line</th>
<th>[TAM] (μM)</th>
<th>VBL</th>
<th>ADR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.77 (0.55–1.06)</td>
<td>0.97 (0.53–1.77)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.94 (0.73–1.20)</td>
<td>1.05 (0.67–1.63)</td>
</tr>
<tr>
<td></td>
<td>5.0a</td>
<td>1.11 (1.09–1.28)</td>
<td>1.23 (1.02–1.49)</td>
</tr>
<tr>
<td>CL 10.3</td>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.31 (0.26–0.37)</td>
<td>0.37 (0.28–0.50)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.55 (0.43–0.71)</td>
<td>0.56 (0.44–0.71)</td>
</tr>
<tr>
<td></td>
<td>5.0a</td>
<td>0.89 (0.89–1.00)</td>
<td>0.86 (0.80–1.20)</td>
</tr>
<tr>
<td>MCF-7ADR</td>
<td>0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.52 (0.23–1.19)</td>
<td>0.53 (0.34–0.81)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.51 (0.46–0.58)</td>
<td>0.53 (0.34–0.81)</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>0.85 (0.65–1.11)</td>
<td>0.77 (0.46–1.27)</td>
</tr>
</tbody>
</table>

* Data from 2 experiments.
We detect antagonism in some isoeffect curves; for example, 5.0 μM TAM antagonizes the effects of ADR in MCF-7 cells [Fig. 1 (right, bottom)], producing a 1x of 1.23 (Table 2). However, the degree of antagonism at lower concentrations, where TAM alone is also inhibitory, is not significant (Table 2). We do not detect antagonism in the ER-positive CL 10.3 cells [Fig. 2 (right, bottom); Table 2]. Despite potentially adverse perturbations in cell cycle distribution, TAM also obtained from the [3H]azidopine bound to gp170.

c, VBL. TAM produces a similar increase in the intracellular steady state populations. We first determined the ability of TAM to influence the accumulation of [3H]trioxarate and 5-fluorouracil in MCF-7 cells (43). These observations indicate that TAM/drug combinations are unlikely to produce significant adverse interactions in some ER-positive/gp170-negative subpopulations.

The data in Figs. 2 (right) and 3 (right) and Table 2 indicate that TAM at certain concentrations can interact synergistically with ADR and VBL in MCF-7ADR and CL 10.3 cells. This has important mechanistic implications. TAM can inhibit calmodulin activity (36), and trifluoperazine inhibitors of calmodulin function can increase sensitivity to ADR by mechanisms other than alterations in drug accumulation or retention (44). However, gp170 confers resistance by maintaining a low intracellular concentration of substrate. We first determined the ability of TAM to influence the accumulation of [3H]-VBL. TAM produces a similar increase in the intracellular steady state levels of VBL in CL 10.3 and MCF-7ADR cells. These levels do not reach those apparent in the untreated parental MCF-7 cells, where TAM has no significant effect on VBL accumulation. These observations are consistent with the ability of TAM to produce a comparable partial reversal of gp170-mediated drug resistance in CL 10.3 and MCF-7ADR cells.

Because TAM can alter both the intracellular accumulation and cytotoxicity of the non-gp170 substrate Melphalan (42), we wished to determine whether its effects in CL 10.3 and MCF-7ADR cells could be mediated through interactions with gp170. The data in Fig. 5 demonstrate that TAM is able to prevent the binding of the gp170 substrate [3H]azidopine to gp170. While 1 μM TAM interacts synergistically in the cytotoxicity assays, it is ineffective in competing for [3H]azidopine. This probably reflects the relative affinities of TAM and azidopine for gp170, which may be different from those of VBL and ADR.

The extent to which TAM-induced inhibition of [3H]azidopine binding to gp170 represents a direct allosteric or competitive binding interaction remains to be determined. Drug efflux mediated by gp170 is energy dependent and associated with phosphorylation of intracellular residues on the glycoprotein (45). TAM binds directly to protein kinase C with an IC50 of 25 μM (46), and this enzyme may be involved in gp170 phosphorylation (47, 48). However, we observe synergistic interactions at 1 μM TAM. Thus, it is unclear whether these effects are mediated through inhibition of protein kinase C activity. We previously reported the ability of TAM (1–10 μM) to decrease membrane fluidity, and we suggested that these effects may alter the transport of drugs that use membrane-associated transport proteins (35). TAM-induced effects on membrane fluidity occur at concentrations where we observe significant synergy. However, the degree to which this directly or indirectly influences gp170 function remains to be determined.

In cells expressing gp170, the ability of TAM to concurrently increase the cytotoxic effects of ADR and VBL, increase VBL accumulation, and inhibit the binding of azidopine to gp170 strongly implies that the major effect of TAM is mediated through its effects on gp170. However, we cannot exclude the possibility that other mechanisms also influence ADR cytotoxicity, particularly in MCF-7ADR cells.

TAM is an attractive candidate for inclusion in chemohormonal regimens due to the low incidence of serious dose-limiting toxicities (34). However, for TAM to have potential as a clinical agent for reversing gp170 resistance, the doses that reverse resistance must be within the ranges achievable in human patients. TAM is generally administered p.o. in the range of 10–20 mg daily. There is a long mean terminal half-life of 5–11 days (49, 50). TAM steady state serum concentrations of ≥1 μM have been reported using high-dose regimens of 40 mg/m2 TAM twice daily (49, 51). More recently, Trump et al. (52) reported that 150 mg/m2 TAM can produce mean plasma levels of 4 μM TAM without dose-limiting toxicity. While TAM concentrations in the range of 1–5 μM are within the clinically achievable range, the clinical relevance of experimental data generated using TAM concentrations of ≥10 μM remains unclear. TAM and its metabolites are extensively bound to serum proteins (50), and the amount of free drug that may be available to cells is not known.

We demonstrate that 1 μM TAM produces significant synergy with both VBL and ADR and increases the intracellular accumulation of [3H]VBL. Increasing the concentration of TAM from 2.5 μM to 5 μM results in a loss of synergy for ADR and VBL in both CL 10.3 and MCF-7ADR cells. At these concentrations, the apparently nonspecific toxicity of TAM becomes significant. The nonspecific toxicity component of the overall response may antagonize the effects of ADR and VBL, independently of any interactions with gp170. At concentrations of ≥5 μM this antagonism may begin to predominate over the syngentic component of the biological response. It is unlikely that these nonspecific effects are mediated through ER-associated mechanisms such as altered transforming growth factor β secretion (53), because these effects are observed in ER-negative cells (35).

While the clinical relevance of these analyses is unclear, the observed loss of synergy at very high concentrations of TAM is clearly worthy of consideration. The conditions that generate nonspecific toxic effects of TAM in vitro may be biologically/pharmacologically comparable to those that induce dose-limiting toxicities in patients receiving high doses of TAM (52). Thus, the use of very high-dose TAM regimens may prove to be suboptimal, while increasing the

![Fig. 5. Ability of TAM to prevent the binding of [3H]azidopine to gp170. Bands represent the signal obtained from the [3H]azidopine bound to gp170. ctr, [3H]azidopine alone (control lane).](image-url)
potential for generating severe TAM-induced toxicity. It will require further study to determine the degree to which this may apply to other systems. We are currently attempting to address these issues by performing a series of analyses in tumors growing in athymic nude mice.

REFERENCES


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Effect of Tamoxifen on the Multidrug-resistant Phenotype in Human Breast Cancer Cells: Isobologram, Drug Accumulation, and Mr 170,000 Glycoprotein (gp170) Binding Studies

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