Effect of Tamoxifen on Cell Lines Displaying the Multidrug-Resistant Phenotype

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We examined the effect of tamoxifen (Tmx), verapamil, and daunorubicin (DNR) in two cell lines that displayed the multidrug-resistant (MDR) phenotype and used laser flow cytometry to quantitate intracellular DNR content. In the vinblastine-resistant human lymphoblastic lymphoma cell line CEM-VBL, simultaneous incubation of DNR with Tmx 10 μmol/L or Tmx 50 μmol/L increased intracellular DNR fluorescence in a dose-dependent manner and demonstrated an uptake pattern similar to that seen with DNR and verapamil. Similar results were obtained in the vincristine-resistant human myeloid leukemia cell line HL-60/RV+. Cellular retention of DNR was also measured in both cell lines and results suggested that continuous exposure of the cells to Tmx resulted in higher intracellular DNR content compared with cells resuspended in fresh medium. No effect of Tmx or verapamil was observed in the drug-sensitive parent cell lines CEM or HL-60. Clonogenic experiments were then performed to determine whether Tmx was itself inhibitory to cell growth or whether Tmx potentiated DNR cytotoxicity. Tmx 10 μmol/L did not significantly inhibit either CEM-VBL or HL-60/RV+ cells after a 3-hour exposure followed by culture in methycellulose. Tmx 50 μmol/L was significantly more inhibitory in both cell lines. However, cells that had been incubated with DNR and Tmx 10 μmol/L demonstrated a marked increment in growth inhibition compared with cells that had been incubated with DNR alone or Tmx 10 μmol/L alone. Based on the data presented here, we suggest that clinical testing of Tmx and DNR be pursued in the setting where MDR may play a role.

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tion of $2 \times 10^6$ cells/mL were exposed to DNR 1 $\mu$g/mL alone or in combination with the test drug. At time 0, 5, 30, 45, 60, and 90 minutes cells were washed twice in ice-cold phosphate-buffered saline (PBS) without Ca$^{2+}$ or Mg$^{2+}$ (GIBCO) and resuspended in 0.5 mL ice-cold PBS with 5% FCS. DNR fluorescence was immediately measured using the FACSscan.

Drug retention studies were performed in separate experiments. Cells were first incubated with either DNR 1 $\mu$g/mL or the combination of DNR and the test drug for 1 hour. All samples were then washed twice in ice-cold medium and resuspended at 37°C in fresh medium containing the specific parameter, and DNR fluorescence was measured at time 0, 5, 15, 30, 60, and 120 minutes. DNR fluorescence was measured using the same FACSscan instrument as in the uptake experiments.

All drug uptake and retention experiments were performed in duplicate.

Clonogenic assays. Cells at a concentration of $2 \times 10^6$ were incubated with either medium, 0.1% DMSO, DNR 1 $\mu$g/mL, Tmx 10 $\mu$mol/L, Tmx 50 $\mu$mol/L, or the combination of DNR 1 $\mu$g/mL plus Tmx 10 $\mu$mol/L or Tmx 50 $\mu$mol/L for 1 hour. Aliquots of these test samples were then washed twice and plated in triplicate at a concentration of 250 cells per plate. To determine whether a more prolonged exposure to Tmx had an enhanced effect on cytotoxicity, separate aliquots were simultaneously resuspended in either medium, 0.1% DMSO, DNR 1 $\mu$g/mL, Tmx 10 $\mu$mol/L, or Tmx 50 $\mu$mol/L for an additional 2 hours, washed twice, and plated in triplicate at the above concentration.

The effect of verapamil on clonogenic capacity was also examined. Cells were incubated with either medium, DNR 1 $\mu$g/mL, verapamil 10 $\mu$mol/L, or the combination of DNR 1 $\mu$g/mL plus verapamil 10 $\mu$mol/L for 1 hour. Aliquots were then washed twice and plated in triplicate at the above concentration. Separate aliquots were simultaneously resuspended in either medium, DNR 1 $\mu$g/mL, or verapamil 10 $\mu$mol/L for an additional 2 hours, washed twice, and plated in triplicate at the above concentration.

Culture medium used for plating consisted of 1 mL of Iscove’s modified Dulbecco’s medium (IMDM; GIBCO) containing 24% FCS, 0.8% deionized bovine serum albumin (BSA; Sigma Chemicals, St Louis, MO), $10^{-4}$ mol/L 2-mercaptoethanol (Sigma), and methylcellulose at a final concentration of 1.3% in 35-mm Lux tissue culture dishes (Miles Scientific, Naperville, IL). Culture plates were incubated in a humidified atmosphere of 5% CO$_2$ in air for 10 to 12 days and scored using an inverted microscope. Aggregates $\geq 40$ cells were considered colonies.

Controls for each experiment were as follows: medium alone served as controls for experiments using DNR, medium with 0.1% DMSO served as controls for experiments using Tmx, and medium with 0.008% DMSO served as controls for experiments using verapamil.

Quantitation of membrane binding with HYB-241. Samples of cells to be studied for PGP expression were suspended at a concentration of $1 \times 10^6$ in 0.5 mL PBS. Two microliters of the MoAb HYB-241 (kindly provided by Dr L. Rittmann-Grauer, Hybritech Corp, San Diego, CA) was added to the test sample and 2 $\mu$g of the isotypic control antibody anti-gardenella GDJ352 (Hybritech) was added to the control sample. Both samples were processed simultaneously. The cells were first incubated on ice for 1 hour and then washed in PBS. Fifty microliters of a 1:64 dilution of ice-cold fluorescein isothiocyanate (FITC) conjugated goat antimouse IgG (Sigma) was then added and incubated for 1 hour on ice. Samples were then washed three times in PBS and fluorescence was measured immediately on the FACSscan. Consort 30 and Lysis software (Becton Dickinson, San Jose, CA) was used for data analysis.

Estrogen and progesterone receptor analysis. Estrogen and progesterone receptor analysis was performed using the dextran-coated charcoal assay.

RESULTS

HYB-241 binding to HL-60/RV+ and CEM-VBL cells. Figure 1A shows that the CEM-VBL cells incubated with MoAb HYB-241 strongly fluoresce, indicating the presence of PGP. Background fluorescence was determined by incubation with the control MoAb (GDJ352). No PGP-related fluorescence was detected in the parent cell line CEM.

Figure 1B shows the same experiment performed with the MDR subline HL-60/RV+ and its parent line HL-60. The HL-60/RV+ cells strongly fluoresce after incubation with HYB-241; no PGP-related fluorescence was detected.
in the HL-60/RV+ cells incubated with the control MoAb or on the parent cell line HL-60 incubated with HYB-241.

Effect of different concentrations of verapamil on DNR uptake. DNR plus verapamil 10 μmol/L, 25 μmol/L, and 50 μmol/L were incubated for 1 hour in both CEM-VBL and HL-60/RV+ cells and analyzed for intracellular DNR concentration. No significant differences were seen among the different concentrations of verapamil used (data not shown). The lowest dose of verapamil, 10 μmol/L, was used as a control in the Tmx experiments.

Effect of Tmx on DNR uptake and retention in CEM-VBL and HL-60/RV+ cell lines. Figure 2A shows the results of uptake studies in CEM-VBL cells after exposure to DNR 1 μg/mL alone or in combination with Tmx 10 μmol/L, Tmx 50 μmol/L, or verapamil 10 μmol/L. Cells incubated with Tmx alone or verapamil alone served as controls. The simultaneous incubation of DNR and verapamil 10 μmol/L demonstrated a fivefold increase in DNR uptake compared with control. Compared with the control, simultaneous incubation of DNR and Tmx 10 μmol/L increased DNR uptake nearly threefold. Uptake was increased nearly fivefold with the combination of DNR and Tmx 50 μmol/L.

Intracellular retention of DNR was also studied in CEM-VBL cells. Cells were incubated with DNR alone, DNR with Tmx, or DNR with verapamil. After 1 hour of incubation cells were resuspended in either medium or medium containing the initial incubation concentration of Tmx or verapamil, and DNR intracellular content was subsequently measured at various time points.

The results are shown in Fig 3A. Cells incubated initially with DNR and resuspended in medium for up to 2 hours demonstrated a rapid decrease in intracellular DNR content. Similar results were observed when the combinations of DNR plus Tmx 10 μmol/L and DNR plus verapamil were incubated for 1 hour and resuspended in medium. The combination of DNR plus Tmx 50 μmol/L incubated and
resuspended in medium led to a threefold increase in DNR retention.

To determine whether continuous exposure to verapamil or Tmx was important to maintain increased intracellular levels of DNR, a second set of samples was analyzed after resuspension in fresh medium containing the same concentration of drug that was used for the initial 1 hour of incubation; e.g., cells that were incubated for 1 hour with DNR plus Tmx 10 μmol/L were washed twice and resuspended in fresh medium containing Tmx 10 μmol/L. Retention was measured at specific time points and results from these experiments are also shown in Fig 3A. Cells that were exposed to DNR and Tmx 10 μmol/L and resuspended for 2 hours in Tmx 10 μmol/L had DNR retention similar to that of cells exposed to Tmx 50 μmol/L for 1 hour and resuspended in medium. Cells exposed to DNR and verapamil and resuspended in verapamil retained a high intracellular DNR content as shown.

CEM-VBL cells that had been incubated with DNR plus Tmx 50 μmol/L and then resuspended in medium containing Tmx 50 μmol/L demonstrated the highest intracellular concentration of DNR. As shown in Fig 3A, intracellular DNR concentration was nearly 10 times that of cells resuspended in medium alone.

**Effect of Tmx on DNR uptake and retention in HL-60/RV+ cells.** DNR uptake studies with Tmx and verapamil were also performed using HL-60/RV+ cells. Figure 2B shows that cells incubated for 1 hour with DNR and Tmx had increased intracellular DNR levels compared with cells incubated with DNR alone. Cellular uptake appeared dose-dependent with Tmx 50 μmol/L demonstrating the highest accumulation.

DNR retention experiments were also performed after 1 hour of exposure to DNR alone, DNR with Tmx, or DNR with verapamil. Cells were then resuspended in either fresh medium alone or fresh medium containing the initial incubation concentration of Tmx or verapamil. The pattern of DNR retention in these cells was similar to that observed with the CEM-VBL cell line and is shown in Fig 3B. Cells incubated for 1 hour with DNR and either Tmx 10 μmol/L or verapamil and then resuspended in fresh medium had rapid efflux of intracellular DNR. Cells incubated with DNR and Tmx 10 μmol/L and resuspended in Tmx 10 μmol/L had intracellular DNR concentrations similar to cells incubated with DNR and Tmx 50 μmol/L and resuspended in medium. Intracellular DNR levels were lower in both instances than cells incubated with DNR and verapamil and resuspended in verapamil.

As observed in the CEM-VBL line, HL-60/RV+ cells incubated with DNR and Tmx 50 μmol/L and resuspended in Tmx 50 μmol/L maintained high intracellular DNR levels throughout the 2-hour period of reincubation.

**Effect of Tmx on the drug-sensitive parent cell lines CEM and HL-60.** CEM and HL-60 cells were studied for DNR uptake as described above. Tmx did not increase DNR uptake in either of these cell lines (Figs 4A and B, respectively). Additionally, incubation of these cells with verapamil did not increase DNR uptake.

**Effect of Tmx and verapamil on clonogenic capacity of CEM-VBL and HL-60/RV+ cells.** Clonogenic assays were then performed to determine the relationship between DNR retention and cell kill. The first set of experiments was performed after 60 minutes of incubation with DNR and the drug under study. To determine whether prolonged exposure to the drug resulted in increased cell kill over that observed when cells were resuspended in fresh medium alone, a second set of clonogenic experiments was performed after an additional 2 hours of resuspension in either drug or fresh medium.

Figure 5A outlines the effect of Tmx on the clonogenic
growth of CEM-VBL cells. Cells incubated in control medium alone for 1 hour served as controls (100%) (panel 1). Tmx 10 μmol/L had no effect on the growth of these cells after 1 hour of incubation (panel 3); an additional 2 hours of resuspension in Tmx 10 μmol/L did not result in any further inhibition (panel 12). Clonogenic growth after 1 hour of incubation with DNR 1 μg/mL demonstrated 22% inhibition (panel 2). However, after CEM-VBL cells were exposed to the combination of DNR 1 μg/mL and Tmx 50 μmol/L for 1 hour, approximately 50% growth inhibition was noted (panel 6). Resuspension for 2 additional hours in medium (panel 17) or Tmx 10 μmol/L (panel 18) led to a greater degree of inhibition, 62% and 72%, respectively.

Tmx 50 μmol/L was more inhibitory to CEM-VBL cells than Tmx 10 μmol/L; 36% inhibition was noted after 1 hour of incubation of CEM-VBL cells (panel 4), which increased to 72% after an additional 2 hours of exposure in fresh medium containing Tmx 50 μmol/L (panel 14). Cells exposed to the combination of DNR 1 μg/mL and Tmx 50 μmol/L demonstrated a strong degree of inhibition, 86%, after 1 hour of incubation (panel 7); an additional 2 hours of resuspension in either fresh medium (panel 19) or fresh medium containing Tmx 50 μmol/L (panel 20) also resulted in a strong degree of growth inhibition, 82% and 99%, respectively.

Figure 5B outlines clonogenic experiments performed using HL-60/RV+ cells. As observed with the CEM-VBL cells, Tmx 10 μmol/L had no inhibitory effect after 1 hour of exposure (panel 3). After an additional 2 hours of resuspension in either fresh medium (panel 11) or fresh medium containing Tmx 10 μmol/L (panel 12), a small amount of inhibition was noted, 21% and 29%, respectively. Incubation of HL-60/RV+ cells with the combination of DNR 1 μg/mL and Tmx 10 μmol/L resulted in a significant degree of inhibition after 1 hour of exposure, 65% (panel 6); resuspension for an additional 2 hours in either fresh medium with 0.1% DMSO alone (panel 17) or fresh medium with 0.1% DMSO containing Tmx 10 μmol/L (panel 18), a similar degree of inhibition was noted, 76% and 89%, respectively.

As noted with the CEM-VBL cells, incubation with Tmx 50 μmol/L resulted in ~50% inhibition after 1 hour of exposure (panel 4). Resuspension in fresh medium containing Tmx 50 μmol/L for 2 hours resulted in ~95% inhibition (panel 14). The combination of DNR plus Tmx 50 μmol/L was also strongly inhibitory after 1 hour of exposure (panel 7) and after resuspension in either fresh medium (panel 19) or fresh medium containing Tmx 50 μmol/L (panel 20); in the latter instance, 100% inhibition was noted.

Growth inhibition after exposure to verapamil alone and in combination with DNR was also examined in CEM-VBL cells after 1 hour of exposure (Fig 5A, panels 5 and 8) and
after 2 hours of resuspension in either medium alone (panel 15) or medium containing verapamil (panel 16). Results are similar to those seen with Tmx 10 μmol/L; the combination of DNR and verapamil was much more inhibitory to cell growth than either drug alone (panels 21 and 22). A similar effect was noted in HL-60/RV+ cells (Fig 5B, panels 5 and 8 after 1 hour of incubation and panels 15, 16, 21, and 22 after 2 hours of resuspension).

**Estrogen and progesterone receptor analysis of CEM-VBL and HL-60/RV+ Cells.** To determine whether the toxicity of Tmx correlated with expression of either estrogen or progesterone receptors on the cells, receptor analysis was performed. CEM cells had no detectable estrogen or progesterone receptors. CEM-VBL cells had no detectable estrogen receptor but were positive for the progesterone receptor (9.5 femtomole [fm]/mg protein). The HL-60 parent line and the HL-60/RV+ line were negative for both estrogen and progesterone receptors.

**DISCUSSION**

In this report we demonstrate that Tmx can increase DNR uptake and retention in two human leukemia cell lines that display the MDR phenotype. The increase in DNR uptake with Tmx was concentration dependent in both the CEM-VBL cells (Fig 2A) and the HL-60/RV+ cells (Fig 2B) and is unrelated to Tmx's known antiestrogenic properties because estrogen receptors were absent from both cell lines studied. Tmx had no effect on DNR uptake in the drug sensitive parent cell lines (Figs 4A and B).

Ramu et al first recognized that Tmx was able to reverse drug resistance in a murine leukemia cell line in 1984 and postulated that, by virtue of its structure, the hormone interacted with the membrane phospholipid domain in such a manner as to alter the lipid packing density and thereby the diffusion rate of certain drugs. However, the exact nature of its interaction with the cellular membrane has not been clarified. Clarke et al have suggested that the plasticity of the membrane and, secondarily, its transport systems may be affected by highly lipophilic hormones. This group measured fluorescence of the fluorophore 1,6-diphenylhexatriene in the human breast cancer cell lines MCF-7 (estrogen receptor positive) and MDA-MB-436 (estrogen receptor negative) and demonstrated that both estrogen and Tmx can significantly decrease the fluidity of the cellular membranes. Clarke et al have also suggested that these membrane changes account for their own previous observation concerning the dose-dependent reduction of the cytotoxic effects of methotrexate when 17b-estradiol is added to MDA-MB-436 cells and for the decrease in cytotoxicity induced by melphalan or fluorouracil when Tmx is added to both the estrogen receptor positive and negative breast cancer cell lines recently described by Osborne et al.

More recent evidence suggests that Tmx interacts with specific enzyme and calcium transport systems. For example, it has been shown that Tmx antagonizes phospholipid and calmodulin-activated protein kinase C. Significantly, Greenberg et al have also shown that Tmx can inhibit calcium influx and can compete for calcium binding sites in the neurosecretory cell line PC12. It is possible that Tmx binds directly to PGP; that hormones can do so has been recently demonstrated by two separate groups. Yang et al have shown that progesterone significantly inhibited [3H] vinblastine binding to membrane vesicles prepared from MDR cells; hydrocortisone and testosterone did so to a lesser degree. Moreover, progesterone enhanced vinblastine accumulation in these cells and increased their sensitivity to vinblastine. Naito et al subsequently confirmed that progesterone bound specifically to PGP.

That Tmx may also bind to PGP rather than exert a more generalized effect on membrane function is suggested by the results of the experiments presented here. If Tmx altered membrane permeability such that DNR diffused in more rapidly, then intracellular DNR retention would not be expected to remain constant over time as, presumably, efflux would be just as rapid. However, the retention experiments in both the CEM-VBL (Fig 3A) and the HL-60/RV+ (Fig 3B) cell show that intracellular DNR concentrations remain relatively constant over time, suggesting that active efflux of the drug is blocked. Photoaffinity labeling studies are planned that will hopefully determine whether Tmx binds directly to PGP.

Data from the clonogenic experiments with both CEM-VBL (Fig 5A) and HL-60/RV+ cells (Fig 5B) demonstrate that Tmx 10 μmol/L had little inhibitory effect on cell growth. As expected in these drug resistant cell lines, DNR alone did not have a significant inhibitory effect on cell growth. However, incubating cells for 1 hour with the combination of Tmx 10 μmol/L and DNR resulted in a marked enhancement in cytotoxicity compared with cells exposed to DNR alone. When cells were subsequently resuspended in fresh medium containing Tmx 10 μmol/L, inhibition of cell growth increased further. This result would suggest that continued exposure to Tmx results in a higher percentage of cell death. The effect of verapamil on growth inhibition is shown in Figs 5A and B. Similar to Tmx 10 μmol/L, the inhibitory effect of incubating cells with verapamil and DNR is increased compared with incubating cells in DNR alone.

Based on the in vitro data presented here we suggest a potential new role for Tmx in patients with relapsed or refractory leukemia (or any solid tumor that demonstrates the MDR phenotype) whereby the hormone is given in high doses for a short period of time, followed by administration of a drug affected by the PGP pump such as DNR. However, we recognize that significant difficulty may exist in applying these results directly to the clinical setting. First, not all patients with relapsed or refractory disease have PGP expressed on their cells and overexpression of PGP may be heterogeneous within a given population of cells. It is clear that further data regarding PGP expression is needed before defining the extent of this form of drug resistance and it will be extremely important to correlate PGP expression with clinical resistance.

Second, results with the two cell lines tested here suggest that a high concentration of Tmx, in the range of 10 μmol/L, is effective in increasing the intracellular concentration of...
DNR in MDR cells. Both these cell lines are many-fold resistant to DNR, and it may be that lesser amounts of Tmx are needed for high, prolonged serum levels: the half-life of the hormone is biphasic, with an initial serum half-life of 7 to 14 hours and a secondary half-life of more than 7 days.44 With a standard dose of Tmx (20 to 40 mg/d), serum levels are in the range of 300 ng/mL or 0.8 \mu mol/L and may take up to 28 days to achieve.43 Higher doses of Tmx, in the range of 200 mg/d, have been used and serum levels in this instance are \sim 5 \mu mol/L.45 By administering an initial loading dose of Tmx followed by high daily doses for a short period of time it may be possible to achieve high serum levels within 1 week.45 Preliminary results of a phase I trial of high-dose Tmx combined with vinblastine in patients with advanced renal cancer suggest that such an approach is feasible.44

Third, Tmx does generate some degree of toxicity even at standard doses. The main side effects of Tmx are nausea and hot flashes;46 transient leukopenia and thrombocytopenia have also been reported46 as have venous and arterial thromboses, although the incidence of the latter two complications is low.46 High doses administered over a prolonged period of time (240 to 320 mg/d administered for more than a year), can cause abnormalities of the cornea and retina.47 Concern exists, however, that toxicity may be altered when Tmx is administered to reverse the MDR phenotype, given that PGP has been detected on a variety of normal tissues such as normal human kidney, adrenal gland, liver, and colon.48,49 Additionally, Cordon-Cardo et al49 have shown that endothelial cells lining the trachea, lung, endometrium, and prostate as well as acinar cells in the pancreas all express the PGP. However, previous studies combining verapamil with vinblastine,9 adriamycin,20 or a vincristine-adriamycin-decadron regimen11 have not shown excessive toxicity apart from the anticipated cardiac side effects of verapamil (hypotension and heart block). Moreover, Fine et al46 have shown that verapamil in doses high enough to reverse the MDR phenotype does not inhibit normal marrow granulocyte-macrophage colony forming unit. Whether Tmx in similar doses is toxic to normal marrow is currently under study.

Lastly, it may be possible to combine a lower dose of both Tmx and verapamil and achieve the same degree of PGP reversal. Preliminary uptake experiments in HL-60/RV+ cells have shown that the combination of Tmx 5 \mu mol/L and verapamil 2 \mu mol/L are additive in their effect (Berman, unpublished data). Further evaluation of other triphenylethylene derivatives such as toremifene is also warranted as preliminary evidence also suggests that this compound is also able to reverse the MDR phenotype.51

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