Differential modulation of doxorubicin toxicity to multidrug and intrinsically drug resistant cell lines by anti-oestrogens and their major metabolites

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Summary The ability of the anti-oestrogens tamoxifen, toremifene and their 4-hydroxy and N-desmethyl metabolites to modify doxorubicin (dox) toxicity to intrinsically resistant and multidrug resistant cell lines was compared, using human breast and lung cancer, and Chinese hamster ovary cell lines. The anti-oestrogens significantly enhanced dox toxicity to multidrug resistant, P-glycoprotein-positive cell lines, but did not affect toxicity to intrinsically resistant, P-glycoprotein-negative cells. Modification was observed at clinically achievable anti-oestrogen concentrations. Toremifene and tamoxifen would therefore appear to be good candidates for in vivo studies as MDR modulating agents in selected patients with P-glycoprotein-positive tumours.

Doxorubicin (dox) is cytotoxic to many solid tumours and anthracyclines are the most active single agents available for the treatment of advanced breast cancer, with response rates of 43% in previously untreated and 28% in previously treated patients (Tormey, 1975). Unfortunately, chemotherapy is not curative in these patients, and the development of drug resistance is a major problem in clinical management. Tumour cells may become resistant not only to the drug to which they were initially exposed, but also to a range of structurally and functionally unrelated compounds. This phenomenon, known as multidrug resistance (MDR), frequently coincides with expression of a 170 kDa membrane glycoprotein (the mdr1 gene product, P-glycoprotein; Endicott & Ling, 1989). Increased levels of P-glycoprotein (Pgp), associated with resistance to dox and vinblastine, have been detected in a number of human tumours, including breast cancers in patients previously treated with chemotherapeutic drugs (Sanfilippo et al., 1991). MDR-positive cells generally accumulate less drug than their sensitive counterparts (Kessel, 1986; Foster et al., 1988), and the structure of Pgp indicates it may act as an ATP-dependent 'drug efflux pump', reducing intracellular drug concentrations to sub-lethal levels. The mdr1 gene product has also recently been shown to be 'associated' with a volume-activated chloride channel (Valverde et al., 1992), and is a member of the ABC (ATP binding cassette) superfamily of ATP-dependent active transporters of which over 40 members have so far been characterised (Higgins, 1989). The superfamily includes bacterial transport proteins and the cystic fibrosis transmembrane conductance regulator, CFTR (Higgins & Hyde, 1991). Cells expressing the MDR phenotype are typically cross-resistant to large lipophilic 'natural product' cytotoxins such as dox and the Vinca-alkaloids, but not to anti-metabolites or alkylating agents.

Circumvention of MDR could be of great clinical benefit, and many potential resistance modifiers have been evaluated. The calcium channel blocker verapamil was the first to be identified and was shown to enhance vincristine toxicity to MDR-positive P388 leukaemia (Tsuroo et al., 1981). A photo-affinity analogue of verapamil with no calcium channel antagonist activity binds Pgp (Quan & Beck, 1990), indicating that the MDR-modulating activity of verapamil is due to competitive inhibition of Pgp at specific drug binding sites, resulting in inhibition of drug efflux (Kessel, 1986). Indeed, many MDR modifiers have been shown to enhance intracellular drug accumulation in Pgp-positive cells (Ramu et al., 1984; Kessel, 1986), although the increases in drug concentration observed rarely exceed 2- to 3-fold and may not be sufficient to explain the large enhancements of drug toxicity demonstrated (Fairchild & Cowan, 1991). Unfortunately, levels of verapamil achievable in vivo only border on those required to modify drug resistance in vitro and attempts to modulate resistance, particularly in solid tumours, have been relatively unsuccessful. Other compounds demonstrated to enhance drug toxicity to MDR-positive cell lines include calmodulin antagonists (e.g. trifluoperazine; Ganapathi et al., 1991), immuno-suppressants (e.g. cyclosporin A; Twentyman et al., 1987) and the anti-oestrogens tamoxifen (Ramu et al., 1984) and toremifene (DeGregorio et al., 1989).

Tamoxifen and toremifene are used to treat breast cancer, and their ability to bind oestrogen receptors (ER) is well documented (Lerner & Jordan, 1990; Kangas, 1990). Both anti-oestrogens are well tolerated, although toremifene can be administered at higher doses (Kohler et al., 1990; Robinson et al., 1990), and higher plasma concentrations can therefore be achieved (DeGregorio et al., 1989; Kaye, 1990). Tamoxifen and toremifene are metabolised extensively in vivo, primarily to the N-desmethyl, N-didesmethyl and 4-hydroxy derivatives (Jordan et al., 1983; Kangas, 1990; Kaye, 1990; Robinson et al., 1991). These metabolites differ from their parent compounds in their biological activity; for example, 4-hydroxy tamoxifen (OHTx) has 100-fold greater affinity for ER than tamoxifen (Jordan et al., 1980). The cytotoxic and MDR-modulating activity of tamoxifen, toremifene and their major metabolites should therefore be thoroughly investigated if these compounds are to be seriously considered as potential in vivo modifiers of MDR.

The effects of tamoxifen, toremifene and their two major metabolites on cell growth and on dox toxicity have been studied. The panel of cell lines used includes three Pgp-positive MDR cell lines and their drug-sensitive parental lines (Table I). In addition, a range of human breast and lung cancer cell lines of varying histological type were included in this study. They differ markedly in intrinsic sensitivity to dox, and this effect is unrelated to P-glycoprotein expression.

Materials and methods

Cell lines and tissue culture

Wild type cell lines and their drug-resistant sublines used in this study were (i) the human non-small cell lung carcinoma S1 (Baas et al., 1990) and its mdr1-transfected subline S1/1.1 which has mdr1 levels at least 100-fold higher than the wild
were ing days. The list in Table 1 show...eas of materials and methods. Strong positive staining (+ + + +); weak positive staining (+ +); weak staining (+); negative staining (−); heterogeneous results e.g. most cells strongly positive with others weakly positive (+ + + +).

**Table 1** Characteristics of cell lines

| Cell line | Characteristics | References | Density* (cells/well) | Antibody staining† | MRK16 | C219 |
|-----------|----------------|------------|-----------------------|--------------------|-------|------|
| CHO       |                |            |                       |                    |       |      |
| CHO-K1    | Wild type      | Puck et al. (1938) | 1,000  | + | − | /+ |
| CHO-K1<sup>mdr</sup> | MDR + ve | Chatterjee & Harris (1990) | 1,000  | + | + | |
| Breast cancer |     |            |                       |                    |       |      |
| MCF-7<sup>ndmt</sup> | ER + ve | Soule et al. (1973) | 5,000 | − | − | /+ |
| MCF-7<sup>ndmt</sup> | ER + ve, MDR + ve | Batist et al. (1986) | 5,000 | + + + + | + + /+ |
| MDA-468   | ER − ve        | Cailleau et al. (1974) | 5,000 | − | − | |
| T47D      | ER + ve        | Freake et al. (1981) | 5,000 | − | − | + |
| Lung cancer |     |            |                       |                    |       |      |
| S1        | Non-small cell | Baas et al. (1990) | 1,500 | − | − | − |
| S1/1.1    | mdr1-transfected | Baas (unpublished, 1991) | 1,500 | + + | + | + |
| NCI-H 322 | Bronchio-alveolar | Carmichael et al. (1987) | 10,000 | − | − | − |
| NCI-H 358 | Bronchio-alveolar | Carmichael et al. (1987) | 10,000 | − | − | − |
| NCI-H 460 | Large cell     | Carmichael et al. (1987) | 1,000 | − | − | − |
| NCI-H 841 | Small cell (variant) | Carmichael et al. (1987) | 10,000 | − | − | − |

*Density at which cells were plated in wells of 96 well microtitre plates for cytotoxicity assays. †Cells were stained with anti-Pgp monoclonal antibodies MRK16 and C219 as described in Materials and methods. Strong positive staining (+ + + +); weak positive staining (+ +); weak staining (+); negative staining (−); heterogeneous results e.g. most cells strongly positive with others weakly positive (+ + + +).

**Table 2** Sensitivity of cell lines to tamoxifen and toremifene. Anti-oestrogen IC<sub>50</sub> values are mean values from 6 determinations ± s.e.m.

| Cell line | Anti-oestrogen IC<sub>50</sub> (µM) | MNC (µM)† |
|-----------|----------------------------------|-----------|
| CHO       | 20 ± 4                           | 18 ± 2    | 10        |
| CHO-K1<sup>mdr</sup> | 16 ± 2  | 14 ± 1 | 10        |
| MCF-7     | 11 ± 1                           | 12 ± 1   | 1         |
| MCF-7<sup>ndmt</sup> | 31 ± 2 | 27 ± 1 | 20        |
| MDA-468   | 15 ± 2                           | 15 ± 1   | 10        |
| T47D      | 19 ± 2                           | 18 ± 1   | 5         |
| S1        | 22 ± 2                           | 23 ± 1   | 10        |
| S1/1.1    | 21 ± 1                           | 21 ± 2   | 10        |
| NCI-H 322 | 27 ± 3                           | 25 ± 1   | 10        |
| NCI-H 358 | 26 ± 2                           | 27 ± 2   | 10        |
| NCI-H 460 | 14 ± 2                           | 15 ± 2   | 5         |
| NCI-H 841 | 28 ± 3                           | 27 ± 2   | 10        |

†MNC (maximum non-toxic concentrations) are the same for all anti-oestrogens.

Dox, formulated for clinical use (Farmitalia UK, St Albans), was stored as a 5 mM solution in normal saline at −20°C and diluted as required in saline. Tamoxifen and metabolites were provided by ICI Pharmaceuticals (Macclesfield, UK) and toremifene and metabolites by Orion Corporation (Turku, Finland). Tamoxifen was prepared as a 50 mM stock solution in ethanol and stored at 4°C. N-desmethyl tamoxifen (NdMTx), OHTx, toremifene, N-desmethyl toremifene (NdMTI) and 4-hydroxy tamoxifen (OHTI) were dissolved in DMSO to give 50 mM stock solutions, which were diluted as required in PBS. Anti-oestrogens were tested in each cell line at a range of concentrations (0.5, 1, 2, 5, 10, 20, 25, 30, 50 and 100 µM). The highest level which was reproducibly found to alter control cell optical density by less than 5% was defined as the maximum non-toxic concentration (MNC, listed in Table II), and this concentration was used in drug toxicity modification experiments. Organic solvent levels did not exceed 0.1% by volume of the cell suspension, a concentration of vehicle demonstrated not to affect cell growth.

**Cytotoxicity assays**

Exponentially growing cells were trypsinised, centrifuged and reseeded in fresh medium (10% FCS, 2 mM glutamine) at the appropriate cell density. Cell suspension (180 µl) was aliquoted into 96 well microtitre plates at a seeding density previously demonstrated to allow exponential growth for 4 days. Anti-oestrogen modifiers, dox and/or vehicle (10 µl) were added in quadruplicate at appropriate concentrations. Cells were incubated continuously with drug and/or modifier at 37°C (5% CO<sub>2</sub>, 100% humidity) for 4 days. Cytotoxicity was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide] assay (Mosmann, 1983; Carmichael et al., 1987). MTT (50 µl, 2 mg ml<sup>−1</sup>) was aliquoted into all wells and the cells incubated for a further 4 h. Plates were inverted to discard medium and formazan crystals solubilised in 100 µl DMSO with 25 µl glycine buffer (0.1 M glycine in 0.1 M NaCl, pH 10.5; Plumb et al., 1989). Plates were agitated for 5 min, and optical densities determined immediately at 540 nm using a Titertek Multiskan Plus MKII ELISA plate reader. Data were analysed using Deltasoft Elisa Analysis software (BioMetallics Inc., Princeton, NJ).

Cytotoxicity was expressed as the IC<sub>50</sub> value; the concentration of drug causing a 50% reduction of control cell optical density. IC<sub>50</sub> values are presented as the means of those determined from at least six experiments ± the standard error of the mean (s.e.m.). The number of repeats is indicated in the Table and Figure legends. IC<sub>50</sub> values determined in the presence and absence of anti-oestrogens were compared using paired t-tests. Modification of dox toxicity by anti-oestrogens was expressed as a modification factor (MF), calculated by dividing the dox IC<sub>50</sub> value determined in the absence of anti-oestrogen by that determined in the presence of anti-oestrogen. A MF value of 1 therefore indicates that anti-oestrogens do not affect dox toxicity, while values greater than 1 indicate enhancement of, and values less than 1 protection from, dox toxicity.
**Immunocytochemical staining**

Cells in exponential growth phase were removed from flasks by trypsinisation, washed three times with PBS and resuspended in PBS to a density of 10^5 cells ml^{-1}. Cell suspension (0.5 ml) was applied to microscope slides by cytospanning. Cells were incubated first with a monoclonal antibody (MRK16 or C219) for 30 min, then with peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark). The peroxidase reaction was developed using diaminobenzidine (Sigma Chemical Co.) and hydrogen peroxide.

**Results**

Cell lines were stained with monoclonal antibodies, MRK16 and C219, which recognise different epitopes of Pgp. In general, similar results were obtained with both antibodies, although MRK16 stained cells more strongly (Table I). Both antibodies stained MCF-7Adr cells positively for Pgp with \( \sim 70\% \) of cells showing very strong staining, while MCF-7 cells stained weakly with C219 only. CHO-K1Adr cells were positive for Pgp with both antibodies. CHO-K1 cells stained weakly and uniformly with MRK16, while \( \sim 40\% \) of cells stained with C219. The *mdr1* transfectant S1/1.1 was positive with both antibodies, with \( \sim 5\% \) of cells staining more strongly with MRK16, and wild type S1 cells were Pgp-negative with both antibodies. Results for the remaining wild type lung and breast cancer cell lines were negative or weakly positive and are summarised in Table I.

Tamoxifen and its structural analogue toremifene were equitoxic (Table II). The metabolites of these anti-oestrogens had similar toxicities to their parent compounds (data not shown), although their reported affinities for ER vary (Jordan *et al*., 1980). ER-positive MCF-7 cells were most sensitive to anti-oestrogens, exhibiting biphasic dose response curves, while ER-negative cell lines were in general more resistant, with steep, monophasic dose response curves (data not shown). Although the MCF-7Adr cell line was more resistant to anti-oestrogens than its ER-positive parental line, the remaining MDR-positive cell lines were not, indicating that such resistance is not part of the MDR phenotype.

MCF-7Adr cells were 180-fold resistant to dox relative to wild type MCF-7 cells (Table III). Figure 1 shows the effect of tamoxifen on dox toxicity to wild type MCF-7 and MCF-7Adr cells. Increasing concentrations of tamoxifen (up to a MNC of 20 \( \mu \)M) shifted the dox dose response curve for MCF-7Adr cells progressively leftwards, indicating enhancement of drug toxicity, while similar dose response curves for

| Modifier | MCF-7 | MF | MCF-7Adr | MF |
|----------|-------|----|----------|----|
| None     | 64 ± 5 | 1  | 11600 ± 1100 | 1  |
| Tamoxifen| 70 ± 10| 0.92| 1410 ± 230  | 8.2***|
| OHTx     | 71 ± 18| 0.9 | 1020 ± 80   | 11.4***|
| NdMTx    | 60 ± 7 | 1.1 | 2320 ± 330  | 5.0***|
| Toremifene| 74 ± 11| 0.9 | 972 ± 146   | 11.9***|
| OHTT     | 58 ± 10| 1.1 | 853 ± 121   | 13.6***|
| NdMTT    | 82 ± 17| 0.8 | 1300 ± 230  | 8.9***|

*Anti-oestrogens were added to cells at the appropriate MNC (Table II). **Where MF = 1, *P* values of 0.05–1.0 were obtained. ***P* = 0–0.001.

**Figure 1** Effect of tamoxifen on dox toxicity to **a**, wild type MCF-7 and **b**, MDR-positive MCF-7Adr cells (results from one representative experiment). **a**, 0 (○), 0.01(▲), 0.5 (▲) and 1 \( \mu \)M (▲) tamoxifen; **b**, 0 (○), 1 (▲), 10 (▲) and 20 \( \mu \)M (▲) tamoxifen.

**Figure 2** Effect of anti-oestrogens on dox toxicity to MCF-7Adr cells. Modification of dox toxicity is expressed as a modification factor (ratio of dox IC50 values determined in the absence and presence of modifier). Tamoxifen (○), OHTx (▲), NdMTx (■), toremifene (◇), OHTT (△) and NdMTT (□). Results are mean MF calculated from ten identical experiments ± s.e.m.
wild type MCF-7 cells overlaid, indicating that tamoxifen had no effect on drug toxicity. However, it should be noted that the MNC of anti-oestrogens for MCF-7 was 1 μM, a dose causing no significant modification of dox toxicity to either cell line. The effects of anti-oestrogens were not altered by addition of oestradiol (unpublished data). The effects of the MNC of tamoxifen, toremifene and their metabolites on dox toxicity to wild type MCF-7 and MCF-7\textsuperscript{AAdr} cells are summarised in Table III. All anti-oestrogens significantly enhanced dox toxicity to Pgp-positive MCF-7\textsuperscript{AAdr} cells (5- to 14-fold), but had no effect on wild type cell sensitivity. The relative efficacy of the anti-oestrogens as modifiers of dox toxicity to MCF-7\textsuperscript{AAdr} cells is compared in Figure 2, where the degree of modification observed is expressed as a function of anti-oestrogen concentration. Modification of dox toxicity was clearly a dose-dependent effect, although curves plateaued at anti-oestrogen concentrations approached 20 μM. All compounds significantly enhanced dox toxicity and OHTF was apparently the most effective modifier at all concentrations. The maximum modification observed was a 14-fold reduction in the IC\textsubscript{50} value from 11.6 μM to 0.85 μM in the presence of 20 μM OHTF, a value still 13-fold in excess of that obtained for wild type cells (0.064 μM); sensitivity was not reduced to wild type levels.

Wild type MCF-7 cells were more sensitive to anti-oestrogens than MCF-7\textsuperscript{AAdr} cells, and equimolar doses of modifiers therefore could not be compared. The effects of tamoxifen and toremifene on dox toxicity to wild type CHO-K1 and MDR-positive CHO-K1\textsuperscript{AAdr} cells, which are equally sensitive to anti-oestrogens, were therefore determined. CHO-K1\textsuperscript{AAdr} cells showed a marked (18-fold) resistance to dox relative to its parental line, CHO-K1. Tamoxifen and toremifene (10 μM) enhanced dox toxicity to the wild type cell line 3- and 4-fold, respectively, but caused more substantial 7- and 9-fold increases in toxicity to CHO-K1\textsuperscript{AAdr} cells (Table IV). Lesser modifications were observed with 5 μM tamoxifen and toremifene. A mean 7-fold degree of resistance therefore remained between MDR-positive and wild type cells in the presence of anti-oestrogens.

Surprisingly, the lung cancer cell lines S1 and the mdr1 transfectant S1/1.1 were equally sensitive to dox (although S1/1.1 cells were 5-fold resistant to vinblastine relative to S1 cells, unpublished data), despite different levels of mdr1 expression. Dox toxicity to wild type cells was not enhanced by tamoxifen and toremifene, however a slight but significant degree of modification (2-fold; \( P < 0.01 \)) was observed for the transfectant, to below wild type sensitivity (Figure 3).

The effects of anti-oestrogens on dox toxicity to Pgp-negative cell lines were further investigated by determining dox toxicity to 6 MDR-negative lung and breast cancer cell lines; IC\textsubscript{50} values ranging from 22 to 335 nM were deter-

| Table IV | Effects of tamoxifen and toremifene on dox toxicity to CHO, lung cancer and breast cancer cell lines. Dox IC\textsubscript{50} values are presented as the mean of at least 6 determinations ± s.e.m. |
| Cell line | + PBS | + Tamoxifen\textsuperscript{a} | MF | + Toremifene\textsuperscript{b} | MF |
| CHO-K1 | 76 ± 6 | 29 ± 5 | 2.6** | 17 ± 2 | 4.5** |
| CHO-K1\textsuperscript{AAdr} | 1360 ± 140 | 182 ± 37 | 7.5** | 145 ± 37 | 9.4*** |
| S1 | 71 ± 4 | 65 ± 3 | 1.1b | 62 ± 4 | 1.1 |
| S1/1.1 | 61 ± 6 | 37 ± 3 | 1.6** | 30 ± 3 | 2.0** |
| MCF-7\textsuperscript{c} | 64 ± 5 | 70 ± 10 | 0.9 | 74 ± 11 | 0.9 |
| MCF-7\textsuperscript{AAdr} | 11600 ± 1100 | 1410 ± 230 | 8.2*** | 972 ± 146 | 11.9*** |
| MDA-468 | 182 ± 8 | 170 ± 11 | 1.1 | 180 ± 3 | 1.0 |
| T47D | 187 ± 30 | 236 ± 14 | 0.8 | 206 ± 22 | 0.9 |
| NCI-H 322 | 335 ± 64 | 343 ± 15 | 1.0 | 387 ± 53 | 0.9 |
| NCI-H 358 | 169 ± 7 | 156 ± 46 | 1.1 | 158 ± 30 | 1.1 |
| NCI-H 460 | 22 ± 2 | 25 ± 2 | 0.9 | 21 ± 2 | 1.0 |
| NCI-H 841 | 118 ± 6 | 115 ± 9 | 1.0 | 117 ± 13 | 1.0 |

\( ^{a} \)Tamoxifen and toremifene were added to cells at the appropriate MNC (Table II). \( ^{b} \)Where MF = 1, \( P \) values of 0.05-1.0 were observed. \( ^{c} \)These data duplicated from Table III. \( * P = 0.01-0.05. ** P = 0.001-0.01. *** P = 0.0001. \)

**Figure 3** Effect of 0 (●), 1(O), 5 (△) and 10 μM (▲) tamoxifen on dox toxicity to a, wild type S1 and b, mdr1-transfected S1/1.1 cells (results from one representative experiment).
menced. The effects of the MNC of tamoxifen and toremifene (5 and 10 μM) on drug sensitivity was investigated (Table IV). Dox toxicity in the presence and absence of anti-oestrogens was not significantly different, indicating that no modification of drug toxicity was achieved.

Discussion

Despite their different affinities for ER, tamoxifen, toremifene and their metabolites were equtoxic to a range of breast and lung cancer cell lines. This results differs from that described by DeGregorio et al. (1989), who found MCF-7MDR cells to be more sensitive to OHTX and NdMTx than to tamoxifen. Cytotoxicity was assessed using methotazine blue staining after 48 h exposure to anti-oestrogens. Differences between their results and those presented here may arise from different assay systems employed. Tamoxifen, toremifene and their metabolites are structurally similar, and it is perhaps not surprising that toxicity to ER-negative cells does not vary greatly between these compounds. Higher resistance of MCF-7MDR cells to anti-oestrogens relative to wild type MCF-7 cells was probably due to different ER status rather than mdr1 expression; CHO-K1 and CHO-K1mdr1 cells were equally sensitive to anti-oestrogens, as were S1 and S1/1.1 cells. Resistance to anti-oestrogens therefore does not appear to be characteristic of the MDR phenotype, consistent with the report by Kessel (1986) that tamoxifen is not transported by Pgp.

Toremifene and toremifene substantially modified dox toxicity to Pgp-positive CHO-K1 and MCF-7MDR cells. Modification of dox toxicity by anti-oestrogens occurred irrespective of ER status and was unaffected by oestradiol (unpublished data), indicating that enhanced dox toxicity is not an anti-oestrogenic effect. In fact, substantial modification of drug toxicity is more likely to be observed with ER-negative cell lines which can tolerate higher doses of anti-oestrogens, because MDR-modification occurs in a dose dependent manner (Figure 2).

No enhancement of dox toxicity to MDR-negative lung and breast cancer cell lines exhibiting a 15-fold range of sensitivities to the drug was observed and modification of drug toxicity by anti-oestrogens would therefore appear to be an MDR-specific effect. However, dox toxicity to CHO-K1 cells was enhanced. Gupta (1988) demonstrated that wild type CHO cells display intrinsic resistance to drugs associated with MDR, which can be reversed by verapamil. CHO-K1 cells stained weakly with anti-Pgp antibodies (Table I), and intrinsic MDR in CHO cells may therefore be mediated by Pgp. This is supported by the discovery that Pgp is associated with a volume-activated chloride channel (Valverde et al., 1992), as CHO cells are known to respond to increases in volume with enhanced chloride channel activity (Sarkadi et al., 1984).

The ability of tamoxifen, toremifene and their metabolites to modify dox resistance in MCF-7MDR cells was compared. Maximal modification of MDR was observed at 10–20 μM. The relative ranking of efficacy for both tamoxifen and toremifene appeared to be: hydroxy metabolites > parent compounds > N-desmethyl metabolites. DeGregorio et al. (1989) also observed modification of dox toxicity to MCF-7MDR cells by toremifene and its metabolites, but found OHTX to be less effective than toremifene and NdMTF. Differences between their results and those presented here may arise from differences between experimental procedures. DeGregorio and co-workers described synergy between toxic concentrations of anti-oestrogens and a single (toxic) dose of dox (1 μM), whilst in the present study, the effects of non-toxic doses of anti-oestrogens on a range of dox concentrations were determined. The ability of anti-oestrogen metabolites to reverse drug resistance is highly significant as these compounds are major products of tamoxifen and toremifene metabolism. Both state serum hydroxy and 4-hydroxy metabolites are, respectively, ~140% and ~400% of parent compound levels in patients receiving tamoxifen (Lien et al., 1989) and ~25% in patients treated with toremifene (DeGregorio et al., 1989; Kohler et al., 1990).

Overexpression of Pgp is generally associated with decreased intracellular drug concentration, and there is evidence that anti-oestrogens increase drug accumulation in MDR-positive cells (Kessel, 1986; Ramu et al., 1984). For example, 10 μM tamoxifen caused a ~3-fold increase in daunorubicin accumulation in the MDR-positive cell lines, HL-60/RV + and CEM-VBL, but did not alter accumulation of other drug levels in wild type lymphoblastic leukaemia CEM cells or in myeloid leukaemia HL60 cells (Berman et al., 1991). The mechanism by which anti-oestrogens alter cellular drug retention is not fully understood. Tamoxifen inhibits protein kinase C (PKC) (O’Brian et al., 1985; Su et al., 1985; Horgan et al., 1986), an enzyme implicated in the phosphorylation and activation of Pgp (Chambers et al., 1990). Inhibition of PKC by staurosporine reduces the sensitivity of MDR-positive cells to doxorubicin, increases daunorubicin accumulation and enhances drug toxicity in MDR-positive HL-60 cells (Ma et al., 1991). Staurosporine also increases vincristine accumulation in MDR-positive human myelogenous leukaemia (K562/ADM) cells (Sato et al., 1990). Tamoxifen may therefore modulate MDR through inhibition of PKC, leading to inactivation of Pgp and enhanced drug accumulation. However, while staurosporine is the most active inhibitor of PKC (O’Brian et al., 1988) it is the least effective modifier of MDR in the present study.

Tamoxifen is also a calmodulin antagonist (Lam, 1984) and it has been suggested that this activity may be responsible for modulation of MDR (Chatterjee & Harris, 1990). However, Tsuruo et al. (1982) compared the effects of a range of calmodulin inhibitors on the accumulation and toxicity of vincristine and dox to MDR-positive P388 leukaemia, and found no correlation between antagonism of calmodulin and either drug accumulation or modulation of drug toxicity.

Hindenburg et al. (1987) report intra-lysozomal localisation of dox in dox-resistant HL60/AR, but not in drug sensitive HL60 cells. They suggest a wide range of resistance modifiers (e.g. chloroquine, clomiphene, toremifene, verapamil) alter drug solubility in subcellular compartments, allowing drug to redistribute within the cell and gain access to intracellular targets.

Interestingly, dox was equally toxic to the non-small cell lung carcinoma cell lines S1 and S1/1.1, although the mdrl-transfected cells were shown to be Pgp-positive with antibody staining (Table I). The low levels of resistance in S1/1.1 cells described in this study are typical of some mdrl transfectants, and occur despite elevated levels of Pgp expression. Fairchild et al. (1990) transfected wild type MCF-7 cells with the mdrl gene isolated from the MDR-positive subline, MCF-7MDR, and achieved levels of Pgp in transfectants equal to, or exceeding, those observed in MCF-7MDR cells. However, although the same pattern of MDR was displayed, the transfected cells did not exhibit the high degree of resistance observed in MCF-7MDR cells. It therefore appears that while expression of mdrl can confer the MDR phenotype, it is not sufficient to generate the very high levels of resistance observed in cell lines which have been exposed to high drug concentrations in vitro. The possible interaction between PKC and Pgp has been discussed above. PKC levels are frequently elevated in highly resistant MDR-positive cells (Fine et al., 1988) and may be necessary for activation of Pgp and expression of the full MDR phenotype.

It is also apparent that chronic drug-treatment activates multiple, independent mechanisms of resistance. Dox exerts multiple cellular effects including topoisomerase II inhibition (Tewey et al., 1984), DNA binding (Neidle, 1985), membrane disruption (Triton, 1991) and production of oxygen radicals (Bachur et al., 1979), and may induce resistance via any of these pathways.

Patients treated with tamoxifen or toremifene accumulate stable serum concentrations of parent compounds and metabolites (Kohler et al., 1990; Langan-Fahy et al., 1990). Patients receiving 'high' dose MDR modulators (e.g. P-gp or MDR1 (Stuart et al., 1992) achieved plasma tamoxifen levels of 3.5 μM, while total levels of tamoxifen and metabolites were
~7 µM. Although anti-oestrogens have been demonstrated to be ~99% bound to plasma proteins such as alpha; acid glycoprotein (Chatterjee & Harris, 1990), these compounds are lipophilic cations and it is therefore likely that they accumulate to higher concentrations within the cell. Lien et al. (1991) determined anti-oestrogen levels in patients treated with 20–80 mg tamoxifen daily. A mean serum tamoxifen concentration of 0.2 µM was achieved; however, mean levels in brain were 4.5 µM and in metastases, 6.6 µM. The same trend was observed with NdMTx and OHTx, and on average the concentration of anti-oestrogen accumulated in tissues was 16- to 30-fold higher than in serum. The total concentration of tamoxifen, OHTx and NdMTx achieved in metastases was ~16 µM. The accumulation of anti-oestrogen which would enhance dox toxicity to MCF-7Adr cells ~1-fold in vitro. This result suggests that low dose therapy may generate anti-oestrogen levels sufficient to modulate MDRI in vivo. Alternatively, if the same degree of compartmentalisation between serum and tissues occurs during high dose tamoxifen treatment, very high intracellular anti-oestrogen concentrations may be reached, and significant enhancement of cytotoxic drug activity achieved.

Tamoxifen, toremifene and their 4-hydroxy and N-desmethyl metabolites are effective in vitro modifiers of MDRI at achievable serum concentrations. They are clinically well-tolerated and may therefore be of great benefit in the treatment of tumours which characteristically express high levels of Pgp, such as renal, colorectal and adrenal carcinomas. However, it should be stressed that little effect was observed in intrinsically resistant MDR-negative cell lines, making patient selection an important parameter in the clinical evaluation of this class of modifier.

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Abbreviations: Dox, Doxorubicin hydrochloride; ER, oestrogen receptor; GST, glutathione-S-transferase; MDR, multidrug resistance; MNC, maximum non-toxic concentration; MTX; 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NdMT, N-desmethyl toremifene; NdMTx, N-desmethyl tamoxifen; OHT, 4-hydroxy toremifene; OHTx, 4-hydroxy tamoxifen; PBS, phosphate-buffered saline; Pgp, P-glycoprotein; PKC, protein kinase C.

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