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REVERSAL OF P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE BY PURE ANTI-OESTROGENS AND NOVEL TAMOXIFEN DERIVATIVES

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Abstract—In this study the ability of five novel anti-oestrogens [4-iodotamoxifen, pyrrolidino-4-iodotamoxifen, ethyl bromide tamoxifen (EBTx), ICI 164,384 (ICI 164) and ICI 182,780] to alter drug toxicity to multidrug resistant cell lines have been compared. The effect of these compounds on ATP-dependent vinblastine (VBL) transport was also tested using inside-out vesicles (IOV) prepared from highly P-glycoprotein (Pgp)-expressing CCRF-CEM/VBL_{1000} cells. The pure anti-oestrogen ICI 164 was most effective, enhancing doxorubicin and VBL toxicity to MCF-7^{Adr} cells 25- and 35-fold, respectively, and was also the best inhibitor of ATP-dependent [^{3}H]VBL accumulation by IOV. Pure anti-oestrogens, tamoxifen and iodotamoxifens completely reversed VBL resistance in the *mdr*1 transfected lung cancer cell line, S1/1.1, where resistance relative to wild-type cells was mediated solely by Pgp. The membrane impermeant tamoxifen derivative EBTx did not modify drug resistance, yet was as effective an inhibitor of VBL accumulation by inside-out Pgp-positive vesicles as tamoxifen. This indicates an intracellular role for tamoxifen and its derivatives in the modulation of Pgp-mediated drug resistance.

Key words: P-glycoprotein; multidrug resistance; anti-oestrogens; doxorubicin; vinblastine

Cancer patients treated with chemotherapeutic agents may initially respond to treatment but frequently relapse as tumour cells develop resistance, or as treatment selects for intrinsically drug-resistant cells. In general, such tumours become resistant not only to the drug with which they were initially treated, but also to other hydrophobic natural compounds. This phenomenon, known as MDR,¶ is frequently characterized by resistance to structurally and functionally diverse drugs, decreased drug accumulation and overexpression of the 170 kDa membrane protein, Pgp [1]. Pgp has been detected in drug resistant breast tumours [2], and the level of expression in cancer cell lines found to correlate with the degree of cellular resistance to DOX and VBL [3]. Pgp has putative intracellular drug and nucleotide binding sites [4], and has been identified as an ATP-dependent drug efflux pump which reduces intracellular drug concentrations. Its normal function is not fully understood, although its presence in normal tissues, such as kidney, liver and adrenal glands, provides circumstantial evidence supporting a secretory role.

Reversal of MDR is clearly an important clinical aim. Many compounds capable of modifying drug resistance *in vitro* have been identified, including the calcium-channel blocker verapamil [5], the immunosuppressant cyclosporin A [6], and calmodulin antagonists [7]. Many such compounds have undesirable side effects *in vivo*, and levels of compound which modify MDR *in vitro* frequently cannot be achieved safely. The anti-oestrogen tamoxifen has, however, been reported to reverse MDR *in vitro* at clinically achievable levels [8–10].

Tamoxifen competes with oestrogens for binding to ER and has been used since the 1970s to treat hormone-responsive breast cancer [11, 12]. It is not a pure anti-oestrogen as it exerts some oestrogenic effects, and there has been interest in developing new and more potent compounds with increased anti-oestrogenic activity. Tamoxifen is metabolised *in vivo* to OHTx, which has 100-fold greater affinity for ER than its parent compound [13], but is rapidly inactivated *in vivo* by glucuronidation of the 4-hydroxyl group. In ITx and PITx (Idoxifene) the hydroxyl substituent of OHTx has been replaced with an iodine atom resistant to conjugation [14]

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¶ Abbreviations: DOX, doxorubicin; EBTx, ethyl bromide tamoxifen; ER, oestrogen receptor(s); FCS, foetal calf serum; ICI 164, ICI 164,384; ICI 182, ICI 182,780; IOV, inside-out vesicles; ITx, 4-iodotamoxifen; MDR, multidrug resistance; MF, modification factor (ratio of IC_{50} values determined in absence and presence of modifier); MNC, maximum non-toxic concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; OD, optical density; OHTx, 4-hydroxy tamoxifen; Pgp, P-glycoprotein; PITx, pyrrolidino-4-iodotamoxifen; ROV, rightside-out vesicles; TBS, Tris-buffered sucrose; VBL, vinblastine.

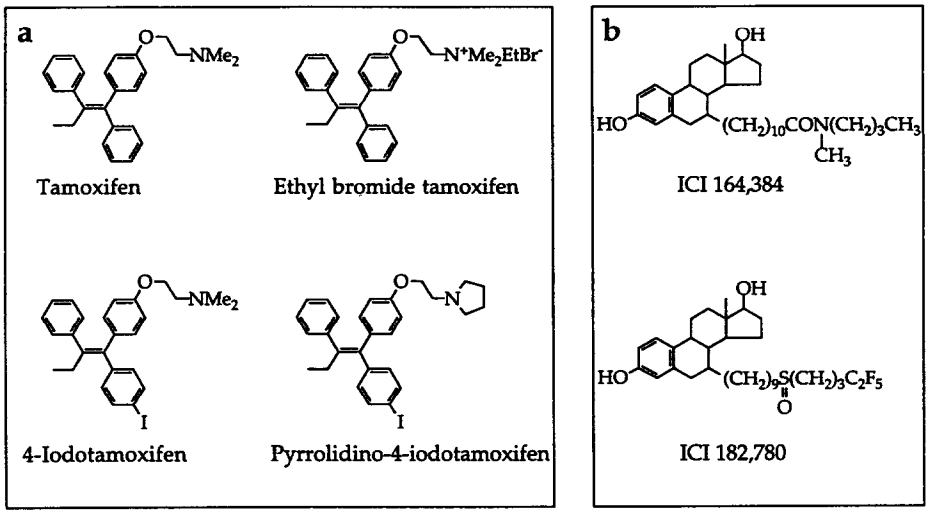

Fig. 1. Structures of (a) non-steroidal and (b) steroidal anti-oestrogens.

(Fig. 1), and PITx has greater anti-oestrogenic and anti-tumourigenic activity than tamoxifen or ITx in rat models [15]. Steroidal compounds with greater structural homology to oestrogen have also been developed. ICI 164 and ICI 182 are 7-α-alkyl amide analogues of oestradiol (Fig. 1) and, unlike tamoxifen, are pure anti-oestrogens [16–18].

In this study the effect of ICI 164, ICI 182, ITx, PITx and a novel membrane-impermeant tamoxifen derivative (EBTx [19]) on DOX and VBL toxicity to MDR-positive and MDR-negative cells were investigated. Resistance to both VBL (a microtubule assembly inhibitor [20]) and DOX (which has multiple mechanisms of toxicity including inhibition of topisomerase II [21] and membrane disruption [22]) is mediated by Pgp [23]. Following on from this, the effects of these compounds on VBL accumulation by IOV prepared from a highly Pgp-positive cell line were tested. There is evidence that anti-oestrogens may increase drug accumulation in MDR-positive cells [8, 24–26]. However, while the potency of MDR modulating agents such as calcium channel blockers has been correlated with their ability to inhibit VBL transport in Pgp-positive plasma membrane vesicles [27], the effects of anti-oestrogens in such a vesicle system, which allows more direct determination of the effects of inhibitors on Pgp drug pumping, have not previously been reported.

MATERIALS AND METHODS

Materials. DOX (Farmitalia UK, St Albans, U.K.) was stored at −20° as a 5 mM solution in normal saline and VBL sulphate (David Bull Laboratories Pty Ltd, Victoria, Australia) at 4° as a 1 mM solution in PBS. ICI 164, ICI 182, ITx, PITx and EBTX were stored at 4° as 20 mM solutions in ethanol and diluted in PBS. [^{3}H]VBL (11.7 Ci/mmol) was obtained from Amersham International (Amersham, U.K.).

Cell lines and tissue culture. MDR-positive cell lines used in this study were: (1) ER-negative MCF-7^{Adr} [28], selected by chronic exposure of the ER-positive epithelial breast cancer cell line MCF-7 [29] to DOX and (2) S1/1.1, derived from the non-small cell lung cancer cell line S1 [30] by transfection with an *mdr*1 expression vector [31]. CCRF–CEM/VBL$_{1000}$ (CEM/VBL$_{1000}$) cells are a human lymphoblastic leukaemia cell line made 1000-fold resistant to VBL by *in vitro* exposure to this drug [32].

Cell lines were maintained as suspension (CEM/VBL$_{1000}$) or monolayer cultures in HAMS F12 (S1 and S1/1.1) or RPMI 1640 media (CEM/VBL$_{1000}$, MCF-7 and MCF-7^{Adr}) supplemented with 10% FCS and 2 mM glutamine, incubated at 37° (5% CO_2/100% humidity). Cultures were maintained in exponential growth by passaging twice weekly. All cell lines were regularly shown to be *Mycoplasma*-free.

Cytotoxicity assays. Exponentially growing cells were trypsinized, centrifuged and resuspended in fresh medium (10% FCS, 2 mM glutamine). Cell suspension (180 μL) was aliquoted into 96-well microtitre plates at a density previously demonstrated to allow exponential growth for 4 days. Cells were incubated continuously with anti-oestrogens, drug and/or vehicle (10 μL) at appropriate levels (in quadruplicate) at 37° for 4 days. Cytotoxicity was determined using the MTT assay [33]. MTT (50 μL, 2 mg/mL) was aliquoted into all wells and the cells incubated for 4 hr. Plates were inverted to discard medium, and formazan crystals were solubilized in 100 μL DMSO with 25 μL glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) [34]. Plates were agitated for 5 min and OD determined immediately at 540 nm using a Titertek Multiskan Plus MKII ELISA plate reader. For drug resistance modification experiments anti-oestrogens were added to cells at non-toxic concentrations, i.e. concentrations which were consistently found to alter control (anti-oestrogen-free) cell OD by less than 5%.

Data was fitted to a four parameter equation $\{y = (A - D)/[1 + (\text{concn}/C)^B] + D\}$ and IC_{50} values (concentration of drug causing a 50% reduction in control cell OD) were calculated using DeltaSoft ELISA Analysis software (BioMetallics Inc., Princeton, NJ, U.S.A.). IC_{50} values quoted were mean values determined from at least three separate, identical experiments ± SEM. Values determined in the presence and absence of anti-oestrogens were compared using Student's paired *t*-test. Modification of drug toxicity by anti-oestrogens was expressed as an MF.

Membrane vesicle preparation. Membrane vesicles were prepared as described previously [35]. CEM/VBL_{1000} cells (2 L, 8×10^5 cells/mL) were harvested, washed three times with PBS and resuspended in 50 mL cavitation buffer (10 mM Tris–HCl, 250 mM sucrose, 0.2 mM $CaCl_2$, 10 μM leupeptin, 10 μM aprotinin, 1 mM phenylmethyl-sulphonyl fluoride, pH 7.4). Cells were disrupted by gaseous cavitation under nitrogen pressure (900 psi, 20 min) on ice then supplemented with EDTA (1 mM final concentration). The homogenate was centrifuged (1000 *g*, 10 min) to remove cellular debris, layered over a 35% sucrose cushion [35% sucrose (w/v), 1 mM EDTA, 10 mM Tris–HCl, pH 7.4] and centrifuged (16,300 *g*, 40 min). Vesicles at the interface were collected, diluted in 5 vol. TBS (250 mM sucrose, 10 mM Tris–HCl, pH 7.4), centrifuged (100,000 *g*, 1 hr) then resuspended in TBS, snap frozen and stored at −70°. Vesicles prepared by this technique have been shown to comprise 13% IOV, 60% ROV and 27% leaky vesicles [35].

[^{3}H]*VBL uptake by membrane vesicles.* Determination of VBL uptake by membrane vesicles has been described previously [27, 35]. Vesicles (20 μL, 5 mg/mL) were added to 30 μL of nucleotide buffer (10 mM Tris–HCl, 250 mM sucrose, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/mL creatine phosphokinase, pH 7.4) containing either ATP (1 mM) or AMP (1 mM) at 25°. Inhibitor solutions (1 μL, to give final concentrations of 0.1–100 μM), or solvent (1 μL) were added in duplicate and the reaction was started by addition of 50 μL [^{3}H]VBL (60 nM in TBS, 11.7 Ci/mmol). Ice-cold TBS (4 mL) was added to duplicate samples after appropriate times (10 min for dose–response experiments) and passed immediately through cellulose acetate filters (0.45 μm) under vacuum. Filters were washed with 4 mL TBS, air-dried and counted in 6 mL scintillation cocktail (Emulsifier-Safe, Packard, U.S.A.). [^{3}H]-VBL association with vesicles in the absence of ATP indicated the level of non-specific binding. Comparative experiments were carried out simultaneously, using vesicles from the same preparation.

Determination of membrane integrity. The integrity of IOV on exposure to anti-oestrogens was tested by measuring $^{45}Ca^{2+}$ accumulation by vesicles in the presence and absence of these inhibitors. In intact cells calcium ions are rapidly extruded by the ATP-dependent pump, while in IOV this mechanism leads to the accumulation of calcium, providing that vesicles are intact. This system therefore offers a simple test of vesicle integrity. Uptake of calcium was measured by a rapid filtration technique [36] at 25°. Vesicle suspension (20 μL, 5 mg/mL) was incubated with 70 μL of reaction medium (final concentration in 100 μL: 250 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES–Tris buffer, 1 mM ouabain, 0.1 mM $CaCl_2$, 3 mM ATP, pH 7.4). Transport was started by addition of 10 μL tracer $^{45}Ca^{2+}$ (0.5 μCi) diluted in reaction medium. Anti-oestrogens (50 μM final concentration) or alamethicin (20 μg/mL final concentration) were added either before initiation of transport or after steady-state accumulation of $^{45}Ca^{2+}$ was achieved. The reaction was stopped by adding 3 mL ice-cold "stop" solution (300 mM sucrose, 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES–Tris buffer, 1 mM EGTA, pH 7.4) 1, 5, 10 and 15 min after initiation. Vesicles were immediately collected on cellulose acetate filters (0.45 μm pore size) under suction, washed with 3 mL ice-cold stop solution and prepared for scintillation counting.

RESULTS

Effects of anti-oestrogens on cell growth

ICI 164 and ICI 182 inhibited MCF-7 cell growth by 50% at 30 nM, but caused no further decrease in cell growth at 50 μM. Inhibition was completely

Table 1. Sensitivity of cell lines to anti-oestrogens

	Anti-oestrogen IC_{50} (μM)						
Cell line	Tamoxifen*	ITx	MNC (μM)	PITx	MNC (μM)	EBTx	MNC (μM)
MCF-7	11 ± 1	8 ± 1	0.5	11 ± 2	0.5	–	–
MCF-7^{Adr}	31 ± 2	23 ± 3	10	16 ± 2	10	>50	–
S1	22 ± 2	15 ± 2	10	16 ± 4	10	43 ± 7	–
S1/1.1	21 ± 1	15 ± 2	10	15 ± 3	10	>50	–

Cells seeded into 96-well microtitre plates were grown continuously in the presence of anti-oestrogen for 4 days, then incubated for 4 hr with MTT (50 μL, 2 mg/mL). Medium and unconverted MTT were discarded and the formazan product solubilized in DMSO (100 μL) and glycine buffer (25 μL of 0.1 M glycine/0.1 M NaCl, pH 10.5). OD were determined at 540 nm. IC_{50} values are mean values from at least four determinations ± SEM.

* From Ref. [10].

Table 2. Effects of anti-oestrogens on VBL toxicity.

Cell line	Vinblastine IC_{50} (nM)					
	+PBS	+ITx†	+PITx†	+EBTx‡	+ICI 164§	+ICI 182§
MCF-7	1.2 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	–	–	–
MF	–	1‖	1	–	–	–
MCF-7^{Adr}	321 ± 17	48 ± 4	26 ± 6	146 ± 6	9.2 ± 1.1	25 ± 2
MF	–	7**	12**	2***	35***	13***
S1	2.4 ± 0.2	2.4 ± 0.4	2.4 ± 0.3	2.5 ± 0.4	2.6 ± 0.1	2.5 ± 0.2
MF	–	1	1	1	1	1
S1/1.1	11.3 ± 0.6	3.7 ± 0.6	3.5 ± 0.4	6.2 ± 1.1	1.1 ± 0.2	3.1 ± 0.3
MF	–	3*	3*	2*	10***	4**

Cells were grown continuously in the presence of VBL and/or anti-oestrogen for 4 days. Cytotoxicity was assessed as described in the legend to Table 1. Results are mean values from 10 determinations ± SEM.

† ITx and PITx were added to cells at their MNC (Table 1).

‡ EBTx was added to MCF-7^{Adr}, S1 and S1/1.1 cells at 20 μM.

§ ICI 164 and ICI 182 were added to MCF-7^{Adr}, S1 and S1/1.1 cells at 20 μM.

‖ MF = 1, P = 0.05-1.

* P = 0.01–0.05.

** P = 0.001–0.01.

*** P = 0–0.001.

antagonized by oestradiol (100 nM) and 50 μM ICI 164 and ICI 182 were non-toxic to ER-negative S1, S1/1.1 and MCF-7^{Adr} cells, indicating that the blockade of growth in the ER-positive cells was oestrogen-specific. ITx and PITx were approximately equitoxic and were more toxic than tamoxifen to ER-negative cells (Table 1), while EBTx was non-toxic to MDR-positive MCF-7^{Adr} and S1/1.1 cells at concentrations up to 50 μM and inhibited growth of S1 cells with an IC_{50} value of 43 μM.

Modification of VBL toxicity

MCF-7^{Adr} cells were 270-fold resistant to VBL relative to wild type cells (Table 2). The effects of ICI 164 (0–50 μM) on VBL toxicity to the MDR-positive cell line are shown in Fig. 2. Dose–response curves for VBL were shifted to lower concentrations of VBL with increasing anti-oestrogen concentration, indicating substantial enhancement of drug toxicity. The effects of pure anti-oestrogens and tamoxifen derivatives at their MNC, where applicable, on VBL toxicity are compared in Table 2. ICI 164 (20 μM) was most effective, causing a 35-fold decrease in the VBL IC_{50} value, but did not restore wild-type sensitivity. ICI 182, ITx, and PITx also caused substantial modification, while EBTx enhanced toxicity only 2-fold at 20 μM. Drug sensitive wild-type MCF-7 cells tolerated concentrations of ITx and PITx up to 0.5 μM, a dose which did not alter VBL toxicity. Figure 3 shows modification of VBL toxicity to MDR-positive MCF-7^{Adr} cells as a function of anti-oestrogen concentration and allows comparison of the effects of anti-oestrogens over their non-toxic ranges. Data for tamoxifen (from [9]) are also included for comparison. Tamoxifen and ICI 164 at 20 μM were equally effective, however, at lower concentrations, ICI 164 was the best modifier of VBL toxicity. ITx and PITx were as effective as tamoxifen at 1–10 μM, but MDR-modulation by the iodotamoxifens was limited by their toxicity at higher concentrations. EBTx was least effective.

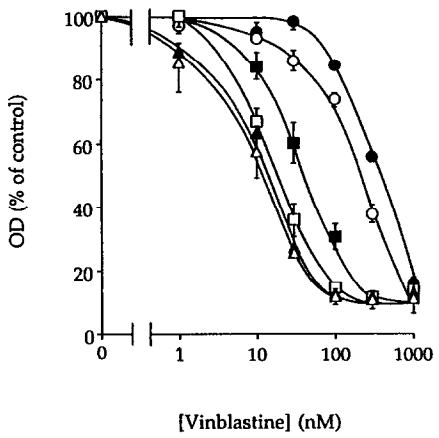

Fig. 2. Effect of 0 (●), 1 (○), 5 (■), 10 (□) 20 (▲), and 50 (△) μM ICI 164 on VBL toxicity to MCF-7^{Adr} cells. The viability of cells grown continuously for 4 days with VBL and/or ICI 164 was assessed using a semi-automated MTT assay. Points, means of four replicates from one representative experiment; bars, SD.

The *mdr*1-transfected cell line, S1/1.1, was 5-fold resistant to VBL relative to its parental line (P = 0.0002, Table 2). VBL IC_{50} values for S1 cells, and for S1/1.1 cells treated with pure anti-oestrogens and iodotamoxifens, were not significantly different

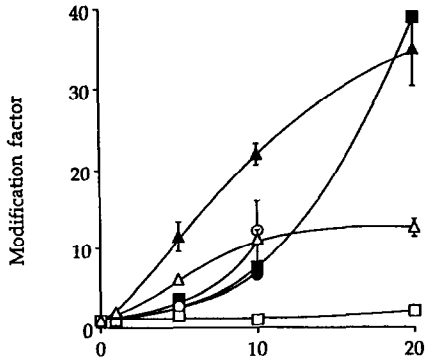

Fig. 3. Effect of anti-oestrogens on VBL toxicity to MCF-7^{Adr} cells. Modification of VBL toxicity is expressed as an MF. Tamoxifen (■), EBTx (□), ITx (●), PITx (○), ICI 164 (▲) and ICI 182 (△). Points, mean MF calculated from 10 identical experiments; bars, SEM (where no bar is present, errors were smaller than the symbols).

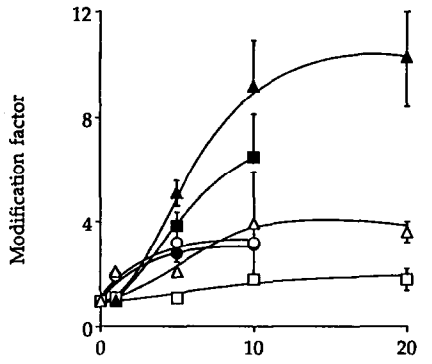

Fig. 4. Effect of anti-oestrogens on VBL toxicity to S1/1.1 cells. Modification of VBL toxicity is expressed as an MF. Tamoxifen (■), EBTx (□), ITx (●), PITx (○), ICI 164 (▲), and ICI 182 (△). Points, mean MF calculated from 10 identical experiments; bars, SEM, (where no bar is present, errors were smaller than the symbols).

($P > 0.05$); drug resistance was therefore completely abolished under these conditions. EBTx (20 μM) caused a smaller (2-fold) increase in VBL toxicity to S1/1.1 cells, and no modifier altered toxicity to parental S1 cells. The pattern of modification achieved by anti-oestrogens at non-toxic levels (Fig. 4) was similar to that observed for MCF-7^{Adr} cells (Fig. 3), with ICI 164 once again the most, and EBTx the least, effective modifier at all concentrations tested. Tamoxifen (from [9]) was more effective than ITx and PITx.

Modification of DOX toxicity

MCF-7^{Adr} cells were 170-fold resistant to DOX relative to the parental cell line, MCF-7 and the effects of all anti-oestrogens at their MNC, where applicable, on DOX toxicity are compared in Table 3. ICI 164 (20 μM) was most effective, causing a 25-fold decrease in the DOX IC_{50} value, but did not reduce resistance to wild-type levels. A 7-fold degree of resistance remained between MCF-7^{Adr} cells treated with ICI 164 and wild-type cells. ITx, PITx and ICI 182 also enhanced drug toxicity, while EBTx

Table 3. Effects of anti-oestrogens on DOX toxicity

	Doxorubicin IC_{50} (nM)					
Cell line	+PBS	+ITx†	+PITx†	+EBTx‡	+ICI 164§	+ICI 182§
MCF-7	58 ± 5	56 ± 4	57 ± 5	–	–	–
MF	–	1‖	1	–	–	–
MCF-7^{Adr}	9650 ± 810	1020 ± 40	910 ± 41	8720 ± 930	384 ± 17	760 ± 80
MF	–	9**	11**	1	25**	13**
S1	51 ± 5	55 ± 3	50 ± 6	53 ± 8	32 ± 5	31 ± 3
MF	–	1	1	1	2*	2**
S1/1.1	57 ± 7	19 ± 4	22 ± 3	58 ± 9	27 ± 4	22 ± 2
MF	–	3*	3*	1	2*	3*

Cells were grown continuously in the presence of DOX and/or anti-oestrogen for 4 days. Cytotoxicity was assessed as described in the legend to Table 1. Results are mean values from 10 determinations ± SEM.

† ITx and PITx were added to cells at their MNC (Table 1).

‡ EBTx was added to MCF-7^{Adr}, S1 and S1/1.1 cells at 20 μM.

§ ICI 164 and ICI 182 were added to MCF-7^{Adr}, S1 and S1/1.1 cells at 20 μM.

‖ MF = 1, P = 0.05-1.

* P = 0.01–0.05.

** P = 0.001–0.01.

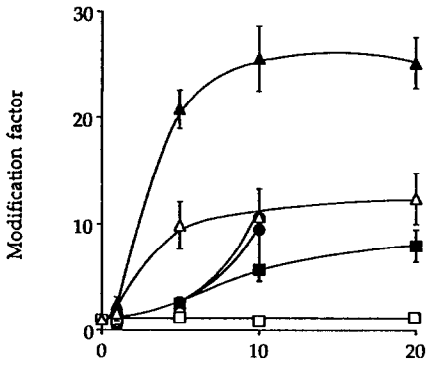

Fig. 5. Effect of anti-oestrogens on DOX toxicity to MCF-7^{Adr} cells. Modification of DOX toxicity is expressed as an MF. Tamoxifen (■), EBTx (□), ITx (●), PITx (○), ICI 164 (▲), and ICI 182 (△). Points, mean MF calculated from 10 identical experiments; bars, SEM (where no bar is present, errors were smaller than the symbols).

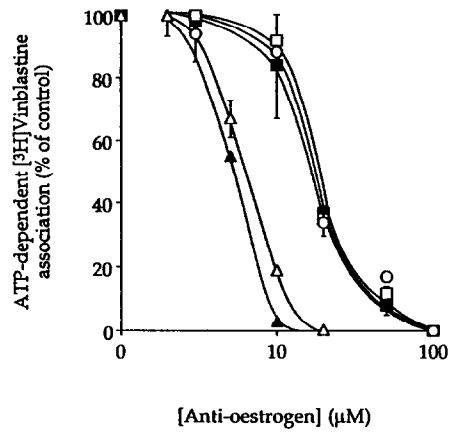

Fig. 7. Inhibition of ATP-dependent [^{3}H]VBL accumulation by vesicles prepared from the highly Pgp-positive cell line, CEM/VBL_{1000}, by ICI 164 (▲), ICI 182 (△), tamoxifen (■), PITx (○) and EBTx (□). Points, means of values determined in duplicate from a single representative experiment; bars, SD.

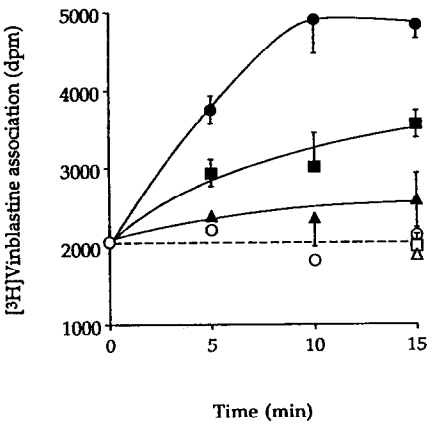

Fig. 6. Effect of ICI 164 (▲, △) and tamoxifen (■, □) on accumulation of [^{3}H]VBL in the presence of ATP (filled symbols) or AMP (open symbols) by vesicles prepared from Pgp-positive CEM/VBL_{1000} cells. Vesicles were incubated for 0, 5, 10, 15 or 20 min at 25° with [^{3}H]VBL (30 nM, 11.7 Ci/mmol) and ICI 164 (10 μM) or tamoxifen (10 μM). Solvent controls (●, ○). Points, means of values determined in duplicate from a single representative experiment; bars, SD.

(20 μM) was ineffective. Figure 5 shows modification of DOX toxicity as a function of anti-oestrogen concentration, including data for tamoxifen (from [10]) for comparison. ICI 164 was the best modifier of DOX toxicity at all concentrations tested. ITx and PITx were more effective than tamoxifen at 10 μM, but MDR-modulation by the iodotamoxifens was limited by toxicity at higher concentrations. EBTx did not alter DOX toxicity at any concentration tested (1–50 μM).

The toxicity of DOX to wild-type, ER-positive MCF-7 cells was not altered by ITX and PITx at their MNC of 0.5 μM. Because MCF-7 cells were exquisitely sensitive to ICI 164 and ICI 182, the effects of pure anti-oestrogens alone on DOX toxicity could not be investigated. However, 100 nM oestradiol completely antagonized growth inhibition by 10 μM ICI 164, and this combination did not modify DOX toxicity to MCF-7 cells. Modulation of DOX resistance in MCF-7^{Adr} cells by ICI 164 was unaffected by 100 nM oestradiol, and oestradiol alone was unable to enhance drug toxicity (not shown).

S1 cells and their *mdr*1-transfected sub-line, S1/1.1, were equally sensitive to DOX (Table 3). ITx, PITx, ICI 164 and ICI 182 caused small (2- to 3-fold) but significant ($P < 0.05$) modifications of DOX toxicity to S1/1.1 cells, to below wild-type levels. ICI 164 and ICI 182 caused a slight (2-fold) enhancement of DOX toxicity to wild-type S1 cells. EBTx did not affect drug toxicity to either cell line.

Effect of anti-oestrogens on [^{3}H]*VBL accumulation in vesicles*

The observation that the novel anti-oestrogens tested here modified drug resistance in Pgp-positive cells suggested that these compounds might inhibit the Pgp-mediated efflux of drugs from cells. In order to test directly the effects of these compounds on Pgp drug pumping, a vesicle system that has been shown previously to allow measurement of the effects of inhibitors on energy-dependent drug transport was used. ATP hydrolysis is an absolute requirement for Pgp-mediated translocation of drug across the membrane. ATP binding sites are located on the outer surface of the IOV membrane, and addition of ATP and VBL results in accumulation of drug within the vesicle. In ROV, however, Pgp cannot be activated; the vesicle membrane is impermeable to ATP, which cannot access binding

Table 4. Effects of anti-oestrogens on ATP-dependent [^{3}H]VBL accumulation in Pgp-positive vesicles

Anti-oestrogen	IC_{50} (μM)
ICI 164	2.8 ± 0.9
ICI 182	4.1 ± 1.1
Tamoxifen	16.4 ± 1.9
PITx	17.7 ± 1.0
EBTx	16.3 ± 1.1

Plasma membrane vesicles prepared from Pgp-positive CCRF-CEM/VBL_{1000} cells were incubated with [^{3}H]VBL (30 nM, 11.7 Ci/mmol) and anti-oestrogen (0.1–100 μM) and/or vehicle with ATP or AMP (1 mM). After 10 min ice-cold "stop" solution (25 mM sucrose, 10 mM Tris–HCl, pH 7.4) was added. Vesicles were collected on 0.45 μm cellulose acetate filters under vacuum then taken for scintillation counting. IC_{50} values indicate the concentration of anti-oestrogen which inhibited by 50% the ATP-dependent accumulation of VBL and are mean values from four determinations ± SEM.

sites situated on the luminal side of the membrane. Figure 6 shows a time–course for association of [^{3}H]VBL with vesicles prepared from CEM/VBL_{1000} cells in the presence and absence of 10 μM tamoxifen and ICI 164. This cell line is 1000-fold resistant to VBL [32] and expresses the very high levels of Pgp necessary for ATP-dependent drug accumulation to be detectable. The high level of [^{3}H]VBL association observed in the absence of ATP, which did not vary with time, was due to non-specific binding of the drug to vesicles. Tamoxifen and, to a greater degree, ICI 164 inhibited ATP-dependent VBL accumulation, but association in the absence of ATP was unaffected by anti-oestrogens, indicating that these compounds did not affect non-specific association of VBL with vesicles.

Tamoxifen, PITx, EBTx, ICI 164 and ICI 182 were tested at 0.1–100 μM to generate the representative dose–response curves for inhibition of ATP-dependent drug accumulation shown in Fig. 7. No anti-oestrogen significantly altered non-specific association of VBL with vesicles. Pure anti-oestrogens were the best blockers of active VBL accumulation, while dose–response curves for tamoxifen, PITx and EBTx overlayed. Table 4 lists mean IC_{50} values for inhibition determined from four separate experiments. ICI 164 was slightly more effective than ICI 182 ($P < 0.05$). EBTx, which did not enhance drug toxicity to Pgp-positive cell lines, was as effective an inhibitor of ATP-dependent VBL accumulation as tamoxifen and PITx (IC_{50} values = 16–18 μM).

The effects of anti-oestrogens on ATP-dependent $^{45}Ca^{2+}$ accumulation by vesicles was also investigated to determine whether reduced [^{3}H]VBL accumulation was due to vesicle lysis. Similar results were obtained when inhibitors were added before initiation of transport, or after steady-state accumulation was achieved. ICI 164 and ICI 182 (50 μM) did not affect tracer accumulation while alamethicin, an ionophore known to make the membrane leaky, reduced $^{45}Ca^{2+}$ accumulation by 97%. Inhibition of ATP-dependent [^{3}H]VBL accumulation by pure anti-oestrogens was therefore not due to lysis of the vesicle membrane. Tamoxifen at 50 μM inhibited steady-state $^{45}Ca^{2+}$ accumulation by 32%, indicating that tamoxifen caused either a degree of membrane lysis at this concentration or inhibited the calcium ATPase. However, 50 μM tamoxifen caused a greater reduction in ATP-dependent [^{3}H]VBL accumulation (84%), and membrane lysis was clearly insufficient to account entirely for inhibition of VBL accumulation.

DISCUSSION

ER-positive MCF-7 cells were more sensitive than ER-negative cell lines to all anti-oestrogens tested. In particular, ICI 164 and ICI 182 were not toxic to ER-negative cells but inhibited MCF-7 cell growth by 50% at 30 nM. ITx and PITx were equitoxic, and both were more toxic than tamoxifen to ER-negative MCF-7^{Adr}, S1 and S1/1.1 cells (Table 1). ITx and PITx are more potent calmodulin antagonists than tamoxifen and this activity correlates with inhibition of cell growth [37].

The MDR-modifying effects of anti-oestrogens were determined using drug-resistant cell lines in which overexpression of Pgp had been induced by two distinct methods. MCF-7^{Adr} cells were derived by chronic *in vitro* exposure to DOX; such treatment may activate other mechanisms of resistance as DOX has multiple mechanisms of cytotoxicity [22]. Indeed, MCF-7^{Adr} cells have increased glutathione-*S*-transferase and peroxidase activity [28], which may mediate resistance to DOX by protecting against damage caused by hydroxyl radicals. By contrast, Pgp-overexpression was induced in S1/1.1 cells by transfection with the *mdr*1 gene. Overexpression of Pgp is therefore the only mechanism of MDR active in the transfected cells but absent from parental S1 cells. Reversal of VBL resistance in S1/1.1 cells to wild-type levels by ICI 164, ICI 182, ITx and PITx therefore indicates that these anti-oestrogens completely abolish Pgp-dependent MDR. By contrast, VBL toxicity to MCF-7^{Adr} cells was only partially reversed, highlighting the presence of non-Pgp-mediated mechanisms of resistance in this highly drug resistant cell line.

Modification of MDR was unaffected by oestradiol and occurred irrespective of ER status. It was therefore not an anti-oestrogenic effect. Oestradiol did not alter drug toxicity to MDR-positive cell lines, although its close structural analogues ICI 164 and ICI 182 were highly effective modifiers. The presence of the bulky 7-α-alkyl substituent was therefore crucial for modification of MDR. ITx and PITx also substantially enhanced DOX and VBL toxicity to Pgp-positive cell lines, but were less effective than tamoxifen as their MDR-modulating activity was limited by non-specific toxicity. ICI 164 was a better modifier of resistance to DOX and VBL than tamoxifen. This is in agreement with the work of Hu *et al.* [26], who first demonstrated that ICI 164 enhances DOX toxicity to Pgp-positive cell lines and that the pure anti-oestrogen reverses drug resistance and enhances whole-cell accumulation of [^{3}H]-daunomycin more effectively than tamoxifen.

In this study the effects of anti-oestrogens on

ATP-dependent [^{3}H]VBL accumulation by vesicles prepared from a highly drug-resistant cell line were determined, as such measurements provide direct evidence for inhibition of drug pumping by Pgp. The order of effectiveness of the anti-oestrogens tested was:

$$\text{ICI 164} \geq \text{ICI 182} > \text{tamoxifen} = \text{PITx} = \text{EBTx},$$

compared to their activity as modulators of MDR:

$$\text{ICI 164} > \text{ICI 182} \geq \text{tamoxifen [9, 10]} \approx \text{PITx} \approx \text{ITx} > > \text{EBTx}.$$

The most interesting point to arise from the comparison is that EBTx was unable to substantially modify drug resistance, yet was as potent an inhibitor of active drug accumulation as tamoxifen. EBTx is a quaternary derivative of tamoxifen with reduced ability to diffuse into cells [19], and this result suggested that tamoxifen derivatives interact with intracellular site(s) in drug-resistant cells. IOV lack intracellular organelles and cytoplasmic enzymes, and it may be concluded that the site of action for inhibition of VBL accumulation by tamoxifen derivatives is located at the cytoplasmic surface of the plasma membrane. Mechanisms of action could include competition with VBL for binding to Pgp. The possibility that reduced drug accumulation results from membrane lysis is ruled out for pure anti-oestrogens by their inability to alter $^{45}Ca^{2+}$ accumulation within vesicles, although the possibility that these compounds enhanced leakage of drug (but not $^{45}Ca^{2+}$) from vesicles due to altered membrane fluidity cannot be excluded. Tamoxifen slightly reduced calcium accumulation by vesicles, indicating that it may lyse the vesicle membrane. However, tamoxifen (50 μM) caused a much greater reduction in [^{3}H]VBL accumulation than in $^{45}Ca^{2+}$ accumulation, and vesicle lysis would have been insufficient to account solely for inhibition of VBL transport. Alternatively, tamoxifen may inhibit the calcium ATPase. Tamoxifen is known to inhibit other calcium-binding proteins, including calmodulin [37], protein kinase C [38] and voltage-dependent Ca^{2+} channels of the PC12 neurosecretory cell line [39].

Similar concentrations of pure anti-oestrogens were required to inhibit active drug accumulation in the vesicle system and alter drug resistance in cell-growth assays. Tamoxifen and PITx, however, enhanced drug toxicity to MDR cell lines at concentrations somewhat lower than those required to inhibit ATP-dependent accumulation of [^{3}H]-VBL. This may indicate that tamoxifen derivatives enhanced VBL toxicity to MDR cell lines via mechanisms in addition to direct interaction with the plasma membrane and associated proteins. However, there were many differences between the assay systems, for example the time scales over which they took place and the concentrations of VBL used. In addition, the membrane potential in intact cells is dispelled when cells are disrupted and would be negligible in vesicles, perhaps affecting the extent to which tamoxifen and its derivatives interacted with the plasma membrane. Direct comparison cannot be made, therefore, between the concentrations of anti-oestrogens effective in these very different systems, although it would appear valid to comment on the patterns of inhibition observed.

In summary, the new pure anti-oestrogen ICI 164 is a highly effective modifier of MDR, able to completely reverse Pgp-dependent VBL resistance in an *mdr*1 transfected cell line and to block VBL transport by Pgp. Tamoxifen has been tested in phase I clinical studies as an MDR reversing agent in combination with VBL, and at high doses (300–400 mg tamoxifen/m^2/day), plasma levels of 4 μM were achieved [40]. Such doses of tamoxifen do not increase VBL-induced myelotoxicity, but can cause mild neurotoxicity and cardiac abnormalities [40]. Pure anti-oestrogens were designed to treat hormone-responsive breast cancer with no oestrogenic side-effects and, as more effective modifiers of MDR than tamoxifen, may be ideal candidates for combination treatment with drugs transported by Pgp.

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