ORIGINAL ARTICLE

Taylor&Francis healthsciences

Differential Effects of Toremifene on Doxorubicin, Vinblastine and Tc-99m-sestamibi in P-glycoprotein-expressing Breast and Head and Neck Cancer Cell Lines

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Acta Oncologica Vol. 43, No. 5, pp. 443–452, 2004

The effect of toremifene on P-glycoprotein-mediated multidrug resistance (MDR) in breast and head and neck cancer cell lines was measured in vitro and in vivo. Pgp expression was low and high, respectively, in drug-sensitive (MCF7-S, KB) and drug-resistant (MCF7-R, MCF7-R1, KBV1) cell lines. Toremifene (7.5 μM) significantly enhanced cytoplasmic and nuclear accumulation of doxorubicin in drug-resistant cells. Toremifene (10 μM) increased the in vitro cytotoxicity of doxorubicin in drug-resistant breast cancer cells (13-fold and 21-fold for MCF7-R and MCF7-R1, respectively) without affecting the sensitivity of MCF7-S cells. Similarly, toremifene (10 μM) caused a 12-fold increase in the sensitivity of KBV1 cells to vinblastine. In contrast, toremifene (5 μM) reduced the net uptake of the radiolabelled Pgp substrate, Tc-99m-sestamibi, in the Pgp-overexpressing cell lines by factors of 0.32 and 0.42 for MCF7-R1 and KBV1 cells, respectively ($p < 0.01$), and, to a lesser extent, by corresponding factors of 0.89 and 0.86 in the drug-sensitive cell lines ($p < 0.05$ and $p > 0.05$, respectively). In nude mice bearing both KB and KBV1 xenograft tumours, significantly higher tumour levels of Tc-99m-sestamibi were recorded in KB tumours compared with KBV1 tumours. After 3 days of treatment with intraperitoneal toremifene (25 mg/kg), tumour levels of Tc-99m-sestamibi were reduced in KB and KBV1 tumours but only statistically significantly for KB tumours. Toremifene is a potent MDR modulating agent with respect to chemotherapeutic agents but has the opposite effect with respect to Tc-99m-sestamibi. This finding is of importance in view of the widespread use of Tc-99m-sestamibi as an imaging surrogate for a chemotherapeutic agent.

Received 15 November 2002
Accepted 7 January 2004

Resistance to cytotoxic drugs is a significant barrier to successful chemotherapy for solid cancers. Cross-resistance to a wide range of seemingly unrelated drugs can exist either intrinsically or as an acquired characteristic after administration of cytotoxic chemotherapy (1). In most instances, this multidrug resistance (MDR) is due to overexpression of P-glycoprotein (Pgp) by the tumour (1–4). Pgp acts as an efflux pump for a diverse range of compounds and affects the response of many solid cancers to drugs, including anthracyclines, vinca alkaloids, epipodophyllotoxins and taxanes, that are key components of combination chemotherapy regimens (1, 5). Attempts have been made to overcome MDR by means of agents that can block the efflux function of Pgp (1, 5). The success of this approach has been limited by the toxicity of the first generation of MDR modulators. For example, the cardiovascular effects of the calcium channel antagonists and the immunosuppression, nephro-, neuro-, and hepatotoxicity of cyclosporin A impose dose limits below the threshold for effective reversal of MDR in vivo (5–8).

There has therefore been an active search for alternative agents that can be administered safely at effective doses. Anti-oestrogenic drugs have been shown to increase the cytotoxicity of anthracyclines, vinca alkaloids, and taxanes in Pgp-positive human breast, lung, gastrointestinal, and bladder cancer cell lines (9–18). Most attention has focused on tamoxifen and its metabolites that appear to act by binding to Pgp in competition with cytotoxic agents (12, 19). However, Phase I and II studies of moderate or high dose tamoxifen in patients have demonstrated significant toxicity with little evidence of increased tumour sensitivity to cytotoxic agents which are Pgp substrates (7, 8, 20, 21).

The tamoxifen analogue, toremifene, has recently received attention as a potential MDR modulating agent (13,

 ISSN 0284-186X
DOI: 10.1080/02841860410031048

22). A Phase I trial has shown that it can be delivered safely at doses that achieve plasma concentrations in the range required for MDR reversal in vitro. A mean serum concentration of 7.8 μM (range 2.5–14.7 μM) was achieved in patients treated with toremifene at a dose of 780 mg a day for 3 days (22). In these studies, we have assessed the effect of toremifene at such clinically achievable concentrations (5–10 μM) in Pgp-expressing breast and head and neck cell lines, in terms of doxorubicin uptake and cytotoxicity in vitro. In addition, the effect of toremifene on the net cellular uptake of the radiolabelled Pgp substrate, Tc-99m-sestamibi, was studied in vitro with a view to assessing the potential of using this agent to predict the functional Pgp status of tumors in vivo. Finally, the effect of toremifene was assessed in nude mice bearing both Pgp non-expressing (KB) and overexpressing (KBV1) tumors.

MATERIAL AND METHODS

Cell culture

The human breast adenocarcinoma cell line, MCF7, was obtained from the European Collection of Animal Cell Culture. Both the doxorubicin-sensitive parental cell line, MCF7-S (23), and the doxorubicin-resistant derivative, MCF7-R (24), were studied. In addition, a subclone of MCF7-R, called MCF7-R1, was generated by continuous passage in 3 μM doxorubicin. The human squamous cell head and neck cancer cell line, KB (25), which is vinblastine-sensitive, and its vinblastine-resistant variant, KBV1 (26), were obtained from the American Tissue Culture Collection. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, penicillin 10000 IU/ml, streptomycin 10000 μg/ml and amphotericin B 25 μg/ml (all supplied by Gibco, Paisley, UK) at 37°C in a humidified atmosphere containing 10% CO_2.

Immunocytochemistry

Cells were grown to 60–70% confluence on 8-well plastic or 4-spot glass tissue culture slides (Falcon, New Jersey, USA) under the growth conditions described above, washed gently with PBSA (Dulbecco's phosphate buffered saline (without Ca^{2+} or Mg^{2+}) containing 0.2 M Na_2HPO_4), and fixed in methanol:acetone (1:1 v/v at −20°C) for 10 min and air dried. Two murine IgG2a monoclonal antibodies, C219 and C494 (Signet Laboratories, London, Canada) that recognize different epitopes on Pgp were used for immunostaining. Non-specific binding sites were blocked with a 1:20 dilution of normal rabbit serum. Wells were then covered with monoclonal antibodies C494 (1:40 dilution) or C219 (1:10 dilution) and incubated in a humid atmosphere for 16 h, washed with PBS and incubated for 1 h in 50 μl biotinylated rabbit anti-mouse IgG secondary antibody (Dako A/S, Glostrup, Denmark) (1:150 dilution). Horseradish peroxidase (HRP)-conjugated streptavidin (ID Labs Inc, Glasgow, UK) (1:150) was applied as a third layer for 1 hour at room temperature and 3,3′-diaminobenzidine (0.05% (w/v) solution in 0.03% (v/v) hydrogen peroxide) was added as a chromogen. Cells were counterstained with fresh Cole's haematoxylin (Pioneer Research Chemicals, Colchester, UK), dehydrated through sequential alcohol and xylene washes and mounted using Pertex solution (Cell Path House, Hemel Hempstead, UK).

Western blotting

Western analysis was performed on cell lysates to assess Pgp expression. Cells were grown on 90 mm plastic dishes (Falcon) to 70–90% confluency, washed thrice with PBSA, harvested by scraping, and pelleted at centrifugation. The pellet was resuspended in 2 ml of lysis buffer (715 mM 2-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulphate (SDS), 40 mM Tris (pH 6.8), 1 mM EDTA) and boiled for 10 min. The lysate was incubated on ice for 5 min and centrifuged to pellet insoluble debris. This step was repeated and the supernatant was transferred to a clean microfuge tube and stored at −70°C after an aliquot had been taken for protein estimation (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 1 mm thick 5–10% resolving gel (5–10% acrylamide, 0.5 M Tris (pH 8.8), 0.1% SDS) overlaid with a 4.5% acrylamide stacking gel. Twenty μg of protein was loaded into each well under SDS-PAGE running buffer (50 mM Tris, 0.4 M glycine, 0.2% SDS) and electrophoresed at 90V for 5–10 min. Protein samples were electro-blotted onto a nitrocellulose membrane at 250 mA for 2 hours in transfer buffer (25 mM Tris, 0.2 M glycine, 1% SDS and 20% methanol). After Ponceau S staining, the membrane was incubated with 0.5% (w/v) casein hydrosylate made up in TBS-Tween (0.5% Tween 20 in tris buffered saline), followed by optimally diluted primary antibody for 2 h. The membrane was then washed in TBST and incubated with the secondary antibody diluted 1:1500 in TBST for 2 h. After a final wash in TBST, the membrane was placed in enhanced chemiluminescent solution (ECL, Amersham plc, Amersham, UK) and exposed to Hyperfilm (Amersham plc). Optimum exposure times were determined for each experiment.

Fluorescent microscopy

Cells were grown on 4-well glass slides to 50–60% confluence. Four hours before fluorescent microscopy, the medium was changed to contain the following additives: (1) no drug; (2) doxorubicin; (3) toremifene; (4) doxorubicin plus toremifene. The cells were washed with ice-cold saline and viewed under an Olympus BX 60 fluorescence microscope (Olympus, Tokyo, Japan) using a 575 nm filter.

FACS analysis

Cells were grown in 75 cm^2 flasks (Falcon) in the same growth conditions as described above. Cells were harvested, resuspended in 5 ml counting tubes at 5×10^5 cells/ml and incubated for 4–6 h in the presence of 1 μM doxorubicin with or without toremifene (5 μM and 10 μM). Thereafter, the cells were pelleted at centrifugation, washed with ice-cold normal saline, and resuspended in phenol red-free DMEM supplemented with 1% FCS and 1 mM $CaCl_2$. The fluorescence of 10 000 events was measured at flow cytometry (Coulter Model EPIC XL-MCL, UK) at an excitation wavelength of 575 nm using a band pass filter at 560–590 nm.

Cytotoxicity assay

Tumor cells were harvested by brief trypsinisation, resuspended and plated at 4000–5000 cells per well in 96-well microtitre plates. Doxorubicin or vinblastine (with or without toremifene) was added to wells in quadruplicate and the cells were incubated for 4 days. Cytotoxicity was determined using the MTT assay (27). Briefly, 50 μl of MTT reagent (2.5 mg/ml) was added to each well and incubated for 4 h, after which the medium was drained. Formazan crystals were solubilized by adding a mixture of 25 μl glycine buffer (0.1 M glycine in 0.1 M NaCl, pH 10.5) and 100 μl dimethyl sulphoxide to each well and agitating for 5 min. Absorption was read on a Bio-Rad Model 550 microplate reader at 540 nm and data were analysed using specifically designed software (Microplate Manager, Bio-Rad, UK). Drug dose–response curves and EC50 values were generated for each agent and, in the case of doxorubicin and vinblastine, the effect of adding toremifene was expressed as a modification factor (MF).

Preparation of Tc-99m-sestamibi

For the preparation of Tc-99m-sestamibi, a one step Cardiolite kit (Dupont Pharma, Letchworth Garden City, UK) containing 0.075 mg stannous chloride and sestamibi as the Cu (MIBI).4BF salt was obtained and 15–20 mCi of pertechnetate (Radiopharmacy, Hammersmith Hospitals NHS Trust) in approximately 2 mL normal saline was added. The resulting solution was mixed and heated in a water bath at 100°C for 10 min, cooled and the radiolabelled sestamibi was separated on a reverse-phase Sep-Pak C18 cartridge (Waters Associates, Milford, Mass, USA) pre-wetted with 3 ml of 90% ethanol and then 5 ml distilled water. Hydrophilic impurities were eluted from the column with 10 ml of 0.15 M sodium chloride and the Tc-99m-sestamibi was eluted with 2 ml of a 9:1 mixture of ethanol and saline. Radiochemical purity was measured prior to use by means of aluminium oxide strip thin layer chromatography using ethanol as the solvent.

Measurement of accumulation of Tc-99m-sestamibi

Cells were grown in 60 mm plastic culture dishes (Falcon) under the conditions described above until 70–80% confluent. Cells were washed with PBSA and 5 ml fresh DMEM supplemented with 1% FCS was added. For experiments involving toremifene, the drug was added 15 min before 0.1 MBq/ml of Tc-99m-sestamibi. Subsequently, duplicate 60 mm dishes were removed at 1, 5, 15, 30, 45, and 90 min and the cells were washed with ice-cold medium to remove extracellular radioactivity. The cells were harvested, transferred to 5 ml tubes (Falcon) and counted in a Packard Cobra II Auto-Gamma counter (Canberra Packard Ltd, Pangbourne, Berks, UK). Standard dilutions (10^{-1}, 10^{-2}, and 10^{-3}) of Tc-99m-sestamibi were counted simultaneously to allow correction for physical decay of the radioisotope. Thereafter, the number of cells in each sample was determined by counting in a Neubauer haemocytometer and the radioactive counts were expressed as percentage of added radiotracer per 10^7 cells.

In vivo model

Female nude mice of mixed genetic background that had been bred under specific pathogen-free conditions at the Imperial Cancer Research Fund Animal Breeding Unit, South Mimms, Herts, UK were used. The animals were housed in sterile filter-top cages on sterile bedding and maintained on an irradiated diet and autoclaved, acidified water (pH 2.8) ad libitum. KB and KBV1 tumour cells were grown as detailed above and harvested by brief incubation with a 1:3 ratio of trypsin/versene (EDTA 0.02%). Bilateral xenograft tumours were established in the nude mice by injecting single cell suspensions of 5×10^6 KB and KBV1 tumour cells in 0.1 ml of culture medium subcutaneously into the right and left flanks, respectively. These cell lines grew well in nude mice with no significant differences in growth rates. Human xenograft tumours composed of cell culture injections were used for the following reasons: (1) ready availability of cell lines generated in tissue culture (in contrast with tumour explant samples); (2) ease of bilateral tumour set-up with tumours differing only in the expression of Pgp; (3) ability to generate bilateral tumours of similar sizes simultaneously. Animals were used for experiments at approximately 14 days after tumour inoculation. The animals were divided into two groups. Toremifene-treated mice received twice-daily intraperitoneal injections of toremifene (25 mg/kg in 100 μl, total dose 1.25 mg/day) for 3 days and control mice received no treatment. This dose of toremifene was chosen because it was estimated that it represented a similar dose level (approximately 10 mg/kg) to that used in the clinical study by Braybrooke et al. (2000) (22). No attempt was made to confirm similar serum levels by pharmacokinetic analysis. At the end of the 3-day period, mice received 10 MBq of Tc-99m-sestamibi as an intravenous tail vein injection. At 20, 50, and 90 min after

the intravenous injection, groups of 4–6 mice were anaesthetized with isofluorane (Abbott Laboratories Ltd, Queenborough, UK) and killed by exsanguination at cardiac puncture followed by cervical dislocation. A 0.5 ml sample of blood was decanted into a scintillation vial (Sterilin, Stone, UK) and, at the time of cervical dislocation, any urine voided was collected. The bilateral tumours, spleen, liver, kidneys, heart, and lungs were removed, blotted dry and weighed in pre-weighed scintillation vials. The levels of contained radioactivity were determined using a Canberra Packard Minaxi 5550 gamma-counter. A standard of 1% of the injected dose was counted in parallel with the tissue samples. Tissue activity was expressed as a percentage of the total injected dose per gram (%ID/g). In view of the unequivocal data from the immunocytochemistry in vitro, immunohistochemistry of tumour xenografts was not performed. In addition to this consideration, immunohistochemistry has a number of general limitations including technical failure, false-negative results due to sampling error, heterogeneous staining patterns that may fail to give an accurate global estimate of protein expression, and an inability to convey information on function.

Statistical analysis

The effects of toremifene on net doxorubicin uptake, EC50, and sestamibi accumulation in drug-sensitive and drug-resistant cell lines were evaluated by Student's unpaired t-test, taking 0.05 as the cut-off for significance. With regard to accumulation of the radiotracer in tumour xenografts in vivo, unpaired t-testing was used to compare accumulation of sestamibi between drug-sensitive and drug-resistant

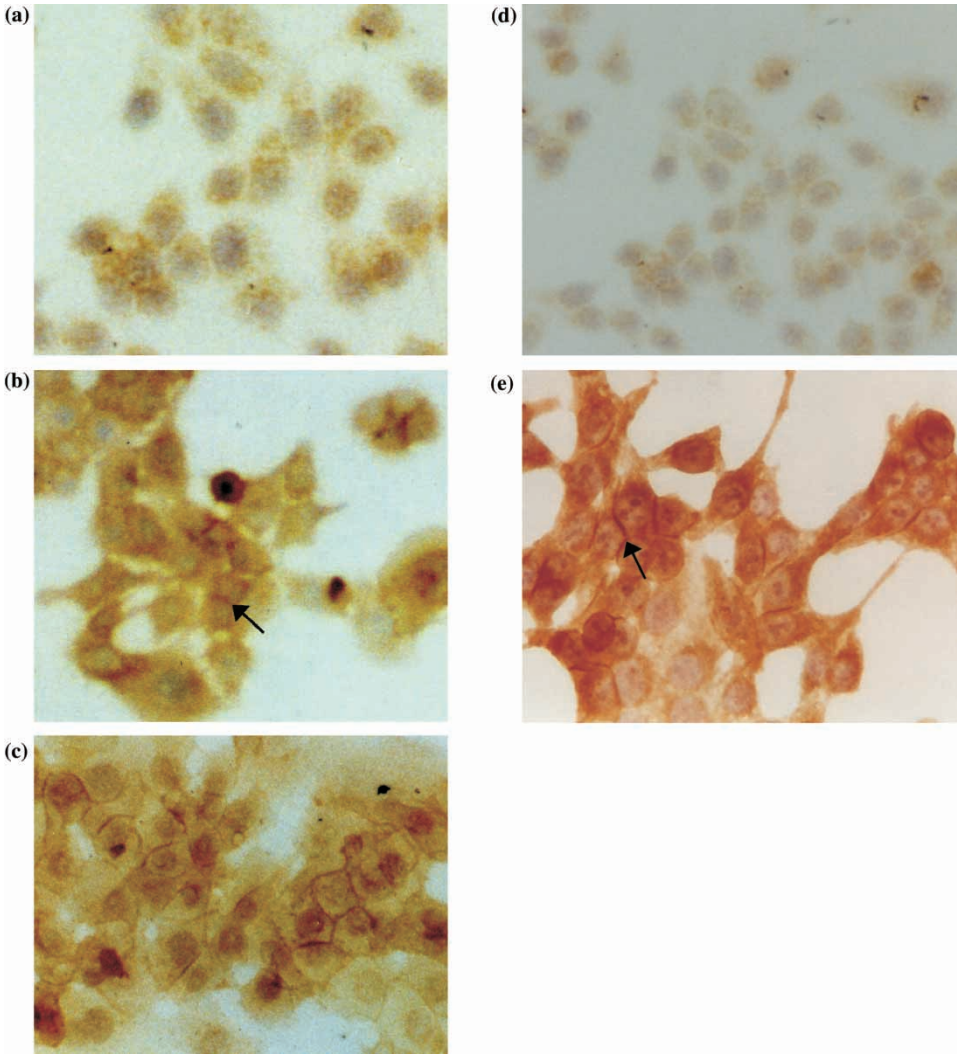

Fig. 1. Immunocytochemistry of drug-sensitive and drug-resistant breast and head and neck cancer cell lines immunostained for Pgp expression with monoclonal antibody C494 (magnification 200x): (a) MCF7-S cells showing weak granular cytoplasmic Pgp immunoreactivity; (b) MCF7-R cells showing diffuse moderate to strong cytoplasmic Pgp expression. Membranous Pgp expession is also evident in closely adherent cells (see arrow); (c) MCF7-R1 cells showing greater levels of Pgp expression than MCF7-R; (d) KB cells showing absence of Pgp immunoreactivity; (e) KBV1 cells showing strong cytoplasmic and membranous immunoreactivity (see arrow).

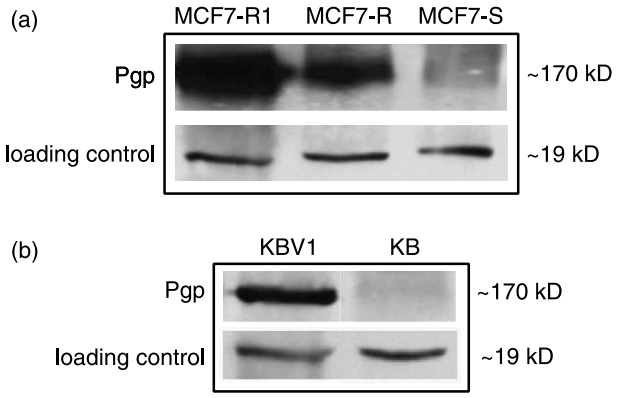

Fig. 2. Western blot for Pgp expression in drug-sensitive and drug-resistant breast and head and neck cancer cell lines: (a) the strength of the Pgp reactive band at approximately 170 kDa shows a progressive increase in MCF7-S, MCF7-R and MCF7-R1 cells; (b) there is no detectable Pgp expression in KB cells, whereas KBV1 cells show a prominent Pgp reactive band. In each blot, the 19kDa control confirms equal protein loading.

xenografts and to assess the effects of toremifene on sestamibi accumulation in both tumour types.

RESULTS

Immunocytochemistry

The parental cell lines (MCF7-S and KB) showed either weak or absent Pgp staining with both monoclonal antibodies. In contrast, the drug-resistant cell lines (MCF7-R, MCF7-R1 and KBV1) demonstrated strong immunoreactivity for Pgp (Fig. 1a–e).

Western analysis for Pgp expression

The results of Western analysis were consistent with those described above for immunocytochemistry (Fig. 2). MCF7-S and KB cell lines showed minimal and no detectable Pgp, respectively. The drug-resistant cell lines (MCF7-R, MCF7-R1, and KBV1) showed a single, relatively broad 170 kDa band on 6% SDS-PAGE. Densitometric analysis revealed 3–5-fold greater expression of Pgp in MCF7-R1 cells compared with MCF7-R. Western analyses of both MCF7-R1 and KBV1 cells after exposure to doxorubicin and vinblastine, respectively, showed a significant increase in Pgp expression (Fig. 3).

Fluorescence microscopy

Fluorescence microscopy revealed significant accumulation of doxorubicin in drug-sensitive MCF7-S and KB cell lines. Furthermore, there was significant localization of doxorubicin in the nucleus of these cell lines (Figs 4a and 4e). Drug accumulation was not altered by addition of 7.5 μM toremifene (Figs 4b and 4f). In contrast, there was very little accumulation of doxorubicin in the drug-resistant MCF7-R1 and KBV1 cell lines and this was restricted to the

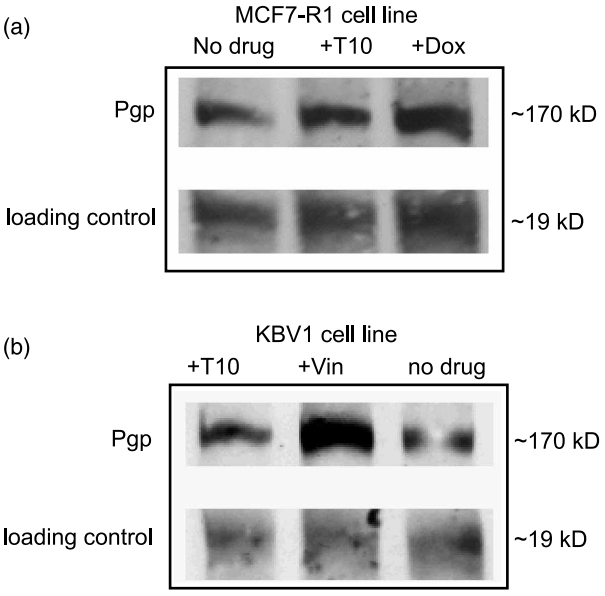

Fig. 3. Western blot for Pgp expression in drug-resistant breast and head and neck cancer cell lines after exposure to cytotoxic agents (doxorubicin or vinblastine) or toremifene: (a) Pgp expression increases significantly in MCF7-R1 cells after exposure to doxorubicin; (b) Pgp expression increases significantly in KBV1 cells after exposure to vinblastine. In each cell line, there was minimal or no significant change after exposure to 10 μM toremifene. Dox = doxorubicin; Vin = vinblastine; T10 = 10 μM toremifene.

cytoplasm (Figs 4c and 4g). In the presence of 7.5 μM toremifene, however, there was obvious cellular (and nuclear) accumulation of doxorubicin (Figs 4d and 4h).

FACS analysis

Data on the accumulation of doxorubicin in MCF7-S, MCF7-R1, KB, and KBV1 cells in the presence of toremifene are presented in Table 1. Both drug-resistant cell lines demonstrated a significant increase in drug accumulation in a toremifene dose-dependent manner. In contrast, there was no evidence of increased doxorubicin accumulation in the drug-sensitive cell lines.

In vitro cytotoxicity

The EC50 values for doxorubicin against the various cell lines studied as assessed using the MTT assay are presented in Table 2. MCF7-S cells were significantly more sensitive than either MCF7-R or MCF-7R1 cells and this sensitivity was not increased by the addition of toremifene at 5 μM or 10 μM (Fig. 5). In contrast, on addition of toremifene, the EC50 for the MCF7-R1 cell line was significantly reduced in a dose-dependent fashion (Fig. 6). Similar data were obtained for KB and KBV1 cells with vinblastine, in that toremifene significantly decreased the EC_{50} of the drug-resistant, but not the drug-sensitive, cell line (Table 3).

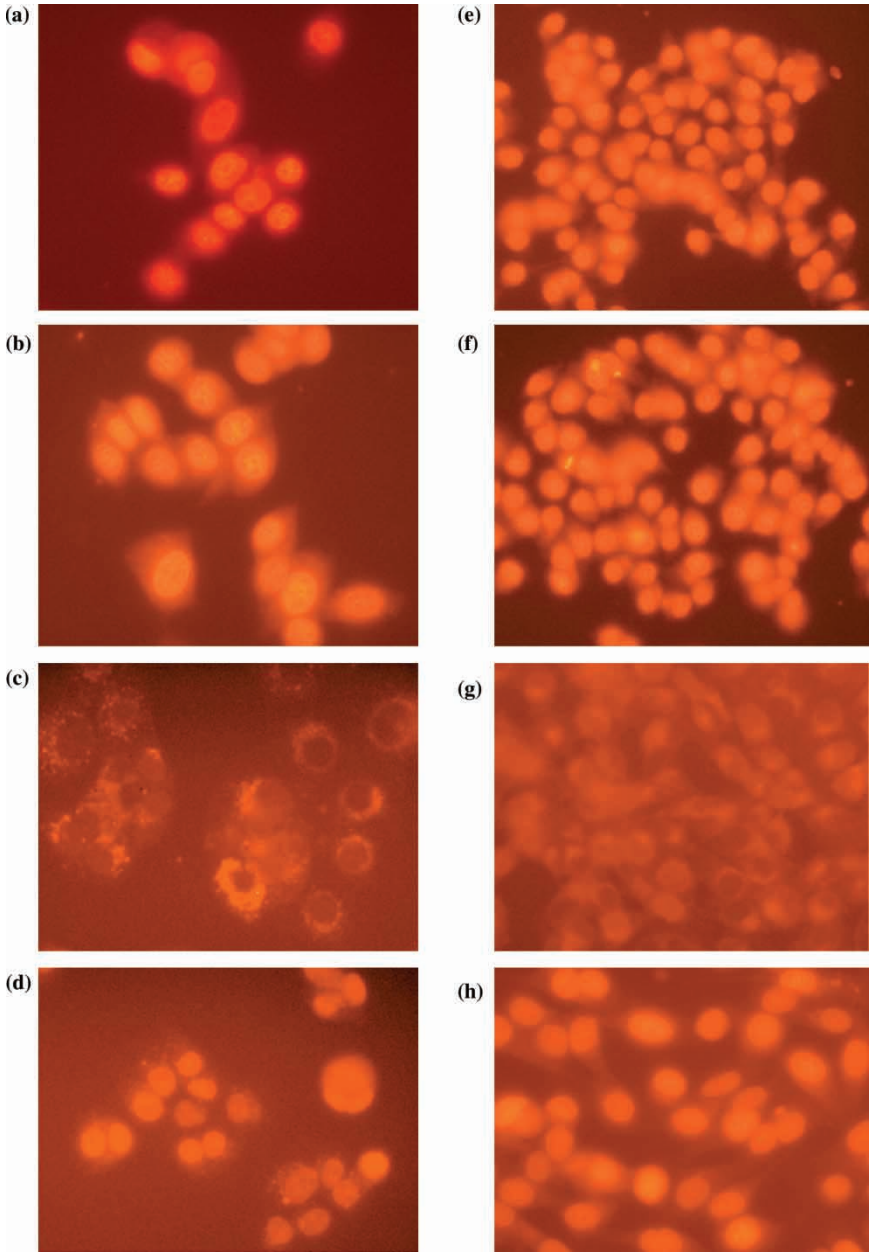

Fig. 4. Fluorescent microscopy in drug-sensitive and drug-resistant breast and head and neck cancer cell lines after exposure to doxorubicin, with or without toremifene (5–7.5 μM) for 4 hours (Magnification –100x): (a) MCF7-S cells plus doxorubicin. Marked fluorescence is seen with prominent nuclear drug localization; (b) MCF7-S cells plus doxorubicin and toremifene 7.5 μM. The addition of toremifene has little effect on drug localization; (c) MCF7-R1 cells plus doxorubicin. Minimal intracytoplasmic fluorescence is seen, consistent with active extrusion of the drug in these Pgp overexpressing cells; (d) MCF7-R1 cells plus doxorubicin and toremifene 7.5 μM. A significant increase in intracellular fluorescence is seen with a predominantly nuclear distribution; (e) KB cells plus doxorubicin. Marked fluorescence is seen with prominent nuclear drug localization; (f) KB cells plus doxorubicin and toremifene 5 μM. The addition of toremifene has little effect on drug localization; (g) KBV1 cells plus doxorubicin. No intracellular fluorescence is seen, consistent with active extrusion of the drug in these Pgp overexpressing cells; (h) KBV1 cells plus doxorubicin and toremifene 7.5 μM. A marked increase in intracellular fluorescence is seen with a predominantly nuclear distribution.

Tc-99m-sestamibi accumulation studies in vitro

The accumulation of Tc-99m-sestamibi was determined for MCF7, MCF7-R, MCF7-R1, KB, and KBV1 cell lines in the absence and presence of toremifene. In its absence, there was a progressive increase in Tc-99m-sestamibi accumulation between 5 and 60 min followed by a plateau between 60 and 90 min in all cell lines. The levels of accumulation were significantly higher in the parental drug-sensitive cell lines

Table 1

Effect of toremifene on the accumulation of doxorubicin in drug-sensitive and drug-resistant MCF7 and KB cells. Data are expressed in terms of the percentage of fluorescence on FACS analysis relative to cells incubated with doxorubicin in the absence of toremifene. Data shown are the mean (±SD) of at least 3 experiments. Toremifene 10 μM was not used with MCF7-S or KB cells

Cell line	toremifene 5 μM	toremifene 10 μM	p-value
MCF7-S	95±12	–	
MCF7-R1	129±14	175±16	< 0.02
KB	98±9	–	
KBV1	133±18	181±20	< 0.05

than in their drug-resistant counterparts. Addition of toremifene unexpectedly resulted in a consistent decrease in Tc-99m-sestamibi accumulation in KBV1 and MCF7-R cell lines (Table 4). This effect was less marked in the drug-sensitive cell lines, with a significant decrease only in KB cells.

Accumulation of Tc-99m-sestamibi in vivo

After intravenous injection, blood activity was maximal at 20 min and then showed a progressive decline at 50 and 90 min. Accumulation of Tc-99m-sestamibi in all non-tumour tissues was slightly higher at 50 min compared with 20 min but clearly decreased at 90 min (Table 5). Toremifene had no obvious effect on the localization and retention of Tc-99m-sestamibi in any native tissues.

In both xenografts, sestamibi accumulation was maximal at 50 min before and after toremifene (Table 5). Accumulation of sestamibi in tumours before toremifene administration was greater in KB compared with KBV1 xenografts but only significantly at 20 min. After toremifene, KB tumours still accumulated more sestamibi than KBV1. There was significant washout of sestamibi from both tumours between 50 and 90 min both in the presence and absence of toremifene. Treatment with toremifene did not increase the uptake or retention of sestamibi in either KB or KBV1 xenograft tumours but instead showed a tendency to reduce it, although reaching statistical significance only in the case of KB (drug-sensitive) tumours. These data must be considered in light of the fact that the animal numbers in each group were relatively small, although the findings were consistent within the various groups (see Table 5).

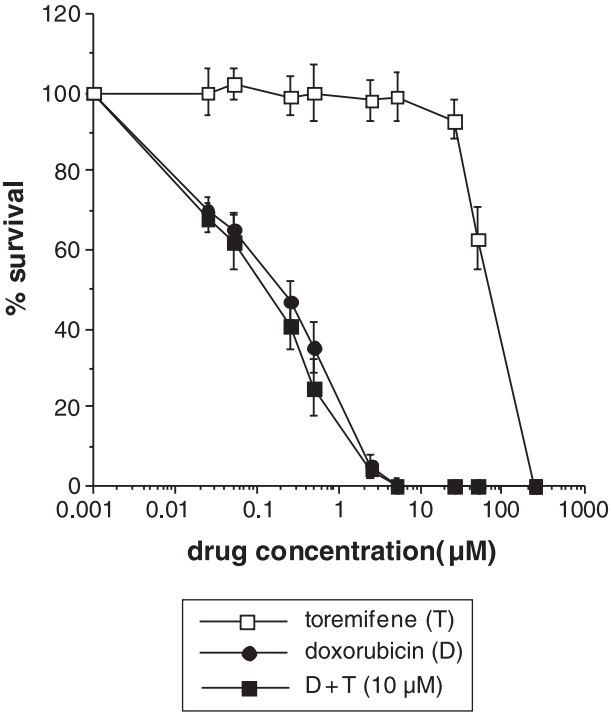

Fig. 5. MTT cell survival assay for MCF7-S cells in response to doxorubicin in the presence or absence of toremifene 10 μM. Data are expressed as % cell survival relative to untreated controls and represent the mean (±SD) of at least 3 experiments. MCF7-S cells were sensitive to doxorubicin (EC50 0.175±0.06 μM) and this effect was not significantly enhanced in the presence of toremifene (EC50 0.142±0.04 μM).

DISCUSSION

The level of Pgp expression has been correlated with sensitivity to cytotoxic chemotherapy and prognosis in patients with breast cancer (4, 28–32). As such, modulation of the function of Pgp represents a legitimate target for therapeutic intervention. These studies confirmed the activity of toremifene as a modulator of MDR in vitro.

Table 2

Effect of toremifene on the EC50 of doxorubicin in drug-sensitive (MCF7-S) and drug-resistant (MCF7-R and MCF7-R1) breast cancer cell lines. The EC50 values are expressed in μM and the data shown represent the mean (±SD) from at least 3 separate experiments. Modification factors (in parentheses) were calculated by dividing the EC50 in the absence of toremifene by the EC50 obtained at either 5 μM or 10 μM toremifene

Cell line	EC50 values for doxorubicin		
	Control	toremifene 5 μM	toremifene 10 μM
MCF7-S	0.175±0.06 (1.00)	0.169±0.05 (1.04)[1]	0.142±0.04 (1.23)[1]
MCF7-R	4.98±0.84 (1.00)	0.58±0.12 (8.6)[2]	0.38±0.09 (13.1)[2]
MCF7-R1	33.6±2.5 (1.00)	2.36±0.31 (14.2)[2]	1.58±0.19 (21.3)[2]

[1]Not significant, [2]$p < 0.001$ versus control.

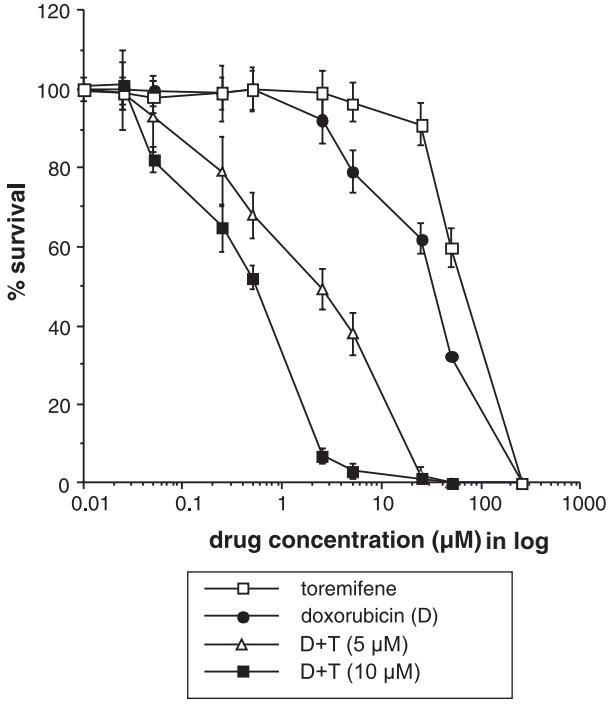

Fig. 6. MTT cell survival assay for MCF7-R1 cells in response to doxorubicin in the presence or absence of toremifene 5 μM or 10 μM. Data are expressed as % cell survival relative to untreated controls and represent the mean (±SD) of at least 3 experiments. MCF7-R1 cells were resistant to doxorubicin (EC50 33.6±2.5 μM). The addition of toremifene significantly increased the toxicity of doxorubicin (EC50 2.36±0.31 μM and 1.58±0.19 μM with 5 μM and 10 μM toremifene, respectively).

Increased cellular accumulation of doxorubicin in a dose-dependent fashion was seen by FACS analysis in Pgp-expressing MDR cell lines. Moreover, fluorescence microscopy demonstrated a clear shift in the intracellular pattern of doxorubicin localization, with a significant increase in drug entering the nuclei of Pgp-expressing cell lines. Such a phenomenon may reflect efflux activity of Pgp in the nuclear membrane itself. These effects were shown to correlate with increased sensitivity to doxorubicin and vinblastine in MCF7-R and KBV1 cells, respectively. Indeed, for the two drug-resistant MCF7 cell lines, the ability of toremifene to reverse the MDR phenotype was directly related to the level of Pgp expression documented on immunocytochemistry and Western analysis. Therefore, the EC_{50} modification factors with both 5 μM and 10 μM toremifene were significantly greater for MCF7-R1 cells than for MCF7-R cells. Such data strongly support future clinical evaluation of toremifene as an MDR modulator, a conclusion that is further strengthened by the data from the recent phase I trial which showed that toremifene can be administered safely at high doses (22), in contrast with tamoxifen (7, 8, 20, 21).

However, the impressive in vitro localization and cytotoxicity data obtained with toremifene plus doxorubicin or vinblastine were in direct contrast with those obtained with Tc-99m-sestamibi. Toremifene reduced the accumulation of Tc-99m-sestamibi both in vitro and in vivo, despite the fact that this agent has previously been shown to be a substrate for Pgp (33, 34). The current data, showing significantly lower levels of radiotracer uptake in the control drug-resistant, as opposed to drug-sensitive, cell lines in vitro and the lower levels of radiotracer uptake in the KBV1, as compared with KB, control xenograft tumours in vivo confirmed that Tc-99m-sestamibi did, indeed, serve as a Pgp substrate in these models. Interestingly, the effect of toremifene in vitro was greater in drug-resistant cell lines whereas the effect of toremifene in vivo was significant only in drug-sensitive xenografts. Moreover, there was no evidence that administration of toremifene caused increased accumulation of Tc-99m-sestamibi in the normal tissues, including those which express Pgp at high levels. These data

Table 4

Effect of toremifene on the accumulation of Tc-99m-sestamibi in drug-sensitive (MCF7-S and KB) and drug-resistant (MCF7-R1 and KBV1) breast and head and neck cancer cell lines. The uptake values are expressed as a percentage of the added radioactivity and the data shown represent the mean (±SD) from at least 3 separate experiments. The modification factor (MF) was calculated by dividing the % uptake in the presence of toremifene by the % uptake in the absence of toremifene

Cell line	Control	toremifene 5 μM	MF	p-value
MCF7-S	4.31±0.54	3.70±0.90	0.86	>0.05
MCF7-R1	1.06±0.19	0.34±0.06	0.32	<0.01
KB	11.40±0.52	9.92±0.29	0.89	<0.05
KBV1	1.51±0.23	0.63±0.04	0.42	<0.01

Table 3

Effect of toremifene on the EC50 of vinblastine in drug-sensitive (KB) and drug-resistant (KBV1) head and neck cancer cell lines. The EC50 values are expressed in μM and the data shown represent the mean (±SD) from at least 3 separate experiments. The modification factor (in parentheses) was calculated by dividing the EC50 in the absence of toremifene by the EC50 obtained at either 5 μM or 10 μM toremifene

Cell line	EC50 values for vinblastine (μM)		
	Control	toremifene 5 μM	Toremifene 10 μM
KB	0.172±0.017 (1.00)	0.153±0.018 (1.12)[1]	–
KBV1	7.88±0.96 (1.00)	1.05±0.09 (7.5)[2]	0.65±0.07 (12.1)[2]

[1]Not significant, [2]$p < 0.001$ versus control.

suggest that the interaction of toremifene with Pgp is complex and paradoxical when viewed alongside chemotherapeutic agents. The in vivo findings echo recent findings in a clinical study of ours on the effect of toremifene on sestamibi uptake in breast cancer in which the modulator decreased uptake of radiotracer in Pgp-negative cancers but had no effect on Pgp-positive cancer (35). This suggests a dual effect of toremifene: an indirect effect promoting net accumulation through inhibition of Pgp and a direct effect opposing uptake, perhaps through cellular depolarization and interference with the electrostatic trapping of positively charged sestamibi in the negative environment of mitochondria, an effect previously observed for tamoxifen (36). It is not clear why toremifene had a quantitatively different impact on Tc-99m-sestamibi accumulation in vitro as compared with in vivo. Obvious factors present in vivo which would be absent in vitro and which may have modified comparative accumulation include competition for sestamibi by native tissues, a shorter period of availability for uptake as a result of sestamibi's fairly rapid blood clearance, variations in tissue blood flow (of which sestamibi is a general marker), and variable microvascular permeability to toremifene along with different operating intracellular toremifene concentrations.

In addition, it must be remembered that the Pgp efflux pump is only one mechanism by which breast cancers can manifest drug resistance. Multidrug resistance-associated proteins (MRP1 and MRP2) and breast cancer resistance protein (BCRP/MXR/ABCP) have also been shown to be involved in this process (37–39). The clinical significance of these other mechanisms remains uncertain at present but a recent clinical study suggested that only overexpression of MDR1 (and hence Pgp) was associated with a worse prognosis (40). The role of toremifene (and other MDR modulators) in reversing other non-Pgp mechanisms of drug resistance has not yet been systematically studied but is certainly worthy of further examination.

In summary, toremifene is able to reduce the Pgp-dependent efflux of doxorubicin and vinblastine from tumour cells but, in contrast, appears to reduce Tc-99m-sestamibi accumulation even in the presence of Pgp overexpression. These data have a number of implications that are potentially clinically relevant. The differences in the levels of Tc-99m-sestamibi uptake between the Pgp-negative and Pgp-positive tumour cells in the control setting support the notion that tumour imaging may provide a means of predicting functional Pgp status of tumours in vivo. In contrast, the conflicting data obtained for the effect of toremifene on uptake of the cytotoxic drugs and the radiotracer raise questions about the ability of nuclear medicine imaging techniques to predict reliably the therapeutic effect of MDR modulator drugs in vivo. More importantly, the fact that toremifene can have opposite effects on different Pgp substrate molecules suggests that this agent may reduce the uptake of certain cytotoxic agents if they are handled in the same way as sestamibi. Although this matter has not been directly tested in this study, such results may have some bearing on the generally negative results obtained in the Phase II clinical trials that have examined MDR reversal strategies and underline the need for preliminary in vitro studies.

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