of 26.5 months in the present study following CT is not very different from the figure of 24 months predicted by A'Hern *et al.* [15]. In fact, our figure of 26.5 months does exaggerate the survival expectations after CT in RBC somewhat due to the fact that patients with early death were excluded from the study population.

The use of historical controls has always created much debate [16, 17]. However, the present study is population-based which means that a number of the selection factors which are operative in hospital-based studies are largely eliminated. Given the basic assumption that the natural history of breast cancer has not changed during the two decades covered by the study, our data suggest that CT in RBC does prolong survival with 9.5 months in patients who survive more than 2 weeks from the start of treatment for their recurrence.

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Selective Reversal of Vinblastine Resistance in Multidrug-resistant Cell Lines by Tamoxifen, Toremifene and their Metabolites

Julie Kirk, Sue Houlbrook, Nicholas S.A. Stuart, Ian J. Stratford, Adrian L. Harris and James Carmichael

In this study we describe the effects of tamoxifen, toremifene and their 4-hydroxy and *N*-desmethyl metabolites on the toxicity of a range of drugs to human breast and lung cancer and to Chinese hamster ovary cell lines, determined using a tetrazolium-based semi-automated colorimetric assay. Vinblastine resistance was completely abolished in an *mdr*1-transfected lung cancer cell line (S1/1.1), indicating that P-glycoprotein-mediated multidrug resistance can be fully reversed by anti-oestrogens. A substantial (14- to 39-fold) enhancement of vinblastine toxicity to highly multidrug-resistant (MCF-7^{Adr}) cells expressing P-glycoprotein was also observed in the presence of tamoxifen, toremifene and their metabolites, while *m*-amsacrine, cisplatin and melphalan toxicity was unaffected.

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INTRODUCTION

TUMOURS TREATED with chemotherapeutic drugs may become resistant not only to subsequent treatment with the same drug, but to treatment with structurally and functionally dissimilar natural products such as anthracyclines, epipodophyllotoxins and vinca alkaloids [1]. This phenomenon, known as multidrug resistance (MDR), is a major clinical problem, rendering a wide range of drugs redundant in the treatment of some tumours. MDR is frequently associated with overexpression of the *mdr*1 gene product [2], the 170 kD P-glycoprotein (Pgp). Cellular

resistance appears to correlate with Pgp density in the cell membrane [3]. Overexpression of Pgp is associated with decreased intracellular drug accumulation [4] and the protein has been identified as an ATP-dependent drug efflux pump. MDR levels are generally higher in tumours derived from tissues which normally express Pgp, e.g. kidney and liver [5, 6]. Circumvention of Pgp-dependent MDR has been demonstrated *in vitro* with a range of compounds, including the calcium channel blockers, e.g. verapamil [7] and anti-oestrogens, e.g. tamoxifen [8]. Unfortunately, many compounds which reverse MDR *in vitro* cannot be administered to patients at doses sufficient to generate resistance-modifying plasma concentrations without causing unacceptable toxicity (e.g. verapamil, [9]).

Tamoxifen has been used in the treatment of breast cancer for 25 years [10]. It is well tolerated and is frequently used clinically for prolonged periods. Patients can receive up to 480 mg tamoxifen daily without suffering severe toxicity and such doses yield plasma concentrations of 3–5 μmol/l [8]. Toremifene, a close structural analogue of tamoxifen, appears to be better tolerated and higher plasma concentrations may be achieved; patients treated with 400 mg toremifene daily achieved mean serum levels of 13 μmol/l [11]. In addition, both tamoxifen and toremifene are extensively metabolised to a range of compounds, including the *N*-desmethyl and 4-hydroxy metabolites [12, 13]. Although anti-oestrogens in plasma are 99.7% bound to serum proteins such as alpha 1 acid glycoprotein [14], high levels have also been found to accumulate intracellularly. For example, levels of parent compound and metabolites in metastases of patients receiving low doses of tamoxifen (20–80 mg/day) are up to 30-fold higher than in plasma, giving total anti-oestrogen levels of ~16 μmol/l [15]. Tissue anti-oestrogen concentrations may be even greater in patients receiving high-dose tamoxifen or, in particular, toremifene therapy.

This study compares the effects of tamoxifen, toremifene and their metabolites on the *in vitro* cytotoxicity of structurally and functionally diverse drugs to a panel of cell lines (listed in Table 1). Previous studies have analysed the MDR-modifying effects of anti-oestrogens on cells in which high levels of resistance were induced by chronic *in vitro* exposure to drugs. However, such treatment activates multiple resistance mechanisms in addition to Pgp. Our panel of cell lines included an *mdr*1 transfectant in order to specifically investigate effects of anti-oestrogens on Pgp-mediated drug resistance. Cell lines with intrinsic drug resistance, and those where high levels of MDR had been induced through drug exposure, were also included.

MATERIALS AND METHODS

Cell lines and tissue culture

Cell lines are listed in Table 1 [14, 16–21]. These included three wild type cell lines and their drug-resistant sublines: (a) CHO-K1 and its MDR-positive subline CHO-K1^{Adr} [14], (b) the human epithelial breast cancer cell line MCF-7 [16] and its highly drug-resistant subline MCF-7^{Adr} [17] and (c) the human non-small cell lung cancer cell line S1 [20], from which the Pgp-positive subline S1/1.1 was derived by transfection with an *mdr*1 expression vector. Levels of *mdr*1 in S1/1.1 cells are at least 100-fold higher than in the parental cell line (Dr F. Baas, University of Amsterdam, The Netherlands). MCF-7^{Adr} and CHO-K1^{Adr} sub-lines were selected by chronic exposure to doxorubicin and express Pgp. A range of breast and lung cancer cell lines of varying histological origin [16, 18, 19, 21] were also included in this study.

Table 1. *Characteristics of cell lines*

Cell type	Cell line	Ref.	Characteristics	MNC (μmol/l)
CHO	CHO-K1	14	Wild type	10
	CHO-K1^{Adr}	14	MDR phenotype	10
Breast cancer	MCF-7	17	ER+ve	1
	MCF-7^{Adr}	18	ER−ve, MDR phenotype	20
	MDA-468	19	ER−ve	10
	T47D	20	ER+ve	5
Lung cancer	S1	21	Non-small cell carcinoma	10
	S1/1.1	*	*mdr*1 transfectant of S1	10
	NCI-H 322	22	Non-small cell carcinoma	10
	NCI-H 460	22	Large cell carcinoma	5
	NCI-H 841	22	Small cell carcinoma	10

*Dr F. Baas (University of Amsterdam, The Netherlands).
MNC = maximum non-toxic concentration.

Cell lines were maintained as monolayer cultures in continuous exponential growth by bi-weekly passage. S1, S1/1.1 and CHO cell lines were grown in HAMS F-12 medium and other cell lines in RPMI 1640 medium, each supplemented with 10% fetal calf serum (FCS) and 2 mmol/l glutamine. All cell lines were incubated at 37°C (5% CO_2, 100% humidity) and regularly shown to be mycoplasma free.

Drugs

Vinblastine sulphate, formulated for clinical use (David Bull Laboratories Pty Ltd, Mulgrave, Victoria, Australia), was stored at 4°C as a 1 mmol/l solution in phosphate buffered saline (PBS); *M*-amsacrine (AMSA) (Parke-Davis, Michigan, U.S.A.), at −20°C as a 1 mmol/l solution in dimethylsulphoxide (DMSO)/PBS (50% v/v); cisplatin (Farmitalia U.K., St Albans), at −20°C as a 200 mmol/l solution in DMSO. Stock solutions were diluted in PBS as required. Melphalan (Wellcome, Kent, U.K.) was freshly prepared as a 100 mmol/l solution in cold HCl/ethanol (2% v/v) and diluted in saline. Tamoxifen and metabolites were provided by ICI Pharmaceuticals (Macclesfield, U.K.) and toremifene and metabolites by the Orion Corporation (Turku, Finland). Tamoxifen (50 mmol/l in ethanol), toremifene (50 mmol/l in DMSO) and anti-oestrogen metabolites (50 mmol/l in DMSO) were stored at 4°C and diluted in PBS. Anti-oestrogens were added to cells at their maximum non-toxic concentration (MNC), defined as the highest concentration tested which reduced control cell optical density (OD) by <5%. Organic solvent levels did not exceed 0.1% by volume of the cell suspension, a concentration which did not affect cell growth.

Cytotoxicity assays

Exponentially growing cells were trypsinised, centrifuged and resuspended in fresh medium (10% FCS, 2 mmol/l glutamine). Cell suspensions (180 μl) were aliquoted into 96-well microtitre

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plates at a seeding density previously demonstrated to allow exponential growth for 4 days. Anti-oestrogen modifiers, drug (at eight different concentrations) and/or vehicle (10 μl) were added in quadruplicate at appropriate concentrations. Cells were incubated continuously with drug and/or modifier at 37°C for 4 days. Cytotoxicity was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay [21]. MTT (50 μl, 2 mg/ml) was aliquoted into all wells and the cells incubated for 4 h. Plates were inverted to discard medium and formazan crystals were solubilised in 100 μl DMSO with 25 μl glycine buffer (0.1 mol/l glycine in 0.1 mol/l NaCl, pH 10.5; [22]). Plates were agitated for 5 min, and OD immediately determined at 540 nm using a Titertek Multiskan Plus MKII ELISA plate reader under computer control (Macintosh SE/30 microcomputer).

DeltaSoft enzyme-linked immunosorbent assay (ELISA) analysis software (BioMetallics Inc., Princeton, New Jersey, U.S.A.) was used to fit raw data to a four parameter curve fitting equation. IC_{50} values (concentration of drug causing a 50% reduction in control cell OD) were calculated using the same software, through interpolation of a value 50% of control cell OD into the curve fitting equation. IC_{50} values quoted in text and Tables are mean values determined from at least three identical experiments (performed on different days) ± S.E.M. IC_{50} 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 is expressed as a modification factor (MF), calculated by dividing the IC_{50} value determined in the absence of modifier by that determined in its presence. MF < 1, therefore, indicates protection from, MF > 1 synergism with, and MF = 1 no effect on drug toxicity.

RESULTS

MCF-7^{Adr} cells were 300-fold resistant to vinblastine relative to wild type MCF-7 cells (Table 2). Tamoxifen caused a large leftward shift in the vinblastine dose–response curve for Pgp-positive MCF-7^{Adr} cells (Fig. 1) but had no effect on drug toxicity to wild type cells, although it should be noted that the MNC of tamoxifen for MCF-7 cells was 1 μmol/l compared with 20 μmol/l for the oestrogen receptor-negative MCF-7^{Adr} cells (Table 1). Similar effects were observed using toremifene and its derivatives, and tamoxifen metabolites (Table 2). Modification of drug toxicity, ranging from 13-fold (20 μmol/l *N*-desmethyl tamoxifen, NdMTx) to 40-fold (20 μmol/l tamoxifen), was dose-related as shown in Fig. 2. Tamoxifen, toremifene and their 4-hydroxy metabolites were equally effective modifiers ($P > 0.05$), while *N*-desmethyl metabolites were significantly less effective ($P < 0.05$). The maximum MF achieved was 40 (20 μmol/l tamoxifen); wild type sensitivity was therefore not restored, a residual 8-fold degree of resistance remaining.

CHO-K1^{Adr} cells were 13-fold resistant to vinblastine relative to CHO-K1 cells (Table 3). The cell lines were equally sensitive to anti-oestrogens, and the effect of equimolar doses of tamoxifen and toremifene could, therefore, be compared. Figure 3 shows the effects of 0, 1, 5 and 10 μmol/l tamoxifen on vinblastine dose–response curves. Tamoxifen enhanced vinblastine toxicity towards both cell lines in a dose-dependent manner (Figs 3 and 4). CHO-K1^{Adr} sensitivity to the drug was substantially increased, while a smaller effect was seen with parental CHO-K1 cells. In the presence of 10 μmol/l anti-oestrogen, vinblastine toxicity was increased ~17-fold to CHO-K1^{Adr} cells and ~7-

Table 2. Effects of tamoxifen, toremifene and their metabolites on vinblastine toxicity to MCF-7 and MCF-7^{Adr} cell lines. Results are mean values from six identical experiments ± S.E.M.

	Vinblastine IC_{50} value (nmol/l)			
Modifier	MCF-7 +Modifier*	MF	MCF-7^{Adr} +Modifier*	MF
None	1.2 ± 0.2	—	359 ± 16	—
Tamoxifen	1.2 ± 0.1	1†	9.3 ± 1.5	39‡
OHTx	—	—	16 ± 4	22‡
NdMTx	—	—	28 ± 3	13‡
Toremifene	1.1 ± 0.3	1	11 ± 1	33‡
OHTf	—	—	17 ± 2	21‡
NdMTf	—	—	26 ± 3	14‡

*Anti-oestrogens were added at their MNC (Table 1).
†Where MF (modification factor) = 1, *P* values of 0.05–1 were obtained.
‡P = 0–0.001.

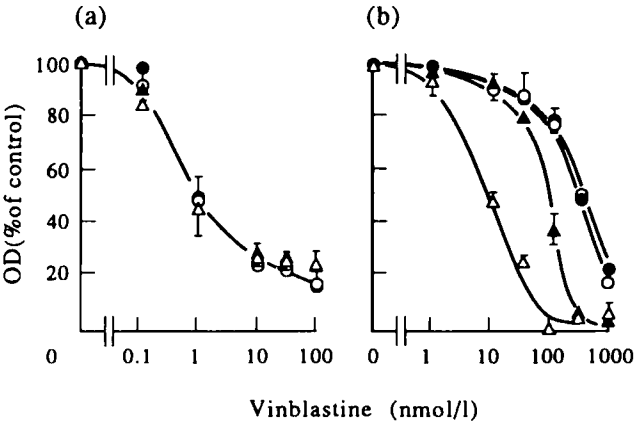

Fig. 1. Effect of tamoxifen on vinblastine toxicity to (a) wild type MCF-7 and (b) MDR-positive MCF-7^{Adr} cells. (a) 0 (●), 0.1 (○), 0.5 (▲) and 1 μmol/l (△) tamoxifen. (b) 0 (●), 1 (○), 10 (▲) and 20 μmol/l (△) tamoxifen. Results are from one representative experiment ± S.D. calculated from four replicates; where not shown, error bars fall within the area of plot symbols.

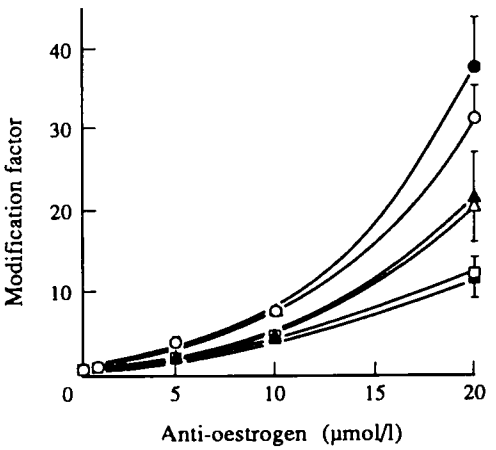

Fig. 2. Effect of anti-oestrogens on vinblastine toxicity to MCF-7^{Adr} cells. Modification of vinblastine toxicity is expressed as a modification factor (MF; ratio of vinblastine IC_{50} values determined in the absence and presence of modifier). Tamoxifen (●), OHTx (▲), NdMTx (■), toremifene (○), OHTf (△) and NdMTf (□). Results are mean MF calculated from six identical experiments ± S.E.M.

Table 3. Effects of tamoxifen and toremifene on vinblastine toxicity. Results are mean values from three identical experiments ± S.E.M.

Cell line	Vinblastine IC_{50} value (nmol/l) +PBS	Tamoxifen*	MF	Toremifene*	MF
CHO-K1	8.6 ± 0.3	1.3 ± 0.4	7§	1.1 ± 0.2	8§
CHO-K1^{Adr}	112 ± 13	7.0 ± 0.9	16‡	6.5 ± 0.6	17§
S1	2.0 ± 0.2	1.2 ± 0.2	2‡	1.1 ± 0.1	2†
S1/1.1	9.6 ± 0.8	1.4 ± 0.3	7§	0.9 ± 0.2	10§
MDA-468	1.9 ± 0.1	1.5 ± 0.4	1†	1.8 ± 0.3	1
T47D	6.0 ± 0.3	7.2 ± 0.5	1	5.1 ± 0.6	1
NCI-H 322	4.1 ± 0.7	3.7 ± 0.4	1	4.5 ± 0.5	1
NCI-H 460	2.8 ± 0.3	2.5 ± 0.2	1	2.4 ± 0.3	1
NCI-H 841	2.0 ± 0.4	1.6 ± 0.3	1	1.8 ± 0.4	1

*Tamoxifen and toremifene were added to cells at the appropriate MNC (Table 1).
†Where MF (modification factor) = 1, P values of 0.05–1 were obtained.
‡P = 0.001–0.01; §P = 0–0.001.

fold to wild type cells (Table 3). CHO-K1^{Adr} cells were, therefore, more highly sensitised to vinblastine by anti-oestrogens than were parental cells, although a 6-fold degree of resistance still remained.

Figure 5 shows dose–reponse curves for S1 and S1/1.1 cells determined in the presence of 0, 1, 5 and 10 μmol/l tamoxifen. A marked dose-dependent leftward shift was observed for the *mdr*1 transfectant S1/1.1, and a slight shift was also seen with parental cells. S1/1.1 cells were 5-fold resistant to vinblastine relative to wild type cells (Table 3). This resistance was completely reversed by tamoxifen and toremifene, which caused 7- and 10-fold modifications of drug toxicity, respectively (P = 0.0001). Although a slight modification (2-fold, $P < 0.01$) of wild type sensitivity occurred, vinblastine IC_{50} values for S1 and S1/1.1 cells determined in the presence of 10 μmol/l tamoxifen or toremifene did not differ significantly (0.9–1.4 nmol/l, $P > 0.5$). The dose-dependent effects of tamoxifen and toremifene on vinblastine IC_{50} values are compared in Fig. 6.

A range of wild type human breast and lung cancer cell lines

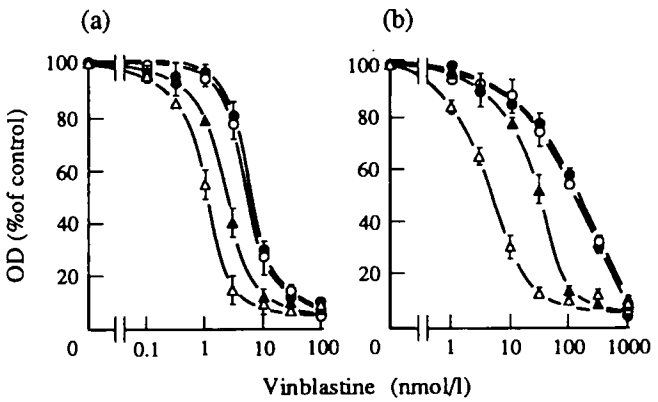

Fig. 3. Effect of 0 (●), 1 (○), 5 (▲) and 10 μmol/l (△) tamoxifen on vinblastine toxicity to (a) CHO-K1 and (b) CHO-K1^{Adr} cells. Results are from one representative experiment ± S.D. calculated from four replicates; where not shown, error bars fall within the area of plot symbols.

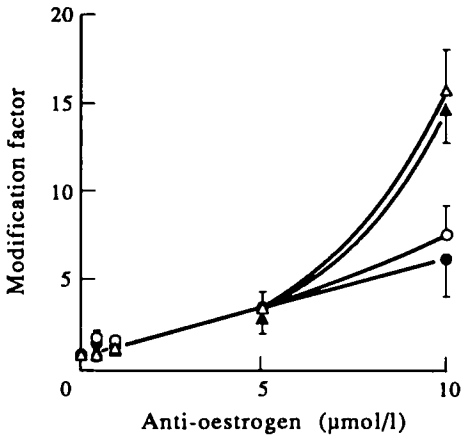

Fig. 4. Effect of tamoxifen (closed symbols) and toremifene (open symbols) on vinblastine toxicity to CHO-K1 (●, ○) and CHO-K1^{Adr} cells (▲, △). Modification of vinblastine toxicity is expressed as a modification factor (MF; ratio of vinblastine IC_{50} values determined in the absence and presence of modifier). Results are mean MF calculated from six identical experiments ± S.E.M.

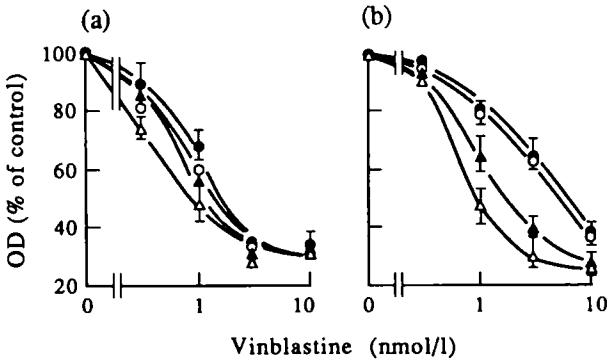

Fig. 5. Effect of 0 (●), 1 (○), 5 (▲) and 10 μmol/l (△) tamoxifen on vinblastine toxicity to (a) wild type S1 and (b) *mdr*1-transfected S1/1.1 cells. Results are from one representative experiment ± S.D. calculated from four replicates; where not shown, error bars fall within the area of plot symbols.

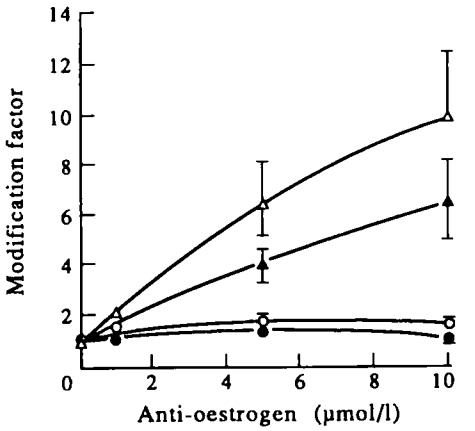

Fig. 6. Effect of tamoxifen (closed symbols) and toremifene (open symbols) on vinblastine toxicity to S1 (●, ○) and S1/1.1 cells (▲, △). Modification of vinblastine toxicity is expressed as a modification factor (ratio of vinblastine IC_{50} values determined in the absence and presence of modifier). Results are mean MF calculated from six identical experiments ± S.E.M.

showed a 6-fold range in sensitivity to vinblastine. Table 3 lists IC_{50} values for vinblastine determined in the absence and presence of tamoxifen and toremifene; no modification was observed.

Resistance to AMSA was observed in two cell lines, MCF-7^{Adr} (12-fold) and CHO-$K1^{Adr}$ (5-fold), but was not modified by tamoxifen of toremifene. Likewise, no modulation of melphalan toxicity was observed although moderate resistance to the drug was observed in MCF-7^{Adr} (4-fold) and CHO-$K1^{Adr}$ cells (5-fold), relative to parental cells. Sensitivity to cisplatin was also assessed using the panel of breast and lung cancer cell lines. Despite a wide variation in sensitivity (34-fold), no modification of drug toxicity to any cell line by its MNC of tamoxifen or toremifene occurred.

DISCUSSION

Anti-oestrogens substantially enhanced vinblastine toxicity to a range of MDR-positive, ER-negative cell lines, although drug-sensitive wild type breast and lung cancer cell lines were unaffected. Anti-oestrogen-sensitive resistance to vinblastine therefore correlated with Pgp overexpression, and modification of MDR was clearly not an anti-oestrogenic effect. Vinblastine resistance was completely abolished in *mdr*1 transfected S1/1.1 cells, indicating that tamoxifen and toremifene can fully reverse Pgp-mediated resistance. Vinblastine toxicity to wild type CHO-K1 cells was also enhanced, reflecting the intrinsic drug resistance characteristic of wild type CHO cell lines which is sensitive to MDR-modulating agents [23]. Tamoxifen, toremifene and their 4-hydroxy metabolites substantially modified vinblastine toxicity, and were more effective than the *N*-desmethyl derivatives.

S1/1.1 cells have over 100-fold greater levels of *mdr*1 than parental S1 cells, but were only 5-fold resistant to vinblastine. Transfection with the *mdr*1 gene has been demonstrated to confer the MDR phenotype without generating high degrees of resistance [24], suggesting that overexpression of Pgp alone is insufficient to account for the extreme resistance of cell lines chronically exposed to drugs. The inability of anti-oestrogens to affect AMSA and melphalan toxicity, or to fully reverse vinblastine resistance in highly resistant MCF-7^{Adr} and CHO-$K1^{Adr}$ cells, also indicates the presence of additional resistance mechanisms.

Tamoxifen and toremifene completely reversed Pgp-mediated vinblastine resistance, but the mechanism by which this occurs is not fully understood. Unlike other MDR modulators (e.g. verapamil), tamoxifen is though not to inhibit drug efflux through competitive binding to Pgp [4]. There is increasing evidence that Pgp is phosphorylated and activated by protein kinase C (PKC), which is overexpressed in MDR-positive cells [25]. Modification of PKC activity has been shown to alter cellular levels of, and sensitivity to, doxorubicin and vincristine [26, 27]. Tamoxifen and its metabolites inhibit PKC [28] and may, therefore, reverse Pgp-mediated resistance indirectly.

The panel of cell lines displayed a 34-fold range in sensitivity to cisplatin. Resistance was clearly independent of Pgp-overexpression, and could not be modified by anti-oestrogens. This is in contrast to the results of a recent study [29], describing enhancement of cisplatin toxicity towards a wild type melanoma cell line (T-289) and its cisplatin-resistant subline by clinically achievable concentrations of tamoxifen. Anti-oestrogen modulation of cisplatin toxicity may, therefore, be a tissue-specific effect.

In summary, Pgp-mediated resistance to vinblastine was completely reversed by clinically achievable concentrations of anti-oestrogens. The MDR-modulating activity of the major metabolites of tamoxifen and toremifene is highly significant as these compounds accumulate within tissues to levels demonstrated to modify MDR *in vitro*. Treatment with anti-oestrogens or vinblastine alone has limited activity against renal carcinoma, a tumour type displaying a very high degree of intrinsic Pgp-mediated drug resistance [5]. The ability of tamoxifen and toremifene to reverse vinblastine resistance makes them prime candidates for the therapy of renal carcinoma in combination with this drug. Such a clinical trial, evaluating combined toremifene and vinblastine treatment, is currently in progress.

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Potentiation of Tumour Necrosis Factor-mediated Cell Killing by VP16 on Human Ovarian Cancer Cell Lines. *In vitro* Results and Clinical Implications

Monica Valenti, Guido Cimoli, Silvio Parodi, Gabriella Lucia Mariani, Marco Venturini, PierFranco Conte and Patrizia Russo

Synergism between recombinant human tumour necrosis factor (rHuTNF) and DNA topoisomerase II inhibitor VP16 during the killing of cells has been studied in six human ovarian cancer cell lines (A2774, A2780, SW626, IGROV-1, SKOV3, Pa1) and a cervical carcinoma cell line (Me180). Studies were performed using an assay of colony formation inhibition (drug treatment for 1 h) and a growth inhibition assay (continuous exposure for 20 h). Concomitant treatment of cells with VP16+rHuTNF enhanced cell killing in all the cell lines tested—an effect observed in both short- and long-term cytotoxicity assays. This study suggests that the activity of VP16 in ovarian cancer cell lines might be enhanced by rHuTNF in *in vitro* models.
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INTRODUCTION

TUMOUR NECROSIS FACTOR (TNF) was originally described as a polypeptide producing acute and haemorrhagic necrosis of tumour nodules 24–28 h after local or systemic injection in experimental animals [1]. However, this cytokine has a broad spectrum of biological activities including osteolysis, collagenase secretion and angiogenesis [2, 3]: it also plays an important role in the pathophysiology of such diverse conditions as cachexia, endotoxaemia, malaria, arthritis, alcoholic hepatitis and acute respiratory distress syndrome [4–7]. *In vitro* studies indicate both cytostatic and cytotoxic effects against a wide range of human tumour cells [8, 9]. However, TNF is cytotoxic in less than one-third of cell lines tested [10].

A synergistic cytotoxic effect has been reported in cell lines exposed to combinations of TNF and DNA topoisomerase II-targeted anti-cancer drugs, such as etoposide (VP16) and mitoxantrone, *in vitro* [11–14] and *in vivo* [15–17]. In contrast to

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