secretion in malignant cells of the gastrointestinal tract but not in breast carcinoma may further allow the speculation that a common regulatory pathway exists for pS2 and hSP in these steroid hormone receptor negative cells.

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Circumvention of Doxorubicin Resistance in Multi-drug Resistant Human Leukaemia and Lung Cancer Cells by the Pure Antioestrogen ICI 164384

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ICI 164384, a new steroidal antioestrogen, entirely devoid of oestrogenic activity, modulates doxorubicin resistance *in vitro*. At non-cytotoxic concentrations, ICI 164384 potentiated the cytotoxicity of doxorubicin in a dose-dependent manner in both the classical multi-drug resistant (MDR) human leukaemia cell lines CEM/VLB 100 and CEM/VLB 1000 and the human small cell lung cancer cell line H69 LX4. ICI 164384 had no effect on the two respective parental cell lines, CEM/CCRF and H69 P. None of these cell lines expressed the oestrogen receptor. In comparative studies at concentrations ranging from 1.25 to 10 μmol/l, ICI 164384 was significantly more effective (1.2–6-fold) than tamoxifen in reducing the IC_{50} of doxorubicin in the CEM/VLB 100 line. In resistant cells, ICI 164384 increased ^{3}H-daunomycin accumulation in a dose-dependent manner and was significantly more effective than tamoxifen at concentrations ranging from 2.5 to 10 μmol/l. ICI 164384 reduced the efflux of daunomycin from resistant cells more effectively than tamoxifen. These studies suggest that ICI 164384 is an effective modulator of MDR.
Eur J Cancer, Vol. 27, No. 6, pp. 773–777, 1991

INTRODUCTION

IT IS widely accepted that multi-drug resistance (MDR), described in several tumour types [1] and numerous cell lines [2–4], is a major obstacle in cancer chemotherapy [5]. Based on *in vitro* studies, the classical MDR phenotype may be defined by the cross-resistance of cells to a number of structurally unrelated cytotoxic drugs [3] and the increased expression of P-glycoprotein [6, 7]. Although the exact function of this membrane protein is unknown, it is thought to be responsible for the reduced cellular accumulation of drugs in resistant cells [8, 9] as a result of increased energy-dependant drug efflux [10, 11].

A number of biochemical modulators capable of reducing the level of resistance in tumour cell lines have been reported. These include a diverse group of structurally unrelated agents such as cyclosporin [12, 13], calcium channel blockers [14] and antioestrogens such [15] as tamoxifen.

Tamoxifen, a non-steroidal antioestrogen, was first noted to modulate the resistance of P388/ADR, a doxorubicin-resistant murine leukaemia cell line [15]. More recently, tamoxifen has also been shown to increase the drug sensitivity of a human doxorubicin-resistant breast cancer line [16]. Although its mechanism of action remains unclear, early studies suggested that

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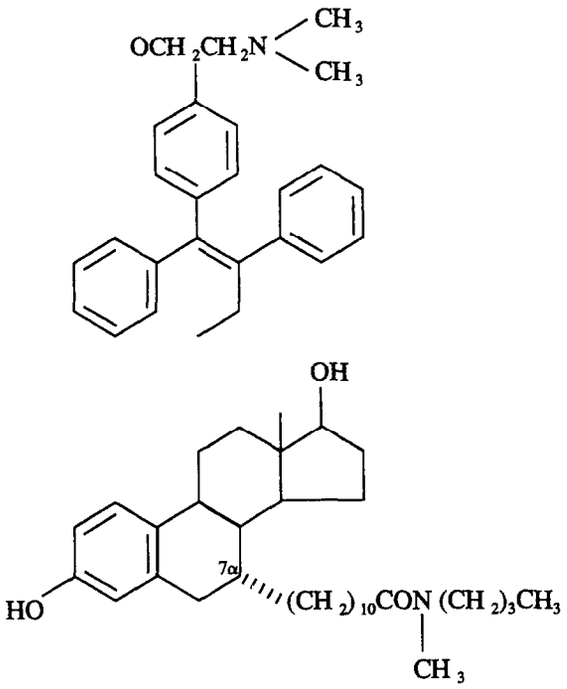

Fig. 1. Chemical structures of the non-steroidal antioestrogen, tamoxifen (upper) and the steroid antioestrogen, ICI 164384 (lower).

tamoxifen was not acting as a biochemical modulator by oestrogen-mediated regulation of tumour cell growth [15], but due to inhibition of drug transport [17]. However, more recent studies have suggested that its mechanism of action was related to inhibition of protein kinase C [18, 19] and not increased drug accumulation [16].

ICI 164384, a new, pure antioestrogen that differs from triphenylethylene antioestrogens such as tamoxifen (Fig. 1) in both structure and biological activity [20], is thought to act via the oestrogen receptor and independently of protein kinase C. Tamoxifen has been shown to inhibit the enzymatic activity of the B_1 isoform of protein kinase C with an IC_{50} of 15 μmol/l [21] but ICI 164384 had no activity in this assay (S. Rotenberg, New York).

We have compared the effects of ICI 164384 on the MDR phenotype with those of tamoxifen and verapamil.

MATERIALS AND METHODS

Cell lines

The following human tumour cell lines were used in these experiments.

The moderately vinblastine resistant, CEM/VLB 100, and highly resistant, CEM/VLB 1000, lines are variants of the parental cell line CEM/CCRF, originally derived from a patient with a T cell lymphoblastic leukaemia [22]. The two resistant lines, maintained in the presence of sublethal concentrations of vinblastine (0.01 and 0.1 μg/ml, respectively) represent classic MDR cell lines exhibiting cross-resistance to doxorubicin and enhanced expression of P glycoprotein proportional to the degree of drug resistance [23].

H69 LX4 is a doxorubicin-resistant variant of the anthracycline-sensitive, human small cell lung cancer line, H69 P. H69 LX4 overexpressed P glycoprotein [24] and was maintained in the presence of 0.4 μg/ml doxorubicin [25].

The lines were generously provided by Dr D.R. Bell (Sydney, Australia) and Dr P.R. Twentyman (Cambridge, UK) respectively. All cell lines used in the studies were tested for oestrogen receptor using the immunoperoxidase technique [26], a method shown to correlate highly with standard binding assays [27]. Neither cell line expressed the oestrogen receptor.

Materials

Doxorubicin, verapamil and tamoxifen were obtained commercially from Farmitalia, Schering, and Sigma, respectively. ICI 164384 was kindly donated by ICI Pharmaceuticals, Macclesfield, UK. Tamoxifen and ICI 164384 were dissolved initially in absolute alcohol to give stock solutions of 2.6 and 1.9 mmol/l respectively and stored at −20°C. ^{3}H-thymidine (Amersham) was used at a concentration of 1 μCi/ml (specific activity 83 Ci/mmol). ^{3}H-daunomycin (specific activity 1.9 Ci/mmol) was purchased from New England Nuclear, RPMI 1640 was purchased as a powder (Gibco) and supplemented with 10% fetal calf serum (Flow), gentamicin (80 μg/ml), minocycline (1 μg/ml), HEPES (20 mmol/l), sodium bicarbonate (0.21%) and glutamine (0.8 mmol/l).

Assays

^{3}H-thymidine incorporation assay. The ^{3}H-thymidine assay has been shown previously to correlate with the results of clonogenic test systems [28]. It has also been shown to correlate with assays using cell number as a measure of cell growth [29].

Table 1. The effect of ICI 164384 and tamoxifen on modulating doxorubicin resistance in three resistant cell lines, CEM/VLB100, CEM/VLB1000, H69/LX4 and their parental cell lines CEM/CCRF and H69 P

	Doxorubicin IC_{50} (μg/ml)*†								
	ICI 164384 (μmol/l)					Tamoxifen (μmol/l)			
Cell lines	0	1.25	2.5	5	10	1.25	2.5	5	10
CEM/CCRF	0.80	0.84	0.86	—	—	0.76	0.82	0.79	0.80
CEM/VLB100	84.0	58.0	25.8	5.4	1.6	72.5	60.0	23.5	9.6
CEM/VLB1000	133.0	77.4	46.0	20.0	6.2	84.5	69.6	37.0	14.8
H69	0.25	0.31	0.10	—	—	0.30	0.28	0.32	—
H69/LX4	10.1	2.30	1.48	0.45	—	2.70	2.30	1.43	—

*These results are representative of similar experiments from which identical conclusions were drawn.
†Doxorubicin IC_{50} in the absence or presence of various concentrations of each modulator. The IC_{50} level is derived from a graph relating ^{3}H-thymidine incorporation to drug concentration.

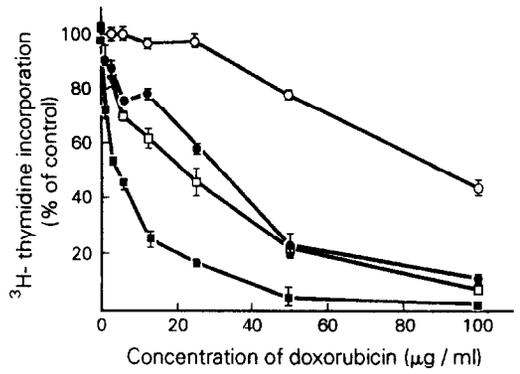

Fig. 2a. Change in the percentage incorporation of ^{3}H-thymidine in the moderately resistant cell line CEM/VLB100 as a function of increasing concentration of doxorubicin in absence (○—○) or presence of 5 μmol/l tamoxifen (●—●); verapamil (□—□) or ICI 164384 (■—■). Error bars represent the standard deviation in triplicate experiments.

Cell numbers were counted using a haemocytometer and the concentration adjusted to 5×10^6/ml; viability was determined using the trypan blue exclusion technique. The assay was performed in 96-well tissue culture plates (Flow). For CEM cells, 5×10^5 cells were incubated overnight at 37°C in 5% CO_2 in air with varying concentrations of doxorubicin and the appropriate modulators.

For H69 cells, 2.5×10^5 cells were placed into 96-well plates and resuspended overnight to enable cells to recover from the preparative process requiring the isolation of single cells. Drugs were added on the following day. After an overnight incubation with various concentrations and combinations of doxorubicin and/or the modulators, the cells were pulsed for four hours with ^{3}H-thymidine (1 μCi/ml). The cells were then harvested onto glass fibre filters (Titertek) using an automated cell harvestor (Titertek).

The filters were dried, dissolved in 5 ml of scintillant (Beckman's solution) and radioactivity was measured in a beta counter. All assays were performed in triplicate and under sterile conditions.

The IC_{50} was defined as the concentration of any one agent that inhibited the incorporation of ^{3}H-thymidine by 50% compared to controls.

Cell growth assay. After determining cell viability and adjusting the final concentration of cells in 12-well plates (Flow) to 1×10^5 cells/well, varying concentrations of doxorubicin and

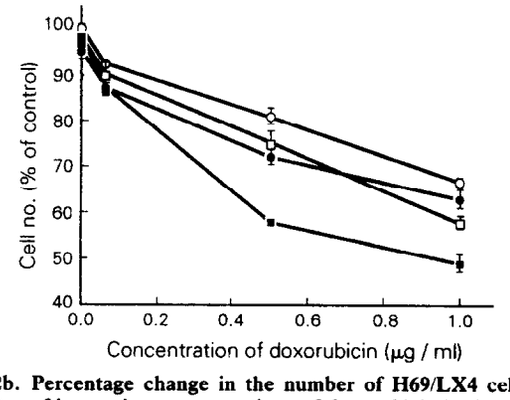

Fig. 2b. Percentage change in the number of H69/LX4 cells as a function of increasing concentrations of doxorubicin in the absence (○—○) or presence of 1 μmol/l tamoxifen (●—●); verapamil (□—□) or ICI 164384 (■—■).

or the biochemical modulators were added to each well. The cells were incubated in humidified chambers at 37°C for 4 days and counted using an automated Coulter counter. Results are expressed as the percentage change in cell number of the drug-exposed cells relative to the change in control cells. All assays were carried out in triplicate and under sterile conditions.

Drug accumulation and drug efflux. Cells were counted and viability determined as mentioned above. For drug accumulation studies, 7.5×10^5 cells were placed in 96-well tissue culture plates and incubated at 37°C in 5% CO_2 in air with varying concentrations of ICI 164384 or tamoxifen and tracer amounts of ^{3}H-daunomycin (final concentration, 0.5 μCi/ml, 0.05 μg/ml). The cells were then harvested at designated times, in the same manner as described above. For drug efflux studies, cells were incubated with tracer quantities of ^{3}H-daunomycin in 96-well plates for 60 min at 37°C before plates were centrifuged at 4°C and cells washed rapidly 3 times in cold RPMI 1640. The cells were then reincubated with 10 μmol/l ICI 164384, tamoxifen or verapamil in the presence of a gross excess of unlabelled doxorubicin (25 μg/ml). At designated time points, cells were harvested and radioactivity determined as previously described. All assays were performed in triplicate. We have previously demonstrated that the accumulation of ^{3}H-daunomycin in MDR vinblastine resistant CEM cells varies relative to the degree of drug resistance [30].

Statistics

The effects of ICI 164384 and tamoxifen on drug accumulation and efflux were compared using an analysis of covariance. This method was used to compare the slopes of the lines as well as individual points obtained in the presence of the two modulators.

RESULTS

Effect of ICI 164384 *and tamoxifen on modulating doxorubicin resistance*

The effects of various concentrations of ICI 164384 and tamoxifen on reversing doxorubicin resistance in the CEM/VLB 100 and CEM/VLB 1000 line are illustrated in Table 1. At the concentrations of tamoxifen or ICI 164384 shown, there was an obvious decrease in expression of the MDR phenotype. Similar modulation was seen in the H69 LX4 line (Table 1). No effect of either modulator was seen in the parental cell lines. The IC_{50} values were derived from the appropriate dose–response curves.

A dose–response effect was observed over a wide range of concentrations of the two modulators. However, as the concentrations of ICI 164384 or tamoxifen were increased, ICI 164384 appeared to be a more effective modulator of drug resistance (Table 1). Thus, comparing relative resistance in the presence of either reagent at concentrations ranging from 1.25 to 10 μmol/l, there was a significantly greater effect obtained with ICI 164384 compared to tamoxifen in the three resistant cell lines ($P=0.015$). For example in the CEM/VLB 100 cells, the doxorubicin IC_{50} of the resistant line was less than 2-fold greater than the sensitive line in the presence of 10 μmol/l of ICI 164384 compared to 12-fold with 10 μmol/l tamoxifen. In both assays, at the concentrations used, neither ICI 164384 nor tamoxifen inhibited thymidine incorporation or cell growth by more than 10%. In addition, all experiments were performed with simultaneous alcohol controls at equivalent concentrations to those used for the modulators.

These experiments were also performed using verapamil, the prototype biochemical modulator of the MDR phenotype (Fig.

*Table 2. The effect of ICI 164384 or tamoxifen on ^{3}H-daunomycin accumulation in the CEM/VLB 100 cell line**

	Concentration (μmol/l)			
Modulators	0	2.5	5	10
Tamoxifen	18.9(1.3)	20.2(0.9)	24.6(1.8)†‡	42.6(3.2)†‡
ICI 164384§	20.3(1.7)	31.0(1.3)†‡	41.4(2.7)†‡	58.1(2)†‡

*Drug accumulation was determined as described in the Methods section. Results are expressed as counts per minutes (S.D.) × 10^{-3} of intracellular ^{3}H-daunomycin.

†In the presence of modulator, the accumulation of ^{3}H-daunomycin was significantly higher ($P < 0.02$) than observed in the absence of this agent (based on a two sample t test).

‡The mean accumulation at a given concentration of modulator was significantly higher than the effect of the adjacent lower concentration of modulator ($P < 0.05$–0.003).

§Using analysis of covariance, the accumulation of ^{3}H-daunomycin in the presence of each concentration of ICI 164384 was significantly higher than the accumulation of ^{3}H-daunomycin in the presence of equivalent concentrations of tamoxifen ($P < 0.001$).

2). At a concentration of 5 μmol/l verapamil, tamoxifen or ICI 164384, verapamil and tamoxifen demonstrated a similar effect on the modulation of doxorubicin resistance in the CEM/VLB 100 cells. ICI 164384 was the most effective ($P < 0.0001$) of the 3 reagents (Fig. 2a). In a cell growth assay, a similar relative effect of the three modulators was also demonstrated ($P < 0.005$) in the H69 LX4 cell ine (Fig. 2b) using lower concentrations of the three agents.

Drug accumulation studies

Potential mechanisms by which ICI 164384 and tamoxifen modulated drug resistance were investigated by performing drug accumulation studies in the resistant and sensitive CEM sublines. Neither modulator had any effect on ^{3}H-daunomycin accumulation in the parental CEM/CCRF cells (data not shown). In the CEM/VLB 100 line, both ICI 164384 and tamoxifen dramatically increased the accumulation of radiolabelled daunomycin measured after incubation with tracer quantities of drug for 150 min (Table 2). At concentrations of 2.5 μmol/l ICI 164384 ($P < 0.002$), and 5 μmol/l tamoxifen ($P < 0.02$) drug accumulation was significantly increased when compared to untreated controls exposed to the tracer alone. Not only was there a significantly increased effect on drug accumulation with increasing concentrations of either modulator ($P < 0.05$–0.003) but ICI 164384 appeared to be significantly more effective than tamoxifen at equivalent concentrations of each drug ($P < 0.001$).

The accumulation of ^{3}H-daunomycin at various time points in the presence of a fixed concentration (10 μmol/l) of verapamil, tamoxifen, or ICI 164384 is represented in Fig. 3. Verapamil and tamoxifen similarly significantly increased the accumulation of ^{3}H-daunomycin. However, using analysis of covariance to compare the curves, ICI 164384 seemed the most effective of the three agents tested ($P < 0.001$).

Further studies were undertaken to compare the effect of the three modulators on the efflux of ^{3}H-daunomycin (Fig. 4). Using analysis of covariance, tamoxifen, verapamil and ICI 164384 (at a concentration of 10 μmol/l) each significantly inhibited ^{3}H-daunomycin efflux ($P < 0.001$) compared to the control (absence of modulator). In these studies, verapamil was the most

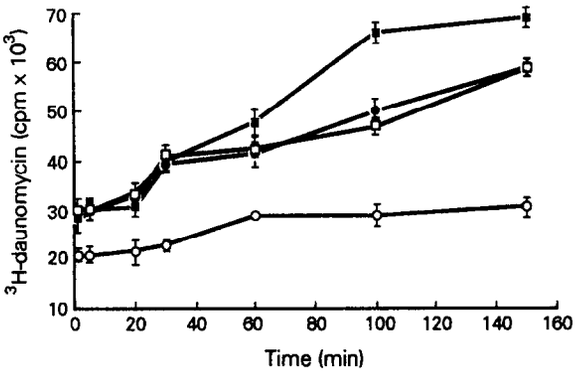

Fig. 3. The increase in the intracellular levels of ^{3}H-daunomycin in the moderately resistant cell line CEM/VLB100 in the absence (○—○) or presence of 10 μmol/l of tamoxifen (●—●); verapamil (□—□); or ICI 164384 (■—■). Error bars represent the standard deviation in triplicate experiments.

effective agent ($P < 0.001$) but both verapamil and ICI 164384 were significantly more active than tamoxifen ($P < 0.001$).

DISCUSSION

This study has demonstrated that the new steroidal antioestrogen ICI 164384 is an efficient modulator of multi-drug resistance in MDR cell lines. It acts in a dose-dependent manner in resistant lines, but had no effect on the growth (measured by either standard growth assays or by ^{3}H-thymidine incorporation) of parental, drug sensitive cell lines. At concentrations ranging from 1.25 to 10 μmol/l, ICI 164384 is significantly more efficient than tamoxifen in reducing the IC_{50} of doxorubicin in the CEM/VLB 100 and CEM/VLB 1000 lines. In the H69 LX4 small cell lung cancer line, ICI 164384 is also significantly more efficient than tamoxifen. Similar experiments were performed using verapamil, the prototype biochemical modulator of MDR. Tamoxifen appeared to be as effective as verapamil in reducing the IC_{50} of doxorubicin in both the CEM/VLB 100 and H69 LX4 lines but ICI 164384 was the superior agent.

Verapamil is thought to modulate the MDR phenotype by promoting drug accumulation and inhibiting drug efflux [31, 32]. Similar studies were performed using tamoxifen and ICI 164384. Our studies confirm that both tamoxifen and ICI 164384 alter drug accumulation in multi-drug resistant cells by increasing drug uptake and reducing the efflux of radiolabelled

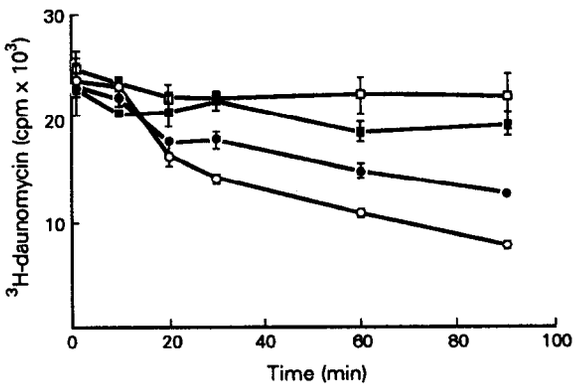

Fig. 4. The decrease in the intracellular levels of ^{3}H-daunomycin in moderately resistant cell line CEM/VLB100 in absence (○—○); or presence of 10 μmol/l of tamoxifen (●—●); ICI 164384 (■—■); or verapamil (□—□). Error bars represent the standard deviation in triplicate experiments.

daunomycin. However, ICI 164384 is the most efficient of the three modulators with respect to drug uptake and verapamil the most active in reducing drug efflux (Figs 3,4).

As discussed previously, the mechanisms by which tamoxifen modulates the MDR phenotype remains uncertain. Initial reports suggested that tamoxifen circumvented daunomycin resistance by inhibiting the outward transport of daunomycin, presumably by a direct membrane interaction [17], leading to increased drug accumulation in drug-resistant cells [16]. This theory was strengthened by the fact that tamoxifen only reversed drug resistance in MDR cell lines [15–17] and not in atypical MDR tumour cells characterised by the failure to overexpress P-glycoprotein [33]. Furthermore, Naito *et al.* [34] recently observed that steroid hormones inhibited the specific binding of vinca alkaloids to P-glycoprotein.

In contrast, it has recently been suggested that the mechanism of action of tamoxifen as a biochemical modulator was due to stimulation of protein kinase C [19] and independent of drug accumulation [16]. Our data strongly support the former model [17] as all three modulators tested, verapamil, tamoxifen and ICI 164384 enhance drug uptake and reduce drug efflux in three classical MDR cell lines, none of which express as the oestrogen receptor. Our study suggests that both tamoxifen and ICI 164384 modulate the MDR phenotype by reducing the cellular accumulation of cytotoxics by a membrane interaction in MDR cell lines. Further clarification of the mechanism of action of tamoxifen and ICI 164384 will depend on confirmation of data suggesting that ICI 164384 has no effect on intracellular signal processes such as protein kinase C.

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