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Differential effects of estrogen, tamoxifen and the pure antiestrogen ICI 182,780 in human drug-resistant leukemia cell lines

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Abstract. ICI 182,780, a potent, new steroidal antiestrogen without apparent agonist activity, appears to be a potent modulator of the classic multidrug resistance (MDR) phenotype in the CEM/A7, CEM/VLB$_{100}$ and K562/VIN100 MDR cell lines. This reagent had no effect on the respective parental CCRF-CEM and K562 cell lines. The use of 1.25 μ*M* ICI 182,780 resulted in a 6- to 7-fold decrease in doxorubicin resistance in the CEM/A7 and CEM/VLB$_{100}$ cell lines. A dose-response effect was observed at ICI 182,780 concentrations of up to 5 μ*M*. As compared with tamoxifen (TAM), ICI 182,780 was 2 and 4 times more effective in the K562/VIN100 and CEM/A7 cell lines, respectively. ICI 182,780 at 0.625 μ*M* increased [^{3}H]-daunomycin uptake (P <0.0001) as effectively as 5 μ*M* TAM in the resistant CEM/A7 line. Drug-efflux studies showed that 5 μ*M* ICI 182,780 significantly decreased drug efflux as compared with 5 μ*M* TAM (P <0.0001). Estradiol (EST) at 10 μ*M* increased doxorubicin resistance by 1.2–1.3 times in the CEM/A7 and CEM/VLB$_{100}$ cell lines and significantly decreased drug accumulation (P = 0.002) and retention (P <0.001) in the CEM/A7 cell line. However, the addition of 10 μ*M* EST to 1–2 μ*M* ICI 182,780 did not inhibit the ability of ICI 182,780 to modulate doxorubicin resistance in the two resistant cell lines. Using reverse-phase high-performance liquid chromatography (HPLC) to measure lipophilicity, we found no apparent association between the ability of ICI 182,780, TAM or EST to modulate resistance and their relative hydrophobicity.

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Introduction

Drug resistance is a major factor limiting the effectiveness of currently available cytotoxic drugs in the treatment of human tumours [17]. Based on studies conducted in vitro, this clinical phenomenon is thought to have principally a genetic basis [9, 17]. Studies in a large number of cell lines have defined the classic multidrug-resistant (MDR) phenotype, characterised by cross-resistance to several structurally and functionally unrelated cytotoxic drugs [1] and the expression of P-glycoprotein [2, 14]. The MDR phenotype can be partially reversed by a wide range of structurally diverse compounds such as verapamil [28] and cyclosporin A [11, 26] at concentrations that are not toxic to cells expressing this phenotype. P-glycoprotein, a 170-kDa membrane phosphoglycoprotein encoded by the *mdr*1 gene [4], is thought to function as a drug-efflux pump in MDR cells [21].

A number of antiestrogens have been shown to reduce the resistance of MDR cell lines to the growth-inhibitory effects of cytotoxic agents. Thus, both non-steroidal, partial agonists such as tamoxifen [20] and toremifene [5] and the steroidal pure antiestrogen ICI 164,384 modulate the MDR phenotype [13]. Unlike the classic hormonal actions of these agents, this effect does not depend on the presence of the estrogen receptor (ER) in MDR cells. Initially, it was thought that tamoxifen may act on MDR via protein kinase C (PKC) in ER-negative cells [10, 18], but since ICI 164,384 is more effective than tamoxifen and does not inhibit PKC [23], alternative mechanisms need to be considered.

In an attempt to elucidate the potential mechanism of action of these antiestrogens in modulating the MDR phenotype in ER-negative cell lines, the relative lipophilicity of these agents was specifically examined in view of a number of reports suggesting that the hydrophobicity of both steroids and forskolins is important in determining their effect on the inhibition of drug binding to P-glycoprotein [30, 33].

This report describes studies with a novel antiestrogen, ICI 182,780. This reagent [31], a pure antiestrogen, is more

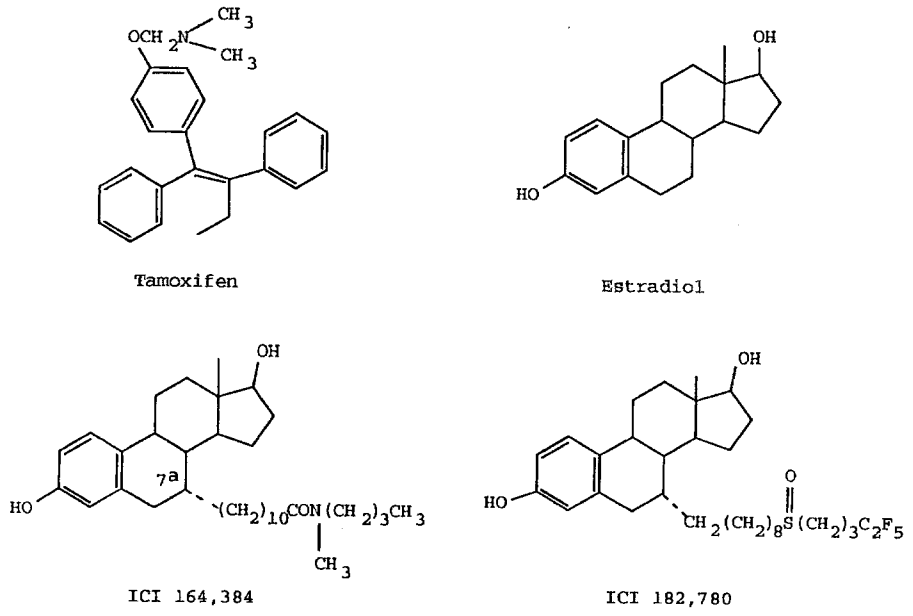

Fig. 1. Chemical structures of estradiol, the pure antiestrogens ICI 182,780 and ICI 164,384 and the non-steroidal antiestrogen tamoxifen

potent than ICI 164,384 (both are 7α-alkyl analogues of 17β-estradiol; Fig. 1) in hormone-sensitive cells and, consequently, is a candidate for clinical evaluation in the treatment of breast cancer. In view of the observation that a significant number of breast carcinomas express P-glycoprotein (Pgp) de novo or after exposure to cytotoxic drugs [15, 24, 29, 32], a clearer understanding of the mechanism of action of ICI 182,780 in modulating the function of Pgp may enhance the therapeutic potential of this class of drugs.

Materials and methods

Cell lines

The parental human leukemia cell line CCRF-CEM was originally derived from a patient with T-cell lymphoblastic leukemia [7]. Two resistant variants of this cell line, CEM/A7 and CEM/VLB$_{100}$, of low-level and moderate resistance, respectively, were used in these experiments. The CEM/A7 cell line is a classic MDR cell line with decreased anthracycline accumulation, overexpression of mdr1 mRNA and increased Pgp expression. However, the *mdr*1 gene is not amplified (Zalcberg et al., manuscript in preparation). The moderately resistant variant CEM/VLB$_{100}$ has previously been characterised [2]. This line is similar to the CEM/A7 line, although it does have amplification of the *mdr*1 gene.

The two resistant cell lines were maintained in the presence of sub-lethal concentrations of doxorubicin (0.12 μ*M*) and vinblastine (0.01 μ*M*), respectively. Cells were maintained in a humidified chamber containing 5% CO_2 (in air) and were regularly screened for mycoplasma contamination (and discarded accordingly). The multidrug-resistant K562/VIN100 line, an MDR variant of the K562 erythroleukemia line, was also used in these studies. Both the MDR variant and the parental line were provided by Prof. M. Van Der Weyden (Melbourne, Australia).

Materials

Doxorubicin and TAM were obtained commercially from Farmitalia Carlo Erba (Australia) and Sigma (St. Louis, Mo.), respectively. ICI 182,780 and ICI 164,384 were kindly donated by ICI Pharmaceuticals (England). ICI 182,780, ICI 164,384, TAM and EST were initially dissolved in absolute alcohol to give stock solutions of 3.3, 1.9, 2.6 and 7.3 m*M*, respectively, and were stored at –20° C. [^{3}H]-Thymidine (Amersham) was used at a concentration of 1 μCi/ml (specific activity, 89 Ci/mmol). [^{3}H]-Daunomycin (specific activity, 2.3 Ci/mmol) was purchased from New England Nuclear (Australia). RPMI 1640 was purchased as a powder (Gibco Laboratories) and supplemented with 10% fetal calf serum (Flow Laboratories, Australia), gentamicin (80 μg/ml), minocycline (1 μg/ml), HEPES (20 m*M*), sodium bicarbonate (0.21%) and glutamine (0.8 m*M*).

[^{3}H]-Thymidine incorporation assay

The nucleotide incorporation assay has previously been shown to correlate with the results of clonogenic test systems [27]. It has also been shown to correlate with assays using cell number as a measure of cell growth [11]. Using a hemocytometer, cell numbers were counted and the concentration was adjusted to 5 × 10^6/ml. Cell viability was determined using the trypan blue exclusion technique. Cells (5 × 10^5) were placed into 96-well tissue-culture plates and incubated overnight in varying concentrations of doxorubicin and the appropriate modulators at 37° C in 5% CO_2. After being pulsed with [^{3}H]-thymidine for 4 h on the next day, the cells were harvested with an automated cell harvester (Titertek) onto glass-fibre filters and the radioactivity was determined. All assays were performed in triplicate and under sterile conditions.

Results are expressed as the percentage of incorporation of [^{3}H]-thymidine by drug-treated cells relative to appropriate controls. The IC_{50} was defined as the concentration of drug that, relative to controls, inhibited the incorporation of [^{3}H]-thymidine by 50%. These values were determined by calculating the graphical intercepts. Relative resistance represents the ratio of the IC_{50} of doxorubicin in the resistant cells to the IC_{50} level in the parental cell line.

Cell growth assay

The sensitivity of each of the cell lines (in the presence/absence of each modulator) to doxorubicin was also determined by a standard growth inhibition assay [11]. After determination of cell viability and adjustment of the final cell number to 2 × 10^5/well in 12-well plates (Flow Laboratories), varying concentrations of doxorubicin and/or the biochemical

Table 1. Effect of ICI 182,780 on doxorubicin resistance in MDR cell lines

Cell lines	Doxorubicin IC_{50}[a] ICI 182,780 (μ*M*)				
	0	0.5	1.25	2.5	5.0
CCRF-CEM	2.9 ± 0.3	2.6 ± 0.5	2.2 ± 0.2	2.4 ± 0.3	2.2 ± 0.3
CEM/A7	82.9 ± 3.1(28)	31.4 ± 1.4(12)	11.7 ± 1.0 (5)	5.3 ± 0.9(2)	4.7 ± 1.0(2)
CEM/VLB_{100}	172.4 ± 5.7(71)	97.9 ± 2.9(38)	30.9 ± 2.6(14)	9.7 ± 2.4(5)	8.7 ± 1.2(5)
K562	1.6 ± 0.2	1.9 ± 0.2	1.6 ± 0.2	1.4 ± 0.2	–
K562/VIN100	23.4 ± 1.2(15)	8.6 ± 0.5(5)	5.2 ± 0.5(3)	4.0 ± 0.7(3)	–

Data represent mean values ± SD derived from triplicate experiments. Data in parentheses represent the relative resistance (see Materials and methods)
[a] Results are expressed as the concentration of doxorubicin (μ*M*) required to inhibit the incorporation of [^{3}H]-thymidine by 50% as compared with controls

modulators were added to each well. The cells were incubated in humidified chambers at 37° C for 3 days and counted using an automated Coulter counter. All assays were carried out in triplicate and under sterile conditions. The IC_{50} for each drug tested was determined by calculating the drug concentration required to inhibit growth relative to controls by 50%.

Drug kinetics studies

A change in [^{3}H]-daunomycin kinetics was used as a functional assay of Pgp [12].

Accumulation of [^{3}H]-daunomycin. Cells were adjusted to a concentration of 5×10^6/ml and viability was assessed with trypan blue. Cells were pipetted into 96-well plates (Flow Laboratories) to give a final number of 7.5×10^5 cells/well and were incubated at 37° C with tracer amounts of [^{3}H]-daunomycin (final concentration, 1.85×10^4 Bq/ml, 0.086 μ*M*). The cells were harvested and the radioactivity was determined as described above. All assays were performed in triplicate.

Drug efflux. Drug efflux was measured after incubation of cells with tracer quantities of [^{3}H]-daunomycin (final concentration, 1.85×10^4 Bq/ml, 0.086 μ*M*) for 60 min at 37° C. Cells were washed three times in cold RPMI 1640 containing doxorubicin (34.5 μ*M*). The labeled cells were pipetted into 96-well plates to give a final number of 7.5×10^5 cells/well and were then reincubated at 37° C in 5% CO_2 in various concentrations of ICI 182,780, TAM or 10 μ*M* EST in the presence of a gross excess of unlabeled doxorubicin (34.5 – 86.2 μ*M*). Excess cold (unlabeled) doxorubicin was used to reduce re-uptake of the isotope. At designated time points, cells were harvested in the same manner described above. All assays were performed in triplicate.

Measurement of lipophilicity

The lipophilicity of EST (66.1 μ*M*), TAM (10.8 μ*M*), ICI 182,780 (23.1 μ*M*) and ICI 164,384 (45.7 μ*M*) was measured by reverse-phase HPLC after injection of the amounts specified. The mobile phase consisted of 80% methanol and 20% buffer (10 m*M* sodium phosphate, pH 7.4) pumped at 3 ml/min. A μ Bondapak C_{18}, 10-μ*M* (8 cm × 10 mm) column was used (Waters Associates, Milford, Mass.) and the detection wavelength was 280 nm. Samples of each drug (4 – 7 m*M*) in ethanol were injected into the chromatograph. The capacity factor (K′) was measured directly from the chromatogram [3] as:

$$K' = \frac{t_r - t_0}{t_0}$$

where t_r is the retention time of the drug and t_0 is the retention time of an unretained compound.

Statistical analysis

The effects of ICI 182,780, TAM and EST on drug accumulation and efflux were analysed using analysis of covariance to test for significant differences in the pattern of drug efflux and accumulation over time. In the presence of the antiestrogens and/or EST, this method was used to compare the lines over periods of 20 – 180 min for accumulation and 15 – 90 min for efflux, adjusting for the initial value. Analyses were performed on the package GLIM. A *P* value of less than 0.05 was considered significant.

Results

Effect of ICI 182,780 and TAM in modulating doxorubicin resistance

The effect of various concentrations of ICI 182,780 in modulating doxorubicin resistance is illustrated in Table 1. As ICI 182,780 led to more than a 15% reduction in [^{3}H]-thymidine incorporation at concentrations greater than 5 μ*M*, higher doses were not used in this particular assay. A dose-response effect was demonstrated over the range of concentrations of ICI 182,780 used in these studies. ICI 182,780 had no effect on the IC_{50} of doxorubicin in the parental cell line (CCRF-CEM). With increasing concentrations of ICI 182,780 there was a dramatic decrease in the IC_{50} of doxorubicin in the two resistant cell lines. In the CEM/A7 and CEM/VLB_{100} lines, there was a 6- to 7-fold decrease in doxorubicin resistance in the presence of 1.25 μ*M* ICI 182,780. A further increase in the concentration of ICI 182,780 led to a smaller decrease in doxorubicin resistance (Table 1). Similar effects were seen in the K562/VIN100 cell line. Overall, ICI 182,780 and the related, pure antiestrogen ICI 164,384 had a similar efficacy in modulating doxorubicin resistance except in the CEM/VLB_{100} line, in which case ICI 182,780 seemed to be the more potent reagent (data not shown).

The effects of ICI 182,780 and TAM in modulating doxorubicin resistance were measured at low concentrations of both drugs (Fig. 2, Table 2). The raw data describing the effect of increasing doxorubicin concentrations in the presence of ICI 182,780 on cell survival (as determined in the [^{3}H]-thymidine assay) in the CEM/A7 line are represented in Fig. 2a. The relative effects of ICI 182,780 and TAM were also demonstrated in a [^{3}H]-thymidine incorpo-

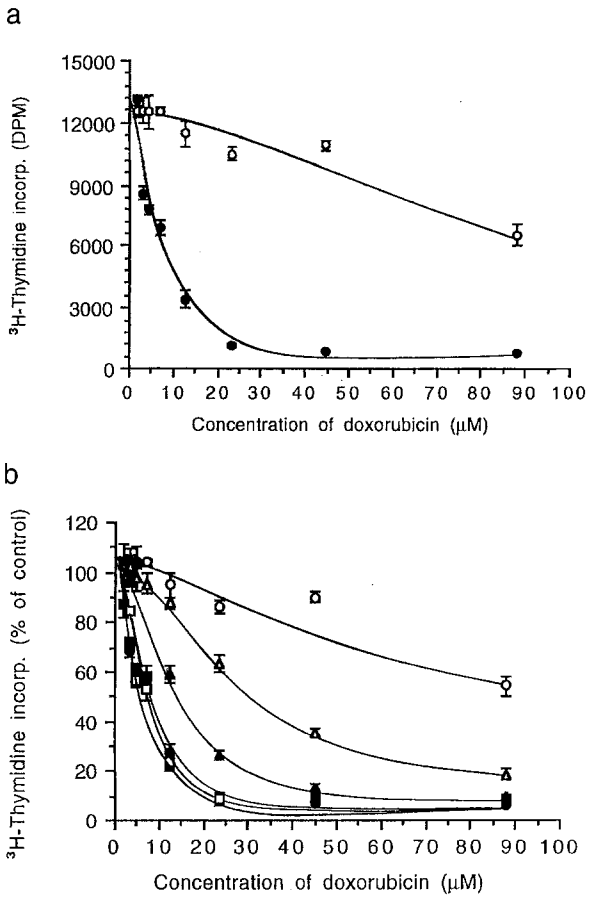

Fig. 2. a Incorporation of [^{3}H]-thymidine at different concentrations of doxorubicin in the CEM/A7 line in the absence (○) or presence of 2.5 μ*M* ICI 182,780 (●). **b** Incorporation of [^{3}H]-thymidine relative to untreated controls in the CCRF-CEM (□) and doxorubicin-resistant CEM/A7 cell line as a function of increasing concentrations of doxorubicin. The dose of ICI 182,780 was progressively increased from 0 (○) to 1.25 (▲), 2.5 (●) and 5 μ*M* (■) in the CEM/A7 line. TAM at 5 μ*M* (△) was also used in this experiment. Neither ICI 182,780 nor TAM had any effect on [^{3}H]-thymidine incorporation in the CCRF-CEM line. *Points* represent the mean value derived from triplicate experiments and *error bars*, the standard deviation

ration assay (Fig. 2b). At a concentration of 1.25 μ*M*, ICI 182,780 decreased the resistance profile of the CEM/A7 line more effectively than a 4-fold greater concentration (5 μ*M*) of TAM (Fig. 2b). Similar results were obtained in both this line and a second MDR cell line, K562/VIN100 (Table 2). In these experiments, the data were derived from a cell growth assay and, as a result, the IC_{50} levels were substantially lower than those seen previously (Table 1). However, the relative efficacy of the two modulators is clearly demonstrated. In the CEM/A7 line, a 4-fold greater concentration of TAM (4 μ*M*) relative to ICI 182,780 (1 μ*M*) was required to reduce the doxorubicin IC_{50} level to the same degree. In the K562/VIN100 cell line, ICI 182,780 appeared to be about twice as potent as TAM, with 1 μ*M* of the former compound producing an IC_{50} level similar to that produced by 2 μ*M* TAM.

Table 3. Effect of EST on the efficacy of ICI 182,780 in modulating MDR

ICI 182,780 (μ*M*)	Doxorubicin IC_{50} (μ*M*)[a]		
	CCRF-CEM	CEM/A7	CEM/VLB_{100}
0	2.8±0.2	86.6±7.1	175.9±3,4
1	2.4±0.2	15.2±1.0	32.4±2.2
2	2.6±0.3	10.3±1.4	11.9±1.4
0 + EST 10	2.9±0.2	105.3±2.0	226.7±2.6
1 + EST 10	2.6±0.2	16.9±1.7	38.4±4.1
2 + EST 10	2.2±0.3	12.6±1.7	15.3±2.2

Data represent mean values ± SD derived from triplicate experiments
[a] Doxorubicin IC_{50} levels were derived from graphs relating [^{3}H]-thymidine incorporation to increasing drug concentrations (see Materials and methods)

Effect of EST on doxorubicin resistance

In view of the similarity in structure between EST and ICI 182,780 (Fig. 1), the effect of EST in modulating doxorubicin resistance was also investigated. At concentrations as high as 10 μ*M*, EST failed to reduce doxorubicin resistance in any of the drug-resistant cell lines tested, and the addition of EST to ICI 182,780 did not have any significant effect on the ability of ICI 182,780 to modulate doxorubicin resistance in the [^{3}H]-thymidine incorporation assay (Table 3).

Table 2. Effect of ICI 182,780 and TAM on doxorubicin resistance in two MDR cell lines

Modulators (μ*M*)		Doxorubicin IC_{50} (n*M*)[a]			
		CCRF-CEM	CEM/A7	K562	K562/VIN100
ICI 182,780	0	27.6±1.6	703.4±27.8(26)	24.7±0.9	448.3±19.3(18)
	1	26.4±0.9	246.6± 7.9 (9)	22.2±0.2	229.3± 7.7(10)
	2	25.0±0.7	155.2± 7.0 (6)	23.3±0.7	163.8± 8.4 (7)
TAM	1	–	–	23.1±1.4	310.3±14.1(13)
	2	28.3±1.4	310.9± 7.8(11)	22.4±1.0	220.3± 5.5(10)
	4	27.2±1.0	237.9±11.9(9)	–	–

Data represent mean values ± SD derived from triplicate experiments. Data in parentheses represent the relative resistance (see Materials and methods)
[a] IC_{50} levels represent the concentration of doxorubicin (n*M*) required to inhibit cell proliferation by 50% as compared with controls (modulators alone)

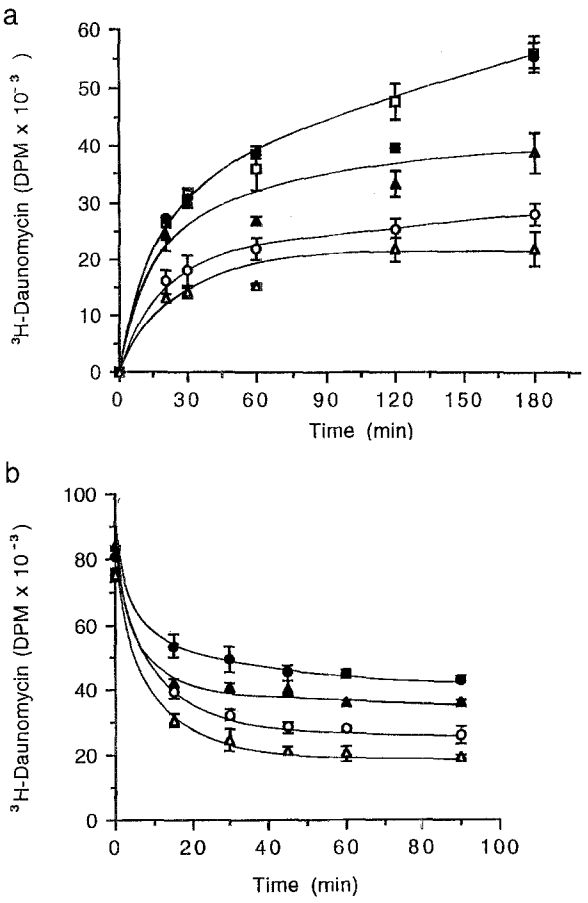

Fig. 3. a Increase in the intracellular levels of [^{3}H]-daunomycin measured in the CCRF-CEM line (□) over time. Levels detected in the CEM/A7 cell line are plotted in the absence (○) or presence of 5 μ*M* ICI 182,780 (●), 5 μ*M* TAM (▲) or 10 μ*M* EST (△). Neither ICI 182,780, TAM nor EST had any effect on drug accumulation in the sensitive parental line CCRF-CEM. *Error bars* represent the standard deviations in triplicate experiments. **b** Decrease in the intracellular levels of [^{3}H]-daunomycin measured in the CEM/A7 cell line in the absence (○) or presence of 5 μ*M* ICI 182,780 (●), 5 μ*M* TAM (▲) or 10 μ*M* EST (△). *Error bars* represent the standard deviation in triplicate experiments

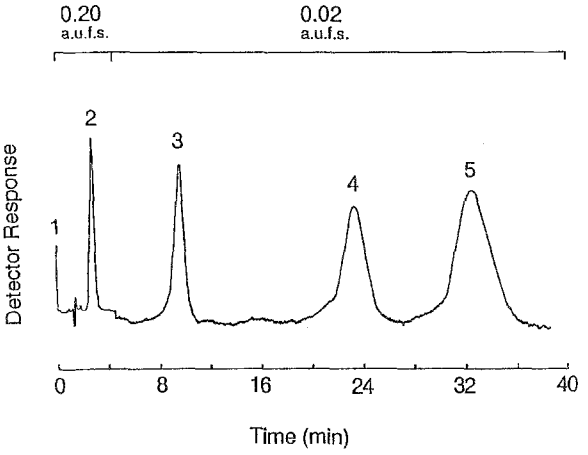

Fig. 4. HPLC chromatogram of EST and antiestrogens. The peaks are as follows: *1*, injection event; *2*, EST; *3*, ICI 182,780; *4*, ICI 164,384; and *5*, TAM. *a. u. f. s. represents the absorbance units, full scale*

Drug kinetics

In MDR cell lines, previous studies have demonstrated that decreased drug accumulation and/or increased drug efflux corresponds to the degree of drug resistance [6, 12]. The role of ICI 182,780, TAM and EST was compared in drug accumulation studies using [^{3}H]-daunomycin. Neither ICI 182,780, EST nor TAM had any effect on drug accumulation in the parental CCRF-CEM cells. However, ICI 182,780 dramatically increased [^{3}H]-daunomycin accumulation in both the CEM/A7 (Fig. 3a) and the CEM/VLB$_{100}$ (data not shown) resistant cell lines in a dose-dependent manner. At an ICI 182,780 concentration as low as 0.625 μ*M*, drug accumulation was significantly increased in the CEM/A7 line as compared with untreated controls (P <0.0001), an effect that was virtually equivalent to that seen with 5 μ*M* TAM (Fig. 3a; the two curves were virtually superimposable such that only the TAM data are shown). At a concentration of 5 μ*M*, ICI 182,780 increased drug accumulation to the level seen in the CCRF-CEM cell line (Fig. 3a). The use of 10 μ*M* EST significantly decreased drug accumulation in this cell line (P = 0.002, Fig. 3a).

Similar conclusions were drawn when drug-efflux studies were performed in the presence of ICI 182,780, TAM or EST. ICI 182,780 reduced drug efflux in a dose-dependent manner. Equimolar concentrations (5 μ*M*) of ICI 182,780 reduced drug efflux more effectively than did TAM (P <0.0001, Fig. 3b). However, 10 μ*M* EST significantly increased drug efflux in the CEM/A7 cell line (P <0.0001).

Lipid solubility

Using reverse-phase HPLC, we obtained K′ values of 1.2 for EST, 6.5 for ICI 182,780, 16.9 for ICI 164,384 and 24.1 for TAM (Fig. 4). This sequence should be contrasted with the relative efficacy of these agents as modulators of drug resistance. ICI 182,780 and ICI 164,384 were equieffective modulators (in the CEM/A7 cell line), TAM was moderately effective and EST failed to modulate at all. In contrast, EST was the least hydrophobic and TAM, the most hydrophobic of the four compounds. Lipid solubility did not correlate with the relative efficacy of the three antiestrogens as biochemical modulators.

Discussion

ICI 182,780 appears to behave as a classic modulator of MDR. It reduced the doxorubicin resistance of three MDR cell lines, CEM/A7, CEM/VLB$_{100}$ and K562/VIN100, at concentrations that had no effect on cell survival when used alone (Tables 1–3; Fig. 2). The use of ICI 182,780 was also associated with increased drug accumulation and decreased drug efflux as compared with untreated controls (Fig. 3). ICI 182,780 was significantly more active than TAM in modulating the MDR phenotype (Table 2), al-

though it was virtually equieffective to ICI 164,384 (data not shown), another pure antiestrogen not currently targeted for clinical development. Despite the similarity in structure of ICI 182,780 and the parent compound (Fig. 1), EST did not sensitise any of the three MDR cell lines to doxorubicin. In fact, there was a suggestion that EST actually reduced drug accumulation and increased drug efflux as compared with untreated controls (Fig. 3). The explanation for this effect is unclear.

The structural features of series of phenothiazines capable of modulating the MDR phenotype have previously been described [8]. Substitutions on the phenothiazine ring that increased hydrophobicity appeared to increase the efficiency of several chemical analogues as biochemical modulators of MDR [27]. In our case, substitutions at two sites on the steroid nucleus dramatically increased the capacity of a steroid to modulate doxorubicin resistance in three classic MDR cell lines. However, although these substitutions on the estrogen nucleus were associated with an increase in lipophilicity, the relative hydrophobicity of EST, TAM, ICI 182,780 and ICI 164,384 as determined by separation by reverse-phase HPLC on a C18 column (Fig. 4) did not correlate with their relative efficacy as modulators of drug resistance. Presumably, the mechanism of action of ICI, 182,780 is similar to that of other biochemical modulators and is related to interference with the function of P-glycoprotein (Pgp) preliminary experiments have suggested that verapamil and ICI 182,780 compete for binding to Pgp, although the exact nature of this interaction requires further clarification.

The MDR phenotype has been described in a number of breast-cancer cell lines [19] as well as in fresh tumour specimens obtained from patients with locally advanced or metastatic breast cancer [14, 25]. Expression of Pgp in clinical samples obtained from previously untreated patients has been shown to correlate with the response to chemotherapy and the progression-free interval [1, 22]. Reports that breast tumours intrinsically express the MDR phenotype suggest that treatment with antiestrogens (used as biochemical modulators) should be considered when chemotherapy is first introduced, independent of whether individual tumours are ER-negative or ER-positive. There may be obvious theoretical advantages in using a modulator that is also an effective antiestrogen, although, given the potential complexity of this interaction with respect to changes in cell-cycle kinetics, among other factors, careful study will be needed to determine whether there is at least an additive, if not a synergistic, interaction between these effects in ER-positive tumours. One difficulty that will need to be addressed in animal models involves the observation that the dose required to modulate the MDR phenotype is 10^{-6} *M*, which is significantly greater than the expected serum levels of ICI 182,780 (10^{-8} *M*). However, tumour levels of antiestrogens may significantly exceed those measured in the peripheral circulation [16].

The data reported herein may have important implications for the use of combined endocrine/chemotherapy programs with this new antiestrogen. The mechanism by which this fluorinated antiestrogen modulates the resistance profile of three ER-negative MDR leukemic cell lines is the subject of further study in our laboratory.

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