The synergistic anti-tumour activity of ICI 182,780 in combination with docetaxel is mediated by P-glycoprotein inhibition

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Abstract
Docetaxel is a semi-synthetic drug that has been shown to be effective in refractory advanced breast cancer. Its main mechanism of action seems to be to block microtubule depolymerisation. In this study we investigated the interaction between docetaxel and the pure anti-oestrogen ICI 182,780 on different oestrogen receptor-negative cancer cells, some of which express the classical multi-drug resistance (MDR) phenotype. ICI 182,780 potentiated the anti-proliferative effect of docetaxel only in the MDR-positive cells. Isobolic analysis demonstrated that this chemosensitising effect was a synergism. In the same conditions where synergism was detected, cell cycle analysis demonstrated an augmentation of cells blocked at the G2/M phase of the cell cycle, suggesting that ICI 182,780 increases the activity of docetaxel. In order to test this hypothesis, we performed bcl-2 western blot analysis and demonstrated that the addition of ICI 182,780 to docetaxel induced bcl-2 phosphorylation only in MDR-positive cells. The functional inactivation of bcl-2 is probably responsible for the commitment to apoptosis, since the combined docetaxel/ICI 182,780 treatment was able to foster a massive apoptosis in MDR-bearing cells as demonstrated by morphological analysis.

Our results suggest that the synergism between docetaxel and ICI 182,870 is due to a block in P-glycoprotein activity, thus determining cell cycle block, bcl-2 inactivation and apoptosis induced by docetaxel accumulation.

Introduction
Docetaxel, a member of the taxanes family, is a new anticancer drug effective in refractory human breast cancer (Seidman et al. 1996). Its main mechanism of action is by inhibiting tubulin depolymerisation, thus causing a block at the G2/M phase of the cell cycle (Schlichenmyer & Von Hoff 1991). Disruption of the microtubule organisation is able to activate a c-raf-1-dependent metabolic pathway leading to the functional inactivation of the anti-apoptotic bcl-2 protein (Haldar et al. 1995, 1996, Blagosklonny et al. 1996). Through this mechanism, all the compounds able to disrupt normal microtubule turnover are able to induce apoptotic cell death (Blagosklonny et al. 1997).

Drug resistance is a major problem in the clinical application of most chemotherapeutic agents. Two major resistance factors for taxanes have been demonstrated by in vitro studies: the first concerns a mutation in the target protein tubulin that is responsible for a 20- to 30-fold resistance with respect to the parental cell line with unchanged tubulin; the second is dependent on the expression of P-glycoprotein (P-gp) on the plasma membrane of cancer cells with the consequent decreased retention of the drug within the cell (Bhalla et al. 1994). This latter mechanism seems to be the most important for the taxanes, as it is responsible for a resistance of 500- to 1000-fold with respect to the parental cell line not expressing P-gp protein. As demonstrated for other anti-tumour agents which are substrates of the P-gp, such as doxorubicin (Kirk et al. 1993), vinblastine (Trump et al. 1992) and docetaxel (Ferlini et al. 1997), the use of anti-
oestrogens as down-modulators of the P-gp function is an attractive approach to improve the efficacy of docetaxel-based anti-tumour therapy.

The aim of this study was to investigate the in vitro efficacy of docetaxel in combination with the pure anti-oestrogen ICI 182,780 on different oestrogen receptor (ER)-negative cancer cell lines, some of which express the multi-drug resistant (MDR) phenotype. Results indicate that, in MDR-bearing cells, the combination treatment of docetaxel/ICI 182,780 presents a significant synergism in terms of growth inhibition, cell cycle block, bcl-2 phosphorylation and induction of apoptosis.

Materials and methods

Drugs
Docetaxel (kindly provided by Dr Bombardelli, Indena, Italy) was solubilised in dimethyl sulphoxide (DMSO; stock solution 10 mM) and used within 7 days. The control cells were treated with the same amount of vehicle alone. ICI 182,780 and tamoxifen (TAM) stock solutions (100 mM) (kindly provided by Dr Isola, Zeneca, Italy) were made up in absolute DMSO, and were used at concentrations ranging from 0.1 to 100 µM. The final DMSO concentration never exceeded 0.2% (v/v) in either control or treated samples.

Cell cultures
Two breast cancer cell lines (MDA-MB 231 and MCF-7 ADRr) and two leukaemia cell lines (CEM and CEM VBLr) were used. The MDA-MB 231 cell line was purchased from the American Type Culture Collection (Rockville, MD, USA); the MDR-positive MCF-7 ADRr cell line, selected for adriamycin (ADR) resistance as previously described (Scambia et al. 1994), was kindly provided by Dr Kenneth H Cowan (National Cancer Institute, NIH, Bethesda, MD, USA). MDA-MB 231 cells were grown in minimum essential medium (MEM) and MCF-7 ADRr cells in RPMI-1640 medium supplemented with 10 µM ADR. The media were complemented with 10% fetal calf serum (FCS) and 200 U ml⁻¹ penicillin (Sigma, St Louis, MO, USA). Cells, propagated as monolayer cultures in 75-cm² tissue culture flasks, were trypsinised weekly and plated at a density of 8×10⁴ cells/ml. The CEM and CEM VBLr cell lines were grown in RPMI 1640 supplemented with 10% FCS and 200 units penicillin. CEM VBLr cells were grown in the presence of 100 ng/ml vinblastine (VBL). Cells were seeded at 2-3×10⁴ cells/ml and split in a ratio of 1:3 every day. All cultures were incubated at 37°C under 5% carbon dioxide, 95% air in a high humidity atmosphere. The MCF-7 ADRr and CEM VBLr cell lines exhibit the classical MDR phenotype (mdr-1 mRNA and P-gp over-expression) and were ER-negative (Berman et al. 1991, Scambia et al. 1991). For the experiments described herein, drugs (ADR and VBL) were removed 2 weeks prior to an experiment.

Growth experiments
Cells were plated in six-well flat-bottom plates (Falcon, Lincoln Park, NJ, USA) at a density of 8×10⁴ cells/ml in complete medium. After 24 h, the medium was replaced with fresh medium containing ICI 182,780 and/or docetaxel and incubated for an additional 72 h. Control cells were treated with vehicle alone. Triplicate counts of triplicate cultures were performed after 3 days of exposure to the drugs.

Evaluation of drug interaction
In the synergy experiments, dose-response curves for the single agents were first generated. The effect of the combined treatment was analysed by the isobole method (Berenbaum 1981) for a combination of drugs A and B, applying the equation: Ac/Ae+Bc/Be=D, where Ac and Bc correspond to the concentrations of drugs used in the combination treatment, and Ae and Be correspond to the concentrations of drugs able to, by themselves, produce the same magnitude of effect. If D (combination index) is <1 the effect of the combination is synergistic, whereas if D=1 or D is >1 the effect is additive or antagonistic respectively (Berenbaum 1981).

Cell cycle analysis
Cells were plated in the specific medium supplemented as above. After 24 h, the medium was replaced with fresh medium containing the compounds to be tested or vehicle alone. After different times (from 12 to 72 h) cells were harvested and nuclei isolated and stained using a solution containing 0.1% (w/v) sodium citrate, 0.1% (v/v) NP40, 4 mM EDTA and 50 µg ml⁻¹ propidium iodide (PI) as a DNA dye (Ferlini et al. 1996). Cells were incubated with the staining solution for a minimum of 24 h at 4°C. Flow cytometric DNA analysis was performed by acquiring a minimum of 20000 nuclei with an Epics-XL flow cytometer (Coulter Immunology, Miami, FL, USA). DNA fluorescence was collected in linear mode and pulse signal processing was used to set a doublet discrimination gate. Cell cycle analysis was performed using the Multicycle software package (Phoenix, San Diego, CA, USA).

Morphological analysis
For cells growing in monolayer, treated and control samples were seeded in eight sterile chamber slides (Nunc, Naperville, IL, USA) coated with 0.01% (v/v) poly-L-lysine (Sigma). Poly-L-lysine was used as a cell adhesive to minimise the release of dead cells from the monolayer of vital adherent cells. After 24 h of culture, chambers...
Figure 1 Line charts showing the anti-proliferative effect of various concentrations of ICI 182,780 alone on (A) MDA MB-231, (B) MCF-7 ADRr and (C) CEM VBLr cell lines. Bar charts represent the growth inhibiting effect of docetaxel in combination with increasing doses of ICI 182,780 on (D) MDA MB-231, (E) MCF-7 ADRr and (F) CEM VBLr cell lines. Each point is the mean of three separate experiments performed in triplicate. Standard deviations were less than 10% and have been omitted.
were removed and slides were stained with May-Grunwald-Giemsa. For cells growing in suspension, morphological evidence of apoptosis was obtained by staining chromatin with the fluorescent DNA dye YOYO-1 (Molecular Probes, Eugene, OR, USA). Briefly, cultures were fixed in 70% cold ethanol, stored overnight at −20 °C and then resuspended in PBS containing 50 µg/ml Ribonuclease (Sigma). After 1 h incubation cells were washed and stained with YOYO-1 at a concentration of 4 µM. Slides were examined using an inverted microscope, Diavert (Leica, Wetzlar, Germany) to score cells with features of apoptotic chromatin. Image analysis was performed using the IAS2000 system (Delta Sistemi, Rome, Italy).

**Bcl-2 western blots**

The pellet obtained from 1×10^7 cells was washed twice in PBS and then dissolved in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 2 U/ml of the kallikrein inhibitor, aprotinin. After addition of SDS sample buffer, 200 µg total proteins were separated by 15% SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) (Millipore Co., Bedford, MA, USA). The membranes were incubated with 6% non-fat dry milk in TBST (0.1 M Trizma base, 0.15 M NaCl, 0.05% Tween 20, pH 7.4) for blocking and then with a 1:100 dilution of the mouse monoclonal anti-bcl-2 antibody clone 124 (Dako, Glostrup, Denmark) in TBST. The membranes were then incubated with a biotinylated secondary antibody (ABC Vectastain Elite, Vector Labs, Burlingame, CA, USA) and detection was performed with the DAB kit (Vector Labs) in TBST. Images of the blots were acquired with a CCD camera and quantification of the bcl-2 bands was performed by Phoretix 1D Gel Analysis software (Phoretix International Ltd, Newcastle-upon-Tyne, UK).

**Results**

**Growth inhibitory effect of the combined treatment with docetaxel/ICI 182,780 on ER-negative cancer cells**

The anti-proliferative activity of ICI 182,780 (range 0.1-100 µM) was assessed after 72 h of single drug exposure on ER-negative MDA-MB 231, MCF-7 ADRr and CEM VBLr cell lines (Fig. 1). In particular, the IC₅₀ (the concentration giving the half-maximal growth inhibition) values were 10, 13 and 10 µM for MDA-MB 231, MCF-7 ADRr, and CEM VBLr cells respectively, indicating that the expression of the MDR phenotype did not noticeably affect the response to the anti-oestrogen ICI 182,780. Similarly, the growth inhibition of docetaxel (range 0.1-0.5 µM) was determined and the IC₅₀ values were 0.0014, 1.9 and 1.4 µM on MDA-MB 231, MCF-7 ADRr and CEM VBLr cells respectively, thus confirming that expression of the classical MDR phenotype is the major cause of resistance to taxanes. In order to evaluate the interaction between docetaxel and ICI 182,780, cancer cells were cultured in the presence of increasing doses of ICI 182,780 (range 0.1-10 µM) in combination with a concentration of docetaxel able to induce a 25% growth inhibition. ICI 182,780 potentiated the anti-proliferative effect of docetaxel on MDR-positive cells, whereas it was ineffective on MDR-negative MDA-MB 231 cells (Fig. 1). To understand better the effect of the combined

<table>
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<th>ICI 182,780 (µM; Ac)</th>
<th>Docetaxel (µM; Bc)</th>
<th>% of control</th>
<th>ICI 182,780 (µM; Ae)</th>
<th>Docetaxel (µM; Be)</th>
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Ac and Bc, concentrations of drugs used in the combination treatment; Ae and Be concentrations of drugs able to produce, alone, the same magnitude of effect; D (combination index)<1 = synergistic effect; D≥1 = additive or antagonistic effect.
Figure 2  Cell cycle analysis of MCF-7 ADRr cells after 24 h of drug exposure. Cultures were treated with 0.2, 0.5 and 1μM docetaxel (left-hand, middle and right-hand columns respectively) and with TAM and ICI 182,780 as indicated.
docetaxel/ICI 182,780 treatment in growth experiments, the isobole method was used to analyse the above data (Berenbaum 1981). This analysis showed that the chemosensitising effect in MDR-positive cells was a synergism (D value <1), and is shown in Table 1.

**Synergistic anti-proliferative activity of docetaxel and ICI 182,780 is mediated by an increase in cell cycle block at the G2/M phase**

Our previous study showed a synergistic effect of TAM when used in combination with low doses of docetaxel in both MDR-positive and MDR-negative human cancer cells (Ferlini et al. 1997). This combination was found to cause a G2/M cell cycle block. Therefore, cell cycle perturbations induced by either docetaxel/TAM or docetaxel/ICI 182,780 combinations have been assessed. In Fig. 2 we show the cell cycle analysis of MCF-7 ADRr cells treated with increasing doses of docetaxel (0.2, 0.5 and 1 µM) and the anti-oestrogens ICI 182,780 and TAM (0.1, 1 and 10 µM) after 24 h of exposure. An augmentation of cells blocked in the G2/M phase of the cell cycle is evident at an ICI 182,780 concentration of 10 µM in combination with increasing doses of docetaxel. Table 2 summarises data from Fig. 2, reporting only the effects observed at the highest doses of TAM and ICI 182,780. After 24 h of exposure to 0.5 µM docetaxel in

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Combination with equimolar doses (10 µM) of ICI 182,780 and TAM, the percentage of cells blocked in the G2/M phase was 70% versus 23% respectively. The same difference, albeit less dramatic, was observed at 1 µM docetaxel (67% versus 46%). These data were confirmed after 48 and 72 h (data not shown) of drug exposure, when ICI 182,780 also potentiated the effect at a lower concentration of docetaxel (0.2 µM). Taking all these data together, we concluded that ICI 182,780 is more effective than TAM as a chemosensitising agent in MDR-bearing cells.

In order to confirm this finding, flow cytometric DNA analysis was performed on the ER-negative human leukaemia cancer cell line CEM VBLr; these cells also express the MDR phenotype. ICI 182,780 was also able to produce, in combination with docetaxel, a relevant cell cycle block in these cells (Table 3). After 24 h of exposure to 0.2 µM docetaxel in combination with 10 µM ICI 182,780 and TAM, the percentage of cells blocked in the G2/M phase was 72% versus 47% respectively.

When the same experiments were performed on the MDR-negative cell lines (MDA-MB 231 and CEM), using the same concentrations of anti-oestrogens and docetaxel doses ranging from 0.0001 to 0.0005 µM, no potentiating effect was observed for ICI 182,780, while TAM, in keeping with previous observations (Ferlini et al. 1997), was still able to increase the cell cycle blocking activity of docetaxel (data not shown).

Combined treatment of docetaxel and ICI 182,780 induces bcl-2 phosphorylation only in MDR-bearing CEM cells

The inactivation of bcl-2 by phosphorylation has been shown to play a pivotal role in taxane-induced apoptosis (Haldar et al. 1995, 1996, Blagosklonny et al. 1996, 1997). Therefore, the synergism between docetaxel and ICI 182,780 in terms of inducing functional inactivation of bcl-2 protein was investigated. Western blot analysis was performed on CEM VBLr and CEM treated cells (Fig. 3).

Combined treatment of docetaxel and ICI 182,780 induces apoptosis in MDR-bearing cells

In order to evaluate if the combined treatment was able to induce apoptosis, classical changes related to the apoptotic process, such as reduction in nuclear size, chromatin condensation, marginalisation and nuclei fragmentation (Ferlini et al. 1996) were investigated by morphological analysis. MCF-7 ADRr cells were treated with either 0.5 µM docetaxel or 10 µM ICI 182,780 alone, or with a combination of both drugs. Untreated control cells exhibited only 12% of cells with condensed and marginalised chromatin. Treatment with 0.5 µM docetaxel increased anisonucleosis, but did not induce consistent changes in chromatin structure, while ICI 182,780 administered alone induced a slight increase in cells with features of apoptosis (up to 24%). The combined treatment with the two drugs fostered massive apoptosis with formation of binucleated cells with typical fragmented chromatin polarised in the tails of cellular poles. These data are consistent with the cell cycle analysis, showing that this combination induced a marked block in the G2/M phase of the cell cycle. Interestingly, in some cells there is evidence of the presence of apoptotic bodies, suggesting that, in this model, when massive apoptosis occurs some cells retain the ability to take up apoptotic bodies from the medium.

In order to confirm the occurrence of apoptosis in another MDR-positive model, we scored the apoptosis...
index in CEM VBLr cells. Again, treatment with docetaxel (0.5 µM) and ICI 182,780 administered as single agents did not produce an apoptosis rate higher than 15%. On the other hand, the combined exposure to both drugs resulted in a dramatic increase in the apoptotic rate (60%). Thus, these data undoubtedly confirmed that the marked synergism observed with the combined docetaxel/ICI 182,780 treatment is mediated by the activation of the apoptotic programme, starting in a large part from the M checkpoint of the cell cycle.

Discussion

Adriamycin is the most active single agent currently used in the treatment of advanced breast cancer. However, the disease often relapses during or after the course of therapy. In such patients, it has been proved that docetaxel is still able to maintain a high response rate (Burris et al. 1995, Ravdin 1995, Seidman et al. 1996). Unfortunately, this treatment may also become ineffective due to the primitive presence and/or the positive selection of MDR-bearing cancer cells (Ravdin 1995, Trock et al. 1997).

In this study we have demonstrated that the anti-oestrogen, ICI 182,780, is able to produce a marked synergism in combination with docetaxel. This synergism is mediated by an increase in the blocking activity of docetaxel at the M checkpoint of the cell cycle with the consequent functional inactivation by serine-phosphorylation of the anti-apoptotic bcl-2 protein. This metabolic event fosters massive entry of cancer cells into the apoptotic pathway, starting maximally from the M phase of the cell cycle as demonstrated by morphological and DNA analysis. The ability of ICI 182,780 to synergise with docetaxel is specifically confined to P-gp-positive cell lines, since synergism, increased cell cycle arrest and induction of bcl-2 phosphorylation are not present in MDR-negative cells exposed to the combined drug treatment for two drugs. The strict relationship between the occurrence of apoptosis and bcl-2 phosphorylation observed here confirms previous observations that bcl-2 is the key molecule involved in the anti-tumour activity of all the drugs able to disrupt normal microtubule organisation (Blagosklonny et al. 1997).

The synergism between docetaxel and ICI 182,780 on MDR-bearing cells is in good agreement with the notion that anti-oestrogens are effective modulators of P-gp function. Hu et al. (1991) previously reported that the pure anti-oestrogen, ICI 164,384, increases the uptake and reduces the efflux of radiolabelled daunomycin, suggesting that it has a specific role in modulating P-gp function. More recently, our group suggested that ICI 182,780 may also modulate P-gp activity without affecting P-gp expression levels, thereby producing a synergism in combination with adriamycin in MCF-7 ADRr cells (De Vincenzo et al. 1996). The ability of anti-oestrogens to modulate P-gp function has been shown to be shared by the non-steroidal partial anti-oestrogen, TAM, and we have demonstrated previously that TAM in combination with docetaxel produced a good synergism (Ferlini et al. 1997). However, this synergism is also present in MDR-negative cells and in this study we noticed that, in MDR-bearing cells, the cell cycle arrest induced by the addition of TAM to docetaxel is comparatively lower than that observed with the addition of ICI 182,780. Such differences suggest that, in mediating synergism with docetaxel, TAM and ICI 182,780 act through not fully overlapping mechanisms.

The minimal dose of ICI 182,780 required to obtain the synergism with docetaxel in MDR-bearing cancer cells was 1 µM. Preclinical studies have shown that ICI 182,780 serum levels exceeding 5 µM must be attained to obtain a therapeutic effect in advanced breast cancer patients (Dukes et al. 1993). These values could be achievable with minimal side effects, as it has been demonstrated that drug accumulation occurs during therapy with ICI 182,780 (Howell et al. 1996a). Moreover, it is possible that ICI 182,780 levels inside tumour tissues may exceed considerably those in the peripheral circulation, thus resembling the behaviour of TAM (Lien et al. 1991).

The improved understanding of the mechanisms underlying drug resistance has led to therapeutic strategies devoted to the inhibition of P-gp function. In particular, clinical trials have been performed using verapamil and cyclosporine as inhibitors of P-gp (reviewed in Yuen & Sikic 1994). Although these studies demonstrated a partial activity in modulating drug resistance, both verapamil and cyclosporine showed considerable side effects at a dosage lower than that required for the complete inhibition of P-gp function. Thus, due to the minimal side effects shown in preclinical studies, ICI 182,780 appears to represent an effective modulator of P-gp function. The clinical importance of our data is further strengthened by the recent demonstration that, in breast cancer, failure of neoadjuvant chemotherapy can be predicted by the presence of cancer cells bearing the MDR-phenotype (Chevillard et al. 1996, Trock et al. 1997). Furthermore, in this study we used only ER-negative cellular models and it is reasonable to hypothesise that additional anti-tumour effects should be expected on ER-positive cells eventually present inside the tumour (Howell et al. 1995, 1996b), because ICI 182,780 is capable of producing complete inhibition of signalling through the ER-receptor. Therefore, the use of ICI 182,780 as a chemosensitising agent in advanced breast cancer could add further advantages with respect to the use of docetaxel alone.

In summarising our results, we have demonstrated that the addition of ICI 182,780 to a docetaxel-based therapy
is able to improve the \textit{in vitro} anti-neoplastic performance of the taxane in MDR-bearing cell lines. Moreover, our data suggest that ICI 182,780 in breast cancer may be utilised not only as a pure anti-oestrogen effective on TAM-resistant patients but also in a wider way and independent of ER status as a chemosensitizer in patients relapsing after treatment with anthracyclines. Thus, if these results are confirmed in \textit{in vivo} experimental models, prospective clinical studies will be needed to verify whether the combined use of docetaxel and ICI 182,780 is able to ameliorate the response rate of advanced breast cancer.

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