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The aromatase inhibitor letrozole enhances the effect of doxorubicin and docetaxel in an MCF7 cell line model

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Abstract

Introduction: Post-menopausal women with estrogen receptor (ER) positive breast cancer receive adjuvant chemotherapy and endocrine therapy sequentially since tamoxifen may antagonise the cytotoxicity of chemotherapy drugs. With increased use of aromatase inhibitors (AIs) in place of tamoxifen, the potential use of concomitant chemo-endocrine treatments with the AI letrozole, before clinical trials are undertaken, requires evaluation.

Methods: MCF7-aro cells expressing the aromatase gene were treated with letrozole, doxorubicin and docetaxel. The effects of different drug concentrations, drug combinations and scheduling on cytotoxicity and aromatase activity were investigated. Key receptor, cell cycle regulation and apoptosis proteins were examined by immunoblotting.

Results: Administration of letrozole with either doxorubicin or docetaxel resulted in increased levels of cytotoxicity under all treatment schedules (add in, sequential or simultaneous drug administration) with the greatest anti-proliferative effect observed using concomitant treatment (letrozole first with chemotherapy added in). The inhibitory effect of letrozole on aromatase activity was unchanged by the addition of doxorubicin or docetaxel. Letrozole treatment resulted in decreased HER2 expression and addition of doxorubicin and docetaxel to letrozole led to elevated ER-β levels.

Conclusions: *In vitro*, letrozole, unlike tamoxifen, enhances the cytotoxicity of both doxorubicin and docetaxel. This supports the prospect of trials using letrozole with chemotherapy in postmenopausal women with ER positive breast cancer.

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Introduction

Breast cancer accounts for almost one in three (31%) of all cancer cases in UK women (excluding non-melanoma skin cancer) and causes over 12,100 deaths each year [1]. However, despite a rising incidence, survival from breast cancer is increasing, due to earlier detection and improved treatment.

Two thirds of breast cancers occur in post-menopausal women and within this group, estrogen receptor alpha

(ERα) positive cancers constitute some 80%. Advances in treatment including the endocrine agent tamoxifen, chemotherapies and more recently aromatase inhibitors [2, 3] have contributed significantly to the disease free survival of such patients.

Tamoxifen is a selective estrogen receptor modulator (SERM), antagonizing estrogen receptor function by competitively binding to the ERα, blocking estrogen binding and thus inhibiting cellular proliferation [4]. However, due to the partial agonistic properties of tamoxifen on ERα, tamoxifen treatment is associated with a number of adverse side effects including endometrial cancer and also an increased risk of thromboembolism [5, 6]. Although a greater disease free survival was observed after 10 years of tamoxifen compared to 5 years adjuvant therapy [7], prolonged tamoxifen use may also lead to acquired tumour resistance and coupled with the significant side effects has led to increasing interest in aromatase inhibitors (AIs) [8].

Aromatase inhibitors (AIs) block the aromatase enzyme, preventing the conversion of androstenedione to estrone and subsequently estradiol [4, 9, 10], without any direct activity on ERα. AIs thus block estrogen synthesis including the autocrine and paracrine estrogen production from peri-tumoral stromal cells, which may play a role in tumour proliferation [11, 12]. The AIs anastrozole and letrozole have demonstrated an increased disease free survival compared to tamoxifen [13, 14]. Subsequent trials have demonstrated increased activity and/or survival advantage of letrozole over tamoxifen in the neoadjuvant [15], adjuvant [14], extended adjuvant [16] and advanced disease [17] settings . Moreover, letrozole given subsequent to 5 years of tamoxifen therapy also improved disease free survival [18]. Letrozole is currently the only AI licensed for use in the neo-adjuvant, adjuvant, extended adjuvant and metastatic settings in post-menopausal ERα positive patients.

With this increased use of letrozole for postmenopausal women in the neoadjuvant, adjuvant and advanced disease settings, the sequencing of AIs with chemotherapy agents requires further appraisal. However, meta-analyses have demonstrated that tamoxifen, added to chemotherapy, was of marginal benefit in older women [19]. Furthermore, consensus trials data [20, 21] , and *in vitro* experimental evidence [22-24] suggested that tamoxifen may be interacting antagonistically with some cytotoxic agents. The phase III SWOG-8814 / INT-0100 trial, comparing cyclophosphamide, doxorubicin and fluorouracil (CAF) followed by tamoxifen (CAF-T) or CAF with concurrent tamoxifen (CAFT), reported sequential chemotherapy followed by tamoxifen was superior to concurrent tamoxifen and anthracycline based therapy [25].

Given this background, tamoxifen treatment has been prescribed post-chemotherapy in the adjuvant setting; despite the differing modes of action, aromatase inhibitors are also prescribed in a similar manner. Given recent interest in combining AIs with chemotherapy, this study sought to test in an in vitro model system whether chemo-endocrine therapy using a widely prescribed AI, letrozole, in combination with two standard breast cancer chemotherapy agents, doxorubicin and docetaxel, holds clinical potential. To test this hypothesis, using MCF7-aro cells over-expressing the aromatase gene [26], letrozole, docetaxel or doxorubicin were administered under a range of experimental conditions. The cytotoxic effects, aromatase activity, and proteins involved in ERα signalling, in cell cycle regulation and apoptosis were studied to elucidate and compare the effects of letrozole in this setting.

Materials and methods

Cell culture

The MCF7-aro cell-line, stably transfected with the human aromatase gene and thus dependent on androgens for proliferation, was maintained in Dulbecco's Modified Eagle Media +phenol-red (+DMEM) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 700μg/ml G418 (Invitrogen Ltd., Renfrewshire, UK) [26][21]. For experiments the cells were purged of any residual steroids and the estrogenic effects of phenol-red by sub-culturing for 4 days in DCC media (Dulbecco's Modified Eagle Media -phenol-red [-DMEM] supplemented with 3% charcoal stripped-FCS [Hyclone, Thermo-Fisher, Leicestershire, UK], 2% HEPES[1M] and 1% L-Glutamine[200mM]. Media was changed after 2 days. All cells were cultured at 37°C in humidified incubator with 5% CO_2 and periodically checked for Mycoplasma according to manufacturer's instructions (MycoAlert®, Cambrex, Berkshire, UK).

Cell Viability Assays

MCF7-aro cells at a confluency of 80% were steroid deprived for 4 days, trypsinised with trypsin/EDTA lacking phenol-red (Invitrogen Ltd., Renfrewshire) and seeded into 96-well plates (1.2×10^4 cells/well) in 200μl DCC media. After 24 hours the media was removed and steroid depleted media (DCC) containing 25nM 4-Androstenedione (AD) (Sigma-Aldrich, Dorset, UK) was added to the cells to induce proliferation. Vehicle control (EtOH) or compounds either singly (letrozole, doxorubicin, docetaxel, tamoxifen) or in combinations (letrozole, doxorubicin, docetaxel) were added in media for either 2 days (48 hours), 4 days (96 hours) or 6 days depending on the experiment (Table 1). Tamoxifen (4-Hydroxytamoxifen) and docetaxel were

Table 1. Three drug regimens using letrozole, doxorubicin and docetaxel in a simultaneous, sequential or add-in approach.

Regimen	First 48 hours (2 days)	Subsequent 96 hours (4 days) to total 6 days
Simultaneous	Letrozole	Letrozole
	Letrozole + doxorubicin	Letrozole + doxorubicin
	Letrozole + docetaxel	Letrozole + docetaxel
	Letrozole + doxorubicin + docetaxel	Letrozole + doxorubicin + docetaxel
Sequential	Letrozole	Letrozole
	Letrozole	Doxorubicin
	Letrozole	Docetaxel
	Letrozole	Doxorubicin + docetaxel
Add in	Letrozole	Letrozole
	Letrozole	Letrozole + doxorubicin
	Letrozole	Letrozole + docetaxel
	Letrozole	Letrozole + doxorubicin + docetaxel

obtained from Sigma-Aldrich, Dorset, UK, doxorubicin from Pharmachemie B.V, Haarlem, Netherlands, and letrozole from Novartis, UK. Pre-treatment exposure was for 2 days followed by media removal and further treatment for 4 days. After the appropriate length of time, cells were assayed for cell viability utilizing the CellTiter 96® Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instructions (Promega, Southampton, UK). Briefly, media was removed and 100µl of AD media containing 20µl MTS added to each well and incubated for 3 hours at 37°C 5% CO_2. Absorbance was read at 490nm with a 96-well SpectraMax 190 UV/Vis plate reader. Cells treated with AD were taken to represent 100% proliferation and all drug-treated cells were expressed relative to this.

Western Blotting

Cells were seeded at $1x10^7$ in 60mm tissue-culture plates in 4ml media and cultured for 24 hours. Cells were then treated in DCC media containing 25nM AD +/– drug (IC_{50} concentrations) for either 24h, or 2 days pre-treatment with letrozole followed by a further 24h treatment. Cells were harvested by washing twice in ice cold PBS prior to lysis in 180ml of complete protein lysis buffer (150mM NaCl, 1% NP40, 50mM Tris HCL pH 8.0, 50mM NaF, 5mM EDTA pH 8.0 with freshly added complete protease inhibitor [Roche, Sussex, UK]). Cell lysates were vortexed for 2 min then centrifuged at 14,000rpm for 20 min at 4°C. Protein concentrations in the supernatants were determined by Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Herts, UK) and equal amounts of protein used for western blot analysis. Immunoblot analysis was performed using the following primary antibodies: Anti-ER-a, anti-EphA2 (Sigma-Aldrich, Dorset, UK); anti b-Actin, anti-Bax (Abcam Ltd, Cambridge, UK); anti-$P21^{wafl}$ (Calbiochem, Nottingham, UK); anti-Bcl_2 (Dako Ltd, Cambridgeshire, UK) anti-HER-2, anti-c-Fos, anti-VEGF, anti-ER-β (Novacastra Laboratories Ltd, Newcastle Upon-Tyne); anti-p53 (DO-1) (Lane Group, Ninewells Hospital, Dundee, UK). Polyclonal rabbit-anti-mouse IgG HRP and polyclonal swine-anti-rabbit IgG were used as secondary antibodies (Dako Ltd, Cambridgeshire, UK). Samples were separated by SDS-PAGE on 4-12% Bis-Tris NuPage gels with either MOPS or MES buffers using the Novex mini cell system (Invitrogen, Glasgow, UK), after which they were transferred to Immobilon PVDF membranes (Millipore, Buckinghamshire, UK) and blocked in 0.1% (v/v) PBS-Tween-20 (PBST) with 5% (w/v) dry skimmed milk (PBSTM) for 1h at RT. The membrane was washed 3 times for 15 min with PBST before incubation with primary antibodies at 4°C overnight. Membranes were further washed (3 x 5 min) in PBST before incubation with the appropriate HRP labeled secondary antibody in PBSTM for 1 h at room temperature. Membranes were washed (3 x 5 min) in PBST, developed by ECL™ Solution (Amersham, Buckinghamshire, UK) and exposed to autoradiography film (Fuji x-ray film) prior to development in a Fuji X-ray Imaging Developer 800A.

Aromatase Assay

Aromatase enzymatic activity was assayed using a modification of the method described by Zhou *et al.* [26] and exploits release of $[^3H]H_2O$ from the substrate ^{3}H-19, hydroxyl-stenedione (^{3}H-AD) in the presence of aromatase. Cells were seeded at 3.5 x 10^4 per well in 96-well tissue-culture plates in 200µl DCC media and cultured for 24 hours. Cells were then treated in DCC media containing 25nM AD +/– drug (IC_{50} concentrations) for 72h. The media was removed and 120µl of 4.8 pmol of 1b-^{3}H(N)-androst-4-ene-3, 17-dione (NET-926; NEN PerkinElmer, Bucks, UK) as substate (specific activity, 23.5 Ci/mmol) in DCC media was added. Controls of 120µl DCC media and vehicle (0.0012% EtOH) were also included. After 6 hours of incubation 110µl of this media was removed and extracted into 220µl of chloroform. Samples were vortexed for 30 seconds and centrifuged at 1700g for 10 min. Residual steroids were then removed by addition of 100µl of the aqueous phase to 100µl of 0.5% charcoal vegetal active/0.5% dextran T70 suspension.

Samples were again vortexed for 30 seconds and centrifuged at 15,000g for 5 min before 150µl of the resultant upper-phase was removed and added to 4ml of OPTI-FLUOR scintillation fluid (Anachem Ltd, Bedfordshire, UK). The [^{3}H]H_2O released was counted on a ^{3}H channel scintillation counter reading at 10 min intervals on a Beckman LS6500 scintillation counter (Beckman Coulter UK Ltd., High Wycombe, UK). The protein concentration of the cells in each well was also determined, using the Bradford assay. Briefly, cells were washed twice in PBS, prior to addition of 20µl of 50mM Tris-HCl, pH 7.2 followed by 200µl of Bradford reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories, Herts, UK). Samples were incubated for 5 min at room temperature and the absorbance was read at 595nm on a 96-well SpectraMax 190 UV/Vis plate reader with SOFTmax® PRO Softwear, Molecular Devices Ltd. Aromatase activity was calculated from the disintegrations per minute as picomoles of estrogen produced by milligrams of protein per hour (pmol/mg/h).

Statistical analysis

Statistical analysis was performed using Minitab statistical software (Minitab Inc., version 15.1.0.0) for descriptive statistics (mean, standard deviation, 95% confidence interval, etc.). 2-sample t-tests were used to test the differences in mean values. A p-value of <0.05 was considered statistically significant.

Results

To establish the drug concentrations that produced a 50% inhibition of cellular growth when steroid deprived MCF7-aro cells were stimulated to proliferate with 25nM AD and then treated with the drug for 96 hours, MTS assays were performed with the letrozole, doxorubicin, docetaxel and tamoxifen. IC_{50} values were compiled from 3 separate experiments, each done in quadruplicate (Table 2), then the IC_{50} values used in all subsequent experiments.

Table 2. Drug IC_{50} values. IC_{50} values for doxorubicin, docetaxel, letrozole and tamoxifen in MCF7-aro cells stimulated to proliferate with 25nM AD and then drug treated for 96 hours. Values shown were derived from 3 separate experiments, each done in quadruplicate ± SEM.

Drug	IC_{50} (µM)
Docetaxel	5.0 ± 1.0
Doxorubicin	6.0 ± 0.7
Letrozole	1.0 ± 0.03
Tamoxifen	2.5 ± 0.2

As expected, letrozole inhibited cellular growth and significantly inhibited aromatase activity (p=0.006) (Figure 1). Conversely, tamoxifen did not inhibit aromatase activity and indeed an increase in aromatase activity was noted in the MCF7-aro cells (p=0.044) (data not shown).

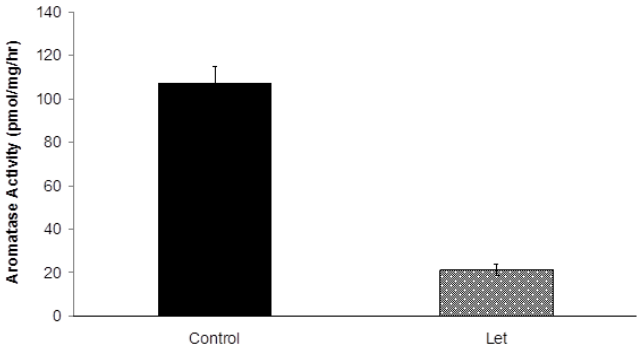

Figure 1. Aromatase activity of MCF7-aro cells in DCC media containing 25nM AD and treated with 1µM letrozole for 72 hours. Error bars ± 95% CI.

Combining chemo-endocrine therapies in different schedules

The effects of combining letrozole with the chemotherapeutic agents doxorubicin and/or docetaxel and the effects of scheduling of the agents was examined. Cells were pre-treated with letrozole for 48 hours and this was then followed by chemotherapy alone (Sequential) or chemotherapy in the presence of further endocrine agent (Add-in). In addition, cells were not pre-treated with any agent and allowed to proliferate to 80% confluence prior to treatment with chemotherapy and endocrine agent together (Simultaneous). For each combination, three separate MTS assays were each done in quadruplicate after a total of 6 days in culture (Figure 2).

Chemotherapy effects

Cell survival with doxorubicin alone or docetaxel alone was reduced by combining the two drugs (Figure 2a).

Simultaneous treatment (letrozole + chemotherapy together)

Simultaneous treatment against growing cells with letrozole and either doxorubicin or docetaxel, or the combination chemotherapy, displayed enhanced cytotoxicity compared to letrozole alone ($p<0.0001$ in each case)(Figure 2b).

Sequential treatment (letrozole alone followed by chemotherapy alone)

Sequential treatment showed that letrozole followed by either doxorubicin or docetaxel or doxorubicin and

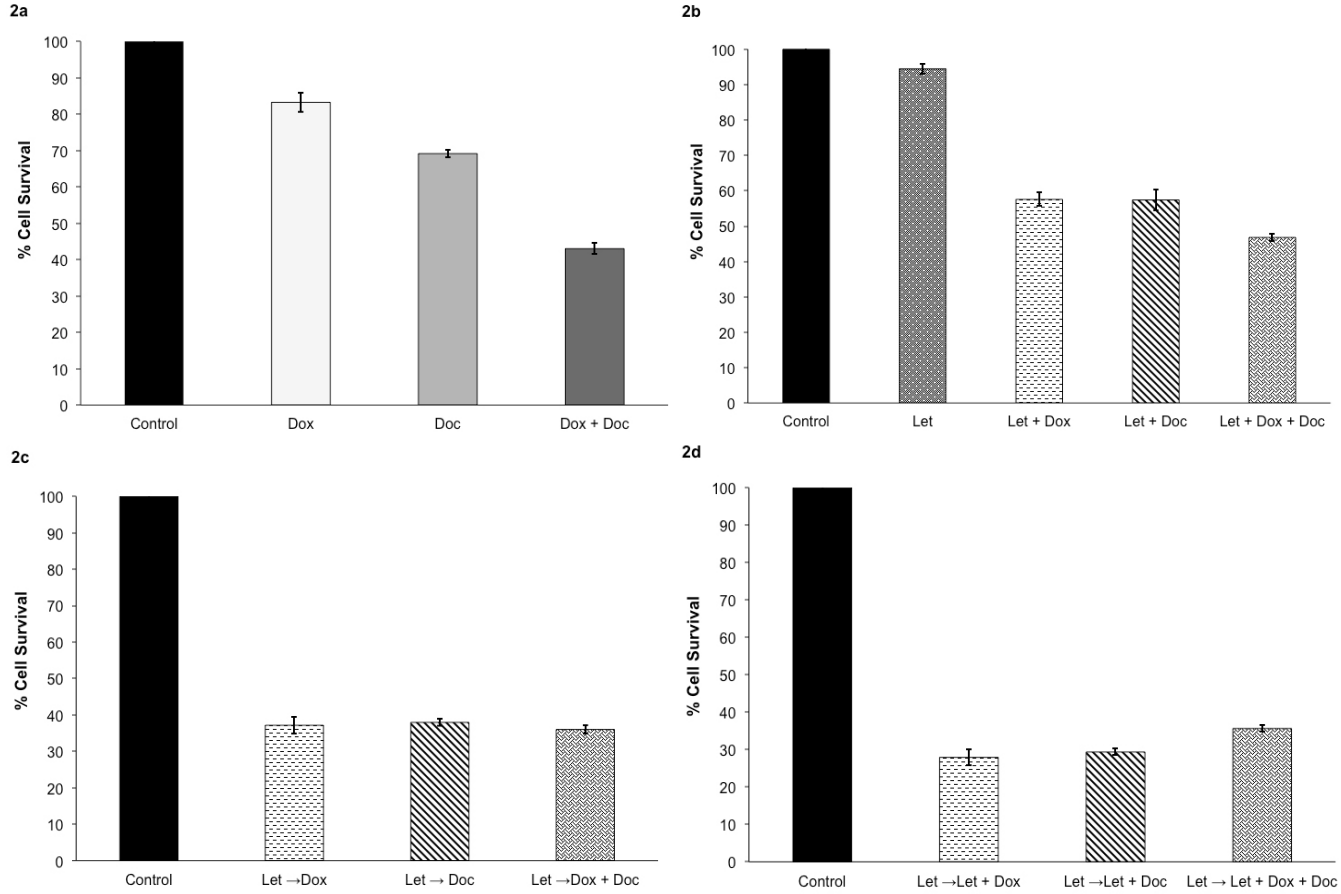

Figure 2. Combining chemo-endocrine therapies in different schedules. MCF-7aro cells in DCC media containing 25nM AD were seeded into 96 well plates and then after a total of 6 days in culture MTS cytotoxicity assays were performed. The percentage of cells surviving relative to the cells treated solely with AD was calculated and plotted. Results shown are the average of three separate MTS assays each done in quadruplicate. Error bars ± 95% CI. Drugs were applied at their IC_{50} values: doxorubicin – 6μM, docetaxel – 5μM, letrozole – 1μM. **(2a) Chemotherapy controls** – Cells were grown for 48 hours and then treated solely with chemotherapy agents for 96 hours. **(2b) Simultaneous** – Cells were grown for 48 hours and then treated with both chemotherapy and endocrine agents together for a further 96 hours as indicated. **(2c) Sequential** – Cells were pre-treated with letrozole for 48 hours and then treated with chemotherapy for 96 hours. **(2d) Add-in** – Cells were pre-treated with letrozole for 48 hours and then treated with chemotherapy in the presence of further letrozole for 96 hours.

docetaxel combined, resulted in significantly enhanced cytotoxocity compared to letrozole alone ($p<0.0001$ for each comparison) (Figure 2c).

Add-in pre-treatment (letrozole first + chemotherapy added in)

Add-in pre-treatment with letrozole followed by further letrozole and the addition of either doxorubicin or docetaxel or combined doxorubicin and docetaxel significantly enhanced the cytotoxic effects of the drugs ($p<0.0001$) (Figure 2d).

In all the treatment schedules tested, letrozole provided a greater cytotoxic effect when combined with chemotherapy, either doxorubicin, docetaxel or the two agents combined, than if letrozole was given alone. Add-in treatment of letrozole with chemotherapy provided the most efficacious method for reducing cell survival followed by sequential treatment and lastly by simultaneous treatment. Add-in treatment (Figure 2d) also displayed the biggest differential between chemotherapy alone (Figure 2a) and a chemotherapy-letrozole combinations.

Chemo-endocrine therapy with letrozole does not alter aromatase activity

As letrozole appeared to synergize with both doxorubicin and docetaxel to promote increased efficacy of these agents, aromatase activity was re-examined. Letrozole with either doxorubicin or docetaxel, alone or in combination, did not significantly alter the inhibitory effect of letrozole on aromatase activity (Figure 3).

Molecular changes

To examine the effects of letrozole at a molecular level, a series of western blots for proteins involved in estrogen receptor signaling (ER-α, ER-β, HER-2, VEGF and EphA2; Figure 4a) and key proteins involved in cell cycle and apoptosis were also examined (p21, p53, Bax,

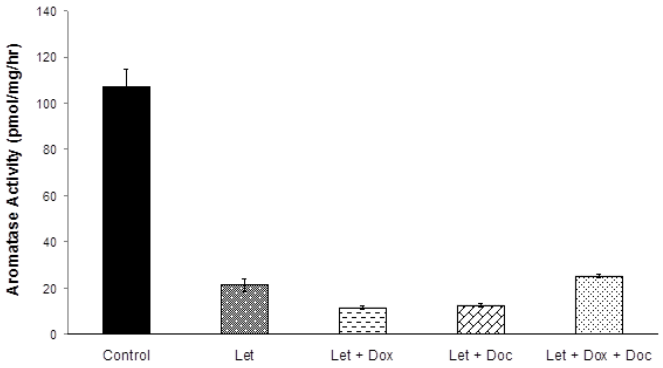

Figure 3. Aromatase activity of MCF7-aro cells in DCC media containing 25nM AD treated with 1μM letrozole in the presence of either 6μM doxorubicin, 5μM docetaxel or both chemotherapeutics. Assays were carried out after 72 hours of incubation. Error bars ± 95% CI.

Bcl-2 and c-fos; Figure 4b)(Table 3).

ER-α increased and HER-2 levels decreased with letrozole. Protein levels of VEGF decreased but EphA2 and ER-β did not alter. p21, p53, bax, bcl2 and c-fos levels were unchanged with letrozole treatment.

Cells treated with doxorubicin or docetaxel, as predicted from their different mechanisms of action, displayed very different protein profiles. Doxorubicin treated cells showed increased p21, p53 and EphA2 expression and reduced expression of ER-α, c-fos and VEGF was noted. Docetaxel treated cells displayed decreased EphA2 expression. When the two agents were combined, smaller increases in p21 were observed, ER-β, and EphA2 were elevated but ER-α levels decreased.

Treatment with letrozole and doxorubicin resulted in increased levels of ER-β but neither drug added individually altered ER-β expression. However, when docetaxel was added with letrozole the expression of most proteins examined was increased (Table 3).

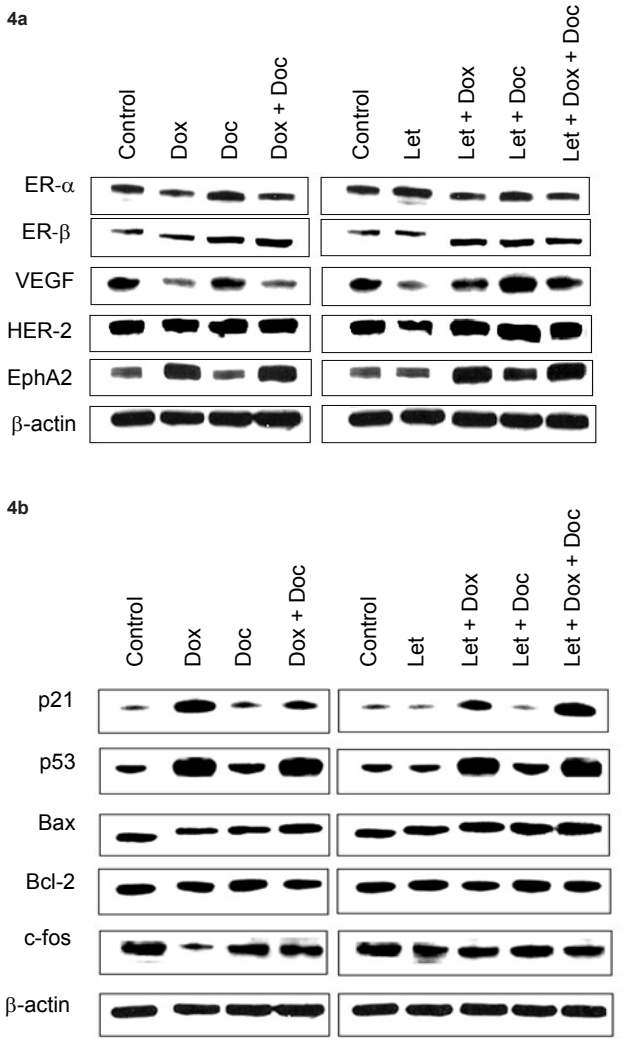

Figure 4. Examples of western immunoblotting of MCF7-aro cells in DCC media containing 25nM AD treated with 1μM letrozole, 6μM doxorubicin, 5μM docetaxel or drug combinations as indicated, for 24 hours. (4a) Expression of proteins involved in estrogen receptor signalling. (4b) Expression of cell cycle and apoptosis related proteins. β-actin was used as a loading control in all cases.

Table 3. Summary of changes in protein expression with drug treatment of MCF7-aro.

	Letrozole	Doxorubicin	Docetaxel	Doxorubicin+ Docetaxel	Letrozole+ Doxorubicin	Letrozole+ Docetaxel	Letrozole+ Doxorubicin+ Docetaxel
ER	↑	↓	↑	↓		↑	
ER	↔	↔	↑	↑	↑	↑	↑
HER-2	↓	↔	↔	↔	↔	↔	↔
VEGF	↓	↓	↔	↓	↔	↑	↑
EphA2	↔	↑	↓	↑	↑	↑	↑
P21	↔	↑	↔	↑	↑	↑	↑
P53	↔	↑	↔	↑	↑	↑	↑
Bax	↔	↔	↔	↑	↑	↑	↑
Bcl-2	↔	↔	↔	↔	↔	↔	↔
c-fos	↔	↓	↔	↔	↔	↔	↔

Application of the two chemotherapeutics with letrozole resulted in a similar pattern of protein expression with increases in p21, p53, EphA2 and ER-β expression.

Discussion

Using estrogen receptor positive, aromatase enzyme transfected MFC7-aro cells as a model system and cytotoxicity assays, we have demonstrated that letrozole, when combined with the commonly used chemotherapeutic agents doxorubicin and/or docetaxel, displayed increased cytotoxicity compared to letrozole alone. This was true whether the chemo-endocrine cocktail of drugs were applied sequentially, simultaneously or added-in, although add-in application (letrozole first + chemotherapy added in) was most potent in terms of effect. Furthermore, the addition of doxorubicin, docetaxel or both chemotherapies along with letrozole did not compromise aromatase inhibition by letrozole. If extrapolated into an *in vivo* clinical situation, these data suggest at least no detriment in using letrozole contemporaneously with doxorubicin and/or docetaxel chemotherapy, with the potential clinical convenience of starting letrozole therapy while awaiting the add-in of chemotherapy. Indeed, a combination of letrozole with these agents may have enhanced clinical potential in the neoadjuvant, adjuvant or advanced disease settings. This contrasts with the established clinical trials evidence that concurrent tamoxifen and anthracycline based therapy is not beneficial [25].

Letrozole treatment alone resulted in modest changes in the proteins examined with only HER-2 and VEGF showing downregulated expression. However, given the cross-talk between HER-2 with several signal transduction pathways, alterations in HER-2 expression may underpin the increased cytotoxicity observed with letrozole [27].

When letrozole was added to docetaxel the effects on protein expression were usually additive. In contrast, combining letrozole with doxorubicin gave data dissimilar to that predicted from the drugs applied individually. With either letrozole or doxorubicin, ER-β protein expression was unchanged, but when combined together ER-β levels increased. Again, this highlights the complex cross-talk pathways operating at cellular levels and may also reflect the multiple mechanisms of action of doxorubicin compared with those of docetaxel [28, 29].

Application of both chemotherapies together with letrozole resulted in similar changes in the protein profiles compared to the untreated controls. Given that the proteins examined are primarily involved in cell cycle control or apoptosis, and a substantial reduction in cell viability was observed, this result may not be unexpected. However, this effect was not simply due to the actions of the combined chemotherapies as the protein profiles obtained in the presence of letrozole were different compared to the doxorubicin plus docetaxel regimen. For example, with letrozole + doxorubicin + docetaxel, ER-β levels were increased reflecting a potential role in ER signaling [30, 31].

ER-β is expressed in both normal and malignant breast tissue, although it tends to show lower expression in tumours [30]. Re-introduction of ER-β into ER-α positive breast cancer cells has demonstrated that it has opposing actions to ER-α, causing an anti-proliferative effect [30]. High expression of ER-β has also been correlated with good prognosis and prolonged disease free survival, albeit in response to tamoxifen (reviewed in [31]). Thus, up-regulation of ER-β, by combining letrozole with doxorubicin and docetaxel, may provide one plausible explanation at the molecular level for the enhanced efficacy of this regime.

Combining the AI letrozole with the chemotherapy agents doxorubicin and/or docetaxel *in vitro* enhances the cytotoxic and therapeutic potential. In contrast to the clinical trials experience with tamoxifen, the potential use of letrozole sequentially, or concurrently, with doxorubicin and/or docetaxel chemotherapy within the neoadjuvant, adjuvant and advanced disease setting warrants further investigation in the clinical setting. As a first step, a trial of docetaxol and doxorubicin +/– letrozole for ER positive primary breast cancer in postmenopausal women is attractive to detect the potential enhanced *in vivo* efficacy of adding letrozole to a known active chemotherapeutic regimen. Clinical endpoints of disease response and biomarker studies (informed by the data presented here) would be informative for subsequent adjuvant trials combining letrozole with chemotherapy rather than extrapolating from historic, tamoxifen-based data.

Acknowledgments

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