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RESEARCH PAPER

Increasing doxorubicin activity against breast cancer cells using PPARγ-ligands and by exploiting circadian rhythms

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BACKGROUND AND PURPOSE
Doxorubicin is effective against breast cancer, but its major side effect is cardiotoxicity. The aim of this study was to determine whether the efficacy of doxorubicin on cancer cells could be increased in combination with PPARγ agonists or chrono-optimization by exploiting the diurnal cycle.

EXPERIMENTAL APPROACH
We determined cell toxicity using MCF-7 cancer cells, neonatal rat cardiac myocytes and fibroblasts in this study.

KEY RESULTS
Doxorubicin damages the contractile filaments of cardiac myocytes and affects cardiac fibroblasts by significantly inhibiting collagen production and proliferation at the level of the cell cycle. Cyclin D1 protein levels decreased significantly following doxorubicin treatment indicative of a G_1/S arrest. PPARγ agonists with doxorubicin increased the toxicity to MCF-7 cancer cells without affecting cardiac cells. Rosiglitazone and ciglitazone both enhanced anti-cancer activity when combined with doxorubicin (e.g. 50% cell death for doxorubicin at 0.1 μM compared to 80% cell death when combined with rosiglitazone). Thus, the therapeutic dose of doxorubicin could be reduced by 20-fold through combination with the PPARγ agonists, thereby reducing adverse effects on the heart. The presence of melatonin also significantly increased doxorubicin toxicity, in cardiac fibroblasts (1 μM melatonin) but not in MCF-7 cells.

CONCLUSIONS AND IMPLICATIONS
Our data show, for the first time, that circadian rhythms play an important role in doxorubicin toxicity in the myocardium; doxorubicin should be administered mid-morning, when circulating levels of melatonin are low, and in combination with rosiglitazone to increase therapeutic efficacy in cancer cells while reducing the toxic effects on the heart.

Abbreviations
CI, combination index; Cig, ciglitazone; DOX, doxorubicin; MEL, melatonin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide; Rosi, rosiglitazone; TB, trypan blue; TZD, thiazolidinedione

Introduction

The clinical use of doxorubicin (DOX) when treating breast cancer is compromised by the associated dose-dependent and cumulative myocardial damage that often results in congestive heart failure (Lipshultz *et al.*, 2002). The anti-tumour activity of DOX is primarily due to inhibition of topoisomerase II and the induction of oxidative stress, resulting in cell

necrosis and apoptosis (Minotti *et al.*, 2004). Despite the extensive use of DOX, its mechanisms of cardiotoxicity are yet to be fully explained. Efforts have been made to minimize or prevent this toxic response by, for example, combination with other anti-cancer drugs such as conatumumab (Demetri *et al.*, 2012) or gemcitabine (Sahar *et al.*, 2011) to improve its therapeutic index or by using targeted formulations containing DOX such as the liposomal system, doxil (Fiegl *et al.*, 2011).

The PPARγ-ligands (thiazolidinedione derivatives, TZDs) display anti-tumour activity in various cancers including epithelial (Allred and Kilgore, 2005), prostate (Lyles *et al.*, 2009), gastric and colon (Sato *et al.*, 2000), and lung (Tsubouchi *et al.*, 2000). The anti-proliferative activity of TZDs is receptor-independent and many pathways have been proposed as plausible explanations for this activity. The use of TZDs has been controversial due to conflicting reports concerning possible negative effects on the CVS (Palee *et al.*, 2011a). Ren *et al.* (2009) showed that PPARγ-ligands, such as rosiglitazone (Rosi), protect cardiomyocytes from H_2O_2-induced apoptosis by up-regulating Bcl-2 expression. Rosi has also been shown to reverse the remodelling associated with fibrosis in rats (Torres *et al.*, 2010) and can reduce the size of infarcts in pig hearts (Palee *et al.*, 2011b). Others have shown that Rosi can enhance the efficiency of cardiac contractility (How *et al.*, 2007). Considering the above, it is plausible that combining PPARγ agonists with DOX could reduce cardiac toxicity and enhance the therapeutic index of DOX in cancer cells.

A more recent approach in treating cancer patients is the use of chronotherapy, which exploits the diurnal variation in cell division, tissue turnover and drug metabolism (Innominato *et al.*, 2010). The main mediator of systemic and cellular circadian activity is the light–dark cycle associated with cyclic changes in many circulating hormones. One of the main mediators of circadian activity is the hormone melatonin (MEL), which peaks at the end of the dark period in both diurnal and nocturnal mammals (Ohdo *et al.*, 2011). MEL has therefore been used to mimic the dark period in humans and as a means of treating sleep disorders and jetlag (Altun and Ugur-Altun, 2007). The therapeutic index of many drugs is significantly affected by the time of administration and is of particular significance in cancer therapies (Zmrzljak *et al.*, 2012).

Here, we determine the mechanisms of doxorubicin cardiotoxicity by examining its effects on myocytes and cardiac fibroblasts. We show that doxorubicin damages the contractile filaments, which would lead to a loss of cardiac function. The drug also affects cardiac fibroblasts by inhibiting collagen production and cell proliferation at the level of the cell cycle. By combining PPARγ agonists with doxorubicin, the toxicity to MCF-7 cells can be increased without a concomitant increase in the damage to cardiac cells. Our data show that the dose of doxorubicin could be reduced by 20-fold in combination with the PPARγ agonists during cancer treatment, thereby reducing the toxic effects on the heart. We also show that the presence of melatonin increases doxorubicin toxicity, hence doxorubicin should be given mid-morning when circulating levels of melatonin are low to further reduce cardiotoxicity from DOX treatment.

Methods

Cell culture

MCF-7 human breast cancer cells were kindly provided by the Tenovus Centre for Cancer Research (Cardiff, UK). The cells were cultured in RPMI 1640 containing 5% FBS in a humidified atmosphere (37°C, 5% CO_2, 95% air). Myocytes and fibroblasts were isolated from neonatal rat pups of mixed sexes (2–3 days) by sequential digestion with collagenase, as previously described (Boateng *et al.*, 2003). Briefly, the cells were isolated and then pre-plated to separate the myocytes from non-myocytes before the myocytes were seeded into 6-well plates (500 000 cells per well) coated with fibronectin (25 $\mu M \cdot mL^{-1}$) in PC1 medium for 24 h. The medium was then changed to DMEM: M199 serum-free medium. The fibroblasts were kept in the flasks 3–4 days before seeding into 6-well plates (500 000 cells per well) using DMEM-F12 with 5% FBS. The fibroblasts were kept for 24 h in serum-free medium before starting the different treatments.

General treatment protocol

In preliminary work, we investigated the appropriate dosing and treatment regimens, varying the duration of cell exposure to DOX and altering DOX concentrations. From these studies, it was found that treating cells with DOX for 2 h generated effects that were reproducible, and following which, the effects of subsequent treatments could be discriminated. While some studies applied TDZs for 24 h before DOX dosing, the onset of DOX application was taken as time zero. The total time taken before cell assays were performed [MTT for MCF-7 cells or trypan blue (TB) for cardiac cells] was 72 h after DOX was applied.

Drug cytotoxicity against MCF-7 cells

Cytotoxicity of DOX and of the TZDs, alone and in combination, was assessed by MTT assay as previously described (Greco *et al.*, 2007). Briefly, cells were treated with different concentrations of DOX for 2 h (MCF-7 cells, 0.025–6 μM) and the cells were then washed with PBS (three times, 1 mL). Fresh RPMI supplemented with 5% FBS (i.e. with no DOX) was added and cells were allowed to grow for a further 70 h (72 h in total) before MTT assays were performed (MTT reagents added 5 h before the assay was performed; Greco *et al.*, 2007). For the TDZs, a range of concentrations (1–200 μM) was applied to the cells and were incubated in the presence of these drugs as above. For combination treatments, cells were incubated for 24 h with the TDZ (at the IC_{50} values) before treatment with DOX (also at the IC_{50}) for 2 h. The cells were washed as above and then re-incubated with the TDZ (i.e. no DOX) before MTT assays were performed.

To investigate interactions on combining DOX with the PPARγ-ligands and to assess the combined growth-inhibitory effect at 50% cell death, the combination index (CI) was calculated to determine if the combination resulted in synergism, antagonism or gave an additive interaction (Fischel *et al.*, 2001). The CI is defined by

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}$$

where $(D)_1$ is the concentration of drug necessary to cause a defined effect (50% cell death in our case) in the combina-

tion; $(Dx)_1$ is the concentration of the same drug which will produce the same level of effect by itself (50% cell death); $(D)_2$ is the concentration of the second drug which will produce the particular effect in combination (50% cell death); and $(Dx)_2$ is the concentration of the second drug which produce the same level of effect by itself (50% cell death). CIs > 1 indicate antagonism, CIs < 1 synergism, while CIs = 1 show an additive effect (Fan *et al.*, 2010).

Effects of MEL on drug toxicity against MCF-7 cells

MCF-7 cells were incubated with stripped FBS (sFBS) for 24 h for synchronization. Control cells were then treated with 1 nM oestradiol (E2), while test cells were treated with sFBS plus E2 with DOX (at IC_{50}), or with MEL (1 pM, 1 nM, 1 μM) plus DOX (IC_{50}) for 2 h prior to washing and growth as above.

Drug cytotoxicity against cardiac myocytes and fibroblasts

Myocytes and fibroblasts were treated according to the same protocol used for MCF-7 cells; DOX (0.6–10 μM) was applied for 2 h before washing, growth in fresh DMEM/M199 medium (with no DOX) and then the percentage of viable cells was assessed by TB exclusion. As before, Rosi and ciglitazone (Cig) were added to both cell types at a range of concentrations (2.5–40 μM), while MEL was added to the cells at 1 pM, 1 nM or 1 μM (for 12 h prior to treatments) for 72 h before cell viability was assessed by TB exclusion.

Cytotoxicity of combination treatment

The myocytes and fibroblasts were treated with Rosi or Cig (2.5–20 μM) or MEL (1 pM, 1 nM and 1 μM) for 24 h and then the IC_{50} value of DOX was added for 2 h. Cells were then washed with PBS (three times) and re-incubated with the TDZs or MEL (as with MCF-7 cytotoxicity assessment), and 72 h after the DOX treatment, TB exclusion assays were performed.

Immunocytochemistry and image analysis

After the various treatments, cells for immunocytochemical staining were fixed in 4% paraformaldehyde for 5 min and then covered with 70% ethanol, sealed with parafilm and kept at –20°C. Fixed cells were immunostained with alpha actinin (Abcam, Cambridge, UK) antibody as described previously (Boateng *et al.*, 2003). Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes, Life Technologies Ltd., Paisley, UK) were used to visualize the specific proteins. Fluorescently labelled cells were then viewed using a Leica DMIRE2 laser scanning confocal microscope (Fisher Scientific, Loughborough, UK).

Assessment of sarcomere integrity

Sarcomeres are the basic contractile unit in the myocytes and directly correlate with the efficiency of the myocardium contractility. Immunochemistry analysis was performed on the myocytes to assess sarcomere integrity after treatment with DOX (IC_{50} = 2.6 μM), Rosi (5, 10 and 20 μM) and Cig (5, 10 and 20 μM). The cells were seeded in 6-well plates coated with fibronectin and treatment with the above compounds commenced after 24 h of attachment. Fixation, freezing and immunostaining of the cells was the same as above. The cells were probed for α-actinin with α-actinin mouse antibody and the fluorescent cells were viewed with a Leica TCS SP5 confocal microscopy system. The percentage of intact sarcomeres was assessed relative to the control (untreated controls with media only) group. Three random spots were selected in each slide and intact sarcomeres were counted using a square of 10 μm × 10 μm. The experiments were repeated three times independently on different cultures. Only intact sarcomeres were counted in a region and the treated samples were presented as a percentage of untreated controls.

Western blotting

Cultured cells were scraped from the wells using lysis buffer containing 1% SDS, protease inhibitor and phosphatase inhibitor (Sigma, Poole, UK). The amount of protein present in the cell lysate was assessed using the DC (Bio-Rad, Hercules, CA, USA) protein assay. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (ThermoFisher, Loughborough, UK). The blots were probed for cyclin D1 (Abcam), procollagen (Hybridoma Bank, Iowa City, IA, USA) and actin (Abcam). HRP-conjugated antibodies (anti-mouse–HRP or anti-rabbit–HRP; Research Diagnostics, Inc., Flander, NJ, USA) were used to visualize proteins by enhanced chemiluminescence (ECL; Amersham, Middlesex, UK). Protein bands were standardized to total protein loading relatively to actin by using a Gel-Pro analyzer 4.5.

Statistics

Statistical analysis was carried out against the control group by one-way ANOVA followed by Bonferroni's post hoc test or by Student's *t*-test relative to the control (untreated).

Results

Cytotoxicity of doxorubicin and PPAR-γ ligands against MCF-7 cells

To assess the relative toxicity of DOX and PPARγ-ligands on cancer cells, MCF-7 cells were treated with DOX (Figure 1A), Rosi and Cig (Figure 1B,C); all showed dose-dependent decreases in cell viability compared with the control (untreated) group. The IC_{50} values against MCF-7 cells were: DOX = 0.1 μM; Rosi = 22.3 μM; Cig = 20.5 μM. After assessing the individual cytotoxicities, the activity of PPARγ-ligands in combination with DOX was explored.

Combining DOX with both TDZs enhanced toxicity against MCF-7 cells (Figure 1D). The CI values were significantly lower than 1, indicating synergism between DOX and the TDZs in MCF-7 cells; CI values were: DOX + Rosi = 0.44; DOX + Cig = 0.5.

Cytotoxicity of doxorubicin and PPARγ-ligands in cardiac cells

To assess cardiotoxicity, primary rat cardiac myocytes and fibroblasts were treated with DOX, Rosi and Cig. The results show a dose-dependent decrease in cell viability following DOX treatment in both myocytes (Figure 2A; IC_{50} 2.6 ± 0.4 μM) and fibroblasts (Figure 2B; IC_{50} 2.2 ± 0.6 μM). Rosi did not induce any significant decrease in the viability of either

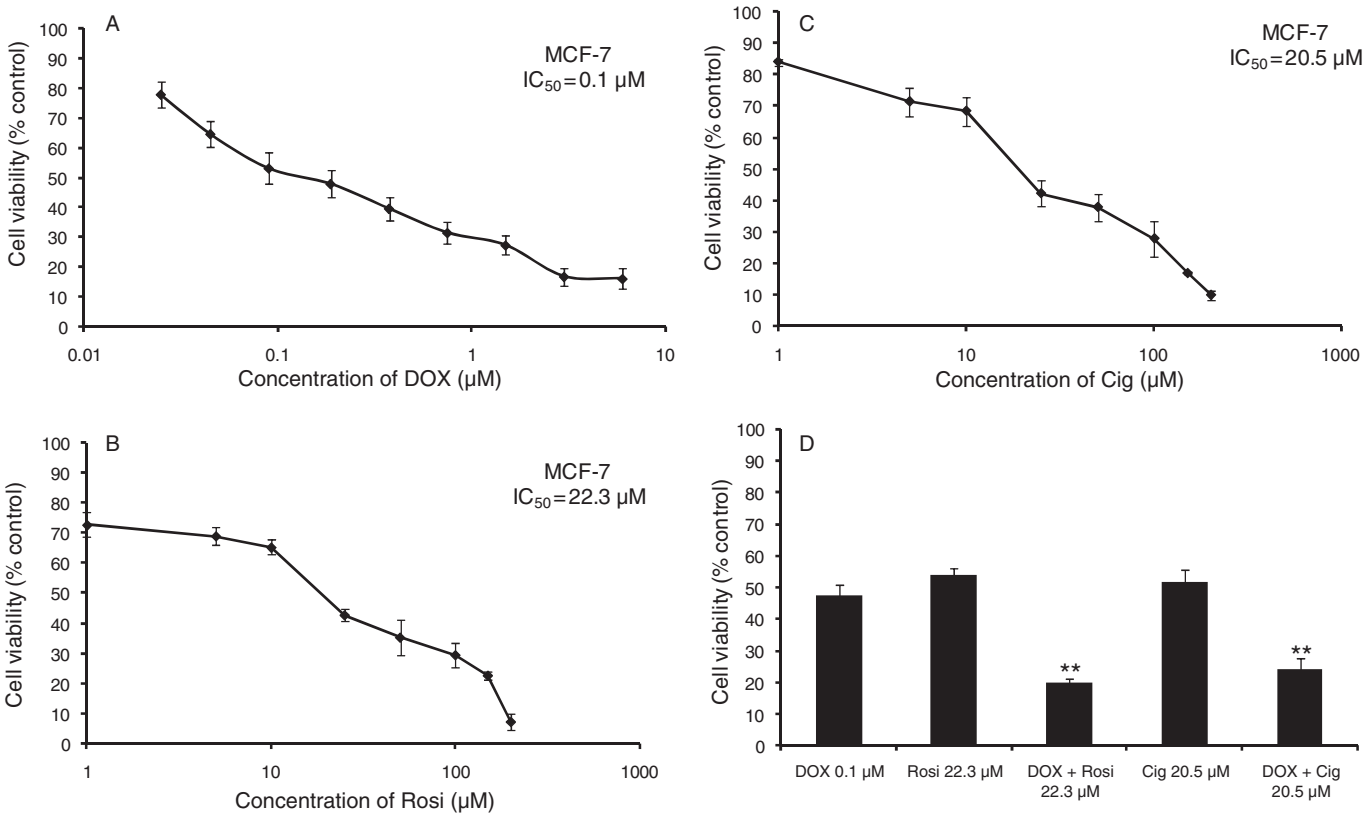

Figure 1

The effect of different concentrations of (A) DOX, (B) Rosi and (C) Cig on the viability of MCF-7 cells using MTT assay. (D) The effect of combining DOX (IC_{50}) with Rosi or Cig (IC_{50} values) on the growth of MCF-7 cells. The data were expressed as mean ± SEM, $n = 3$ independent experiments. Statistical analysis compared DOX with treated groups. $^{**}P < 0.01$.

myocytes (Figure 2C) or fibroblasts (Figure 2D) over all concentrations used but Cig caused a dose-dependent decrease in the viability of myocytes (Figure 2E; IC_{50} 11 ± 0.4 μM) but not fibroblasts (Figure 2F).

PPARγ-ligands potentiate the effects of doxorubicin against MCF-7 cells but not on myocytes

Considering the synergism in toxicity to MCF-7 cells, the effects of Rosi and Cig combined with DOX against cardiac cells was explored. As described earlier, cardiac cells were initially treated with the TDZs (at 2.5, 5, 10 or 20 μM) for 24 h before DOX was included at the IC_{50} (2.6 μM) for 2 h. Following washing, the cells were re-incubated with the TDZs (with no DOX). As can be seen from Figure 2G,H, cardiac cells treated with DOX at the IC_{50} resulted in the expected 50% cell viability. Combining DOX with Rosi did not change the viability of either myocytes or fibroblasts at all concentration used (Figure 2G), consistent with the data in Figure 2C,D which showed that Rosi alone had no influence on cell viability. In contrast, using Cig alongside DOX potentiated cytotoxicity in both myocytes and fibroblasts (Figure 2H). In myocytes, combining DOX with 20 μM Cig significantly enhanced cytotoxicity (only 25.4% cell viability) compared with DOX alone (Figure 2H; $P < 0.05$),while in fibroblasts, the inclusion of both 10 and 20 μM Cig significantly potentiated cytotoxicity (22.7 and 27.3% viability, respectively, Figure 2H; $P < 0.01$).

Doxorubicin effects on myocyte myofilament and sarcomere integrity

The above cytotoxicity studies determine cell death but significant numbers of myocytes remain after drug treatment. To determine the potential function of the remaining myocytes post-treatment, cell morphology was investigated for structural damage. In myocytes, the striated sarcomere is the smallest unit of contraction and is the site at which contractile force is generated. Any loss of sarcomeres would reflect a potential loss of contractile function as a result of the drug treatments. Sarcomere integrity was analysed by confocal microscopy following immunocytostaining for the protein α-actinin antibody. Figure 3A–D shows myocytes without and with doxorubicin, rosiglitazone and ciglitazone treatment. The percentage of intact sarcomeres compared with untreated controls is shown in Figure 3E,F. DOX at 2.6 μM (IC_{50}) significantly reduced the number of intact sarcomeres by 26% ($P < 0.05$) in the remaining viable cells. Rosi alone (5–20 μM) did not adversely affect myocyte sarcomere integrity, while Cig reduced sarcomere numbers at 20 μM but not at 5 or 10 μM. Combinations of DOX with Rosi or Cig only

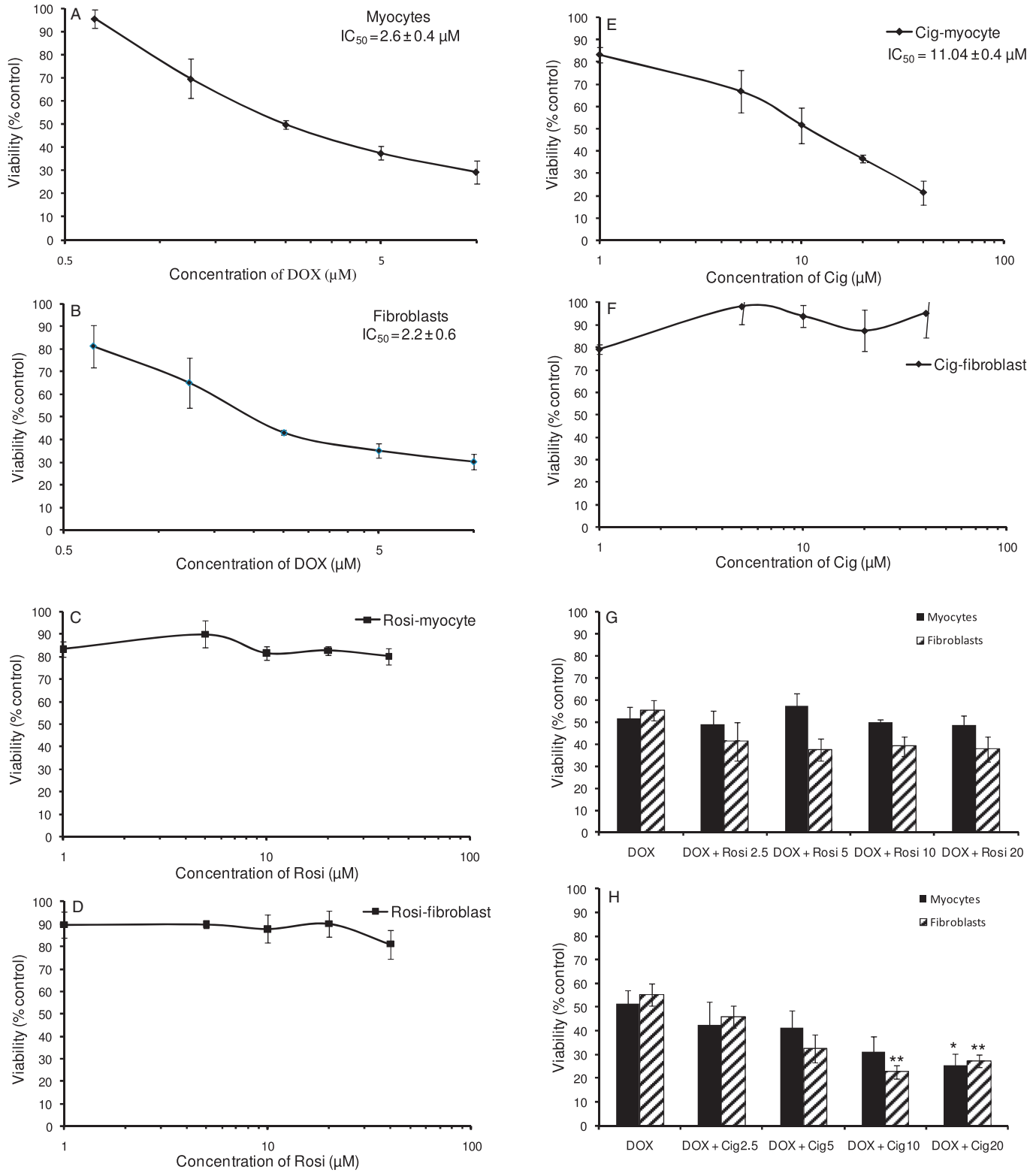

Figure 2

The effect of (A) DOX on myocytes, (B) DOX on fibroblasts, (C) Rosi on myocytes, (D) Rosi on fibroblasts, (E) Cig on myocytes, (F) Cig on fibroblasts and the effect of combining DOX (IC_{50}) with Rosi and Cig (2.5 μM, 5 μM, 10 μM, 20 μM) and (G and H, respectively) on the viability of myocytes and fibroblasts. Each value represents the mean ± SEM (n = 6 cultures) by using TB exclusion after 72 h of the treatment compared with DOX alone. The x-axis is logarithmic. Statistical analysis carried out by one-way ANOVA test followed by Bonferroni's post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

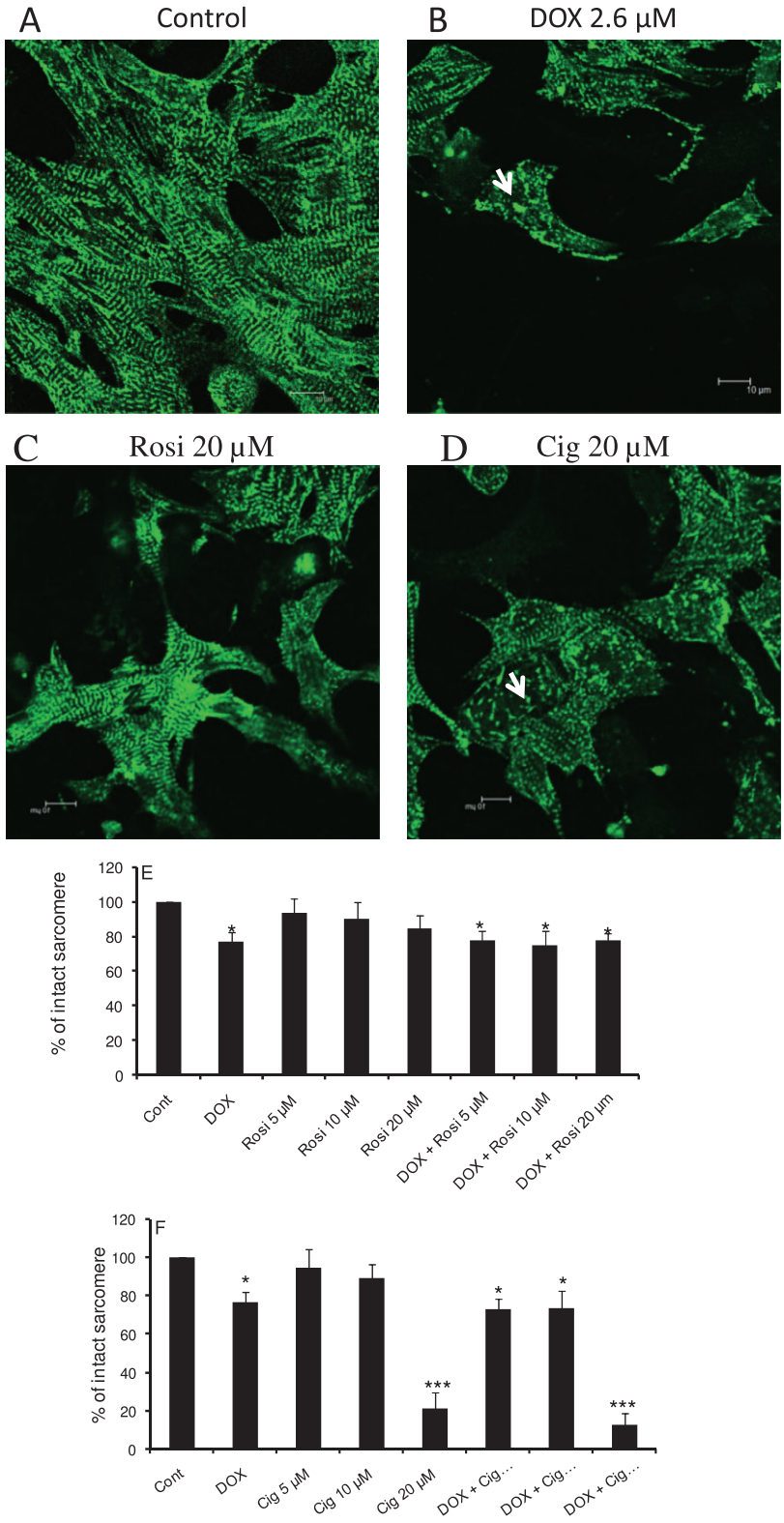

Figure 3

The integrity of sarcomeres in the myocytes after 24 h of the treatment observed by staining for alpha actinin. Cells were visualized with confocal microscopy. (A) Control untreated, or with (B) DOX (IC_{50} = 2.6 µM), (C) Rosi (20 µM), (D) Cig (20 µM). The white arrows highlight the damaged sarcomeres. The percentage of intact sarcomeres in the myocytes after 24 h of the treatment with DOX (IC_{50} = 2.6 µM) with (E) Rosi (5, 10 and 20 µM) and (F) Cig (5, 10 and 20 µM). The data are expressed as mean ± SEM, n = 9 cultures. Statistical analysis carried out by one-way ANOVA test followed by Bonferroni's post hoc test compared with the control (untreated) group. *$P < 0.05$, **$P < 0.01$.

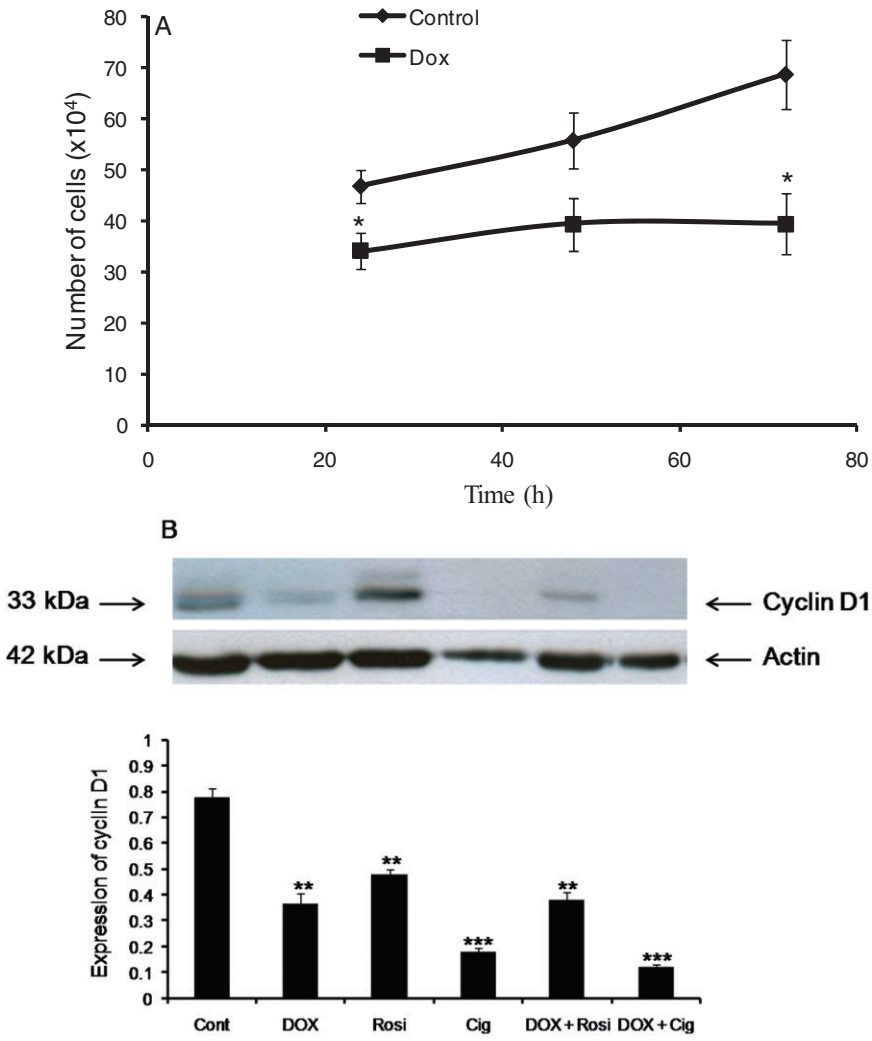

Figure 4

(A) The growth of fibroblasts after treatment with the IC_{50} of DOX (2.2 μM). Data were expressed as the mean ± SEM, $n = 6$ cultures by using TB exclusion. Control (untreated) vs the treated group (*$P < 0.05$). (B) The effect of DOX (IC_{50}), Rosi (20 μM) and Cig (20 μM) on the expression of cyclin D1 by fibroblasts. Data (normalized to actin protein) are expressed as mean ± SEM ($n = 3$ cultures). Statistical analysis carried out by one-way ANOVA test followed by Bonferroni's post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

reduced sarcomere integrities to the same extent as DOX alone, illustrating no synergistic adverse effects.

Influence of doxorubicin and PPARγ-ligands on the cell cycle of cardiac fibroblasts

To determine whether the reduced number of fibroblasts present following DOX treatment (at 2.2 μM, IC_{50}) was due to cell death or reduced cell proliferation, cells were counted at 24, 48 and 96 h after DOX treatment; as shown in Figure 4A, the drug inhibited cell proliferation, suggesting that the cells had become cytostatic. To determine whether inhibition of fibroblast proliferation was regulated at the level of the cell cycle, we measured cyclin D1 levels in fibroblasts after treatment with DOX, Rosi and Cig by Western blotting. All treatments significantly reduced the levels of cyclin D1 compared with untreated controls (Figure 4B). The combination of DOX with the TDZs decreased the levels of cyclin D1, suggesting a direct effect on the cell cycle.

Influence of doxorubicin on cardiac fibroblast pro-collagen expression

The expression and secretion of collagen is one of the main activities of cardiac fibroblasts and so changes to these cells will be reflected in their ability to synthesize this extracellular matrix protein. To assess collagen production by cardiac fibroblasts, we measured the precursor pro-collagen by Western blotting, as shown in Figure 5. The data illustrate that treatment with the IC_{50} for DOX resulted in almost no procollagen being detected, demonstrating severe inhibition of cell function and activity. Rosi did not affect procollagen synthesis at low concentrations, but at 20 μM, it decreased the protein synthesis by 50% ($P < 0.01$); but treatment

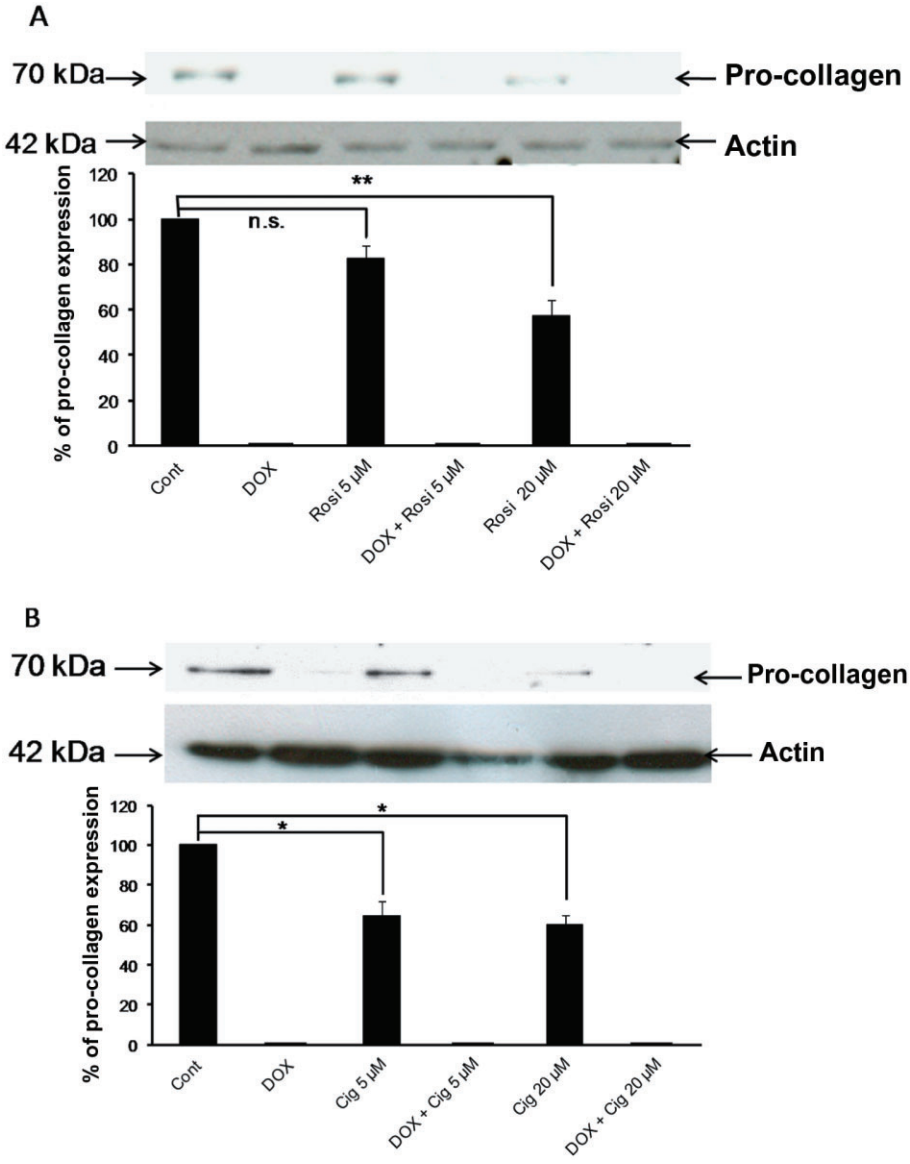

Figure 5

The expression of procollagen by fibroblasts after the treatment with DOX (IC_{50} = 2.2 µM) combined with (A) Rosi (5 µM, 20 µM) and (B) Cig (5 µM, 20 µM). The data (normalized to actin) expressed as the mean ± SEM, n = 3 cultures by using Western blotting analysis. Statistical analysis carried out by one-way ANOVA test followed by Bonferroni's post hoc test compared with the control untreated group. *P < 0.05, **P < 0.01.

with Cig at 5 µM, procollagen levels decreased by 40% (P < 0.05).

Melatonin regulates doxorubicin toxicity in cardiac cells

Chronotherapy is an increasingly important factor to consider in treatment regimens as circadian biology influences drug pharmacokinetics and hence therapeutic dose and toxicity *in vivo* (Innominato *et al.*, 2010). It is known that cells maintain a circadian clock that can be regulated by drug or hormonal treatment (Ohdo *et al.*, 2011). To mimic the diurnal cycle, cells were treated with different doses of MEL prior to drug treatment to assess whether this would alter the cellular toxicity of DOX. In MCF-7 cells, MEL had no effect on DOX cell toxicity when compared to cells treated with DOX but no MEL (Figure 6A). However, in cardiac fibroblasts, MEL at both 1 and 10 µM potentiated the effects of DOX toxicity (Figure 6B; P < 0.05, P < 0.01 respectively). In contrast, for cardiac myocytes, MEL did not potentiate the effects of DOX in myocytes (Figure 6B), suggesting that it is the cardiac fibroblasts that are most sensitive to the presence of this circadian hormone.

Discussion

Cardiotoxicity of DOX, resulting in congestive heart failure and left ventricular dysfunction, has been reported over the

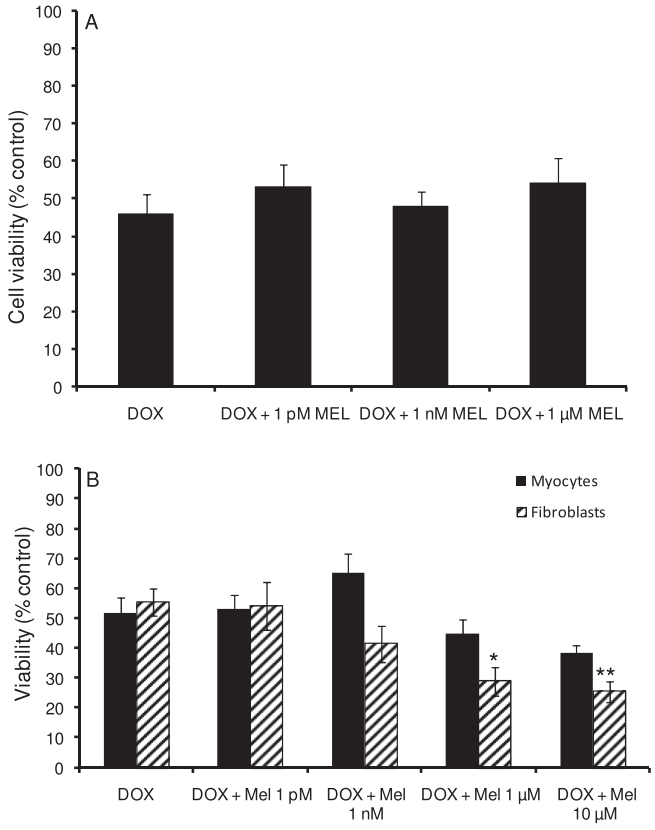

Figure 6

(A) The effect of DOX (IC_{50}) and MEL on the viability of MCF-7 cells. Data are expressed as mean ± SEM (n = 6 cultures) by using MTT assay after 72 h of the treatment. Cells were treated with MEL for 12 h prior to DOX treatment. (B) Effect of DOX (IC_{50}) and MEL on the viability of myocytes and fibroblasts. Statistical analysis carried out by one-way ANOVA test followed by Bonferroni's post hoc test compared with the DOX-treated group. *P < 0.05, **P < 0.01.

last few decades and presents a major obstacle to the clinical use of this drug for the treatment of cancer (Yeh *et al.*, 2004). Our results show for the first time that the combination of PPARγ agonist Rosi with DOX can significantly increase the toxicity of the drug towards MCF-7 cancers cells without concomitant adverse effects in cardiac cells. Cig also potentiates the effects of DOX on MCF-7 cells, but at relatively high doses that also adversely affect cardiac cells. We also found that MEL potentiates DOX toxicity in cardiac fibroblasts but not in MCF-7 cells. These results show that a combination of DOX and Rosi, given when circulating levels of melatonin are low, would provide the most effective treatment for patients with breast cancer. This combination and timing allows significantly lower doses of DOX to be used to obtain the equivalent desired toxicity against the cancer cells while reducing cardiotoxic effects in patients.

The IC_{50} for MCF-7 cells treated with DOX was comparable to the values reported by others (Fornari *et al.*, 1996) and is comparable to the clinical peak plasma concentration of ~5 µM (Gewirtz, 1999; Minotti *et al.*, 2004). The IC_{50} for DOX on the cardiac cells *in vitro* is also close to the peak plasma concentration observed clinically and explains why these concentrations are cardiotoxic. Clinical treatment that used a combination with Rosi would enable the DOX dose to be reduced by 20-fold, thereby significantly reducing cardiotoxicity. DOX induces necrosis, apoptosis, autophagy and mitotic errors in cancer cells through increased oxidative stress induced by free radical production (Oz *et al.*, 2006). The cardiotoxicity of DOX has been investigated previously (Minotti *et al.*, 2004; Oz *et al.*, 2006), but our present work is the first to consider toxicity and mechanisms towards both myocytes and fibroblasts; previous studies have only examined the cardiotoxicity of DOX on cardiac myocytes. Although myocytes are the source of contractile activity, fibroblasts are the more numerous cells and they provide a suitable environment for the myocytes that maintain heart function.

The mechanism of DOX toxicity in myocytes is reportedly through apoptosis, necrosis and autophagy (Kostin *et al.*, 2003), primarily correlated with the induction of oxidative stress (Minotti *et al.*, 2004). Our work shows that the integrity of the contractile filaments is compromised on exposure to doxorubicin; in the heart, loss of sarcomeres decreases contractile function and leads to subsequent heart failure. These findings are in agreement with previous studies that reported a decrease in myofibrillar bundles (Arola *et al.*, 2000) and degradation of sarcomeric titin (Lim *et al.*, 2004) following DOX treatment. The myofilaments are the site at which contractile force is generated and any loss or damage would impair cardiac function. DOX also damages mitochondria by binding to cardiolipin, a lipid involved in mitochondrial function (Minotti *et al.*, 2004). Here, we show for the first time that DOX also inhibits cardiac fibroblasts at the level of the cell cycle by decreasing cyclin D1 protein expression, indicative of arrest in the G_1/S phase of the cycle. It was also important to assess the function of the surviving fibroblasts for which we measured procollagen, a precursor of the extracellular matrix protein collagen. Procollagen levels were significantly decreased by DOX treatment, suggesting a functional loss in these cells. Collagen production is a valuable measure of cardiac fibroblast function as this is one of their major roles in the heart (Camelliti *et al.*, 2005). Decreased collagen synthesis suggests that other functions of the cardiac fibroblast may also be impaired by DOX treatment.

A difference in anti-proliferative activity was observed between Rosi and Cig and correlated with the differences present in the molecular mechanisms of Rosi and Cig (Kim *et al.*, 2006). The anti-proliferative activity of Cig was due to disruption of the oestrogen receptor α (ERα) signalling through proteosomal degradation, whereas Rosi operated through a different mechanism (Lecomte *et al.*, 2008). Our results show that both Rosi and Cig when combined with DOX significantly increased toxicity in MCF-7 cells. However, at higher concentrations, Cig combined with DOX adversely affect cardiac cells, whereas Rosi had no such additional effects. The TZD derivatives, including Rosi, regulate glucose and lipid homeostasis and are involved in cell differentiation (Weng *et al.*, 2006). Other studies have shown that the TZDs have anti-proliferative activity through apoptosis, and inhibition of histone deacetylase activity and the ERα pathway (Kim *et al.*, 2006). Moreover, the TZDs are reported to be cardioprotective by reducing harmful ventricular remodelling

and gene expression, in part through effects on the NF-κB pathway (Liang *et al.*, 2003). The mechanism for increased Cig cardiotoxicity compared with Rosi is unclear at present but could be due to structural differences or differential uptake by cardiac cells. In MCF-7 cells, our study showed synergism in toxicity between DOX and Rosi with a CI that was significantly less than 1. The degree of synergism has major clinical implications as the concentration (hence administered dose) of DOX could be reduced 20-fold while producing the same degree of toxicity when combined with Rosi. Clearly, that the IC_{50} values for DOX in MCF-7 cells and cardiac cells differ, but our work highlights the importance of measuring cell function as well as cytotoxicity. However, a 20-fold reduction in DOX dose would protect the heart significantly while maintaining cytotoxicity against the cancer cells. Very high concentrations of Rosi did reduce both cyclin D1 and procollagen in cardiac fibroblasts but this is above the clinical plasma TZD concentrations of 1–7 μM used clinically for non–insulin-dependent diabetes mellitus (Sahi *et al.*, 2003). The effect of TZDs on cyclin D1 expression is thought to be due to increased ubiquitination and subsequent proteosome-dependent degradation (Alao, 2007). Some side effects on other highly proliferative cells, such as fibroblasts, are likely to be an unavoidable consequence of this type of cancer therapy, although our experiments would have magnified these effects. *In vivo*, cardiac fibroblasts divide less rapidly than they do *in vitro* due to contact inhibition and so would be affected to a lesser degree than the rapidly dividing cancer cells (Boateng *et al.*, 2003).

Importantly, we have also shown that the diurnal cycle influences the cellular toxicity of DOX. Previous work supports our finding where DOX was best tolerated during the rest period in mice (Granda *et al.*, 2001). However, in nocturnal mammals like rodents, the sleeping period coincides with daylight, while in human, it coincides with the active period (Liu *et al.*, 2007). However, in both mammals, melatonin levels are regulated by light and always peak at the end of the dark phase regardless of whether the animal is active or not (Altun and Ugur-Altun, 2007). This evidence indicates that it is the presence of melatonin that determines cellular toxicity rather than the phase of activity. In mammals, MEL has been shown to coordinate the phase of the peripheral circadian cellular clocks including those in the heart (Hardeland, 2009). This circadian timing directly and indirectly regulates endobiotic and xenobiotic detoxification pathways that alter drug pharmacokinetics and toxicity (Zmrzljak *et al.*, 2012). Tamoxifen has been shown to be more effective in the treatment of breast cancer when used in combination with MEL (García *et al.*, 1998). Melatonin signalling through GPCRs MT1 and MT2 leads to effects on cellular cAMP, activation of PL C and PK C. This, in turn, modulates MAPK, PI3K and Akt pathways, leading to effects on cell growth, proliferation and metabolism (Hardeland, 2009). Circadian signalling is complex and MEL is one of a number of hormones that coordinate biology rhythms. Our findings show that DOX should be administered when melatonin levels are at their minimum, which in human corresponds to mid-morning.

In conclusion, we suggest two approaches to improve the therapeutic index of DOX in treating of breast cancer. Firstly, we demonstrate that DOX activity against breast cancer cells can be significantly increased when used in combination with the TDZs Rosi and Cig (Rosi being the preferred adjunct therapy due to its lower cardiotoxicity), with no additional toxicity on the myocardium. Secondly, the cardiotoxic effects of DOX can be reduced by administering the drug mid-morning when plasma melatonin levels are at their lowest. These simple strategies may improve drug efficacy during treatment while reducing potential cardiovascular side effects by allowing DOX to be used at significantly lower concentrations.

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Conflict of interest

The authors of this manuscript have no conflicts of interest to declare.

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