

Increased anti-tumour effects of doxorubicin and zoledronic acid in prostate cancer cells in vitro: supporting the benefits of combination therapy

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Received: 17 June 2009 / Accepted: 30 July 2009 / Published online: 3 September 2009
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Abstract

Purpose Combination treatment using the chemotherapy drug doxorubicin and the anti-resorptive agent zoledronic acid has shown to be very effective in inducing apoptosis in breast cancer cells, and also to eradicate breast tumour growth in vivo. Here, we investigated whether apoptotic cell death is increased when zoledronic acid and doxorubicin are given in sequence or in combination in prostate cancer cells in vitro.

Methods PC3, DU145 and LNCaP prostate cancer cells were treated with zoledronic acid or doxorubicin alone, in sequence or in combination, and apoptosis was measured by evaluation of nuclear morphology following staining with Hoechst and PI. The involvement of the mevalonate pathway in the induction of apoptosis was assessed through the addition of the mevalonate pathway intermediate geranylgeraniol.

Results Both agents induced PC3 cell death, with 5 μ M zoledronic acid inducing 1.73% apoptosis and 50 nM doxorubicin 3.60% apoptosis following 24 h of exposure. In contrast, sequential exposure (doxorubicin followed by zoledronic acid) caused 8.87% apoptosis. Doxorubicin followed by zoledronic acid induced 4.77% apoptosis in LNCaP cells, compared to 1.53% caused by zol alone, 2.23% by dox alone and 2.5% following the reverse sequence ($P < 0.001$ in

all cases). In DU145 cells doxorubicin followed by zoledronic acid induced 5.73% apoptosis, compared to 1.8% following zol alone, 2.93% by dox alone, and 3.20% following the reverse sequence ($P < 0.001$ in all cases).

Conclusions This is the first detailed study to show that an increased anti-tumour effect is generated when doxorubicin and zoledronic acid are given in sequence in both hormone-sensitive and insensitive prostate cancer cells in vitro. Our results suggest that combined treatment with these agents is superior to single agent therapy, and should be explored in a tumour model of prostate cancer.

Keywords Prostate cancer · Chemotherapy · Combination therapy · Bisphosphonates

Abbreviations

BPs Bisphosphonates
FPP Farnesyl diphosphate
GGPP Geranylgeranyl diphosphate
N-BPs Nitrogen-containing bisphosphonates

Introduction

Prostate cancer is the most common cancer in men in the UK, with approximately 35,500 men diagnosed annually. Localised prostate cancer can be successfully treated with a combination of surgery, androgen ablation and radiotherapy, but 20–30% of patients will present with metastatic disease. Prostate cancer frequently spreads to the skeleton, resulting in considerable morbidity and mortality, and at this stage only palliative treatment is available [1]. As single agent therapy has proven inadequate in the treatment of cancer, there is increasing use of combination therapy where one agent targets the tumour cells and the other agent

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modifies the tumour microenvironment, thereby mounting a two-pronged attack on developing tumours. This may also be a treatment option for prostate cancer patients with metastatic bone disease. Zoledronic acid is a potent third-generation nitrogen-containing bisphosphonate, approved for use in patients with cancer-induced bone disease from prostate cancer. Although shown to reduce skeletal-related events and improve quality of life, zoledronic acid does not increase survival of patients with advanced cancer [2]. Recent studies using *in vivo* models of breast cancer have shown that sequential treatment with doxorubicin followed by zoledronic acid reduces tumour growth both in peripheral sites [3] and in bone [4], leading to increased survival. We therefore investigated the potential for increased anti-tumour effect of combination treatment in prostate cancer cells *in vitro*, to establish whether a similar treatment regimen may also potentially be beneficial in prostate cancer.

Bisphosphonates are a group of stable synthetic analogues of pyrophosphate that possess a strong affinity for bone. Based on their molecular mechanism of action, they can be separated into two groups, the non-nitrogen containing bisphosphonates (BPs) and the nitrogen-containing bisphosphonates (N-BPs). N-BPs reduce osteoclastic bone resorption through inhibition of the mevalonate pathway, which is an important and diverse metabolic pathway in eukaryotic cells, which is principally involved in the synthesis of cholesterol [5]. N-BPs like zoledronic acid have been shown to inhibit the enzyme farnesyl diphosphate synthase [6], leading to the decreased production of the isoprenoid lipids farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) which are required for the prenylation of small GTP-ases [7]. Loss of prenylation impairs membrane localisation of a number of these GTP-ases required for osteoclast function, eventually leading to osteoclast apoptosis [2]. There is increasing preclinical data suggesting that in addition to their well-documented effect on osteoclasts, N-BPs may have a direct inhibitory effect on a range of cancer cell types [reviewed in 8]. These anti-tumour effects include the induction of apoptosis and inhibition of cellular proliferation [9–11], the inhibition of cellular adhesion, invasion and migration [12–14] and the inhibition of angiogenesis [15–17]. Zoledronic acid has been shown by numerous studies to be the most potent inhibitor of bone resorption among N-BPs [18], and this potency is reflected in its anti-tumour effects.

Zoledronic acid is increasingly given to patients with bone metastases to prevent skeletal related events that occur as a consequence of cancer-induced bone disease. These patients may also be treated with chemotherapy agents to target the tumour cells. The effect of combining zoledronic acid with anti-cancer agents has been investigated in a number of cancer cell lines. Zoledronic acid has been shown to have an anti-tumour effect, including

increased tumour cell death and inhibition of cell proliferation, when given with numerous chemotherapy agents [reviewed in 19], including doxorubicin in breast cancer cells [20], docetaxel in prostate cancer cells [21] and dexamethasone in myeloma cells [22] *in vitro*. Zoledronic acid has been shown to enhance the effects of docetaxel on prostate cancer cell growth in bone *in vivo*, causing significant reduced tumour growth compared to that achieved with single agent therapy [23].

Doxorubicin belongs to the anthracycline group of chemotherapeutic drugs that is commonly used to treat early and metastatic breast cancers [24]. The mechanism of action of doxorubicin is complex, but involves DNA damage occurring predominantly in the G2 phase [25, 26]. Proposed mechanisms of cytotoxicity include the induction of DNA double strand breaks, interference with topoisomerase II [27], interference with DNA unwinding [28], induction of differentiation [29] and the generation of free radicals [30]. Doxorubicin has been used in the treatment of hormone-refractory prostate cancer [31], but owing to poor response rate and problems with adverse events, this is currently not the standard therapy [32]. There is a persistent need to improve the therapeutic options for prostate cancer, and anthracyclins are considered as possible options as part or combinations with other anti-cancer agents.

As both breast and prostate cancer have a preference for metastasising to bone, it is logical to transfer positive findings from the studies of advanced breast cancer to the prostate cancer setting. Patients with metastatic breast cancer to bone may receive both zoledronic acid and doxorubicin as part of therapy. Early *in vitro* studies revealed that administration of doxorubicin, followed by zoledronic acid, induced a synergistic anti-tumour effect in breast cancer cell lines at doses where the individual agents had no significant effect [20]. The sequence in which the drugs were given was found to be very important, as giving the two drugs together, or in reverse sequence (zoledronic acid followed by doxorubicin), did not cause increased levels of tumour cell apoptosis. In this original study, PC3 prostate cancer cells were reported to be more sensitive to the sequential therapy than the breast cancer cell lines. Subsequent studies showed that treatment with doxorubicin followed 24 h later by zoledronic acid eliminated breast tumour growth *in vivo* [3], supporting that sequential therapy elicits superior anti-tumour effects compared to either combined therapy or administration of the single agents.

The purpose of this study was therefore to carry out a detailed investigation of the effects of zoledronic acid and doxorubicin, given alone, in combination and in sequence, on apoptosis in different prostate cancer cells *in vitro*. The possible mechanism of action of zoledronic acid's anti-tumour effect when given with doxorubicin was also investigated.

Materials and methods

Cell lines

The hormone-dependent LNCaP and hormone-independent Du145 and PC3 prostate cancer cell lines were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). PC3 cells were cultured in RPMI-1640 media, supplemented with 10% Foetal Calf Serum (FCS). LNCaP and Du145 cells were cultured in DMEM media, supplemented with 10% FCS (Gibco, Invitrogen Corporation, Paisley, UK). Cells were harvested using trypsin (PAA laboratories).

Drugs and chemicals

Zoledronic acid ([1-hydroxy-2-(1H-imidazol-1-yl) ethylidene] bisphosphonic acid) was supplied as the hydrated di-sodium salt by Novartis Pharma AG, Basel, Switzerland. A stock solution of 10 mM was prepared in PBS (Invitrogen Life Technologies). Doxorubicin (adriamycin hydrochloride) was supplied by Sigma-Aldrich, Poole, UK as a dry powder. A stock solution of 10 mM was made up with sterile water. Geranylgeraniol (all *trans*-3,7,11-15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol) (GGOH) was supplied by Sigma-Aldrich. All drugs and chemicals used in experiments were diluted in culture medium and sterile filtered (using a 0.13-mm filter) prior to use.

Treatment of cells

PC3, Du145 and LNCaP cells were seeded at a density of 20,000 cells/ml per well into 24-well plates and allowed to adhere to the bottom of the wells for 24 h before the beginning of treatment. For the single agent experiments, cells were exposed to increasing doses of zoledronic acid or doxorubicin for 24, 48 or 72 h. DMEM medium (+10% FCS) was used as a control for the Du145 and LNCaP cell lines and RPMI medium (+10% FCS) for the PC3 cell line. For the short exposure period experiments, cells were exposed to 25 μ M zoledronic acid for 2, 4 or 8 h. Following completion of treatment, the cells were incubated with fresh medium for the remainder of the 72-h incubation period. In the sequence experiments, five different treatment regimes were used: (1) 5 μ M zoledronic acid for 4 h, which was removed and replaced with fresh medium for 24 h; (2) 50 nM doxorubicin for 24 h, followed by fresh medium for 4 h; (3) 5 μ M zoledronic acid for 4 h, followed by 50 nM doxorubicin for 24 h; (4) 50 nM doxorubicin for 24 h, followed by 5 μ M zoledronic acid for 4 h; (5) 5 μ M zoledronic acid and 50 nM doxorubicin in combination for 28 h. Medium alone was used as a control. Following completion of treatment, drug solutions were removed from the wells,

and the cells were incubated with fresh medium for 44 h, to give a total incubation time of 72 h. For the sequence experiments in which GGOH was added, five different treatment regimes were used: (1) 5 μ M zoledronic acid for 24 h, which was removed and replaced with RPMI medium (+10% FCS) for 24 h; (2) 50 nM doxorubicin for 24 h, followed by RPMI medium (+10% FCS) for 24 h; (3) 50 nM doxorubicin for 24 h, followed by 5 μ M zoledronic acid for 24 h; (4) 50 nM doxorubicin for 24 h, followed by 5 μ M zoledronic acid and 10 μ M GGOH in combination for 24 h; (5) RPMI medium (+10% FCS) for 24 h, followed by 10 μ M GGOH for 24 h. RPMI (+10% FCS) was used as control. Following completion of treatment, drug solutions were removed from the wells, and the cells were incubated with fresh RPMI medium (+10% FCS) for 24 h, to give a total incubation time of 72 h.

Apoptosis assay

Following completion of the treatment with the drug(s) as indicated, 1 ml of RPMI culture medium (+10% FCS) with Hoechst 33342 (Sigma-Aldrich, Poole, UK) and propidium iodide (Molecular Probes, Cambridge, UK) (final concentration of 1 μ M) was added to each well followed by incubation for 15 min at 37°C. Cells were then examined using a fluorescent-inverted Leica DMIRB microscope and a UV filter (355-nm excitation and 465-nm emission), and PI negative cells determined to be apoptotic based on their characteristic nuclear morphology and presence of apoptotic bodies. PI positive cells were considered to be necrotic, but other forms of cell death leading to loss of membrane integrity are included in this category. The number of viable, apoptotic, necrotic and mitotic cells was determined by counting using a Whipple graticule in five different locations in each of the three wells for each treatment, containing between 250 and 300 cells. Each experiment was then repeated a minimum of three times ensuring that a minimum of 750 cells were counted in each treatment group. Regular assessment by a second operator ensured consistency of scoring, and the counting was carried out in a blinded fashion with respect to the treatment.

Statistical analysis

Statistical analysis was performed using Graph pad Prism software, version 5. For comparison between means with multiple time points, two-way analysis of variance (ANOVA) was used with a Bonferroni post-test. For comparison between means, one-way analysis of variance (ANOVA) was used with post-hoc analysis using Tukey's multiple comparison tests. In all the cases, a *P*-value of <0.05 was considered significant.

Results

Effect of zoledronic acid on PC3 cells after 24, 48 or 72 h of exposure

Zoledronic acid has been shown in previous studies to induce apoptosis in PC3 cells at high doses of 100 μM after exposure periods of 24 h or greater [10, 33]. Before investigating the effect of doxorubicin and zoledronic acid given in sequence/combination, the effect of zoledronic acid alone on prostate cancer cells was determined. PC3 cells were treated with zoledronic acid (0–5 μM) for 24, 48 or 72 h, and the percentage of apoptotic and necrotic cells was assessed by evaluation of nuclear morphology as described in the materials/methods section. Treatment of PC3 cells with 2.5 μM zoledronic acid for 24 h induced a significantly raised percentage of both apoptotic cells (1.93% vs. 0.53%, $P < 0.05$, Fig. 1) and necrotic cells after 24 h (4.2% vs. 0.47%, $P < 0.05$, Fig. 1) compared to control. The 2.5 μM zoledronic acid also induced significant levels of apoptosis, but not necrosis, after 48 and 72 h (2.1% and 2.67% vs. 0.47%, $P < 0.05$ and 0.001). There was no significant difference in the levels of apoptosis induced at the different time points. These results show that 2.5 μM zoledronic acid is able to induce significant levels of apoptosis in PC3 cells within 24 h of exposure and that incubation beyond this time does not lead to further induction of apoptosis. The effect of zoledronic acid on DU145 and LNCaP cells was determined in separate experiments, which showed that 5 μM for 24 h induced around 4.5% apoptosis in DU145 and 3% apoptosis in LNCaP cells (data not shown). Based on these results, 5 μM zoledronic acid was chosen as the standard dose for the combination experiments with doxorubicin.

Effect of short-term exposure to zoledronic acid in PC3 cells

As the half-life of zoledronic acid in the circulation is 1–2 h following intravenous infusion, tumour cells outside of bone will not be exposed to the drug for long periods of time. In order to determine whether zoledronic acid also had an effect following shorter exposure times, the levels of apoptosis and necrosis in PC3 cells after 2, 4 and 8 h of exposure to 25 μM zoledronic acid was investigated. 25 μM zoledronic acid was found to induce a significantly raised level of apoptosis in PC3 cells after 2 h (1.9% vs. 0.67%, $P < 0.05$, Fig. 2), which did not increase further after 4 h of exposure. The levels of necrosis were not significantly elevated compared to control at any of the time points (data not shown). These results show that 25 μM zoledronic acid is able to induce apoptosis in PC3 cells after short exposure periods.

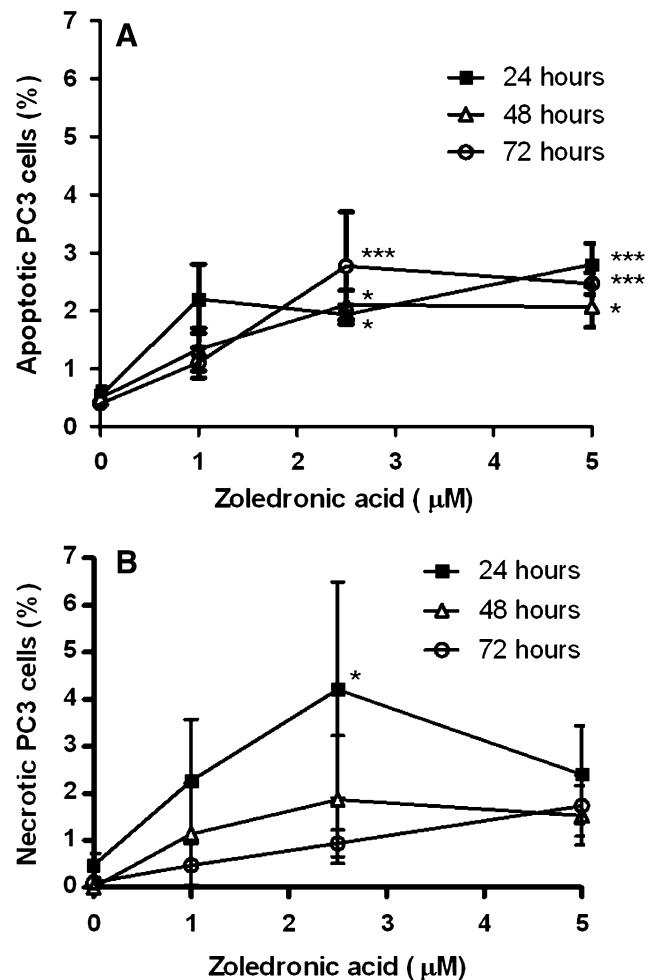


Fig. 1 Effect of zoledronic acid on PC3 cells after 24, 48 or 72 h of exposure. PC3 cells were treated with zoledronic acid (0–5 μM) for either 24, 48 or 72 h, and the percentage of apoptotic (a) and necrotic cells (b) was determined by evaluation of nuclear morphology following staining with hoechst 33342 and propidium iodide. Results are the mean \pm SEM of three experiments in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control

Effect of doxorubicin on PC3 cell death

The effect of doxorubicin on PC3 cells has not been investigated in previous studies. In order to establish the effect of doxorubicin alone on prostate cancer cells, PC3 cells were treated with increasing concentrations of doxorubicin (0–100 nM) for 24, 48 or 72 h. Following completion of treatment, the nuclear morphology of the cells was assessed to calculate the number of apoptotic and necrotic cells. Doxorubicin induced a dose-dependent increase in apoptosis after 24, 48 and 72 h of exposure (Fig. 3). A significant increase in apoptotic PC3 cells occurred after 24 h of exposure to 50 nM doxorubicin (1.96% vs. 0.63%, $P < 0.05$). The highest level of apoptosis was induced by 100 nM

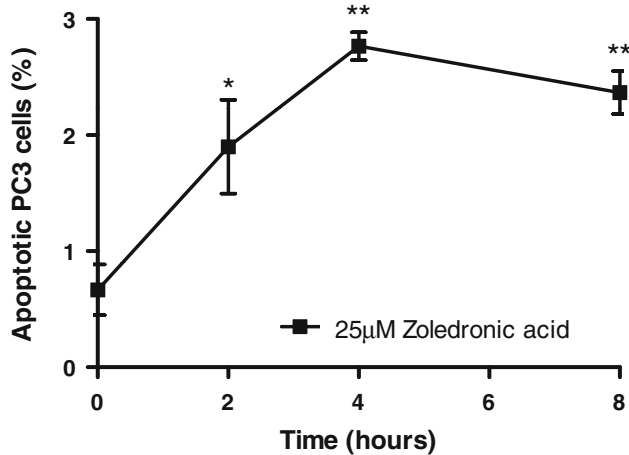


Fig. 2 Effect of short-term exposure to zoledronic acid in PC3 cells. PC3 cells were treated with zoledronic acid (25 μ M) for either 2, 4 or 8 h, followed by incubation with fresh medium for the remainder of the 72-h treatment period. The percentage of apoptotic cells were determined following completion of treatment. Results are the mean \pm SEM of three experiments in triplicate. * $P < 0.05$, ** $P < 0.01$ compared to control

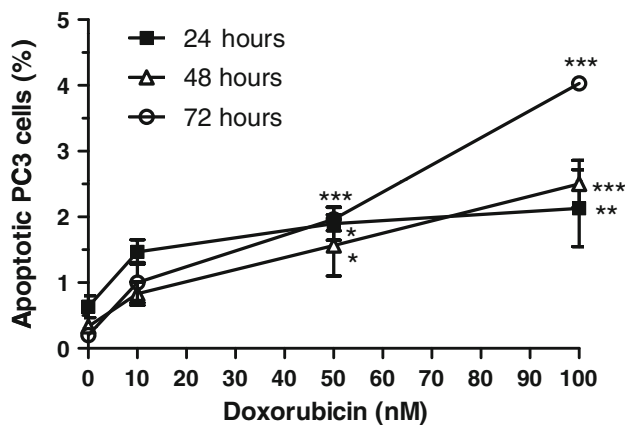


Fig. 3 Effect of doxorubicin on PC3 cell death after 24, 48 or 72 h. PC3 cells were treated with doxorubicin alone (0–100 nM) for either 24, 48 or 72 h, and the percentage of apoptotic (a) and necrotic (b) cells determined. Results are the mean \pm SEM of three experiments in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to control

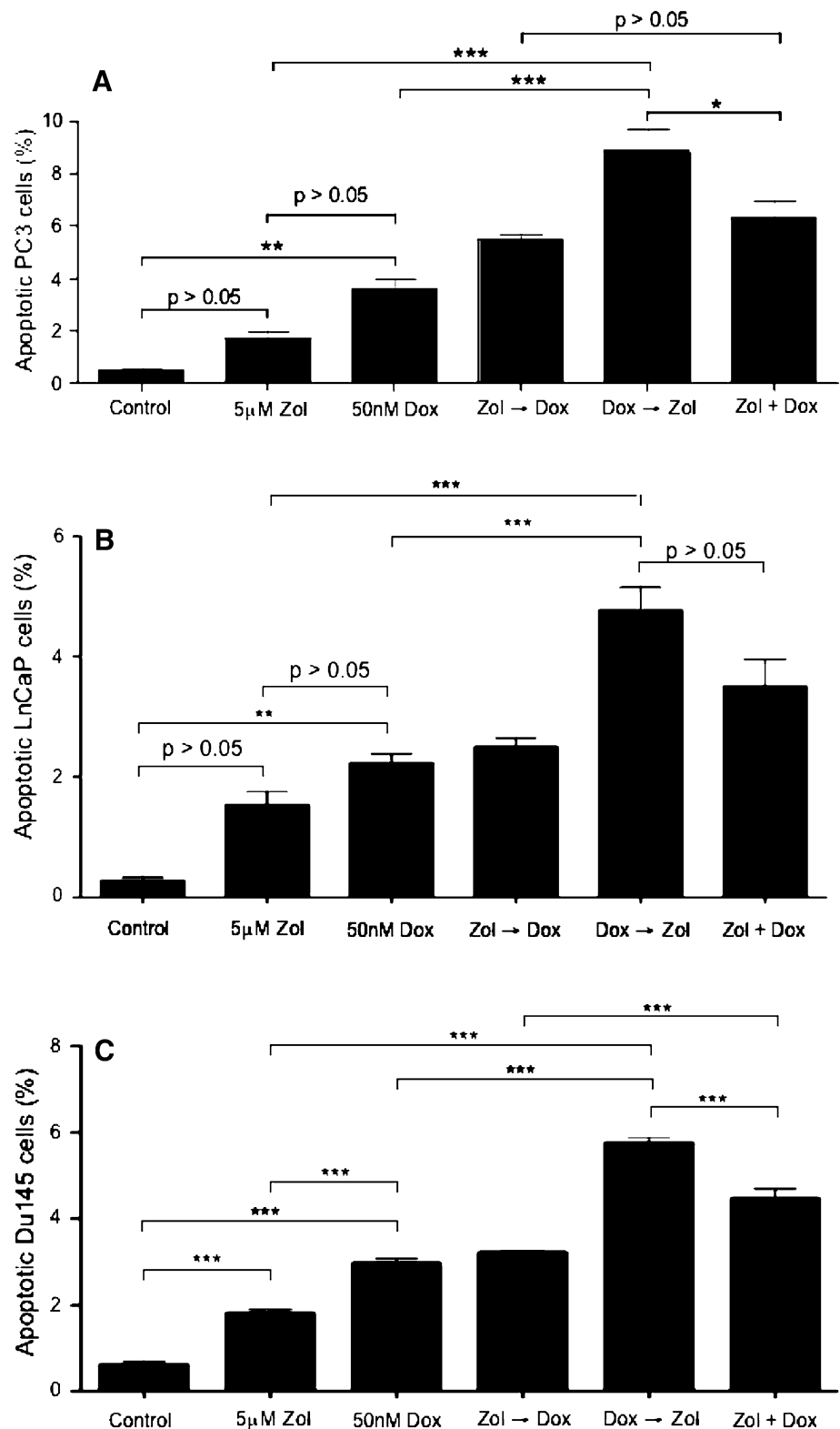
doxorubicin after 72 h of exposure (4.03% vs. 0.2%, $P < 0.001$). There was no significant difference in the levels of apoptosis induced by 50 nM doxorubicin at each time point. Doxorubicin did not induce an increase in necrosis (data not shown). These results show that 50 nM doxorubicin induces moderate levels of apoptosis in PC3 cells after 24 h of exposure, and subsequent experiments showed that 50 nM doxorubicin induced a maximum level of 7% apoptosis in DU145 and LNCaP cells (data not shown). Subsequent combination experiments were therefore performed using this dose and exposure period for all the three cell lines.

Effect of doxorubicin and zoledronic acid in sequence and in combination

We next determined the effect of combination treatment using doxorubicin and zoledronic acid. A dose of 5 μ M zoledronic acid was chosen as it had been shown by our previous experiments to induce significant but moderate levels of apoptosis in PC3 cells after 24 h (Fig. 1a). This was combined with 50 nM doxorubicin, which in our pilot experiments induced low levels of apoptosis in prostate cancer cells. PC3 cells were treated with 5 μ M zoledronic acid for 4 h, or 50 nM doxorubicin for 24 h, alone. The cells were also exposed to the agents in sequence and in combination, as described in the materials and methods section. When the agents were given in sequence (doxorubicin followed by zoledronic acid), we saw a significantly raised level of apoptosis compared to the agents given alone (8.87% vs. 3.6% (dox) and 1.73% (zol), $P < 0.001$, Fig. 4a). This sequence also induced a significantly raised level of apoptosis compared to the level when the drugs were given in the reverse sequence (zol followed by dox) or in combination (8.87% vs. 5.47% and 6.3%, $P < 0.05$). Giving the agents in the reverse sequence (zol followed by dox), or in combination (dox and zol together), did not induce a significantly raised level of apoptosis compared to doxorubicin alone (5.47% and 6.3% vs. 3.6%, $P > 0.05$). There was no significant difference in the levels of apoptosis between the agents given in reverse sequence (zol followed by dox) and those in combination (5.47% vs. 6.3%, $P > 0.05$). Further, 50 nM doxorubicin given alone induced a significantly raised level of apoptosis compared to control (3.6% vs. 0.5%, $P < 0.01$), but 5 μ M zoledronic acid did not (1.73% vs. 0.5%, $P > 0.05$).

The experiments were repeated using DU145 and LNCaP cells to determine whether the increased levels of apoptosis caused by sequential treatment could be replicated in different types of prostate cancer cells. The data in Fig. 4b and c show that there is a sequence-specific increase in apoptosis in both DU145 and LNCaP cells, again showing that the highest levels of apoptosis are generated when doxorubicin is given first, followed by zoledronic acid. In LNCaP cells (Fig. 4b), doxorubicin followed by zoledronic acid induced 4.77% apoptosis, which was significantly different from that caused by either single agent (1.53% zol alone, $P < 0.001$ and 2.23% dox alone, $P < 0.001$) or the reverse sequence (zol then dox 2.5%, $P < 0.001$). When the cells were treated with both drugs in combination, intermediate levels of apoptosis were observed, and these were not significantly lower than those when doxorubicin was given prior to zoledronic acid (3.5 and 4.77%, respectively, $P > 0.05$). In DU145 cells (Fig. 4c), doxorubicin followed by zoledronic acid induced 5.73% apoptosis, which was signifi-

Fig. 4 Effect of doxorubicin and zoledronic acid in sequence and in combination on prostate cancer cells. PC3 (a), LNCaP (b) and Du145 (c) cells were treated with doxorubicin alone (50 nM for 24 h followed by fresh medium for 4 h), zoledronic acid alone (5 μ M for 4 h followed by fresh medium for 24 h), the drugs in sequence and in combination. Following completion of the treatment, cells were maintained in fresh medium for the remaining period of the total incubation time of 72 h, and the % of apoptotic cells was determined. Results are the mean \pm SEM of three experiments in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$



cantly different from that caused by either single agent (1.8% zol alone, $P < 0.001$ and 2.93% dox alone, $P < 0.001$) or the reverse sequence (zol then dox 3.2%, $P < 0.001$). When cells were treated with both drugs in

combination, intermediate levels of apoptosis were observed, significantly lower than those when doxorubicin was given prior to zoledronic acid (4.47 and 5.73%, respectively, $P < 0.001$).

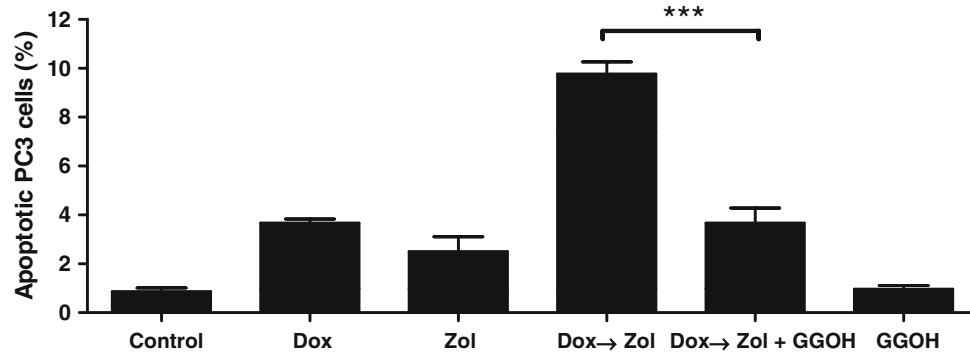


Fig. 5 The reversal of the apoptotic effect of doxorubicin and zoledronic acid by geranylgeraniol. PC3 cells were treated with 50 nM doxorubicin alone, 5 μ M zoledronic acid alone, 10 μ M GGOH alone or 50 nM doxorubicin and 5 μ M zoledronic acid (alone and combined

with 10 μ M) in sequence. Following completion of the treatment, cells were maintained in fresh medium for 24 h, and the % of apoptotic PC3 cells was determined. Results are the mean \pm SEM of three experiments in triplicate. *** P < 0.001

The reversal of the apoptotic effect of doxorubicin and zoledronic acid by geranylgeraniol

N-BPs, such as zoledronic acid, exert their anti-resorptive effect by inducing apoptosis in osteoclasts through inhibition of the mevalonate pathway. This mechanism is reported also to be responsible for the induction of apoptosis in prostate cancer cells [33]. In order to investigate what proportion of the increased levels of apoptosis caused by combining doxorubicin and zoledronic acid is achieved through inhibition of the mevalonate pathway, PC3 cells were treated with doxorubicin for 24 h, followed by zoledronic acid in the presence or absence of the mevalonate pathway intermediate geranylgeraniol (GGOH) for 24 h. GGOH has previously been shown to reverse the apoptotic effect caused by doxorubicin and zoledronic acid in sequence in breast cancer cells [20]. Doxorubicin followed by zoledronic acid induced a significantly raised level of apoptosis compared to the agents given alone to a level (9.77% vs. 3.67% (doxorubicin) and 1.73% (zoledronic acid), Fig. 5), comparable to the data shown in Fig. 4. The addition of GGOH along with zoledronic acid completely reversed the increased levels of apoptosis to that caused by doxorubicin alone (Fig. 5). These results show that addition of GGOH is able to reverse the increased levels of apoptosis induced by doxorubicin and zoledronic acid in sequence, and that approximately 60% of the additive apoptotic effect generated by sequential treatment (doxorubicin followed by zoledronic acid) is caused by inhibition of the mevalonate pathway.

Discussion

Bone metastases is a common feature of advanced prostate cancer, and the moment the cancer has spread to the skeleton, the disease is considered up to now as incurable. The

levels of bone markers in prostate cancer patients with skeletal metastases reflect increased bone resorption, also in the cases where the bone lesions appear to be predominantly osteosclerotic, and high levels of N-telopeptide is associated with poor prognosis [34]. In vivo studies investigating the effects of zoledronic acid treatment in advanced prostate cancer models have reported a clear reduction of the frequency and severity of bone lesions, whereas tumour burden is unaffected [35, 36]. We have found that in a breast cancer model, zoledronic acid does inhibit lytic bone disease, but not intra-osseous tumour growth [4]. Taken together, these reports show that simply preventing the development of cancer-induced bone disease is not sufficient to inhibit the progression of bone metastases from solid tumours.

As single agent therapy has proven ineffective, there is a clear need for a multi-dimensional approach to the treatment of prostate cancer bone metastases [37]. One option is to combine therapies that directly target tumour cells with agents that affect the bone microenvironment. Zoledronic acid modifies the local tumour microenvironment in bone by reducing tumour cell-stimulated bone resorption, thereby reducing the release of cytokines and growth factors that are known to stimulate tumour cell growth [38]. A potential further benefit of the use of zoledronic acid is that it may be able to enhance the anti-tumour effects of chemotherapy agents such as doxorubicin and docetaxel. This potential has been clearly demonstrated using in vivo models of prostate cancer growth in bone, where a combination of docetaxel and zoledronic acid caused the highest degree of inhibition of tumour growth [23]. Combining zoledronic acid with imatinib mesylate and paclitaxel has also been shown to cause a significant reduction in prostate tumour growth in bone, compared to administration of the single agents [39]. Similar data have been reported from models of breast tumour growth in bone, using combinations of zoledronic acid and doxorubicin [4], or ibandronate and

paclitaxel [40]. There have been no reports of investigations of the potential beneficial effects of combined anti-resorptive agents and doxorubicin in *in vivo* models of prostate cancer, possibly because anthracycline therapy has not been successful in the treatment of prostate cancer patients [31, 32]. The data in this report indicate that a better outcome may be achieved if doxorubicin is combined with zoledronic acid, as this clearly leads to increased tumour cell death compared to single agent therapy.

Zoledronic acid has been shown to induce raised levels of apoptosis in prostate cancer cells [10], breast cancer [41], renal cancer [42] and lung cancer [43], among others, *in vitro*. On the whole, these results have required the exposure of cells to high concentrations of zoledronic acid for long periods of time (up to 96 h). Following a clinical infusion, zoledronic acid reaches a peak plasma concentration of around 1 μM that lasts for 1–2 h. We found zoledronic acid alone was able to induce apoptosis in PC3 prostate cancer cells at 2.5 μM after 24 h of exposure, and also that high a dose of zoledronic acid (25 μM) induces significantly increased apoptosis after 2 h of exposure. It remains to be determined whether clinically achievable doses of zoledronic acid cause apoptosis following these exposure periods.

In an early study, Neville-Webbe et al. reported that the administration of doxorubicin, followed by zoledronic acid, generates a synergistic anti-tumour effect in PC3 prostate cancer cells *in vitro*, but the reverse sequence or giving the drugs in combination was not tested [13]. In this study, the administration of doxorubicin followed by zoledronic acid also induced a significantly raised level of apoptosis, which appeared to be additive when compared to the single agents. In agreement with reports using MCF7 breast cancer cells [20], we show here that treating PC3, Du145 or LNCaP cells with the reverse drug sequence (zoledronic acid followed by doxorubicin) did not induce a significant increase in apoptosis compared to the agents given alone. Combining the two drugs together induced elevated levels of apoptosis compared to the single agents in all the three prostate cancer cell lines, but not to the same extent as that was induced due to sequential administration of doxorubicin followed by zoledronic acid. The effect was independent of the hormone responsiveness of the prostate cancer cells, as apoptosis was induced to a similar extent in both androgen sensitive LNCaP and androgen-independent (DU145 and PC3) cells.

The effects on LNCaP cells are in contrast to those reported by Budman and Calabro [44] who compared the effects of combined treatment with epirubicin and zoledronic acid on the rate of proliferation of the same three prostate cancer cell lines. They found that whereas treating PC3 and DU145 cells with a combination of both agents for 72 h caused a synergistic inhibition of proliferation, the

same schedule caused an antagonistic effect on LNCaP cells. The reasons for this discrepancy between the reported effects could partly be explained by the different methodology, doses and incubation periods used. The clinical relevance of our results remains to be established, but data from breast cancer models have shown that initial positive findings from similar *in vitro* studies of sequential treatment with doxorubicin and zoledronic acid [20] were subsequently confirmed in several *in vivo* models [3, 4]. As a result there is now an ongoing clinical trial exploring this treatment schedule in breast cancer patients who require neoadjuvant chemotherapy (ANZAC trial—EUDRACT number 2007-001526-27).

The results of this study suggest that doxorubicin may in some way prime or sensitise the cells to zoledronic acid, increasing its apoptotic effect. We have detected increased uptake of a fluorescently labelled bisphosphonate following treatment of breast cancer cells with doxorubicin (PD Ottewell, unpublished data). Another possibility is that doxorubicin has an effect on some of the genes involved in zoledronic acid-induced apoptosis, increasing its apoptotic effect, or that doxorubicin leads to a synchronisation of the cells in a particular phase of the cell cycle, leaving them more prone to a second insult (provided by zoledronic acid). Although the exact molecular mechanism underlying the sequence-specific induction of cancer cell apoptosis observed in this study remains to be identified, the reversal experiments using GGOH clearly show that the increased levels of apoptosis depend on zoledronic acid inhibition of the mevalonate pathway. Our data suggest that the majority of the increased effect (approx. 60%) was reversed by the addition of GGOH and is therefore due to zoledronic acid.

In conclusion, sequential treatment with doxorubicin followed by zoledronic acid induces significantly elevated levels of apoptosis in both androgen-sensitive and -insensitive prostate cancer cells, compared to that caused by the single agents or the reverse drug sequence. While doxorubicin is not commonly used in treatment of prostate cancer, this study suggests that the combination of zoledronic acid with doxorubicin may have a beneficial effect in the clinical management of advanced prostate cancer and should be investigated further using *in vivo* model systems.

Acknowledgments R.D. Clyburn was sponsored by bursary by Yorkshire Cancer Research, UK. P. Reid is funded by a grant from Weston Park Hospital Cancer Charity, Sheffield, UK.

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