Combined effects of zoledronic acid and doxorubicin on breast cancer cell invasion *in vitro*

Julia K. L. Woodward[a], Helen L. Neville-Webbe[a], Robert E. Coleman[a] and Ingunn Holen[a]

The bisphosphonate zoledronic acid and the cytotoxic drug doxorubicin induce synergistic levels of apoptosis in breast cancer cells. As zoledronic acid and doxorubicin have been shown to reduce cell invasion and migration, we have investigated if these drugs also act synergistically on breast cancer invasion *in vitro*. MCF7 cells were treated with 0.05 μM doxorubicin/4 h followed by 1 or 10 μM zoledronic acid/24 h (or the reverse sequence). To study invasion, MCF7 cells were either grown on Transwell membranes coated with Matrigel or in a 24-well plate. Cells were treated sequentially using the above drug combinations, prior to starting the invasion assays for 48 h. Cell growth and death were also assessed under the same conditions. We found that invasion of MCF7 cells treated with zoledronic acid and doxorubicin was significantly reduced when compared with control, but the effect was dependent on drug sequence. At 1 μM, zoledronic acid significantly reduced invasion only if cells were pre-treated with doxorubicin, but cell growth was unaffected. For 10 μM zoledronic acid, invasion was reduced when administered before or after the doxorubicin, but this dose of zoledronic acid caused a significant reduction in MCF7 growth. Apoptosis was not induced by any of the drug doses and combinations. We conclude that pre-treatment with 0.05 μM doxorubicin followed by 1 μM zoledronic acid reduces invasion when cells were grown on Matrigel. For 10 μM zoledronic acid, pre- or post-doxorubicin also reduces invasion, but for this combination inhibition of cell growth may contribute to the reduction in invasion observed. *Anti-Cancer Drugs* 16:845–854 © 2005 Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2005, **16**:845–854

Keywords: adhesion, bisphosphonate, breast cancer, cytotoxic drug, invasion, metastatic bone disease, mevalonate pathway, migration

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Sponsorship: Weston Park Cancer Appeal has supported this work. Novartis kindly donated the zoledronic acid.

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Received 28 May 2005 **Accepted** 14 June 2005

Introduction

For tumor cells to invade away from the primary tumor mass, they must adhere to and migrate through the complex network of proteins and polysaccharides comprising the extracellular matrix (ECM) and basement membrane, and ultimately the endothelium [1]. To assist motility, degradation of the individual components of the ECM and basement membrane is essential, facilitated by secretion and activation of proteolytic enzymes, producing protein fragments for cells to migrate towards and adhere to [1].

Once present in the circulation (or lymphatics), metastatic tumor cells have the propensity to spread to a secondary site, invading into the target tissue through similar mechanisms. Metastatic breast carcinomas, for example, frequently target the bone, growing within the bone marrow cavity, stimulating osteoclast-mediated bone resorption and leading to increased osteolysis [2,3]. At this stage, anti-resorptives are commonly administered, binding to the bone mineral to inhibit osteoclast activity and improve the patient's quality of life through prevention of skeletal-related events [4,5]. Of this class of drugs, the third-generation nitrogen-containing bisphosphonate zoledronic acid has been found to be most potent at reducing bone resorption [5,6]. By inhibiting a key enzyme in the mevalonate pathway (farnesyl diphosphate synthase), nitrogen-containing bisphosphonates such as zoledronic acid prevent protein prenylation (geranylgeranylation and farnesylation) of a wide variety of small intracellular G-proteins, including Ras and Rho [7–10]. As a consequence, the subcellular distribution of small GTPases is altered, preventing them from acting at the correct spatial location within the osteoclast, thus affecting normal cellular function.

Similar to the effects on osteoclasts, preclinical data have now demonstrated that bisphosphonates have anti-tumor effects, inhibiting processes including cell proliferation, apoptosis, angiogenesis, adhesion to the ECM and bone, and invasion, with zoledronic acid again proving to be the most potent bisphosphonate studied [11–22]. When considering tumor cell invasion for example, Boissier *et al.* [17,22] originally demonstrated that pre-treatment of the breast cancer cell lines MCF-7 and MDA-MB 231

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with a number of bisphosphonates, including zoledronic acid, can reduce adhesion to both mineralized and unmineralized matrices. Subsequent invasion through an artificial basement membrane was also reduced by affecting attachment through effects on the tumor cells themselves as opposed to changing the matrix surface protein structure [17,22]. A number of groups have reported that treatment with bisphosphonates including zoledronic acid can reduce both breast and prostate cancer invasion *in vitro*, potentially through inhibition of the mevalonate pathway [13,17,20,21].

Evidence is now available to suggest that the anti-tumor effect of bisphosphonates can also be enhanced by co-administration of chemotherapeutic agents, including paclitaxel and doxorubicin. Combined treatment with alendronate and paclitaxel has, for example, a greater effect on bone and non-bone metastatic development than treatment with each alone [23], whilst a synergistic effect on apoptosis is reported after co-administration of MCF-7 breast cancer cells *in vitro* with paclitaxel [24]. Recent data from our group have additionally demonstrated that the order in which the drugs are given may be important, as apoptosis of the breast cancer cell lines MCF7 and MDA-MB 436 was significantly enhanced when cells were treated with zoledronic acid after treatment with doxorubicin [25]. For tumor invasion, a small number of investigations have indicated that treatment with cytotoxic drugs alone can reduce this process [23,26–30]. Magnetto *et al.* reported an additive effect of the nitrogen-containing bisphosphonate ibandronate and paclitaxel on MDA-MB 231 breast cancer cell adhesion to bone matrices and invasion [23]. There are, however, no studies available to date investigating the effects of combining the most potent and commonly used bisphosphonate in oncology, zoledronic acid, with chemotherapy drugs such as doxorubicin on tumor cell invasion and migration. As zoledronic acid and doxorubicin have a synergistic effect on tumor cell apoptosis [25], this present investigation was carried out to assess whether zoledronic acid and doxorubicin would similarly have a synergistic effect on breast cancer cell invasion and migration *in vitro*.

Materials and methods

Drugs and chemicals

A stock 10 mM solution of zoledronic acid ([1-hydroxy-2-(1H-imidazol-1-ly) ethylidene] bisphonic acid; Novartis, Basel, Switzerland) was prepared in phosphate-buffered saline (PBS) (pH 7.4) and stored at −20°C until required. Doxorubicin (adriamycin hydrochloride; Sigma-Aldrich, Poole, UK) was reconstituted with sterile distilled water at a concentration of 10 mM and kept at 4°C. A stock solution of geranylgeraniol [all *trans*-3,7,11–15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol (GGOH); Sigma-Aldrich] was made up with 100% ethanol. For use in all experiments, drugs were diluted using full growth medium and filter-sterilized (0.22 μm filter).

Cell culture

The estrogen-dependent human breast cancer MCF7 cell line was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells were grown in RPMI 1640 culture medium containing 10% fetal calf serum, fungizone (5 μg/ml) (all obtained from Invitrogen Life Technologies, Paisley, UK), penicillin (100 U/ml) and streptomycin (100 μg/ml) (both from GlaxoSmithKline, Uxbridge, UK) in a 5% CO_2 incubator. Trypsin (Invitrogen Life Technologies) was used to enzymatically dissociate the cells when required.

Drug treatment of cells

After initial optimization, for each experiment, cells were treated with 0.05 μM doxorubicin for 4 h and zoledronic acid (1–10 μM) for 24 h. After drug treatment, migration and invasion assays were run for 20 and 48 h, respectively. For growth and apoptosis assays, cells were further incubated following treatment in fresh medium for 48 h to allow comparison between invasion, growth and apoptosis assays. The different assays used are illustrated in Figure 1.

The drug sequences used were as follows (time lines shown in Fig. 2 summarize these treatments).

Drug sequence 1 ('zoledronic acid then doxorubicin') (Fig. 2A)

Day 1: Cells were treated with zoledronic acid (1 or 10 μM) for 24 h.
Day 2: Zoledronic acid was removed, cells were washed and media replaced with doxorubicin (0.05 μM) for 4 h. Cells were again washed free of the drug and medium replaced with fresh medium for a further 48 h before counting living or dead cells. For invasion and migration assays, the assays were set up at this point.

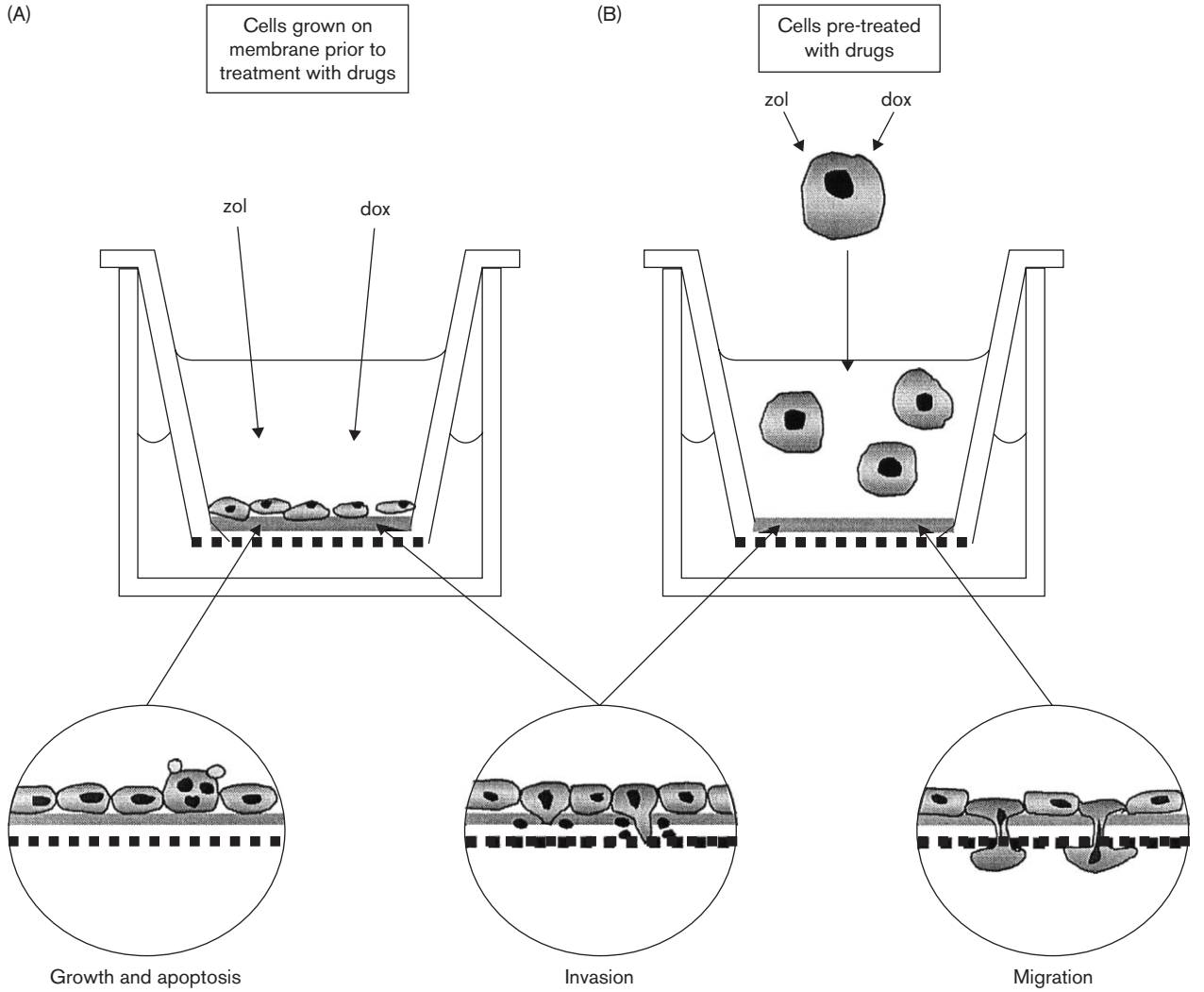
Drug sequence 2 ('doxorubicin then zoledronic acid') (Fig. 2B).

Day 1: Cells were treated with doxorubicin (0.05 μM) for 4 h. Cells were then washed and the medium replaced with zoledronic acid (1 or 10 μM) for a further 24 h.
Day 2: Zoledronic acid was removed, cells were washed and media replaced with fresh medium for a further 48 h before counting living or dead cells. For invasion and migration assays, the assays were set up at this point.

Matrigel invasion assays

Cell invasion assays were performed using Transwell chambers (Costar, Corning, New York, USA), placing inserts with polycarbonate membranes (8 μm pore size) into a 24-well companion plate. Membranes were coated

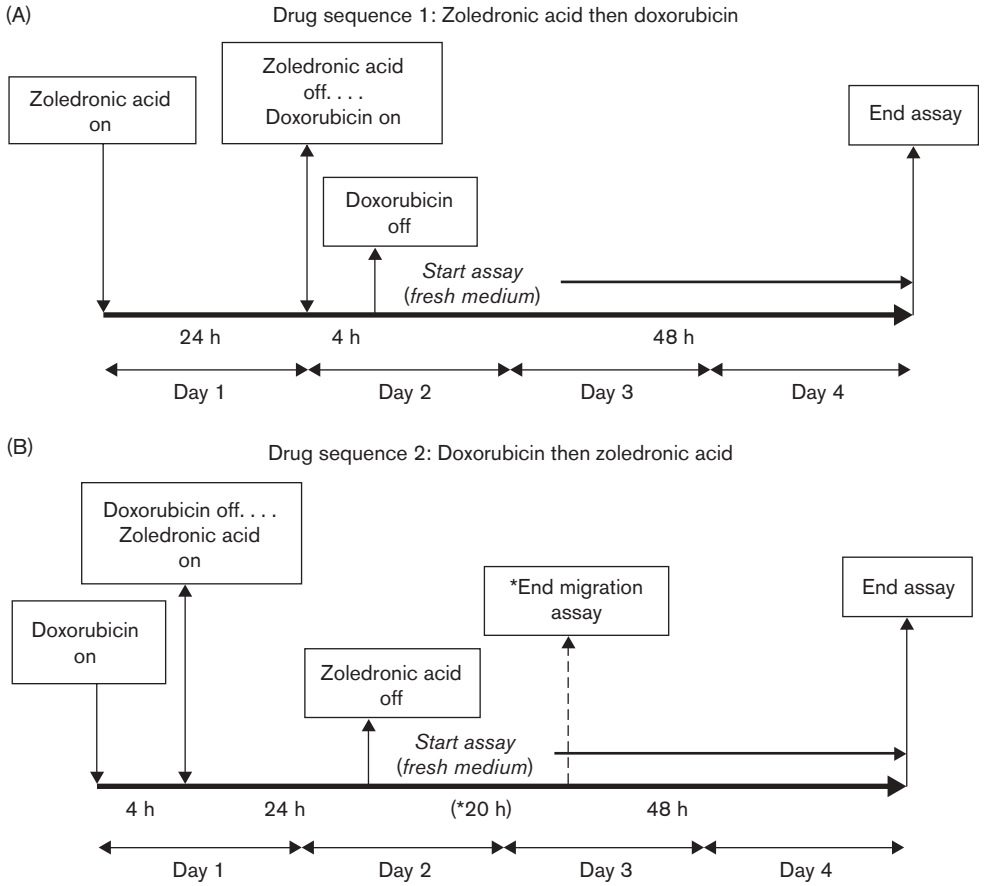
Fig. 1



Schematic diagram of the methods used to study MCF7 invasion, migration, growth and apoptosis. MCF7 cells were either grown and subsequently treated on Matrigel-coated Transwell membranes (represented by a solid grey line and a hashed black line, respectively) (A) or were pre-treated before setting up the invasion or migration assay (B). Growth and apoptosis assays were also set up in parallel.

with the artificial basement membrane Matrigel (Becton Dickinson, Plymouth, UK) (10 µl/membrane), used at a concentration of 1.5 mg/ml and allowed to air dry in a sterile environment for at least 1 h. Cells were seeded on the Matrigel-coated filters at a concentration of 8×10^3/well for 72 h in normal growth medium. Medium was absent from the lower chamber during this period to prevent cells from invading onto the under side of the membrane whilst undergoing drug treatment. To initially establish an optimum treatment time with zoledronic acid, cells were treated with zoledronic acid for 1, 4 or 24 h. In a brief experiment to investigate the involvement of the mevalonate pathway, 50 µMGGOH was included with the zoledronic acid treatment. After establishing the optimum treatment time, cells were sequentially treated on the membranes as described above. In combination experiments, after treating with the second drug, cells were washed with PBS and the medium in the upper chamber replaced with 200 µl serum-free medium [with 0.1% bovine serum albumen (BSA)]. Then, 600 µl of standard growth medium (the medium used to grow the cells in) was added to the lower chamber as a chemoattractant (Fig. 1). Plates were then incubated for 48 h. Any non-invaded cells were then carefully removed with a cotton bud, before fixing and staining the invaded cells, using a Hema Gurr Rapid Staining Kit (BDH, Poole, UK). The membranes were mounted on glass slides using an aqueous mount (Dako, Carpinteria, California, USA) and cells from 10 random fields of view per membrane were counted ($\times$ 400 magnification). All experiments were set up in duplicate and repeated at least once. Levels of invasion are expressed as the

Fig. 2



Time lines to illustrate the treatment schedule. Tumor cells were either treated with zoledronic acid (1 and 10 μM) for 24 h and doxorubicin (0.05 μM) for 4 h (A) or the reverse sequence (B). In both cases, cells were washed between and after the final treatment and the invasion assays set up for 48 h. For growth and apoptosis assays, the medium was replaced with fresh growth medium for 48 h before analyzing cell numbers or levels of apoptosis. *For migration assays, plates were incubated for 20 h.

percentage invasion of the untreated controls (fresh medium alone).

To compare whether treatment of the cells prior to setting up the invasion assay would have a similar effect on invasion as the treatment of cells on the Matrigel-coated membranes, cells were seeded in a 24-well plate at a concentration of 4×10^4/ml for 72 h and sequentially treated as described previously. After treatment with the last drug, cells were washed and trypsinized to dissociate. Cells were resuspended in serum-free medium (with 0.1% BSA) at a concentration of 1.25×10^6/ml and added to the upper chamber of the Matrigel-coated inserts (200 μl/well). Standard growth medium was again added to the lower chamber and the assay continued as described above (Fig. 1).

Migration assays

Migration assays were carried out using Transwell chambers (8 μm pore size) (Costar Corning, High Wycombe, UK), coated with fibronectin (6.5 μg/ml) (Sigma-Aldrich, Poole, UK). As for the invasion assays, MCF7 cells were seeded in a 24-well plate at a concentration of 4×10^4/ml for 72 h. Cells were then sequentially treated with doxorubicin and zoledronic acid, prior to dissociating the cells from the plate, and resuspending in serum-free medium (with 0.1% BSA) at a concentration of 1.25×10^6/ml. Then, 200 ml of cell suspension was added to each well and standard growth medium added to the lower chamber as previously described (Fig. 1). Plates were incubated for 20 h before removing any non-migrated cells, fixing, staining and counting migrated cells as detailed above.

Growth assays

To assess the effects of the above drug combinations on cell growth, cells were seeded at a concentration of 4×10^4/well for 72 h, and sequentially treated with zoledronic acid and doxorubicin as described above (Fig. 2). After 48 h in fresh medium, cells were washed twice with PBS,

trypsinized and resuspended in Coulter Isoton II diluent (Beckman, Hialeah, Florida, USA). Cells were then counted using a Beckman Coulter Z2 Series counter (Beckman), assessing three counts for each well and a mean value taken. Triplicate wells were assessed for each treatment; experiments were repeated and the mean value calculated. Results were compared with untreated cells (fresh medium alone) and with cells treated with each drug alone (treating cells at the same time points as those treated in the combined groups).

Apoptosis assays

Cells were seeded in a 24-well plate and treated as described for the growth studies (Fig. 2). After 48 h in fresh medium, viable, necrotic and apoptotic cells were stained with 8 μM Hoechst 33341 (Sigma-Aldrich) and 5 μM propidium iodide (Molecular Probes, Cambridge, UK) for 15 min at 37°C. Analysis of apoptosis was assessed as previously described [25] using a fluorescent inverted microscope with a UV filter. Cell counts were made using a Whipple graticule, assessing five random grid areas in three wells for each treatment. Assays were repeated and the mean taken, expressing the percentage of viable, apoptotic, necrotic and viable cells, comparing treated cells with untreated cells (fresh medium alone) and cells treated with one drug only (Fig. 1).

To additionally assess apoptosis, apoptotic and necrotic cells were labeled with Annexin-V–FITC (BD Pharmingen, Plymouth, UK) and propidium iodide (Sigma-Aldrich), respectively and detected by flow cytometry (FACSCalibur; Becton Dickinson). Annexin-V–FITC binds to phosphatidylserine residues, which are translocated from the inner to the outer surface of the membrane during early events in apoptosis. In brief, treated cells were dissociated and washed twice in a binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$). Cells were then labeled with 5 μl Annexin-V–FITC/50 μl binding buffer for 15 min in the dark and at room temperature. Immediately prior to analysis on the flow cytometer, samples were also labeled with 250 μl of a 10 mg/ml stock propidium iodide solution. For each sample, 1×10^4 events were acquired. Analysis was carried out in triplicate and repeated. Results are presented as scatter graphs; plotting FL3 (propidium iodide) against FL1 (FITC) values on the *x*- and *y*-axes, respectively. For analysis, scatter plots are divided into quadrants to distinguish viable, early and late apoptotic and necrotic cells. Viable cells do not stain with Annexin-V–FITC or propidium iodide and are detected in the R2 quadrant. Early apoptotic cells can be distinguished from necrotic cells as the former will stain with Annexin-V–FITC alone and can be seen in the R3 quadrant. Late apoptotic cells are located in the R4 quadrant. Necrotic cells in contrast stain positively with both Annexin-V–FITC and propidium iodide and are located in the R5 region.

Statistical analysis

Statistical analysis was carried out using SPSS software (version 11). One-way ANOVA was used to assess the difference between mean values, using a Dunnett's two-sided test or Tukey's HSD test (both assuming equal variances) for *post-hoc* analysis. $p < 0.05$ was considered significant in all cases.

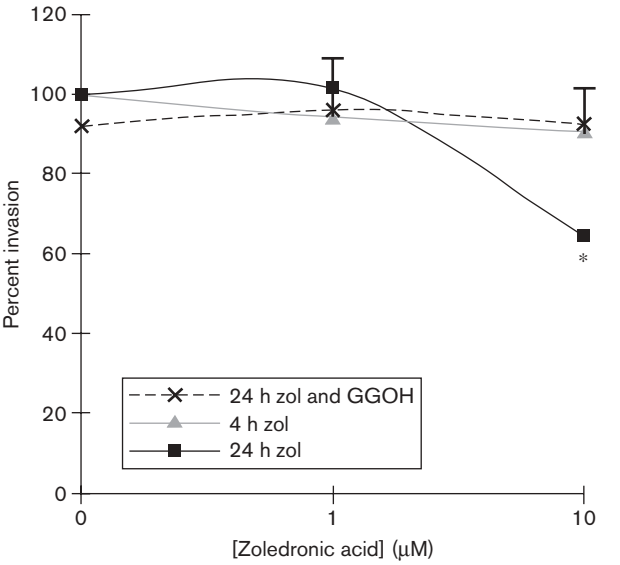
Results

Effect of treatment with zoledronic acid and doxorubicin on MCF7 invasion and migration

Treatment with drugs alone

When MCF7 cells were grown on Matrigel, a significant reduction in the number of invaded cells was only seen when cells were treated with 10 μM zoledronic acid for 24 h [$p < 0.01$ versus control (fresh medium alone)] (Fig. 3), with levels of invasion reduced to 64.9 ± 3.09% of control levels. No significant reduction of invasion was observed when cells were treated with the lower dose of 1 μM or when cells were treated for shorter time periods (1 or 4 h) ($p > 0.05$). As bisphosphonate treatment is reported to prevent tumor cell invasion through inhibition of enzymes of the mevalonate pathway [13,17,20,21], GGOH, a mevalonate pathway metabolite,

Fig. 3



Effect of 4- and 24-h zoledronic acid (zol) treatment and 24 h zoledronic acid treatment with GGOH, on MCF7 invasion after 48 h. MCF7 cells were grown on Matrigel on Transwell membranes and then treated with 1 or 10 μM zoledronic acid for 4 or 24 h, or with zoledronic acid and GGOH for 24 h. Zoledronic acid was then washed off and the invasion assay set up for 48 h. The *y*-axis shows the percent invasion, relating levels of invasion in treatment wells with untreated controls. Results are the means ± SEM of three experiments*. Treatment with 10 μM zoledronic acid for 24 h only caused a significant reduction in invasion [$p < 0.01$ versus control (fresh medium alone)]. Inclusion of 50 μM GGOH with the zoledronic acid reversed the inhibitory effect on invasion ($p > 0.05$).

was included with the zoledronic acid treatment to determine whether protein prenylation was also responsible for the inhibitory effect of zoledronic acid in this system. Inclusion of 50 μM GGOH with the zoledronic acid reversed the inhibitory effect of the 24-h treatment with 10 μM zoledronic acid on invasion (restoring levels to 92.9 ± 8.85% of control values) (Fig. 3). Treatment with doxorubicin or GGOH alone had no significant effect on invasion ($p > 0.05$).

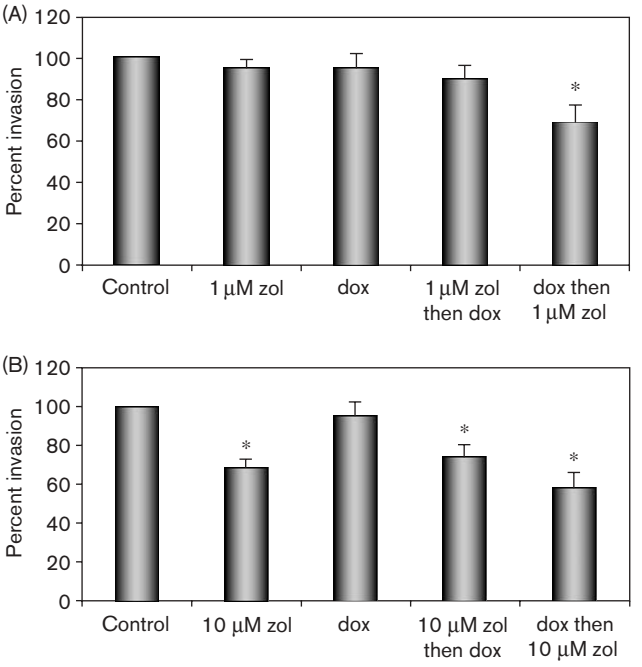
Combined treatment with zoledronic acid and doxorubicin

As treatment of cells with zoledronic acid for 24 h has also been previously reported to reduce invasion [13,17], this time point was selected for the combined treatment experiments. When cells were sequentially treated with zoledronic acid (1 or 10 μM) for 24 h and 0.05 μM doxorubicin for 4 h, invasion of MCF7 cells (grown on Matrigel) was significantly reduced when compared with the untreated control, but the effect was dependent on drug sequence (Fig. 4). When cells were treated with 1 μM zoledronic acid, invasion was only significantly reduced if cells were treated with doxorubicin prior to zoledronic acid ($p < 0.001$, versus control (fresh medium alone)) (Fig. 2B), with levels reduced to 69 ± 4.64% of control levels (Fig. 4A). For 10 μM zoledronic acid, invasion was also significantly reduced, but the effect was independent of the sequence (for both sequences, $p < 0.001$, versus control) (Fig. 2). When treated with 10 μM zoledronic acid before doxorubicin, invasion levels were reduced to 74 ± 6.76% of the untreated controls, whilst treatment with doxorubicin followed by 10 μM zoledronic acid reduced invasion to 58 ± 8.5% of controls levels (Fig. 4B).

To assess whether pre-treating the cells in the absence of Matrigel with doxorubicin followed by 1 μM zoledronic acid (Figs 2B and 4A) (the drug sequence shown to significantly reduce invasion when cells were grown on Matrigel) would have a similar effect on invasion, cells were treated with this drug combination in a 24-well plate prior to assessing invasion. As invasion involves the processes of attachment to the ECM, degradation and migration, the effect of pre-treating the cells with this drug sequence on migration through fibronectin was also studied. However, no significant reduction in invasion through Matrigel or migration was observed after treating the cells in this manner ($p > 0.05$) (data not shown). Doxorubicin pre-treatment of MCF7 cells (with or without zoledronic acid) similarly did not significantly reduce MCF7 migration or invasion ($p > 0.05$).

As circulating levels of zoledronic acid following i.v. infusion are approximately 1–3 μM [31] and are maintained for a few hours only, the treatment time with zoledronic acid was reduced to 4 h to assess if invasion could be reduced with combined treatment with doxorubicin. Treatment of MCF7 cells grown on Matrigel with zoledronic acid (1 or 10 μM) for 4 h with the lower dose of 0.01 μM doxorubicin for 24 h did not, however, cause any significant reduction in invasion ($p > 0.05$), when compared to untreated controls (data not shown).

Fig. 4



Effect of (A) 1 and (B) 10 μM zoledronic acid (zol) and doxorubicin (dox) treatment on MCF7 invasion. MCF7 cells were grown on Matrigel on Transwell membranes and then treated with (A) 1 and (B) 10 μM zoledronic acid for 24 h (drug sequence 1) or 0.05 μM doxorubicin for 4 h (drug sequence 2). Cells were then washed and the cells treated with either doxorubicin (drug sequence 1) or zoledronic acid (drug sequence 2) for the appropriate length of time. The *y*-axis shows the percent invasion, relating levels of invasion in treatment wells with untreated controls. Results are the means ± SEM of three experiments. *Significant results [$p < 0.001$ versus control (fresh medium alone)] obtained when cells were treated with 10 μM zoledronic acid (alone or in combination with doxorubicin) or with doxorubicin followed by 1 μM zoledronic acid.

Effect of combined treatment with zoledronic acid and doxorubicin on MCF7 growth

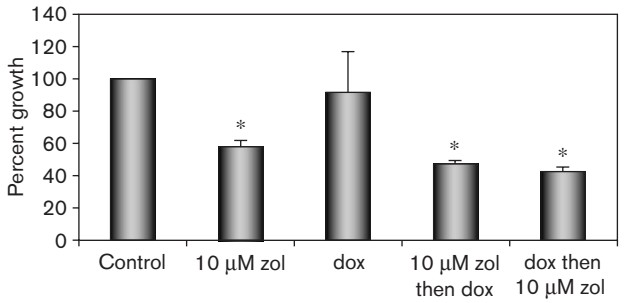
As previous studies have revealed that incubation of cells in fresh medium for 24–48 h post-treatment were required for detection of optimum levels of apoptosis [25], this was included in our assays. Treatment with 10 μM zoledronic acid for 24 h alone caused a significant reduction in MCF7 growth [$p < 0.05$ versus control (fresh medium alone)] as determined after 48 h in fresh medium (Fig. 5), decreasing cell numbers to 69.1 ± 7.17% of the untreated control. Sequential treatment with doxorubicin did not additionally affect this result (Fig. 2). In contrast, treatment with 1 μM zoledronic acid for 24 h, either alone or when combined

with doxorubicin, did not significantly reduce cell growth after 48 h in fresh medium ($p > 0.05$) (data not shown).

Effect of combined treatment with zoledronic acid and doxorubicin on MCF7 apoptosis

Use of both cell counting and flow cytometry apoptosis assays revealed that treatment of MCF7 cells with zoledronic acid (1 or 10 μM) for 24 h caused only minimal apoptosis determined after a further 48 h in fresh medium [$p > 0.05$ versus control (fresh medium alone)] (Table 1). This result was unaffected by the combined treatment with doxorubicin (Fig. 2), establishing that these conditions were not affecting tumor cell invasion through increasing cell death. Results of the Hoechst and propidium iodide assays are not shown; the results of the Annexin-V–FITC staining are shown in Table 1.

Fig. 5



Effect of 10 μM zoledronic acid (zol) and doxorubicin (dox) treatment on MCF7 growth. MCF7 cells were treated with 0.05 μM doxorubicin for 4 h followed by 10 μM zoledronic acid for 24 h. Samples were also set up with the reversed drug sequence. The *y*-axis shows the percent growth, relating cell numbers in treatment wells with untreated controls. Results are the means ± SEM of two experiments. *Significant results [$p < 0.05$ versus control (fresh medium alone)] obtained when cells were treated with 10 μM zoledronic acid (alone or in combination with doxorubicin).

Discussion

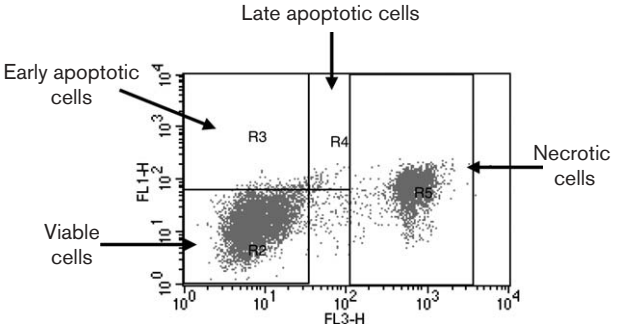
Much preclinical evidence now exists to suggest that bisphosphonates not only inhibit osteoclast function, but also tumor cell activity. Experiments carried out *in vitro* have reported that zoledronic acid, for example, inhibits tumor cell growth [13,16,32], apoptosis [12], adhesion [21], invasion [13,17,21] and angiogenesis, targeting cells of the tumor vasculature [11]. Once administered, bisphosphonates rapidly accumulate in the bone, binding strongly to hydroxyapaptite on the bone mineral surfaces via a region known as the 'bone hook'. The active side-chain of the bisphosphonate is left exposed and hence available to affect tumor cells within the resorption lacunae. The rapid migration of bisphosphonates to the bone results in very low circulating plasma levels, with a concentration estimated at 1–3 μM, maintained for a few hours only following i.v. administration [31,33]. In contrast, the concentration within the bone microenvironment is estimated at approximately 0.1–1 mM [31] with the highest levels present in the resorption pits. To affect tumor cells in sites other than the bone, clinically relevant concentrations of zoledronic acid would be between 1 and 3 μM.

Table 1 **Effect of 1 and 10 μM zoledronic acid and doxorubicin treatment on MCF7 apoptosis**

Treatment	Region of cells			
	Viable (R2)	Early apoptotic (R3)	Late apoptotic (R4)	Necrotic (R5)
Control	71.5 ± 2.73	0.49 ± 0.33	0.41 ± 0.32	26.6 ± 1.98
1 μM zoledronic acid alone	69.1 ± 0.15	1.01 ± 0.56	0.35 ± 0.16	28.5 ± 0.67
10 μM zoledronic acid alone	71.9 ± 1.94	1.28 ± 0.60	0.525 ± 0.30	24.4 ± 3.74
Doxorubicin alone	73.9 ± 8.27	0.88 ± 0.31	0.34 ± 0.25	28.1 ± 0.64
1 μM zoledronic acid then doxorubicin	69.8 ± 1.46	0.83 ± 0.7	0.41 ± 0.36	28.1 ± 0.64
10 μM zoledronic acid then doxorubicin	73.7 ± 3.25	2.31 ± 0.44	1.24 ± 0.475	21.7 ± 4.24
Doxorubicin then 1 μM zoledronic acid[a]	64.5 ± 4.00	0.79 ± 0.53	0.43 ± 0.27	33.3 ± 3.09
Doxorubicin then 10 μM zoledronic acid	71.1 ± 0.38	0.94 ± 0.015	0.7 ± 0	25.9 ± 0.43

MCF7 cells were treated with 0.05 μM doxorubicin for 4 h followed by 1 or 10 μM zoledronic acid for 24 h. Samples were also set up with the reversed drug sequence. Following drug treatment, cells were incubated in fresh medium for 48 h prior to counting the number of apoptotic cells by staining cells with Annexin-V–FITC and propidium iodide. Values shown are the mean ± SEM of two experiments.

[a]A representative dot plot of these results (doxorubicin then 1 μM zoledronic acid) as no significant increase in early apoptosis (region R2) was seen after any of the treatments ($p > 0.05$).



In support of this theory, reports have now shown that concentrations of zoledronic acid of 1 μM and below can reduce the invasion of aggressive MDA-MB 231 breast and PC3 prostate cancer cells *in vitro* through an artificial basement membrane when cells are pre-treated for 24 h prior to assessing invasion [13,17,20]. The invasive process nevertheless involves a number of stages, including attachment and degradation of the ECM, and subsequent migration through the matrix. Any one of these processes could therefore be affected by the bisphosphonate treatment. Further investigation of these individual components by Boissier *et al.* [17,22] revealed that at 1 μM, zoledronic acid primarily appeared to be affecting breast and prostate cancer cell adhesion and not migration or degradation; activity of proteolytic enzymes was only reduced when cells were treated with much higher does of zoledronic acid (concentrations of 40 μM and above) [13,17,20]. Pre-treatment of the prostate cancer cell line PC3 with the nitrogen-containing bisphosphonate alendronate, however, revealed that this bisphosphonate reduces invasion by principally inhibiting migration and not cell adhesion [13,17,20], suggesting that the precise effect of the bisphosphonate may be compound specific. In this present investigation, invasion of the estrogen-dependent MCF7 breast cancer cells could only be significantly reduced when cells were pre-treated with 10 μM zoledronic acid for 24 h, after growing cells on the artificial basement membrane Matrigel. Invasion was not significantly reduced when lower doses of zoledronic acid or treatment times of 4 h or less were used in this system, possibly relating to differences in the sensitivity of the cell type studied to these drugs.

In clinical practice, metastatic breast cancer patients would rarely be treated with bisphosphonates alone, and may instead be given a combination of drugs, including bisphosphonates and cytotoxic chemotherapy drugs such as anthracyclines and taxoids. A small number of reports suggest that cytotoxic drugs alone, when given at low doses, can affect tumor cell migration and invasion. The taxoid paclitaxel for example, is described to inhibit the invasion, migration and degradative activity of PC3-ML prostate cancer cells, Ovcar-3 ovarian cancer cells, glioma cells and B16F10 melanoma cells *in vitro* [23,27–29]. Treatment for 1 h with clinically relevant doses [0.5–1.5 μg/ml (around 0.9–2.6 μM)] of the anthracycline doxorubicin has also been described to reduce the invasion and migration of A2058 melanoma and MDA-MB 231 breast cancer cells, respectively, *in vitro* [26,28,30]. However, apoptosis was not assessed under identical conditions in these studies and thus the inhibitory effect may have been attributed to an increase in cell death rather then reduced invasion or migration. In this present study, treatment of MCF7 cells with the lower dose of 0.05 μM doxorubicin for 4 h nevertheless did not cause a significant effect on invasion or migration, suggesting that this comparatively low dose and short treatment time is not sufficient to induce a functional change within these cells.

Despite a lack of response of MCF7 cells to low doses of bisphosphonates and cytotoxic drugs alone, we and others have shown synergistic and additive effects on tumor apoptosis, adhesion and invasion when bisphosphonates and cytotoxic drugs are administered in combination or sequentially [24,25]. The significant reduction of invasion of MCF7 cells grown on Matrigel after treatment with 10 μM zoledronic acid for 24 h was unaffected by additional treatment with 0.05 μM doxorubicin for 4 h, independent of the sequence. Assessment of apoptosis with these drug treatments revealed only minimal apoptosis in all treatment groups, suggesting that any reduction in invasion by treatment with 10 μM zoledronic acid was not due to increased cell death. Although our group has recently reported a synergistic effect on breast cancer cell apoptosis when treated with doxorubicin for 24 h followed by zoledronic acid for 1 h [25], the results here suggest that doxorubicin and zoledronic acid do not act synergistically on MCF7 apoptosis when given at these doses and treatment times. Pre-treatment of the tumor cells with doxorubicin for 24 h instead of 4 h prior to zoledronic acid treatment must therefore be required to induce this synergistic apoptotic response. However, when cell growth is investigated, it was evident that treatment with 10 μM zoledronic acid for 24 h (with or without doxorubicin treatment) caused a significant reduction in cell numbers when compared with untreated controls, supporting published data on other breast cancer cell types and indicating a growth inhibitory effect at this concentration [13,16,32]. The reduction of invasion observed by these treatments may therefore be accredited to inhibition of cell growth as opposed to reduced invasive potential.

Following reduction of the concentration of zoledronic acid to 1 μM, treatment of MCF7 cells for 24 h did not cause any significant reduction in invasion when cells were treated in the presence or absence of Matrigel. These results differ from the low zoledronic acid concentrations previously shown to reduce invasion, but as these reports have used the MDA-MB 231 breast cancer cell line [13,17], this again may relate to difference in the sensitivity of the cell type studied to zoledronic acid. When cells grown on Matrigel were sequentially treated with 0.05 μM doxorubicin for 4 h, invasion was significantly reduced, but was dependent upon the sequence. If zoledronic acid was administered after doxorubicin in this invasion assay system there was no significant reduction on invasion, whilst when doxorubicin was given prior to zoledronic acid, invasion was significantly reduced when compared with untreated controls. As cell growth and apoptosis were unaffected by all drug combinations, this would indicate that the significant effect observed was actually

due to a reduction of invasive activity by the combined doxorubicin and zoledronic acid treatment. In support, Denoyelle *et al.* [13] have also reported that zoledronic acid only affects cell proliferation and apoptosis at concentrations above 10 μM, whilst Magnetto *et al.* [21] describe an additive effect of the nitrogen-containing bisphosphonate ibandronate and paclitaxel on breast cancer cell adhesion to bone matrices and invasion.

Our study was extended to assess whether treating the cells with doxorubicin followed by 1 μM zoledronic acid without the presence of Matrigel would have a similar effect on invasion, to more closely reproduce the work of Magnetto *et al.* [21]. Cells were therefore treated in a culture plate prior to setting up the assay. However, treating cells in this manner did not result in a similar inhibitory effect on invasion nor migration, perhaps suggesting that the presence of Matrigel was in some way contributing to this effect on invasion. As bisphosphonates readily bind to bone [34] and can be detected for a number of years post-treatment, gradually being released from the skeleton [35], it is feasible that the ECM proteins present in Matrigel are sequestering zoledronic acid and possibly doxorubicin, and releasing the drugs slowly over the entirety of the assay. In the study by Neville-Webbe *et al.* [25], maximal effect on apoptosis was seen when cells were treated with doxorubicin for 24 h prior to zoledronic acid treatment. If Matrigel can bind doxorubicin, then it is possible that the cells are in contact with the drug for periods of longer than 4 h, which may be sufficient to induce this synergistic effect. Moreover, whilst growing on the Matrigel, MCF7 cells could have begun to degrade the matrix proteins, thus aiding drug release. Bound within the matrix, however, are a number of growth factors and thus any degradation could have also resulted in release of these factors, potentially influencing these results. Carrying out comparable growth and apoptosis measurements on cells grown on Matrigel is therefore required to clarify this point.

The mechanism of bisphosphonate action is now well documented, with nitrogen-containing bisphosphonates such as zoledronic acid affecting cellular behavior through inhibition of the mevalonate pathway. By inhibiting a key enzyme in the mevalonate pathway (farnesyl diphosphate synthase), this class of bisphosphonates prevents protein prenylation (geranylgeranylation and farnesylation) of a wide variety of small intracellular G-proteins, including Ras and Rho—both of which are reported to determine tumor cell motility and invasion [36]. Loss of prenylation consequently prevents these proteins from acting at the correct spatial location within the cell, thus ultimately affecting their normal behavior. Denoyelle *et al.* [13] have, for example, demonstrated that inhibition of MDA-MB 231 breast cancer cell invasion following 1 μM zoledronic acid treatment can be reversed by inclusion of the artificial isoprenoid GGOH in the assay to restore geranylgeranylation, indicating the involvement of the mevalonate pathway in tumor invasion. In this present study, the reduction of invasion observed after treatment with 10 μM zoledronic acid was similarly prevented by the addition of GGOH. The importance of Rho proteins was further demonstrated in the study by Denoyelle *et al.* [13], after analysis of the cytosol following zoledronic acid treatment identified prevention of RhoA, but not Ras, membrane localization ultimately leading to cytoskeletal disorganization to inhibit cell motility. The synergistic mechanism of zoledronic acid and doxorubicin is currently unknown. Anthracyclines such as doxorubicin primarily act as DNA-damaging agents, inhibiting topoisomerases, but their precise effect appears to vary between cell types and the concentrations administered. Precisely how doxorubicin may enhance zoledronic acid treatment here and in our previous study [13] is under investigation. As invasion and apoptosis are two distinct processes, utilizing separate intracellular pathways, the different treatment times required to achieve an enhanced response on each process would suggest that these drugs are acting together, but in a different manner, on apoptosis and invasion.

In summary, the results of this study would suggest that doxorubicin might enhance the effect of zoledronic acid on breast cancer cell invasion. At concentrations of 10 μM, zoledronic acid is likely to affect cell growth in addition to invasion, whilst at lower, clinically relevant doses, the effect on cell growth is minimal, indicating that 1 μM zoledronic can reduce invasion only if cells have been previously treated with doxorubicin.

Acknowledgements

We would like to thank Mrs Alsyon Evans and Ms Diane Lefley for their technical support.

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