Microvascular Endothelial Cell Responses in vitro and in vivo: Modulation by Zoledronic Acid and Paclitaxel?

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Key Words

Anti-angiogenic therapy • Anti-vascular therapy • Dorsal microcirculation chamber • Endothelial cells • Paclitaxel • Zoledronic acid

Abstract

Background/Aims: The cytotoxic agent paclitaxel and the anti-resorptive drug zoledronic acid are used in the early and advanced breast cancer setting, respectively. Both agents have been demonstrated to have anti-tumour and anti-endothelial actions. Combining paclitaxel with zoledronic acid induces a synergistic increase in apoptotic breast cancer cell death in vitro, suggesting an increased anti-tumour effect in vivo, but any specific effects on the normal microvasculature and potential side-effects of this combination remain to be established. **Methods:** The effects of zoledronic acid and paclitaxel were investigated, alone and in combination, on human microvascular endothelial cells in vitro, using functional assays including proliferation, migration, tubule formation and apoptosis. The in vivo effect of the drugs on the normal microvasculature was determined using the dorsal microcirculation chamber model. **Results/Conclusion:** Zoledronic acid reduced human dermal microvascular endothelial cell (HuDMEC) proliferation, caused accumulation of cells in S phase, and inhibited migration, tube formation and Rap1a prenylation. Paclitaxel significantly inhibited tube for-

mation and proliferation, and increased endothelial necrosis; the combination induced HuDMEC apoptosis and further enhanced the inhibition of tube formation and migration. The combination caused minimal effects on the normal microvasculature in vivo, suggesting that this potential therapeutic strategy is not associated with deleterious microvascular side-effects.

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Introduction

Over the past decade there has been an increasing research effort to develop inhibitors of tumour angiogenesis as anti-cancer therapies. Tumours up to ~1 mm in diameter obtain oxygen and nutrients by passive diffusion. Small tumour cell populations subsequently stimulate the angiogenic switch, recruiting endothelial cells (EC) from surrounding tissues, as well as endothelial progenitor cells that home from the bone marrow to the site of active angiogenesis via the circulation. This leads to the formation of a new vascular network that infiltrates and nourishes the tumour mass, facilitating tumour expansion.

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Anti-angiogenic agents may inhibit expansion of the tumour mass, or induce growth suppression to a pre-angiogenic volume, which may be further reduced by administration of chemotherapy. Conventional anti-cancer therapies, such as cytotoxic drugs [1–4] and anti-resorptive agents [5], may also affect the tumour microvasculature. Zoledronic acid is used in the treatment of cancer-induced bone disease [6], and has been shown to have anti-tumour effects both in vitro [5] and in metastasis models in vivo [5, 7, 8]. Anti-angiogenic effects of zoledronic acid have been demonstrated on EC in vitro [9], and in vivo there is reduced angiogenic growth factor-induced vascularisation of normal tissue or subcutaneous implants [9, 10]. In addition, zoledronic acid reduces the levels of circulating angiogenic factors, such as vascular endothelial, platelet-derived [11] and basic fibroblast growth factors [12], in patients with advanced cancer [11]. Previous studies have shown that the anti-tumour effect of zoledronic acid in vitro is enhanced when administered in a specific sequence with the cytotoxic agent paclitaxel [13, 14].

Paclitaxel is a cytotoxic agent that disrupts normal microtubule function, causing accumulation of the cells in the G2/M phase ultimately leading to apoptotic cell death [15]. Studies have shown that paclitaxel interferes with the microtubule network of EC [16] at concentrations lower than those required to affect tumour cells, but without inducing apoptotic EC death [3, 17]. Anti-angiogenic properties of both zoledronic acid and paclitaxel have been described, but the majority of studies have used macrovascular EC, e.g. human umbilical vein EC (HUV-EC) rather than microvascular EC. Previous in vitro studies have generally used high concentrations of zoledronic acid and paclitaxel to generate anti-angiogenic effects compared to the clinically achievable doses [3, 9, 17, 18]. Although both drugs may be used in the treatment of advanced breast cancer they are not routinely administered in combination and are often given some time apart. There is no information on potential vascular side-effects associated with combining the two agents, and no previous studies have investigated the effects on the normal microvasculature following co-administration of paclitaxel and zoledronic acid in vivo, an important indicator of potential adverse effects associated with combination therapy.

In this study, we investigated the effects of zoledronic acid and paclitaxel, alone and in combination, on human dermal microvascular EC (HuDMEC) in vitro, followed by in vivo studies determining responses on the normal microvasculature using the dorsal microcirculation

chamber (DMC) model. Our data show that the combination of paclitaxel and zoledronic acid increases inhibition of HuDMEC migration, apoptosis and tubule formation in vitro compared to administration of the single agents. Microvascular diameters remained unchanged in the DMC, with no evidence of endothelial disruption in other normal tissues on histological analysis, following in vivo administration of the combination regime. This suggests that zoledronic acid and paclitaxel may not cause adverse short-term (up to 5 days) damage on the normal microvasculature if used as a combination therapy.

Methods

Cell Culture

HuDMEC were extracted from excess tissue following routine breast surgery (kindly provided by Prof. M.W.R. Reed, Royal Hallamshire Hospital, Sheffield, UK; Ethics No. SSREC98/198). All patients gave written informed consent for the use of their tissue for research. HuDMEC were isolated using CD31 Dynabead® selection (Invitrogen, UK) to ensure a homogenous population and cultured in EBM-2 containing 5% v/v of foetal bovine serum with supplements (standard culture medium; Cambrex, UK) at 37°C and 5% CO₂. Cells were used between passages 3 and 7, with approximately 20 different cell isolates used in the study.

Drugs

Zoledronic acid [1-hydroxy-2-(1H-imidazol-1-yl)ethylidene] bisphosphonic acid (supplied as the hydrated disodium salt by Novartis Pharma AG, Switzerland) was diluted in phosphate-buffered saline (PBS). For in vitro use, paclitaxel (Sigma, UK) was dissolved in DMSO at 10 mM according to the manufacturer's instructions and then diluted to 1 mM in PBS. For the experiments nanomolar concentrations were used hence the final DMSO concentration is approximately 10⁻⁶ M. For in vivo use paclitaxel was diluted in 1:1 anhydrous ethanol:Cremophor EL (BDH, UK). Drug dilutions were sterile filtered using Acrodisk® syringe filters 0.8/0.2 µm (Pall, UK) before use. The final concentrations used for the combination experiments were based on preliminary dose-response and time-course studies performed for each of the assays allowing us to establish the doses of each agent that produced no greater than 50% inhibition. Wherever feasible, the observer analysing the data was blinded to the treatment groups.

Cell Cycle Analysis

HuDMEC were cultured in T25 flasks (Nunc, UK) under standard conditions until approximately 20% confluent and washed with PBS before addition of drugs. To determine the effect of zoledronic acid on S phase, cells were treated with 25 or 50 µM zoledronic acid for 24–72 h. In the combined treatment, cells were incubated for 24 h with 25 µM zoledronic acid and 4 nM paclitaxel, followed by 48-hour incubation in drug-free standard culture medium. Floating and adherent cells were harvested and centrifuged to remove the supernatant. HuDMEC were resuspended

in 2 ml PBS, then pelleted, fixed by dropwise addition of 1 ml of 70% ice-cold ethanol and incubated at 4°C overnight. For cell cycle analysis, cells were washed twice in 1 ml of PBS and resuspended in 50 µg/ml propidium iodide and 100 µg/ml RNase A (Sigma). Following overnight incubation at 4°C, cell cycle analysis was performed using an FACSsort analyser (BD Biosciences, UK) and the CellQuest (BD Biosciences) software programme was used to plot flow charts and to quantify the results using the parameters FL3 (x-axis, which represents the signal value of the parameter) and FL1 (y-axis, which represents the number of events of the parameter). The experiment was carried out three times in triplicate.

Cell Proliferation

HuDMEC were seeded into 24-well plates at 10⁴ cells/well and incubated overnight at 37°C and 5% CO₂. Following washing with PBS, 1, 10, 25 and 50 µM zoledronic acid (24, 48 and 72 h) or 2, 4 and 10 nM paclitaxel (48 and 72 h) were added to the standard culture medium, and cells were counted after 24, 48 and 72 h of incubation using a Coulter Counter (Becton-Dickinson, UK). The experiment was carried out three times in triplicate.

Cell Death Assay: Apoptosis and Necrosis

Following treatment with 50 µM zoledronic acid and 2 nM paclitaxel for 24 h with 48-hour incubation in drug-free medium, HuDMECs were incubated with 8 µM Hoechst 33342 (Sigma RBI, UK) and 5 µM propidium iodide (Molecular Probes, UK) in the dark for 15 min at 37°C and 5% CO₂. Detection of cell death was performed by manual counting of necrotic (propidium iodide positive), viable and apoptotic (Hoechst 33342+) nuclei, the apoptotic nuclei displaying features of karyorrhexis. Evaluation of nuclear characteristics was made using an inverted fluorescent-phase Leica DMIRB microscope with an UV filter (355 nm excitation and 465 nm emission). The numbers of viable, apoptotic and necrotic cells were determined by manual counting using a Whipple graticule (100 squares in a 7-mm² area from Pyser-SGI, UK). The percentage of each type of cell death was established by counting 5 different areas per well. Each experiment was carried out three times and included quadruplicate samples.

Detection of Unprenylated Rap1a

HuDMEC were cultured in T25 flasks under standard conditions and treated with 10–50 µM of zoledronic acid for 24, 48 and 72 h. HuDMEC lysates were generated using a mammalian cell lysis kit (Sigma) according to the manufacturer's instructions, and 10 µg of cell lysate and 10 µl of molecular weight marker (Bio-Rad, UK) were loaded in each lane of a 10% polyacrylamide-SDS gel, followed by blotting onto polyvinylidene difluoride membranes (PVDF Immobilisation Transfer Membranes; Millipore, UK) under reducing conditions. Membranes were then incubated with goat polyclonal antibody specific for unprenylated Rap1a (1:2,000, Cat. No. sc1482, clone C-17; Santa Cruz Biotechnology, USA) in blocking buffer (5% dried milk in distilled water) at 4°C overnight, followed by a 1-hour incubation with anti-rabbit IgG-HRP conjugate in blocking buffer (1:15,000, Cat. No. NA934; Amersham Biosciences, UK). The protein bands were visualised using Supersignal West Dura (Pierce, UK) and developed on Kodak Biomax MR film (Anachem, UK). Each experiment was performed three times.

Tube Formation Assay

HuDMEC were pre-treated in T25 flasks with 10 µM zoledronic acid for 24 h and 0.25 nM paclitaxel for 4 h, with further 48-hour incubation in drug-free culture medium. Cells were trypsinised and transferred onto 24-well plates coated with full growth factor Matrigel® (30 µl Matrigel/well, BD Biosciences) at 2 × 10⁴ cells/well, including a control of untreated cells, followed by incubation at 37°C and 5% CO₂ for 8 h, fixed with ice-cold ethanol and stained with haematoxylin (BDH) for 5 min followed by eosin (BDH) for 2 min. Tubules were photographed in 3 randomly chosen fields of view per well using a ×4 objective, and their number manually determined. Tubules assessed in this study could be defined as belonging to one of the three following categories: (1) intercellular tubule: a single tube formed between two single cells; (2) 'chain-like' tubule: tubes formed by multiple single cells arranged in a row connecting two cell 'islands', and (3) 'bridge-like' tubule: tubes formed by two tube chains arranged in a thick bundle that connects two cell 'islands'. Each experiment was performed three times in quadruplicate.

Migration Assay

HuDMEC were seeded in 6-well plates pre-coated with 0.2% gelatin at 6.6 × 10⁵ cells/well, and incubated overnight at 37°C and 5% CO₂. HuDMEC were then incubated with 10 µg/ml/well of mitomycin C (Sigma) in serum-free RPMI for 3 h on ice to inhibit cell proliferation during the migration period, followed by a 20-min recovery incubation in standard culture medium at 37°C and 5% CO₂. Two 1-mm scratches/well were made and two fields of view/scratch were marked on the external lower surface of the plate to indicate the exact position for the assessment of migration. HuDMEC were then thoroughly washed with PBS before 50 µM zoledronic acid and 500 pM paclitaxel were added to standard culture medium for 48 h. Following marking of the cell-free area, migration was determined as percentage of scratch closure, quantified by use of Image J software (National Institutes of Health, USA). The experiment was carried out three times in quadruplicate.

Animals and DMC Implantation

Male CD1 nude mice (8 weeks old, n = 19) were obtained from Charles River Laboratories (UK). Housing, handling, and surgical and treatment procedures were conducted in accordance with Home Office UK legislation under PPL 40/2972 (N.J.B.). DMC was manufactured from titanium by the Medical Workshops (University of Sheffield, UK). DMC consists of a pair of window frames, a pair of C-clips, a pair of glass slips (Ø 8 mm), 3 screws, 3 nuts and 3 spears. Mice were anaesthetised with 150 µl of 1:1 Hypnorm (VetaPharma, UK)/diazepam (Alpharma, UK) in two parts of water (Braun, Germany). A circular section of dermis and the most superficial layer of the panniculus carnosus muscle with underlying fascia was removed from one side of the dorsal skin flap, which allowed recording of microvessels on the contralateral side. The two chamber halves were sutured onto each side of the skin flap, filled with saline, sealed with a circular glass slide and secured with a titanium clip [19].

Administration of Zoledronic Acid and Paclitaxel in vivo

Mice were allowed to recover from surgery for approximately 36 h prior to drug administration. Before surgery the animals were trained to sit in a Plexiglas restrainer mounted onto the microscope,

Fig. 1. Effects of zoledronic acid and paclitaxel on HuDMEC proliferation and cell cycle. **a** The effect of zoledronic acid on HuDMEC proliferation. HuDMEC were treated with 1–50 μM of zoledronic acid for 24–72 h. Data were normalised against untreated control and presented as percentage of cell number against increasing concentration (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **b** The effect of zoledronic acid on accumulation of HuDMEC in S phase. HuDMEC were treated with 25 and 50 μM of zoledronic

acid for 24–72 h. Data are presented as percentage of cell number in S phase against total percentage of events in S phase with increasing time (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **c** The effect of paclitaxel on HuDMEC proliferation. HuDMEC were treated with 2–10 nM of paclitaxel for 48 and 72 h. Data were normalised against untreated control and presented as percentage of cell number against increasing concentration (* $p < 0.05$). **a–c** Error bars indicate $\pm$ SEM ($n = 3$).

which minimised any effects on the microvasculature due to stress. The horizontally mounted microscope (Nikon, UK) was connected to a video recorder and 30-second recordings of the microvasculature were taken from 3 randomly selected arterioles and venules per animal [19]. The same vessels were monitored at each time point following drug administration, and then again on each individual day of treatment. On days of drug administration, a 30-second video recording was taken before treatment (baseline), and immediately following treatment at 0 min, at 30 min and finally at 120 min. Each arteriolar and venular diameter was determined in triplicate along a 30-μm length of vessel at each time point, using CapiScope image analysis software (KK Technologies, UK). Three administration schedules were used: (A) zoledronic acid alone – administered intraperitoneally at 50, 100 and 150 μg/kg once per day (days 1, 3 and 5; $n = 8$); (B) paclitaxel alone – administered at 10, 20 and 30 mg/kg i.p. once per day (days 1, 3 and 5; $n = 5$), and (C) zoledronic acid combined with paclitaxel. Zoledronic acid was administered at 100 μg/kg i.p. with 20 mg/kg i.v. paclitaxel on day 1, followed by 20 mg/kg i.v. paclitaxel on days 3 and 5 ($n = 6$). The regimes reflect the clinical administration of these agents. Animals were killed by schedule 1 at the end of the study and tissues removed for morphological and histological assessment.

Histological Assessment of EC Morphology

Evaluation of whether the treatments caused overt disruption of EC barriers or acute toxicity, in addition to the morphology of the endothelium and microvasculature, were assessed in a separate experiment, using the combination therapy protocol but without DMC implantation. After culling, brain, spleen, lungs and liver were isolated from 2 animals from each treatment group. Tissues were fixed, paraffin embedded and processed for histo-

logical analysis using standard methodology. Sections were stained using a rat antibody specific for mouse CD34 (MCA-1825CA 1:50, ABDSerotec, UK) and an IgG2A isotype control, EC and vessel morphology were assessed by an experienced histopathologist (S.S.C.) who was blinded to the treatment groups.

Statistical Analysis

Statistical analysis was carried out by GraphPad Prism (version 5.00) for Windows (GraphPad Software, USA) with 95% confidence intervals. For in vitro studies, dose- and time-response data with zoledronic acid and the migration study were analysed by two-way ANOVA with Bonferroni post hoc test. Proliferation data using paclitaxel, and apoptosis and tube formation data were analysed by Kruskal-Wallis test with Dunns post hoc test. Cell cycle data using combined treatment were analysed by Kruskal-Wallis test with Bonferroni post hoc test. For in vivo studies, data with single agents were analysed by Kruskal-Wallis test with Dunn's post hoc test, combined treatment was analysed by two-way ANOVA with Bonferroni post hoc test. $p < 0.05$ was considered statistically significant.

Results

Effects of Zoledronic Acid and Paclitaxel on HuDMEC Proliferation and Cell Cycle

It remains to be established how zoledronic acid affects HuDMEC proliferation, and whether this is associated with induction of apoptosis. We therefore deter-

Fig. 2. The effect of combined treatment with zoledronic acid (ZOL) and paclitaxel (PAC) on accumulation of HuDMEC in S phase and induction of apoptosis. Error bars indicate $\pm$ SEM ($n = 3$). **a** HuDMEC were treated with 25 μ M of zoledronic acid and 4 nM of paclitaxel for 24 h, followed by a 48-hour incubation in drug-free culture medium. Percentage of cells (percent events) accumulated in S phase was detected by flow cytometry. ** $p < 0.01$; *** $p < 0.001$, vs. untreated control per incubation time point.

b The effect of combined treatment with zoledronic acid and paclitaxel on EC apoptosis. HuDMEC were treated with 50 μ M zoledronic acid and 2 nM paclitaxel sequentially or simultaneously combined for 24 h, followed by a 48-hour incubation in drug-free standard cell culture medium. Apoptosis was determined by evaluation of nuclear morphology using UV light microscopy. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, vs. all treatments.

mined the effect of zoledronic acid (1, 10, 25 and 50 μ M) on HuDMEC proliferation and cell cycle progression, following treatment for 24, 48 and 72 h (fig. 1a). Zoledronic acid (25 μ M) inhibited cell proliferation after 24 h of treatment to $28.3 \pm 6.3\%$ ($p < 0.05$) and 50 μ M to $30.7 \pm 17.1\%$ ($p < 0.05$) compared to untreated cells. Longer incubation periods with 50 μ M zoledronic acid did not cause an additional reduction in proliferation, with $46.3 \pm 9.2\%$ ($p < 0.001$) at 48 h and $34.0 \pm 7.4\%$ ($p < 0.01$) at 72 h compared to untreated cells. This decrease in cell proliferation was accompanied by an increased accumulation of cells in S phase of the cell cycle, with 50 μ M zoledronic acid for 48 h ($12.4 \pm 1.3\%$; $p < 0.001$) and 72 h ($12.7 \pm 5\%$; $p < 0.001$) compared to untreated cells (fig. 1b), with a corresponding decrease in cell number in G1 and G2/M phases, but there was no induction of apoptotic cell death (data not shown). Paclitaxel (0.01–10 nM) has been shown to affect HUVEC proliferation [16, 20], whereas effects on HuDMEC have

not been reported. Paclitaxel caused a dose-dependent inhibition of HuDMEC proliferation, with maximal effects at 10 nM for 72 h ($44.8 \pm 18.6\%$; $p < 0.05$ vs. control, fig. 1c).

Combining Zoledronic Acid and Paclitaxel Induces HuDMEC Apoptosis and Increases Accumulation of S-Phase Cells

We next determined whether combining paclitaxel and zoledronic acid modulated EC cycle distribution and apoptosis levels, following treatment with either the single agents, or the drugs given in sequence or simultaneously. Simultaneous treatment with 25 μ M of zoledronic acid and 4 nM of paclitaxel attenuated HuDMEC cycle at S phase ($10.1 \pm 0.3\%$) compared with zoledronic acid alone ($3.99 \pm 0.3\%$; $p < 0.01$), paclitaxel alone ($2.6 \pm 7.7\%$; $p < 0.001$) or the sequential treatment of zoledronic acid followed by paclitaxel ($2.8 \pm 0.5\%$; $p < 0.001$) and paclitaxel then zoledronic acid ($4.0 \pm 1.0\%$; $p < 0.01$;

Fig. 3. The effect of zoledronic acid on prenylation of Rap1a. HuDMEC were treated with 10–50 μM zoledronic acid for 24, 48 and 72 h and unprenylated Rap1a levels were determined by Western blot using an antibody specific for unprenylated Rap1a. Actin was used as a positive control.

fig. 2a). No significant accumulation of cells in G1 and G2/M phases was observed compared to the respective controls (data not shown). Inhibition of cell cycle progression was accompanied by induction of apoptosis, with a maximal increase following simultaneous treatment with zoledronic acid and paclitaxel. Simultaneous treatment with both agents increased apoptosis levels ($11.7 \pm 0.8\%$) compared to the untreated control ($0.6 \pm 0.06\%$; $p < 0.001$), the single agents (zoledronic acid: $2.2 \pm 0.3\%$; paclitaxel: $6.2 \pm 0.5\%$; $p < 0.001$) and to both sequential treatments (zoledronic acid followed by paclitaxel: $8.5 \pm 0.5\%$; paclitaxel then zoledronic acid: $7.2 \pm 0.9\%$; $p < 0.001$; fig. 2b).

Zoledronic Acid Inhibits Rap1a Prenylation in HuDMEC

Zoledronic acid acts by inhibition of farnesyl diphosphate synthase, a key enzyme in the mevalonate pathway, blocking prenylation of small GTPases, e.g. Rap1a [21], causing intracellular accumulation of their unprenylated forms [22, 23]. Detection of increased levels of unprenylated Rap1a is therefore commonly used as a surrogate measurement of zoledronic acid uptake by cells in vitro. The levels of unprenylated Rap1a were determined fol-

lowing 24–72 h of zoledronic acid treatment (fig. 3). Zoledronic acid increased accumulation of unprenylated Rap1a in HuDMEC following exposure to 25 or 50 μM for 24 h, with further increases following 48 and 72 h of exposure.

Combining Zoledronic Acid and Paclitaxel Causes Increased Inhibition of HuDMEC Tube Formation

We used the Matrigel assay to investigate whether exposing HuDMEC to zoledronic acid for short periods affected tubule formation in vitro; a 4-hour exposure to 50 μM attenuated tube formation at 48 h, reducing tubule number to $36.2 \pm 8.6\%$ of control. Low doses of paclitaxel for 24 h reduced tubule number to 53.3 ± 6.7 ($p < 0.05$) and $54.3 \pm 11.1\%$ ($p < 0.05$) following treatment with 0.5 and 1 nM, respectively, compared to untreated control (data not shown). Using either single agents or drug combinations we demonstrated that 0.5 nM of paclitaxel significantly inhibited tubule formation compared to control when given either before (35.5 ± 5.33 vs. $0.8 \pm 4.6\%$, $p < 0.01$) or after 10 μM of zoledronic acid (41.0 ± 6.6 vs. $0.8\% \pm 4.6\%$, $p < 0.001$), which was not further enhanced by changing the drug sequence (fig. 4). In contrast, when both agents were given simultaneously, tubule number was reduced by $78.6 \pm 1.5\%$ compared to control, both single-agent ($p < 0.001$) and sequential treatments ($p < 0.001$). In contrast to previous studies in breast cancer cells [14], the most effective combination of paclitaxel and zoledronic acid in EC appears to be simultaneous exposure to the two drugs.

Combining Zoledronic Acid and Paclitaxel Causes Increased Inhibition of HuDMEC Migration

Migration of EC is one of the fundamental processes occurring during angiogenesis, and several studies have reported significant inhibition of HUVEC migration by zoledronic acid [9, 18, 28]. Paclitaxel (5–20 nM) has also been shown to cause inhibition of chemotactic migration of human aortic EC [24]. We therefore investigated whether combining zoledronic acid and paclitaxel induced increased inhibition of EC migration compared to the single agents. Treatment with 50 μM zoledronic acid or paclitaxel 0.5 nM for 48 h did not significantly affect HuDMEC migration compared to untreated control (zoledronic acid 41.3 ± 3.7 vs. $52.9 \pm 3.0\%$, and paclitaxel 49.6 ± 5.6 vs. $52.9 \pm 3.0\%$; both $p > 0.05$). In contrast, zoledronic acid combined with paclitaxel significantly inhibited migration compared to control (32.98 ± 5.6 vs. $52.9 \pm 3.0\%$; $p < 0.01$; fig. 5).

Fig. 4. The effect of combined treatment with zoledronic acid (ZOL) and paclitaxel (PAC) on HuDMEC tube formation. HuDMEC were pre-treated with 10 μ M zoledronic acid and 0.25 nM paclitaxel for 4 h with a 48-hour incubation in drug-free medium, followed by incubation on full growth factor Matrigel for 8 h. **a** Representative views of tubular network (magnification:

$\times 4$). Scale bar: 25 μ m. **b** Percentage inhibition of tube formation normalised against control. Error bars indicate $\pm$ SEM ($n = 3$). ** $p < 0.01$; ^a $p < 0.01$ or ^a $p < 0.001$ vs. respective single-agent treatments; ^b $p < 0.001$ vs. untreated control, single-agent and combination treatments.

Zoledronic Acid Alone or in Combination with Paclitaxel Does Not Affect Normal Microvascular Diameter in vivo

Acute effects of zoledronic acid alone, or in combination with paclitaxel, on the normal microvasculature have not been described previously. To investigate this, we administered zoledronic acid alone at increasing doses on days 1, 3 and 5, paclitaxel alone at increasing doses on days 1, 3 and 5, or in combination by administration of 100 μ g/kg i.p. zoledronic acid (day 1) with 20 mg/kg i.v. paclitaxel (days 1, 3 and 5) and determined venular (fig. 6) and arteriolar diameter. 100 μ g/kg of zoledronic acid is equivalent to the 4-mg intravenous infusion ad-

ministered to patients with skeletal complications associated with metastatic breast cancer [8]. Increasing doses of zoledronic acid did not significantly modulate venular (fig. 6) or arteriolar diameters compared to the baseline [on day 1 (106.9 ± 9.1 vs. 99.6 ± 8.8 μ m), day 3 (98.91 ± 0.4 vs. 90.5 ± 9.8 μ m) or day 5 (96.8 ± 9.6 vs. 97.7 ± 10.5 μ m), baseline vs. 120 min, respectively, each day of study (1, 3 and 5)]. Paclitaxel at increasing doses from 10 mg/kg did not modulate venular diameters (fig. 6). Arteriolar diameter on day 1 (10 mg/kg), day 3 (20 mg/kg) and day 5 (30 mg/kg) demonstrated no significant differences between baseline and 120-min readings following paclitaxel treatment (baseline vs. 120 min: day 1,

Fig. 5. The effect of combined treatment with zoledronic acid and paclitaxel on migration of HuDMEC. The cells were incubated with 25 and 50 μ M zoledronic acid and 500 pM paclitaxel in standard culture medium for 48 h. **a** Representative views of exposed endothelium immediately after scratch was produced (0 h) and

then 48 h following treatment (magnification: $\times 10$). Scale bar: 25 μ m. **b** Representative example of data normalised against untreated control at 0 h and presented as percentage of scratch closure. * $p < 0.05$, ** $p < 0.01$. Error bars indicate $\pm$ SEM ($n = 3$).

104.2 ± 15.2 vs. 101 ± 15.7 μ m; day 3, 83.1 ± 14 vs. 82.5 ± 11.4 μ m, and day 5, 85.4 ± 18 vs. 79.9 ± 16.6 μ m). There was a transient but significant ($p < 0.01$) decrease in arteriolar diameter at both the 0- and 120-minute time points between the high (day 5, 30 mg/kg) and the

low (day 1, 10 mg/kg) paclitaxel concentrations. When zoledronic acid (100 mg/kg), was combined with paclitaxel (20 mg/kg), there were no significant changes in venular (fig. 6) or arteriolar diameters either acutely or over the 5-day experimental evaluation (day 1 vs. day 3

Fig. 6. The effect of zoledronic acid or paclitaxel alone and in combination on normal vessel diameters in CD1 nude mice. Vessel diameters were determined 0, 30 and 120 min following administration of treatments on days 1, 3 and 5. Data at each time point were normalised against the diameter at baseline on days 1, 3 and 5. Data are presented as means $\pm$ SEM. Venular diameter follow-

ing zoledronic acid administration (**a**; $n = 8$), paclitaxel administration (**b**; $n = 5$) and following combination treatment (**c**; $n = 6$). **d** Schematic representation of calculation of vessel diameter by Capiscope® software. Vessel diameters [arteriole (a) and venule (v)] were determined in triplicate per vessel (---). Scale bar: 50 μ M.

vs. day 5, 72.0 ± 7.2 vs. 60.9 ± 8.5 vs. 52.3 ± 8.5 μ m). There was no morbidity associated with any of the treatment schedules.

We also determined whether any of the treatments caused overt disruption of EC barriers or acute toxicity,

by tissue morphology and microvascular architecture following treatment assessed in a separate experiment. Following resection and processing, histological assessment of the spleen, liver, lung and brain of tissue from animals in each treatment group was carried out by an

Fig. 7. The effect of treatment with zoledronic acid or paclitaxel on spleen vasculature. CD1 nude mice were treated with saline, 100 $\mu\text{g}/\text{kg}$ i.p. zoledronic acid 20 mg/kg i.v. paclitaxel, alone or in combination. Animals were sacrificed and the spleen isolated and processed for histological analysis of the vasculature following staining with an antibody to CD34. **a** Control. **b** Paclitaxel only. **c** Zoledronic acid only. **d** Paclitaxel and zoledronic acid.

experienced histopathologist. No disruption of the endothelial barrier or tissue architecture was observed in any of the treatment groups (fig. 7).

Discussion

Treatment of cancer frequently involves a combination of different agents, some of which target the tumour vasculature, to eradicate tumour growth and progression. In vitro studies using tumour cell lines have shown that combination treatment with zoledronic acid and cytotoxic agents, including paclitaxel, results in increased anti-tumour effects compared to the single agents [13, 14, 25]. These responses are seen at lower doses when drugs are used in combination in vitro, which may ultimately induce fewer or less severe side-effects in vivo. In addition, drugs may have unexpected adverse effects when given in combination, causing systemic toxicity or affecting normal tissue in addition to the tumour [26, 27]. Prior to combining agents for therapeutic use, it is therefore important to determine the effects on both tumour cells and the normal cell types likely to be affected, both in vitro and in vivo. Although the anti-resorptive agent zoledronic acid is used extensively in the treatment of advanced cancers (including breast cancer), few studies have focused on the potential anti-angiogenic effects of

this drug [9, 11], despite evidence that zoledronic acid modulates responses to EC in vitro [9, 10]. Patients receiving zoledronic acid may also be receiving chemotherapy, including paclitaxel, but the consequence of the combination on the normal microvasculature or indeed the tumour microvascular network has not been described. To our knowledge this is the first study to focus on any endothelial-related events and aimed to determine whether combining paclitaxel and zoledronic acid increased anti-angiogenic responses in vitro and/or caused adverse effects on the normal microvasculature in vivo.

Previous studies have shown that zoledronic acid inhibits HUVEC proliferation [9, 10] causing S-phase cell cycle arrest accompanied by increased levels of apoptosis [9]. Zoledronic acid also causes S-phase accumulation of a range of different tumour cell types [14, 23], which is associated with changes in cyclin expression and upregulation of cyclin-related kinase inhibitors [23], indicating specific effects on cell cycle progression. Reported dosing ranges and incubation times affecting HUVEC proliferation vary from 3–30 μM zoledronic acid for 24 h [9] to 100 μM for 48 h [10]. To investigate whether zoledronic acid modulates proliferation of microvascular EC, HuDMEC were exposed to increasing doses of zoledronic acid for up to 72 h. Zoledronic acid at 50 μM inhibited HuDMEC proliferation after 24 h, and this effect was further enhanced after 48 and 72 h. Inhibition of cell proliferation was ac-

accompanied by an accumulation of cells in S phase of the cell cycle after 48 and 72 h. There was no evidence of apoptotic EC death, suggesting that zoledronic acid affects cell cycle progression via an apoptosis-independent pathway. The data suggest that the agents induce EC cytostasis without killing the cells, supporting previous studies showing that high doses of zoledronic acid [9, 28] and paclitaxel [17] are required for induction of apoptotic cell death. An apoptosis-independent mechanism of action has previously been described in cancer cell lines [29], which does not appear to be tumour cell specific, with similar results demonstrated in leukemic and colorectal carcinoma cell lines of wild-type p53 and p53-negative status [30]. In the current study, treatment with paclitaxel demonstrated that prolonged incubation periods (72 h) and high doses (10 nM) were required to modulate HuDMEC proliferation. The same dose of paclitaxel was previously found to significantly inhibit HUVEC proliferation after only 24 h of treatment [20], which suggests a difference in sensitivity between macro- and microvascular EC. The reasons for this difference remain to be identified but could relate to a differential proliferation rate and/or uptake of the drug [31]. In support of this we found that at 48 h, 10 μ M zoledronic acid caused accumulation of unprenylated Rap1a in HUVEC (data not shown), whereas in HuDMEC, this was only detectable following exposure to 25 μ M. This is the first study showing that HuDMEC internalise zoledronic acid, causing accumulation of unprenylated Rap1a in a dose- and time-dependent manner. Our main finding is that a high zoledronic acid dose (50 μ M) is required to modulate prenylation by 24 h. In comparison, studies in tumour cells have shown accumulation of unprenylated Rap1a following exposure to lower doses of zoledronic acid [32, 33], probably reflecting their higher endocytic activity and hence likely increased rate of drug uptake. The function of Rap1a in EC remains to be established, and we have no evidence to suggest that there is a link between accumulation of unprenylated forms of this protein and inhibition of any of the *in vitro* angiogenic processes investigated. With a large number of cellular proteins undergoing post-translational prenylation [34], inhibition of this process by zoledronic acid is likely to affect a number of key molecules resulting in a range of cellular responses.

Previous studies have shown that the effects of zoledronic acid on breast cancer cell lines are enhanced by addition of a chemotherapeutic agent in a sequence-specific manner [14, 25, 35]. In the current study we therefore used the cytotoxic agent paclitaxel in combination with zoledronic acid to target HuDMEC, administering the drugs either in sequence or simultaneously. Our data show that

compared to treatment with single agents, the combinations cause increased accumulation of cells in S phase and failure of HuDMEC to form tubules on Matrigel. In breast cancer cells the highest levels of apoptosis were induced by treatment with the cytotoxic agent prior to zoledronic acid [14], whereas in EC, simultaneous drug treatment was more effective than any of the sequential treatments. This differential EC and tumour cell response may be due to the targeting of different signalling pathways in addition to variations in the rate of drug uptake. Variations in treatment-induced HuDMEC sensitivity were evident, and were dependent on the functional process under investigation. Zoledronic acid and paclitaxel, both alone and in combination, disrupted basic EC functions, modulating the cytoskeletal-dependent processes of tubule formation and migration following 24 h of exposure, with tubule formation demonstrating an enhanced sensitivity to the combination regime. Cell viability, proliferation or cell cycle progression was less sensitive to the treatments. Breast cancer cells have previously been shown to demonstrate increased sensitivity to sequential drug exposure (paclitaxel followed by zoledronic acid) [14], whereas, in contrast, increased inhibition of tubule formation was seen following simultaneous drug exposure, thus highlighting the differential responses exhibited by different cell types.

The molecular mechanisms involved remain to be identified, although rapidly proliferating cancer cells with high metabolic activity are likely to have a different rate of drug uptake compared to EC. The dose of 100 μ M zoledronic acid which inhibited EC migration is high compared to that achievable in the clinical setting (serum levels of maximally 1–2 μ M for 1–2 h following the standard 4-mg infusion), and therefore is unlikely to affect EC during standard patient therapy. However, there are no corresponding published data regarding the effects of zoledronic acid on microvascular EC migration like HuDMEC. Paclitaxel (10 pM) has been shown to inhibit migration of HUVEC through Matrigel membranes *in vitro* [16], whereas later similar studies report variable effects using doses ranging from 0.1 to 10 nM [20, 36].

Having found that combined treatment with paclitaxel and zoledronic acid modulated a number of processes involved in angiogenesis *in vitro*, we next studied the effects of these drugs, alone and in combination, on the normal microvasculature in DMC *in vivo*. Both paclitaxel [37–39] and zoledronic acid [5, 7, 8, 40] demonstrate anti-tumour growth effects *in vivo*, and further investigation has established zoledronic acid is anti-angiogenic in non-tumour models *in vivo*. Previous studies reported a 35% reduction in prostate weight in testosterone-treated

castrated rats compared to controls following subcutaneous administration of 20 µg/kg/day zoledronic acid [10], which was due to inhibition of testosterone-induced vascularisation of the ventral prostate. In addition to bone, bisphosphonates also accumulate in the prostate, probably due to the high concentration of zinc, which may increase specific targeting of prostatic vascularisation. Another study reported that daily administration of 10 and 100 µg/kg zoledronic acid for 6 days inhibited angiogenesis induced by basic fibroblast and vascular endothelial growth factor in a growth factor implant model of angiogenesis in mice [9]. In agreement with this study, we found that high and prolonged dosing with zoledronic acid was required to reduce HuDMEC proliferation in vitro. Anti-angiogenic properties of paclitaxel have been demonstrated in pre-coated stents, with a dose-dependent inhibition of neointimal hyperplasia, accompanied by a reduction in the mean luminal diameter in the stented segment reported [41]. In addition, a significant reduction in the haemoglobin content of subcutaneous Matrigel pellets has been demonstrated following administration of 28 mg/kg paclitaxel, but not with 10 mg/kg [16].

In the current study, the DMC model was used to determine any treatment-induced responses of the normal, established, quiescent microvasculature, which may be less sensitive to the therapeutic agents than vessels undergoing endothelial proliferation. Zoledronic acid was administered between 50 and 150 µg/kg, as 100 µg/kg is the standard used in in vivo tumour models and is equivalent to the 4-mg clinical dose [8]. Increasing doses of zoledronic acid did not modulate basal arteriolar or venular diameters, whereas increasing doses of paclitaxel demonstrated small transient decreases in arteriolar diameters in a proportion of vessels. The combination of 100 µg/kg zoledronic acid with 20 mg/kg paclitaxel had no significant effects on arteriolar or venular diameters on days 1, 3 and 5 of treatment. No treatment morbidity was observed. Histological assessment revealed normal microvascular and tissue morphology and architecture in skeletal muscles, brain, spleen, lungs and liver.

Any successful anti-angiogenic/anti-vascular therapy must mainly target the proliferating tumour microvasculature, whilst inducing minimal disruption to the normal vessels. It is therefore of crucial importance to determine whether combining potent anti-cancer agents results in structural or functional disruption of the healthy microvasculature. To our knowledge, this is the first study to investigate any early responses (up to 5 days) on the normal microvasculature in vivo using paclitaxel and/or zoledronic acid. Our data show that the combination of

paclitaxel and zoledronic acid modulates proliferating EC in vitro, but high concentrations and prolonged exposure times are required to initiate significant inhibition. In contrast, combined treatment with clinically achievable doses of both agents did not induce acute (2 h) or short-term responses (up to 5 days) in the normal quiescent microvasculature in the DMC in vivo. The normal tissue architecture and different cell types in a physiological in vivo microenvironment may influence the availability, binding and uptake of paclitaxel and zoledronic acid, when compared to the single cell type in vitro models where combined effects of the drugs were demonstrated. Further studies determining responses to prolonged drug treatment in tumour models in vivo will identify whether this combination regime demonstrates potential therapeutic efficacy.

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