Pilot studies of the effect of zoledronic acid (Zometa®) on tumor-derived cells *ex vivo* in the ATP-based tumor chemosensitivity assay

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There is debate regarding the direct effect of bisphosphonates against visceral metastases from solid tumors, despite their proven efficacy against the skeletal complications of metastasis. The aim of this study was to determine whether zoledronic acid showed direct activity against five ovarian cell lines and tumor-derived cells, and whether addition of zoledronic acid to cytotoxic agents increased their cytotoxicity. In this study we used a standardized ATP-based tumor chemosensitivity assay (ATP-TCA) to measure the activity of alendronate, clodronate and zoledronic acid in five ovarian carcinoma cell lines and human solid tumors (breast, lung, ovarian, unknown primary carcinoma, and cutaneous and uveal melanoma) ($n=34$). We also tested the combination of zoledronic acid with paclitaxel and cisplatin in tumor-derived cells. All five cell lines exhibited greater sensitivity to bisphosphonates than the tumor-derived cells and in all five the IC_{50} for zoledronic acid was less than 4 μM. In the tumor-derived cells, zoledronic acid showed concentration-dependent inhibition with a median IC_{50} for all tumors tested of 17 μM and evidence of apoptosis (caspase activation). Simultaneous addition of zoledronic acid to cisplatin or paclitaxel showed no major increase in cytotoxicity. We conclude that the activity of bisphosphonates was greater in cell lines than in tumor-derived cells. However, the pattern of activity of bisphosphonates was the same in cell lines and tumor derived cells. This study suggests a direct, or possibly an indirect, effect of zoledronic acid and other nitrogen-containing bisphosphonates against neoplastic cells, but simultaneous addition with cisplatin or paclitaxel does not substantially increase the activity of the cytotoxic agent. *Anti-Cancer Drugs* 16:969–976 © 2005 Lippincott Williams & Wilkins.

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Introduction

Bisphosphonates are potent inhibitors of osteoclast-mediated bone resorption, and are used to treat tumor-associated bone disease, osteoporosis and Paget's disease [1]. They are analogs of endogenous pyrophosphates in which a carbon atom replaces the central atom of oxygen and are divided into two classes, each having a different molecular mechanism of action.

The non-nitrogen-containing bisphosphonates (first-generation drugs), e.g. clodronate and etidronate, are metabolized intracellularly to a β-γ-methylene (AppCp-type) analog of ATP, which is cytotoxic to macrophages *in vitro* [2]. The main mechanism of action for the nitrogen-containing bisphosphonates (third-generation drugs), e.g. alendronate and zoledronic acid, is thought to be through inhibition of the mevalonate pathway, by inhibition of the enzyme farnesyl pyrophosphate (FPP) synthase [1,2]. Inhibition of the mevalonate pathway leads to loss of prenylated proteins needed for post-translation lipid modification of signaling GTPases, such as Ras, Rho and Rac [3]. This in turn causes a loss of osteoclast function and apoptotic cell death [4,5].

Until recently it was thought that nitrogen-containing bisphosphonates did not affect ATP metabolism. However, work by Monkkonen *et al.* [6] suggests otherwise. The group observed that nitrogen-containing bisphosphonates induce formation of a novel cytotoxic ATP-analog, triphosphoric acid 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester (ApppI) via inhibition of the mevalonate pathway in mammalian cells, such as macrophages, gliomas and osteoclasts. ApppI correlates well with the ability of nitrogen-containing bisphosphonates to inhibit FPP synthase and, consequently, to increase isopentenyl diphosphate and may underlie its visceral effects [6].

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Zoledronic acid has demonstrated superior anti-tumor, anti-metastatic and anti-angiogenic activity compared to other bisphosphonates. Senaratne *et al.* [7] demonstrated that the viability of three breast cancer cell lines, MCF-7, Hs 578T and MDA-MB-231, was dramatically reduced after a 4-day incubation with zoledronate, with IC_{50} values of 20, 3 and 15 μM, respectively. This compares to the IC_{50} values for clodronate of 2000, 900 and 700 μM, respectively [7]. Boisser *et al.* [8] demonstrated, using a Matrigel-based assay, that zoledronic acid acted directly on the prostate carcinoma cell line, PmPC3, and the breast carcinoma cell line, MDA-MB-231, to inhibit tumor cell invasion in a dose-dependent manner. Yaccoby *et al.* [9] demonstrated cessation of osteolysis and reduced tumor burden in severe combined immunodeficient human (SCID-hu) host systems treated with zoledronic acid. Bezzi *et al.* [10] showed that zoledronic acid sensitizes human umbilical vein endothelial cells to tumor necrosis factor-mediated apoptosis. Finally, it has been reported that zoledronic acid exhibits synergistic activity against cell lines when combined with paclitaxel [11] and specifically when cell are exposed to paclitaxel for 24 h before the zoledronic acid is introduced [12]. In addition, recent clinical trials suggest a direct anti-neoplastic effect of bisphosphonates independent of their well-known anti-osteolytic activity, although this remains controversial. In three out of four clinical trials in operable breast cancer, adjuvant clodronate or pamidronate have shown both a protection from skeletal complications and a sustained prolongation of overall survival [13–16]. The latter effect is unlikely to be attributable to the bisphosphonate induced osteoclast inhibition, but argues in favor of an intrinsic, although possibly indirect, anti-tumoral action on visceral metastases.

We have previously shown that the ATP-based tumor chemosensitivity assay (ATP-TCA) can be used to measure the effects of cytotoxic agents and antibodies against human tumor-derived cells, and that this matches clinical outcome in a number of tumor types [17,18]. The assay system has also been used to assist the development of a number of new agents and combinations [19,20].

Methods

Cell Lines

1874, 1874ad, OVCAR-3, OVCAR433 and SKOV ovarian cell lines (obtained from Cancer Research UK, Sutton, UK) were cultured in DMEM (Sigma, Poole, UK; D6171) supplemented with 10% heat-inactivated FCS (Biowest, Ringmer, UK; S185H), 2 mM L-glutamine (Sigma; G7513) and 50 μg/ml penicillin/streptomycin (Sigma; P0781), and maintained at 37°C in a humidified atmosphere with 5% CO_2. Growth and morphology were monitored and cells were passaged when they had reached 90% confluence.

Tumors

A total of 34 tumors (31F:3M) were tested in this study consisting of the following tumor types; breast carcinoma ($n = 5$), carcinoma of unknown primary site ($n = 1$), cutaneous melanoma ($n = 8$), lung carcinoma ($n = 1$), ovarian carcinoma ($n = 18$) and uveal melanoma ($n = 1$). Fourteen of 18 of the ovarian carcinomas were recurrent stage 3/4 tumors and had been previously treated with chemotherapy (11 with carboplatin and three with carboplatin plus paclitaxel). Of the remaining four ovarian carcinomas, three were untreated and for one there was no further clinical data available at the time of writing. Of the remaining 16 samples, three had no further clinical data, one had been treated with tamoxifen (breast carcinoma) and the remaining 12 were untreated. The median age of the patients was 62 (range 32–80). In each case only tumor material not required for diagnosis was used in the study, and in all cases consent had been obtained and permission granted by the local ethics committee.

ATP-TCA

The ATP-TCA was performed as previously published [17,21,22]. First, solid tumor samples were dissociated by enzymatic digestion using collagenase (Sigma; C8051) to obtain a single-cell suspension. Cells from the solid tumor, ascites or cell lines were then plated in 96-well polypropylene plates (Corning Life Sciences, High Wycombe, UK) at 20 000, 10 000 or 2000 cells/well respectively in a serum-free complete assay medium (CAM; DCS Innovative Diagnostik Systeme, Hamburg, Germany). Drugs were added to triplicate wells at serial dilutions corresponding to 6.25–200% of a test drug concentration (TDC) estimated from pharmacokinetic data, including the degree of protein binding. All TDCs were within clinically achievable levels. Two controls were included in each plate: a no drug control consisting of media only (MO) and a maximum inhibitor (MI) control which killed all cells present. The plates were incubated for 6 days at 37°C with 5% CO_2. At the end of the incubation period, remaining cells were lysed by addition of cell extraction reagent (DCS Innovative Diagnostik Systeme). An aliquot of the lysate from each well was added to the corresponding wells of a white 96-well microplate (Thermo Life Sciences, Basingstoke, UK), followed by addition of luciferin–luciferase reagent. The light output corresponding to the level of ATP present was measured in a luminometer (MPLX; Berthold Detection Systems, Hamburg, Germany).

Apoptosis detection assay

The Caspase-Glo 3/7 kit (Promega, Southampton, UK; G8091) is a homogenous, luminescent assay that measures caspase-3 and -7 activities. The general method is described here and follows the manufacturer's instructions. The Caspase-Glo 3/7 buffer and lyophilized Caspase-Glo 3/7 substrate reagents were equilibrated to room temperature. The Caspase-Glo 3/7 buffer was

transferred to the Caspase-Glo 3/7 substrate and the contents mixed gently by inverting the bottle until the substrate had dissolved thoroughly. Samples were set up in the same way as an ATP-TCA, but instead of an MO control containing cells and media, a media only control without cells was set up and used for measuring the background. Following a 6-day incubation, Caspase-Glo 3/7 reagent was added to the samples. The plate was covered with silver foil to protect it from light and gently mixed on a plate shaker at 300 r.p.m. for 30 s. The plate was incubated at room temperature and then luminescence measured using a luminometer (MPLX; Berthold Detection Systems).

Drugs

Zoledronic acid (hydrated sodium salt) was obtained from Novartis (Basel, Switzerland), and clodronate and alendronate from Calbiochem (Nottingham, UK), and were made up according to manufacturer's instructions. Each drug was tested at a range thought to be achievable clinically; zoledronic acid was tested at 2.2–69.0 μM (100% TDC = 34.5 μM), alendronate at 1.9–61.6 μM (100% TDC = 30.8 μM) and clodronate at 1.7–55.4 μM (100% TDC = 27.7 μM). Paclitaxel and cisplatin were obtained from the pharmacy of Queen Alexandra Hospital (Portsmouth, UK) as vials for injection and stored at room temperature. They were tested at concentrations ranging from 1.0 to 31.9 μM (100% TDC = 15.9 μM) and 0.62 to 20.0 μM (100% TDC = 10.0 μM), respectively. Combinations were tested by simultaneous addition.

Data analysis

Data from the luminometer was transferred automatically to an Excel spreadsheet where the results were expressed as a percentage inhibition at each of the six TDCs tested. Inhibition was calculated using the equation: $1-(\text{test}-\text{MI})/(\text{MO}-\text{MI})\times 100$. IC_{50} and IC_{90} values were determined, and area under the concentration–inhibition curve ($Index_{AUC}$) values were calculated from the data using the trapezoidal rule. Previous ATP-TCA studies have found that a natural logarithmic sum index ($Index_{SUM}$) calculated by direct addition of the percentage survival at each concentration tested ($Index = 600 - \Sigma\%Inhibition_{6.25\ldots200}$) provides a better indication of sensitivity or resistance to different drugs in different tumor types [22]. The total inhibition of growth resulted in an index of 0 and no inhibition of growth at any concentrations produces an index of 600. The results were entered into an Access database for further analysis. Statistical tests were performed using non-parametric methods. Additive or synergistic effects were assessed as previously published [19,23,24].

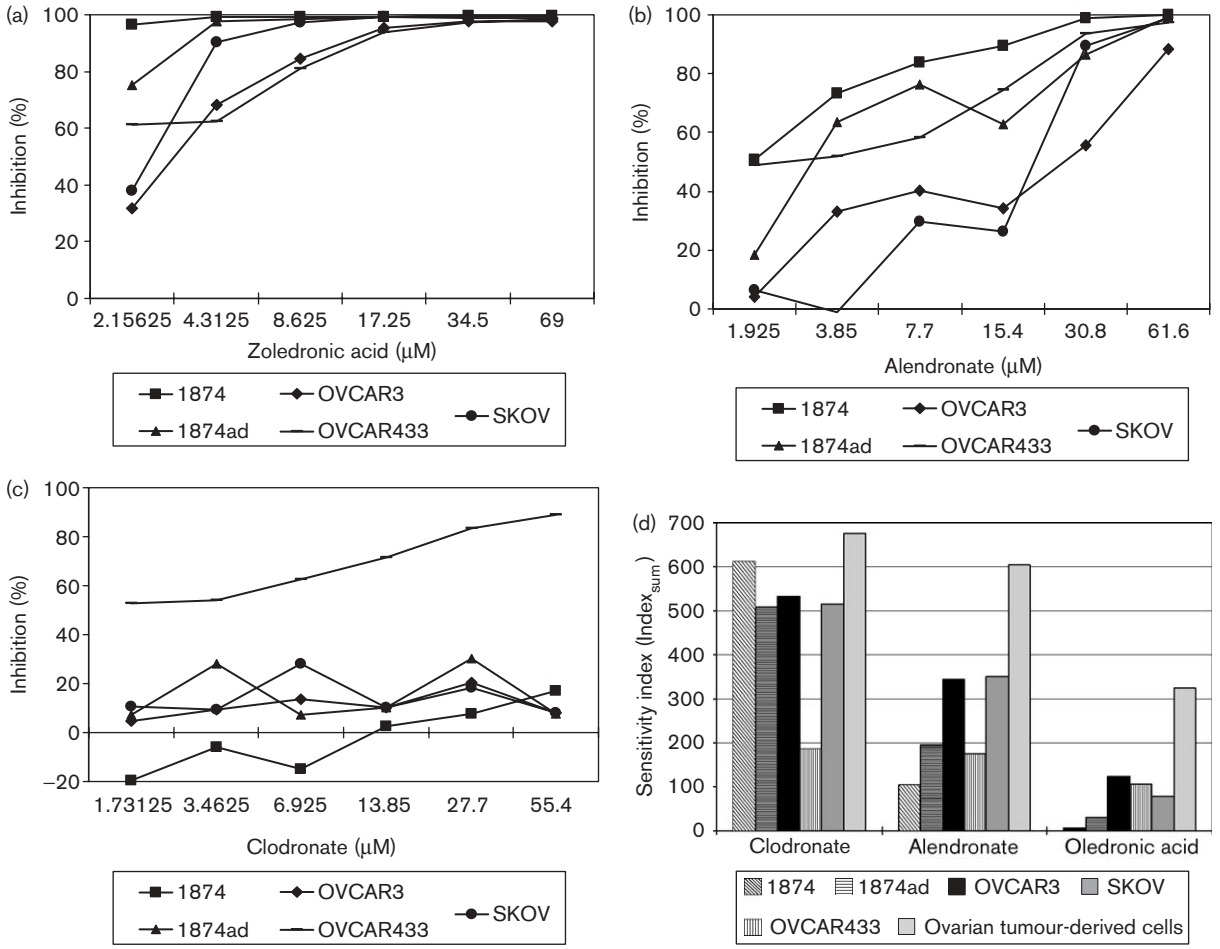
Results

Zoledronic acid (Fig. 1a) and alendronate (Fig. 1b) exhibited greater inhibition in the ovarian carcinoma cell lines compared to clodronate (Fig. 1c). All five cell lines were sensitive to zoledronic acid as measured by an $Index_{SUM} < 300$; three cell lines were sensitive to alendronate (1874, 1874ad and OVCAR-433), one was equivocal (OVCAR-3) and only one cell line (OVCAR-433) was sensitive to clodronate. However, all five cell lines tested exhibited greater sensitivity to clodronate, alendronate and zoledronic acid compared with the tumor-derived cells; Fig. 1(d) summarizes the differences in sensitivities between each cell line, and highlights the difference in comparison to the median sensitivity of ovarian tumor-derived cells tested with clodronate ($n = 9$), alendronate ($n = 9$) and zoledronic acid ($n = 17$). The IC_{50} and IC_{90} values for the bisphosphonates in the cell lines tested are summarized in Table 1. Zoledronic acid exhibited the greatest activity with $IC_{50} < 4$ μM in all five cell lines. This compares to the IC_{50} and IC_{90} values obtained when the cell lines were tested with a second nitrogen-containing bisphosphonate, alendronate; three of five cell lines (1874, 1874ad and OVCAR433) had $IC_{50} < 4$μM. The non-nitrogen-containing bisphosphonate, clodronate, was active in just one of the five cell lines (OVCAR433; $IC_{50} = 1.3$ μM), with the remaining four cell lines all having $IC_{50} > 290$ μM. Clodronate and alendronate both showed greatest activity in OVCAR433 compared to zoledronic acid, which was most active in 1874.

Zoledronic acid showed the greatest concentration-dependent inhibition in tumor-derived cells compared to clodronate and alendronate (Fig. 2) with a median IC_{50} and IC_{90} for all tumors tested of 17 and 88 μM, respectively; for alendronate, the median IC_{50} and IC_{90} were 67and 115 μM, respectively, and for clodronate, 105and 189 μM (Table 2). There was considerable heterogeneity between individual tumors in their response to bisphosphonates (Fig. 2). Using an $Index_{SUM} < 300$ as a threshold for sensitivity, tumor-derived cells were sensitive to zoledronic acid in 37% (11 of 30) of samples compared to 14% (three of 21) in both cases for clodronate and alendronate (Fig. 3). In tumor types where more than one sample had been tested, zoledronic acid and alendronate were both found to be most active in the breast carcinoma samples (median $IC_{50} = 17.8$ and 29.4 μM, respectively); clodronate had little or no activity in all tumor types tested. In the caspase assay, zoledronic acid demonstrated increasing caspase-3/7 activity with an increasing concentration of drug (Fig. 4).

In 56% (19 of 34) of cases, material was available for testing zoledronic acid in combination with paclitaxel (Fig. 5a). The graph shows that the paclitaxel was active in the samples as a single-agent with a median $Index_{SUM}$ value of 304 (76–823), it was therefore expected that only small increases in sensitivity to it would be observed. Of the samples tested with the combination, 42% (eight of 19) showed an increase in sensitivity to paclitaxel (five

Fig. 1



Inhibition by (a) zoledronic acid, (b) alendronate and (c) clodronate in five ovarian cell lines, and (d) the comparison with ovarian tumor-derived cells. Number of human ovarian samples tested: zoledronic acid $n=17$, alendronate $n=9$ and clodronate $n=9$.

Table 1 **IC_{50} and IC_{90} values (μM) for clodronate, alendronate and zoledronic acid in five ovarian carcinoma cell lines**

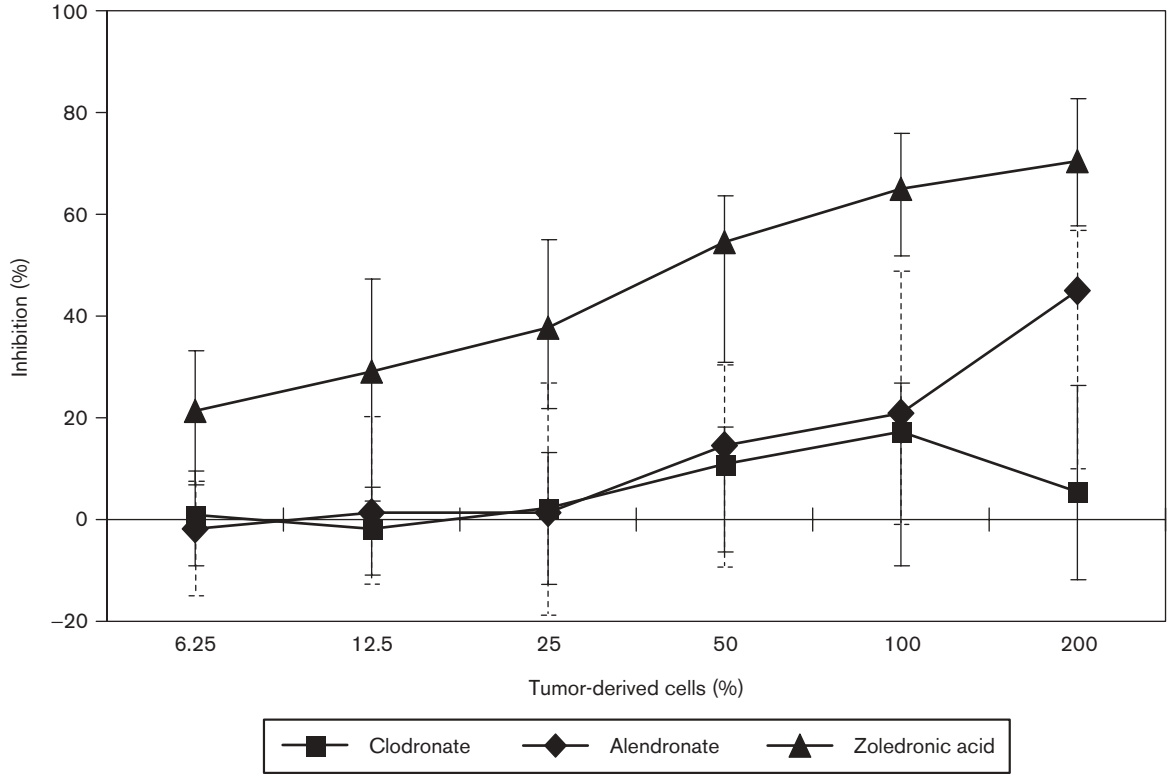
Cell line	Clodronate		Alendronate		Zoledronic acid	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
1874	163.5	294.3	1.9	16.1	1.1	2.0
1874ad	358.3	644.9	3.3	39.3	1.4	3.6
OVCAR3	335.9	604.6	26.8	62.8	3.2	12.9
OVCAR433	1.6	56.1	2.7	27.8	3.2	14.8
SKOV	345.7	622.2	21.2	32.6	2.7	4.3

ovarian and three breast carcinoma samples) measured by a decrease in the $Index_{SUM}$ value. However, of the samples showing increased sensitivity, none exhibited a more than 50% decrease in the $Index_{SUM}$ value. The remaining 58% (11 of 19) showed decreased sensitivity to paclitaxel when combined with zoledronic acid as measured by an increase in the $Index_{SUM}$ value. In 21% (seven of 34) of cases, material was available for testing zoledronic acid in combination with cisplatin (Fig. 5a). The addition of zoledronic acid to cisplatin had a more obvious effect in comparison to the previous combination, as there was greater resistance to single-agent cisplatin; 86% (six or seven) samples showed an increase in sensitivity to cisplatin following addition of zoledronic acid, but none showed a greater than 50% decrease in their $Index_{SUM}$ values. The remaining sample showed a decrease in sensitivity.

Discussion

Cell lines are generated from primary carcinoma cells and adapt to their cell culture environment, thereby developing different characteristics to the primary tissue from which they were formed. As a result, cell lines are usually much more sensitive to chemotherapeutic agents than

Fig. 2



Median inhibition by clodronate ($n=21$), alendronate ($n=21$) and zoledronic acid ($n=30$) in a variety of human solid tumors. Error bars show 25th and 75th interquartile range.

Table 2 Median IC_{50} and IC_{90} values (μM) for all tumors tested

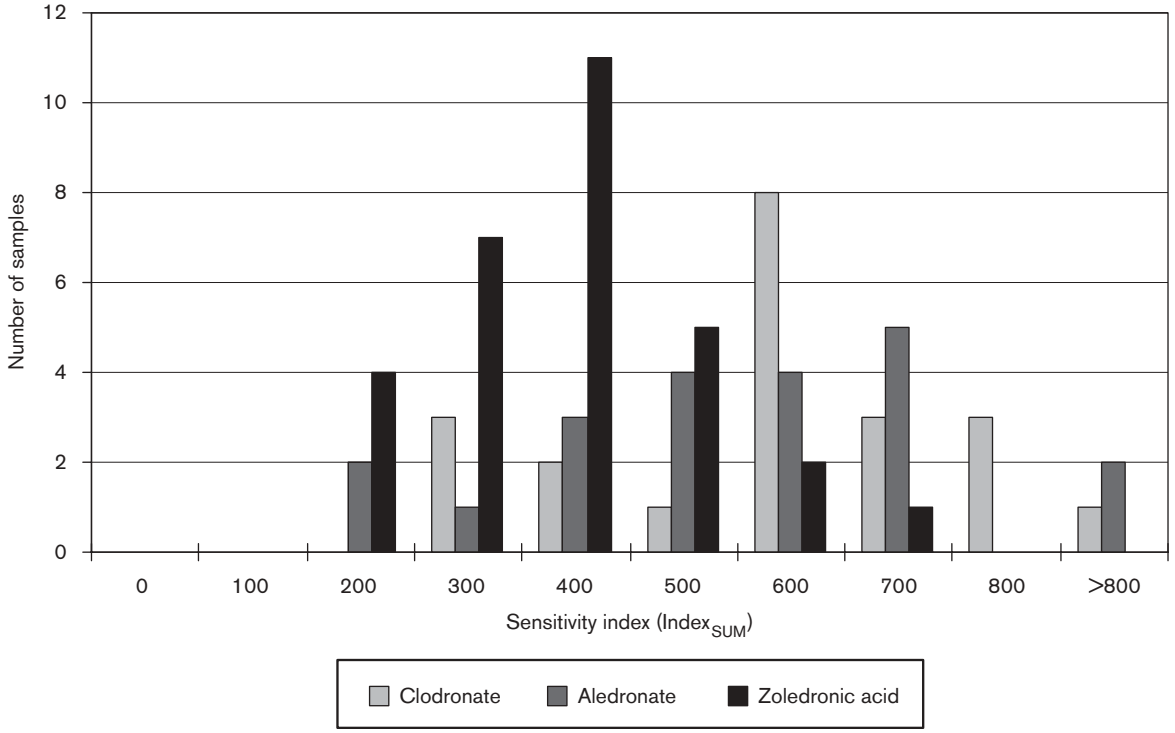
Tumor type	Clodronate ($n=21$)		Alendronate ($n=21$)		Zoledronic acid ($n=30$)	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Breast carcinoma	103 (102–105)	186 (183–189)	29 (24–35)	93 (89–97)	18 (17–18)	99 (90–108)
Cutaneous melanoma	134 (1–502)	241 (65–903)	152 (11–298)	197 (74–537)	19 (2–184)	87 (56–331)
Lung carcinoma	–	–	60	106	61	115
Ovarian carcinoma	105 (80–1406)	189 (144–2530)	69 (15–1856)	124 (79–3341)	14 (2–1912)	78 (70–208)
Unknown primary	–	–	–	–	4	66
Uveal melanoma	–	–	46	109	31	108
Median	105 (1–1406)	189 (65–2530)	67 (11–1856)	115 (74–3341)	17 (2–1912)	88 (56–331)

Range shown in brackets.

tumor-derived cells [25]. However, the use of a serum-free CAM reduces the growth rate of cell lines, causing them to respond in a manner more similar to tumor-derived cells. In this study, the cell lines exhibited greater sensitivity to the bisphosphonates compared to the tumor-derived cells. However, the order of activity from most active to least active bisphosphonate was the same in cell lines as in the tumor-derived cells; zoledronic acid activity > alendronate activity > clodronate activity, with both of the nitrogen-containing bisphosphonates having greater effects than clodronate.

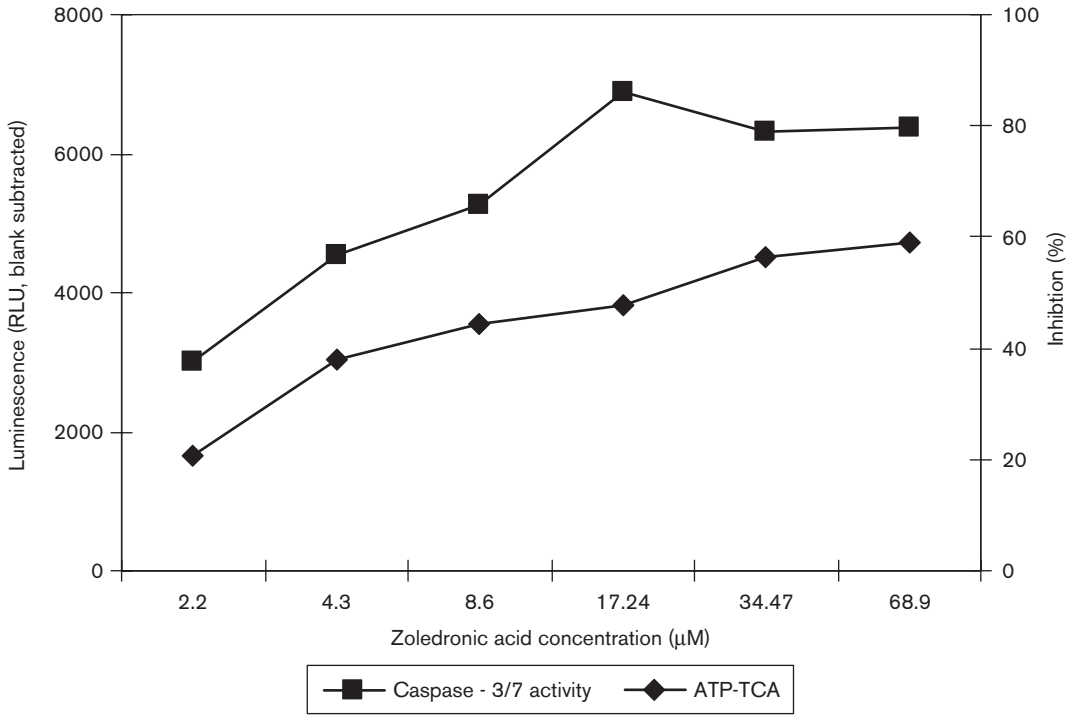
Zoledronic acid showed activity in tumor-derived cells from breast, ovarian and melanoma samples, with a similar sensitivity profile to that seen for cell lines in this and previous studies [7,26]. Zoledronic acid was significantly more active than alendronate and clodronate ($P = 0.0152$ and 0.0022, respectively; Mann–Whitney test). Alendronate showed moderate activity in breast tumor-derived cells and even though it was more active, it was not significantly different to clodronate, which showed little activity in any tumor type. The results of this study suggest a direct effect of zoledronic acid and other

Fig. 3



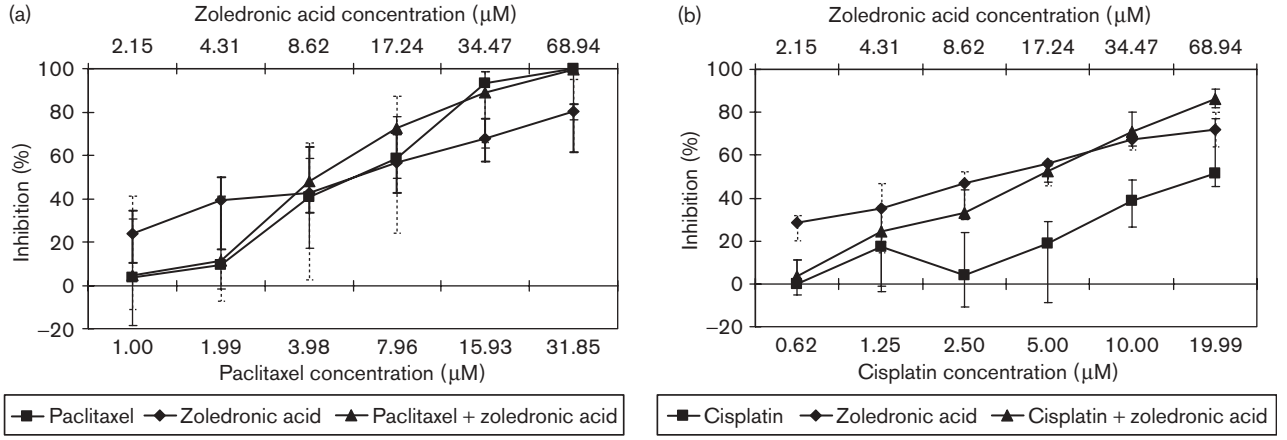
Frequency histogram showing sensitivity to three different bisphosphonates against tumor-derived cells: clodronate ($n=21$), alendronate ($n=21$) and zoledronic acid ($n=30$).

Fig. 4



Caspase-3/7 activity and tumor inhibition in ovarian tumor-derived cells exposed to zoledronic acid.

Fig. 5



Median tumor growth inhibition by zoledronic acid versus (a) paclitaxel + zoledronic ($n = 19$) and (b) cisplatin + zoledronic acid ($n = 7$) in a variety of tumor-derived cells. Error bars show 25th and 75th interquartile range.

nitrogen-containing bisphosphonates against tumor-derived cells.

Zoledronic acid inhibits FPP synthase, thereby preventing synthesis of FPP and geranyl geranyl diphosphate, and subsequently inhibiting prenylation of GTP-binding proteins involved in signal transduction and cell adhesion. Another consequence of loss of signaling proteins is the induction of apoptosis. It was previously thought that nitrogen-containing bisphosphonates were not metabolized to an ATP analog as is the case with non-nitrogen-containing bisphosphonates; however, Mönkkönen *et al.* [6] have identified a novel ATP analog (ApppI) produced via inhibition of the mevalonate pathway by *N*-bisphosphonates *in vitro* in cells such as macrophages, gliomas and osteoclasts. Further investigation of the relevance of these mechanisms in tumor-derived cells would be helpful.

A study by Oades *et al.* [27] demonstrated capase-3 activity in prostate cancer cell lines incubated with zoledronic acid. Riebeling *et al.* [26] and Senaratne *et al.* [7] demonstrated pamidronate, another nitrogen-containing bisphosphonate, activated caspase-3 in melanoma and breast cancer cell lines, respectively. However, Tassone *et al.* [28] found that zoledronic acid activated apoptosis via a caspase-9- and capsase-6-dependent, and caspase-3-independent pathway, suggesting tissue-specific executioners of apoptosis. At the time of writing, there are no published data on the induction of apoptosis in ovarian cancer cell lines exposed to zoledronic acid, although a study by Sawada *et al.* [29] reported that alendronate inhibited Rho activation, thereby inhibiting lysophosphatidic acid-induced migration of ovarian cancer cell lines. In this study, we have observed a direct, or possibly an indirect, effect of zoledronic acid on cells derived from an ovarian tumor and a concentration-dependent increase in caspase-3 activity, although correlation between ATP-TCA inhibition values and caspase-3 results did not quite reach significance ($P = 0.0583$; Spearman rank correlation).

When zoledronic acid was tested in combination with paclitaxel in a variety of tumor-derived cells we observed no significant increase ($P = 0.2935$; Wilcoxon matched-pairs test) in activity, although paclitaxel was very active as a single agent in this study. When zoledronic acid was tested in combination with cisplatin in a variety of tumor-derived cells, the combination did have an increased activity compared to the single agents; however, analysis using the Poch method [23] confirmed there was no synergism between the two drugs. A recent paper by Vogt *et al.* [30] has suggested that zoledronic acid could potentiate both taxane- and non-taxane-based regimens in primary breast carcinomas. In addition, Jagdev *et al.* [11] reported synergy between zoledronic acid and paclitaxel in breast cancer cell lines, although this study used lower concentrations of paclitaxel. Since both agents induce apoptosis, some degree of additive activity seems likely. However, this synergy was only found for sequential rather than simultaneous addition and further studies are required to clarify the effects of these combinations on tumor-derived cells.

Conclusion

This study shows a direct, or possibly an indirect, effect of zoledronic acid cytotoxic/cytostatic effect of zoledronic acid against ovarian cancer cell lines and tumor-derived cells in the ATP-TCA, with increased apoptosis. The use

of zoledronic acid may have a direct anti-tumor effect and clinical trials to examine this question are required.

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