SPANDIDOS PUBLICATIONS

Changes in bone resorption and vascular endothelial growth factor after a single zoledronic acid infusion in cancer patients with bone metastases from solid tumours

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Abstract. Zoledronic acid (Zometa, ZOL) is increasingly used to treat tumour-induced bone disease, and is also reported to have antiangiogenic properties *in vivo*. In this study, we have investigated the correlations between changes in the proangiogenic cytokine, vascular endothelial growth factor (VEGF), and markers of bone resorption in a cohort of patients with metastatic bone disease, following a single infusion of ZOL. Twenty-four consecutive selected cancer patients with scintigraphic and radiographic evidence of bone metastases were treated for the first time with a single infusion of 4 mg ZOL. Patients were considered ineligible if they had received any steroid therapy, radiotherapy, chemotherapy, immunotherapy or haemopoietic growth factors in the 4 weeks before or during the study period. Circulating levels of VEGF and ß crosslinked type I collagen C-telopeptide (ßCTX) were measured at base-line and at 1, 2, 7 and 21 days following ZOL infusion. The majority of our patients (23/24) developed a significant reduction in circulating levels of ßCTX at just 1 day after the single zoledronic acid infusion, median percentage decrease 67.05% (95% CI, 52.39%; 76.27%). This reduction persisted at all following time points in almost all subjects in our patient population (day 2, 95.8%; day 7, 100%; day 21, 91.7%). The median decrease at day 2 was 85.67% (95% CI, 78.23%; 90.16%); at day 7, 67.38% (95% CI, 67.38%; 86.98); and at day 21, 76.89% (95% CI, 35.00%; 83.16%). Moreover, a linear regression model with variance analysis demonstrated a statistically significant correlation between median VEGF and ßCTX circulating levels at each of the time points (1, 2, 7 and 21 days after ZOL infusion). The present work demonstrates that a single infusion of ZOL was able to induce a rapid and long lasting decrease of ßCTX plasma levels in the majority (23/24) of the included cancer patients. Furthermore, we found that there is a correlation between the levels of VEGF and ßCTX following ZOL treatment. Future clinical trials should be designed to prospectively evaluate the prognostic role of reduction of ßCTX and VEGF in response to ZOL to predict clinical and skeletal outcome.

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Introduction

Bone metastases are common in many advanced cancers and are clinically a considerable source of skeletal morbidity. Bisphosphonates are potent inhibitors of osteoclast activity that have demonstrated efficacy in the treatment of bone metastases (1,2). Bisphosphonates are extensively used for the treatment of metabolic bone disease because they bind avidly to the bone mineral at sites of active bone remodelling, where they achieve therapeutic concentrations (1-3). Bound bisphosphonates are subsequently released during bone resorption and are internalized by osteoclasts, leading to induction of osteoclast apoptosis and inhibition of bone resorption (4-6). Type I collagen is the predominant protein in bone and its breakdown products are being increasingly investigated as markers of bone resorption in metastatic bone disease (7). Recent studies on the effect of ZOL on bone resorption in patients with tumour-induced bone disease have shown significant and rapid decreases in the bone resorption markers,

crosslinked N- and C-terminal type I collagen telopeptides, NTX and CTX (8-10). Several urinary and serum markers of bone resorption have recently been demonstrated to be predictive of skeletal complications in prostate cancer, lung cancer and other solid tumours and to be useful for monitoring responses to bisphosphonate therapy (11-13). In cancer patients with lytic bone disease, suppression of NTX correlated with a reduction in fracture risk and a significant reduction in bone disease progression (14). There is now extensive preclinical evidence that bisphosphonates also have antitumour activity, by causing reduced proliferation and viability of tumour cell lines *in vitro* as well as reduced skeletal tumour burden and slower progression of bone lesions in animal models (15-23). Several mechanisms have been proposed to explain these observations. Bisphosphonates may render the bone a less favorable microenvironment for tumour cell growth by reducing bone resorption and local release of growth factors. Bisphosphonates may also have direct antitumour effects, as they have been shown to inhibit proliferation and induce apoptosis of a variety of human tumour cell lines *in vitro* (15-23). In addition, bisphosphonates have been reported to inhibit tumour cell adhesion to the extracellular bone matrix and tumour cell invasion, and to have antiangiogenic and immunomodulatory activities. The mechanisms responsible for the observed antitumour effects of bisphosphonates are beginning to be elucidated.

Recent evidence suggests that part of the antitumour activity of bisphosphonates may be attributed to an anti-angiogenic effect. Wood *et al* (24; Proc ASCO 19: abs. 664, 2000) reported that fetal calf serum, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation is inhibited by ZOL in an *in vitro* model of angiogenesis. Moreover, ZOL was shown to reduce vessel sprouting in cultured aortic rings and in the chicken egg chorioallantoic membrane assay. Finally, Fournier *et al*, in a subcutaneous testosterone implant model in castrated rats, demonstrated that zoledronic acid treatment strongly inhibits the angiogenic response induced by bFGF and VEGF (25). Our research group was the first to report a significant decrease of circulating levels of VEGF in bone metastatic cancer patients receiving a single, first dose of pamidronate or ZOL (26,27). Collectively, these data suggest that ZOL and other bisphosphonates may play a clinical role in the reduction of skeletal tumour burden and the prevention of bone metastasis.

The aims of the present study were, firstly, to confirm the early changes in bone resorption in response to a single ZOL infusion in consecutive patients with bone metastases and, secondly, to investigate the correlations between the modifications of markers of bone resorption and the modifications of circulating levels of VEGF in these patients.

Patients and methods

Patients. Twenty-four consecutive patients (9 males, 15 females), aged 44-76 years (median age, 65), with advanced solid cancer and bone metastases, were included in the study (patients' characteristics are shown in Table I). Patients were considered eligible for the study if they had a histologically confirmed solid cancer associated with scintigraphic identification and radiographic confirmation of bone metastases. In addition, patients were required to have, at study entry, a neutrophil count $\geq 1.5 \times 10^9$/l, a platelet count $>100 \times 10^9$/l, normal hepatic and renal function and no acute or chronic infections or inflammatory diseases. Patients were considered ineligible if they had reported fever (body temperature >38.0˚C) in the last week before study entry or had received any radiotherapy, chemotherapy, immunotherapy or haemopoietic growth factors in the 4 weeks before entry into the study. Patients who recently (<1 week) or simultaneously received steroid treatment were considered ineligible for the study. Patients previously treated with any bisphosphonates were excluded from the study. Hormonal therapy was allowed only if it had started at least 3 months prior to accrual. All patients received ZOL on an outpatients basis. All patients were >18 years of age and had given their informed consent according to local guidelines with the approval of the appropriate ethics committee.

Table I. Patients' characteristics.

Total (%)	24 (100)
Median age in years (range)	65 (44-76)
Male/female (%)	37.5/62.5
Median performance status	
ECOG score (range)	1 (0-2)
0-1 score/2 score (%)	62.5/37.5
Cancer type (%)	
Breast carcinoma	14 (58.3)
Prostate adenocarcinoma	4 (16.7)
Non-small cell lung cancer	3 (12.5)
Other primary cancers	3 (12.5)
Bone segments involved by metastases (range)	1-7
Previous chemotherapy (%)	15 (62.5)
Concurrent hormonal therapy (%)	4 (16.7)
Metastases other than bone locations (patients) (%)	
No other locations	10 (41.6)
Lung metastases	3 (12.5)
Liver metastases	6 (25.0)
Lung + liver metastases	3 (12.5)
Other locations	6 (25.0)

Treatment and follow-up investigation. All patients received 4 mg of ZOL (Zometa®, Novartis) in 100 ml of 0.9% saline over a period of 15 min as an intravenous infusion starting at 10:00 a.m. Venous blood for VEGF and ßCTX assessment was drawn into an EDTA anticoagulant tube before 10:00 a.m. after an overnight fast at baseline, just before the beginning of drug infusion, and again at 1, 2, 7 and 21 days after the ZOL infusion. After drawing, the venous blood sample was

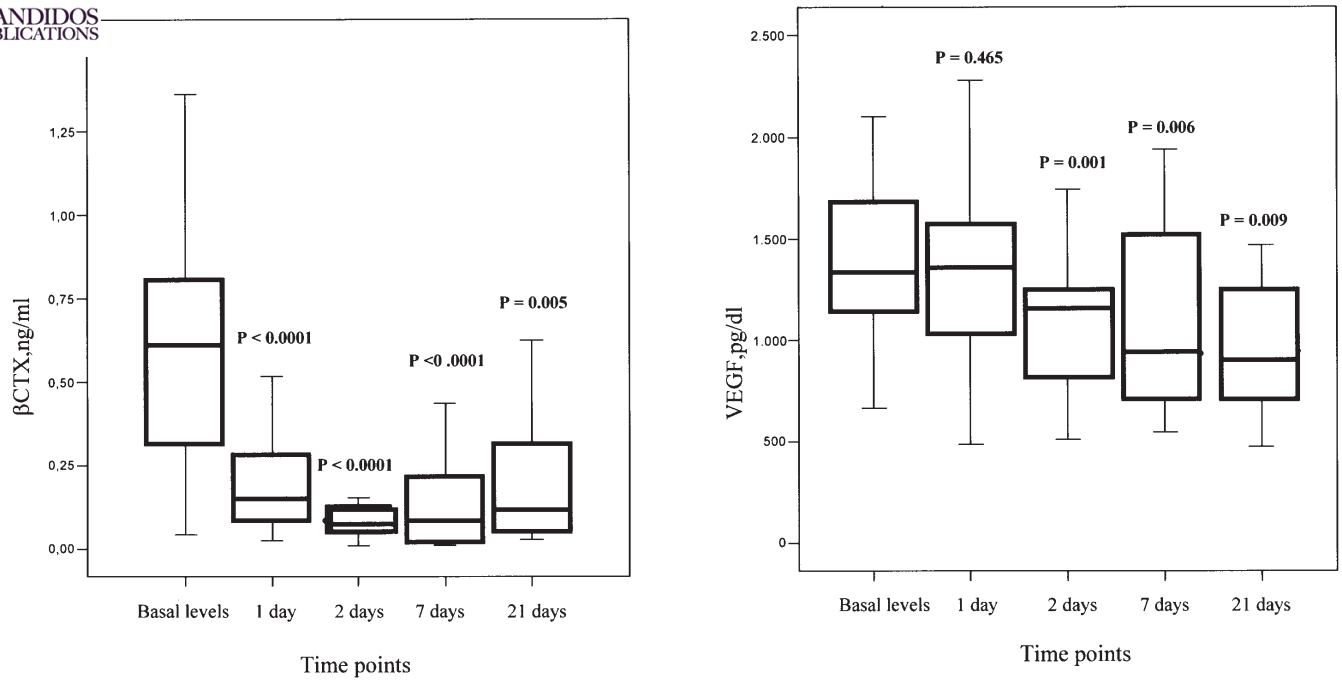

Figure 1. ßCTX and VEGF levels at days 1, 2, 7 and 21 after ZOL administration. Red boxes represent 95 percentiles. Horizontal black bars in the red boxes represent the median value. Bottom and top horizontal bars indicate minimum and maximum values. P-values are calculated according to Wilcoxon's test for non-parametric dependent continuous variable.

Table II. ßCTX and VEGF circulating level modifications after a single infusion of zoledronic acid.

ßCTX	Median value (ng/ml)	95% confidence interval (ng/ml)	P-value
Basal levels	0.6417	0.408-1.010	-
1 day	0.216	0.122-0.455	<0.0001
2 days	0.104	0.066-0.162	<0.0001
7 days	0.127	0.105-0.272	<0.0001
21 days	0.200	0.145-0.366	0.005
VEGF	**Median value (pg/dl)**	**95% confidence interval (pg/dl)**	**P-value**
Basal levels	1371.500	1200.240-1681.764	-
1 day	1368.000	1081.336-1540.442	0.390[a]
2 days	1065.000	854.967-1220.589	0.005
7 days	965.500	907.717-1346.173	0.016
21 days	960.500	814.291-1275.578	0.006

[a]Not significant.

rapidly centrifuged for 10 min at 10,000 rpm and plasma stored at -80˚C until tested for VEGF and ßCTX levels. White blood cell and platelet counts, haemoglobin levels and serum total calcium levels were also determined at the same time points.

VEGF and ßCTX analysis. VEGF was assayed using the R&D quantitative kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The detection limit of the VEGF was 62.5 pg/ml.

Plasma ßCTX was assayed by electrochemiluminescent assay on an Elecsys 2010 auto analyser (Roche Diagnostics GmBH, Penzberg, Germany). The detection limit of the assay was 0.01 ng/ml. The interassay CV was 6.5% at 0.7 ng/ml. All samples were centrifuged at 1200 x g for 5 min before analysis. In both assays, all samples from the same individual were analysed in the same analytical batch.

Statistical analysis. Basal VEGF and ßCTX levels were compared to the values observed at 1, 2, 7 and 21 days after ZOL infusion using the Wilcoxon's test for non-parametric dependent continuous variables. A linear regression model with variance analysis was used to correlate VEGF and ßCTX circulating levels at the different time points. At the last time point, 6 samples were missing; no correction was made for missing data. A two-tailed P-value was considered significant when <0.05. SPSS software (version 11.5, SPSS, Chicago, IL) was used for statistical analysis.

Results

ßCTX analysis. Table II and Fig. 1 show the median circulating levels after a single infusion of zoledronic acid. The median ßCTX basal level was 0.6417 pg/dl (95% CI, 0.408-1.010). These levels significantly decreased 1 day after ZOL infusion to 0.216 ng/ml (95% CI, 0.122-0.455) (P<0.0001). This effect persisted throughout the 21-day follow-up period.

In the majority of patients (23/24), circulating levels of ßCTX were significantly reduced 1 day after the single zoledronic acid infusion, median decrease 67.05% (95% CI, 52.39%; 76.27%). This reduction was found at all of the time points

Table III. Median percentage changes in circulating ßCTX and VEGF levels after zoledronic acid infusion at different time points.

	Median reduction (%)	95% CI (%)
ßCTX		
1 day	-67.05	-52.39; -76.27
2 days	-85.67	-78.23; -90.16
7 days	-67.38	-67.38; -86.98
21 days	-76.89	-35.00; -83.16
VEGF		
1 day	-6.71	+8.70; -16.08
2 days	-23.82	+1.47; -30.88
7 days	-27.75	-2.03; -34.69
21 days	-39.42	-15.09; -44.13

Table IV. Correlation between ßCTX and VEGF circulating levels at different time points.

Time points	ß regression coefficient	P-value
Basal	0.322	0.125[a]
1 day	0.627	0.002
2 days	0.655	0.001
7 days	0.872	<0.0001
21 days	0.511	0.018

[a]Not significant.

in almost all our patients (day 2, 95.8%; day 7, 100%; day 21, 91.7%). The median percentage changes in circulating ßCTX levels after ZOL infusion at different time points are summarized in Table III.

VEGF analysis. The median VEGF basal value was not significantly reduced 1 day after the single infusion of ZOL (1371.50 pg/dl; 95% CI, 1200.24-1681.76) vs 1368.00 pg/dl; 95% CI, 1081.33-1540.44) (P=0.390). However, 2 days after ZOL infusion, VEGF levels showed a statistically significant decrease compared to the basal levels (1065.00 pg/dl; 95% CI, 854.96-1220.58) (P=0.005). This effect on circulating VEGF levels persisted throughout the study. These results are summarized in Table II and Fig. 1. The median reductions in percentage (%) of circulating VEGF levels after ZOL infusion at different time points are summarized in Table III.

Correlation between ßCTX and VEGF circulating levels. A linear regression model with variance analysis failed to show a significant positive correlation between basal VEGF and ßCTX values (ß regression coefficient = 0.322; P=0.125) (Table IV). In contrast, a statistically significant correlation between median VEGF and ßCTX circulating levels was found at each of the time points. In particular, a strong direct correlation was identified just 1 day after the ZOL single infusion (ß regression coefficient = 0.627; P=0.002) which persisted for 2 days (ß regression coefficient = 0.655; P=0.001) and reached the highest level of significance at 7 days after infusion (ß regression coefficient = 0.872; P<0.0001). Furthermore, the correlation persisted for 21 days after the infusion (ß regression coefficient = 0.511; P=0.018). These results are summarized in Table IV and Fig. 2.

Therefore, we arbitrarily decided to divide the patients into 'responders' and 'non-responders' on the basis of ßCTX and VEGF concomitant decrease at 7-day and 21-day time points. We defined 'responder' patients as those who concomitantly showed a VEGF reduction >25% of the basal levels (28) and a ßCTX reduction >50% of the basal values (29,30) 7 or 21 days after the first ZOL infusion. According to these definitions, 45.8% of patients (11 patients) were 'responders' at the 7-day time point and 58.3% (14 patients) were 'responders' at the 21-day time point. We did not demonstrate any correlation between basal levels of ßCTX and response to ZOL (data not shown).

Secondary parameters. No significant differences in platelet level, white blood cell count, haemoglobin concentration and renal function were recorded before and 1, 2, 7 and 21 days after ZOL infusion. However, as expected, a statistically significant decrease in serum total calcium levels was observed after bisphosphonate administration: the median calcium level before ZOL administration was 11.31 mg/dl (range: 9.08-13.1 mg/dl), while the median value 7 days after was 8.38 mg/dl (range: 7.2-10.8 mg/dl) (P=0.0002). Moreover, 4 of 24 patients showed hypocalcemia 21 days after the infusion of ZOL and a second administration had to be changed from 1 to 3 weeks. The changes in serum total calcium did not correlate with the observed changes in circulating VEGF levels following ZOL infusion at any time point. As expected, a significant correlation was identified between serum total calcium concentration and ßCTX level 7 days (ß regression coefficient = 4.980; P=0.009) and 21 days (ß regression coefficient = 5.521; P=0.006) after ZOL infusion. Only a borderline statistical significance was noted between ßCTX and calcium levels at the basal time (ß regression coefficient = 3.357; P=0.061).

A significant correlation in a linear regression model was noted between basal VEGF level and basal platelet count (ß regression coefficient = 4.653; P=0.014).

Discussion

Tumour cells release a variety of growth factors that promote bone resorption and increase the risk of skeletal complications. Recently, bone marker levels have been demonstrated to be representative of neoplastic osteolysis and predictive of negative clinical outcomes in patients with bone metastases secondary to prostate cancer and to NSCLC and other solid tumours. In particular, urinary N-telopeptide level was shown to be a consistent prognostic indicator for all tumour types, reflecting the key role of osteolysis in the development of skeletal complications (7,11). Bisphosphonates are potent inhibitors of osteoclast activity that have demonstrated efficacy in the treatment of tumour-induced bone disease. Bisphosphonates bind avidly to the bone matrix, are released during

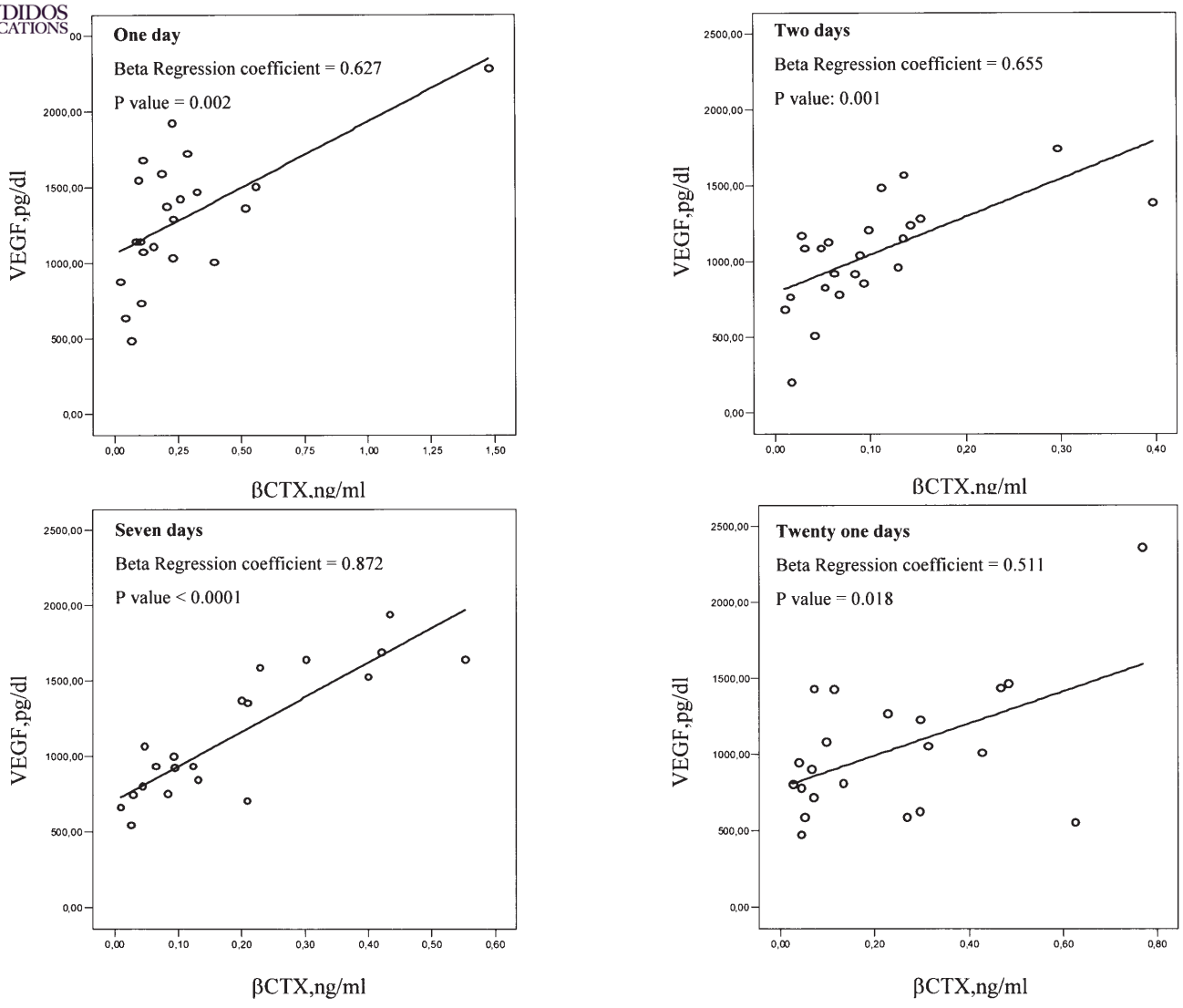

Figure 2. Correlations between median VEGF and ßCTX levels 1, 2, 7 and 21 days after ZOL administration. A statistically significant correlation between median VEGF and ßCTX circulating levels was found at each of the evaluated time points.

bone resorption, and are subsequently internalized by osteoclasts, where they interfere with biochemical pathways and induce osteoclast apoptosis. Bisphosphonates also antagonize osteoclastogenesis and promote the differentiation of osteoblasts. As a result, bisphosphonates inhibit tumour-induced osteolysis and reduce skeletal morbidity. Garnero *et al* (31) analysed the changes in serum markers of bone resorption during ZOL therapy in a cohort of patients with Paget's disease. In detail, the urinary excretion of non-isomerized type I collagen C-telopeptide (αCTX-I) decreased within 5 days with a maximal decrease of 51% at day 10 (P<0.001 vs. baseline and placebo), and levels remained suppressed during the 2 months of the study. Moreover, Chen *et al* (8) analysed the effects of ZOL on markers of bone disease in a sample of cancer patients without any selection regarding the concomitant antineoplastic therapies or the concomitant comorbidities. In this study (8), ZOL produced significant declines from baseline in serum and/or creatinine-corrected urine C-telopeptide, N-telopeptide, pyridinium cross-links, and calcium. The current study is the first to report the early modifications of the levels of markers of bone resorption induced by a single infusion of ZOL in a well characterised cohort of metastatic cancer patients. The present work demonstrates that a single infusion of ZOL is able to induce a rapid and long lasting decrease of ßCTX plasma levels in the majority of the patients studied. The significant reduction of ßCTX plasma levels is a clear reflection of the inhibition of bone resorption induced by ZOL, including that at sites of bone metastasis. It has been demonstrated that ZOL administered by intravenous infusion (4 mg over 15 min), results in an abrupt increase of its concentration in peripheral blood, as shown by estimations of the early distribution and elimination of the drug, which are resulting in population half-lives of the drug of about 15 min (t1/2α) and of 1.75 h (t1/2ß), respectively. Moreover, approximately 55% of the initial administered dose of the drug is retained in the skeleton and is slowly released back in circulation, resulting in a terminal elimination half-life (t1/2γ) of about 7 days in population studies (8,32). It has also been reported that a 15-min pulse with ZOL induces either apoptosis or growth

inhibition in both human prostate and pancreas adenocarcinoma cell lines, starting with osteoclasts after 48-72 h (23,33). Tumour cells secrete themselves or stimulate the production of VEGF by the expression of other growth factors such as TGFα or EGF (34,35). Furthermore, cancer cells can produce bone resorption factors, such as M-CSF or rank ligand, which favour collagen destruction (36,37). Therefore, it is conceivable that reduction of both VEGF and ßCTX could be recorded from 2 days after ZOL administration. Both the prolonged ZOL release from the bone and the long recovery from cell death induced by ZOL on both tumour cells and osteoclasts could be the cause of the sustained effects of the lowering of VEGF and CTX plasma levels.

Reports from *in vitro* systems and animal models have demonstrated that ZOL has antiangiogenic effects. Moreover, as previously described, growing evidence has accumulated in the past years supporting the anticancer effect of ZOL in humans. The *in vivo* antiangiogenic effects of ZOL have also been established by the reduction of circulating levels of pro-angiogenic cytokines in humans induced by a single infusion of the drug (26,27). Recently, a molecular correlate between molecular target of ZOL actions and angiogenesis has been described. In fact, ZOL can induce apoptosis in cancer cells by inactivation of ras (38). The latter is, in turn, involved in the regulation of secretion of angiogenic factors, such as interleukin-8, by both tumour and endothelial cells (39). On the basis of this evidence, we designed this study to evaluate if a correlation between the reduction of VEGF and ßCTX circulating levels, a marker of bone resorption, may exist. The present study clearly demonstrates that a statistically significant correlation exists between circulating levels of VEGF and ßCTX concentration 1 day after a single infusion of ZOL. This correlation is lasting and persists for 21 days after the infusion. The molecular mechanisms of these correlated events are not known. It is proposed that a 'vicious cycle' is created whereby metastatic tumour stimulates bone turnover and bone turnover promotes local tumour growth (40). ZOL has been shown to inhibit the bone turnover, antagonizing the osteoclastogenesis and promoting the differentiation of osteoblasts. As a consequence, the zoledronic-induced inhibition of bone turnover may lead to inhibition of tumour growth in the bone environment; but it is proposed that the tumour growth inhibition induced by ZOL is in part dependent on angiogenesis inhibition. The changes in ßCTX and VEGF in this study may reflect this dual effect of ZOL on tumour growth. In accordance with these results, we identified the percent of decrease of VEGF and ßCTX circulating levels in each patient and at each time point and we observed two types of behaviour. We identified a group of patients with a concomitant significant decrease in VEGF and ßCTX plasma levels as 'responders' and a second group, in which the decreases in VEGF and ßCTX serum levels were not concomitant, as 'non-responders'. This study emphasizes that the biological response to ZOL is not the same in all patients, although the majority had reduced ßCTX following a single infusion of the drug. Whereas the molecular mechanisms underlying the reduction in bone resorption markers (inhibition of osteoclast activity) are well established, it is unclear by which mechanisms ZOL causes reduced levels of circulating VEGF, and further studies are required to establish this. The biological and clinical significance of this differential response to ZOL is not clear, and the number of patients included in this study was not large enough to correlate these changes in ßCTX and VEGF with the clinical and skeletal outcome of the disease. Future clinical trials should be designed to prospectively evaluate the role of changes in ßCTX and VEGF, separately and in combination, in predicting the clinical and skeletal outcome of ZOL treatment in patients with tumour-induced bone disease.

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