Repeated Intermittent Low-Dose Therapy with Zoledronic Acid Induces an Early, Sustained, and Long-Lasting Decrease of Peripheral Vascular Endothelial Growth Factor Levels in Cancer Patients

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Abstract

Purpose: On the basis of stimulating data on animals reporting that weekly regimens of zoledronic acid (ZA) were effective in reducing skeletal tumor burden, we designed a study on humans to investigate the potential antiangiogenic role of a weekly low-dose therapy with ZA in patients with malignancies.

Experimental Design: Twenty-six consecutive patients with advanced solid cancer and bone metastases received 1 mg of ZA every week for four times (days 1, 7, 14, and 21) followed by 4 mg of ZA with a standard 28-day schedule repeated thrice (days 28, 56, and 84). Patients were prospectively evaluated for circulating levels of vascular endothelial growth factor (VEGF) just before the beginning of drug infusion (0) and again at 7, 14, 21, 28, 56, and 84 days after the first ZA infusion.

Results: The median VEGF basal value showed an early statistically significant ($P = 0.038$) decrease 7 days after the first 1-mg infusion of ZA. This effect on VEGF-circulating levels persisted also after the following 1-mg infusions at 14 ($P = 0.002$), 21 ($P = 0.001$), and 28 days ($P = 0.008$). Interestingly, the decrease of VEGF-circulating levels persisted also at each programmed time point during the second phase of the study (ZA 4 mg every 4 weeks). No significant differences were recorded in platelet levels, WBC count, or hemoglobin concentration before and after each ZA infusion.

Conclusions: In the present study, we report that a repeated low-dose therapy with ZA is able to induce an early significant and long-lasting decrease of VEGF levels in cancer patients.

Bisphosphonates are PP_i analogues and differ from one another based on their substituted side chains (1). Nitrogen-containing bisphosphonates (N-BP), including pamidronate and zoledronic acid (ZA), inhibit protein prenylation, which in turn interferes with osteoclast function inducing apoptosis of osteoclasts as well as of myeloma and breast cancer cells *in vitro* (2–4). Bisphosphonates have a widely recognized role in the treatment of patients with multiple myeloma and bone metastases secondary to breast cancer (5, 6). ZA recently received broad regulatory approval for the treatment of bone metastases secondary to all solid tumor types and bone lesions from multiple myeloma based on the results of three large, randomized, phase III clinical trials enrolling more than 3,000 patients (7, 8). Compelling studies suggest that, besides the strong and well-known antiosteoclastic activity, the efficacy of such compounds in oncology could also be due to their antitumor effect, which is exerted at different levels (9). In detail, growing preclinical evidence supports that at least part of the antitumor activity of bisphosphonates may be attributed to an antiangiogenic effect (10). These findings have been confirmed *in vivo*; systemic administration of 3 μg/kg ZA to mice resulted in a potent inhibition of angiogenesis induced by s.c. implants impregnated with β-fibroblast growth factor (11). ZA and ibandronate, but not clodronate, decreased revascularization of the ventral prostate gland in castrated rats treated with testosterone (12). Moreover, ZA exerts an inhibitory effect on endothelial cell adhesion and migration processes by a modulation of adhesion molecules such as integrins that are involved in angiogenesis (13). For the first time in humans, we have recently shown a significant decrease of circulating levels of vascular endothelial growth factor (VEGF) in bone metastatic cancer patients receiving a single dose of either ZA or pamidronate (14, 15). Although the precise mechanism of this

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effect is not completely clear, we can suppose that ZA may elicit several antiangiogenic-related cytokine patterns and cascades that can be likely mediated by the inhibition of isoprenylation of intracellular molecular targets (likely small GTP-binding proteins). Most previous animal studies have used high doses of bisphosphonates that, when translated in the clinical setting, are incompatible with the clinical dosing regimens approved for the treatment of cancer patients with skeletal metastases. Moreover, the doses of bisphosphonates currently used in clinical trials do not show any convincing antitumor effect. For all these reasons, there is much debate about the clinical relevance of these experimental findings.

Frequent (daily or weekly) administration of low-dose chemotherapy agents, referred to as metronomic chemotherapy, reveals profound antiangiogenic effects. The metronomic use of these agents has been suggested to be very active in terms of antitumor efficacy and prevention of drug resistance. Interestingly, some metronomic-chemotherapy regimens induce sustained suppression of circulating proangiogenic cytokines and increase the levels of the endogenous angiogenesis inhibitor thrombospondin 1 and, hence, exert an inhibitory effect on neovascularization (16–18).

On the basis of these considerations, a very recent paper by Daubinè et al. showed that clinically relevant doses of bisphosphonates produced meaningful antitumor effects in an animal model of bone metastasis caused by MDA-MB-231/B02 breast cancer cells as long as the bisphosphonate was administered at a low dosage on a daily or weekly dosing schedule. Importantly, in this study, daily and weekly regimens of ZA (with total doses similar to that used in the clinical setting) were effective in reducing bone destruction as well as skeletal tumor burden, whereas monthly dosing was not.

Interestingly, these findings suggest that a continuous dosing regimen of ZA or clodronate or a frequent intermittent dosing regimen of ZA reduced the progression of osteolysis and the homing of tumor cells to bone (19). These data are supported by the pharmacokinetic profile of ZA. In fact, studies on ZA pharmacokinetics show that after i.v. administration (4 mg over 15 min), an abrupt increase of its concentration in peripheral blood is recorded, as shown by estimations of the early distribution and elimination of the drug, which results in plasma half-lives of the drug of about 15 min ($t_{1/2\alpha}$) and of 105 min ($t_{1/2\beta}$), respectively. Moreover, ~55% of the initially administered dose of the drug is retained in the skeleton and is slowly released back into circulation, resulting in a terminal elimination half-life ($t_{1/2\gamma}$) of about 7 days. Therefore, ZA is prevalently accumulated in the bone, and its circulating plasma levels are low and short lasting. Repeated pulses of ZA could be useful in the maintenance of active plasma concentrations of ZA (20). Taken together, these experimental and preclinical observations represent the rationale of using the weekly, low-dose regimen instead of the monthly bolus dosing.

Therefore, on the basis of these stimulating data on animals, we designed a third study on humans to investigate the effect on VEGF serum levels, and hence, the potential antiangiogenic role, of a repeated intermittent low-dose therapy with ZA in patients with malignancies.

We report that a low-dose schedule of ZA (1 mg for 1 week) is able to induce a significant decrease of VEGF serum levels in cancer patients.

Materials and Methods

Patients. Twenty-six consecutive patients (19 males and 7 females), ages 44 to 80 years (median age, 66 years), with advanced solid cancer and bone metastases, were included in the study (patients' characteristics are shown in Table 1). Patients were considered eligible for the study if they had a histologically confirmed solid neoplasm associated with bone scan identification and radiographic confirmation of bone metastases. Moreover, patients were required to have at study inclusion a baseline Eastern Cooperative Oncology Group (ECOG) performance status 2, a neutrophil count 1.5×10^9/L, a platelet count $>100 \times 10^9$/L, normal hepatic and renal function as determined by serum creatinine <1.5 times the upper limit of normal and creatinine clearance >60 mL/min, and no acute or chronic infections or inflammatory diseases. Patients were considered ineligible for accrual when they had reported fever (body temperature >38.0°C) during the last week before study entry or had received any radiotherapy, chemotherapy, immunotherapy, or growth factors during the last 4 weeks before study accrual. Any chemotherapy was excluded during the metronomic phase of the study (first 4 weeks of ZA administration). Patients recently (<1 week) or simultaneously treated with steroids were considered ineligible for the study. Hormonal therapy was allowed only when started at least 3 months before accrual. Patients were excluded if they had previously been treated with bisphosphonates for the current disease or for previous non-neoplastic diseases. Patient participation was limited to 21 weeks. All patients received ZA on an outpatients basis and provided written informed consent before screening.

Treatment and follow-up investigations. All patients received 1 mg of ZA (Zometa; Novartis) in 100 mL of 0.9% saline over a period of 15 min as an i.v. infusion starting at 10:00 a.m. The treatment was scheduled every week for four times (days 1, 7, 14, and 21) and followed by 4 mg of ZA with a standard 28-day schedule repeated thrice (days 28, 56, and 84). During the study, patients also received calcium (500 mg daily) and vitamin D3 (400-500 IU daily) supplements. Venous peripheral blood for cytokine assessment was drawn into an EDTA anticoagulant-containing tube just before the beginning of drug infusion (0) and again at 7, 14, 21, 28, 56, and 84 days after the first ZA infusion. The samples were collected just before the subsequent ZA infusion in correspondence to the different time points. After drawing, the samples were rapidly centrifuged for 10 min at 18,000 rpm, and plasma was stored at -80°C until assessed for VEGF levels. Moreover, WBC and platelet counts, hemoglobin, and serum total calcium levels were also determined at the same time points.

Table 1. Patients' characteristics

Total number, *N* (%)	26 (100)
Median age (range)	66 (44-80)
Male/Female	19/7
Median performance status ECOG score (range)	1 (0-2)
0-1 score/2 score (%)	62.5/37.5
Cancer type (%)	
Breast carcinoma	4
Prostate adenocarcinoma	6
Non–small cell lung cancer	6
Renal cancer	3
Other primary cancers	7
Bone segments involved by metastases (range)	1-9
Previous chemotherapy (%)	11
Concurrent hormonal therapy	3
Metastases other than bone locations (patients; %)	
No other locations	15
Lung metastases	3
Liver metastases	5
Lung + liver metastases	3
Other locations	6

Table 2. VEGF-circulating level modifications after a single infusion of ZA

Time points (days)	VEGF median value (pg/mL)	95% CI	*P*
Basal	286.11	258.56-603.35	—
7	201.60	174.32-381.13	0.038
14	190.94	124.86-297.96	0.002
21	172.85	118.35-248.24	0.001
28	194.61	123.42-293.80	0.008
56	173.71	95.97-298.99	0.002
84	206.60	83.45-352.13	0.014

Cytokine analysis. Serum levels of VEGF were assayed by using a solid-phase sandwich ELISA kit, designed to measure $VEGF_{165}$ levels (R&D Systems).

The assay was done following the manufacturer's instructions. Absorbance of each sample was measured at 450 nm. Standard absorbance (*A*) curve was derived from serial dilutions of known concentration of human VEGF provided in the manufacturer's kit. The assay was done in duplicate, and the results were expressed in picograms per milliliter (mean of two wells). The minimum detectable level of VEGF was <9.0 pg/mL.

Statistical analysis. Basal cytokine levels were compared with the values observed at 1, 7, 14, 21, 56, and 84 days after ZA infusion using the Wilcoxon's test for nonparametric-dependent continuous variables. A two-tailed *P* was considered significant when ≤0.05. SPSS software (version 10.00; SPSS) was used for statistical analysis.

Results

VEGF analysis. The effects on median circulating VEGF levels observed at 0, 7, 14, 21, 56, and 84 days after ZA infusion are summarized in Table 2. The effects in terms of percent reduction of VEGF levels as compared with basal values and considered at the same time points after ZA infusion are summarized in Table 3. The median VEGF basal value [286.11 pg/mL; 95% confidence interval (95% CI): 258.56-603.35] showed an early statistically significant ($P = 0.038$) decrease already 7 days after the first 1-mg infusion of ZA (201.60 pg/mL; 95% CI: 174.32-381.13). However, after two weekly administrations of 1 mg ZA (14 days), VEGF levels showed an additional decrease to 33.2% below the basal value (190.94 pg/mL; 95% CI: 124.86-297.96; $P = 0.002$). This effect on VEGF-circulating levels persisted also after three weekly infusions of 1 mg ZA with an additional decrease to 39.4% below the basal value (172.85 pg/mL; 95% CI: 118.35-248.24; $P = 0.001$). Finally, after four weekly administrations of 1 mg of ZA (day 28), the reduction of VEGF levels was still statistically significant if compared with basal levels (-31.8%; 194.61 pg/mL; 95% CI: 123.42-293.80; $P = 0.008$).

Interestingly, repeated intermittent low-dose ZA induced a significant and long-lasting decrease of VEGF-circulating levels that remained statistically significant also at each programmed time point during the second phase of the study (ZA 4 mg every 4 weeks). In fact, after 56 days (four weekly administrations of 1 mg plus one monthly infusion of 4 mg of ZA), the decrease of VEGF levels persisted significantly compared with basal levels (-33.6%; 173.71 pg/mL; 95% CI: 95.97-298.99; $P = 0.002$). After 84 days (four weekly administrations of 1 mg plus two monthly infusions of 4 mg of ZA), VEGF level reduction was yet statistically significant (-27.9%; 206.60 pg/mL; 95% CI: 83.45-352.13; $P = 0.014$). Figure 1 shows the behavior of VEGF levels at days 0, 7, 14, 21, 28, 56, and 84 after the first ZA administration.

Secondary parameters. No significant differences have been recorded in platelet level, WBC count, or hemoglobin concentration before and after each ZA infusion. However, as expected, a statistically significant decrease in plasma total calcium levels was observed during the metronomic phase of the study: the median calcium level before the first ZA administration was 10.76 mg/dL (range, 9.1-12.3 mg/dL), whereas the median value 28 days after the first ZA infusion was 8.77 mg/dL (range, 8.1-10.3 mg/dL; $P = 0.001$). A statistically significant decrease in plasma total calcium levels, compared with median basal levels, persisted during the following three standard infusions of ZA: the median value 84 days after the first ZA infusion was 8.55 mg/dL (range, 7.4-10.5 mg/dL; $P = 0.001$). Moreover, no patient developed hypocalcemia or the ZA administration during the first 4 weeks of the study had to be delayed. The serum calcium concentration did not correlate with the circulating VEGF levels before or after ZA infusions.

Discussion

This study reports the *in vivo* potential antiangiogenic role of ZA, confirming our previous results and adding new and intriguing data. Of note, we did the study using an innovative dose schedule referred to as metronomic for ZA infusion instead of the standard regimen (4 mg every 28 days).

In the present study, for the first time in the literature, we clearly showed a significant reduction of VEGF serum levels with repeated intermittent low doses of ZA (1 mg every week).

In fact, the median VEGF basal level showed an early statistically significant ($P = 0.038$) decrease only 7 days after the first 1-mg infusion of ZA, and this effect on VEGF-circulating levels persisted at each programmed time point during the metronomic study period. Moreover, this significant and long-lasting decrease of VEGF-circulating levels persisted also at each programmed time point during the second phase of the study (ZA 4 mg every 4 weeks). Previous studies have reported a positive correlation between platelet number and serum VEGF level in cancer patients (21, 22), supporting the hypothesis that platelets may serve the role of storage of VEGF in the circulation. In the present study, no significant differences were recorded in platelet levels during the entire study period. These data support the theory that the long-lasting serum VEGF

Table 3. Median percentage changes in VEGF levels after metronomic ZA infusion at different time points

Time points (days)	Median reduction (%)	95% CI
7	29.7	13.3-32.4
14	33.2	22.6-45.2
21	39.4	26.0-51.4
28	31.8	26.9-52.0
56	33.6	22.9-44.9
84	27.9	24.3-34.5

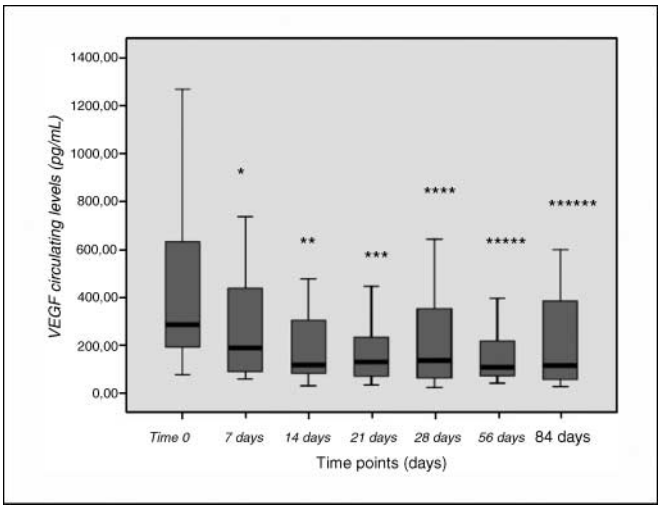

Fig. 1. Behavior of VEGF levels at days 0, 7, 14, 21, 28, 56, and 84 after zoledronic acid administration. This represents 95 percentiles of all VEGF values. Horizontal black bar in the grey boxes, VEGF median value. Bottom and top horizontal bars, minimum and maximum values. *P* is calculated according to the Wilcoxon test for nonparametric-dependent continuous variable: *, $P = 0.038$; **, $P = 0.002$; ***, $P = 0.001$; ****, $P = 0.008$; *****, $P = 0.002$; ******, $P = 0.014$.

decrease is not related to platelet count modifications but is strictly related to ZA action.

Growing *in vitro* and *in vivo* evidence has shown that bisphosphonates exhibit antitumor activity (9). In particular, ZA, which seems to be one of the most effective antiresorptive bisphosphonates *in vitro*, may also exert potent antitumor activity *in vivo* against the progression of visceral metastases (23, 24). Inhibition of several cellular mechanisms such as vascularization, adhesion, invasion, and migration and activation of apoptosis have been proposed to explain this phenomenon (25). Among these antitumor effects, recent evidence suggests that part of the antitumor activity of bisphosphonates may be attributed to an antiangiogenic effect (11).

However, most previous animal studies used high doses of bisphosphonates that are considered incompatible with the clinical dosing regimens approved for the treatment of cancer patients with skeletal metastases. The present study clearly shows that a low dose of ZA (1 mg/week) is able to induce a significant decrease of VEGF serum levels in cancer patients. We showed for the first time in humans that ZA is able to significantly decrease VEGF serum levels and, hence, may interfere with the angiogenesis process when a very low dose of bisphosphonate is administered.

Moreover, we may infer that this biological effect persists with repeated weekly low doses of ZA: after four weekly infusions, the VEGF serum levels were significantly decreased compared with basal values (194.61 pg/mL; 95% CI: 123.42-293.80; $P = 0.008$).

Although the precise mechanism of this effect is still not well known, we hypothesize that ZA may elicit several angiogenic-related cytokine patterns and may inhibit several antiangiogenic-related cascades, as shown by preclinical studies (11–13). Moreover, it is known that the inhibitory effect of ZA on endothelial cell adhesion and migration is mediated, at least in part, by the modulation of integrins that are involved in angiogenesis (13). Giraudo et al. (26), in a mouse cervical cancer model, showed that ZA acts as an unconventional matrix metalloproteinase-9 (MMP-9) inhibitor for antiangiogenic therapy; in this tumor model, ZA suppressed MMP-9 expression by infiltrating macrophages and inhibited metalloprotease activity, reducing the association of VEGF with its receptor on angiogenic endothelial cells. Recently, Hasmin et al. showed that ZA inhibits endothelial adhesion, survival, migration, and actin stress fiber formation through the suppression of multiple, prenylation-dependent signaling pathways (27). All these experimental data explain at cellular and molecular level the antiangiogenic activity of ZA. Although the cellular targets of bisphosphonates are not completely elucidated, it can be assumed that the endothelial-tumor-stroma behavior plays a relevant role in the antiangiogenic properties of bisphosphonates. In search of new strategies for the treatment of cancer, the interaction between tumor and stroma is attracting more and more attention. Several well-established drugs (such as cyclooxygenase-2 inhibitors and mTOR antagonists), which had initially been developed for other indications, also have exhibited antitumor activity (28). Current experimental data and clinical experience suggest that these drugs (especially when administered at low repeated doses) target stroma functions as well as tumor-stroma interactions and, above all, angiogenesis in cancer. Similar to these drugs, frequent (daily or weekly) administration of low-dose chemotherapeutics, referred to as metronomic chemotherapy, reveals profound antiangiogenic effects (16–18). The metronomic use of these agents has been suggested to be very active in terms of antitumor efficacy and prevention of drug resistance. Interestingly, some metronomic-chemotherapy regimens induce sustained suppression of circulating proangiogenic cytokines and increase the levels of the endogenous angiogenesis inhibitor thrombospondin 1 and, hence, exert an inhibitory effect on neovascularization (16–18).

All these preclinical and clinical data should represent the rational basis to consider the metronomic administration of bisphosphonates as a new potential therapy targeting the endothelial-tumor-stroma behavior. The significant, early and long-lasting decrease of VEGF serum levels during low and repeated doses of bisphosphonate could represent the first clinical evidence in humans of the antiangiogenic potential of metronomic use of ZA.

The clinical implications and helpfulness of the bisphosphonates' effect on VEGF levels should be investigated and should represent the objective of future clinical trials. Moreover, it is conceivable that the efficacy of metronomic therapy could be significantly increased when administered in combination with antiangiogenic drugs, such as antibodies against VEGF or VEGF receptor 2 or small tyrosine kinase molecules that inhibit multiple angiogenic receptors (PDGFR, VEGFR, and EGFR).

Further preclinical investigations eventually supported by advanced technological platforms are required for the discovery of new angiogenesis-related molecular targets of N-BPs and for their optimization.

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