ANTICANCER RESEARCH *28*: 933-942 (2008)

Antitumoral and Antiangiogenic Efficacy of Bisphosphonates *In Vitro* and in a Murine RENCA Model

JENS SOLTAU[1], UTE ZIRRGIEBEL[3], NORBERT ESSER[3], CHRISTOPH SCHÄCHTELE[3], FRANK TOTZKE[3], CLEMENS UNGER[2], IRMGARD MERFORT[4] and JOACHIM DREVS[1]

[1]*Cancer Hospital Sanafontis, Freiburg;*
[2]*Department of Medical Oncology and* [3]*ProQinase GmbH, Tumor Biology Center, Freiburg;*
[4]*Department of Pharmaceutical Biology and Biotechnology, Albert Ludwigs University, Freiburg, Germany*

Abstract. *Background: Bisphosphonates have shown direct antitumoral activity in vitro, in vivo and even in clinical studies, but the exact mechanism for this has not yet been elucidated. In this study the antiangiogenic potency of zoledronic acid and clodronate were evaluated. Materials and Methods: The effects of zoledronic acid and clodronate on the proliferation of endothelial cells and different tumor cells, and on the activity of protein kinases were investigated. Furthermore in vitro experiments were performed to evaluate the underlying antiangiogenic mechanism of action. Both bisphosphonates were examined in vivo at different doses and in daily subcutaneous application in a murine renal cell carcinoma model (RENCA). The antiangiogenic activity was evaluated by immunohistochemical staining (CD31) and by determination of mouse vascular endothelial growth factor (VEGF) serum concentration. Results: Zoledronic acid and clodronate inhibited proliferation of endothelial cells at lower concentrations than the different tumor cell lines did. This effect was more pronounced for zoledronic acid. The activity of almost all tested kinases was inhibited by zoledronic acid, whereas clodronate showed no effect. In the RENCA model, a significant effect of zoledronic acid on the primary tumor in a bell-shaped dose response curve with the highest efficacy between 100 Bg/kg 2xd and 200 Bg/kg 1xd, was observed. The mean vessel density (MVD) was significantly reduced by both bisphosphonates at different concentrations. This is the first report on increased mouse VEGF serum concentrations in the RENCA model. Conclusion: The results indicate that these bisphosphonates, particularly zoledronic acid, possess antitumoral and antiangiogenic activity.*

Correspondence to: PD Dr. Joachim Drevs, Cancer Hospital Sanafontis, An den Heilquellen 2, 79111 Freiburg, Federal Republic of Germany. Tel: +49 761 888 595959, Fax: +49 761 888 595957, e-mail: drevs@sanafontis.com

Key Words: Bisphosphonates, angiogenesis, RENCA, vessel density, VEGF.

Bisphosphonates have become indispensable for the treatment of malignant and benign bone disease with enhanced osteoclastic bone resorption. They are metabolically stable analogues of endogenous pyrophosphate (P-O-P), in which the central oxygen atom is replaced by a carbon atom (P-C-P). The carbon atom is linked to additional substituents. Bisphosphonates of the first generation (*e.g.* clodronate) contain simple substituents lacking a nitrogen atom. Newer bisphosphosphonates (*e.g.* zoledronic acid) have either an aliphatic or a heterocyclic side chain containing one or two nitrogen atoms.

These drugs inhibit osteoclastic bone resorption *via* different mechanisms. They influence adhesion and activity of osteoclasts, induce apoptosis in osteoclasts and inhibit osteoclast precursor cells (1, 2). Non nitrogen-containing bisphosphonates are metabolized into non hydrolysable, cytotoxic ATP analogues, which influence cell function and induce apoptosis. In contrast, nitrogen-containing bisphosphonates inhibit a key enzyme of the mevalonate pathway, farnesyl diphosphonate synthase. Inhibition of this enzyme prohibits formation of isoprenoids, such as farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP), which are required for regular prenylation of small GTP-binding proteins, like Rho and Ras (3).

In vitro and *in vivo* experiments indicated that bisphosphonates also have direct antitumoral activity (4). Nevertheless, clinical studies with adjuvant oral clodronate administration in patients with primary breast cancer reported contradictory results (5-7). In summary, these results suggest that bisphosphonates might be useful for the treatment and prevention of bone metastases and of non-osseous metastases. Presently, the underlying mechanism of the antineoplastic activity has not been elucidated, but an inhibition of tumor cell adhesion and invasion and the induction of tumor cell apoptosis and immunomodulatory effects appear to be involved in the

mechanism of action (2). Recently, it was assumed that antiangiogenic effects may contribute to the antitumoral activity of bisphosphonates (8).

Angiogenesis is a prerequisite for the progressive growth of solid tumors and their metastases (9). Above a few mm in diameter, the connection to the nutritive system of the body is essential for tumor progression. In malignant tumors, the development and spread of new vessels is directed and regulated by a complex network of endogenous proangiogenic factors, *e.g.* vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). If the tumor tissue is invaded by blood vessels, the solid tumor mass will be supplied with oxygen and an exchange of metabolites occurs. Neovascularisation can be further stimulated by factors being secreted by the tumor itself. These "leaky" vessels enable the tumor to metastasize to various sites. Thus, an inhibition of angiogenesis is a promising strategy in the treatment of malignant tumors (10).

Our aim was to evaluate the antiangiogenic and antitumoral properties of two different bisphosphonates zoledronic acid and clodronate) in different *in vitro* and *in vivo* experiments. We performed comparable proliferation studies on endothelial cells and on six different tumor cell types. To investigate a possible inhibition of tyrosine kinases, including angiogenesis-relevant kinases, we used a high volume throughput screening. Moreover, we studied antitumoral and antiangiogenic effects after daily subcutaneous application in three independent studies of the murine renal cell carcinoma model (RENCA). In order to investigate novel therapeutic strategies, such as antiangiogenic therapy, RENCA is a particularly suitable animal model (11). In this model, primary kidney tumors are induced by subcapsular renal injection of renal carcinoma (RENCA) cells with subsequent development of metastases in the lung, lymph nodes and spleen. As a positive control we administered PTK787/ZK 222584, a potent inhibitor of all three VEGF-receptor tyrosine kinases (including the lymphangiogenic VEGF-R3), specifically targeting the KDR/flk-1 receptor tyrosine kinase. PTK787/ZK 222584 was discovered and profiled in collaboration between Novartis Pharma AG, Schering AG and the Tumor Biology Center Freiburg, Germany. In a variety of rodent models, *e.g.* RENCA model, PTK787/ZK 222584 showed antitumoral, antimetastatic and antiangiogenic activity (12).

Materials and Methods

Drugs. Zoledronic acid ([1-hydroxy-2-(1*H*-imidazol-1-yl)ethylidene bisphosphonic acid], hydrated disodium salt, PTK787/ZK222584 (1-[4-chloranilino]-4-[-4-pyridyl-methyl]) were provided by Novartis Pharma AG (Basel, Switzerland). Clodronate (dichloromethylene bisphosphonic acid, disodium salt tetrahydrate) was obtained from Schering AG (Berlin, Germany). For *in vitro* experiments, stock solutions of each compound (zoledronic acid: 10 mM, clodronate: 50 mM) were prepared in phosphate-buffered saline (PBS), and then diluted with PBS to the appropriate concentrations. For kinase screening, stock solutions of both bisphosphonates (100 mM) were prepared in 100% dimethyl sulfoxide (DMSO). For application in the RENCA model, zoledronic acid, clodronate and PTK787/ZK 222584 were dissolved in 0.9% NaCl solution.

Cell lines and culture conditions. For *in vitro* experiments all tumor cell lines and endothelial cell lines were grown as monolayers in a humidified atmosphere of 95% air and 5% carbon dioxide at 37˚C. MCF7, PC3, LnCAP, CRW22, SAOS (all ATCC) and RENCA (Prof. H. Pahl, University Freiburg, Germany) were maintained in RPMI-1640. All media were supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 Bg/ml streptomycin and 100 Bg/ml L-glutamine. The endothelial cell line HUVEC (human umbilical vein endothelial cells) was maintained in Endothelial Cell Growth Medium 2 Kit (PromoCell GmbH, Heidelberg, Germany), whereas the endothelial cell line HDMVEC (human dermal microvascular endothelial cells) was maintained in Endothelial Cell Growth Medium MV2 Kit (PromoCell GmbH). Media were routinely changed every 3 days. Cells were released from the tissue flasks by treatment with 0.05% trypsin/EDTA, and the respective cell numbers were counted using the cell analyzer system Casy 1 from Schärfe System (Reutlingen, Germany).

For animal experiments, RENCA cells (Prof. H. Pahl, University Freiburg, Germany) were originally obtained from a tumor that arose spontaneously in the kidney of BALB/c mice. Monolayers of murine RENCA cells were grown in RPMI-1640 with phenol red supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin/ml and 100 µg streptomycin/ml. RENCA cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. Media were routinely changed every 3 days. Cells were released from the tissue flasks by treatment with 0.05% trypsin/EDTA and viability was monitored using the cell analyzer system Casy1 from Schärfe Systems Reutlingen. For the experiments, cells were collected during the logarithmic growth phase.

Cell growth assay. All cells were plated onto 96-well plates ($5x10^3$ cells/well; HUVEC and HDMVEC $3x10^3$ cells/well) and incubated in the respective serum overnight at 37˚C in a 5% CO_2 incubator. After removal of the media, 100 Bl of media (2% serum) containing different concentrations of the test compounds (zoledronic acid, clodronate) were added to each well. Plates were incubated for additional 72 h without subsequent medium changes, and 5-bromo-2-deoxyuridine (BrdU) incorporation was measured using cell proliferation ELISA BrdU (Roche Diagnostics GmbH, Germany). For each cell line three similar experiments were performed.

In order to investigate the antiproliferative mechanism three independent experiments were performed to study the effects of both bisphosphonates after addition of *trans*, *trans* farnesol 96% (FOH), geranylgeraniol (GGOH) and D,L- mevalonic acid lactone (MEV) in endothelial cells (HUVEC, HDMVEC), RENCA cells and LnCAP tumor cells. IC_{50} values were determined for zoledronic acid (20 BM + 50 BM GGOH, 50 BM FOH or 0.5 mM MEV) and clodronate (1500 BM + 50 BM GGOH, 50 BM FOH or 0.5 mM MEV) under the same conditions (72 h incubation, 2% FCS) using the cell proliferation ELISA BrdU (Roche Diagnostics GmbH). As controls 100 BM EDTA to exclude chelation, and 50 BM GGOH, 50 BM FOH and 0.5 mM MEV were used. A consecutive

experiment examined higher concentrations of zoledronic acid and higher concentrations of GGOH (200 BM) to increase the observed effects. The experiment was performed under the same conditions, as described.

Kinase screening. The IC_{50} profile of zoledronic acid and clodronate were determined using 12 protein kinases. IC_{50} values were measured by testing 10 concentrations ($3x10^{-09}$ M to $1x10^{-04}$ M) of each bisphosphonate in duplicate (n=2). A radiometric protein kinase assay (^{33}PanQinase® assay, ProQuinase GmbH, Freiburg, Germany) was used for measuring the activity of the protein kinases. All kinase assays were performed in 96-well FlashPlates™ from PerkinElmer (Boston, MA, USA) in a 50 Bl reaction volume, using the recombinant GST-fused kinase domain expressed in infected SF9 (*Spodoptera frugiperda*) cells. The reaction cocktail was pipetted in four steps in the following order: 20 Bl of standard assay buffer, 5 Bl of ATP solution (in water), 5 Bl of test compound (in 10% DMSO), 10 Bl of substrate/10 Bl of enzyme solution (premixed). The assay for all enzymes contained 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 BM Na-orthovanadate, 1.2 mM DTT, 50 Bg/ml PEG_{20000}, 1 Bg [γ-^{33}P]-ATP (approx. $2x10^5$ cpm per well). The reaction cocktails were incubated at 30˚C for 80 min. The reaction was stopped with 50 Bl of 2% (v/v) H_3PO_4, plates were aspirated and washed twice with 200 Bl water. Incorporation of $^{33}P_i$ was determined with a microplate scintillation counter (Wallac Microbeta, PerkinElmer LAS GmbH, Rodgau-Jügesheim, Germany). All assays were performed with a Beckman Coulter/Sagian robotic system (Beckmann Coulter GmbH, Unterschleißheim, Germany).

ATPase activity is dependent on cations, such as magnesium and manganese, hence it is possible that zoledronic acid inhibits kinase activity through chelation. To exclude this mechanism experiments were performed with different concentrations of divalent cations in the assay buffer, using VEGF-R2 kinase. Therefore, different assay buffers were prepared with 0.3 mM Mg^{2+}/Mn^{2+}, 3 mM Mg^{2+}/Mn^{2+} and 30 mM Mg^{2+}/Mn^{2+}. IC_{50} values were measured by testing 10 concentrations ($3x10^{-09}$ M to $1x10^{-04}$ M) of zoledronic acid, using the different assay buffers and the standard assay buffer under the same conditions, as previously described (n=4).

Animal experiments. All experiments were carried out according to the guidelines of the Ethical Committee of the Regierungspräsidium (Freiburg, Germany AZ-Nr.:9185.82/3/277). Female BALB/c mice were used at 6-8 weeks of age (approximate weight 20 g). The injection of 10^6 RENCA cells in 0.2 ml aliquots into the subcapsular space of the left kidney was performed through a flank incision, after the animals were anaesthetized with 0.5-1.5 volume percent isoflurane with an oxygen flow of 1.5 l/min. The tumor cell injection induced progressive development of a primary tumor mass in the left kidney. One week after application, the primary tumor was macroscopically visible. At 10 days, spontaneous metastases developed in the regional lymph nodes, the lung, the peritoneum and the liver. The mean survival time of RENCA-bearing mice was approximately 32 days when 10^6 RENCA cells were injected.

Administration of drug. Therapy was initiated on day 1 after tumor cell inoculation into the subcapsular space of the left kidney. Twelve animals were always included in each group. Oral applications were performed using a gastric tube. Animal weights were taken every other day.

In animal study I, mice received the dose recommended for *in vivo* studies of 100 Bg/kg zoledronic acid (*s.c.*, once daily (1xd)), 2.86 mg/kg clodronate (*s.c.*, 1xd), a five times higher dose of the estimated clodronate portion in bone/kg/d equal to that in humans at a daily oral clodronate dose of 1600 mg (for 70 kg human 1600 mg daily, with 2.5% of absorption equates to approximately 280 mg/week, which is 0.57 mg/kg per day), 100 mg/kg PTK787/ZK 222584 (*p.o.*, twice daily (2xd)), or a combination of 100 Bg/kg zoledronic acid (*s.c.*, 1xd) with 100 mg/kg PTK787/ZK 222584 (*p.o.*, 2xd) until the animals were sacrificed on day 21.

In animal study II different doses of zoledronic acid (100 Bg/kg, 200 Bg/kg *s.c.*, 1xd), clodronate (0.114 mg/kg, 0.572 mg/kg, 2.86 mg/kg, *s.c.*, 1xd) and the positive control PTK787/ZK 222584 (100 mg/kg, *p.o.*, 2xd) were administered.

Based on the results of study I and II, study III investigated the effects of zoledronic acid in particular. The aim was to confirm the promising results of study II and to attempt to enhance the observed effects. Therefore higher doses (200 Bg/kg and 400 Bg/kg, s.c, 1xd) and altered dose schemes of zoledronic acid were applied in study III. Besides a control group without treatment, PTK787/ZK 222584 (100 mg/kg, *p.o.*, 2xd) as positive control was again administered.

Evaluation of tumors. On day 21, all mice were sacrificed for determination of weight and volume of the primary tumor, lung weight and number of lung metastases, and metastases in the abdominal lymph nodes. Volumes of primary tumors were evaluated macroscopically by measuring their extensions in three orthogonal dimensions. The number of metastases in the lung and abdominal lymph nodes were counted with the aid of a dissection microscope. In the abdominal cavity, all visible lymph nodes were assessed for detection of metastasis, knowing that in healthy animals lymph nodes are not usually visible. Lymph nodes were inspected randomly with a microscope in order to confirm tumor-bearing tissue.

Immunohistochemistry. For histological examination of the tumor vasculature, tumor tissues were frozen immediately in liquid nitrogen. Cryosections of tissue with a thickness of 6 Bm were taken from animals of all study groups. To visualize the blood vessels, immunohistochemical staining for CD31 (Pecam-1 and MEC13.3; Becton Dickinson GmbH, Heidelberg, Deutschland) was performed and vessels were microscopically counted using a defined magnification (x200). Two slides from separate areas of each tumor were used for all analysis, therefore sections were representative of the whole tumor. Regions with low, medium and high vessel density (MVD) were determined and counted. The MVD was declared as the mean value of different regions. Furthermore, the determination was performed by two persons blinded with respect to treatment of the animals.

Mouse serum VEGF concentration. Serum VEGF levels were measured in selected study groups of animals from study II and III. Blood samples (150 Bl) were taken by retrobulbar venous plexus punction to determine the baseline serum VEGF concentration and that on day 21 after tumor cell inoculation. Subsequently, the blood samples were rapidly centrifuged for 20 min at 3000 rpm. Sera were separated and stored at –20˚C until assayed. Mouse VEGF was assayed with the R&D Quantikine®M kit according to the manufacturer's instructions (R&D Systems MMV00, Minneapolis, MN, USA).

Statistical analysis. All values reported were expressed as mean±standard error of the mean (SEM). The Mann-Whitney test was applied to all statistical analyses. A level of $p<0.05$ was considered as being statistically significant.

Results

Cell growth. After an incubation time of 72 h, both bisphosphonates were found to be more active against endothelial cells (HUVEC, HDMVEC) compared to tumor cell lines. IC_{50}'s of 14 BM to 22 BM for zoledronic acid and 816 BM to 1233 BM for clodronate, respectively, were found for endothelial cells, compared to the IC_{50}'s of 49 BM to 103 BM for zoledronic acid and 1400 BM to 2400 BM for clodronate, respectively, in different tumor cells. Zoledronic acid showed only an increased antiproliferative activity in LnCAP prostate cancer cell lines, compared to the IC_{50}'s the other tumor cell lines. Table I gives the IC_{50} values for each drug in each cell line. Furthermore it became obvious that the nitrogen-containing bisphosphonate zoledronic acid tends to be more potent than the non nitrogen-containing clodronate. Our results demonstrated that endothelial cells are more sensitive to incubation with bisphosphonates, than different tumor cell lines (RENCA, MCF7, Saos, CRW22, PC3).

Cell growth after incubation with bisphosphonates and isoprenoids. The addition of geranylgeraniol (50 BM) reduced the antiproliferative activity of zoledronic acid in both endothelial cells and the LnCAP prostate cancer cell line (data not shown). The effect was more pronounced in a second study performed with higher concentrations of zoledronic acid (50 BM and 100 BM) and geranylgeraniol (200 BM) (Figure 1). Farnesol and mevalonic acid lactone showed no influence on the effects of zoledronic acid. As expected due to the known different mechanism of action, no differences of antiproliferative effects were observed for clodonate and additional isoprenoids (data not shown). Furthermore, incubation with EDTA (100 BM) revealed no differences in proliferation compared to the control, so that chelation, as a possible antiproliferative mechanism of action could be excluded (data not shown).

Kinase screening. Table II shows the IC_{50} values of both bisphosphonates and for comparision the IC_{50}'s of PTK787/ZK 222584 on different protein kinases (16). Zoledronic acid inhibited almost all protein kinases examined, including angiogenesis-relevant kinases (VEGF-R2, EPHB4, Tie-2). The highest activity of zoledronic acid was observed on IGF1-R kinase (IC_{50}: 3.3 BM), EPHB4 kinase (IC_{50}: 5.5 BM) and VEGF-R2 kinase (IC_{50}: 6.4 BM). In contrast, clodronate showed no effect on the activity of any kinase at the concentrations used.

To exclude the inhibitory activity of zoledronic acid being related to chelation of cations, a further kinase assay was performed with a selected kinase (VEGF-R2) and different concentrations of divalent cations (Mg^{2+}/Mn^{2+}) in the assay buffer used. The results showed that chelation was not responsible for the detected inhibition of VEGF-R2 after incubation with zoledronic acid. The determined IC_{50}'s for zoledronic acid on the VEGF-R2 were 45 BM (0.3 mM assay buffer), 9.2 BM (3 mM assay buffer), 9.2 BM (30 mM assay buffer) and 9.5 BM (3 mM standard assay buffer).

Table I. *Effects of zoledronic acid and clodronate on proliferation of different cell lines. Values are means±SD; n=3.*

Cell line	IC_{50} (BM)	
	Clodronate	Zoledronic acid
HUVEC (endothelial)	1233±189	14±0.5
HDMVEC (endothelial)	816±346	22±4.5
RENCA (renal cell cancer)	1866±309	94±47
MCF7 (breast cancer)	2300±350	103±26
SAOS (osteosarkoma)	2033±329	71±55
CRW22 (prostate cancer)	1933±464	93±26
PC3 (prostate cancer)	1400±170	49±24
LnCAP (prostate cancer)	2400±355	5.6±1.8

Antitumoral activity in vivo. Both bisphosphonates were well tolerated by mice in all experiments and had no significant effects on body weight or the general well-being of the animals (data not shown).

In animal study I, treatment with clodronate (2.86 mg/kg 1xd) and zoledronic acid (100 Bg/kg 1xd) showed no effects on the primary kidney tumor, whereas the administration of PTK787/ZK 222584 (100 mg/kg 2xd) and the combination of PTK787/ZK 222584 (100 mg/kg 2xd) with zoledronic acid (100 Bg/kg 1xd) reduced primary tumor volume: T/C (mean value of treated tumor-bearing animals (Test) divided by the mean value of untreated tumor-bearing animals (Control) multiplied by 100) 53.4%, $p=0.0142$ and T/C 47.3%, $p=0.0017$, respectively. The combination therapy showed an additive antitumoral efficacy, but was not statistically significant ($p=0.4358$, data not shown).

In animal study II, treatment with the highest daily dose of zoledronic acid (200 Bg/kg 1xd) resulted in a significant reduction of primary tumor volume: T/C 72%, $p=0.0393$, whereas clodronate (0.114 mg/kg 1xd, 0.572 mg/kg 1xd, 2.86 mg/kg 1xd) and zoledronic acid at 100 Bg/kg 1xd had no effect on the primary kidney tumor. PTK787/ZK 222584 (100 mg/kg 2xd) showed comparable antitumoral effects as those observed in animal study I (Figure 2A).

Study III investigated the antitumoral effects of zoledronic acid at higher doses and changed dosing regimes. The evaluation demonstrated a reduction in primary

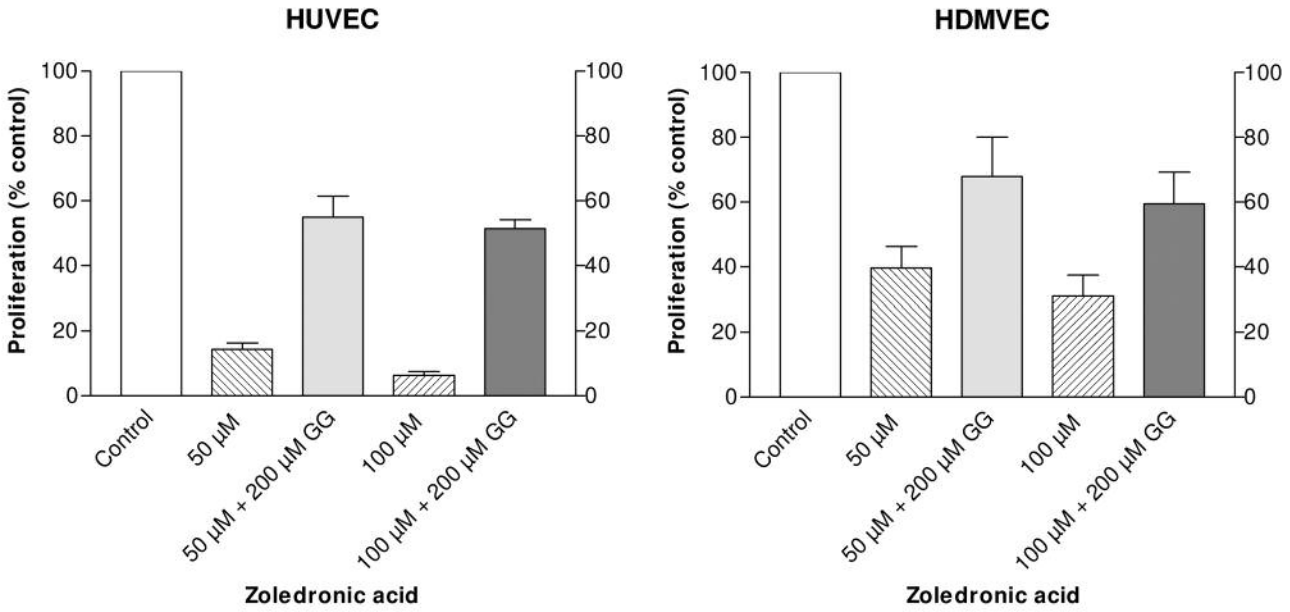

Figure 1. *Effect of additional geranylgeraniol (GG, 200 BM) on antiproliferative effects of zoledronic acid (50 BM and 100 BM) using the cell proliferation ELISA BrdU (Roche Diagnostics GmbH, Germany). Three similar experiments were performed. Values are means; bars, standard deviation (SD); n=3.*

primary tumor volume in animals treated with zoledronic acid (100 Bg/kg 2xd: T/C 62.9%, $p=0.0087$ and 200 Bg/kg 1xd: T/C 66.5%, $p=0.0613$, respectively). Interestingly, a further increase of the administered daily dose abolished the observed antitumoral effects of zoledronic acid. Positive control PTK787/ZK 222584 (100 mg/kg 2xd) again showed comparable antitumoral effects as those observed in animal study I (Figure 2B).

Antiangiogenic activity in vivo

a) Vessel density: For histological examination of tumor vasculature, primary tumor tissues of all study groups were stained for CD31. Initial examination of all tissue sections at low magnification showed homogeneous vessel density with a lack of hot spots. In animal study I, vessel density in primary tumors of animals treated with clodronate (2.86 mg/kg 1xd), zoledronic acid (100 Bg/kg 1xd), PTK787/ZK 222584 (100 mg/kg 2xd) and the combination of PTK787/ZK 222584 (100 mg/kg 2xd) with zoledronic acid (100 Bg/kg 1xd) were significantly lower compared to vessel density in primary tumors of control animals (data not shown).

Histological evaluation of the tumors in animal study II after treatment with clodronate (0.114 mg/kg 1xd, 2.86 mg/kg 1xd), zoledronic acid (100 Bg/kg 1xd, 200 Bg/kg 1xd) and PTK787/ZK 222584 (100 mg/kg 2xd) revealed a significant decrease in vessel density. The highest reductions in the number of vessels could be observed in primary tumors of animals treated with clodronate (2.86 mg/kg 1xd; $p=0.0039$) and in primary tumors of animals treated with zoledronic acid (100 Bg/kg 1xd by 37%, $p=0.0005$) (Figure 3A).

The vessel density in RENCA study III was significantly reduced in almost all treatment groups compared to the control group. The most distinctive effect was seen in animals treated with 200 Bg/kg 1xd with MVD reduced by 33% ($p=0.0002$). Interestingly, the administration of 100

Table II. *IC_{50} values for zoledronic acid and clodronate on 12 protein kinases including angiogenesis-relevant kinases; for comparison the IC_{50}'s of PTK787/ZK 222584 (Proqinase, Freiburg) are given.*

Kinase	IC_{50} (BM)		
	Zoledronic acid	Clodronate	PTK787/ZK222584
CDK2/CycA	15±1	>100	>100
Aurora-B	>100	>100	62.9
PLK1	21±0.5	>100	>100
PDGFR-β	16±0.5	>100	3.21
AKT1	8±0.45	>100	>100
EGF-R	7.3±0.65	>100	>100
IGF1-R	3.3±0.1	>100	>100
VEGF-R2	6.4±0.05	>100	0.1
EPHB4	5.5±0.15	>100	ND
KIT	8.9±0	>100	0.48
TIE2	8.7±0.1	>100	25
VEGF-R3	11±2.1	>100	0.52

ND, not determined.

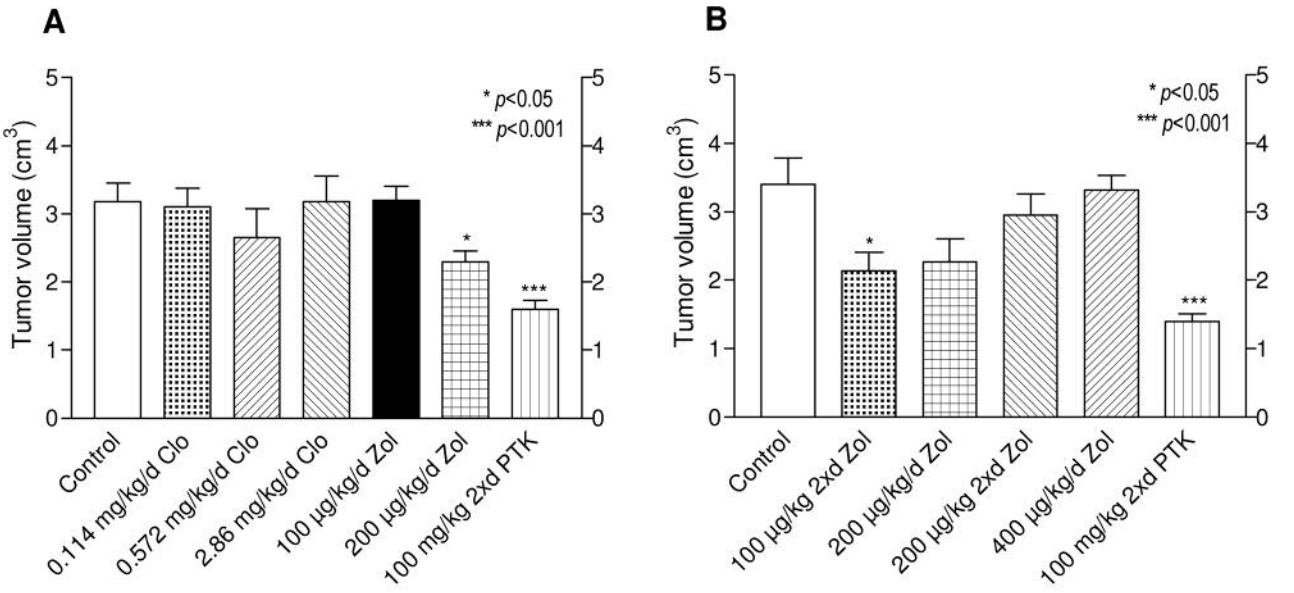

Figure 2. *Effect of bisphosphonates at different doses on tumor volume in the murine renal cell carcinoma model. In study II, zoledronic acid (Zol) and clodronate (Clo) were administered in a daily subcutaneous application. In study III, zoledronic acid was administered in increased daily doses and different dosing regimes. As positive control, PTK787/ZK222584 (PTK) was administered. All therapies were initiated one day after inoculation of RENCA cells into the subcapsular space of the left kidney of syngenic BALB/c mice. Animals were sacrificed after 21 days, and primary tumor weight and volume were assessed. Values are means; bars, SE. P-values were calculated by comparing means of the treated group and means of the untreated group using the Mann-Whitney test. A, Results of animal study II; B, results of animal study III.*

Bg/kg 2xd led only to an insignificant decrease in vessel density of 6% ($p=0.4528$) (Figure 3B).

For PTK/ZK787/ZK222584 as positive control, a comparable, statistically significant efficacy on vessel density was detected in all performed studies.

b) Mouse serum VEGF concentration: Blood samples were collected to determine VEGF levels at baseline and on day 21. Compared to the mean VEGF baseline value of all groups, including the control (19.91±3.35 pg/ml; day 0), the serum VEGF concentration on day 21 was statistically significantly increased in all study groups. This determined increase was found to be lower particularly in animals treated with zoledronic acid (200 Bg/kg 1xd) and PTK787/ZK222584 (100 mg/kg 2xd), by 29% and 28%, respectively (Figure 4). In study III the reduction of the serum VEGF concentration in animals treated with zoledronic acid (200 Bg/kg 2xd; 52%, $p=0.2809$) compared to the control group (data not shown) was confirmed.

Discussion

Recent *in vitro*, *in vivo* and even clinical studies on bisphosphonates clearly indicate, that these drugs might have direct antitumoral activity and may be able to play a role in the treatment of cancer. The aim of our study was to investigate antitumoral and antiangiogenic properties of two structurally different bisphosphonates *in vitro* and in the RENCA model after daily subcutaneous application.

Our studies on proliferation provide a comprehensive overview about the effects of zoledronic acid and clodronate on a wide spectrum of different cell lines. We demonstrated that both investigated drugs are more active against endothelial cells than against different tumor cells, which clearly point to the antiangiogenic potential of bisphosphonates. Likewise, the nitrogen-containing zoledronic acid proved to be more potent than the non nitrogen-containing clodronate in our studies. Initial studies by Wood *et al.* showed similar IC_{50}'s for zoledronic acid on human endothelial cells stimulated with FCS, bFGF or VEGF (4.1 BM, 4.2 BM and 6.9 BM, respectively) (8). In other studies with different bisphosphonates, the proliferation of different tumor cell lines was also reduced at concentrations ranging from 5-2000 BM (2).

Protein kinases are functionally involved in human cancer. They regulate cell proliferation (*e.g.* cyclin dependent protein kinase (CDK2/CycA)), cell survival (*e.g.* insulin like growth factor 1 receptor tyrosine kinase (IGF1-R)) and tumor angiogenesis (*e.g.* VEGF-R2 tyrosine kinase) (13). Therefore, we performed a high volume throughput screening of 12 selected protein kinases, emphasizing angiogenesis-relevant kinases, to assess the effects of bisphosphonates.

Zoledronic acid inhibited almost all tested kinases. However, this compound showed its highest activities on

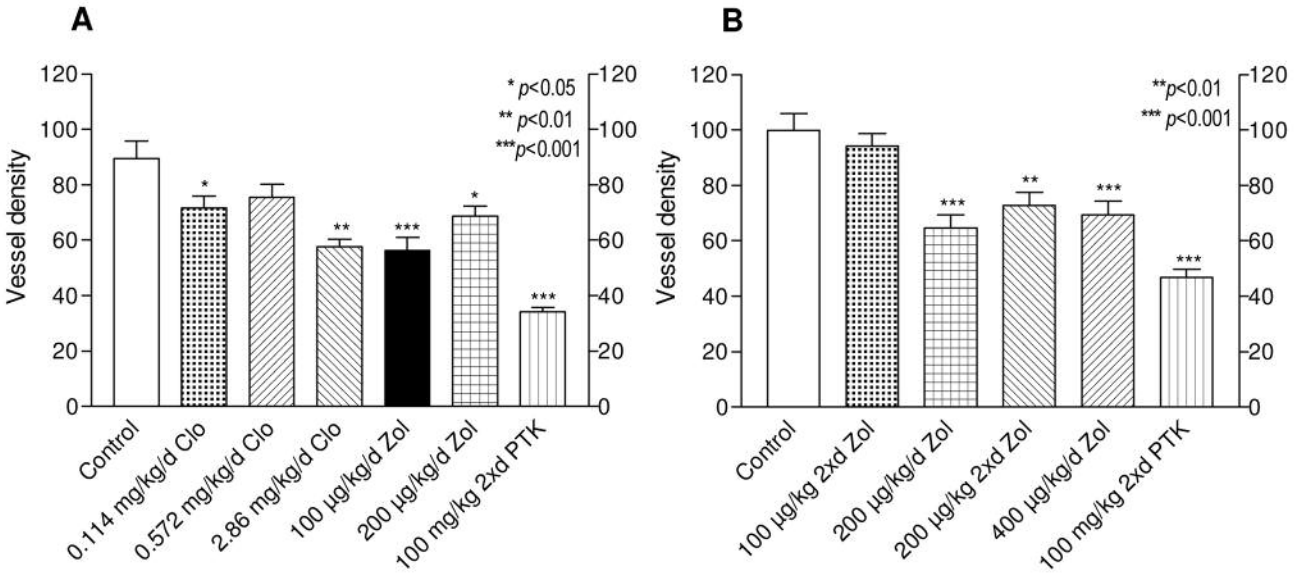

Figure 3. *Effect of zoledronic acid (Zol) and clodronate (Clo) on vessel density in primary tumors of the murine renal cell carcinoma model (assessed at magnification x200). As positive control, the antiangiogenic acitive drug PTK787/ZK 222584 (PTK) was administered. To visualize blood vessels immunohistochemical staining for CD31 was performed. A, Results of animal study II; B, results of animal study III. Values are expressed as the percentage that of the control; bars, SE. P-values were calculated by comparing means of the treated group and means of the control group using the Mann-Whitney test.*

IGF1-R, erythropoietin-producing hepatoma-amplified sequence (EPH) B4 kinase and VEGF-R2 kinase. IGF1-R and its ligand are overexpressed in breast cancer, prostate cancer and osteosarcoma. The activation of IGF1-R led to increased proliferation and blockade of apoptosis. EPH receptors are expressed on endothelial cells and play an important role in the remodeling of vasculature (13). VEGF is one of the most potent endothelial cell-specific factors playing a key role in tumorangiogenesis. Its specifity for endothelial cells is explained by the enhanced expression of its two receptors, VEGF-R1 and VEGF-R2, on endothelial cells of tumor vessels. Recently, it was reported by Bezzi *et al.* that zoledronic acid inhibits the sustained phosphorylation of focal adhesion kinase (FAK), a regulator of endothelial cell adhesion and migration, and protein kinase B (PKB/Akt), which promotes cell survival and protects cells against death induced by cytotoxic stimuli (14).

Our findings indicate that the nitrogen-containing zoledronic acid interferes with angiogenesis-relevant kinase signaling pathways, as a novel possible antiangiogenic mechanism of action. In comparison, PTK787/ZK 222584, a VEGF-receptor antagonist, inhibits the VEGF-R2 at ~100-fold lower concentrations than zoledronic acid. In contrast, the non nitrogen-containing clodronate showed no effects on tested kinases, indicating a different mode of action (Table II).

In the RENCA model, we were able to show, for the first time, that daily application of zoledronic acid resulted in a significant reduction in primary kidney tumor growth in a bell-shaped dose-response with the highest efficacy between 100 Bg/kg 2xd and 200 Bg/kg 1xd. However, the administration of higher doses of zoledronic acid abolished

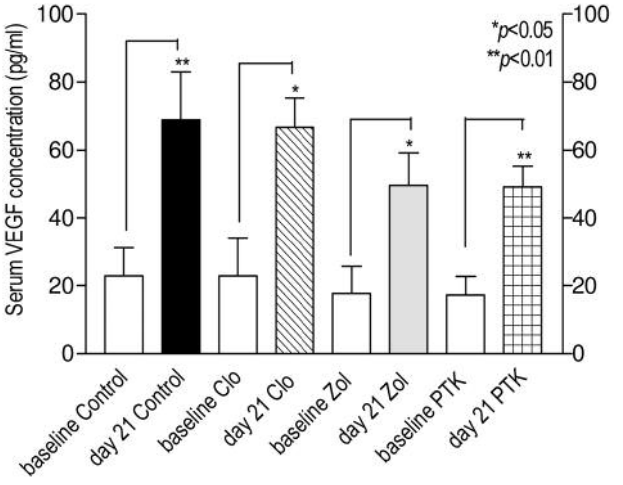

Figure 4. *To determine serum VEGF concentrations, blood of animals on day 0 (baseline) and on day 21 were collected from 4 selected study groups (control, clodronate - Clo (2.86 mg/kg 1xd), zoledronic acid - Zol (200 Bg/kg 1xd) and PTK787/ZK222584 - PTK/ZK (100 mg/kg 2xd)) and rapidly centrifuged afterwards. Sera were separated and stored at –80°C until assayed. Mouse VEGF was assayed with the R&D Quantikine® kit (MMV00) according to the manufacturer's instructions. Values are means; bars, SE. P-values were calculated by comparing means of the baseline values and means of the VEGF values on day 21 using the Mann-Whitney test.*

these antitumoral effects. Thus, there is a small therapeutic window for zoledronic acid in this tumor model. Further investigations are required to elucidate this phenomenon. The antitumoral activity can be described as moderate, compared to PTK787/ZK 222584.

In summary, the effect of bisphosphonates on non-osseous primary tumors and visceral metastases is currently controversial. Besides described antitumoral and antimetastatic effects on lung and liver in other animal studies (15, 16), we showed an antitumoral efficacy in the RENCA model. In particular a combination of zoledronic acid with established cancer drugs or novel targeting therapies could be a promising option to enhance tumor therapy. In contrast to existing dose schedules, we administered zoledronic acid and clodronate in a daily subcutaneous application. Both drugs were well tolerated and showed no side-effects in mice. Due to rapid elimination of bisphosphonates from the body and their accumulation in bone tissue, it appears to be necessary to administer these drugs more frequently at lower doses (metronomic therapy) to optimize antiangiogenic and antitumoral efficacy. Pharmacokinetic studies are needed to investigate how the established dose regime of zoledronic acid (4 mg/15 min every 4 weeks) can be changed and if there is a tolerable toxicity profile (renal side-effects, osteonecrosis) under the changed conditions. Currently it remains unclear, whether it will be possible to transfer the promising preclinical effects to a clinical setting.

First studies with bisphosphonates, particularly zoledronic acid, indicated an antiangiogenic potency (8, 17-19). Thus, our results clearly confirm, that bisphosphonates have antiangiogenic activity. The immunohistochemical evaluation of the primary tumor tissue with the endothelial selective CD31 antibody revealed a significant decrease of the vessel density in almost all animals treated with bisphosphonates, but the tumor size was mostly not affected. This finding indicates a partial angiogenesis-independent growth of the fast growing RENCA tumor. Previous results suggested that the proliferation rate of RENCA cells is superior to the proliferation rate of endothelial cells, resulting in the decreased vessel density in the RENCA model (11). Thus, an even greater antitumoral efficacy might be obtained with slower growing tumors. Furthermore, we determined, for the first time, mouse serum VEGF concentrations in the RENCA model. There was a significant increase in serum VEGF concentration during tumor progression (day 0 - day 21). But we showed in animal study II and III that this increase was reduced in animals treated with zoledronic acid (200 Bg/kg 1xd), compared to that of untreated animals. To reach statistical significance a higher number of animals is surely required. Studies in breast cancer patients confirmed a significant decrease in serum VEGF levels after infusion of zoledronic acid (20, 21). These findings suggest that bisphosphonates may have an impact on VEGF release and/or interact with the VEGF signaling pathway.

At the moment, the underlying antiangiogenic mechanism of action remains unclear. Both bisphosphonates act *via* different molecular mechanisms on osteoclasts and tumor cells. Non nitrogen-containing bisphosphonates are metabolized into non-hydrolysable, cytotoxic ATP analogues, which accumulate intracellularly (22), whereas nitrogen-containing bisphosphonates inhibit the activity of farnesyl diphosphonate synthase, a key enzyme of the mevalonate pathway (3). This enzyme inhibition leads to a disruption of the regular prenylation of small GTP-binding proteins, such as Rho and Ras. Several studies reported that after the addition of geranylgeraniol and farnesol, two intermediates of the mevalonate pathway, growth inhibition and induction of apoptosis of tumor cells induced by nitrogen-containing bisphosphonates were prevented. In our *in vitro* studies we were able to show that geranylgeraniol abolished the inhibition of endothelial cell growth. These reversing effects of geranygeraniol suggest that Rho-proteins in particular play a crucial role in the antiangiogenic mechanism of action of zoledronic acid, because these proteins will be modified posttranslational mostly *via* geranylgeranylation. Further studies determined the important role of RhoA proteins in the VEGF-induced VEGFR2/FAK/PI3K/Akt signaling pathway, which controls endothelial cell invasion, proliferation and migration (23). Similar antiangiogenic effects were seen with statins, which also inhibit a key enzyme of the mevalonate pathway (24, 25). It was shown that these drugs inhibit RhoA-dependent VEGF-, FAK- and Akt signaling. Statins and nitrogen-containing bisphosphonates possibly mediate the antiangiogenic effects by inhibition of the regular posttranslational prenylation of RhoA. Studies by Hasmim *et al.* showed an inhibition of zoledronic acid on HUVEC adhesion, survival, migration and actin stress fiber formation by interfering with protein prenylation and identified ERK1/2, JNK, Rock, FAK and PKB as kinases affected by zoledronic acid in a prenylation-dependent manner (26). Another recently published study with alendronate, a nitrogen-containing bisphosphonate, reported that inhibitory effects on endothelial cells were associated with reduced Rho activation and suppression of the formation of actin stress fibers and focal adhesions. Furthermore, the inhibition by alendronate was reversed by geranylgeraniol, which abrogated the inhibition of Rho geranylgeranylation (27).

In conclusion, our results confirm the very promising potential of bisphosphonates for direct antitumoral efficacy, especially in selective combination therapies, that is explained by a specific antiangiogenic mode of action. Further investigations are urgently required.

References

1 Fleisch H: Development of bisphosphonates. Breast Cancer Res *4*: 30-34, 2002.

2 Green J: Bisphosphonates in cancer therapy. Curr Op Oncol *14*: 609-615, 2002.

3 Luckman SP, Hughes DE, Coxon FP, Graham R, Russell G and Rogers MJ: Nitrogen-containing bisphosphonates inhibit the mevalonate pathway and prevent post-translational prenylation of GTP-binding proteins, including Ras. J Bone Miner Res *13(4)*: 581-589, 1998.
4 Padalecki SS and Guise TA: Actions of bisphosphonates in animal models of breast cancer. Breast Cancer Res *4(1)*: 35-41, 2002.
5 Diel IJ, Solomayer EF, Costa SD, Gollan C, Goerner R, Wallwiener D, Kaufmann M and Bastert G: Reduction in new metastases in breast cancer with adjuvant clodronate treatment. N Engl J Med *339*: 357-363, 1998.
6 Powles T, Paterson S, Kanis JA, McCloskey E, Ashley S, Tidy A, Rosenqvist K, Smith I, Ottestad L, Legault S, Pajunen M, Nevantaus A, Mannisto E, Suovuori A, Atula S, Nevalainen J and Pylkkanen L: Randomized, placebo-controlled trial of clodronate in patients with primary operable breast cancer. J Clin Oncol *20*: 3219-3224, 2002.
7 Saarto T, Blomqvist C, Virkkunen P and Elomaa I: Adjuvant clodronate treatment does not reduce the frequency of skeletal metastases in node-positive breast cancer patients: 5-year results of a randomized controlled trial. J Clin Oncol *19*: 10-17, 2001.
8 Wood J, Bonjean K, Ruetz S, Bellahcene A, Devy L, Foidart JM, Castronovo V and Green JR: Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. J Pharmacol Exp Ther *302*: 1055-1061, 2002.
9 Folkman J: Tumor angiogenesis: therapeutic implications. N Engl J Med *285*: 1182-1186, 1971.
10 Drevs J, Laus C, Medinger M, Schmidt-Gersbach C and Unger C: Antiangiogenesis: current clinical data and future perspectives. Onkologie *25*: 520-527, 2002 (English).
11 Drevs J, Hofmann I, Hugenschmidt H, Wittig C, Madjar H, Muller M, Wood J, Martiny-Baron G, Unger C and Marme D: Effects of PTK787/ZK 222584, a specific inhibitor of vascular endothelial growth factor receptor tyrosine kinases, on primary tumor, metastasis, vessel density, and blood flow in a murine renal cell carcinoma model. Cancer Res *60*: 4819-4824, 2000.
12 Drevs J: PTK/ZK (Novartis). IDrugs 6: 787-794, 2003.
13 Sachsenmaier C: Targeting protein kinases for tumor therapy. Onkologie *24(4)*: 346-355, 2001 (English).
14 Bezzi M, Hasmim M, Bieler G, Dormond O and Ruegg C: Zoledronate sensitizes endothelial cells to tumor necrosis factor-induced programmed cell death: evidence for the suppression of sustained activation of focal adhesion kinase and protein kinase B/Akt. J Biol Chem *278*: 43603-43614, 2003.
15 Hiraga T, Williams PJ, Ueda A, Tamura D and Yoneda T: Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model. Clin Cancer Res *10*: 4559-4567, 2004.
16 Alvarez E, Westmore M, Galvin RJ, Clapp CL, Considine EL, Smith SJ, Keyes K, Iversen PW, Delafuente DM, Sulaimon S, Zambrano C, Ma L, Sato M, Martin TJ, Teicher BA and Galbreath EJ: Properties of bisphosphonates in the 13762 rat mammary carcinoma model of tumor-induced bone resorption. Clin Cancer Res *9*: 5705-5713, 2003.
17 Croucher PI, De Hendrik R, Perry MJ, Hijzen A, Shipman CM, Lippitt J, Green J, Van Marck E, Van Camp B and Vanderkerken K: Zoledronic acid treatment of 5T2MM-bearing mice inhibits the development of myeloma bone disease: evidence for decreased osteolysis, tumor burden and angiogenesis, and increased survival. J Bone Miner Res *18*: 482-492, 2003.
18 Bellahcene A, Chaplet M, Bonjean K and Castronovo V: Zoledronate inhibits alphavbeta3 and alphavbeta5 integrin cell surface expression in endothelial cells. Endothelium *14*: 123-130, 2007.
19 Hamma-Kourbali Y, Di Benedetto M, Ledoux D, Oudar O, Leroux Y, Lecouvey M and Kraemer M: A novel non-containing-nitrogen bisphosphonate inhibits both *in vitro* and *in vivo* angiogenesis. Biochem Biophys Res Commun *310*: 816-823, 2003.
20 Vincenzi B, Santini D, Dicuonzo G, Battistoni F, Gavasci M, La Cesa A, Grilli C, Virzi V, Gasparro S, Rocci L and Tonini G: Zoledronic acid related angiogenesis modifications and survival in advanced breast cancer patients. J Interferon Cytokine Res *25*: 144-151, 2005.
21 Ferretti G, Fabi A, Carlini P, Papaldo P, Cordiali Fei P, Di Cosimo S, Salesi N, Giannarelli D, Alimonti A, Di Cocco B, D'Agosto G, Bordignon V, Trento E and Cognetti F: Zoledronic acid induced circulating level modifications of angiogenic factors, metalloproteinases and proinflammatory cytokines in metastatic breast cancer patients. Oncology *69*: 35-43, 2005.
22 Frith JC, Monkkonen J, Blackburn GM, Russell RG and Rogers MJ: Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5'-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells *in vitro*. J Bone Miner Res *12*: 1358-1367, 1997.
23 Gingras D, Lamy S and Beliveau R: Tyrosine phosphorylation of the vascular endothelial-growth-factor receptor-2 (VEGFR-2) is modulated by Rho proteins. Biochem J *348 Pt 2*: 273-280, 2000.
24 Park HJ, Kong D, Iruela-Arispe L, Begley U, Tang D and Galper JB: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors interfere with angiogenesis by inhibiting the geranylgeranylation of RhoA. Circ Res *91*: 143-150, 2002.
25 Vincent L, Albanese P, Bompais H, Uzan G, Vannier JP, Steg PG, Soria J and Soria C: Insights in the molecular mechanisms of the anti-angiogenic effect of an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Thromb Haemost *89*: 530-537, 2003.
26 Hasmim M, Bieler G and Ruegg C: Zoledronate inhibits endothelial cell adhesion, migration and survival through the suppression of multiple, prenylation-dependent signaling pathways. J Thromb Haemost *5*: 166-173, 2007.
27 Hashimoto K, Morishige K, Sawada K, Tahara M, Shimizu S, Ogata S, Sakata M, Tasaka K and Kimura T: Alendronate suppresses tumor angiogenesis by inhibiting Rho activation of endothelial cells. Biochem Biophys Res Commun *354*: 478-484, 2007.

Received October 9, 2007
Revised December 14, 2007
Accepted January 25, 2008