

The Bisphosphonate, Zoledronic Acid Reduces Experimental Neuroblastoma Growth by Interfering with Tumor Angiogenesis

ULRIKA BÄCKMAN¹, ÅSA SVENSSON¹, ROLF H. CHRISTOFFERSON^{1,2} and FARANAK AZARBAYJANI¹

Departments of ¹Medical Cell Biology and ²Surgical Sciences, Children's Hospital, Uppsala University, Sweden

Abstract. *Background: Zoledronic acid is a new member of the bisphosphonate (BP) class of compounds, a family of closely related synthetic molecules originally derived from the naturally occurring pyrophosphate. These compounds that are potent inhibitors of bone resorption, have been shown to reduce the growth of several cancer cell lines in vitro, and can act as inhibitors of angiogenesis. The angiogenesis inhibitor TNP-470, a synthetic analogue of the fungal antibiotic fumagillin, has been shown to inhibit the growth of multiple tumors in vivo, and is currently in Phase II clinical trials for cancer. Materials and Methods: The effects of daily subcutaneous (s.c.) administration of zoledronic acid (0.1 mg/kg) were compared with those of TNP-470 (15 mg/kg/day and 30 mg/kg every other day, s.c.) in a nude mouse xenograft model for the childhood cancer, neuroblastoma (NB). Results: Zoledronic acid reduced the tumor growth by 33% whereas TNP-470 was less effective and reduced the tumor growth by 26% and 11% for animals treated with 15 mg/kg/day and 30 mg/kg every other day, respectively. Analysis of angiogenesis showed a significant reduction of the number of vessels per grid and in vessel length in all the treatment groups. Conclusion: Zoledronic acid shows tumoristatic and angiostatic properties that might be beneficial in the treatment of solid tumors such as neuroblastoma.*

Neuroblastoma (NB) is a childhood cancer that originates from immature neuroblasts in the peripheral nervous system. The tumors show great heterogeneity with respect to

location, responsiveness to treatment and prognosis (1). Low stage tumors respond to surgical treatment and have a tendency to undergo regression. High-risk NB has a long-term survival rate of less than 40 percent despite intensive treatment protocols involving high-dose chemotherapy, usually with bone marrow rescue, aggressive surgery and radiotherapy (2, 3). Neuroblastoma is the second most common solid tumor among children and there is a clinical need for new treatment strategies, based on, for instance, the induction of differentiation and apoptosis, or the inhibition of tumor angiogenesis (4).

The bisphosphonates (BPs) are a family of pyrophosphate analogues that are structurally related to inorganic pyrophosphates (PPi) which are endogenous regulators of bone mineralization (5). Like PPi, BPs form three-dimensional structures capable of binding divalent metal ions such as Ca^{2+} , Mg^{2+} and Fe^{2+} , by coordination of oxygen from each phosphonate group with the divalent cation (6). Hence, these compounds have high affinity for bone mineral to which they locally bind and are released at high concentrations during active bone remodelling causing osteoclastic apoptosis through their production of nonhydrolyzable cytotoxic ATP analogues (6-9). They are therefore effective in the treatment of a variety of conditions associated with increased bone resorption, including malignant bone disease, Paget's disease and osteoporosis (10). However, high doses of some of the earlier BPs can lead to impaired mineralization (11, 12). By adding an OH to the central carbon atom together with a second nitrogen-containing substituent more potent BPs such as zoledronic acid have been developed which are up to 10,000-fold more potent than etidronate which is one of the first BPs discovered (13). In contrast to BPs, nitrogen-containing bisphosphonates (N-BPs) affect osteoclastic activity and survival through inhibition of a key enzyme involved in the biosynthetic mevalonate pathway. Several intermediates in this pathway, including farnesyl pyrophosphate (FPP) and geranyl-geranyl pyrophosphate (GGPP), are required for the post transitional modification (*i.e.* prenylation) of different signalling molecules (such as Ras, Rac and Rho) which are not only essential for normal cellular functions such as

Abbreviations: CgA, chromogranin A; FDA, fluorescein diacetate; TH, tyrosine hydroxylase; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

Correspondence to: Faranak Azarbayjani, Department of Medical Cell Biology, Box 571, S-751 23 Uppsala, Sweden. Tel: +46 18 471 42 69, Fax: +46 18 471 41 13 or +46 18 55 11 20, e-mail: Faranak.Azarbayjani@mcb.uu.se

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proliferation, survival and cytoskeleton organization, (14, 15) but also play important roles in induction of apoptosis and inhibition of cell cycle progression in different malignancies (16, 17). Another mechanism could be that BPs inhibit protein tyrosine phosphatases (PTP). PTP control crucial events in the regulation of normal cellular processes such as proliferation and differentiation and are also involved in malignant transformation (18, 19). Many studies have demonstrated anti-proliferative effects of BPs on several cancer cell lines *in vitro* and emerging data suggest these compounds may also exert anti-tumor effects *in vivo* (20). Several structurally related BPs have been approved worldwide for the treatment of benign and malignant bone disease. The BPs vary greatly in potency, zoledronic acid, a new generation compound containing a heterocyclic imidazole moiety, is considered the most potent (13, 21). However, zoledronic acid is not yet approved for use in children with cancer.

TNP-470 is a synthetic analogue of fumagillin, a compound secreted by the fungus *Asperigillus fumigatus fresenius*, which inhibits endothelial cell migration and proliferation (22-25). TNP-470 has been shown to reduce the growth and vascularization in several xenografted animal experimental models (26, 27), but no tumor regression has been shown. These findings have been confirmed in our earlier study (28). It has also been shown that TNP-470 induced reduction in angiogenesis causing metabolic stress, chromaffin differentiation and apoptosis in experimental neuroblastoma (29). TNP-470 is currently in Phase II clinical trials for cancer and reported side-effects are confusion, motor ataxia, fatigue and nausea (30).

In this study the effects of zoledronic acid on both tumor growth and tumor angiogenesis was examined in experimental neuroblastoma. Its efficacy was compared to that of TNP-470, a well characterized inhibitor of angiogenesis.

Materials and Methods

Drugs. All the substances were prepared in their respective media immediately before injection. Zoledronic acid (supplied as the hydrated disodium salt by Novartis Pharma AG, Basel, Switzerland) was dissolved in Ca^{2+} free phosphate-buffered saline (PBS), pH 6.5 and only plastic ware was used to avoid binding to glass and loss of substance activity. TNP-470 (Takeda Chemical Industries Ltd., Osaka, Japan) was suspended in 1% ethanol and 5% gum arabic in 0.9 mg/ml sodium chloride.

Cells. The human neuroblastoma cell line SH-SY5Y (31) was kindly provided by Dr. June Biedler of The Memorial Sloan-Kettering Cancer Centre, New York, NY, USA. The cells were cultured in Eagle's minimum essential medium (prepared by The National Veterinary Institute, Uppsala, Sweden), supplemented with 10% fetal calf serum, 1 mM L-glutamine, penicillin (100 IU/ml) and streptomycin (50 µg/ml) (Sigma Chemical Co., St Louis, MO,

USA). The cells were grown at 37°C, in a humidified 95% air/5% CO_2 -atmosphere. The medium was changed twice a week and subconfluent cultures were harvested after 5 min of treatment with 0.25% trypsin and 0.02% EDTA. The SH-SY5Y cells used throughout these experiments were from passage 32 and were shown to be free from Mycoplasma.

Bovine capillary endothelial cells (BCE cells; prepared in our laboratory (32)) were cultured in Dulbecco's modified Eagle's medium containing L-alanyl-L-glutamine, sodium pyrovate, 4,500 mg/l D-glucose and pyridoxine (SVA) supplemented with 10% newborn calf serum (Sigma-Aldrich, Sweden), penicillin and streptomycin (1 µl/ml) and 3 ng/ml recombinant human basic fibroblast growth factor (Sigma-Aldrich). The cells were grown in 75 cm³ culture flasks (Sarstedt, Sweden), coated with sterile 1.5% gelatine in PBS without Ca^{2+} and Mg^{2+} (SVA). The cells were subcultivated before reaching confluence as described below.

All the cells were tested negative for mycoplasma and were grown in humidified air (95%) and 5% CO_2 at 37°C. The medium was changed every second day. The cells were subcultivated after 5 min. of treatment with 0.25% trypsin and 0.02% EDTA and were spun at 130 ×g for 8 min, resuspended and seeded onto newly prepared culturing flasks. Only endothelial cell passages 10-12 were used in the experiment.

Fluorometric microculture cytotoxicity assay (FMCA). Drugs cytotoxicity on the endothelial cells was determined using FMCA. The FMCA procedure is based on measurements of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes as previously described (33). Briefly, the BCE cells (10⁴/well in a 96-well plate) were incubated with serum from either untreated control, TNP-470 or zoledronic acid treated mice for 72 h at 37°C. On each plate, six wells containing only medium were used as blank. At the end of the incubation, the plates were centrifuged and the medium was aspirated followed by one wash with PBS and the addition of 100 µl/well of FDA dissolved in PBS (10 µg/ml). The plates were incubated for 40 min and the generated fluorescence from each well was measured at 538 nm in a 96-well scanning fluorometer (Fluoroscan II, Labsystems Oy, Helsinki, Finland). The fluorescence is proportional to the number of viable cells in the well. Cell survival is expressed as survival index (SI) in percent, calculated from [fluorescence in (test wells – blank wells)/fluorescence in (control wells – blank wells)] × 100. Each experiment was repeated four times.

Animals. Forty-six nude NMRI nu/nu mice (B & M, Ry, Denmark), 33 males and 13 females, were used for xenografting at the age of 7-8 weeks (body weight 25-30 g). The mice were housed in an isolated room at 24°C with a 12-h light, 12-h dark cycle. They were fed *ad libitum* with water and food pellets. The weight and general appearance of the animals were recorded every other day throughout the experiments. The experiments were approved by the regional ethics committee for animal research.

Xenografting and measurement of tumor volume. The tumor cells (30×10⁶ cells in 0.2 ml medium) were implanted subcutaneously (*s.c.*) in the hind leg of the animal using a 23 G needle and syringe. Tumor volume measurement began when the tumor became palpable (~100 mm³) and was then repeated every second day. When the tumor had reached a volume of 300 mm³, treatment with the test compounds was initiated. The tumor volume was calculated from the formula $0.44 \times \text{length} \times \text{width}^2$ (29). For weighing,

measurement of tumor volume and drug injection, the animals were anesthetized with 2% Fluothane (Zeneca Ltd., Macclesfield, UK) supplemented with 50% N₂O in oxygen.

Administration of test compounds. The animals were treated every day with zoledronic acid given *s.c.* at a dose of 0.1 mg/kg body weight or with TNP-470 given *s.c.* at two different doses, 15 mg/kg every day or 30 mg/kg every other day (e.o.d). The controls received the vehicle without the active drug. The treatment was given for 14 days, when the tumor burden in the control animals approached 4 ml and these animals had to be killed according to the protocol.

Perfusion fixation and autopsy. At the termination of the experiments, the animals were anesthetized with an *i.p.* injection of 25 g/kg 2, 2, 2-tribromoethanol in 2.5% 2-methyl-2-butanol (Sigma) in 0.9 mg/ml sodium chloride. A cannula was inserted in the thoracic aorta, and the animal was perfusion-fixed with 4% paraformaldehyde in 1.47 mg/ml NaH₂PO₄, 12.62 mg/ml Na₂HPO₄ × 2 H₂O, and 4.09 mg/ml sodium chloride in distilled H₂O (Millonig's buffer, pH 7.3-7.4, +37°C). The perfusion system was calibrated to give an intra-arterial perfusion pressure of 100 mm Hg. The thoracic and abdominal viscera were examined for macroscopic metastases.

Tissue analyses. After perfusion fixation, the tumors were dissected out and their absolute weights and volumes were recorded. The tumors were then immersion-fixed in 4% formaldehyde for approximately one week prior to dehydration and paraffin embedding. Sections were cut at 3 µm and placed on 3-aminopropyltriethoxy-silane treated glass slides (Sigma).

Immunohistochemistry. To quantify tumor cell proliferation, staining for the Ki-67 nuclear antigen was performed using the MIB 1 which is a monoclonal mouse anti-Ki-67 antibody (Dianova, Hamburg, Germany), as previously described (34). Staining for the neuroendocrine and adrenergic cells, *i.e.*, the neuroblastoma cells, was performed with chromogranin A (CgA), monoclonal mouse anti-human CgA, No. 1199021, and tyrosine hydroxylase (TH), monoclonal mouse anti-TH, No. 1017 381 (Boehringer Mannheim GmbH, Mannheim, Germany). Immunohistochemistry was carried out as described previously (34). Apoptosis was determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay by using the Apoptag[®] kit (Oncor, Gaithersburg, MD, USA) according to the manufacturer's instructions. Immunohistochemistry was performed as described previously (34).

To quantify angiogenesis Bandeira simplifolica lectin histochemistry was used for highlighting endothelial cells. After dewaxing and rehydration and blocking in H₂O₂ for 20 min, neuraminidase solution, N-2133 (Sigma) was applied to the slides overnight at 37°C (approximately 16 hours). The biotinylated lectin, L3759 (Sigma) was applied, 1:100 and incubated at room temperature (RT) for 1 h. The sections were developed, counterstained, and mounted as above. As a positive control BCE cells were used, omission of the neuraminidase solution served as a negative control. For a definition of the vascular parameters, see Wassberg *et al.*, 1999 (29).

Stereological quantifications. A representative section from the geometrical center of each tumor was used. One observer quantified all sections in a blinded fashion. Structures were counted at x400

with an eyepiece grid (506800, Leica, Singapore, Singapore) of 10x10 squares (0.25x0.25 mm). The grid was placed at random at the upper left corner of a section, and then systematically advanced every 1 to 3 mm (depending on the tumor size) in both directions with use of the microscope's goniometer stage. Morphological parameters from 20-26 grids were quantified for each tumor. Although areas with hemorrhage and apoptotic or necrotic cells were excluded from analysis of other parameters because they were considered as non-viable, they were used for calculation of the viable tissue fraction (nVC, number of grids with "viable corner"). The length of vessels per tumor volume (Lv), volume of vessels per tumor volume (Vv), and surface area of vessels per tumor volume (Sv) were quantified (29).

For Ki67, TUNEL, CgA and TH- positive tumor cells, a minimum of 2,000 cells per section were counted using the right upper quarter of the counting grid described above. The fraction of stained cells in these grids is presented as the percentage (%) of proliferative, apoptotic and differentiated cells.

Statistical methods. The data were processed by Statistica 5.1 (StatSoft Inc., Tulsa, OK, USA). Differences between groups were analysed with the Mann-Whitney *U*-test. The level of statistical significance was set at *p*<0.05.

Results

***In vitro* cytotoxicity of zoledronic acid and TNP 470 on endothelial cells.** The effect of the serum from zoledronic acid and TNP-470 treated mice on the SI of the endothelial cells was a moderate decrease for both zoledronic acid (0.1 mg/kg/day) (*p*=0.14), and TNP-470 (15 mg/kg/day and 30 mg/kg e.o.d) (*p*=0.12) compared to serum from vehicle treated control mice. There were no significant differences between the effects of sera from the zoledronic acid and TNP-470 treated animals or between the sera from the two TNP-470 treated groups on the SI of endothelial cells.

Neuroblastoma growth. No toxicity and no metastases were seen in any of the treated or control nude mice. The weight gain in all the treatment groups was similar to that in the control group. The control animals exhibited a mean tumor volume of 3.47 ml at day 14 (~10% of body weight). No tumor regression was observed in either of the treatment groups. At day 14 of therapy the mean treated tumor volume/mean control volume (T/C ratio) was 0.67 for zoledronic acid (Figure 1, Table I), 0.74 for TNP-470 15 mg/kg/day, and 0.89 for TNP-470 30 mg/kg e.o.d (Figure 2, Table I). There was no significant difference between the two groups treated with TNP-470. In both TNP groups the tumors in the females (2.4 ml, n=12) were smaller compared to those in the males (3.4 ml, n=4) but these differences were not significant.

Angiogenesis. The results of the stereological quantification of angiogenesis are presented in Table II. At day 14 the total number of vessels per grid (Qv), length of

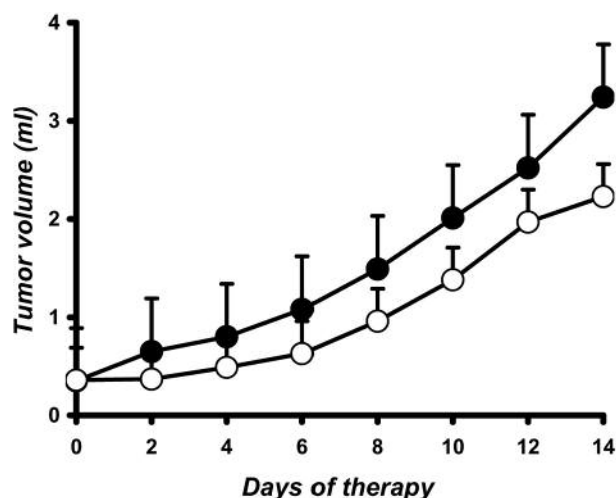


Figure 1. Tumor growth during zoledronic acid therapy (0.1 mg/kg/day). ● — controls (n=10), ○ — zoledronic acid (n=10) Mean±1 S.D. T/C at day 14 was 0.67.

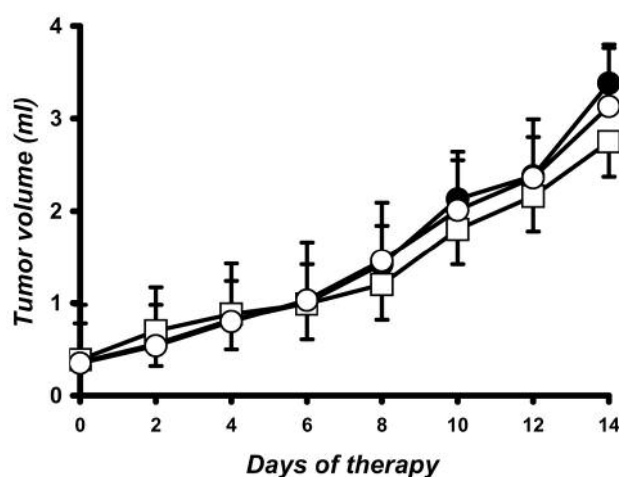


Figure 2. Tumor growth during TNP-470 therapy. ● — controls (n=10), ■ — TNP-470 15 mg/kg/day (n=8), ○ — TNP-470 30 mg/kg e.o.d. Mean±1 S.D. T/C day 14 at therapy was 0.74 for 15 mg/kg daily and 0.89 for 30 mg/kg TNP-470 e.o.d.

vessels per tumor volume (Lv), volume of vessels per tumor volume (Vv), and surface area of vessels per tumor volume (Sv) were all lower in the TNP-470 treatment groups compared to the controls. In the zoledronic acid treated group the total number of vessels per grid (Qv), the length of vessels per tumor volume (Lv), were reduced in comparison to the controls. Surprisingly zoledronic acid appeared to increase the vessel volume (Vv) by 20.2% and the surface area (Sv), by 22.5% but this was not statistically significant. The mean blood vessel diameter was similar in the two TNP-470 treatment groups (25.3 ± 3.4), but significantly larger in the zoledronic acid treated animals (48.9 ± 2.1).

Tumor cell proliferation. The fraction of proliferating cells did not differ significantly in any of the treatment groups. There was however a tendency towards a reduced fraction of proliferating cells in all the treatment groups compared to the controls (Table I).

Tumor cell apoptosis. The fraction of apoptotic neuroblastoma cells was significantly increased at day 14 of treatment with zoledronic acid (28.9%) and both doses of TNP-470 (30.6%, 46.9%) (Table I).

Tumor cell differentiation. The tumor exhibited cells staining positive for CgA and TH, confirming that they were of neuroblastoma origin. The proportion of stained cells was not significantly affected by the treatment. The CgA and TH immunoreactivity was most pronounced in the peripheral parts of the perivascular cuffs of the viable tumor cells.

Discussion

In this study zoledronic acid reduced the growth rate of neuroblastoma in a nude mouse xenograft model by 33% (T/C=0.67). It was more effective in this respect than the well characterized angiogenesis inhibitor TNP-470 implying that BPs may have additional beneficial properties in patients with cancer apart from the inhibition of osteoclastic bone resorption.

The tumor growth rate reduction might possibly have been more pronounced if zoledronic acid was not rapidly cleared from the circulation by binding to bone mineral. The tumors in the zoledronic acid treated animals exhibited a tendency of reduction in number and length of vessels, while the vessel volume (20.2%) and area (22.5%) were both increased. The mean blood vessel diameter was significantly larger in the zoledronic acid treated animals (48.9 ± 2.1) compared to controls (24.2 ± 4.6). The mechanism for this blood vessel dilation is not understood, but it could reflect interference with endothelial calcium homeostasis. Our *in vitro* data showed that the SI of BCE cells was moderately decreased upon 72 hours exposure of these cells to serum from zoledronic acid and TNP-470 treated animals. Interestingly, *in vitro* assays with human umbilical vein endothelial cells (HUVECs) have also shown that zoledronic acid dose-dependently inhibited the calf serum and bFGF induced proliferation of these cells and these findings have been confirmed *in vivo* (13, 35). Recently, Castronovo and his group (26) have shown that zoledronic acid had antiangiogenic properties which were dependent on its effect on decreasing the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins surface expression, thereby altering the endothelial cell integrin-

Table I. Quantification of tumor dynamics after 14 days of treatment.

	Tumor volume (ml)	Viable tissue (%)	Proliferative cells (%)	Apoptotic cells (%)	TH (%)
Control (n=20)	3.47±0.84	83.4±9.4	48.1±5.1	35.6±8.2	14.1±4.5
TNP-470 15 mg/kg/day (n=8)	2.47±0.38	64.5±6.7	44.3±3.0	46.5±5.5	12.3±3.3
‡Change (%)	-28.8*	-22.7*	-7.9	+30.6*	-12.8
TNP-470 30 mg/kg e.o.d (n=8)	2.98±0.61	78.0±8.8	42.4±4.4	52.3±2.0	11.4±1.4
‡Change (%)	-14.1	-6.4	-11.9	+46.9*	-19.1
Zoledronic acid 0.1 mg/kg/day (n=10)	2.32±0.92	68.1±3.3	44.9±7.9	45.9±3.6	12.9±3.6
‡Change (%)	-33.1**	-18.3*	-6.7	+28.9*	-8.5

The ratio of grids with viable corner to the total counted grids per tumor section is shown as the viable tissue. Proliferative cells, apoptotic cells, and TH (Tyrosine hydroxylase) are presented as the ratio of stained cells to the total number of cells in upper right quarter of the counting grid. Mean±1 S. D. Mann-Whitney *U*-test, where * $p \leq 0.05$, ** $p \leq 0.01$. ‡Change compared to controls.

Table II. Quantification of angiogenesis after 14 days of treatment.

	Qves	Lv (mm ⁻²)	Vv (10 ⁻³)	SV (mm ⁻¹)	d (ves) (μm)
Control (n=20)	89.9±36.1	93.7±31.9	25.3±7.3	4.0±1.1	24.8±1.2
TNP-470 15 mg/kg/day (n=8)	32.8±20.1	75.8±33.5	10.4±3.3	2.5±0.9	25.3±2.9
‡Change (%)	-63.5**	-19.1*	-58.9**	-37.5*	+2.0
TNP-470 30 mg/kg e.o.d (n=8)	44.3±18.9	69.5±39.7	23.6±5.8	2.5±1.2	25.3±3.7
‡Change (%)	-50.7**	-25.8*	-6.7	-37.5*	+2.0
Zoledronic acid 0.1 mg/kg/day (n=10)	56.1±24.4	60.3±33.4	30.4±2.3	4.9±2.7	48.9±2.1
‡Change (%)	-37.7*	-35.6*	+20.2	+22.5	+97.1**

Q ves: Number of vessels; Lv, length of vessels per tumor volume (length density); Vv, volume of vessels per tumor volume (volumetric density); Sv, surface area of vessels per tumor volume (surface density); d(ves), mean section diameter of vessels. Mean±1SD, Mann-Whitney *U*-test, where * $p \leq 0.05$, ** $p \leq 0.01$. ‡Change compared to controls.

mediated adhesion which is important during both tumor angiogenesis (5) and cancer cell adhesion and migration to bone favouring their propensity to metastasize to skeleton (36-39). Neither the viable tumor tissue fraction nor the chromaffin differentiation was affected by treatment with zoledronic acid whereas the fraction of apoptotic cells was significantly increased. Zoledronic acid may therefore interfere with tumor angiogenesis either by a direct effect on the tumor endothelium signalling pathways that are involved in endothelial cell migration (such as Ras, Rac and Rho) or by chelating divalent cations necessary for example for the metalloproteinases participating in angiogenesis (35, 40). The decrease in the SI of the endothelial cells *in vitro* when exposed to serum from zoledronic acid treated animals, as well as reduction of tumor angiogenesis and increase in tumor cell apoptosis in the xenografted tumors treated with zoledronic acid, shown in the present study, support this hypothesis. Our findings demonstrate a tumorstatic effect of a BP in a relevant non-toxic dose on rapidly growing, well-vascularized xenografted tumors. This new anti-tumor activity of BPs in solid tumors merits further investigation.

In the present study, TNP-470 reduced the tumor growth rate by 11 to 26%. The difference in tumor growth between

the two dosage groups, even though not significant, suggested that TNP-470 may be more efficient upon frequent administration. The blood vessel parameters and fraction of proliferating cells were not significantly different between the two dosage groups. However the fraction of apoptotic cells was increased by 30.6% and 46.9% compared to the controls. The proliferating cells were mainly located in the inner and middle layers of the perivascular cuffs, while the apoptotic cells were found in the outer layers, the pattern of "agonal differentiation" as reported by Wassberg *et al.* (29). It is concluded that the well characterized angiogenesis inhibitor TNP-470 has a different anti-tumor activity in otherwise similar rat and murine xenograft models, and that daily administration of the drug seems to be more efficient than every other day.

The non-metastatic model, used in the present study was advantageous, since it was easy to measure the whole tumor burden without surgery or imaging techniques. *In vivo* models of metastatic neuroblastoma have been described (41). Such models are elegant, but a reproducible, intentionally non-metastatic model such as ours may be used first to determine the efficacy of a new therapy before a metastatic model is applied. In conclusion, the BP,

zoledronic acid is a potent inhibitor of neuroblastoma growth, and that it may exert its action by interference with tumor angiogenesis. These results merits further investigations concerning zoledronic acid and new experimental neuroblastoma models.

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