In vitro toxicity of bisphosphonates on human neuroblastoma cell lines

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Neuroblastoma is the commonest extracranial solid tumor of childhood and frequently metastasizes to the bone. Bisphosphonates are standard treatment of osteolytic lesions by bone metastasis. Since recent studies suggested direct antitumor effects of bisphosphonates, we screened the toxicity of different bisphosphonates on neuroblastoma cell lines. The nitrogen-containing bisphosphonate pamidronate was significantly more toxic on a panel of eight neuroblastoma cell lines than the non-nitrogen-containing bisphosphonates, clodronate and tiludronate. After 72 h, GI_{50} concentrations (inhibiting cell growth by 50% compared to untreated controls) for pamidronate ranged from 12.8 to >500 μM. CHLA-90 and SH-SY5Y were the most sensitive cell lines. In CHLA-90, zoledronate was the most cytotoxic bisphosphonate, followed by alendronate, pamidronate and ibandronate. In SH-SY5Y, alendronate was the most cytotoxic bisphosphonate, followed by ibandronate, pamidronate and zoledronate. The GI_{50} values after 72 h were 34.1 (SH-SY5Y) and 3.97 μM (CHLA-90) for zoledronate, and 22.4 (SH-SY5Y) and 9.55 μM (CHLA-90) for alendronate. Neuroblastoma cells treated with bisphosphonates showed signs of differentiation and finally underwent apoptosis. The observed GI_{50} concentrations suggest that local nitrogen-containing bisphosphonate concentrations at the bone interface can directly target neuroblastoma cell penetration into the bone matrix. In summary, these observations warrant the investigation of adjuvant bisphosphonate treatment in controlled clinical trials.
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Introduction

Neuroblastoma is the second commonest solid tumor of childhood and responsible for nearly 15% of deaths in childhood malignancies. Despite continuously optimized therapy the 5-year survival rates in children with metastatic disease are still less than 40% [1,2]. About 39% of advanced neuroblastomas metastasize to the bone and the bone matrix [3,4].

In patients with multiple myeloma, breast or prostate cancer, tumor-associated bone disease is successfully treated by bisphosphonates, which reduce skeletal events like fractures, lytic lesions, bone pain and hypercalcemia [5–8]. Bisphosphonates are stable analogs of naturally occurring pyrophosphates. They are absorbed to bone mineral and inhibit bone resorption by osteoclasts [9]. Bisphosphonates interfere with a number of signaling pathways, which contribute to the impairment of osteoclast function and the induction of apoptosis in osteoclasts. Clodronate and etidronate are metabolized to non-hydrolysable toxic adenosine triphosphate analogs [10]. The nitrogen-containing bisphosphonates inhibit the enzymes farnesyl- and geranylpyrophosphate synthetase [11–13]. Farnesyl- and geranylpyrophosphate are intermediates in the mevalonic acid pathway, and necessary for post-translational protein prenylation and sterol synthesis. Prenylation of small guanine triphosphatases (GTPase) like Ras and Rho is necessary for activation of these key regulatory proteins. Bisphosphonates also inhibit protein tyrosine phosphatases which play a major role in the regulation of cell growth, proliferation, metabolism, differentiation and locomotion [14–18]. Since activation of Ras and Rho signaling and dephosphorylation of protein phosphotyrosines might also be deregulated in tumors, bisphosphonates are supposed to exert direct antitumor effects. In prostate cancer, breast cancer, and multiple myeloma cell lines bisphosphonates inhibited proliferation and induced apoptosis in a dose-dependent fashion [19–23]. Bisphosphonates also reduced the adhesion and invasion of breast and prostate tumor cells to the extracellular matrix [24–28].

Recently, Sohara *et al.* demonstrated that the nitrogen-containing bisphosphonate ibandronate significantly delayed the generation of osteolytic lesions by neuroblastoma cells in nude mice [29]. Since bisphosphonates

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interfere with a number of signaling pathways deregulated in tumors and accumulate in bones, favorable effects of bisphosphonates in the treatment of neuroblastomas are possible. We, therefore, examined the cytotoxic effects of two non-nitrogen containing bisphosphonates (clodronate and tiludronate) and four nitrogen-containing bisphosphonates (pamidronate, alendronate, ibandronate and zoledronate) on human neuroblastoma cell lines, which have not been reported so far.

Materials and methods

Reagents

RPMI 1640 medium together with L-glutamine, penicillin, streptomycin and fetal calf serum were obtained from Invitrogen (Karlsruhe, Germany). Clodronate (CLO) and phenylacetate (PA) were purchased from Sigma-Aldrich (Taufkirchen, Germany). The other bisphosphonates were kindly provided by the respective manufacturers, i.e. tiludronate (TIL) by Sanofi-Synthelabo (Berlin, Germany), alendronate (ALE) by MSD Sharp & Dohme (Haar, Germany), ibandronate (IBA) by Roche Diagnostics (Mannheim, Germany), and zoledronate (ZOL) and pamidronate (PAM) by Novartis Pharma (Basel, Switzerland). Stock solutions of bisphosphonates were prepared by dissolution in water. The protein tyrosine phosphatase (PTP) inhibitor α-bromo-4-(carboxymethoxy)-acetophenone, the farnesyltransferase inhibitors *N*-[2(*S*)-[2α-amino-3-mercaptopropyl-amino]-3-methyl-butyl]-Phe-Met-OH (FTI-I) and H-Cys-4-Abz-Met-OH (FTI-II) were purchased from Merck Biosciences (Bad Soden, Germany). The PTP inhibitor was dissolved in dimethylsulfoxide and the FTI inhibitors were dissolved in water. All stock solutions were further diluted with complete cell culture medium.

Cell culture

CHLA-90 and LAN-6 were kindly provided by C. P. Reynolds (Division of Hematology-Oncology, Childrens Hospital Los Angeles and Department of Pediatrics, University of Southern California Keck School of Medicine, Los Angeles). SHEP-SF, KCN and NGP were kindly supplied by C. Poremba (Institute of Pathology, University of Düsseldorf, Germany). SH-SY5Y, IMR-5 and SK-N-SH were obtained from the DSMZ (Braunschweig, Germany). Cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal calf serum and penicillin/streptomycin in a humidified atmosphere with 5% CO_2 at 37°C.

Cell viability assay

Cytotoxicity was measured by the MTT assay [30,31]. Cells were grown in 96-well tissue culture plates coated with collagen. In each well 5000 cells (IMR-5, KCN, NGP, SHEP-SF, SH-SY5Y and SK-N-SH) or 10 000 cells (CHLA-90 and LAN-6) were seeded in 100 µl RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and penicillin/streptomycin. Cells were allowed to attach to the collagen matrix and to resume exponential growth before 100 µl of complete cell culture medium containing the drugs at different concentrations was added. Ten concentrations of each bisphosphonate ranging from 0.5 to 500 µM were tested. PA was added in concentrations of 0.1 µM to 10 mM. The FTIs were tested in concentrations of 0.005 to 5 µM, and the PTP inhibitor in concentrations of 0.05 to 250 µM. After 24, 48, 72 and 96 h, 20 µl of the yellow MTT reagent [5 mg/ml MTT dissolved in phosphate-buffered saline (PBS), pH 7.4] was added and incubated for another 3 h. Metabolically active cells cleaved the yellow tetrazolium salt to a purple formazan dye. An increase in the number of living cells resulted in an increase in total metabolic activity in the sample, which in turn correlated with the amount of purple formazan crystals formed. The formazan crystals were dissolved and the colored solution was quantified spectrophotometrically at a wavelength of 560 nm and a reference wavelength of 650 nm using a microplate reader.

The drug concentration capable of 50% growth inhibition relative to control cells (GI_{50}) was is calculated with the equation ([% viable cells (> 50%)] – 50)/([% viable cells (> 50%)] – [% viable cells (< 50%)]) × (drug concentration above 50% viable cells – drug concentration below 50% viable cells) + (drug concentration below 50% viable cells).

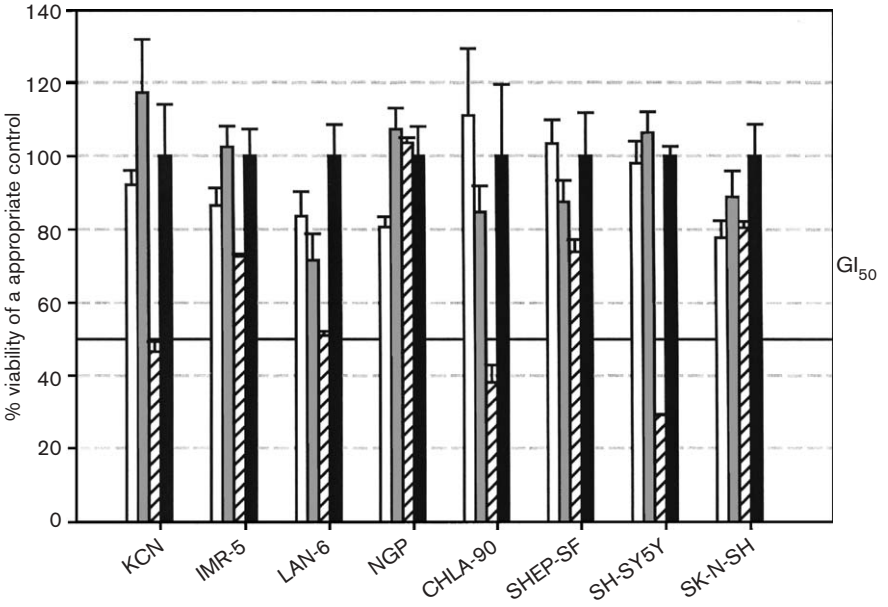
Apoptosis and cell cycle analysis

Apoptosis and necrosis were determined by flow cytometry using Annexin-V and propidium iodide staining [32,33]. Cells were harvested by PBS/EDTA treatment (PBS: 6.8 g sodium chloride, 1.5 g disodium hydrogen phosphate and 0.41 g potassium dihydrogen phosphate were dissolved in distilled water, and the pH was adjusted to 7.4; PBS/EDTA: 5 mM EDTA dissolved in PBS buffer), washed twice in PBS pH 7.4, and diluted with binding buffer at a concentration of 1×10^6 cells/ml. An 100 µl aliquot of this cell suspension ($= 1 \times 10^5$ cells) was incubated with Annexin-V and propidium iodide at room temperature in the dark for 15 min. Cells were diluted with binding buffer and analyzed by flow cytometry using the FACSCalibur with CellQuest software (Becton Dickinson, Heidelberg, Germany).

Results

The non-nitrogen-containing bisphosphonates CLO and TIL and the nitrogen-containing bisphosphonate PAM were tested on eight neuroblastoma cell lines: CHLA-90, LAN-6, NGP, KCN, SHEP-SF, SK-N-SH, SH-SY5Y and IMR-5. The cell lines differed in their response to these bisphosphonates. Compared with CLO and TIL, the nitrogen-containing bisphosphonate PAM reduced neuroblastoma cell growth more effectively (Fig. 1). In

Fig. 1



Cytoreductive effects of CLO, TIL and PAM on eight neuroblastoma cell lines. The cells were exposed to 50 μM CLO (open bars), TIL (shaded bars) and PAM (striped bars) for 72 h. Cell viability was compared to untreated controls (solid bars).

Table 1 GI_{50} concentrations (μM) of neuroblastoma cell lines exposed to PAM

Cell line	24 h	48 h	72 h	96 h
SK-N-SH	>500	>500	34.2	>500
SHEP-SF	>500	>500	>500	284
NGP[a]	>500	>500	>500	123
KCN[a,b]	>500	>500	43.8	22.8
IMR-5[a,b]	>500	>500	403	285
CHLA-90[b]	>500	>500	12.8	16.5
LAN-6[b]	>500	>500	144	13.0
SH-SY5Y	>500	87.2	42.8	39.5

[a]N-*myc* amplified.
[b]1p deleted.

SK-N-SH and SHEP-SF GI_{50} concentrations below 500 μM were not achieved by 72 h and were not seen in SK-N-SH by 96 h. After 72 h GI_{50} was reached in CHLA-90, LAN-6, SH-SY5Y, IMR5 and KCN, and after 96 h also in SHEP-SF. The GI_{50} concentrations ranged from 12.8 μM in CHLA-90 to 285 μM in IMR5 (Table 1). Fifty percent growth inhibition was first observed after 48 h in SH-SY5Y cells at a concentration of 54 μM PAM.

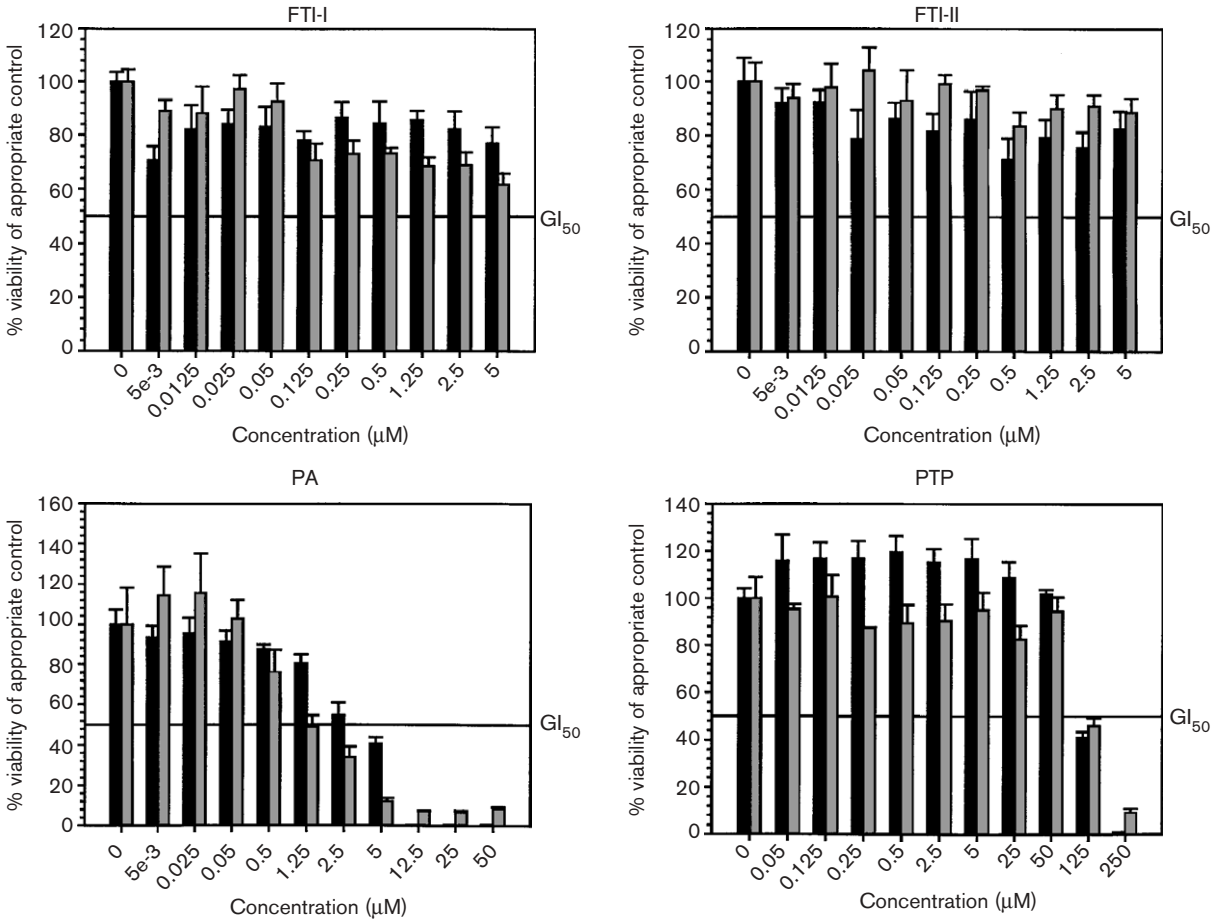
We further studied the effects of IBA, ALE and ZOL in SH-SY5Y and CHLA-90 cells, which were characterized by their good response to PAM. After 72 h, ZOL was the most effective bisphosphonate in CHLA-90 cells followed by ALE. In SH-SY5Y, ALE was the most effective bisphosphonate followed by IBA. In SH-SY5Y, IBA was more potent than PAM, vice versa in CHLA-90 (Table 2).

Table 2 GI_{50} concentrations (μM) of CHLA-90 and SH-SY5Y exposed to bisphosphonates for 72 h

Bisphosphonate	CHLA-90	SH-SY5Y
PAM	12.8	42.8
IBA	19.2	37.8
ALE	9.55	22.4
ZOL	3.97	34.1

Nitrogen-containing bisphosphonates inhibit farnesyl- and geranylpyrophosphate synthase, and thus block the generation of farnesyl- and geranylpyrophosphate, which are required for post-translational prenylation of key regulatory proteins like the small GTPases Ras and Rho. To test whether the interference with protein prenylation contributed to the cytotoxicity of nitrogen-containing bisphosphonates on neuroblastoma cell lines, we examined the effects of the two farnesyltransferase inhibitors FTI-I and FTI-II on SH-SY5Y and CHLA-90. FTI-I inhibits farnesyltransferase with an IC_{50} of 21 nM and FTI-II with an IC_{50} of 50 nM [34]. FTI-I also inhibits the geranyltransferase with an IC_{50} of 760 nM [34]. Within the concentration range tested neither time- nor dose-dependent growth inhibition was observed (Fig. 2). Even at the highest concentrations tested, which were about 100- to 250-fold above the IC_{50} values of FTI-I and FTI-II on farnesyltransferase and about 6-fold above the IC_{50} values of FTI-I on geranyltransferase, growth of CHLA-90 and SH-SY5Y was only reduced by 10–40%. Impairment of protein farnesylation and geranylation, thus, seems unlikely to contribute to the growth inhibition of

Fig. 2



Growth of SH-SY5Y (solid bars) and CHLA-90 (shaded bars) treated with FTI-I, FTI-II, PA or PTP inhibitor for 72 h.

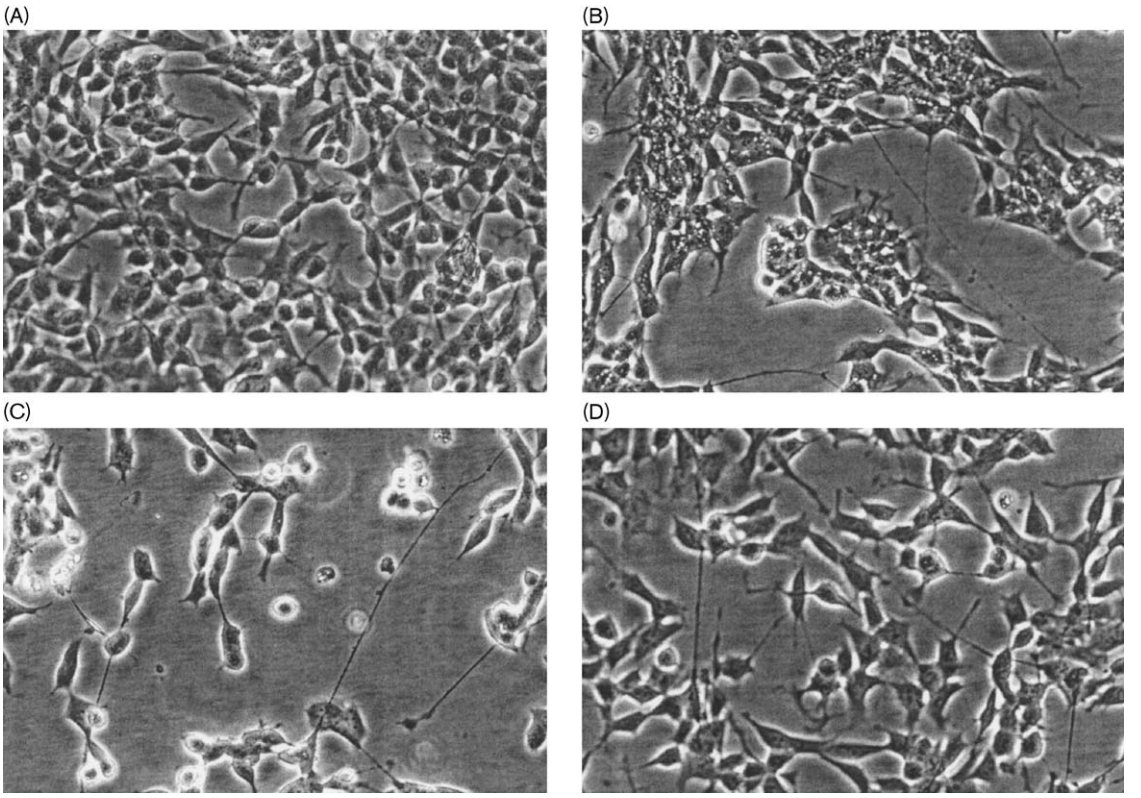
neuroblastoma cell lines by nitrogen-containing bisphosphonates in the first place. PA, which affects the cholesterol synthesis, reduced the viability of SH-SY5Y and CHLA-90 cells at concentrations which inhibited the mevalonate kinase and the mevalonate diphosphate decarboxylase of the mevalonic acid pathway [35,36] (Fig. 2). Bisphosphonates were also reported to inhibit protein tyrosine dephosphorylation [14]. The PTP inhibitor reduced the growth of CHLA-90 and SH-SY5Y cells in a dose- and time-dependent fashion. The GI_{50} concentrations determined after 72 h for CHLA-90 (119 μM) and SH-SY5Y (114 μM) are below the IC_{50} of the PTP inhibitor on the protein tyrosine phosphatase SHP-1 (193 μM) [37].

Under bisphosphonate treatment the neuroblastoma cells displayed signs of differentiation as indicated by neurite outgrowth (Fig. 3). Bisphosphonate treatment also induced apoptosis in neuroblastoma cell lines as determined by annexin staining (Fig. 4).

Discussion

Increased bone absorption by osteoclasts constitutes the primary mechanism of bone degeneration by bone metastases [38–40]. A number of preclinical studies suggest direct antitumor effects of bisphosphonates on multiple myeloma, osteosarcoma, melanoma, breast and prostate cancer cells [41–45]. We examined the antitumor effects of CLO, TIL and PAM on a panel of eight neuroblastoma cell lines, a tumor which frequently metastasizes to the bone. The antiproliferative effects were dependent on the particular bisphosphonate and cell line examined. For the non-nitrogen-containing bisphosphonates CLO and TIL, no sigmoid reduction of cell viability was observed within the concentration range studied, while the nitrogen-containing bisphosphonate PAM inhibited neuroblastoma cell growth in a concentration- and time-dependent manner. Growth inhibition was associated with signs of differentiation and finally resulted in apoptosis. With CLO and TIL the most prominent effects were observed at concentrations

Fig. 3



Morphology of SH-SY5Y in the presence and absence of bisphosphonates. Cultures were treated for 48 h with vehicle (A), 250 μM TIL (B), 250 μM ALE (C) and 250 μM IBA (D). Photographed under phase contrast (× 200).

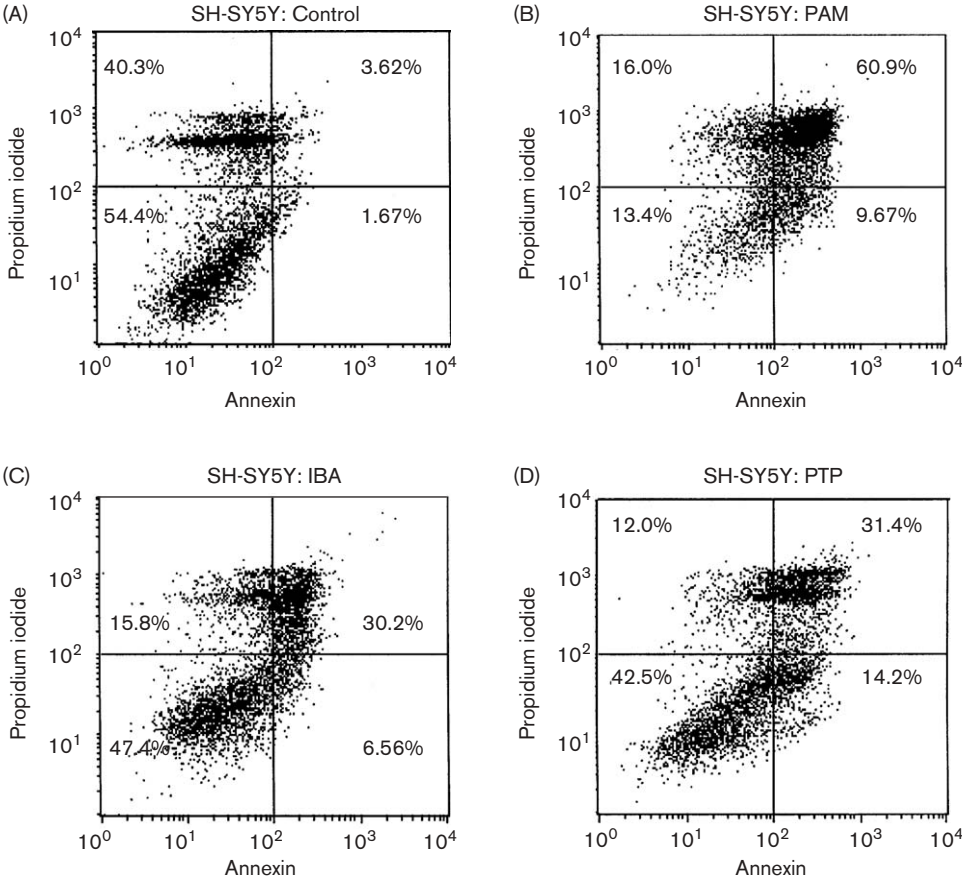
of 500 μM. This concentration is more than 10 times higher than the peak plasma concentrations observed in patients after 3 h infusion of 300 mg CLO [46]. In patients with multiple myeloma, breast and prostate cancer 1-h infusion of 60 mg PAM resulted in peak plasma concentrations of 9.7 ± 3.2 μM (mean ± SD) [47]. This is within the range of the GI_{50} concentration determined for PAM in the most sensitive cell line CHLA-90. After a 15-min infusion of the standard dose of 4 mg, mean peak plasma concentrations of 1 μM ZOL were measured in humans, which is below the GI_{50} concentrations determined for ZOL in CHLA-90 and SH-SY5Y [48]. Bisphosphonates are rapidly cleared from the systemic circulation and mainly distribute to the bone matrix. Dissolution of the bone mineral by tumor cell induced osteoclast activation, however, again releases bisphosphonates and can result in local concentrations of 100 μM or more [49]. For their pharmacokinetics bisphosphonates are thus unlikely to target the primary tumor, but our data do suggest that nitrogen-containing bisphosphonates might directly affect metastasizing neuroblastoma cells.

Subgroups of neuroblastomas are characterized by deletions of chromosome 1p and amplification of the N-*myc* oncogene, which are associated with poor treatment outcome [50]. The cell lines most sensitive to PAM harbored no N-*myc* amplification. Overall there was no association between N-*myc* amplification and sensitivity to PAM. Instead the cell lines with 1p deletion tended to be more sensitive to PAM.

The cytoreductive effects of bisphosphonates did not necessarily reflect their potency in suppressing osteoclast activity as known from osteoporosis therapy [9]. In CHLA-90, ZOL was the most potent bisphosphonate followed by ALE, IBA and PAM, while ALE was more cytotoxic on SH-SY5Y than ZOL. The differences in cytoreductive potency ranged from 1.8 to 4.8. In osteoclasts, ZOL is the most toxic bisphosphonate, followed by IBA, ALE and PAM; it inhibits bone resorption more than 100 times more effectively than PAM [51].

Studies on multiple myeloma, breast and prostate cancer cell lines proposed a number of potential antitumor targets of bisphosphonates. Nitrogen-containing bisphosphonates affected protein prenylation, and thus inhibited proliferation, migration and adhesion, and induced apoptosis in multiple myeloma, breast and prostate

Fig. 4



Annexin-V and propidium iodide staining in SH-SY5Y cells treated with vehicle (A), 125 μM PAM (B), 125 μM IBA (C) and 125 μM PTP III (D) for 96 h.

cancer cells lines [27,42,52]. Nitrogen-containing bisphosphonates impaired protein prenylation by inhibition of the enzyme farnesylpyrophosphate synthase at nanomolar concentrations, with IC_{50} values of 460 nM for ALE and 500 nM for PAM [11]. However, growth inhibition and induction of apoptosis in the neuroblastoma cell lines were not an immediate consequence of impaired protein farnesylation, since both FTI inhibitors failed to induce 50% growth inhibition in CHLA-90 and SH-SY5Y. Farnesylpyrophosphate synthase inhibition also results in reduced cholesterol biosynthesis. Inhibition of the mevalonic acid pathway by PA affected neuroblastoma cell growth at concentrations which inhibited the mevalonate kinase and the mevalonate diphosphate decarboxylase. Affecting the cholesterol biosynthesis by inhibiting farnesylpyrophosphate synthase might thus have contributed to the observed toxicity of nitrogen-containing bisphosphonates on neuroblastoma cell lines.

Phosphatases have a critical function in osteoclast signaling and inhibition of phosphatases induces apoptosis of osteoclasts [14,15]. Nitrogen-containing bisphosphonates inhibited protein tyrosine phosphatases by oxidation of the catalytic cysteine [53]. The IC_{50} values for ALE were 2 μM on PTPε, 0.5–3 μM on PTPσ and 8 μM on CD45. The PTP inhibitor used also blocked the enzyme irreversibly by covalent binding to the SH-group of cysteine in the catalytic domain [37]. The GI_{50} concentrations of the PTP inhibitor on CHLA-90 and SH-SY5Y were close to the IC_{50} values of the inhibitor of protein phosphatase SHP-1. Pathological deregulation of protein tyrosine phosphatases has been associated with cancer development; thus, our own data suggest that apart from farnesylpyrophosphate synthase, increased PTP activity might also be involved in the proliferation of neuroblastomas.

Neuroblastoma is a malignancy of childhood; thus, it is important to consider the safety of bisphosphonates in children. Bisphosphonates are currently used in children with osteopathia (e.g. osteogenesis imperfecta, Paget's disease and juvenile osteoporosis) and calcinosis

(e.g. myositis ossificans), and ALE is approved by the FDA for the treatment Paget's disease and osteogenesis imperfecta in children. A number of studies reported good tolerability of bisphosphonates in children [39,40,54–56]. There are, however, single reports of osteopetrosis in children after prolonged treatment with high doses of bisphophonates [57,58].

Conclusion

Bisphosphonates successfully prevented osteolysis induced by neuroblastoma cells in nude mice [29]. We report direct cytotoxic effects of nitrogen-containing bisphosphonates on the proliferation of neuroblastoma cell lines. The observed GI_{50} concentrations suggest that local nitrogen-containing bisphosphonate concentrations at the bone interface can directly target neuroblastoma cells invading into the bone matrix. In summary, these observations warrant the investigation of adjuvant bisphosphonate treatment in controlled clinical trials.

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