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THE BISPHOSPHONATE ZOLEDRONIC ACID DECREASES TUMOR GROWTH IN BONE IN MICE WITH DEFECTIVE OSTEOCLASTS*

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Abstract

Bisphosphonates (BPs), bone targeted drugs that disrupt osteoclast function, are routinely used to treat complications of bone metastasis. Studies in preclinical models of cancer have shown that BPs reduce skeletal tumor burden and increase survival. Similarly, we observed in the present study that administration of the Nitrogen-containing BP (N-BP), zoledronic acid (ZA) to osteolytic tumor-bearing Tax⁺ mice beginning at 6 months of age led to resolution of radiographic skeletal lesions. N-BPs inhibit farnesyl diphosphate (FPP) synthase, thereby inhibiting protein prenylation and causing cellular toxicity. We found that ZA decreased Tax⁺ tumor and B16 melanoma viability and caused the accumulation of unprenylated Rap1a proteins in vitro. However, it is presently unclear whether N-BPs exert anti-tumor effects in bone independent of inhibition of osteoclast (OC) function in vivo. Therefore, we evaluated the impact of treatment with ZA on B16 melanoma bone tumor burden in irradiated mice transplanted with splenic cells from *src*^{-/-} mice, which have non-functioning OCs. OC-defective mice treated with ZA demonstrated a significant 88% decrease in tumor growth in bone compared to vehicle-treated OC-defective mice. These data support an osteoclast-independent role for N-BP therapy in bone metastasis.

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INTRODUCTION

Bone metastases are a significant cause of morbidity and mortality, resulting in severe bone pain, pathologic fractures, nerve compression syndrome, and hypercalcemia [1]. Tumor cells that infiltrate the bone marrow cavity can directly and indirectly enhance osteoclast-mediated bone resorption by secreting growth factors such as parathyroid hormone (PTH), PTH-related peptide, interleukin-1 (IL-1), IL-6, IL-8, IL-11, macrophage colony-stimulating factor (M-CSF), and receptor activator of NF κ B ligand (RANKL) [2-5]. Bone resorption can lead to the release of a number of bone-derived growth factors, such as transforming growth factor β (TGF β), insulin-like growth factor 1 (IGF-1) and calcium, which in turn may stimulate tumor growth in the marrow cavity [6,7]. Enhanced tumor growth can stimulate the continued release of osteoclastogenic factors, resulting in further bone destruction and tumor bone invasion, a process termed the “vicious cycle” [1,2,8-10].

BPs have been widely used to decrease osteoclast activity in the setting of osteoporosis and bone metastases [11]. All BPs contain two phosphonate groups, which contribute to their specific binding affinity for mineralized bone [11]. The N-BPs, such as ZA, are the most potent class of BPs, and function by inhibiting the activity of FPP synthase [12-14]. This results in a depletion of the FPP and GGPP isoprenoid lipids required for posttranslational lipid modification (prenylation) of proteins [15]. Protein prenylation is essential for the correct function of small guanosine triphosphatases (GTPases). N-BPs thus interfere with the function of GTPases such as Ras, Rac, and Rho, leading to disruption of the actin cytoskeleton, altered trafficking of intracellular components, and impaired integrin signaling within the OC [16]. N-BPs also decrease the number of macrophages, the precursors of OCs, in vitro [17]. In addition, there is evidence that N-BPs inhibit the differentiation of monocytes/macrophages into OCs by increasing production of osteoprotegerin by osteoblasts [18]. Osteoprotegerin binds to RANKL, a key mediator of osteoclastogenesis, and prevents RANKL from promoting macrophage differentiation into OCs [19].

Studies in a number of mouse models have shown that treatment with N-BPs can inhibit skeletal metastasis and reduce tumor burden in bone [2,20-22]. An important question in the field has been whether the anti-tumor action of N-BPs is due only to the direct antagonism of OCs or to the prevention of tumor growth by effects on other cell types. To answer this question, in our current studies, we examined whether weekly treatment of 6 month old tumor-bearing Tax⁺ mice with ZA was able to cause a resolution of skeletal lesions and to reduce soft-tissue tumor burden. Furthermore, to explore whether N-BPs have anti-tumor effects independent of their anti-resorptive action, the effect of N-BP treatment in an intra-arterial tumor injection model using mice with genetically defective OCs (*src*^{-/-}) [23,24] was investigated. We found that irradiated mice transplanted with bone marrow cells from *src*^{-/-} mice, then given tumor and treated with ZA, had an 88% decrease in tumor growth in bone compared to vehicle-treated *src*^{-/-} mice. These data strongly suggest an OC-independent role for N-BP therapy in bone metastasis.

EXPERIMENTAL PROCEDURES

Cells

B16-FL (B16 firefly luciferase-labeled C57BL/6 murine melanoma) cells have been previously described [25]. The TGN-CBR cell line was derived from a subcutaneous tumor that spontaneously developed from a Tax⁺ interferon-gamma null mouse [26]. A single cell suspension was prepared and cultured in 10% RPMI-1640 containing 20% fetal bovine serum (FBS). A single cell clone was selected and termed Tax⁺ interferon-gamma null (TGN), and this cell line grew continuously in vitro in RPMI-1640 media containing 10% FBS and was passaged weekly. TGN cells were stably transduced with the retroviral vector pWZL-blast

containing Click beetle red (CBR)[27] and selected in 2.5 µg/ml blastocidin (Sigma-Aldrich, St Louis, MO) for 1 week.

Cell viability (MTT) assay

5×10³ cells per well were plated in a 96-well plate in quadruplicate for each condition and allowed to adhere overnight. Cells were incubated with vehicle or drug for 72h in fresh media. 10 µl of 5 mg/ml MTT solution was added to each well. Cells were incubated for an additional 4h. The reaction was stopped with 150 µl of isopropyl alcohol/hydrochloric acid mixture and the plate was read at 570 nm and 630 nm. Values are plotted as A570-A630.

In vitro protein prenylation studies

10⁶ B16-FL or TGN-CBR cells per well were plated in 6-well plates. Cells were treated with ZA and lysed at 24 or 48h with lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 10 mM NaF, 0.2 M NaPP_i, 1 mM Na₃VO₄, 1% Triton X, and 1x protease inhibitors (complete protease inhibitor cocktail tablets, Roche, Mannheim, Germany)). 50 µg protein/sample was electrophoresed and transferred to a polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were incubated with a goat polyclonal anti-Rap1A antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that specifically recognizes the unprenylated form of the small GTPase Rap1A [28,29], and rabbit polyclonal anti-β-actin (Sigma, St Louis, MO) as loading control. This was followed by incubation with alexafluor 680-conjugated donkey-anti-goat IgG (Molecular Probes, Leiden, The Netherlands) and IRDye 800-conjugated donkey-anti-rabbit IgG (Rockland Immunochemicals Inc., Gilbertsville, PA), and detection on a LI-COR Infrared Odyssey Imager (LI-COR Biosciences UK Ltd., Cambridge, UK).

Animals and ZA dosing

Immunocompetent C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN). Transgenic mice expressing HTLV-1 Tax under the human granzyme B promoter (Tax⁺ C57B6/SJL) have been previously described [26]. *Src*^{-/-} heterozygous breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under pathogen-free conditions according to the guidelines of the Division of Comparative Medicine and the animal ethics committee of Washington University School of Medicine approved all experiments. ZA (generously provided as the hydrated disodium salt by Novartis Pharma AG, Basel, Switzerland) was administered subcutaneously at a dose of 0.75 µg per mouse per week (approximately 30 µg/kg) or phosphate-buffered saline (PBS) starting at 6 months of age in tumor bearing Tax⁺ mice. This dosing schedule of ZA was designed to produce drug levels similar to those achieved with the clinical dosing regimen of 4 mg Zometa[®] for the treatment of bone metastases. Transplanted animals (see below) were given two 0.75 µg doses (ten and three days prior to tumor cell injection) of ZA or PBS.

In vivo protein prenylation studies

Soft-tissue tumors from Tax⁺ transgenic mice were isolated from the ear, tail, hip and/or foot 48h and 72h after a single injection with 0.75 µg ZA. Tumors were lysed in RIPA buffer using a Precellys homogeniser (Bertin Technologies, Montigny-le Bretonneux, France) and analyzed for the presence of unprenylated Rap1A using Western blotting, as described above.

Hematopoietic transplantation

Five-week-old C57BL/6 mice were lethally irradiated using a ¹³⁷Cs source with 900 rads. Spleen (from *src*^{-/-} mice) or bone marrow (from wildtype (WT) littermates) was harvested from 6-week-old mice. Splenic cells were used from *src*^{-/-} mice because the osteopetrotic phenotype prevents successful bone marrow cell preparation. Single cell suspensions of hematopoietic

cells were prepared in PBS and 10^6 cells in 200 μ l were injected into the lateral tail vein of recipient irradiated mice to generate *src*^{-/-} radiation chimeras and WT controls.

Serum CTX assay

CTX in serum from fasting mice was measured from WT or *src*^{-/-} radiation chimeras at 3 weeks post-transplantation, prior to treatment with ZA or PBS and at 6 weeks post-transplantation, following treatment with either ZA or PBS using a CTX ELISA system (Nordic Bioscience Diagnostics, Herlev, Denmark).

Osteoclast formation assays

Whole bone marrow was extracted from femurs and tibias of transplanted C57BL/6 mice and cells were plated at a concentration of 5×10^5 per ml in CMG-14-12 supernatant (1/10 vol) in α -MEM media containing 10% FBS in petri dishes to generate primary bone marrow macrophages (BMM). BMM were lifted and plated at a concentration of 5×10^5 per ml in quadruplicate in 48-well dishes. Cells were fed every day with α -MEM containing 10% FBS, CMG-14-12 supernatant (1/20 vol) and GST-RANKL (100 ng/ml) and incubated at 37°C in 5% CO₂, 95% room air for five days to generate OCs [30]. TRAP staining was performed according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). A quantitative TRAP solution assay (modified from Tintut et al.) [31] was performed by adding a colorimetric substrate, 5.5 mM *p*-nitrophenyl phosphate, in the presence of 10 mM sodium tartrate at pH 4.5. The reaction product was quantified by measuring optical absorbance at 405 nm.

Image acquisition

Images of cells and isolated tibias were taken at room temperature with a Nikon Eclipse TE300 inverted microscope (Nikon, Japan) connected to a Magnafire camera model S99802 (Optronics, Goleta, CA). The stretch intensity tool in the Magnafire 2.1c software was used to sharpen all images. The 4x Nikon lens with numerical aperture 0.13 or 10x Nikon lens with numerical aperture 0.30 were used.

Radiographs

Animals were placed in the prone position and exposed to 20 KVP for 15 seconds using an X-ray System (Faxitron Corp, Buffalo Grove, IL). Osteolytic lesions were defined as areas of increased radiolucency disrupting bone marrow contour and invading into bone cortex.

Left ventricle (LV or intra-arterial) bone metastasis model

For intra-arterial injections, operators were blinded to genotype. Mice were anesthetized and inoculated intra-arterially with 10^5 B16-FL cells in 50 μ l PBS as previously described [25, 32]. Tumor growth was monitored by in vivo bioluminescence imaging on days 8 and 10 following B16-FL tumor injections.

In vivo bioluminescence imaging and analysis

Imaging was performed using a charge coupled device (CCD) camera (IVIS 100, Xenogen, Alameda, CA) as described [25]. Mice were shaved to minimize attenuation of light by pigmented hair. On the days indicated, mice were injected intraperitoneally with 150 mg/kg of D-luciferin (Biosynth, Naperville, IL) in PBS ten minutes prior to imaging. Mice were then anesthetized using isoflurane and imaged (exposure time one or five minutes, binning 8, FOV 15cm, f/stop 1, no filter). For analysis, total photon flux (photons/sec) was measured from a fixed region-of-interest (ROI) in the bones or whole body using Living Image 2.50 and IgorPro software (Wavemetrics, Portland, OR).

Bone histomorphometry

Mouse tibiae were fixed in formalin and decalcified in 14% EDTA. Paraffin embedded sections were stained with hematoxylin and eosin, and separately for tartrate resistant acid phosphatase (TRAP). Trabecular bone area, OC perimeter, and tumor area were measured according to a standard protocol using Bioquant Osteo V7 10.10 (Bioquant Image Analysis Corporation, Nashville TN) [33]. Bone sections were blinded prior to analysis.

Calcein labeling and calculation of bone formation rate

At six and eleven days following the final dose of ZA or PBS, mice were injected with a 20mg/kg dose of calcein (Sigma-Aldrich, St. Louis MO) in a 2% sodium bicarbonate solution to assess bone formation. Two days following the final calcein injection, mice were sacrificed and calvaria were harvested, stored in 70% EtOH, embedded in methyl methacrylate, and sectioned. An evaluation of bone growth was made on undecalcified calvaria. This labeling allowed for evaluation of mineral apposition rate (MAR) per day, bone surface (BS), single labeled surface (sLS) and double labeled surface (dLS). These measurements were then used to calculate bone formation rate (BFR/BS) per year as previously described[34]. Both the concave and convex surfaces of each bone were evaluated separately using a fluorescence microscope (Olympus BX51, Center Valley, PA). Measurements and calculations were all performed by a single researcher using image analysis software, Image-Pro Plus 5.0 (Media Cybernetics, Bethesda, MD).

Statistical analysis

All experiments were analyzed using Student's t-test, or one-way ANOVA in the case of experiments with greater than three experimental groups. In calculating two-tailed significance levels for equality of means, equal variances were assumed for the two populations.

RESULTS

Sensitivity of tumor cells to ZA treatment in vitro

We evaluated the sensitivity of osteolytic tumor cell lines, TGN-CBR cells, derived from a Tax⁺ mouse tumor, and B16-FL murine melanoma cells, to ZA treatment in vitro. All doses of ZA tested (10, 50, and 100 μ M) significantly decreased both TGN-CBR and B16-FL tumor cell viability at 72h of treatment as assessed by an MTT assay (Fig 1A and 1C). This was confirmed by visual inspection of the cells (Fig 1B and 1D). N-BPs such as ZA act by inhibiting the mevalonate pathway enzyme FPP synthase, leading to inhibition of prenylation of small GTPase proteins such as Ras, Rac, Rho, and Rab. We examined the effect of ZA treatment on inhibition of protein prenylation in our tumor cell lines by detecting the unprenylated form of the small GTPase Rap1A using Western blotting. We detected accumulation of unprenylated Rap1A (uRap1A) in B16-FL cells following treatment with 5 μ M ZA for 24h, and in TGN-CBR cells in response to 10 μ M ZA (Fig 1E; unprenylated Rap1A shown in red). These data demonstrate that ZA can directly decrease viability and inhibit protein prenylation of TGN-CBR and B16-FL tumor cells in vitro from a concentration of 10 μ M.

Resolution of osteolytic lesions but not soft-tissue tumors in Tax⁺ mice treated with ZA

We previously reported that ZA prevented the development of osteolytic bone tumors and soft tissue tumors in the Tax⁺ transgenic mice [30]. Here, we investigated the effect of ZA on established soft tissue and bone tumors in tumor bearing Tax⁺ transgenic mice. Osteolytic tumor-bearing 6-month-old Tax⁺ mice were randomized to receive weekly subcutaneous injections of 0.75 μ g ZA or PBS. X-rays and tumor measurements were obtained at six months of age (baseline) and again at nine months to assess progression of disease. While PBS-injected mice continued to develop new osteolytic lesions, ZA-treated animals had no new bone lesions

and furthermore demonstrated regression of lesions that had been present at the start of treatment (Fig 2A). Histology showed significant tumor infiltration with OC recruitment in the vertebrae of PBS-treated animals while ZA-treated animals had little detectable tumor and virtually no OC recruitment (Fig 2B). We did not observe a statistically significant difference in size or number of pre-existing soft tissue tumors in mice treated with ZA or PBS, with treatment initiated at 6 months of age (data not shown). We analyzed soft-tissue tumors from ZA treated mice for the presence of the unprenylated form of the small GTPase Rap1A, which acts as a marker for intracellular uptake of N-BP and inhibition of its major pharmacological target, FPP synthase. Following a single injection (0.75 μ g) of ZA, soft-tissue tumors from Tax⁺ mice were isolated 48h and 72h post-injection and tumor homogenates were analyzed for unprenylated Rap1A using Western blotting (Fig 2C). While the positive controls (tumor cells treated with ZA *in vitro*) clearly showed accumulation of unprenylated Rap1A, consistent with the results shown in Fig 1E, no unprenylated Rap1A could be detected in the tumors at either 48h or 72h post-ZA. Thus, in tumor-bearing Tax⁺ mice, treatment with ZA prevented the development of new osteolytic lesions and induced resolution of existing osteolytic tumors in bone. However, no significant decrease in soft-tissue tumor burden was detected, and we were unable to detect any unprenylated Rap1A in these tumors following a single injection with ZA.

Src^{-/-} radiation chimeras have dysfunctional osteoclasts

To determine if N-BPs such as ZA could have anti-tumor effects independent of its effects on osteoclasts, we evaluated the effect of ZA treatment on tumor burden in mice with defective OCs. *Src^{-/-}* mice have been shown to have severely defective OCs and are osteopetrotic [23]. *Src^{-/-}* mice have very limited marrow space, which makes histologic assessment of tumor burden difficult. They also have enhanced function of osteoblasts, which could alter tumor growth [35]. We therefore transplanted lethally irradiated wild-type (WT) recipients with *src^{-/-}* bone marrow to generate mice with defective OCs, a normal marrow space and functional osteoblast lineage cells of host (*src^{+/+}*) origin [36,37]. Recent data suggests that bone microarchitecture changes that occur post-irradiation and transplant tend toward the donor phenotype, demonstrating that the bone marrow microenvironment is reconstituted and established by the transplanted cells [38]. We compared *src^{-/-}* marrow recipients to lethally irradiated WT-transplanted control mice. The drastic differences in marrow space seen in X-rays of *src^{-/-}* mice compared to WT [39] were not evident in the radiation chimeras, indicating that the *src^{-/-}* radiation chimeras have comparable marrow space to the WT radiation chimeras (Fig 3A). Additionally, marrow content looked similar in both *src^{-/-}* and WT radiation chimeras, indicating equivalent engraftment (Fig 3B). Further more, *src^{-/-}* OCs did not respond *in vivo* to ZA treatment compared to WT OC; we measured levels of serum CTX, an *in vivo* marker of OC bone resorption, in WT and *src^{-/-}* radiation chimeras, both pre- and post-treatment. *Src^{-/-}* radiation chimeras had significantly lower levels of serum CTX compared to WT radiation chimeras (Fig 3C). To further assess OC function in the radiation chimeras, equal numbers of bone marrow macrophages (BMM) harvested from WT and *src^{-/-}* radiation chimeras were plated in M-CSF and RANKL to generate OCs. OCs derived from *src^{-/-}* radiation chimeras demonstrated the expected defects in spreading (Fig 3D). OCs generated from *src^{-/-}* radiation chimera BMM also produced decreased levels of the OC-specific protein, TRAP, indicative of impaired function (Fig 3E). We investigated the bone formation and mineral apposition rates of ZA and PBS-treated *src^{-/-}* and WT radiation chimeras to determine whether osteoblast function was affected by the transplantation genotype or treatment group. There was no significant difference between the growth rate of WT and *src^{-/-}* radiation chimeras with ZA or PBS (Fig S1A, B).

ZA treatment results in decreased tumor burden in mice with genetically defective osteoclasts

Src^{-/-} and WT radiation chimeras were randomized to receive either ZA or PBS control at ten and three days prior to tumor cell inoculation (Fig 4). 10⁵ B16-FL murine melanoma cells were injected into the arterial system via the left cardiac ventricle to allow B16-FL cells to disseminate to bone and visceral organs. Tumor burden was measured in real time at days eight and ten post-tumor cell injection by in vivo bioluminescence imaging to monitor the occurrence of bone metastasis. *Src*^{-/-} radiation chimeras treated with ZA had a greater than five-fold decrease in bone tumor burden compared to *src*^{-/-} PBS-injected animals as measured by bioluminescence imaging (Fig 5A,B). This was confirmed by histomorphometric analysis of the bones, showing that *src*^{-/-} radiation chimeras treated with ZA had an approximate 88% decrease in tumor area compared to *src*^{-/-} PBS-injected animals (Fig 5C). WT radiation chimeras treated with ZA had an approximate 68% decrease in tumor area compared to WT PBS-injected animals (Fig 5D). Additionally, *src*^{-/-} radiation chimeras had decreased tumor burden in bone when compared to WT radiation chimeras (Fig 5C,D). We have previously observed decreases in bone tumor burden in the OC defective OPG mouse model [25] as well as in non-transplanted *src*^{-/-} mice compared to WT littermates (data not shown). Taken together, these data suggest that ZA could alter tumor burden in bone independent of its effects on OCs.

DISCUSSION

In the present study, we found that treatment of osteoclast-defective *src*^{-/-} mice with ZA can lead to a reduction in tumor burden in bone by both in vivo bioluminescence imaging and histologic analysis of bone tumor burden. These data strongly suggest that ZA can have effects on tumor growth in bone in vivo, independent of its effects on functioning OCs.

Previous studies have demonstrated that N-BP treatment can reduce tumor burden in the bone microenvironment [20-22]. A recent report by Zheng *et al.* demonstrated that treatment with either the N-BP, ibandronate, or the osteoclastogenesis inhibitor, osteoprotegerin, resulted in similar anti-tumor effects in a mouse model of breast cancer bone metastasis [40], suggesting that ibandronate exerts its anti-tumor effects indirectly via inhibition of osteoclastic bone resorption in a manner similar to osteoprotegerin. However, reduction in tumor burden at non-osseous sites in some animal models suggests that N-BPs (in which BP was generally administered at higher doses than those used clinically) may have other anti-tumor properties [30,41,42].

There are several explanations for the decreased bone tumor burden after ZA administration in the OC defective *src*^{-/-} radiation chimeras. ZA may antagonize endothelial cell function, as has been observed in vitro and in vivo using other N-BPs [42-45]. Also, N-BPs can be directly toxic to tumor cells in vitro [17]. We found that ZA reduced cell viability of B16-FL and TGN-CBR tumor cells in vitro. Whether N-BPs directly affect tumor cells, or any other non-OC cells such as endothelial cells, in vivo is highly dependent on the local concentrations of N-BP available, which are presently still unknown.

Following an intravenous infusion of ZOL in cancer patients with bone metastasis, the peak plasma concentration of ZOL is approximately 1 μ M [46]. However, due to the unique bone-binding pharmacology of BPs, it is difficult to translate these circulating concentrations into concentrations that may be present locally in the bone marrow. Once bound to bone, BP is considered to be inert, and only bone-resorbing OCs can efficiently release the BP from the bone surface during the resorption process [47], although small amounts of BP are also thought to be released from bone as a result of natural desorption [48]. It is possible that in the presence of high OC activity, more BP will be released from the bone and hence higher concentrations

of free BP may be present in the bone marrow and thus available for uptake by other cells. In *in vitro* systems, enhanced uptake of BP by macrophages has been observed when cultured on BP-precoated dentine in the presence of resorbing OCs, compared to culturing on BP-precoated dentine in the absence of OCs [47]. However, there is at present no direct evidence that high OC activity *in vivo* results in increased BP concentrations in the bone marrow and enhanced uptake by other cell types. Our findings presented here suggest that OC activity is not required for the anti-tumor activity of BPs *in vivo*, suggesting that the release of BP from the bone surface during resorption may not be a relevant factor in the anti-tumor activity of BPs in animal models.

Due to their potent inhibitory effect on FPP synthase, a ubiquitous effect of N-BPs on any cell type (provided the drug is internalised in sufficient quantities) is an inhibition of protein prenylation, and a resulting accumulation of unprenylated proteins within the cell [17]. Indeed we detected an accumulation of unprenylated Rap1A in tumor cells treated with ZA *in vitro*. However, we were unable to detect an accumulation of unprenylated Rap1A in soft-tissue tumors of Tax+ mice following a single administration of 0.75 µg ZA. Although this finding does not support a direct effect of ZA on the soft-tissue tumors *in vivo*, it should be noted that there are limitations. First, a detection of unprenylated Rap1A, although the most sensitive method we currently have to detect a direct effect of N-BPs such as ZA on tumor cells and other cells, may not be sensitive enough to detect subtle effects on prenylation. In this respect, it is important to note that while *in vitro* unprenylated Rap1A was detected after treatment with 5-10 µM ZA for 24 hours, previous studies have shown that inhibition of tumor cell adhesion can be detected after treatment with approximately 1 µM *in vitro* [49,50], and an inhibition of tumor cell invasion at nanomolar, or even picomolar concentrations [51,52]. Yet these effects on tumor cell adhesion and invasion could largely be prevented by replenishing cells with geranylgeraniol, suggesting that they are mediated by an inhibition of protein geranylgeranylation [52,53]. Secondly, a single injection may not be sufficient to affect cells at extraskeletal sites, but repeated exposure to low circulating levels of ZA with repeated dosing may exert direct effects on the tumors. This notion is supported by a recent study showing that a repetitive ZA dosing regimen was more effective at reducing tumor burden in an animal model of breast cancer metastasis as compared to a single injection, even though the total cumulative dose was the same [22].

Herein, we used a spontaneous model of tumor osteolysis to demonstrate that clinically relevant doses of ZA led to a resolution of skeletal lesions. Furthermore, in an intra-arterial tumor injection model, we demonstrated that ZA reduced tumor burden even in osteoclast-defective *src*^{-/-} mice. These data support an anti-tumor role of N-BP therapy independent of its effects on osteoclasts and suggest that this class of pharmacologic agents may have a clinically beneficial role in decreasing tumor burden in bone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

PTH	parathyroid hormone
IL-1	interleukin-1
IL-6	interleukin-6
IL-11	interleukin-11
M-CSF	macrophage colony-stimulating factor
RANKL	receptor activator of NFκB ligand
TGF-β	transforming growth factor β
IGF-1	insulin-like growth factor
FPP	farnesyl diphosphate
GGPP	geranylgeranyl diphosphate
FL	firefly luciferase
TGN	Tax+ interferon-gamma null
FBS	fetal bovine serum
CBR	click-beetle red
PBS	phosphate-buffered saline
CTX	C-terminus of collagen I
BMM	bone marrow macrophages
CCD	charge-coupled device

ROI	region of interest
TRAP	tartrate resistant acid phosphatase
BMD	bone mineral density
N-BP	nitrogen bisphosphonates
OC	osteoclast
WT	wild-type
h	hour

Fig. 1.

Sensitivity of TGN-CBR and B16-FL tumor cells to ZA treatment *in vitro*. To determine the effect of ZA on cell viability, 5×10^3 TGN-CBR or B16-FL cells were plated in quadruplicate for each condition in a 96-well plate. Cells were allowed to adhere; cell culture media was then replaced with media containing vehicle or increasing concentrations of ZA. Seventy-two hours later, phase-contrast microscopic images were taken and an MTT assay was performed to assess cell viability. A. All doses of ZA led to a significant decrease in TGN-CBR cell viability ($p=0.001$, $p=0.01$, $p<0.0005$ respectively, two-tailed t-test). B. Representative images show decrease in cell number in response to ZA treatment as compared to control (100x magnification). C. All doses of ZA led to a significant decrease in B16-FL cell viability ($p=0.02$, $p=0.0005$, $p=0.0001$ respectively, two-tailed t-test). D. Representative images show decrease in cell number in response to ZA treatment as compared to control (100x magnification). E. To detect an inhibition of protein prenylation in response to ZA treatment, B16-FL and TGN-CBR cells were treated with 0-50 μM ZA for 24 hours, and unprenylated Rap1A (shown in

red) and total Rap1 (shown in green) were detected using Western blot analysis. An accumulation of unprenylated Rap1A was clearly detected in response to 10 μ M ZA in both B16-FL and TGN-CBR cells. Blots shown are representative of two experiments. Beta-actin levels were measured as a loading control.

Fig. 2.

ZA treatment of tumor prone Tax⁺ mice from 6 months of age resolves skeletal lesions but has no effect on established soft-tumor lesions. Baseline measurements of tumor-prone Tax⁺ mice were taken at 6 months of age. Mice were then treated weekly with a subcutaneous injection of 0.75 μ g ZA (n=14) or PBS (vehicle) (n=13) for 3 months. Development of lytic lesions was monitored by x-ray analysis. A. PBS-injected mice continued to develop an average of 1.2 new osteolytic lesions per mouse over this time period. ZA-treated animals did not develop new bone lesions and demonstrated regression of 1 lesion per mouse on average ($p=0.007$ two-tailed t-test). B. Representative histology demonstrates significant tumor infiltration (T) in PBS-treated animals with OC recruitment (indicated with arrows), while the bones of ZA-

treated animals are filled with normal marrow (M) and virtually no tumor infiltrate. C. Tumor-bearing Tax+ mice were injected with a single dose of 0.75 μ g ZA or vehicle control, and soft-tissue tumors were isolated 48 h or 72 h post-injection from the ear, tail, hip, or foot from control mice (1 and 5) or ZA treated mice (2-4; 6-8), as indicated. Unprenylated Rap1A (shown in red) and total Rap1 (shown in green) were detected in whole tumors using Western blotting. No unprenylated Rap1A could be detected in any of the tumor samples. +ve ctls; B16-FL tumor cells treated with 5-20 μ M ZA for 24 hours *in vitro*, as indicated.

Fig. 3.

Src^{-/-} radiation chimeras have dysfunctional osteoclasts. A. Representative radiographs of tail vertebrae from wildtype and *src*^{-/-} radiation chimeras demonstrate that both genotypes of radiation chimeras have a marrow compartment similar to a wildtype mouse of a similar age, while *src*^{-/-} mice of a similar age are osteopetrotic. B. Representative histological sections stained with hematoxylin and eosin to show equivalent engraftment in ZA- and PBS-treated *src*^{-/-} and WT radiation chimeras. C. OC activity measured by serum CTX levels 3 weeks pre- and 6 weeks post-transplant. $p < 0.001$, for WT vs. *src*^{-/-} post-PBS treatment, $p < 0.01$, for WT ZA vs. PBS treatment, $p > 0.05$ for *src*^{-/-} ZA pre- and post- treatment, two-tailed t-test; $N = 4$ for each group. D. Equal numbers of bone marrow macrophages harvested from wildtype radiation chimeras or *src*^{-/-} radiation chimeras were plated in the presence of M-CSF and RANKL to generate OCs. Cultured OCs were stained with TRAP. Representative images demonstrate the inability of *src*^{-/-} OCs to properly adhere and spread compared to wildtype OCs. E. *Src*^{-/-} OCs had decreased levels of TRAP activity by an in vitro TRAP solution assay at all time points examined ($p = 0.01$ Day 2, $p = 0.001$ Day 3, $p = 0.02$ Day 4, $p < 0.0005$ Day 5, two-tailed t-test).

Fig. 4.

Experimental design. In order to generate *src*^{-/-} animals with a normal amount of marrow space, *src*^{-/-} radiation chimeras were generated. Radiation chimeras were given a 0.75μg dose of ZA or PBS (vehicle) ten and three days prior to tumor cell injection. Mice were injected intra-arterially with 10⁵ B16-FL murine melanoma cells three weeks post-transplantation.

Fig. 5. *Src*^{-/-} radiation chimeric mice treated with ZA are partially protected from bone metastasis. A. *Src*^{-/-} radiation chimeras treated with ZA demonstrated greater than five-fold decrease in tumor burden in bone compared to vehicle-treated mice (p=0.007 Day 8 and p=0.03 Day 10, two-tailed t-test; n=5 *src*^{-/-} PBS, n=5 *src*^{-/-} ZA) by in vivo bioluminescence imaging. B. Representative images of tumor burden by bioluminescence imaging are depicted here. C. Histomorphometric analysis confirmed the decreased tumor burden seen in the *src*^{-/-} radiation chimeras treated with ZA (p=0.03, two-tailed t-test; n=5 *src*^{-/-} PBS, n=5 *src*^{-/-} ZA). Representative histology depicted (M=marrow, T=tumor). D. Histomorphometric analysis demonstrated an approximate 68% decrease in tumor burden in wildtype radiation chimeras treated with ZA (p=0.02, two-tailed t-test; n=5 wildtype PBS, n=5 wildtype ZA). Representative histology depicted (M=marrow, T=tumor)