Published in final edited form as:

J Surg Res. 2013 June 15; 182(2): 339–346. doi:10.1016/j.jss.2012.10.033.

Differential Effect of Zoledronic Acid on Human Vascular Smooth Muscle Cells

Hassan Albadawi¹, Mounir J. Haurani¹, Rahmi Oklu², Jordan P. Trubiano¹, Peter J. Laub¹, Hyung-Jin Yoo¹, and Michael T. Watkins^{1,*}

¹Division of Vascular and Endovascular Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA

²Division of Vascular Interventional Radiology, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Boston, MA

Abstract

Introduction—The activation of human vascular smooth muscle cell proliferation, adhesion and migration is essential for intimal hyperplasia formation. These experiments were designed to test whether Zoledronic Acid (ZA) would modulate indices of human smooth muscle cell activation, exert differential effects on proliferating vs. quiescent cells and determine whether these effects were dependent on GTPase binding proteins prenylation. ZA was chosen for testing in these experiments because it is clinically used in humans with cancer, and has been shown to modulate rat smooth muscle cell proliferation and migration.

Methods—Human aortic smooth muscle cells (HASMC) were cultured under either proliferating or growth arrest (quiescent) conditions in the presence or absence of ZA for 48 hours, whereupon the effect of ZA on HASMC proliferation, cellular viability, metabolic activity and membrane integrity were compared. In addition, the effect of ZA on adhesion and migration were assessed in proliferating cells. The effect of increased concentration of ZA on the mevalonate pathway and genomic/cellular stress related poly ADP Ribose polymerase (PARP) enzyme activity were assessed using the relative prenylation of Rap-1A/B protein and the formation of poly ADP-ribosylated proteins (PAR) respectively.

Results—There was a dose dependent inhibition of cellular proliferation, adhesion and migration following ZA treatment. ZA treatment decreased indices of cellular viability and significantly increased membrane injury in proliferating vs. quiescent cells. This was correlated with the appearance of unprenylated Rap-1A protein and dose dependent down regulation of PARP activity.

Conclusions—These data suggest that ZA is effective in inhibiting HASMC proliferation, adhesion and migration which coincide with the appearance of unprenylated RAP-1A/B protein, thereby suggesting that the mevalonate pathway may play a role in the inhibition of HASMC activation.

© 2012 Elsevier Inc. All rights reserved.

*To Whom Correspondence is Addressed: Michael T. Watkins, M.D., 15 Parkman Street, Suite 440, Boston, MA 02114, Phone: 617-726-0908, Fax: 617-726-2560, mtwatkins@partners.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

The incidence of peripheral vascular disease (PVD) continues to increase among our aging population as the risk factors such as diabetes, obesity and hyperlipidemia continue to rise (1). The development of surgical and endovascular based therapies for PVD has been life-saving with increased limb-salvage and decreased disability and represents an important achievement in medicine (2, 3). Despite massive global research efforts, including the development of adjunctive therapies and mechanical techniques, 30–40% of patients develop restenosis within 3 to 24 months of intervention (4). The major processes involved in the development of restenosis are complex and include responses to injury and inflammation (5).

Animal models have shown that bisphosphonates (BP), which are typically used to treat conditions associated with excessive bone resorption, may play an inhibitory role in the development of atherosclerosis and neointimal hyperplasia (6–9). There are also reports of marked BP accumulation in both the healthy aorta and atherosclerotic aorta in rabbits (10, 11). Zoledronic acid (ZA), which is the most potent member of the nitrogen containing BP (12), is currently used in the treatment of osteoporosis and it is being tested in the treatment of bone metastasis in clinical trials (13–15). Recent *in vitro* studies have demonstrated ZA to inhibit proliferation, adhesion and migration of vascular smooth muscle cells derived from rats (16). However, a similar role in human cells has not been shown. These experiments were performed because the effects of drugs on animal tissue do not always correlate with similar effects on human tissue (17–19).

The aim of the present study was to verify whether ZA would sustain an inhibitory effect on activated human vascular smooth muscle cell proliferation, adhesion and migration, which are essential components in the pathogenesis of atherosclerosis and intimal hyperplasia following vascular injury in humans. Experiments were also designed to determine whether ZA exerts distinct effects on growth induced proliferating HASMC viability, metabolic and stress related activities compared to non-induced quiescent cells. BPs are known to modulate the prenylation of GTPase binding proteins of the Ras superfamily, which play a role in several cellular activities including adhesion, growth and survival (20, 21). Therefore, we investigated whether ZA treatment would alter the posttranslational modification of selected members of the Ras superfamily GTPase binding proteins. Additionally, we tested the effect of ZA on PARP enzyme activity, which is an important modulator of cellular stress and smooth muscle cell cellular phenotypic alteration, proliferation and inflammation (5, 22–24).

Materials and Methods

Cell Culture

Human aortic smooth muscle cells (HASMC; Invitrogen Co, Carlsbad, CA, passage 6–7) were serially grown in Medium-231, smooth muscle growth supplement (Invitrogen Co, Carlsbad, CA) containing 100 units/ml penicillin, 0.01 mg/ml streptomycin, 0.25 µg/ml amphotericin-B, 5% fetal bovine serum, recombinant human basic fibroblast growth factor, recombinant human epidermal growth factor and insulin (Growth Media, GM). For experiments performed under growth arrest conditions, cells were exposed to quiescent medium (QM) consisting of medium-231 containing 0.5% fetal bovine serum for 48 hours. Zoledronic acid (Novartis, Basel, Switzerland) was diluted in the either growth medium or quiescent medium prior to each experiment to concentrations of 1, 10 and 100 µM and added to cells in wells or tissue culture flasks.

Cellular Proliferation

HASMCs cultured in 6-well plates (1×10^5 cells per well) were subjected to growth arrest using quiescent medium (QM). After an initial 48 hour period, some of the wells were supplemented with 1, 10 or $100 \mu\text{M}$ of ZA. In separate wells, medium was exchanged to Growth Medium (GM, for proliferating cells) and similar concentrations of ZA were added. The control group was identical to the experimental group except that ZA was not added. After 48 hours incubation, cells were suspended using trypsin/EDTA mixture wherein viable cells were counted with hemocytometer using trypan blue exclusion. Data was expressed as average cells per well $\times 10^6 \pm \text{SEM}$. Each analysis was repeated six times.

Cellular Adhesion

To determine the effect of ZA on cellular adhesion, sub-confluent HASMCs were divided into 1.0×10^4 HASMC aliquots and incubated in GM media containing 1, 10 or $100 \mu\text{M}$ ZA for 30 minutes. Cells were subsequently seeded on to type I collagen coated 24 well plates (BD Biosciences, Bedford, MA) containing the same type of media and incubated for 30 minutes at 37°C in a 5% CO_2 tissue culture incubator. Wells without ZA served as controls for the experiment. Following the 30 minutes incubation period, the wells were rinsed vigorously 3 times with PBS, and the remaining cells in the well were stained using 0.1% crystal violet dye dissolved in ethyl alcohol and counted in six fields in each eight independent wells, as previously described(16). Data were expressed as adherent cells per high power field (hpf).

Cellular Migration

Cellular migration was measured using a 24 transwell tissue culture plates (Corning Corporation, Corning, NY). HASMCs were suspended by 0.05% Trypsin-EDTA in serum free medium supplemented with 0.2% BSA containing 0, 1, 10 or $100 \mu\text{M}$ ZA. Cell suspensions were placed into the upper well at a concentration of 10^4 cells/ $100 \mu\text{l}$. The designated lower compartments were filled with $600 \mu\text{l}$ total volume of GM containing 0, 1, 10 or $100 \mu\text{M}$ ZA each. Following four hours incubation the non-migrating cells on the upper surface were scraped and washed out three times with PBS. Migrating cells that made it through the membrane on the lower surface were fixed and stained with 0.1% crystal violet dye in ethanol and counted in eight independent high power microscopic fields. Each data point was repeated 8 times.

Assessment of viability, metabolic activity and cellular membrane integrity

HASMCs were cultured in 96 solid black wells plates with clear flat bottom at 4000 cells/well density in GM media (Invitrogen, Carlsbad, CA). At 50–60% confluence, cells were subjected to growth arrest for 48 hours using the QM. Following 48 hours, the growth media was exchanged for either GM or QM supplemented with 1, 10 or $100 \mu\text{M}$ ZA for 48 hours. The control group cells received no ZA treatment. Cellular viability was assessed using an analysis of mitochondrial reducing enzyme activities using the WST-1 assay kit (Roche Diagnostics, Indianapolis, IN). In this assay, mitochondrial reducing activity is reduced in direct relationship to decreases in cellular viability. Metabolic activity was determined by measuring the cellular steady state levels of ATP using the ATPlite assay (PerkinElmer, Boston, MA). To measure cellular membrane integrity (MI), the CytoTox-Fluor cytotoxicity assay reagent kit (Promega, Madison, WI) was used. In the CytoTox-Fluor assay, membrane injury is associated with increased detection of dead-cell protease activity released from cells that have lost MI. All kits were used according to their manufacturer's instructions. The assays were repeated 6–8 times and presented as the average percent control (i.e. incubation without ZA in either growth or quiescent medium) $\pm \text{SD}$.

Effect of Geranylgeraniol (GGOH) replacement on viability and metabolic activity

At a 50–60% confluence HASMCs in 96 well plates in GM media were incubated with 1, 10 or 100 μM ZA with and without 25 μM geranylgeraniol (GGOH) an intermediate of the mevalonate pathway (Sigma-Aldrich, Saint Louis, MO) for 48 hours (25, 26). Following 48 hours, the cellular viability (WST-1 assay) and metabolic activity (ATPlite assay) was measured as described above.

Immunoblotting for Rap-1AB prenylation and PARP activity

Cells were rinsed with ice cold PBS, scraped off tissue culture plates then extracted with lysis buffer containing 20 mM Tris, pH 7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM sodium pyrophosphate, 20mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate and protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO) then cleared by centrifugation at 10,000xg for 10 minutes at 4°C. The protein concentration of cell lysates was measured with the bicinchoninic acid protein assay system (Pierce Biotechnology, IL). Twenty micrograms aliquots of cleared protein extracts were separated on a 12% SDS-polyacrylamide gel then transferred to a nitrocellulose membrane (BioRad, Hercules, CA). After blocking with 5% BSA 0.1% Tween-20 TBS solution, membranes were incubated over night at 4°C with primary antibodies that either recognizes the prenylated and unprenylated Rap1A/B protein (Cell Signaling, Danvers, MA) or PAR (Tulip Biolabs, Inc. West Point, PA) followed by incubation with secondary HRP-conjugated antibody. Membranes were developed with the enhanced chemiluminescence detection system (GE, Healthcare, Piscataway, NJ). Chemiluminescence band densities were visualized and quantified using FluorChem HD2 gel-imaging system (ProteinSimple, Santa Clara, CA). The blots were stripped and re-hybridized using β -Actin IgG (Abcam, Cambridge, MA). Data was normalized to β -Actin band densities.

Statistical Analysis

The data are presented as mean $\pm$ SEM. Statistical analyses were performed using the Prism software (Version 3, Graph Pad, San Diego, CA). Comparisons within the same group were performed using a one-way ANOVA with Tukey-Kramer Multiple Comparison Post tests and two-way ANOVA with Bonferroni post tests for absolute data between quiescent and proliferating cells. Student's t test with Welch Correction was used to compare between data points in the quiescent vs proliferating cells when the data was analyzed as percent control, with $P < 0.05$ considered statistically significant.

Results

Zoledronic acid inhibits HASMC proliferation, cellular adhesion and migration

We observed a significant decrease in the level of HASMC proliferation following the treatment of these cells for 48 hours with 1, 10 and 100 μM ZA under stimulated conditions the presence of GM (control: 0.4 ± 0.02 vs. 10 μM : $0.27 \pm 0.004 \times 10^6$ cells, $p < 0.05$; 100 μM : $0.13 \pm 0.008 \times 10^6$ cells, $p < 0.001$, Figure 1). In contrast, ZA treatment had no effect on proliferation in the unstimulated quiescent cell group (Quiescent control: $0.201 \pm 0.017 \times 10^6$ cells, 1 μM : $0.198 \pm 0.017 \times 10^6$ cells, 10 μM : $0.195 \pm 0.019 \times 10^6$ cells and 100 μM : $0.166 \pm 0.009 \times 10^6$ cells, Figure 1). To examine cellular adhesion and migration, proliferating cells were treated in identical ZA concentrations. ZA treatment in all concentrations resulted in a significant dose dependent inhibition of cellular adhesion (Control: 45.33 ± 1.63 vs. 1 μM : 27.86 ± 1.11 cells/hpf, 10 μM : 18.11 ± 1.56 cells/hpf and 100 μM : 9.28 ± 1.28 cells/hpf, $p < 0.001$, Figure 2). When compared to control proliferative conditions, ZA treatment demonstrated significant decrease in cellular migration only at 10 and 100 μM concentrations (Control: 23.7 ± 2.1 vs. 10 μM : 14.0 ± 0.8 , and 100 μM : 10.0 ± 0.8 cells/hpf,

$p < 0.001$, Figure 3). There was no significant difference in cellular migration between control and $1\mu\text{M}$ ZA, or $10\mu\text{M}$ and $100\mu\text{M}$ ZA treatments.

Zoledronic acid treatment reduces viability, metabolic activity and compromises membrane integrity in proliferating HASMC

Cellular viability as measured by reductase enzyme activity was no different between quiescent and proliferating cells at the $1\mu\text{M}$ concentration of ZA (Table I). In contrast, at 10 and $100\mu\text{M}$ concentration ZA significantly decreased cell viability in proliferating rather than quiescent cells. ATP content was greater in proliferating vs quiescent cells at the $1\mu\text{M}$ dose of ZA. At 10 and $100\mu\text{M}$ concentration of ZA, there was no difference between ATP content in proliferating and quiescent cells. MI was marked increased in proliferating cells at all concentrations of ZA.

Effect of ZA on PARP activity and PARP-1 Expression

There was significantly higher relative PARP activity at 10 and $100\mu\text{M}$ ZA treated quiescent cells compared to the proliferating cells (Quiescent $1\mu\text{M}$: 159.56 ± 31.74 vs Proliferating $1\mu\text{M}$: 102.83 ± 28.55 percent untreated control, $p = \text{ns}$, Quiescent $10\mu\text{M}$: 211.32 ± 45.09 vs. Proliferating 94.78 ± 25.74 percent untreated control, $p = 0.015$ and Quiescent $100\mu\text{M}$: 278.31 ± 61.98 vs. Proliferating $100\mu\text{M}$: 47.79 ± 12.3 , percent proliferating control, $p = 0.002$, Figure 4). To determine whether the observed changes in PARP enzyme activity was associated with alteration in the steady state expression of the major PARP isoform, i.e. PARP-1, immunoblotting for the total and fractionated PARP-1 protein were performed. This analysis confirmed significantly lower PARP-1 protein expression at 10 and $100\mu\text{M}$ ZA concentrations in the proliferating cells compared to the corresponding quiescent cells group (Quiescent $1\mu\text{M}$ 105.14 ± 13.84 vs. Proliferating $1\mu\text{M}$ 85.44 ± 12.36 $p = \text{ns}$, Quiescent $10\mu\text{M}$ 109.04 ± 15.56 vs. Proliferating $10\mu\text{M}$ 57.32 ± 13.51 $p = 0.016$, and Quiescent $100\mu\text{M}$; 96.50 ± 10.31 vs. Proliferating $100\mu\text{M}$ 24.67 ± 5.70 percent control, $p = 0.0001$, Figure 5).

Normalization of cellular viability and metabolic activity in ZA treated proliferating cells by Geranylgeraniol (GGOH) substrate

HASMC proliferation, viability and ATP were measured in proliferating cells treated with 1 , 10 and $100\mu\text{M}$ concentrations of ZA with and without $25\mu\text{M}$ GGOH. GGOH replacement led to significant increase in cellular viability and metabolic activity to baseline control levels under all ZA concentrations (Table 2). At the 10 and 100 , but not $1\mu\text{M}$ ZA concentration, GGOH did alter cell counts as compared to untreated cells. These data suggest that the observed effects of ZA on cellular integrity and metabolic activity are mediated in part by the mevalonate pathway activity.

Zoledronic Acid Inhibits Protein Prenylation of the GTPase binding protein Rap1A/B

To understand the mechanism of GGOH rescue of cellular viability and metabolic activity in proliferating cells, the prenylation status of Rap1A/B, which is a member of the GTPase binding super family of proteins known to modulate cellular activity was assessed using immunoblotting. This experimental protocol tested the hypothesis that ZA treatment inhibits Rap1A/B prenylation and that GGOH can lead to reversal of this inhibition. ZA treatment inhibited Rap-1A/B prenylation in proliferating cells at both 10 and $100\mu\text{M}$ concentrations, while quiescent cells showed minimal to undetectable inhibition of prenylation (Figure 6A). In contrast, incubating the ZA treated proliferating cells with $25\mu\text{M}$ GGOH led to loss of detection of the unprenylated Rap1A/B fraction. This data suggests that ZA may modulate the mevalonate pathway by inhibiting prenylation of selected members of the GTPase binding proteins.

Discussion

We hypothesized that ZA would inhibit growth stimulated HASMC proliferation, adhesion and migration and would exert distinct effects on proliferation, cellular viability, metabolic activity and membrane integrity on stimulated cells compared to quiescent cells. Our data suggests that treatment with ZA leads to incremental, dose dependent inhibition of HASMC proliferation, adhesion and migration within the growth-stimulated group (Figures 1–3). The concentrations used in this study are similar to tissue levels achieved in human clinical trials (14, 27) and in vitro studies with animal cells (16, 26). These data are consistent with the reports of Wu and colleagues who studied the effect of ZA on rat smooth muscle cells(16). These experiments were performed because the effects of drugs on animal tissue do not always correlate with similar effects on human tissue(17–19). ZA did not alter cellular proliferation in quiescent HASMC at any concentration studied (Figure 1). Because the number of cells was so low in the quiescent cultures, quantitative analysis of the effect of ZA on cellular migration and adhesion was not meaningful and therefore not reported. This effect of ZA on proliferating cells is consistent with in vitro studies of rat smooth muscle cell function(16) and animal models of atherosclerosis(7) or neointimal hyperplasia(6). Recent data seems to suggest that smooth muscle cell migration may be more important than proliferation in the pathogenesis of intimal hyperplasia(28), however it is clear that ZA treatment modulates both processes.

When evaluating HASMCs cellular viability (Table 1), both proliferating and quiescent cells showed a consistent decrease in viability when treated with ZA. The effect of ZA on proliferating cells was greatest at the 10 and 100 μ M concentration. This preferential effect on proliferating vs. quiescent cells is a necessary and essential therapeutic component of ZA treatment to prevent injury to normal unstimulated smooth muscle cells *in vivo*.

Quiescent and proliferating cells exposed to ZA had lower levels of ATP as compared to non treated HASMC at all concentrations studied (Table 1). These observations are consistent with prior reports on osteoclasts and cancer cells, which suggest that ZA may block mitochondrial ADP/ATP translocase by inducing the formation of an ATP analogue that complexes AMP with isopentenyl pyrophosphate (29–32). For all concentrations except 1 μ M ZA, there was no significant difference in the steady state level of ATP in proliferating vs. quiescent cells.

Surprisingly, the steady state levels of ATP were higher in proliferating vs. quiescent cells only at the 1 μ M concentration. This may mean that at low ZA concentrations in growth stimulated conditions, there are cellular mechanisms which can compensate for the effect of blocking either mitochondrial ADP/ATP translocase or formation of the ATP analogue mentioned earlier.

Despite the overall decrease in cellular ATP content in both quiescent and proliferating cells exposed to ZA, significant compromise of cellular membrane integrity was detected only in the proliferating smooth muscle cells (Table 1). Thus the trend of decreased cellular ATP content in quiescent vs. proliferating cells does not seem to have had a detrimental effect on cellular membrane integrity in the quiescent cells.

To shed light on why ZA treatment might preferentially modulate proliferation and compromise cellular membrane integrity in proliferating cells vs. quiescent cells, experiments were designed to evaluate the expression of PARP enzyme activity and protein expression which plays an essential role in a number of cellular functions including DNA repair and apoptosis. Depending on the degree of PARP activation, PARP can be cytoprotective or cytotoxic(33). The primary member of the PARP family, PARP-1, plays a major role in cellular proliferation and differentiation and the inflammatory process in

smooth muscle cells (34–38). Poly ADP-ribosylation is a form of posttranslational modification of proteins that involves the addition of one or more ADP-ribose moieties by the PARP enzymes that lead to altered function of the acceptor proteins (39, 40). PARP inhibition was previously reported to ameliorate intimal hyperplasia in a rat model of arterial injury (41). In the current experiments, ZA treatment resulted in divergent effect on PARP activity in quiescent and proliferating cells as measured by changes in the expression of Poly ADP ribosylation (PAR, Figure 4). While PAR expression was markedly increased with ZA treatment in the quiescent cells there was a significantly less cellular poly-ADP-ribosylation in the proliferating cells at the 10 and 100 μ M concentrations which correlated by decreased the expression of PARP-1 enzyme (Figure 5). This suggests that ZA treatment prevented PARP activation in proliferating cells and preserved it in quiescent cells. Thus the ability of quiescent cells to resist cytotoxicity associated with ZA treatment may be related to their ability to increase PARP-1 expression, whereas the proliferating cells did not. There was no detectable cleaved PARP-1 to indicate an ongoing apoptosis during 48 hours of ZA treatment. This data is in agreement with the studies by Kabista et. Al, which indicated that ZA inhibited cellular proliferation but failed to induce apoptosis in cultured cholangiocarcinoma cells(26). Another possible explanation might be related to the timing of the endpoint analysis, i.e. PARP-1 cleavage may have happened before cell harvest for immunoblotting at the 48 hour time point.

To determine whether ZA modulates cellular viability by altering the activity of the mevalonate pathway, experiments were performed whereupon the GGOH substrate (an important mediator of mevalonate activity) was administered to ZA treated HASMC. The GGOH substrate significantly reversed ZA mediated alteration in cellular viability (Table 2) and ATP content (Table 2). This is consistent with reports on the effect of *in vitro* ZA treatment with without GGOH on proliferation of prostatic tumor lines(42) and differentiation of human endothelial cell progenitor cells(43).

While it was important to note that GGOH substrate is capable of reversing the effects of ZA on cellular viability and ATP content, strong evidence to support a specific effect of ZA on the mevalonate pathway requires inhibition of RAP1A/B prenylation. ZA treatment did unequivocally inhibit Rap1A/B prenylation in proliferating cells (Figure 6A). Inhibition of Rap1A/B prenylation in proliferating HASMC was successfully reversed by administering the GGOH substrate thereby providing strong evidence that ZA inhibits prenylation of GTPase binding proteins. Similar data were also reported in cancer cells as a consequence of prenylation inhibition, which results in the disruption of signal transduction pathways that regulate proliferation, adhesion and migration (25, 44, 45).

In conclusion, these experimental results support the concept that the bisphosphonate ZA, may play a role in modulating the activity of stimulated human smooth muscle cells while sparing the quiescent cells within the human vessel wall. These findings suggest that the pathways involved in the ZA response may represent attractive targets for the therapy of vascular injury such as atherosclerosis and restenosis, as they would also likely have less detrimental effects on quiescent cells. Consistent with prior reports on the effect of ZA in normal undiseased animal models of vascular injury(46), ZA will likely play an inhibitory role in neointimal hyperplasia. As there have been reports of toxic systemic effects of ZA on renal function(14, 27), it is likely that ZA may work when delivered locally to tissues at risk for intimal hyperplasia, possibly through drug eluting stents, similar to techniques where ZA has been bound to orthopedic implants(47, 48). The ZA effect on vascular smooth muscle cells appear to be mediated by interfering with the mevalonate pathway. The mevalonate pathway and specifically the prenylation of GTPase binding proteins of the Ras superfamily are known to be key players in a variety of cellular activities such as cell adhesion, growth and survival (20, 21). ZA interference of the mevalonate pathway is consistent with multiple

prior reports which have shown that the Ras superfamily contributes to the development of vascular disease.

Acknowledgments

Funding: National Institutes of Health (1R01AR055843)

The authors acknowledge funding from the National Institutes of Health (1R01AR055843), the American Diabetes Association, the Pacific Vascular Research Foundation and the Department of Surgery, Division of Vascular and Endovascular Surgery, Massachusetts General Hospital (The Geneen Fund). Dr. Watkins is the Ronnie Isenberg Scholar in Academic Surgery at the Massachusetts General Hospital.

References

1. Cizek SM, Bedri S, Talusan P, Silva N, Lee H, Stone JR. Risk factors for atherosclerosis and the development of preatherosclerotic intimal hyperplasia. *Cardiovasc Pathol*. 2007; 16:344–350. [PubMed: 18005873]
2. McCann AB, Jaff MR. Treatment strategies for peripheral artery disease. *Expert Opin Pharmacother*. 2009; 10:1571–1586. [PubMed: 19527186]
3. Kiernan TJ, Yan BP, Cruz-Gonzalez I, Cubeddu RJ, Caldera A, Kiernan GD, Gupta V. Pharmacological and cellular therapies to prevent restenosis after percutaneous transluminal angioplasty and stenting. *Cardiovasc Hematol Agents Med Chem*. 2008; 6:116–124. [PubMed: 18473776]
4. Minar E, Pokrajac B, Maca T, Ahmadi R, Fellner C, Mittlbock M, Seitz W, Wolfram R, Potter R. Endovascular brachytherapy for prophylaxis of restenosis after femoropopliteal angioplasty: results of a prospective randomized study. *Circulation*. 2000; 102:2694–2699. [PubMed: 11094034]
5. Mitra AK, Gangahar DM, Agrawal DK. Cellular, molecular and immunological mechanisms in the pathophysiology of vein graft intimal hyperplasia. *Immunol Cell Biol*. 2006; 84:115–124. [PubMed: 16519729]
6. Danenberg HD, Fishbein I, Gao J, Monkkonen J, Reich R, Gati I, Moerman E, Golomb G. Macrophage depletion by clodronate-containing liposomes reduces neointimal formation after balloon injury in rats and rabbits. *Circulation*. 2002; 106:599–605. [PubMed: 12147543]
7. Ylitalo R, Oksala O, Yla-Herttuala S, Ylitalo P. Effects of clodronate (dichloromethylene bisphosphonate) on the development of experimental atherosclerosis in rabbits. *J Lab Clin Med*. 1994; 123:769–776. [PubMed: 8195683]
8. Ylitalo R, Syvala H, Tuohimaa P, Ylitalo P. Suppression of immunoreactive macrophages in atheromatous lesions of rabbits by clodronate. *Pharmacology & Toxicology*. 2002; 90:139–143. [PubMed: 12071335]
9. Cohen-Sela E, Dangoor D, Epstein H, Gati I, Danenberg HD, Golomb G, Gao J. Nanospheres of a bisphosphonate attenuate intimal hyperplasia. *Journal of Nanoscience & Nanotechnology*. 2006; 6:3226–3234. [PubMed: 17048541]
10. Ylitalo R, Monkkonen J, Urtti A, Ylitalo P. Accumulation of bisphosphonates in the aorta and some other tissues of healthy and atherosclerotic rabbits. *J Lab Clin Med*. 1996; 127:200–206. [PubMed: 8636649]
11. Ylitalo R, Kalliovalkama J, Wu X, Kankaanranta H, Salenius JP, Sisto T, Lahteenmaki T, Ylitalo P, Porsti I. Accumulation of bisphosphonates in human artery and their effects on human and rat arterial function in vitro. *Pharmacol Toxicol*. 1998; 83:125–131. [PubMed: 9783331]
12. Green JR. Chemical and biological prerequisites for novel bisphosphonate molecules: results of comparative preclinical studies. *Semin Oncol*. 2001; 28:4–10. [PubMed: 11346859]
13. Doggrell SA. Clinical efficacy and safety of zoledronic acid in prostate and breast cancer. *Expert Rev Anticancer Ther*. 2009; 9:1211–1218. [PubMed: 19761424]
14. Berenson JR, Vescio R, Henick K, Nishikubo C, Rettig M, Swift RA, Conde F, Von Teichert JM. A Phase I, open label, dose ranging trial of intravenous bolus zoledronic acid, a novel bisphosphonate, in cancer patients with metastatic bone disease. *Cancer*. 2001; 91:144–154. [PubMed: 11148571]

15. Vincenzi B, Santini D, Dicuonzo G, Battistoni F, Gavasci M, La Cesa A, Grilli C, Virzi V, Gasparro S, Rocci L, Tonini G. Zoledronic acid-related angiogenesis modifications and survival in advanced breast cancer patients. *Journal of Interferon & Cytokine Research*. 2005; 25:144–151. [PubMed: 15767788]
16. Wu L, Zhu L, Shi WH, Zhang J, Ma D, Yu B. Zoledronate inhibits the proliferation, adhesion and migration of vascular smooth muscle cells. *Eur J Pharmacol*. 2009; 602:124–131. [PubMed: 19000670]
17. Hodkinson DJ, de Groote C, McKie S, Deakin JF, Williams SR. Differential Effects of Anaesthesia on the pHMRI Response to Acute Ketamine Challenge. *Br J Med Med Res*. 2012; 2:373–385. [PubMed: 22737655]
18. Mantovani M, Dooley DJ, Weyerbrock A, Jackisch R, Feuerstein TJ. Differential inhibitory effects of drugs acting at the noradrenaline and 5-hydroxytryptamine transporters in rat and human neocortical synaptosomes. *Br J Pharmacol*. 2009; 158:1848–1856. [PubMed: 19912224]
19. Sordi V, Bianchi G, Buracchi C, Mercalli A, Marchesi F, D'Amico G, Yang CH, Luini W, Vecchi A, Mantovani A, Allavena P, Piemonti L. Differential effects of immunosuppressive drugs on chemokine receptor CCR7 in human monocyte-derived dendritic cells: selective upregulation by rapamycin. *Transplantation*. 2006; 82:826–834. [PubMed: 17006331]
20. Khosravi-Far R, Cox AD, Kato K, Der CJ. Protein prenylation: key to ras function and cancer intervention? *Cell Growth Differ*. 1992; 3:461–469. [PubMed: 1419908]
21. Cox AD, Der CJ. Protein prenylation: more than just glue? *Curr Opin Cell Biol*. 1992; 4:1008–1016. [PubMed: 1485954]
22. Li L, Blumenthal DK, Terry CM, He Y, Carlson ML, Cheung AK. PDGF-induced proliferation in human arterial and venous smooth muscle cells: molecular basis for differential effects of PDGF isoforms. *J Cell Biochem*. 112:289–298. [PubMed: 21069732]
23. Jia G, Mitra AK, Gangahar DM, Agrawal DK. Insulin-like growth factor-1 induces phosphorylation of PI3K-Akt/PKB to potentiate proliferation of smooth muscle cells in human saphenous vein. *Exp Mol Pathol*. 89:20–26. [PubMed: 20471974]
24. Hata JA, Petrofski JA, Schroder JN, Williams ML, Timberlake SH, Pippen A, Corwin MT, Solan AK, Jakoi A, Gehrig TR, Kontos CD, Milano CA. Modulation of phosphatidylinositol 3-kinase signaling reduces intimal hyperplasia in aortocoronary saphenous vein grafts. *J Thorac Cardiovasc Surg*. 2005; 129:1405–1413. [PubMed: 15942585]
25. Kubista B, Trieb K, Sevela F, Toma C, Arrich F, Heffeter P, Elbling L, Sutterluty H, Scotlandi K, Kotz R, Micksche M, Berger W. Anticancer effects of zoledronic acid against human osteosarcoma cells. *J Orthop Res*. 2006; 24:1145–1152. [PubMed: 16602111]
26. Romani AA, Desenzani S, Morganti MM, La Monica S, Borghetti AF, Soliani P. Zoledronic acid determines S-phase arrest but fails to induce apoptosis in cholangiocarcinoma cells. *Biochem Pharmacol*. 2009; 78:133–141. [PubMed: 19464430]
27. Reid IR, Brown JP, Burckhardt P, Horowitz Z, Richardson P, Trechsel U, Widmer A, Devogelaer JP, Kaufman JM, Jaeger P, Body JJ, Brandi ML, Broell J, Di Micco R, Genazzani AR, Felsenberg D, Happ J, Hooper MJ, Ittner J, Leb G, Mallmin H, Murray T, Ortolani S, Rubinacci A, Saaf M, Samsioe G, Verbruggen L, Meunier PJ. Intravenous zoledronic acid in postmenopausal women with low bone mineral density. *N Engl J Med*. 2002; 346:653–661. [PubMed: 11870242]
28. Tigerstedt NM, Savolainen-Peltonen H, Lehti S, Hayry P. Vascular cell kinetics in response to intimal injury ex vivo. *J Vasc Res*. 47:35–44. [PubMed: 19672106]
29. Monkkonen H, Auriola S, Lehenkari P, Kellinsalmi M, Hassinen IE, Vepsäläinen J, Monkkonen J. A new endogenous ATP analog (Apppl) inhibits the mitochondrial adenine nucleotide translocase (ANT) and is responsible for the apoptosis induced by nitrogen-containing bisphosphonates. *Br J Pharmacol*. 2006; 147:437–445. [PubMed: 16402039]
30. Mitrofan LM, Pelkonen J, Monkkonen J. The level of ATP analog and isopentenyl pyrophosphate correlates with zoledronic acid-induced apoptosis in cancer cells in vitro. *Bone*. 2009; 45:1153–1160. [PubMed: 19699819]
31. Raikkonen J, Crockett JC, Rogers MJ, Monkkonen H, Auriola S, Monkkonen J. Zoledronic acid induces formation of a pro-apoptotic ATP analogue and isopentenyl pyrophosphate in osteoclasts in vivo and in MCF-7 cells in vitro. *Br J Pharmacol*. 2009; 157:427–435. [PubMed: 19371349]

32. Monkkonen H, Kuokkanen J, Holen I, Evans A, Lefley DV, Jauhiainen M, Auriola S, Monkkonen J. Bisphosphonate-induced ATP analog formation and its effect on inhibition of cancer cell growth. *Anticancer Drugs*. 2008; 19:391–399. [PubMed: 18454049]
33. Graziani G, Szabo C. Clinical perspectives of PARP inhibitors. *Pharmacol Res*. 2005; 52:109–118. [PubMed: 15911339]
34. Erdelyi K, Bakondi E, Gergely P, Szabo C, Virag L. Pathophysiologic role of oxidative stress-induced poly(ADP-ribose) polymerase-1 activation: focus on cell death and transcriptional regulation. *Cell Mol Life Sci*. 2005; 62:751–759. [PubMed: 15868400]
35. Zerfaoui M, Suzuki Y, Naura AS, Hans CP, Nichols C, Boulares AH. Nuclear translocation of p65 NF-kappaB is sufficient for VCAM-1, but not ICAM-1, expression in TNF-stimulated smooth muscle cells: Differential requirement for PARP-1 expression and interaction. *Cell Signal*. 2008; 20:186–194. [PubMed: 17993261]
36. Dantzer F, Nasheuer HP, Vonesch JL, de Murcia G, Menissier-de Murcia J. Functional association of poly(ADP-ribose) polymerase with DNA polymerase alpha-primase complex: a link between DNA strand break detection and DNA replication. *Nucleic Acids Res*. 1998; 26:1891–1898. [PubMed: 9518481]
37. Simbulan-Rosenthal CM, Rosenthal DS, Hilz H, Hickey R, Malkas L, Applegren N, Wu Y, Bers G, Smulson ME. The expression of poly(ADP-ribose) polymerase during differentiation-linked DNA replication reveals that it is a component of the multiprotein DNA replication complex. *Biochemistry*. 1996; 35:11622–11633. [PubMed: 8794742]
38. Simbulan-Rosenthal CM, Rosenthal DS, Boulares AH, Hickey RJ, Malkas LH, Coll JM, Smulson ME. Regulation of the expression or recruitment of components of the DNA synthesome by poly(ADP-ribose) polymerase. *Biochemistry*. 1998; 37:9363–9370. [PubMed: 9649317]
39. Belenky P, Bogan KL, Brenner C. NAD⁺ metabolism in health and disease. *Trends Biochem Sci*. 2007; 32:12–19. [PubMed: 17161604]
40. Ziegler M. New functions of a long-known molecule. Emerging roles of NAD in cellular signaling. *Eur J Biochem*. 2000; 267:1550–1564. [PubMed: 10712584]
41. Zhang C, Yang J, Jennings LK. Attenuation of neointima formation through the inhibition of DNA repair enzyme PARP-1 in balloon-injured rat carotid artery. *Am J Physiol Heart Circ Physiol*. 2004; 287:H659–666. [PubMed: 15044192]
42. Goffinet M, Thoulouzan M, Pradines A, Lajoie-Mazenc I, Weinbaum C, Faye JC, Seronie-Vivien S. Zoledronic acid treatment impairs protein geranyl-geranylation for biological effects in prostatic cells. *BMC Cancer*. 2006; 6:60. [PubMed: 16539721]
43. Yamada J, Tsuno NH, Kitayama J, Tsuchiya T, Yoneyama S, Asakage M, Okaji Y, Shuno Y, Nishikawa T, Tanaka J, Takahashi K, Nagawa H. Anti-angiogenic property of zoledronic acid by inhibition of endothelial progenitor cell differentiation. *J Surg Res*. 2009; 151:115–120. [PubMed: 18619615]
44. Tanimori Y, Tsubaki M, Yamazoe Y, Satou T, Itoh T, Kidera Y, Yanae M, Yamamoto C, Kaneko J, Nishida S. Nitrogen-containing bisphosphonate, YM529/ONO-5920, inhibits tumor metastasis in mouse melanoma through suppression of the Rho/ROCK pathway. *Clin Exp Metastasis*. 2007; 27:529–538. [PubMed: 20632074]
45. Hamada M, Miki T, Iwai S, Shimizu H, Yura Y. Involvement of RhoA and RalB in geranylgeranyltransferase I inhibitor-mediated inhibition of proliferation and migration of human oral squamous cell carcinoma cells. *Cancer Chemother Pharmacol*.
46. Wu L, Zhu L, Shi WH, Yu B, Cai D. Zoledronate inhibits intimal hyperplasia in balloon-injured rat carotid artery. *Eur J Vasc Endovasc Surg*. 2001; 41:288–293. [PubMed: 21123096]
47. Roshan-Ghias A, Arnoldi J, Procter P, Pioletti DP. In vivo assessment of local effects after application of bone screws delivering bisphosphonates into a compromised cancellous bone site. *Clin Biomech (Bristol, Avon)*. 2006; 26:1039–1043.
48. Stadelmann VA, Terrier A, Gauthier O, Boulter JM, Pioletti DP. Prediction of bone density around orthopedic implants delivering bisphosphonate. *J Biomech*. 2009; 42:1206–1211. [PubMed: 19380139]

Figure 1. Effect of Zoledronic Acid (ZA) on Human Smooth Muscle Cell Proliferation

There was a dose response inhibition of cellular proliferation with ZA treatment in the proliferating cells group compared to control (*P<0.05). ZA did not affect cellular density in the treated quiescent wells compared to quiescent control. Data are presented as the mean of six experiments $\pm$ SEM.

Figure 2. Effect of Zoledronic Acid (ZA) on Human Smooth Muscle Cell Adhesion

ZA treatment inhibited HASMC adhesion 30 minutes after seeding compared to control in a dose dependant manner. The data represents the mean adherent cell counts from six independent wells for each condition $\pm$ SEM. * indicates significant differences compared to control. +, ++ indicates significant difference from 1 or 10µM ZA.

Figure 3. Effect of ZA on HASMC Migration

There was a significant dose dependent effect on proliferating HASMC migration with 10 and 100 μ M ZA compared to control. Results represent the average of six independent chambers each one was counted in eight standardized fields and plotted as mean cell number $\pm$ SEM, * $P < 0.001$ vs. control.

Figure 4. PARP Activity in Proliferating and Quiescent HASMC

PARP activity was not different with 1 μM ZA concentration between the two groups. In contrast there was a significantly higher PARP activity with 10 and 100 μM ZA treatment in the quiescent cells compared to the proliferating cells ($\pm$ SD, * $p < 0.05$, ** $p < 0.01$ respectively).

Figure 5. PARP-1 Expression in Proliferating and Quiescent HASMC

There was no change in the total uncleaved PARP-1 enzyme in the quiescent cells using any ZA concentration. In contrast, there was a significantly lower uncleaved PARP-1 expression with 10 and 100 μM ZA in the proliferating cells compared to the same concentrations in the quiescent group ($\pm$ SD, * $p < 0.05$, ** $P < 0.001$ respectively).

Figure 6. Effect of Zoledronic Acid on prenylation of RAP-1A/B

Panel A shows a representative western blotting for the effect of ZA on the prenylated 20kDa and the unprenylated 23kDa Rap-1A/B protein in quiescent and proliferating HASMC. The 23kDa unprenylated Rap1A/B protein fraction appeared only in the proliferating HASMC at 10 and 100 μ M concentrations. Panel B demonstrates the disappearance of the 23kDa unprenylated Rap1A/B protein in the proliferating cells that were treated with ZA and 25 μ M GGOH.

Table 1

	1 μ M ZA		10 μ M ZA		100 μ M ZA	
	Quiescent	Proliferating	Quiescent	Proliferating	Quiescent	Proliferating
Percent Control						
Viability	66.5 $\pm$ 14.4	64.8 $\pm$ 29.3	51.3 $\pm$ 14.4	26.1 $\pm$ 6.2 [*]	53.5 $\pm$ 19.7	22.9 $\pm$ 8.8 [*]
ATP	53.4 $\pm$ 7.9	76.4 $\pm$ 10.7 ⁺	49 $\pm$ 4.5	53.9 $\pm$ 7.9	36.6 $\pm$ 8.2	36.8 $\pm$ 7.6
Membrane Integrity	99.9 $\pm$ 25.4	128.6 $\pm$ 48.8 ^{**}	73.9 $\pm$ 29.9	205.5 $\pm$ 108.1 ^{**}	122 $\pm$ 46.9	195.6 $\pm$ 72.3 ⁺⁺

 $\pm$ SD,^{*} p<0.01 vs quiescent,⁺ p<0.001 vs quiescent,^{**} p<0.02,⁺⁺ p<0.05 vs quiescent

Table 2

Effect of Geranylgeraniol (GGOH) substrate on HASMC Function

	1 μ M ZA		10 μ M ZA		100 μ M ZA	
	untreated	25 μ M GGOH	untreated	25 μ M GGOH	untreated	25 μ M GGOH
Percent Control						
Viability	86.1 $\pm$ 21.1	110.3 $\pm$ 11.7 [*]	65.9 $\pm$ 26.3	97.6 $\pm$ 17.1 [*]	69.7 $\pm$ 20.9	108.7 $\pm$ 33.1 [*]
ATP	76.4 $\pm$ 10.6	105.3 $\pm$ 20.7 ^{**}	54 $\pm$ 7.2	107.4 $\pm$ 15.6 ^{**}	37 $\pm$ 7.3	89.29 $\pm$ 10.7 ^{**}
Cell Count	78.03 $\pm$ 9.3	78.85 $\pm$ 7.7	65.9 $\pm$ 2.2	77.3 $\pm$ 7.1 ⁺	31.6 $\pm$ 4.7	56.6 $\pm$ 6.5 ⁺

 $\pm$ SD^{*}

p<0.01 compared to untreated,

^{**}

p<0.0001 compared to untreated,

⁺

p<0.01 compared to untreated.