Published in final edited form as:

J Oral Maxillofac Surg. 2011 October ; 69(10): 2564–2578. doi:10.1016/j.joms.2011.03.005.

Novel Therapy To Reverse The Cellular Effects of Bisphosphonates on Primary Human Oral Fibroblasts

Matthew Cozin¹, Bradley M. Pinker¹, Kimberley Solemani¹, Jeremy M. Zuniga¹, Stephen C. Dadaian¹, Serge Cremers², Regina Landesberg³, and Srikala Raghavan^{4,*}

¹Dental Student, College of Dental Medicine, Columbia University, New York, NY 10032.

²Assistant Professor, Department of Medicine-Endocrinology, Columbia University, New York, NY 10032.

³Associate Professor, Division of Oral and Maxillofacial Surgery, University of Connecticut Health Center, School of Dental Medicine, Farmington, CT 06030

⁴Assistant Professor, Division of Oral Surgery, College of Dental Medicine and Department of Dermatology, Columbia University, New York, NY 10032

Abstract

Purpose—Osteonecrosis of the Jaws is a clinical condition that is characterized by a non-healing breach in the oral mucosa resulting in exposure of bone and has been increasingly reported in patients receiving bisphosphonate (BP) therapy. Although the pathogenesis and natural history of ONJ remain ill defined, it appears that the oral soft tissues play a critical role in the development of this condition. In this report we examined the effects of the nitrogen-containing BPs pamidronate and zoledronate on primary human gingival fibroblasts.

Materials and Methods—Primary gingival fibroblasts were exposed to clinically relevant doses of pamidronate and zoledronate. Cellular proliferation was measured using a MTS/PMS reagent-based kit, scratch wound assays were performed to measure cellular migration and apoptosis was measured by using TUNEL and caspase assays. The BP exposed cells were treated with 10ng/mL recombinant human Platelet-Derived Growth Factor-BB (rhPDGF-BB, GEM21™) and 50μM geranylgeraniol (GGOH).

Results—Gingival fibroblasts are significantly more sensitive to inhibition of proliferation by zoledronate compared to pamidronate. Exposure of these cells to pamidronate but not zoledronate resulted in an increase in cellular apoptosis. Furthermore, exposure of gingival fibroblasts to pamidronate or zoledronate resulted in a decrease in cellular migration. We show that these defects are due to a loss of cell-substratum adhesion, and a reduction of F-actin bundles. Finally, we show that the addition of rhPDGF-BB, (GEM21™) and GGOH *in vitro* are able to partially rescue the cell proliferation, migration and adhesion defects.

Conclusion—The cytotoxic affects BPs on oral fibroblasts and its significant reversal by the addition of GGOH and rhPDGF-BB, (GEM21™) provide both the potential mechanism and treatment options for ONJ.

© 2011 The American Association of Oral and Maxillofacial Surgeons. Published by Elsevier Inc. All rights reserved.

*Corresponding Author : Columbia University, College of Dental Medicine and Department of Dermatology, P&S Box 20, 630 W 168th Street, New York, NY 10032, USA. sr2309@columbia.edu .

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

Osteonecrosis of the jaw is a clinical entity that has been defined by a number of professional associations¹⁻⁷. Although there are slight differences in the various definitions, all involve a breach in the mucosa leading to exposed bone that fails to heal in 6 to 8 weeks; furthermore, there should be no history of head and neck radiation in the affected patients. Over the last few years, there have been numerous case reports and series with a limited number of retrospective and prospective studies that strongly suggest a link between intravenous BP therapy and ONJ⁸⁻¹¹. Many questions regarding the incidence, pathoetiology, and natural history of this condition still need to be answered. At the present time, little to no evidence-based treatments for this condition exist making the focus on prevention. Furthermore, it is not known whether the ONJ lesion initiates in the bone or the soft tissue. While many investigators are steadfast in their thinking that ONJ is a solely a disorder of bone, the fact that a breach in the oral mucosa is a requirement for this condition has led us to begin to investigate the role of oral soft tissue in the development and/or progression of this condition. Our group was the first to demonstrate the inhibitory effect of BPs on oral keratinocytes¹². Subsequently others have examined the effects of pamidronate and zoledronate on oral epithelial and fibroblasts¹³⁻¹⁷.

Bisphosphonates are a class of drugs that are synthetic analogue of pyrophosphate and these compounds have a high affinity for hydroxyapatite seen primarily in bone^{18,19}. These drugs are indicated for the prevention and treatment of hypercalcemia of malignancy, Paget's disease of bone, multiple myeloma, and bone metastases associated with breast, prostate, lung, and other soft tissue tumours, as well as postmenopausal and steroid-induced osteoporosis²⁰⁻²⁸. Osteonecrosis of the jaws appears to be associated with the use of nitrogen-containing BPs. Although there are a small number of ONJ cases associated with osteoporotic patients, in the majority of reported cases of ONJ the intravenous administration of BPs zoledronate, pamidronate or both were used to treat skeletal related events in cancer patients. Bisphosphonates are categorized into two major classes; nitrogen containing, and non-nitrogen-containing. Nitrogen-containing BPs act by inhibiting the farnesyl pyrophosphatase enzyme of the mevalonate pathway which is involved in the synthesis of cholesterol, and the prenylation of small G proteins known to be critical in several cellular functions, such as proliferation, cellular migration and ultimately wound healing²⁹⁻³¹ (Fig 1). It has been shown *in vitro* that the effects of BPs can be reversed by the addition of certain biomolecular agents³¹⁻³⁴. One such agent is geranylgeraniol (GGOH, an analogue of geranylgeranyl diphosphate), a molecule that is downstream of the farnesyl pyrophosphatase enzyme³⁵. Another molecule known to be critical to wound healing is Platelet-Derived Growth Factor-BB which has been successfully used in the treatment of non-healing diabetic ulcers and in the regeneration of periodontal defects³⁶. Platelet derived growth factors are critical in early wound healing and are chemotactic, as well as mitogenic for inflammatory cells as well as mesenchymal-derived cells³⁷. Our data in the present as well as previous studies has shown that BPs inhibit several cellular functions of oral epithelial and fibroblastic cells in the wound healing cascade. In this report we have extended our original studies of oral epithelial cells to examine the effects of the nitrogen-containing BPs pamidronate and zoledronate on primary human gingival fibroblasts. Furthermore, we have utilized an *in vitro* model to test whether GGOH and PDGF-BB could overcome the wound healing suppression of oral fibroblasts by BPs.

Materials and Methods

Cell Culture

Primary human oral fibroblasts were obtained from discarded gingival surgical tissue (and exempt from IRB approval). Samples were immediately placed in PBS after surgical

isolation to prevent desiccation. When isolating cells, samples were first disinfected with 7.5% Betadine (Purdue Products, Stamford, CT) and thoroughly washed with PBS. Once disinfected, soft tissue samples were incubated overnight in 5% dispase enzyme (MP Biologicals, Irvine, CA) to remove mucosal layer. A 10mg/mL collagenase enzyme solution (Worthington Biochemical, Lakewood, NJ) was then used to isolate fibroblasts from the extracellular matrix. Fibroblasts were cultured in DMEM/F12 media in a 3:1 ratio with 10% fetal calf serum (Hyclone, Logan, UT) and penicillin/streptomycin antimicrobials. Fibroblasts were grown on 10cm cell culture treated plates (BD Falcon, Bedford, MA) until sufficient cell number was achieved for experiments. All experiments were performed with early passage cells (P0-P6) in a humidified incubator at 37°C with 5% CO₂. Pamidronate was obtained from Sigma (St. Louis, MO) and zoledronate was acquired from Novartis (Basel, Switzerland).

Cellular Proliferation and PDGF and GGOH Rescue Assays

To determine if BPs inhibited cell growth in culture, cellular proliferation experiments were performed using a MTS/PMS reagent-based kit (Promega, Madison, WI). Briefly, 10³ cells were plated in 96 well plates and after 24 hours the media was replaced with fresh media containing 0mM, 0.001mM, 0.01mM, 0.03mM, 0.06mM, 0.1mM, 0.2mM, 0.3mM pamidronate, or 0mM, 0.001mM, 0.002mM, 0.004mM, 0.006mM, 0.008mM, 0.009mM, 0.01mM, 0.02mM, 0.03mM zoledronate (N=4). These concentrations were selected to represent physiologically relevant concentrations of these BPs. The following day, media was replaced with a range of pamidronate or zoledronate concentrations. Cellular proliferation was measured each day after BP exposure for seven days. In addition, 10ng/mL recombinant human Platelet-Derived Growth Factor BB (rhPDGF-BB) and 50μM geranylgeraniol (GGOH) from Sigma was added to cell proliferation assays (N=3) in an attempt to rescue the inhibitory effects of pamidronate and zoledronate. When running the assay, reagents from the kit were added directly into the incubation media and incubated at 37°C for an hour. Absorbance at 490 nm was then measured with a plate reader (Molecular Devices, Sunnyvale, CA). Negative controls were readings obtained for untreated cells. Statistical analysis was performed by ANOVA and Tukey-Kramer post hoc test for all pairwise comparisons (JMP IN 5.1 for Windows; SAS Institute Inc, Cary, NC). Results are presented as a representation from a series of 3 separate experiments.

Terminal deoxynucleotidyl transferase-mediated dUTP-FITC end labelling (TUNEL) Assay

TUNEL assays were performed to visualize apoptotic cells upon exposure to pamidronate and zoledronate. Five thousand cells were seeded into the wells of permanox chamber slides (Nunc, Rochester, NY). The next day, media was exchanged for fresh media containing 0mM, 0.03mM, 0.06mM and 0.1mM pamidronate or 0mM, 0.01mM, 0.03mM and 0.06mM zoledronate. The cells were treated with pamidronate for 96 hours and with zoledronate for 144 hours. Positive controls were obtained by incubating cells with 1μM staurosporine for 6 hours. Negative controls were untreated cells. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. After fixation, slides were washed with PBS and permeabilized with a 0.1% sodium citrate, 0.1% Triton X-100 solution for 2 minutes on ice. Samples were washed with PBS and then incubated with enzyme and labeling solution (Roche Applied Science, Indianapolis, IN) for 1 hour at 37°C in a humidified chamber. The enzyme and labelling solutions catalyze the polymerization of labelled nucleotides to free 3'-OH DNA ends. Slides were then washed 3 times with PBS and mounted with Vectashield mounting solution (Vector Labs, Burlingame, CA). Detection of fragmented DNA was then observed with an Olympus BX53 fluorescence microscope. For each sample, the total number of cells and the number of TUNEL positive cells were quantified in ten representative fields. Statistical analysis was performed by ANOVA and Tukey-Kramer post

hoc test for all pairwise comparisons (JMP IN 5.1 for Windows; SAS Institute Inc, Cary, NC). Results are presented as a representation from a series of 3 separate experiments.

Caspase-3 Assay

To assess the mechanism of pamidronate and zoledronate toxicity on oral fibroblasts, caspase-3 activity was measured in samples using a colorimetric kit (Sigma, St. Louis, MO). Fibroblasts were seeded in the wells of a six-well plate and allowed to grow until confluent at which time they were treated with 0.03mM, 0.06mM and 0.1mM pamidronate for 96 hours, or 0.01mM, 0.03mM and 0.06mM zoledronate for 144 hours. Positive controls were obtained by incubating cells with 1 μ M staurosporine for 6 hours. Negative controls were obtained by incubating untreated cells. Samples were obtained each day following exposure to BPs. Samples were first washed with PBS, then, using a lysis buffer (250mM HEPES, pH 7.4, with 25mM CHAPS and 25mM DTT) included in the kit, cells were dissociated from the culture plates with a cell scraper. If any non-adherent cells were observed with light microscopy in samples at the time of collection, the media was centrifuged at 1,000 RPMs for five minutes and the resulting cell pellet was added to the lysis buffer. The cell lysates were stored at -80°C until assays were performed. When all samples were acquired, protein lysates and reagents were thawed on ice and added to the wells of a 96 well plate according to the manufacturer's instructions. The reaction was left overnight at 37°C in a humidified chamber and the colour change quantified using a plate reader (Molecular Devices, Sunnyvale, CA) at 405nm. Statistical analysis was performed by ANOVA and Tukey-Kramer post hoc test for all pairwise comparisons (JMP IN 5.1 for Windows; SAS Institute Inc, Cary, NC). Results are presented as a representation from a series of 3 separate experiments.

Wounding Assay and rescue with GGOH

The *in vitro* model of wound healing was used to examine the effects of BP treatment on the ability of oral fibroblasts to migrate and proliferate in a monolayer culture. Fibroblasts were first cultured in 6 well cell culture plates. Once confluent, cells were exposed to 0.03mM, 0.06mM and 0.1mM pamidronate, or 0.008mM, 0.03mM and 0.06mM zoledronate for 24 hours. Negative controls were obtained by observing wound closure in untreated cells. For the rescue experiments, 50 μ M GGOH was added to the cells along with the BPs. Twenty four hours after adding the BPs, the center of the confluent cell monolayers was scratched using a P10 pipette tip and fresh media containing the relevant concentrations of pamidronate and zoledronate with or without GGOH was added. Select areas were marked and photographed immediately (0 hours) and subsequently every 24 hours to record cellular migration into the wounded areas. The experiment was stopped when the untreated cells had completely closed the wound (72 hours). Wounding and rescue experiments were done in triplicate.

Visualization of focal adhesions and actin cytoskeleton of cells treated with zoledronate

To study the effects of zoledronate on cell-substratum adhesion, 1,000 cells were plated on fibronectin coated (10 μ g/mL) glass coverslips. Once the cells had attached (approximately 6 hours later) the media was replaced with fresh media containing 0mM, 0.03mM and 0.06mM zoledronate with or without 50 μ M GGOH for 96 or 120 hours. Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, then were blocked with 5% normal donkey serum for 1 hour, and then incubated with a mouse anti-vinculin (Sigma, USA) primary antibody diluted 1:200 in 5% normal donkey serum. Coverslips were then thoroughly washed and exposed to anti-mouse FITC secondary antibody and TRITC-phalloidin to stain the actin cytoskeleton for 1 hour. Secondary antibody alone was used as a negative control and untreated cells were used as positive control. Samples were washed and mounted on slides using Vectashield fluorescent mounting media with DAPI (Vector Labs,

Burlingame, CA). Cells were visualized and photographed using an Olympus BX53 fluorescence microscope. Results are presented as a representation from a series of 3 separate experiments.

Results

The effect of BPs Pamidronate and Zoledronate on Cell Proliferation

For cell proliferation assays, 10^3 cells were plated in 96 well plates and after 24 hours the media was replaced with fresh media containing a range of pamidronate or zoledronate concentrations. Cellular proliferation was measured each day after BP exposure for seven days. Cell proliferation was significantly inhibited after 48 hours of exposure to 0.06mM pamidronate and after 96 hours of exposure to 0.03mM pamidronate (Fig 2A). After 72 hours concentrations of 0.06mM and higher caused cells to detach from the culture plates while concentrations below 0.03mM did not have any noticeable effect. The human gingival fibroblasts were more inhibited in their ability to proliferate upon exposure to zoledronate compared to pamidronate. Exposure to 0.008mM zoledronate resulted in a significant reduction in proliferation after 72 hours while a concentration of 0.004mM zoledronate had a marked effect after 168 hours of exposure compared to controls (Fig 2B). The reduction in proliferation rates upon exposure to pamidronate and zoledronate were statistically significant when compared with untreated controls ($p < 0.05$).

The effect of BPs Pamidronate and Zoledronate on Cell Survival

We tested the effect of pamidronate and zoledronate on cell survival. Approximately 5% of the gingival fibroblasts exposed to 0.03mM pamidronate were TUNEL positive cells compared with controls (0-1%) after 96 hours. In fibroblasts exposed to 0.06mM pamidronate for 72 hours and 96 hours the number of TUNEL positive cells were 15% and 25%, respectively (Fig 3A). Higher concentrations of pamidronate resulted in an increased percentage of TUNEL positive cells and cellular detachment from culture plates (Fig 3D). Interestingly, exposure of oral fibroblasts to 0.03mM of zoledronate which resulted in a significant reduction in cellular proliferation at 72 hours did not show a corresponding increase in the number of TUNEL positive cells and the same time point (Fig 3B). Oral fibroblasts exposed to 0.03mM and 0.06mM of zoledronate for 144 hours had approximately 4% and 15% TUNEL positive cells respectively (Figs 3B, F). We next tested the levels of caspase-3 activity in cells treated with pamidronate and zoledronate. Cells treated with pamidronate had significant levels of caspase-3 activity. Oral fibroblasts exposed to 0.03mM, 0.06mM, and 0.1mM of pamidronate for 96 hours had 25%, 30%, and 40% of caspase-3 activity respectively compared to the positive control which was set at a 100% (Fig 3G). Cell lysates of samples exposed to zoledronate did not have detectable active caspase-3 levels (Data not shown).

Rescuing the cell proliferation defects with growth factors and metabolic intermediates

Cellular proliferation assays were repeated with pamidronate and zoledronate in the presence recombinant human platelet-derived growth factor-BB (rhPDGF-BB), and geranylgeraniol (GGOH) in an attempt to reverse the effects of the BPs on cellular proliferation. (Text removed) In cells treated with 0.03mM pamidronate, addition of 10ng/mL of rhPDGF-BB resulted in the cells being approximately 2.5 fold more proliferative when compared to cells treated with pamidronate alone at 168 hours (Fig 4A). In contrast, the proliferation defects seen in oral fibroblasts exposed to 0.008mM zoledronate for 168 hours were not rescued by the addition of 10ng/ml rhPDGF-BB (Fig 4B). In gingival fibroblasts treated with 0.03mM pamidronate the addition of 50 μ M GGOH restored the proliferation 75% of the control after 168 hours (Fig 4C). However, the cells treated with 0.06mM pamidronate were not rescued by the addition of 50 μ M GGOH (Fig 4C). The

proliferation defects seen in zoledronate treated cells were also rescued by the addition of 50 μ M GGOH. In cells treated with 0.008mM zoledronate, the addition of 50 μ M GGOH restored the proliferation to 75% of the control after 168 hours ($p < 0.05$). The effects were even more dramatic in cells that had been exposed to 0.03mM zoledronate where the addition of 50 μ M GGOH completely rescued the proliferation defects in these cells at 168 hours (Fig 4D).

The effect of pamidronate and zoledronate on wound healing and rescue with GGOH

Concentrations of pamidronate and zoledronate that significantly inhibited proliferation were used in these experiments to determine the migratory ability of BP treated oral fibroblasts. Both pamidronate and zoledronate exposure to fibroblasts resulted in decreased healing after cell cultures were wounded. Control untreated cells and cells exposed only to 50 μ M GGOH were able to completely close the scratch wound after 72 hours (Figs 5B, D, J, L). The addition of GGOH had no effect on wound closure. Additionally no morphological changes were observed. Cells exposed to 0.06mM pamidronate exhibited slower migration into the wounded area at 72 hours (Fig 5F) and the addition of 50 μ M GGOH to these cells was not able to significantly rescue this defect (Fig 5H). In cells treated with 0.03mM zoledronate, there were very few cells that had migrated into the scratch wound after 72 hours (Fig 5N). Interestingly in cells treated with 0.03mM zoledronate and 50 μ M GGOH the cells were completely rescued and were able to close the scratch wound after 72 hours (Fig 5P). This robust rescue of the zoledronate treated cells could be seen in cells treated with cytotoxic levels of zoledronate, 0.1mM for 72 hours (data not shown). Since the proliferation defects seen in cells that were exposed to zoledronate could not be rescued rhPDGF-BB, as well as the fact that pamidronate treated cells exposed to rhPDGF-BB had a change in cell morphology (data not shown) indicating that they were stressed and this prompted us not to perform the scratch wound assays under these conditions.

Focal adhesions and actin cytoskeleton of oral fibroblasts exposed to zoledronate

Our data showed that in cells treated with zoledronate, the cells detached from the plates prior to undergoing apoptosis. This suggests that there may be an underlying defect in cell-substratum adhesion following exposure to zoledronate. In order to address this, we examined the focal adhesions and the actin cytoskeletal network in cells treated with 0.03mM and 0.06mM zoledronate after 120 hours by staining the cells with an antibody to vinculin, a focal adhesion protein and phalloidin, which binds to F-actin. In untreated cells, there was a robust actin cytoskeletal network present that was associated with numerous focal adhesions, as can be seen with the green vinculin staining in Fig 6 panel A. The focal adhesions and actin network were not affected by the addition of 50 μ M GGOH (Fig 6B). In cells that were treated with 0.03mM or 0.06mM zoledronate for 120 hours, very few cells were attached to the coverslips and in those that remained, there were no focal adhesion or bundles of F-actin observed (Figs 6C, E). Interestingly, when cells were treated with 50 μ M GGOH in addition to 0.03mM both the focal adhesions and F-actin bundles were restored (Fig 6D). In cells that were treated with 0.06mM zoledronate, the addition of 50 μ M GGOH was able to substantially (but not completely) rescue both the numbers of focal adhesion and actin stress fibers (Fig 6F).

Discussion

Osteonecrosis of the jaws was first described in 2003³⁸. Several professional societies have since defined the clinical appearance of ONJ and while small differences exist the common element is a breach in the oral mucosa resulting in an area of exposed maxillary and/or mandibular bone¹⁻⁷. A large majority of ONJ cases develop following trauma to the alveolar area, most often after tooth extraction^{39, 40}. The incidence, pathophysiology, and

natural history of ONJ is still not well defined; however, many potential contributory factors have been identified including the use of bisphosphonates (BPs) ⁴¹. It appears likely that ONJ is multifactorial in nature. One key question that remains to be answered is why this condition occurs almost exclusively in the maxillofacial region? It is also unclear where the ONJ lesion initiates and the sequence of events that take place in the progression of the lesion ⁴¹.

Oral wound healing, particularly following a routine tooth extraction where healing is by secondary intention, is a complicated cascade of events that involves many cell types including keratinocytes, gingival fibroblasts, alveolar osteoblasts, osteoclasts, osteocytes, endothelial cells and inflammatory cells ^{42, 43}. Critical to oral wound healing are cellular proliferation, migration, and differentiation. We have previously investigated the effect of BPs (pamidronate) on oral keratinocytes and were the first group to demonstrate that pamidronate inhibits cellular proliferation and migration at doses that are consistent with expected levels in the maxillofacial bones ¹². Furthermore, this effect was not due to cellular apoptosis ¹². In the present paper we have extended these studies to examine the effect of BPs on human gingival fibroblasts, a cell that is critical in the healing of oral wounds; most notably following tooth extraction or healing of ulcerations due to ill-fitting dentures; traumatic events that are associated with the development of ONJ in a majority of cases ^{39, 40}.

In the present report we investigated the effect of pamidronate and zoledronate, the most commonly used nitrogen-containing BPs ^{21, 22, 26, 28} on primary human gingival fibroblasts. We, and others have estimated expected bone concentrations of BPs from published animal studies and have concluded that the concentrations of BPs used in our experiments are within physiologically relevant levels ^{12, 41, 44}. Exposure of gingival fibroblasts to pamidronate concentrations of 0.06mM showed significant inhibition of proliferation after 72 hours. We have previously investigated the effects of pamidronate on oral keratinocytes and showed that a significant inhibition of proliferation was seen at a pamidronate concentration of 0.1mM following a minimum of 72 hours of BP exposure ¹². A significant inhibition of gingival fibroblast proliferation by zoledronate was seen at 72 hours at a concentration of 0.008mM. These results suggest that gingival fibroblasts are significantly more sensitive than oral keratinocytes to inhibition of cellular proliferation following exposure to pamidronate.

Zoledronate is known to be significantly more potent than pamidronate in both *in vitro* and *in vivo* studies ⁴⁵. According to the American Society of Clinical Oncology the potencies (relative to etidronate, a non-nitrogen containing BP with a relative potency of 1) are listed as 100 and 100,000 for pamidronate and zoledronate respectively ²⁰. While our results show an inhibition of proliferation with a slightly lower concentration of zoledronate than pamidronate, it is not anywhere near the expected difference based on the published potencies of these drugs. Our results, are however, consistent with those of several other groups where periodontal ligament fibroblast and gingival fibroblast proliferation was inhibited by zoledronate concentrations ranging from 0.003mM – 0.1mM ¹³⁻¹⁷.

While BPs have been shown to cause apoptosis of osteoclasts ⁴⁶, the mechanism of their toxicity for epithelial and subepithelial cells is less clear. Numerous studies have explored the effect of BPs on a variety of epithelial cells, including gastrointestinal cells, cervical epithelial cells, renal cells, prostate epithelial cells, and oral mucosal cells ^{12, 37, 47-50}. The development of gastric erosions and ulcers is a well-documented side-effect associated with oral administration of nitrogen-containing BPs ⁵¹. Our previous studies failed to show any increase in cellular apoptosis when oral epithelial cells were exposed to pamidronate concentrations that significantly inhibited proliferation ¹². In the present study, human

gingival fibroblasts showed a significant increase in TUNEL positivity over the course of 72 and 144 hours when exposed to pamidronate; however, when exposed to concentrations of zoledronate that caused significant decreases in proliferation gingival fibroblasts did not show a corresponding increase in the number of TUNEL positive cells. While cells exposed to pamidronate had significant increases in caspase-3 activity zoledronate exposure did not show any increase in caspase-3 levels when compared to controls. These results suggest that the mechanisms underlying the reduction in proliferation and increase in cell detachment may be different for pamidronate and zoledronate.

Our results indicate that similar to cell proliferation, gingival fibroblasts are more sensitive to the effects of pamidronate than oral keratinocytes as we were unable to demonstrate apoptosis by TUNEL or caspase-3 levels in oral epithelial cells¹². Several groups have evaluated the inhibitory effect of BPs on cellular viability *in vitro*. Walter et al.,¹⁷ examined the effects of clondronate (a non-nitrogen BP), ibandronate, pamidronate, and zoledronate on human umbilical vein endothelial, gingival fibroblast, and osteogenic cell lines. Similar to our findings, the gingival fibroblasts showed the greatest sensitivity to inhibition by pamidronate and zoledronate although pamidronate toxicity was seen at a lower concentration than zoledronate. It is difficult to compare our results with the results of Scheper et al.,¹⁶ as the conditions and assay methodology for evaluating apoptosis differ considerably. This group evaluated apoptosis of gingival fibroblasts following zoledronate exposure by TUNEL assay at a single time point of 24 hours and found an increase of 5% positivity. Caspase-3 levels were evaluated by western blot analysis without quantification in contrast to our methodology where a colorimetric assay was used. Additionally, many of the cell culture experiments were performed in serum free media which likely causes significant stress to the cells and this may account for the very early apoptosis seen by these investigators. It should also be noted that both these groups utilized cell lines as opposed to our investigation where primary gingival fibroblasts were isolated for each series of experiments. Agis et al.,¹³ measured cell viability following exposure of primary gingival fibroblasts and periodontal fibroblasts to zoledronate at 24, 48 and 72 hours. Their results at 72 hours showed a 35% decrease in viability of gingival fibroblasts following exposure to zoledronate, similar to our findings.

At the present time the pathophysiology of ONJ has not been clearly delineated⁴¹. Consequently, evidence based treatment protocols are not available resulting in the focus of therapy being placed on prevention. We, therefore, have utilized our *in vitro* model systems to test two potential treatments for ONJ. In order to attempt to overcome the inhibition of cellular proliferation of gingival fibroblast by BPs, cells were exposed to PDGF-BB a growth factor known to stimulate proliferation of cells of the fibroblastic lineage. The PDGF family is a group of polypeptide growth factors that are released from platelets following injury and subsequent formation of a blood clot⁵². These factors are critical in early wound healing and are chemotactic, as well as mitogenic for inflammatory cells as well as mesenchymal-derived cells including fibroblasts, osteoblasts, chondrocytes, vascular endothelial cells and smooth muscle cells³⁷. Platelet-Derived Growth Factor-BB has been successfully used in the treatment of non-healing diabetic ulcers and in the regeneration of periodontal defects⁵²⁻⁵⁴. We, therefore, used our proliferation and *in vitro* wound healing models to test the ability of PDGF-BB to overcome the suppression of BPs on gingival fibroblasts. Gingival fibroblasts were cultured with 0.03mM of pamidronate and 10 ng/mL of PDGF-BB for up to 7 days. Cellular proliferation was measured at 24, 72, and 168 hours. There was no effect of PDGF-BB at the 24 and 72 hour time points however at 168 hours the addition of PDGF showed a 2.5 fold increase in proliferation when compared to cells treated with pamidronate without the addition of growth factor. When similar experiments were performed with zoledronate (0.008mM and 0.01mM), PDGF-BB was unable to overcome the BP suppression of proliferation. It should be noted that the concentration of

PDGF-BB used in these experiments was significantly lower than that used clinically were concentrations range from 150–300mg/mL⁵²⁻⁵⁴.

Bisphosphonates are analogues of inorganic phosphate that bind avidly to calcium ions and hence are deposited in bone mineral. These compounds are released from the bone during resorption and subsequently taken up by the osteoclasts⁵⁵. Bisphosphonates are classified according to whether or not they contain nitrogen⁴⁴. The non-nitrogen containing BPs such as clodronate are metabolized intracellularly into ATP analogues that accumulate in the cell and ultimately induce cell death⁵⁶. The more potent nitrogen-containing BPs such as pamidronate and zoledronate act by inhibiting farnesyl diphosphate synthetase (FPP synthetase), an enzyme in the cholesterol pathway that is responsible for generating farnesyl diphosphate and geranylgeranyl diphosphate, isoprenoid lipids required for the post-translational prenylation of small GTPases such as Rho, Ras, and Rab⁵⁷. These small GTPases are critical to osteoclast function as well as survival. Recently it has been demonstrated that certain biomolecular agents can reverse the inhibition of nitrogen-containing BPs.³¹⁻³⁴ We, therefore, examined whether the addition of geranylgeraniol (GGOH, an analogue of geranylgeranyl diphosphate) could reverse the inhibition of BPs on cellular proliferation and wound healing migration of gingival fibroblasts.

As discussed previously, cellular migration is critical to oral wound healing particularly in the closure of extraction sockets and traumatic ulcerations^{39, 40}. Inhibition of soft tissue cell migration by BPs would likely play a key role in the development of ONJ. Gingival fibroblasts were exposed to 0.03mM pamidronate or 0.008mM and 0.03mM of zoledronate and were cultured for up to 168 hours in the presence of 50μM GGOH. Significant inhibition of cellular proliferation was evident at 72 hours and beyond. While PDGF-BB was able to partially reverse the effect of pamidronate only at 168 hours, it was unable to reverse the inhibitory effects of zoledronate on gingival fibroblast proliferation. The addition of GGOH stimulated cell proliferation to 75% of the control levels at the 168 hour time point in both the pamidronate and zoledronate inhibited cells.

Additionally, we tested the ability of GGOH to rescue the suppression of cell migration by BPs using the *in vitro* wound assays system described above. Our results show that both pamidronate and zoledronate resulted in a significant decrease in cell migration into the wounded area; however, while the addition of GGOH resulted in a complete restoration of wound closure in the zoledronate treated cultures, the pamidronate cultures could not be rescued with by GGOH. These results strongly suggest that the mechanism of inhibition of proliferation and cellular migration by zoledronate, but not pamidronate, in primary gingival fibroblasts is through the cholesterol/mevalonate pathway similar to that seen in osteoclasts.

The rescue of the cell adhesion defects in zoledronate treated cells by the addition of 50μM GGOH suggests that this metabolic intermediate GGOH is able to circumvent the inhibitory effects of the BP zoledronate on the prenylation of the small GTPases such Rho (Fig 1), which is critical for the formation of stable focal adhesions. Cells attach to the underlying substratum via focal adhesions, that provide a structural link between the extracellular matrix to the actin cytoskeleton network and a signalling center that regulates growth control⁵⁸. The small GTPase Rho is important for the assembly of the focal adhesions. One of the functions of Rho is to bundle actin by stimulating contractility in adherent cells⁵⁹. This actin bundling in turn, results in the assembly of focal adhesions which are nucleated by integrin cell surface receptors and other integrin associated proteins such as vinculin, talin, paxillin and focal adhesion kinase (FAK)^{60, 61}.

Taken together, these data provide a plausible mechanism for the ability of GGOH to rescue the cell proliferation, migration and adhesion defects seen in cells treated with zoledronate.

Furthermore, in the cells treated with zoledronate the cells detached from the plate prior to apoptosis possibly due to a significant decrease in the number of cell-substratum adhesions and decrease in the number of F-actin stress fibres.

In conclusion, our results have demonstrated that the nitrogen containing BPs pamidronate and zoledronate inhibit gingival fibroblastic wound healing. Furthermore, our *in vitro* model system described herein has allowed us to demonstrate the ability of PDGF-BB and GGOH to partially overcome the inhibitory effects of these BPs. We believe that this is the first demonstration of potential therapeutic modalities for the treatment of the mucosal lesions seen in ONJ. While these *in vitro* results using a single cell type are promising additional investigations are critical to further test the efficacy of PDGF-BB and GGOH for the treatment of ONJ lesions. In the future, we plan to extend these studies by using organotypic systems, as well as *in vivo* mouse or rat models for ONJ to test the efficacy of PDGF-BB and GGOH in reversing the effects of these BPs. We also plan to test other growth factors such as keratinocyte growth factor (KGF) and metabolic intermediates such as farnesyl diphosphate (FPP) in reversing the effects of BPs on oral keratinocytes and fibroblasts.

Acknowledgments

This work was funded by NIH grant 5R21DE17164 to (RL, SC) and Irving Research Scholar Grant (SR).

References

1. Colella G, Campisi G, Fusco V. American Association of Oral and Maxillofacial Surgeons position paper: Bisphosphonate-Related Osteonecrosis of the Jaws-2009 update: the need to refine the BRONJ definition. *J Oral Maxillofac Surg.* 2009; 12:2698. [PubMed: 19925998]
2. Khosla S, Burr D, Cauley J, et al. Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research. *J Bone Miner Res.* 2007; 10:1479. [PubMed: 17663640]
3. Kyle RA, Yee GC, Somerfield MR, et al. American Society of Clinical Oncology 2007 clinical practice guideline update on the role of bisphosphonates in multiple myeloma. *J Clin Oncol.* 2007; 17:2464. [PubMed: 17515569]
4. Patel V, McLeod NM, Rogers SN, et al. Bisphosphonate osteonecrosis of the jaw-a literature review of UK policies versus international policies on bisphosphonates, risk factors and prevention. *Br J Oral Maxillofac Surg.* 2010
5. Ruggiero SL, Dodson TB, Assael LA, et al. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws--2009 update. *J Oral Maxillofac Surg.* 2009; 5(Suppl):2. [PubMed: 19371809]
6. Ruggiero SL, Fantasia J, Carlson E. Bisphosphonate-related osteonecrosis of the jaw: background and guidelines for diagnosis, staging and management. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2006; 4:433. [PubMed: 16997108]
7. Yoneda T, Hagino H, Sugimoto T, et al. Bisphosphonate-related osteonecrosis of the jaw: position paper from the Allied Task Force Committee of Japanese Society for Bone and Mineral Research, Japan Osteoporosis Society, Japanese Society of Periodontology, Japanese Society for Oral and Maxillofacial Radiology, and Japanese Society of Oral and Maxillofacial Surgeons. *J Bone Miner Metab.* 2010; 4:365. [PubMed: 20333419]
8. Dodson TB. Intravenous bisphosphonate therapy and bisphosphonate-related osteonecrosis of the jaws. *J Oral Maxillofac Surg.* 2009; 5(Suppl):44. [PubMed: 19371814]
9. Kos M, Kuebler JF, Luczak K, et al. Bisphosphonate-related osteonecrosis of the jaws: a review of 34 cases and evaluation of risk. *J Craniomaxillofac Surg.* 2010; 4:255. [PubMed: 19592261]
10. Thumigere-Math V, Sabino MC, Gopalakrishnan R, et al. Bisphosphonate-related osteonecrosis of the jaw: clinical features, risk factors, management, and treatment outcomes of 26 patients. *J Oral Maxillofac Surg.* 2009; 9:1904. [PubMed: 19686928]

11. Wang EP, Kaban LB, Strewler GJ, et al. Incidence of osteonecrosis of the jaw in patients with multiple myeloma and breast or prostate cancer on intravenous bisphosphonate therapy. *J Oral Maxillofac Surg.* 2007; 7:1328. [PubMed: 17577497]
12. Landesberg R, Cozin M, Cremers S, et al. Inhibition of oral mucosal cell wound healing by bisphosphonates. *J Oral Maxillofac Surg.* 2008; 5:839. [PubMed: 18423269]
13. Agis H, Blei J, Watzek G, et al. Is zoledronate toxic to human periodontal fibroblasts? *J Dent Res.* 2010; 1:40. [PubMed: 19948943]
14. de Correia FV, Caldeira CL, Marques MM. Cytotoxicity evaluation of sodium alendronate on cultured human periodontal ligament fibroblasts. *Dent Traumatol.* 2006; 6:312.
15. Ravosa MJ, Ning J, Liu Y, et al. Bisphosphonate effects on the behaviour of oral epithelial cells and oral fibroblasts. *Arch Oral Biol.* 2010
16. Scheper MA, Badros A, Chaisuparat R, et al. Effect of zoledronic acid on oral fibroblasts and epithelial cells: a potential mechanism of bisphosphonate-associated osteonecrosis. *Br J Haematol.* 2009; 5:667. [PubMed: 19036117]
17. Walter C, Klein MO, Pabst A, et al. Influence of bisphosphonates on endothelial cells, fibroblasts, and osteogenic cells. *Clin Oral Investig.* 2010; 1:35.
18. Coxon FP, Thompson K, Rogers MJ. Recent advances in understanding the mechanism of action of bisphosphonates. *Curr Opin Pharmacol.* 2006; 3:307. [PubMed: 16650801]
19. Green J, Lipton A. Anticancer properties of zoledronic acid. *Cancer Invest.* 2010; 9:944. [PubMed: 20879838]
20. Hillner BE, Ingle JN, Berenson JR, et al. American Society of Clinical Oncology guideline on the role of bisphosphonates in breast cancer. American Society of Clinical Oncology Bisphosphonates Expert Panel. *J Clin Oncol.* 2000; 6:1378. [PubMed: 10715310]
21. Rosen LS, Gordon D, Kaminski M, et al. Zoledronic acid versus pamidronate in the treatment of skeletal metastases in patients with breast cancer or osteolytic lesions of multiple myeloma: a phase III, double-blind, comparative trial. *Cancer J.* 2001; 5:377. [PubMed: 11693896]
22. Saad F, Gleason DM, Murray R, et al. A randomized, placebo-controlled trial of zoledronic acid in patients with hormone-refractory metastatic prostate carcinoma. *J Natl Cancer Inst.* 2002; 19:1458. [PubMed: 12359855]
23. Santini D, Vincenzi B, Avvisati G, et al. Pamidronate induces modifications of circulating angiogenic factors in cancer patients. *Clin Cancer Res.* 2002; 5:1080. [PubMed: 12006522]
24. Santini D, Vincenzi B, Dicuonzo G, et al. Zoledronic acid induces significant and long-lasting modifications of circulating angiogenic factors in cancer patients. *Clin Cancer Res.* 2003; 8:2893. [PubMed: 12912933]
25. Usui T, Oiso Y, Tomita A, et al. Pharmacokinetics of incadronate, a new bisphosphonate, in healthy volunteers and patients with malignancy-associated hypercalcemia. *Int J Clin Pharmacol Ther.* 1997; 6:239. [PubMed: 9208339]
26. Van Poznak CH. The use of bisphosphonates in patients with breast cancer. *Cancer Control.* 2002; 6:480. [PubMed: 12514566]
27. Vincenzi B, Santini D, Dicuonzo G, et al. Zoledronic acid-related angiogenesis modifications and survival in advanced breast cancer patients. *J Interferon Cytokine Res.* 2005; 3:144. [PubMed: 15767788]
28. Major P. The use of zoledronic acid, a novel, highly potent bisphosphonate, for the treatment of hypercalcemia of malignancy. *Oncologist.* 2002; 6:481. [PubMed: 12490736]
29. Bergstrom JD, Bostedor RG, Masarachia PJ, et al. Alendronate is a specific, nanomolar inhibitor of farnesyl diphosphate synthase. *Arch Biochem Biophys.* 2000; 1:231. [PubMed: 10620343]
30. Coxon FP, Rogers MJ. The role of prenylated small GTP-binding proteins in the regulation of osteoclast function. *Calcif Tissue Int.* 2003; 1:80. [PubMed: 12370802]
31. Goffinet M, Thoulouzan M, Pradines A, et al. Zoledronic acid treatment impairs protein geranylgeranylation for biological effects in prostatic cells. *BMC Cancer.* 2006;60. [PubMed: 16539721]
32. Nogawa M, Yuasa T, Kimura S, et al. Zoledronic acid mediates Ras-independent growth inhibition of prostate cancer cells. *Oncol Res.* 2005; 1:1. [PubMed: 15839301]

33. Suyama K, Noguchi Y, Tanaka T, et al. Isoprenoid-independent pathway is involved in apoptosis induced by risedronate, a bisphosphonate, in which Bim plays a critical role in breast cancer cell line MCF-7. *Oncol Rep.* 2007; 5:1291. [PubMed: 17914587]
34. Zhong WB, Liang YC, Wang CY, et al. Lovastatin suppresses invasiveness of anaplastic thyroid cancer cells by inhibiting Rho geranylgeranylation and RhoA/ROCK signaling. *Endocr Relat Cancer.* 2005; 3:615. [PubMed: 16172195]
35. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. *Nature.* 1990; 6257:425. [PubMed: 1967820]
36. Steed DL. Clinical evaluation of recombinant human platelet-derived growth factor for the treatment of lower extremity diabetic ulcers. Diabetic Ulcer Study Group. *J Vasc Surg.* 1995; 1:71. [PubMed: 7823364]
37. Wallace JL, Dicay M, McKnight W, et al. N-bisphosphonates cause gastric epithelial injury independent of effects on the microcirculation. *Aliment Pharmacol Ther.* 1999; 12:1675. [PubMed: 10594404]
38. Marx RE. Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic. *J Oral Maxillofac Surg.* 2003; 9:1115. [PubMed: 12966493]
39. Kyrgidis A, Vahtsevanos K, Koloutsos G, et al. Bisphosphonate-related osteonecrosis of the jaws: a case-control study of risk factors in breast cancer patients. *J Clin Oncol.* 2008; 28:4634. [PubMed: 18574158]
40. Hoff AO, Toth BB, Altundag K, et al. The Frequency and Risk Factors Associated with Osteonecrosis of the Jaw in Cancer Patients Treated with Intravenous Bisphosphonates. *J Bone Miner Res.* 2008
41. Landesberg R, Woo V, Cremers S, et al. Potential pathophysiological mechanisms in osteonecrosis of the jaw. *Ann N Y Acad Sci.* 2011; 1:62. [PubMed: 21291478]
42. Amler MH. The time sequence of tissue regeneration in human extraction wounds. *Oral Surg Oral Med Oral Pathol.* 1969; 3:309. [PubMed: 5251474]
43. Elsubeihi ES, Heersche JN. Quantitative assessment of post-extraction healing and alveolar ridge remodelling of the mandible in female rats. *Arch Oral Biol.* 2004; 5:401. [PubMed: 15041488]
44. Reid IR, Bolland MJ, Grey AB. Is bisphosphonate-associated osteonecrosis of the jaw caused by soft tissue toxicity? *Bone.* 2007; 3:318. [PubMed: 17572168]
45. Mehrotra B. Bisphosphonates--role in cancer therapies. *J Oral Maxillofac Surg.* 2009; 5(Suppl):19. [PubMed: 19371811]
46. Hughes DE, Wright KR, Uy HL, et al. Bisphosphonates promote apoptosis in murine osteoclasts *in vitro* and *in vivo*. *J Bone Miner Res.* 1995; 10:1478. [PubMed: 8686503]
47. Giraudo E, Inoue M, Hanahan D. An amino-bisphosphonate targets MMP-9-expressing macrophages and angiogenesis to impair cervical carcinogenesis. *J Clin Invest.* 2004; 5:623. [PubMed: 15343380]
48. Suri S, Monkkenen J, Taskinen M, et al. Nitrogen-containing bisphosphonates induce apoptosis of Caco-2 cells *in vitro* by inhibiting the mevalonate pathway: a model of bisphosphonate-induced gastrointestinal toxicity. *Bone.* 2001; 4:336. [PubMed: 11595616]
49. Twiss IM, de Water R, den Hartigh J, et al. Cytotoxic effects of pamidronate on monolayers of human intestinal epithelial (Caco-2) cells and its epithelial transport. *J Pharm Sci.* 1994; 5:699. [PubMed: 8071824]
50. Twiss IM, Pas O, Ramp-Koopmanschap W, et al. The effects of nitrogen-containing bisphosphonates on human epithelial (Caco-2) cells, an *in vitro* model for intestinal epithelium. *J Bone Miner Res.* 1999; 5:784. [PubMed: 10320527]
51. de Groen PC, Lubbe DF, Hirsch LJ, et al. Esophagitis associated with the use of alendronate. *N Engl J Med.* 1996; 14:1016. [PubMed: 8793925]
52. Hollinger JO, Hart CE, Hirsch SN, et al. Recombinant human platelet-derived growth factor: biology and clinical applications. *J Bone Joint Surg Am.* 2008;48. [PubMed: 18292357]
53. Pellegrini G, Seol YJ, Gruber R, et al. Pre-clinical models for oral and periodontal reconstructive therapies. *J Dent Res.* 2009; 12:1065. [PubMed: 19887682]
54. Young CS, Ladd PA, Browning CF, et al. Release, biological potency, and biochemical integrity of recombinant human platelet-derived growth factor-BB (rhPDGF-BB) combined with

- Augment(TM) Bone Graft or GEM 21S beta-tricalcium phosphate (beta-TCP). J Control Release. 2009; 3:250. [PubMed: 19577598]
55. Van Beek ER, Lowik CW, Papapoulos SE. Bisphosphonates suppress bone resorption by a direct effect on early osteoclast precursors without affecting the osteoclastogenic capacity of osteogenic cells: the role of protein geranylgeranylation in the action of nitrogen-containing bisphosphonates on osteoclast precursors. Bone. 2002; 1:64. [PubMed: 11792566]
56. Frith JC, Monkkenen J, Blackburn GM, et al. Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5'-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells *in vitro*. J Bone Miner Res. 1997; 9:1358. [PubMed: 9286751]
57. Hasmim M, Bieler G, Ruegg C. Zoledronate inhibits endothelial cell adhesion, migration and survival through the suppression of multiple, prenylation-dependent signaling pathways. J Thromb Haemost. 2007; 1:166. [PubMed: 17059425]
58. Raghavan S, Vaezi A, Fuchs E. A role for alphabeta1 integrins in focal adhesion function and polarized cytoskeletal dynamics. Dev Cell. 2003; 3:415. [PubMed: 12967561]
59. Ridley AJ. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. Trends Cell Biol. 2006; 10:522. [PubMed: 16949823]
60. Giancotti FG, Ruoslahti E. Integrin signaling. Science. 1999; 5430:1028. [PubMed: 10446041]
61. Zamir E, Geiger B. Molecular complexity and dynamics of cell-matrix adhesions. J Cell Sci. 2001; (Pt 20):3583. [PubMed: 11707510]

Figure 1. Nitrogen Containing BPs: Mechanism of Action

Both pamidronate and zoledronate act by inhibiting the farnesyl pyrophosphatase enzyme in the HMG-CoA reductase pathway. Inhibition of this enzyme prevents the prenylation of proteins which results in cell death of osteoclasts. The addition of exogenous GGOH is able to overcome the block of the geranylgeranylation of Rab and Rho GTPases.

Figure 2. Cell Proliferation of fibroblasts exposed to BPs

Cellular proliferation of oral fibroblasts was strongly inhibited after exposure to 0.06mM pamidronate for greater than 48 hours (A). A dose of 0.03mM pamidronate was sufficient to have an adverse effect on proliferation after 168 hours of exposure. Cellular proliferation of oral fibroblasts was inhibited after exposure to 0.008mM zoledronate for greater than 72 hours (B). Concentrations of 0.002mM zoledronate and higher strongly inhibited cell growth after 168 hours of exposure.

* - $p < 0.05$ when compared to controls

Figure 3. The effects of the BPs on cell survival

The percentage of cells that were TUNEL positive upon exposure to 0.03mM, 0.06mM or 0.1mM pamidronate were 5%, 25% and 35% respectively after 96 hours (A). Cells exposed to 0.06mM zoledronate had approximately 15% TUNEL positive cells, after 144 hours of exposure (B). Cells exposed to 0.1mM pamidronate for 96 hours displayed more number of TUNEL positive cells (D) compared to cells exposed to 0.06mM zoledronate for 144 hours (F) as judged by the number of green cells per field. Non treated cells did not have any TUNEL positive cells (C, E). Cells that were exposed to increasing levels of pamidronate displayed increased caspase activity (G).

Figure 4. Proliferation of primary human fibroblasts treated with bisphosphonates and rescued with PDGF-BB and GGOH

Cells were untreated or treated to 0.03mM or 0.06mM pamidronate and 0.003mM or 0.008mM zoledronate. Platelet-derived growth factor (10 ng/ml) or 50μM GGOH were added to cultures to assess the ability of these agents to overcome the inhibition of the BPs. A 20% increase in proliferation could be seen by the addition of PDGF to the cell cultures (A and B). When added to the pamidronate treated cells at 168 hours, PDGF was able to restore approximately 40% of the proliferation when compared cells treated with pamidronate alone (A). The addition of PDGF was unable to rescue the zoledronate treated cells (B). In cells treated with 0.03mM pamidronate, the addition of 50μM GGOH was able to restore the proliferation to 75% of control after 168 hours (C). The cells treated with 0.008mM and 0.03mM zoledronate the addition of 50μM GGOH restored the proliferation to 75% and 100% of control respectively (D).

* - $p < 0.05$ when compared to controls

^ - $p < 0.05$ when compared to inhibited samples

Figure 5. Wound healing of primary human oral fibroblasts treated with BPs and rescued with GGOH

Cells in a confluent 6 well plate were either untreated (A, I) or treated with 50μM GGOH (C, K). Cells were exposed to 0.06 mM pamidronate alone (E) or 0.06 mM pamidronate plus 50μM GGOH (G) and 0.03mM zoledronate alone (M) or 0.03mM zoledronate plus 50μM GGOH (O) and scratch wound assays were performed. After 72 hours, untreated cells closed the wound (B, J). Cells exposed to 0.06mM pamidronate or 0.03mM zoledronate were inhibited from migrating into the wounded area (F, N). When 50μM GGOH was added to the wounded cultures, minimal rescue of pamidronate treated cultures was seen (H), while the zoledronate cultures showed a complete rescue of wound healing (P). Addition of GGOH to non-wounded cultures had no effect on the cell migration (D, L).

Figure 6. Effect of zoledronate on integrin mediated cell-substratum adhesions of oral fibroblasts
Cells were plated on fibronectin-coated coverslips and were exposed to 0.03mM or 0.06mM zoledronate with or without the addition of 50μM GGOH. The cells were fixed after 120 hours and then stained with vinculin and phalloidin to visualize the focal adhesions and actin cytoskeleton. In cells that were untreated or treated with 50μM GGOH alone, there was robust actin cytoskeletal network associated with numerous focal adhesions (A, B). In cells that were exposed to 0.03mM or 0.06mM zoledronate, there were very few F-actin bundles and a corresponding loss of focal adhesions (C, E). The addition of 50μM GGOH to cells treated with 0.03mM zoledronate was able to completely rescue both the focal adhesions and the actin stress fibres (D). In cells treated with 0.06mM zoledronate and 50μM GGOH, the focal adhesions and actin cytoskeleton network were also restored (F), but not to the same extent as seen in (D).