

# Geranylgeraniol in Bone Cement Rescues Osteoclasts from the Toxic Effects of Pamidronate and Restores Function

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## Research

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# Abstract

## Background

Exposed intraoral bone in medically related osteonecrosis of the jaw (MRONJ) is a devastatingly side effect of treatment with nitrogen containing bisphosphonates (NBPs). The pathogenesis of the condition is thought to be caused by NBP inhibition of farnesyl diphosphate synthase (FDPS), a critical enzyme in the mevalonate pathway. This block suppresses the prenylation of factors necessary for the maintenance and survival of osteoclasts. Geranylgeraniol (GGOH), a metabolite in the mevalonate pathway downstream of FDPS, reverses the block NBPs impose on osteoclasts. However, no effective method currently exists to deliver GGOH locally to NBP-induced lesions in the mouth. The purpose of this study is to develop a biocompatible, resorbable and tunable carrier to deliver GGOH intraorally and thereby reverse the damaging effects of MRONJ.

## Methods

Primary human oral fibroblasts and RAW264.7 osteoclasts were exposed to pamidronate in vitro and GGOH contained within a bone cement pellet and survival of the cells was measured. The kinetics of release of <sup>3</sup>HGGOH from the bone cement was measured. The resorptive function of osteoclasts exposed to pamidronate with or without GGOH was quantitated by measuring the release of bound fluorescent dye from a CaPO<sub>4</sub> coated plate. Resorption areas on this plate were photographed.

## Results

Human gingival fibroblasts exposed to pamidronate (100uM) alone decreased survival by 49% (p<0.001) over 72 hours, 2mM GGOH incorporated into bone cement rescued these cells from the toxic effect of NBP (p=0.0024).

Exposure of osteoclasts to 400 and 800uM pamidronate over 72 hours showed a 49% and 69% loss of viability respectively. (p<0.0001 for each concentration). 2mM GGOH incorporated into bone cement increased survival by 247% (p=0.0001) for the cells treated with 800uM pamidronate and 4mM GGOH by 450% (p<0.0001). Pamidronate at both concentrations reduced osteoclast function and 4mM GGOH completely restored resorptive function. At a powder to liquid ration of 2.5:1 92% of the loaded metabolite was released into medium after in 96 hours.

## Conclusions

GGOH contained in a bone cement carrier can rescue osteoclast function from the toxic effects of pamidronate. This carrier may be the first step in local delivery of this metabolite in the oral cavity.

## Background

Nitrogen containing bisphosphonates (NBPs) are currently used to treat osteoporosis, osteogenesis imperfecta, Paget's disease, multiple myeloma and metastases from breast, prostate and lung cancers. One side effect of these drugs is the development of medically related osteonecrosis of the jaw. (MRONJ) The American Association of Oral and Maxillofacial Surgeons defines MRONJ as a pathologic condition in which exposed bone can be probed through an intraoral or extra-oral fistula in the maxillofacial region that has persisted for 8 weeks in patients who have been treated with anti-resorptive or anti-angiogenic agents who have had no history of radiation therapy. [1] In addition to the morbidity produced by exposed intraoral bone, MRONJ often results in mandibular fractures.

No cause-based treatment for this debilitating disorder currently exists. Current theories suggest that inhibition of angiogenesis, infection, local trauma and oral mucosal toxicity might be causal.[2] The most accepted hypothesis concerning MRONJ etiology focuses on NBPs suppression of osteoclast function.[3] By inhibiting farnesyl diphosphate synthase (FDPS), an enzyme in the mevalonate pathway that generates cholesterol and isoprenoids, NBPs suppress the prenylation of rho, rac and other proteins necessary for osteoclast maturation and function.[4], [5]

Geranylgeraniol (GGOH) is a metabolite downstream of FDPS that rescues epithelial cells, fibroblasts and osteoclasts from NBP toxicity in vitro when freely available in the cell media. It has also been shown to reverse the effect of MRONJ when injected systemically.[6] However, systemic administration of GGOH negates the therapeutic effect of NBPs in non-oral tissues. Daily local administration of high concentrations of GGOH as shown by Koneski in the form of a spray would be impractical for the patient and an inefficient way of delivering this drug.[7] Currently, there is no method to deliver clinically effective doses of GGOH in a controllable manner to cells of the oral cavity.

The aim of this study is to describe a clinically acceptable carrier of GGOH, bone cement that can deliver and release this drug locally to fibroblasts and osteoclasts treated with NBP, without inducing toxicity. This carrier is biocompatible, resorbable and tunable and most importantly appropriate for use in the oral cavity. Specifically we show that bone cement serves as a carrier for GGOH and rescues cells from the toxic effects of therapeutic levels of pamidronate while restoring resorptive function to osteoclasts.

## Materials And Methods

### Cells and materials used:

Primary human gingival fibroblasts were obtained from ATCC (PCS-201-018) and grown under conditions specified by ATCC using the specified low serum growth kit (ATCC PCS-201-041) and fibroblast basal medium (ATCC PCS 201-030) with penicillin, streptomycin and amphotericin B.. After 4 cell divisions cells were harvested and frozen. These frozen stocks were then used for experiments.

Raw 264.7 macrophages were obtained from ATCC (TIB71) and cultured in DMEM with 10% FBS. Initial stocks were grown for 4 cell doublings and frozen. Over a period of 4-6 days these cells were differentiated into osteoclasts using recombinant mouse RANK-L from R&D systems (cat #462-TR-010)

Pamidronate was obtained from Sigma cat # p2371-10mg. Geranylgeraniol (Sigma cat G3278-100mg) was used.

Prism software (Graphpad) version 8 was used for all statistical calculations.

### **Carriers tested and description of cell model system:**

Calcium phosphate bone cement (Stryker Hydroset) was evaluated as a carrier for GGOH.

For Hydroset 5ul of 100mM GGOH was added to a mix of 0.625 gram Hydroset powder (8ul/gram powder)+ 250ul liquid . This mix was sufficient to create 5 bone cement pellets and produced a concentration of 2mM within each pellet. The pellet was allowed to harden after placing mix in 4.7 mm diameterx2.0 mm high sterile plastic tubing (Marina Air Line) at 37C in a humidified chamber for 30' after which plastic tubing was removed with a razor.

### **Survival experiments**

$2.0-4.0 \times 10^4$  primary human gingival fibroblasts or  $1.5-2.0 \times 10^4$  Raw 264.7 osteoclasts were grown on 24 well plates for 24 hours. For fibroblasts pamidronate alone or in combination with GGOH was added and cells were incubated at 37C 5% CO<sub>2</sub> for 72 hours. For Raw 264.7 cells after plating for 24 hours media was replaced with media containing 20ng/ml of RANKL with or without GGOH in pellets and pamidronate and incubated for 72 hours. Cell viability was quantitated, using Cell Titer 96 kit (Promega), by measuring optical density at 490nm after subtracting background absorption at 650nm. Each time point had between 3 and 5 replicates which were averaged and experiment was repeated twice. Controls with no treatment, carrier alone and carrier with GGOH were included in the experiment.

### **Osteoclast Function experiments**

Corning Osteo Assay 24 well plate (3987) was used to measure osteoclast function along with reagents from the Cosmo Bio Bone resorption Assay kit (CSR-BRA-24). The coated calcium phosphate is first bound to fluoresceinamine-labeled chondroitin sulfate (FACS), which is released from the calcium phosphate layer into conditioned medium by osteoclastic resorption activity. Bone resorption activity is evaluated by simply measuring the fluorescence intensity of the conditioned medium. The protocol as outlined in the Cosmo assay kit was followed. Briefly, RAW cells were incubated for 96 hours with or without GGOH and pamidronate in the presence of 100ng/ml RANKL and fluorescence intensity was measured with an excitation at 485nm and emission at 535nm. Each time point had between 3 and 8 replicates which were averaged and the experiment was repeated twice. Controls with no treatment, carrier alone and carrier with GGOH were included in the experiment.

To visualize resorption areas after removal of media, wells were treated with 5% sodium hypochlorite for 5 minutes, rinsed with water and allowed to dry. Areas of resorption were documented qualitatively by photomicrograph at 10 x magnification using a Nikon Eclipse T1 microscope.

## **Kinetics of release of GGOH from the two carriers:**

American Radiolabel Chemicals supplied  $^3\text{H}$  GGOH (all trans) Specific Activity: 50-60 Ci/mmol 1.85-2.22 TBq/mmol. A combination of  $^3\text{H}$  labelled GGOH and unlabeled GGOH was added to the various carriers to achieve internal concentrations 2mM. The carrier was then placed in a scintillation vial filled with 0.5-1.0 ml PBS at 37C on a rocking platform. After various time periods, media was removed, counted and replaced with fresh media. The release of radioactivity from both the 2.5:1 powder to liquid  $\text{CaPO}_4$  (used in cell experiments) and the denser 3.0:1 bone cements were measured after adding scintillation fluid and counted. Background counts were subtracted. Each experiment repeated twice consisted of 3 replicates which were averaged.

## **Statistical Analysis**

Using Prism software version 8, ordinary one way ANOVA analysis with a Bonferroni multiple comparisons test was used to compare experimental conditions. P values <0.05 were considered significant.

# **Results**

## **Survival**

### **Primary human gingival fibroblasts:**

Human gingival fibroblasts were grown alone or in combination with GGOH incorporated into a Hydroset pellet with or without 100uM pamidronate for 72 hours and tested for viability. 100uM pamidronate alone reduced fibroblast viability by 49.4% ( $p<0.0001$ ). 2mM GGOH in the bone cement pellet restored full viability to these cells. 4mM GGOH in the pellet allowed a 78% recovery ( $p=0.0028$ ), inhibiting recovery by about 21% ( $p=0.0455$ ). Calcium phosphate pellets either alone or loaded with 2mM GGOH had no significant effect on fibroblast viability. (figure 1)

### **Raw 264.7 cells**

## **Survival**

Murine transformed macrophages were differentiated into osteoclasts after exposure to RANK L for 5 days. Exposure of these cells to 400 and 800uM pamidronate over 72 hours showed a 49% and 69% loss of viability respectively. ( $p<0.0001$  for each concentration). Including 2mM GGOH in bone cement in cells exposed to 800uM pamidronate increased viability by 247% ( $p=0.0001$ ). For osteoclasts treated with 800uM pamidronate and 4mM GGOH viability increased by 450% ( $p<0.0001$ ). Bone cement alone or containing 2mM GGOH had no significant effect on survival.(figure 2)

## **Function**

Since GGOH was shown to rescue osteoclasts from the toxic effects of physiologic doses of pamidronate experiments were designed to determine if this agent salvaged osteoclast function. To quantitate function fluorescent dye release from a 24 well plate coated with mineralized CaPO<sub>4</sub> bound to fluoresceinamine-labeled chondroitin sulfate (FACS) was measured. Osteoclasts were exposed to varying concentrations of pamidronate and GGOH over a period of 4 days. Concentrations of 400uM and 800 uM reduced osteoclast function by 36% (p=0.0128) and 41%(p=0.0037) respectively. 4mM GGOH loaded into Hydroset pellets increased fluorescent dye release by 215% for cells exposed to 400uM pamidronate. Osteoclasts exposed to 800uM pamidronate showed a 184% increase in resorptive function when this concentration of GGOH was added (p<0.01) (figure 3) Pamidronate had no effect on osteoclast function for doses of 200uM or less (results not shown). Cells treated with the pellet alone or with the pellet containing 4mM GGOH alone showed no significant differences in function compared to the untreated controls.

Representative photomicrographs of of total and partial resorption pits created by the Raw cells maintained on the 24 well plate for each of the conditions listed above are shown in figure 5.

### **Kinetics of release of GGOH from various carriers**

Hydroset cement was tested at both low and high densities (p/l ratios) for the release of GGOH as described in Methods. At a powder to liquid ratio of 2.5:1 (the ratio used in all of the experiments described above), 92% of the loaded metabolite was released into medium after in 96 hours. (fig 4a). At the higher density of 3.0:1, 14% of GGOH was released during this time period. The rate of release for each density peaked at 1 hour.(fig 4b)

## **Discussion**

It is reported here that a commercially available bone cement can serve as a vehicle to supply a metabolite that can restore viability and function to osteoclasts exposed to physiologic levels of pamidronate. This metabolite, geranylgeraniol (GGOH), located downstream of farnesyl diphosphate synthase (FDPS) has previously been shown to rescue osteoclasts, oral fibroblasts and keratinocytes from cell death when freely available in cell culture medium. Results herein show that this metabolite incorporated into a bone cement carrier can rescue primary oral fibroblasts and osteoclasts. Additionally, it is shown that osteoclast function as measured by calcium phosphate resorption can be restored in vitro by GGOH incorporated into biocompatible carrier.

Interestingly results presented confirm that fibroblasts are far more sensitive to the effects of NBPs than are osteoclasts. Other differences include the ability of GGOH to rescue each cell type from the toxic effect of pamidronate. For fibroblasts this rescue effect peaks with pellets containing between 2 and 4mM GGOH while for osteoclasts no such peak rescue effect is seen even at concentrations of 4mM. (figures 1 and 2) Examination of the data also reveals how GGOH's ability to rescue osteoclast survival and function differ in the face of an NBP challenge. Osteoclast survival continues to increase at 800uM

pamidronate when a pellet with 4mM GGOH is added. In contrast function levels of these cells levels off at these levels of NBP and metabolite. (figures 2 and 3)

Others have demonstrated that NBP toxicity can be reversed by GGOH in osteoclasts. GGOH has also been shown to restore TRAP (tartrate resistant acid phosphatase) staining, and other markers of osteoclastic differentiation, cathepsin K, calcitonin receptor, and those of multinucleation related molecules like osteoclast stimulatory transmembrane protein.[6] Additionally the protein prenylation function of osteoclasts has recently been shown to be restored by GGOH in osteoclasts exposed to zoledronate. [8] To our knowledge GGOH induced rescue of osteoclast function as measured by their ability to resorb calcium phosphate from a substrate has not been shown.

Other investigators have also shown that GGOH when delivered as a liquid in vivo directly to the extracted tooth socket “locally” and daily for 35 days resulted in improved socket healing in a rat model of MRONJ. [7] This delivery method would be impractical for human patients. It has been also shown that GGOH delivered systemically in the rat model can reverse the effects of MRONJ. [7] Aside from the issue of GGOH toxicity, systemic administration might negate the anti-metastatic effect of nitrogen containing bisphosphonates in patients receiving chemotherapy for breast, lung and other cancers. The aim in this study is to develop a biocompatible, resorbable and tunable carrier that could be used as a vehicle to deliver GGOH locally to avoid these systemic effects.

Calcium phosphate resorbable cements have been used to deliver antibiotics, analgesics, anticancer, anti-inflammatory drugs and growth factors.[9] Otsuka and his colleagues show that 6 mercapto purine, an anticancer drug, has a kinetic release profile similar to the one found here for GGOH. [10]

Results presented here show that GGOH can be released over a periods of 72-96 hours in sufficient quantity and activity to rescue gingival fibroblasts and osteoclasts from the toxic effects of pamidronate and to restore the ability of osteoclasts to resorb a calcium phosphate substrate. The kinetics of release of GGOH is shown to be affected by the ratio of powder to liquid incorporated into the cement pellet. For both the 2.5:1 and the 3.0:1 (p/l) densities a spike in release occurred within an hour of exposure to medium possibly indicating the release of surface bound drug followed later by slower release of GGOH contained within the pores.

Due to the selective incorporation of nitrogen containing bisphosphonates into bone, this tissue is thought to act like a reservoir for N-BPs. Although not a mainstream theory it is postulated that this store of drug is released to the surrounding connective tissue and epithelial cells and may account of the necrosis of soft tissue seen in MRONJ.[11] It is known that human gingival fibroblasts produce important mediators of osteoclastogenesis including RANKL (receptor activator of nuclear factor kappa-B ligand), osteoprotegerin and interleukin 6. [12] Landesberg et al. showed that bisphosphonate pre-treatment of oral mucosal cells inhibits proliferation and wound healing at clinically relevant doses and that this inhibition is not due to cellular apoptosis [13]. The effect of GGOH released from bone cement on fibroblasts was therefore investigated as a possible further therapeutic option to reverse these toxic effects.

Interestingly possibly because gingival fibroblasts secrete RANKL and depend on it for survival, denosumab a monoclonal antibody directed against this protein, has been found to also be toxic to these cells. Human gingival fibroblasts exposed to denosumab alone exhibited impaired survival and when challenged with the scratch wound healing test showed deficiencies compared to untreated controls. This result occurred with and without the presence of the inflammatory mediator LPS.[14] This result might account for the mucosal lesions seen in MRONJ induced by this therapeutic.

Indications for use of this therapeutic would include MRONJ with exposed necrotic bone that had been debrided and the patient placed on a drug holiday. GGOH in conjunction with other NBP depleting strategies might be of clinical benefit but further *in vivo* studies are necessary. In addition to NBPs RANKL antibodies also produce MRONJ [15]. Denosumab, one such antibody, acts by directly inhibiting RANK-L thus preventing the macrophage-osteoclast transition. This antibody, unlike NBPs, allows the prenylation of Rho, Ras and other factors that permit macrophage maturation but stops osteoclast formation at a later stage than NBPs do. GGOH might also ameliorate denosumab-induced MRONJ by increasing the number of available mature macrophages partially overcoming the denosumab inhibition of RANK-L but this remains to be tested.

One limitation of this study is that in our model system bisphosphonates were added to the culture medium. *In vivo* bisphosphonate exposure is through its release and osteoclast uptake during bone resorption. The *in vitro* uptake from the medium is likely different than its *in vivo* uptake from the actively resorbing osteoclast. Concentrations of GGOH that rescued osteoclasts and restored functions might also be different *in vivo*. Experiments with a rat *in vivo* MRONJ model are ongoing. Finally, human osteoclasts exposed to GGOH might not recover viability and function to the same degree that RAW 264.7 murine transformed macrophages have in these experiments.

## Conclusions

In summary, we have shown that bone cement can deliver a downstream metabolite in the mevalonate pathway that can counteract the inhibitory effect of NBPs on FDPS. In so doing it can rescue primary gingival fibroblasts and osteoclasts from the toxic effects of pamidronate and restore the ability of osteoclasts to resorb a mineralized substrate.

## Abbreviations

MRONJ- Medically Related Osteonecrosis of the Jaw

NBP- Nitrogen containing bisphosphonate

GGOH- Geranylgeraniol

FDPS- Farnesyl diphosphate synthase

RANKL-Receptor activator of nuclear factor kappa-B ligand

TRAP-tartrate resistant acid phosphatase

## Declarations

Ethics approval and consent: No human or animal subjects were involved in this research. Not applicable.

Consent for publication: All authors consent to publication of this data.

Availability of data and material. All data is available on request to the corresponding author

Competing interests: none of the authors has competing interests.

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Authors Contributions:

GF conceived of and helped execute experiments.

AY executed experiments helped design them.

DT helped write manuscript and provide clinical insight

BD helped write and edit manuscript and provided critical clinical background.

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Authors contribution: GF designed and performed experiments wrote manuscript, AY helped design experiments and write manuscript, DT helped design experiments and write manuscript, RD conceived of experiments and provided clinical insight, helped write manuscript

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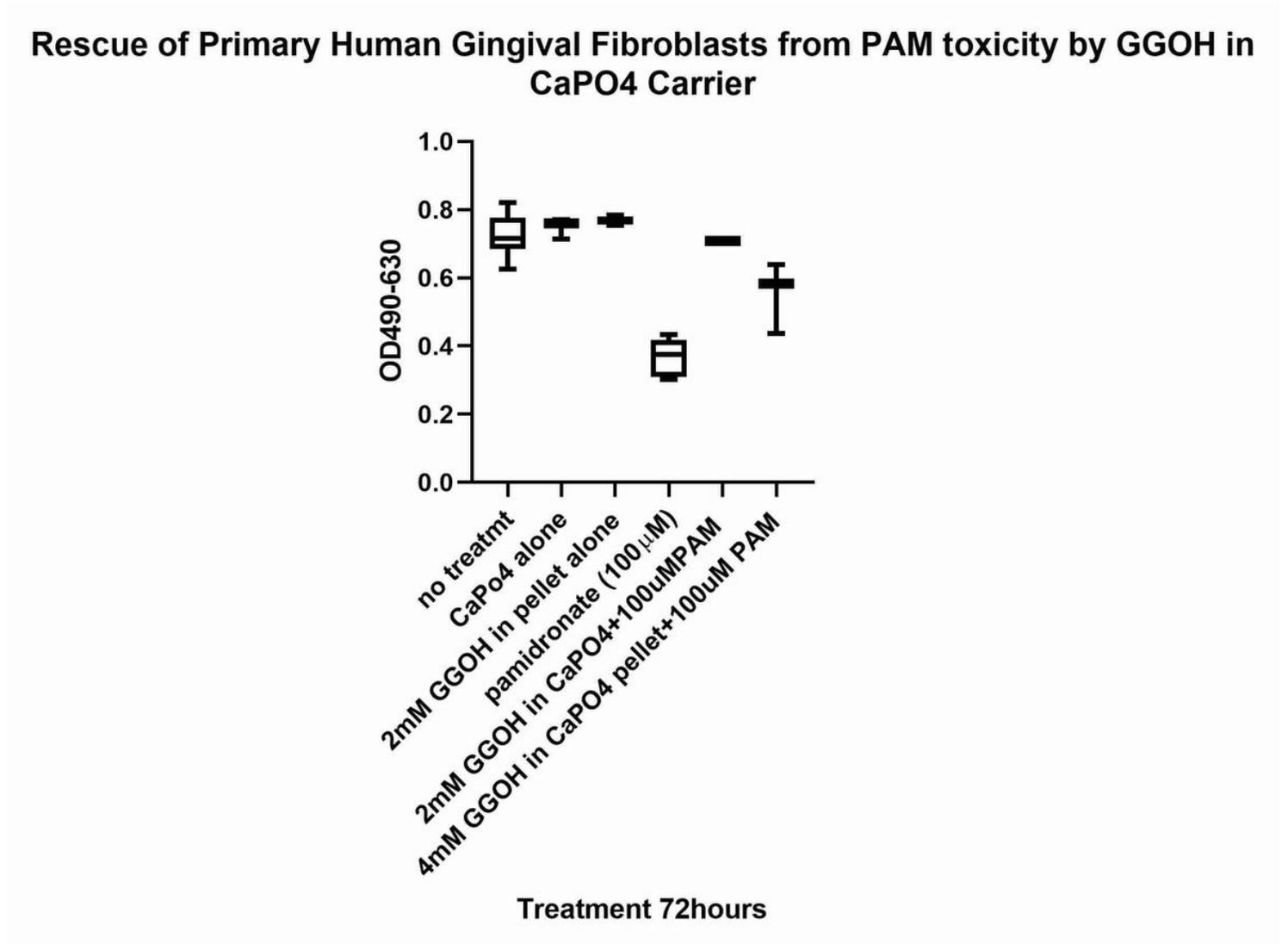
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## Figures

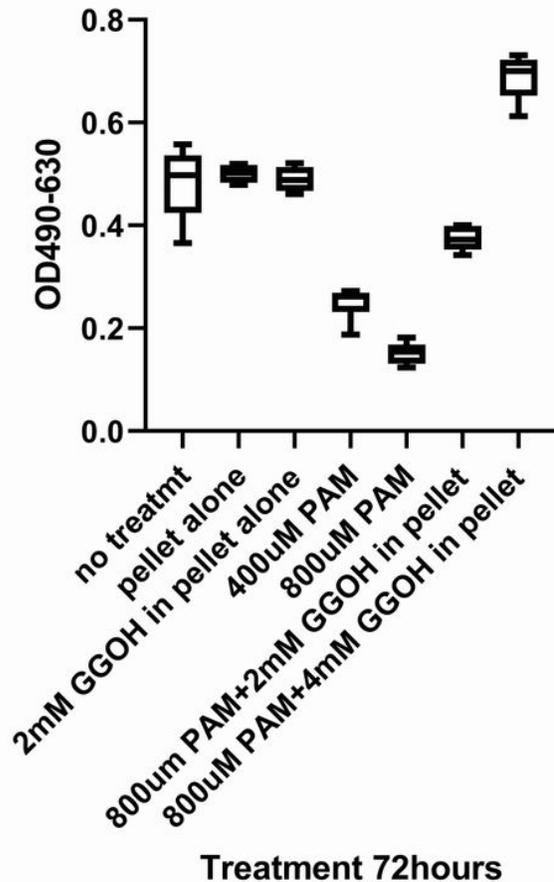


**Figure 1**

Rescue over 72 hours of primary human gingival fibroblasts from toxic effects of 100uM pamidronate by GGOH incorporated into a bone cement pellet. Y axis depicts cell viability based on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose this ability. Error bars show upper and lower limits of data with the data mean shown as a horizontal line. 100uM pamidronate alone reduced fibroblast viability by 49.4%

( $p < 0.0001$ ). 2mM GGOH in the bone cement pellet restored full viability to these cells. 4mM GGOH in the pellet allowed a 78% recovery ( $p = 0.0028$ ), inhibiting recovery by about 21% ( $p = 0.0455$ ). Each time point had between 3 and 5 replicates which were averaged and experiment was repeated twice.

### Rescue of TIB71 osteoclasts from toxic effects of pamidronate by GGOH in CaPO<sub>4</sub> pellet



**Figure 2**

Rescue of RAW 264.7 osteoclasts from various concentrations of pamidronate by GGOH incorporated into a bone cement pellet over 72 hours. Raw 264.7 cells were plated for 24 hours and media was replaced with media containing 20ng/ml of RANKL with or without GGOH and pamidronate and incubated for 72 hours. Cell viability was measured as described in Methods. 400 and 800uM pamidronate over 72 hours showed a 49% and 69% loss of viability respectively. ( $p < 0.0001$  for each concentration). Including 2mM GGOH in bone cement in cells exposed to 800uM pamidronate increased viability by 247% ( $p = 0.0001$ ). For osteoclasts treated with 800uM pamidronate and 4mM GGOH viability increased by 450% ( $p < 0.0001$ ). Error bars show upper and lower limits of data with the data mean shown as a horizontal line

## Rescue of RAW osteoclast function from toxic effect of pamidronate

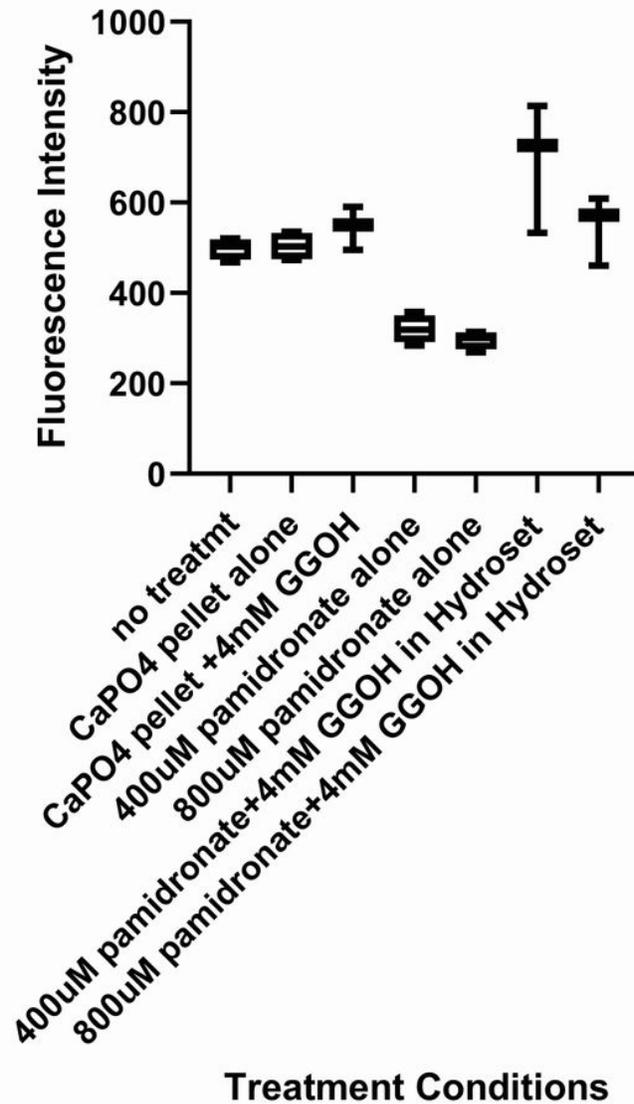
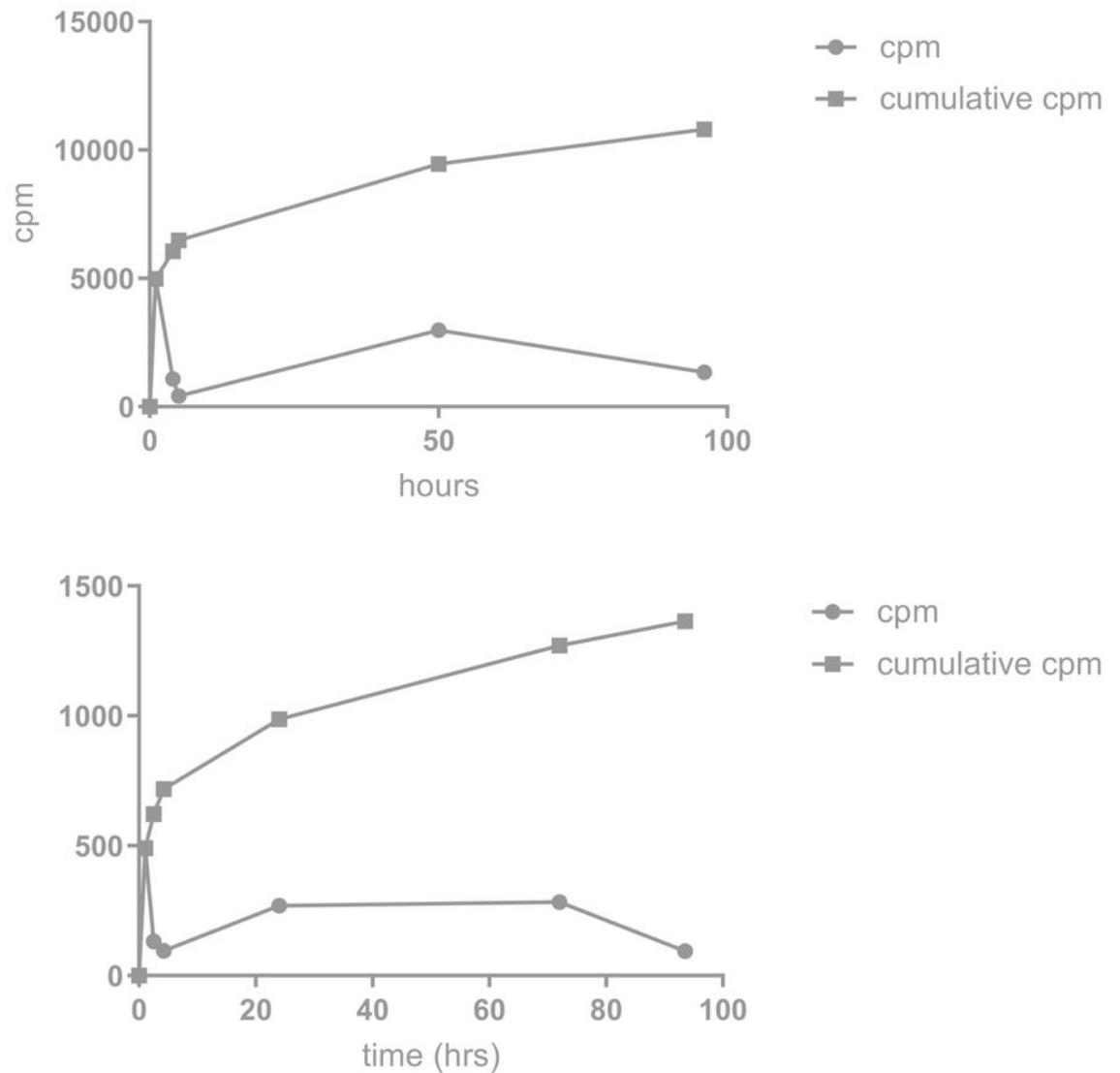


Figure 3

Rescue of RAW 264.7 osteoclast function from various concentrations of pamidronate by GGOH incorporated into cement pellets. Fluoresceinamine-labeled chondroitin sulfate (FACS), was added to a plate coated with calcium phosphate. The release of this fluor into conditioned medium by osteoclastic resorption activity was quantified by measuring the fluorescence intensity of the conditioned medium. , RAW cells were incubated for 96 hours with or without GGOH and pamidronate in the presence of 100ng/ml RANKL and fluorescence intensity was measured with an excitation at 485nm and emission at 535nm. Concentrations of 400uM and 800 uM reduced osteoclast function by 36% ( $p=0.0128$ ) and 41% ( $p=0.0037$ ) respectively. 4mM GGOH loaded into Hydroset pellets increased fluorescent dye release by 215% for cells exposed to 400uM pamidronate. Osteoclasts exposed to 800uM pamidronate showed a 184% increase in resorptive function when this concentration of GGOH was added ( $p<0.01$ ) Each time

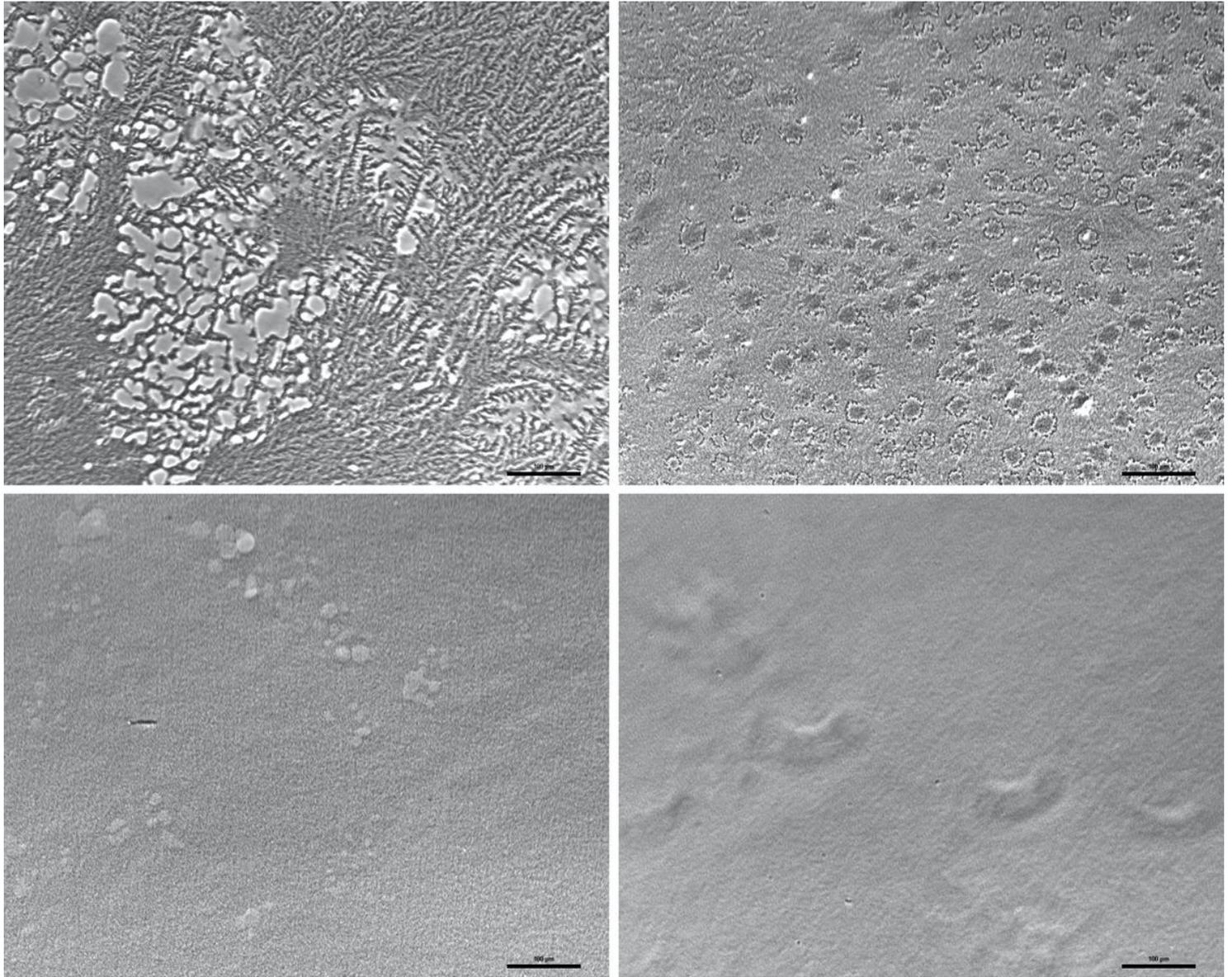
point had between 3 and 8 replicates which were averaged and the experiment was repeated twice. Error bars show upper and lower limits of data with the data mean shown as a horizontal line



**Figure 4**

a) Kinetics of release of  $^3\text{H}$  labelled GGOH bone cement pellets made up with a powder to liquid ratio of 2.5:1. Both labelled and unlabeled GGOH were added to bring concentration to 2mM. Each time point represents an average of 3 replicates. The carrier was placed in a scintillation vial filled with 0.5-1.0 ml

PBS at 37C on a rocking platform. After various time periods, media was removed, counted and replaced with fresh media. The release of radioactivity from both the 2.5:1 powder to liquid CaPO<sub>4</sub> (10,500 counts of 3H GGOH loaded into pellet)(used in cell experiments) and the b) denser 3.0:1 bone cements (9300cpm loaded) were measured. At a powder to liquid ratio of 2.5:1 (the ratio used in all of the experiments described above), 92% of the loaded metabolite was released into medium after in 96 hours. (fig 4a) At the higher density of 3.0:1, 14% of GGOH was released during this time period. The rate of release for each density peaked at 1 hour.(fig 4b)



**Figure 5**

photomicrographs of of total and partial resorption pits created by the Raw cells maintained on the 24 well plate for conditions tested in figure 3. a- No treatment, b- 400uM pamidronate alone, c- 800uM pamidronate alone, d- 400uM pamidronate+4mM GGOH in pellet, e- 800uM pamidronate+4mM GGOH in pellet