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Novel protective function of an isoprenoid, geranylgeraniol (GGOH), in human bone cells treated with zoledronate: an in vitro study

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Eidesstattliche Versicherung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema "**GERANYLGERANIOL (GGOH) AS A LOCAL THERAPY IN THE TREATMENT OF MEDICATION-RELATED OSTEONECROSIS OF THE JAW (MRONJ)"** selbstständig verfasst habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

ABBREVIATIONS	DESCRIPTION
AAOMS	American Association of Oral and Maxillofacial Surgeons
AFF	Atypical femoral fracture
ASBMR	The American Society for Bone and Mineral Research
BE	Barrett's esophagus
BMD	Bone mineral density
BMMSCs	Bone marrow mesenchymal stem cells
BP	Bisphosphonate
BRONJ	Bisphosphonate-related osteonecrosis of the jaw
BSA	Bovine serum albumin
FBS	Fetal bovine serum
FOH	Farnesol
FPPS	Farnesylpyrophosphate synthase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GGOH	Geranylgeraniol
GGPP	Geranylgeranyl pyrophosphate
HA	Hydroxyapatite
HG-DMEM	High glucose Dulbecco's Modified Eagle Medium
HGF	Human gingival fibroblast
HOBs	Human osteoblasts
HOCs	Human osteoclasts
HOK	Human oral keratinocyte
HRT	Hormone replacement therapy
HUVEC	Human umbilical vein endothelial cell
IV	Intravenous
LILT	Low-intensity laser therapy
M-CSF	Macrophage colony-stimulating factor
MRONJ	Medication-related osteonecrosis of the jaw
MVP	Mevalonate pathway
N-BP	Nitrogen-containing bisphosphonate
OH	Hydroxy group
OPG	Osteoprotegerin
Pen/Strep	Penicillin/Streptomycin
PBS	Phosphate buffer saline
PPi	Inorganic pyrophosphate
PRP	Platelet-rich plasma
PRF	Platelet-rich fibrin
PTH	Parathyroid hormone
PTHrP	Parathormon-related peptide receptor
PVDF	Polyvinylidene difluoride
RANKL	Receptor activator of nuclear factor-kappaB ligand
RT	Room temperature
SDS	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERM	Selective estrogen receptor modulator
TBST	Tris-buffered saline (TBS) and polysorbate 20
TRAP	Tartrate-resistant acid phosphatase
WR	Working reagent
WST-1	Water soluble tetrazolium salt
ZA	Zoledronic acid

SUMMARY

Medication-related osteonecrosis of a jaw (MRONJ) [1] is a rare but serious adverse reaction of antiresorptive medications and angiogenesis inhibitors, which can cause an extensive and progressive bone destruction in the maxillofacial region [2-4].

Antiresorptive drugs are administrated to patients suffering from osteoporosis, multiple myeloma, and breast or prostate cancers involving multiple osteolytic metastases in the bone. Among cancer patients under treatment with zoledronate (ZA), the cumulative incidence of MRONJ is around 0.7-6.7% [5-7].

This study investigates the effects on human osteoblasts (hOBs) and human osteoclasts (hOCs) of ZA as a mevalonate pathway (MVP) inhibitor and of geranylgeraniol (GGOH) as its antagonist. Zoledronate (a nitrogen-containing bisphosphonate [N-BP]) affects osteolytic tumor metastases by inhibiting a key enzyme of MVP, namely farnesyl pyrophosphate synthase (FPPS) [8, 9]. The cytotoxic effects of ZA have been attributed to the decreased prenylation of small GTPases such as Rap [10], Ras, and Cdc42 [8, 9, 11, 12]. These proteins are essential for important cell processes such as cell movement, cytoskeletal rearrangement and apoptosis [13, 14].

The following thesis hypothesizes that external local supplementation of GGOH may reverse the negative effects of ZA. Some studies investigated the role of GGOH on other cell lines which were treated with N-BPs, such as human oral keratinocytes (HOKs) and human oral fibroblasts and hOBs [15, 16]. However, the effect of various concentrations of GGOH and ZA has not been sufficiently tested on human bone cell lines (hOBs and hOCs).

In this study, the following methods were used to examine the hypothesis:

cell viability assay (water-soluble tetrazolium salt [WST-1]), live/dead assay (Calcein-AM/ ethidium homodimer [EthD-1]), tartrate-resistant acid phosphatase (TRAP) staining and western blot analyses.

The results of our experiments showed that GGOH, having a dose-dependent effect on bone cells, may be able to reverse the negative effect of ZA in a dose-dependent manner. Despite the positive effects of lower concentrations of GGOH (10-40μM) on bone cells treated with ZA, higher concentrations of GGOH showed cytotoxic effects on cell viability. The obtained results indicate that GGOH may be used as a local therapy in the treatment of early stages of MRONJ, in the form of mouth rinses or appropriate drug delivery systems [17]. However, the systemic administration of GGOH, especially to patients with malignant diseases and bone metastasis who use or have used intravenous (IV) ZA (N-BP), may suppress the antitumor activity of ZA and consequently may lead to the spread of the malignancy.

ZUSAMMENFASSUNG

Medikamenten-assoziierte Kiefernekrosen (MRONJ) stellen seltene, aber ernst zu nehmende Nebenwirkungen hauptsächlich zweier Gruppen von Medikamenten dar. Antiresorptive Medikamente und Angiogenese-Inhibitoren können zu ausgedehnten und progressiven Destruktionen der Ober- und/oder Unterkiefer führen [2-4, 18]. Antiresorptive Medikamente werden bei Erkrankungen wie Osteoporose, Multiplen Myelomen, oder bei Patienten mit Mamma- oder Prostatakarzinom mit osteolytischen Knochenmetastasen gehäuft eingesetzt. Bei Patienten mit Tumorerkrankungen, die eine intravenöse Therapie mit Zoledronat (ZA) erhalten haben, beträgt die Inzidenz von MRONJ circa 0.7 - 6.7% [5-7].

Ziel dieser Untersuchung ist es, herauszufinden, ob die inhibitorische Wirkung von ZA, als ein potenter Inhibitor des Mevalonatweges (MVP), auf Osteoblasten und Osteoklasten durch Geranylgeraniol (GGOH) antagonisiert werden kann. Zoledronat beeinflusst den Mevalonatweg durch Blockade eines Schlüsselenzyms, welches als Farnesyl-Pyrophospat-Synthase (FPPS) bezeichnet [8, 9]. Diese führt zu einer verringerten Prenylierung der kleinen GTPase Proteine wie Rap, Ras und Cdc42, die den zytotoxischen Effekt des Zoledronates erklären [8, 9, 11, 12]. Kleine GTPasen sind integraler Bestandteil unterschiedlicher zellulärer Prozesse mit Einfluß auf Zellmorphologie, Reorganisation des Zytoskelettes und Apoptose [13, 14].

Die vorliegende Promotion basiert auf der Hypothese, dass die lokale Applikation von GGOH den negativen Effekt von ZA antagonisieren könnte und aus diesem Grund in der Zukunft als eine mögliche lokale Therapie in Form von Mundspüllösungen in früheren Stadien von MRONJ eingesetzt werden könnte. Einige Studien haben die Wirkung von ZA/GGOH auf verschiedene Zelllinien wie Endothelzellen der menschlichen Nabelschnurvene und auf die gingivalen Fibroblasten untersucht [19-22]. Trotzdem ist die Wirkung von unterschiedlichen Konzentrationen von ZA/GGOH auf die Knochenzellen (hOBs and hOCs) nicht vollständig erforscht.

Folgende Test-Methoden wurden in unseren Untersuchungen eingesetzt:

WST-1 Assay, L/D Assay (Calcein-AM/ Ethidium-Homodimer (EthD-1), TRAP-Färbung und Western Blot Analysen.

Die Ergebnisse der Experimente haben gezeigt, dass GGOH den negativen Effekt von ZA sowohl auf hOCs als auch auf hOBs antagonisiern kann. Diese Wirkung ist aber stark abhängig von der GGOH-Dosis. Während niedrigere GGOH-Konzentrationen (10µM, 20µM, 40µM) einen positiven Effekt auf die Lebensfähigkeit von hOBs und hOCs (vorbehandelt mit ZA) zeigten, wiesen höhere GGOH Konzentrationen eine Verstärkung des zytotoxischen

Effekts von ZA auf. Die Ergebnisse dieser Arbeit verdeutlichen, dass GGOH in der Zukunft als eine mögliche lokale medikamentöse Therapie in früheren Stadien von MRONJ in Frage kommen könnte.

CHAPTER 1 INTRODUCTION

Bone function and diseases

The bone is a connective tissue that supplies mechanical support for stature and mobility and also protects various organs [23]. It is regulated by the continuous, highly complex mechanism of remodelling in which new bone replaces old ones through bone resorption and bone formation [8]. Any imbalance between these two processes results in bone disorders, which can be associated with great morbidity. Thus, understanding these mechanisms may lead to improvements in bone health [24-26].

Bone disorders are classified as either disorders of excess bone deposition, characterized by elevated bone density caused by failure of bone resorption by hOCs, as in osteopetrosis, or bone resorption disorders, characterized by increased activity of hOCs, as in osteopetrosis or bone resorption disorders characterised by an increased hOCs activity such as in osteoporosis, lytic bone metastases, multiple myeloma [27], and rheumatoid arthritis [28, 29]. Pharmacological substances have begun to be used in drug therapy to optimize bone quality, targeting the processes leading to bone formation and inhibiting bone resorption [30, 31].

Treatment of bone disorders

Bone-forming drugs

Particular interest has been shown to the development of medications able to stimulate bone formation [32]. Anabolic drugs such as teriparatide, a biosynthetic human parathyroid hormone (PTH), have been shown to increase bone mass and cancellous bone volume. Furthermore, teriparatide restores trabecular bone architecture by binding to the G-protein-dependent parathormone-related peptide receptor (PTHrP) type 1 and activating several signaling pathways, stimulating thereby both the formation and resorption of bones, which depend on the duration and periodicity of exposure to PTH [33-35].

Dual-action bone drugs (strontium ranelate)

Strontium ranelate acts on bone cells in two different ways [36]. It increaes bone formation by stimulating osteoblast precursor replication and simultaneously decreases bone resorption by inhibition of osteoclast differentiation and activity [37-39].

Antiresorptive drugs (ARDs)

A better understanding of complex mechanisms involved in bone resorption has allowed the development of therapeutic drugs, namely bisphosphonates (BPs). These drugs interact with specific pathways within the bone environment to ensure adequate bone remodelling and repair of microdamage to the bone; they also increase bone strength and reduce bone resorption without a concomitant decrease in bone formation. Antiresorptive therapies are grouped under five different classes: BPs, selective estrogen receptor modulators (SERMs), estrogens, monoclonal antibodies such as denosumab and calcitonin [30, 37, 40, 41].

Bisphosphonates (BPs)

Bisphosphonates are used as the leading antiresorptive drugs to prevent pathological fractures when treating disorders with elevated bone resorption [42] such as multiple myeloma and bone metastases from breast- or prostate cancers, as well as tumor-related hypercalcemia and osteoporosis [43-45].

Chemical structure of bisphosphonates (BPs)

Bisphosphonates are derivatives of inorganic pyrophosphate (PPi), in which the oxygen molecule has been changed by carbon (P–C–P) [46]. BPs are resistant to hydrolysis and are insusceptible to biological degradation [46]. The central atom also has two side chains, termed R_1 and R_2, which can be replaced with different ligands to produce BPs with different potencies. The presence of a hydroxy (OH) group at the R_1 position enhances the affinity of molecule binding to the bone [47], resulting in tridentate binding rather than bidentate binding. However, the R_2 side chain is responsible for the antiresorptive potency of the drug [48]. Small changes in this part of the structure (the R_2 chain) can result in large differences in their antiresorptive potencies [48-51].

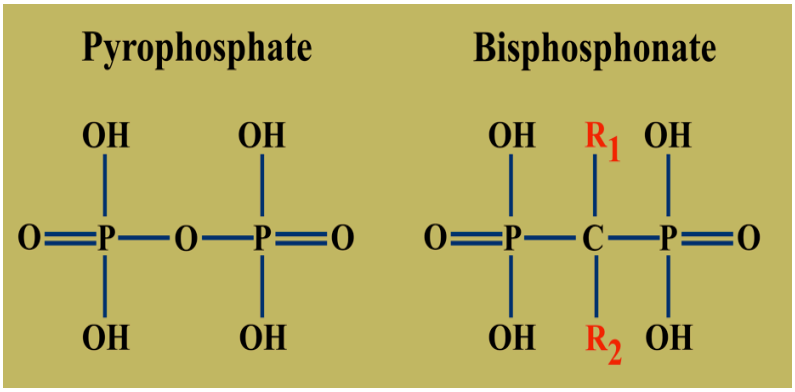
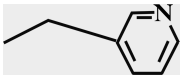


Figure 1: Structure of bisphosphonates [52].

The structure of BPs with its similarity to inorganic pyrophosphate.

According to the R_2 side chain, BPs can be classified into two main classes regarding to the presence or absence of nitrogen [53], and differing in their potencies and kinds of action: the low potency non-nitrogen-bisphosphonates (NN-BPs) and higher potency nitrogen-bisphosphonates (N-BPs) [48, 54-56]. Non-nitrogen BPs have antiresorptive potencies ranging from 1-10, while a nitrogen group increases the antiresorptive potency of N-BPs to a range of between 100 and 10,000 relatively to the NN-BPs **(Table. 1)** [48, 52, 54].

Table 1: Classification of bisphosphonates according to their potencies and routes of administration [52, 57].

Agent	Nitrogen-containing	R_1-side chain	R_2- side chain	Potency	Route of administration
Etidronate	No	-OH	$-CH_3$	1	oral
Clodronate	No	-CL	-Cl	10	oral/iv
Tiludronate	No	-H	-H	10	oral
Pamidronate	Yes	-OH	$(CH_2)_2NH_2$	100	iv
Alendronate	Yes	-OH	$(CH_2)_3NH_2$	500	oral
Ibandronate	Yes	-OH	H_3C $(CH_2)_2N(CH_2)_4CH_3$	1,000	oral/iv
Risedronate	Yes	-OH		2,000	oral/iv
Zoledronate	Yes	-OH		10,000	iv

Bisphosphonates' mechanism of action

The effects of BPs can be analyzed on two levels: cellular and molecular [58]. At the molecular level, NN-BPs act differently than N-BPs. At the cellular level, they act on different kinds of cells; in particular, hOBs, hOCs and bone marrow mesenchymal stem cells (BMMSCs).

Mechanism of action at the molecular level

Bisphosphonates bind to the bone, especially to the bone with high turnover rate, modulating this process and reducing its remodelling when excessive resorption occurs. The phosphate groups gives BPs a high affinity for hydroxyapatite (HA) crystals, whereas the hydroxy group further rises BPs' ability to bind calcium [59].

Non-nitrogen BPs act differently than N-BPs. After NN-BPs are taken in by hOCs, they can be integrated into adenosine triphosphate (ATP). Consequently, ATP accumulates in the cells and these non-hydrolyzable ATP analogues can have cytotoxic effects on hOCs, leading to osteoclast apoptosis due to the inhibition of multiple ATP-dependent cellular processes [60].

In contrast, the nitrogen-bisphosphonates act primarily by inhibition of the farnesyl pyrophosphate (FPP) synthase, a key enzyme in the mevalonate pathway (MVP) [20, 61, 62] **(Fig. 2).** The MVP is a biosynthetic pathway that produces cholesterol and other lipids, and is responsible for posttranslational modification of the small GTPases such as Ras, Rho, Rac and Cdc42 [63]. Those are essential signaling proteins for the normal osteoclasts' morphology, cytoskeleton arrangement, vesicle transport, membrane ruffling and cell survival [17, 51, 52, 63-74].

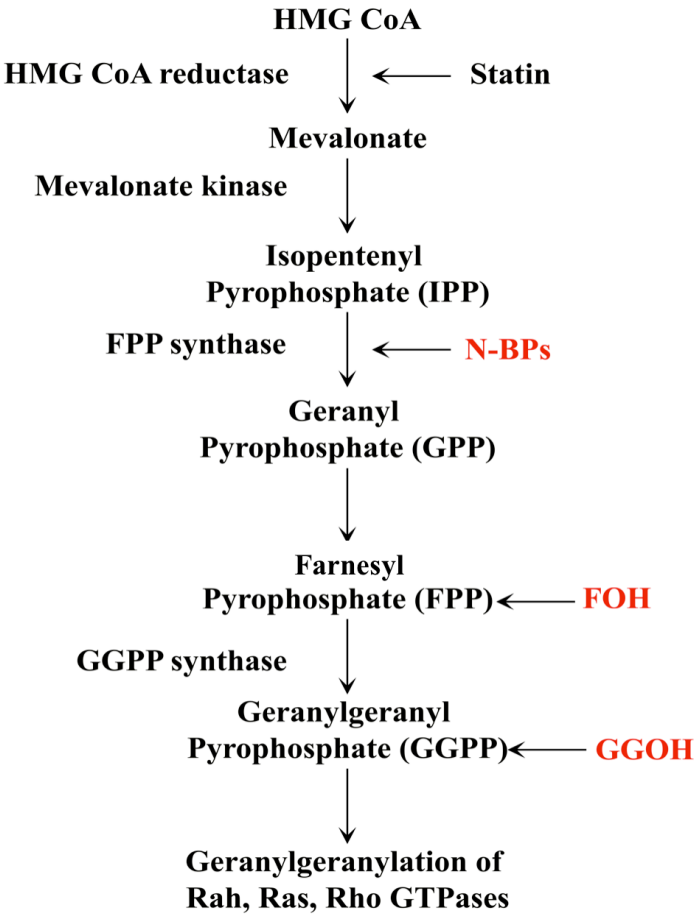


Figure 2: Mevalonate pathway [73].

The role of bisphosphonates (preventing of production small GTPases by inhibition of farnesyl pyrophosphatesynthase (FFPS).

Mechanism of action at the cellular level

At the cellular level, BPs act mainly on hOCs but also on hOBs and BMMSCs.

Potential effects on hOCs

At the cellular level, BPs can reduce bone resorption by different mechanisms, such as inhibiting hOCs retention to the bone surface and reducing osteoclast differentiation [75, 76]. As the bone in which BPs have accumulated starts to be resorbed by hOCs, BPs are released from the bone that will eventually be taken up by hOCs. During bone resorption, the acidic environment created by the hOCs in the resorption area causes a release of more BPs from HA because BPs cannot remain bound to HA at lower pHs. The BPs are then

internalized by the hOCs [77]. This results in an impaired ability of the hOCs to form ruffled borders and to a decreased bone resorption. Eventually, the hOCs become subject to apoptosis. These findings show the negative impact of BPs on osteoclastic differentiation, which results in reduced bone resorption [51, 58, 78-93].

Potential effects on hOBs

The primary pharmacological action of BPs is the inhibition of osteoclast-mediated bone resorption [94], but they can target also hOBs as well as hOCs [95]. Some studies have suggested that BPs stimulate the growth of preosteoblastic cells and thereby increase their differentiation by modulating some of the osteoblastic differentiation markers, such as the ALP activity. In contrast, other studies have suggested that the continuous exposure of hOBs to high-dose BPs could inhibit hOBs function or survival. However, despite the abundant research in this area, demonstrating the direct effects of clinically relevant in vivo doses of BPs on hOBs has proved to be difficult [49, 90, 96-103].

Potential effects on bone marrow mesenchymal stem cells (BMMSCs)

Bisphosphonates have a dose-dependent impact on the proliferation and osteogenic differentiation of BMMSCs. Some studies have shown that ZA at concentrations of 5 μM and 10 μM inhibits the proliferation and osteogenic differentiation of BMMSCs [78]. In contrast, ZA at concentrations of 0.5 μM triggers the proliferation and osteogenic differentiation of BMMSCs and upregulates the ALP activity and the expression of bone morphogenic protein-2 (BMP-2), bone sialoprotein-II, type 1 collagen and osteoprotegerin (OPG) [80, 104-107].

Side effects of bisphosphonates (BPs)

Like any other drug, BPs have advantages and disadvantages. On the one hand, they are widely prescribed and highly effective in limiting bone loss in many disorders that are characterized by increased bone resorption. On the other hand, since BPs are common medications worldwide, the potential side effects of oral and intravenous (IV) administration of these drugs are not be underestimated.

Short-term adverse effects of bisphosphonate therapy

Gastrointestinal side effects, such as erosive esophagitis, are the most common side effects of oral BPs [108]. Patients who receive IV BP therapy may witness inflammatory symptoms

such as fever [108]. Acute-phase reactions occur only when IV aminobisphosphonates are administered, particularly ZA. Ocular side effects associated with BP therapy include conjunctivitis, uveitis, episcleritis, scleritis, and keratitis [108-120].

Long-term adverse effects of bisphosphonate therapy

Bisphosphonates are eliminated renally by glomerular filtration and proximal tubular secretion [121]. Renal complications of BPs have been observed, ranging from focal segmental glomerular sclerosis with nephrotic syndrome to acute kidney injury and tubular necrosis. Current recommendations limit the use of these drugs in patients with renal insufficiency depending on the GFR level [122], and suggest creatinine monitoring of their use, especially in patients who receive them intravenously [121, 123-126].

Atrial fibrillation is another side effect of BPs, seen mainly after IV administration. It may be triggered shortly after an infusion with ZA or other IV BPs [127].

As previously mentioned, BPs can cause erosive esophagitis, especially in patients who do not maintain upright body position after taking BPs. If this condition persists for a long time, it may result in Barrett's esophagus (BE), a very serious side effect, since it is known as the premalignant precursor lesion in most cases of esophageal adenocarcinoma. Patients with BE have higher risks to develop esophageal adenocarcinoma [128].

Another long-term side effect of BP therapy is the occurrence of atypical femoral fracture (AFF). Long-term BP therapy has been found to possibly suppress the bone remodelling. The reduced bone turnover by BP therapy alters bone mineral, leading to a reduced ability to repair skeletal microtraumas and an increased skeletal instability [129]. Saito et al. [130] mentioned that BP therapy increases the advanced glycation end products of the extracellular bone matrix, worsening the mechanical features of the bone [130]. Elongated BP therapy causes repetitive microdamage to the bone and reduces the diversity of organic matrix and mineral features [130-139].

Medication-related osteonecrosis of the jaw (MRONJ) has been widely reported in clinical literature as an adverse side effect of BP- and antiangiogenic therapy [108, 140]. The incidence of MRONJ is higher for patients receiving IV BPs than those receiving them orally [141]. For cancer patients who received ZA intravenously, it is estimated at around 0.7% - 6.7%. However, for osteoporotic patients who have been treated with ZA orally, it is estimated at around 0.017% - 0.04% [5].

Medication-related osteonecrosis of the jaw (MRONJ)

Medication-related osteonecrosis of the jaw is a serious complication of the use of antiresorptive- or antiangiogenic drugs [142]. It involves progressive bone destruction in the mandible or maxilla. Marx et al. [143] reported the first cases of bisphosphonate-related osteonecrosis of the jaw (BRONJ) in 2003. The American Association of Oral and Maxillofacial Surgeons (AAOMS) (update 2014) [5] has defined MRONJ as a necrotic bone exposed to the oral cavity [144] for a period of at least eight weeks without signs of the wound healing, associated with present or previous treatments with antiresorptive- or antiangiogenic drugs and the absence of a radiation therapy or metastatic diseases to the jaw [5, 145, 146]. Medication-related osteonecrosis of the jaw was previously known as BRONJ, related to the use of BPs only, but AAOMS changed the terminology because of the increasing number of ONJ cases related to other antiresorptive- and antiangiogenic medications [147]. These include not only BPs, but also denosumab (a RANKL-inhibitor) and inhibitors of angiogenesis such as bevacizumab. The incidence of MRONJ depends on several factors, including the type of BP used (ZA and pamidronate disodium being the most potent), the route of administration (the IV route is associated with a higher risk), and the dosage and duration of therapy (the longer the duration and the higher the dose, the higher the risk). Medication-related osteonecrosis of the jaw ends in significant morbidity, adversely affecting the quality of life, and representing a major clinical challenge for oral and maxillofacial surgeons [2, 143, 148-153].

Stages of MRONJ

Table 2: Stages of MRONJ [5, 154].

Stage	Description	Management
Stage 0	Asymptomatic patient, radiographic results suggesting necrotic bone, but fistula may be present	Antibacterial mouth rinse Pain treatment and antibiotic therapy if symptomatic Patient education
Stage 1	Exposed necrotic bone without pain or infection	Antibacterial mouth rinse Control every 3-4 months Patient education
Stage 2	Pain and/or infection, exposed necrotic bone or fistula	Antibacterial mouth rinse Pain treatment and antibiotic therapy Debridement Patient education
Stage 3	Exposed necrotic bone or fistula with evidence of infection and at least one of the following: a) Exposed necrotic bone beyond the region of alveolar bone b) Fracture c) Extraoral fistula d) Oral-antral or oral-nasal connection e) Osteolysis lengthening to the inferior border of mandible or sinus floor in the maxilla	Antibacterial mouth rinse Pain medication and antibiotics Debridement Patient education

Why does MRONJ occur almost exclusively in the jaw bones?

The jaw bones are the most predisposed to MRONJ because of the high bone turnover rate, in the alveolar parts [155]. Alveolar bone has a high bone turnover, which means that alveolar bone can incorporate far more BPs than other skeleton sites. It appears that tissue homeostases in the mandible and maxilla bones are disrupted in MRONJ. However, the exact reason why MRONJ specifically affects the jaw is still unknown. The jaw bones are the bones least protected from infection in the human skeleton. They are separated from oral pathogens only by a thin mucoperiosteal cover, as opposed to deep soft tissues and skin that protect other bones. The continuity of the gingiva is interrupted by the presence of teeth, creating a potential entrance for bacterial infection. Medication-related osteonecrosis of the jaw is two times more frequent in the mandible than in the maxilla [156, 157]. The jaws are also subject

to repeated microtraumas due to the presence of teeth and the force of mastication. This may be another reason for the occurrence of MRONJ, since there are reports of exposed bones as a result of chronic local microtraumas from unfit dentures [156, 158-160].

Hypothesis on MRONJ pathophysiology

A single hypothesis is unlikely to explain the pathophysiology of MRONJ: many hypotheses partially explain it from various aspects.

Inhibition of bone remodelling

Bisphosphonates attenuate hOCs and, indirectly, the osteoblast-mediated differentiation of hOCs, which results in a decreased bone turnover. Osteoclast differentiation plays an important role in bone remodeling [161]. Alveolar bone may show a higher remodeling rate than other bones in the body, which could explain the predisposition of the jaw to MRONJ [5, 50, 162-173].

Inflammation and infection

Inflammation and infection are believed to play a role in the development and progress of MRONJ. Tooth extraction is usually the most common occasion associated with MRONJ, but the teeth are most frequently extracted due to periapical and periodontal infections and inflammation [173]. For multiple myeloma and metastatic cancer patients, intensive dental hygiene decreases the incidence of MRONJ [173]. Therefore, it is necessary to treat the teeth-associated inflammation and infection before starting the BP therapy. Following the administration of BPs, it is essential to perform regular and frequent dental checks to prevent any soft or hard tissue inflammation because of a tooth disease [5, 50, 162-172].

Inhibition of angiogenesis

Angiogenesis is a complex process building new blood vessels. These processes involve several signaling molecules which bind to their receptor and activate them. These signals induce new blood vessel formation [174]. The interruption of bone blood supply, which may be a result of treatment with anti-angiogenesis agents, may consequently lead to avascular necrosis of the jaw bones [5, 50, 162-172].

Other hypotheses

Another hypothesis in MRONJ pathophysiology is the soft tissue toxicity of BPs [173]. In vitro studies have demonstrated that N-BPs accumulate not only in the bone, but also in the epithelial tissue [162]. According to the position paper (AAOMS) (update 2014) [5], soft tissue toxicity has not been reported related to the denosumab [5, 50, 162-172].

Treatment of MRONJ

Depending on the stage of MRONJ, AAOMS (update 2014) [5] recommends either non-surgical or surgical treatments. Non-surgical treatment has generally been recommended in preference to surgery for stages 0 and 1 of MRONJ. Non-surgical treatment includes a combination of antiseptic mouth rinses and antibiotics. Bactericidal solutions such as chlorhexidine are indicated for patients who have developed an exposed or necrotic bone without any symptoms or signs of infection. Patients with soft tissue infections require a treatment with antibiotics such as amoxicillin and clavulanic acid or clindamycin [175].
New and innovative treatment strategies for MRONJ have been developed, such as platelet-rich plasma and platelet rich fibrin [176, 177]. Mesenchymal stem cell (MSC) concentrates can be added to the wound before wound closure. Increased knowledge about MRONJ suggests that surgical therapy can stop the MRONJ advancement, allowing a histopathological examination of the necrotic bone tissue [178]. Many MRONJ patients have been treated according to the staging guidelines in the AAOMS position paper, in which surgical intervention was recommended to treat stages II and III. Surgical interventions can reach success rates higher than 90% and the detection of the bone fluorescence can assist in the surgical treatment of the osteonecrosis [5, 79, 178-185].

Geranylgeraniol (GGOH) in the mevalonate pathway (MVP) for treatment of MRONJ

Geranylgeraniol (GGOH) is an acyclic diterpene alcohol and one of the important constituents of essential oils. It is a natural molecule that can be extracted from different plants or synthesized industrially and that has been also found in human beings as a metabolite of the MVP. It is used not only as one of the ingredients in perfumes, but also as a material for synthesis of pharmacological mediators and for synthesis of hydrophobic vitamins, for instance A and E [186]. Recent studies have mentioned the anti-inflammatory, anti-tumorigenic and neuroprotective activities of GGOH [19, 186-198]. Nitrogen-containing

bisphosphonates result in inhibition of GGOH production by inhibition of FPPS in the MVP. Consequently, the decreased synthesis of the metabolite GGOH facilitates the development of MRONJ. Studies have shown that GGOH can reverse the negative effect of BPs on some cell lines. Some animal model studies demonstrated the possible positive effect of GGOH on wound healing in the early stages of MRONJ [19, 186-198].

Mechanism of action of GGOH on bone cells

Farnesyl- and geranylgeranyl pyrophosphate GGPP) are precursors for post-translational maturation of diverse proteins involved in cell growth [199]. Geranylgeraniol is necessary for post-translational maturation and membrane localization of intracellular proteins, particularly small GTPase proteins (G-proteins), such as Ras and Rap, which are involved in several signaling pathways and influence the cytoskeleton [200, 201]. G-proteins stabilize the cytoskeleton and promote the differentiation and formation of hOCs. This finding could be relevant to both the function and survival of hOCs.

Geranylgeraniol can also be used as an antitumor agent inducing apoptosis in different cancer cell lines [197]. For example, Takeda et al. [197] have demonstrated that GGOH induces apoptotic cell death of human hepatoma cells by stimulating caspase-3 activity, and Fernandes et al. [202] showed that it can suppress the viability of human DU145 prostate carcinoma cells [197, 202-205].

AIM OF THE STUDY

Nitrogen-containing BPs inhibit a key enzyme of the MVP namely FPPS [206]. This results in a lower production of Geranylgeranyl pyrophosphate (GGPP), which is an important metabolite for prenylation of small GTPase proteins. Our research begins with the hypothesis that external supplementation of GGOH, which can be converted in the cells to Geranylgeranyl pyrophosphate (GGPP), may reverse the previously described negative effect of BPs. This study investigates the effect of GGOH on hOBs and hOCs that have been treated with various concentrations of ZA/GGOH for a duration of seven days.

CHAPTER 2 MATERIALS AND METHODS

MATERIALS

Table 3: Reagents used in our experiments

Material	Catalogue Number	Company	Country
Acid Phosphatase, Leukocyte (TRAP) Kit	386A-1KT	Sigma-Aldrich	Mannheim, Germany
Acrylamide solution	1610158	BIO-RAD	Munich, Germany
Anti-GAPDH	MAB5718	R&D Systems	Minneapolis, USA
Anti-mouse (H&L) IgG antibody	610-1102	Rockland	Hamburg, Germany
Anti-rabbit IgG, HRP-linked antibody	7074P2	Cell Signaling	Frankfurt am Main
Dulbecco's Modified Eagle Medium (DMEM)	31966-021	Gibco Life Technologies	Munich, Germany
Fetal bovine serum (FBS)	F9665	Sigma-Aldrich	Munich, Germany
Geranylgeraniol (GGOH)	G3278-100MG	Sigma-Aldrich	Munich, Germany
Human Osteoblast Growth Medium	C-27001	Lonza	Heidelberg, Germany
Human osteoblasts (hOBs)	C-12720	PromoCell	Heidelberg, Germany
Human osteoclasts (hOCs)	2T-110	Lonza	Basel, Switzerland
Live/Dead staining	PK-CA707-30002	Promokine	Heidelberg, Germany
M-CSF	300-25-10UG	PeproTech	Hamburg, Germany
Micro BCA Protein Kit	23235	Thermo Fisher Scientific	Munich, Germany
Page Ruler Plus	26619	Thermo Fisher Scientific	Munich, Germany
Penicillin/Streptomycin	A2213	Biochrom GmbH	Berlin, Germany
RANKL	310-01-10UG	PeproTech	Hamburg, Germany
Rap 1A/B antibody	VPA00481	BIO-RAD	Munich, Germany
RIPA buffer	PI89900	Thermo Fisher Scientific	Munich, Germany
TEMED	2367.3	Carl Roth GmbH	Karlsruhe, Germany
Trypsin/EDTA	L2153	Biochrom GmbH	Berlin, Germany
TWEEN 20	SLBR3776V	Sigma- Aldrich	Munich, Germany
Water soluble tetrazolium-1 assay (WST-1)	11644807001	Sigma-Aldrich	Mannheim, Germany
Zoledronate (ZA)	118072-93-8	Chemos	Regenstauf, Germany

METHODS

Culture of cells

Human osteoblasts (hOBs)

Human osteoblasts (hOBs) were cultured at a density of 1.0×10^6 cells in hOB media in T_{225} flasks and incubated at 37°C and 5% CO_2 until they reached 80-90% confluency. The cells were then trypsinized with 0.5% Trypsin/0.2% EDTA, counted by a hemocytometer and cultured in six-well plates at a density of 3.5×10^4 cells/well for the experiments. The culture medium was changed twice per week. For our experiments, cells from two different lots were used between passages 3 and 6.

Human osteoclasts (hOCs)

Human osteoclast precursor cells were cultured in six-well plates in culture media consisting of high glucose Dulbecco's modified Eagle medium (HG-DMEM) supplemented with 15% fetal bovine serum (FBS), 1% penicillin/streptomycin (Pen/Strep: 10000 U/ml/10000 μg/ml), 25 ng/ml macrophage colony-stimulating factor (M-CSF) and 50 ng/ml receptor activator of nuclear factor-kappaB ligand (RANKL) as the essential cytokines responsible for the differentiation of osteoclast precursor cells into mature hOCs [207, 208]. The plates were incubated in an incubator at 37°C and 5% CO_2 for 14 days. Human osteoclasts at passage 1 were used in the experiments. Cells were cultured at a density of 1.0×10^4 cells/well, and the culture media were changed twice per week.

Preparation of drugs

Zoledronate (ZA)

Zoledronate (ZA) was diluted in 0.9% NaCl (sodium chloride; physiological saline) to make stock solutions of 20 μM and 20 mM. The solutions were sterile-filtered and kept at −20°C until they were used in the experiments. The stock solutions were diluted in the appropriate culture media and final concentrations of 0.1 μM, 25 μM and 100 μM ZA were thereby prepared.

Geranylgeraniol (GGOH)

Geranylgeraniol (GGOH) was purchased in liquid form. A stock solution of 5 mM was prepared by diluting GGOH in pure undenatured ethanol. It was then sterile-filtered and kept at −20°C until it was used in the experiments. The stock solution was then diluted in appropriate culture media to gain GGOH concentrations of 10 μM, 20 μM, 40 μM, and 80 μM [17].

Cell culture treatment

Bone cells were cultured in six-well plates in suitable culture media. Cells without administration of any drugs served as a negative control, and those treated with only ZA at various concentrations served as a positive control. The experimental group (test group) comprised the cells which were co-treated with different concentrations of ZA and GGOH [17].

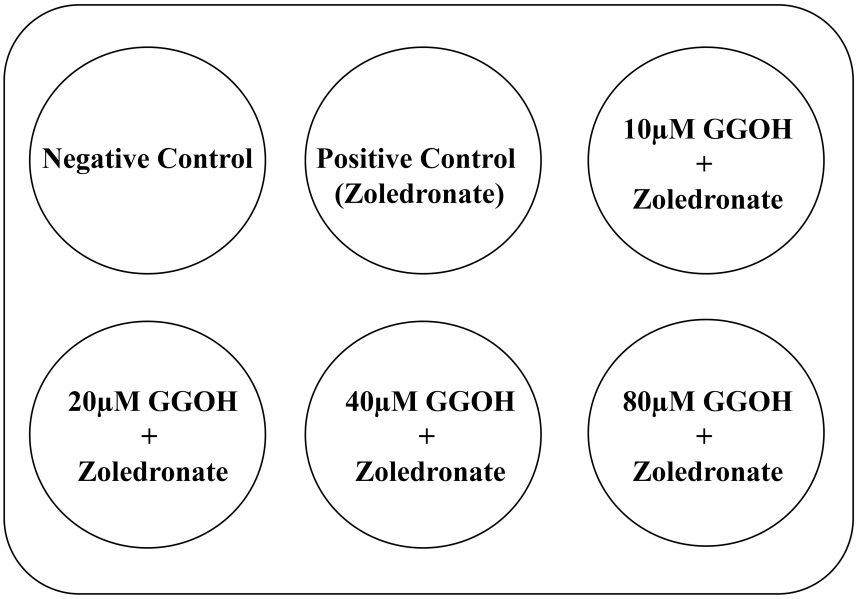


Figure 3: The setup of the cell culture plates for the experiments.

Cell viability by water-soluble tetrazolium-1 assay (WST-1 assay)

Human osteoblasts and osteoclasts (hOBs and hOCs) were cultured in six-well plates as mentioned above. On the next day, the culture medium was changed with fresh media containing drugs with the required concentration and incubated for seven days at 37°C and 5% CO_2. The viability of bone cells was determined by measuring the mitochondrial dehydrogenase activity using WST-1 assay [209] according to the manufacturer's guidelines. In brief, cells were washed with phosphate buffer saline (PBS), after which WST-1 reagent was diluted in fresh medium at a ratio of 1:10. The cells were incubated for 4 h at 37°C and 5% CO_2 in an incubator. The absorbance was determined at 450 nm against a reference wavelength of 620 nm using an ELISA reader [210] (Thermo Fisher Scientific, Munich, Germany) [211]. All measurements were performed in triplicate and repeated three times.

Cell viability by live/dead staining

Cell viability was performed using PromoKine's Live/Dead Cell Staining Kit II according to the instructions of the manufacturer. First, the dye stock solutions were warmed up to room temperature. After adding 5 µl of 4 mM Calcein-AM and 20 µl of 2 mM EthD-III to 10 ml of PBS, a staining solution of 2 µM Calcein-AM/4 µM EthD-III was prepared. The cells were washed twice with PBS. For adherent cells, Calcein-AM/EthD-III staining solution was added to the cell monolayer. The samples were wrapped in foil and incubated for at least 30 min at 37°C. Pictures were taken by fluorescent microscope (AxioObserver Z1; Zeiss, Oberkochen, Germany). The live/dead assay was performed three times from two different donors [17].

Tartrate-resistant acid phosphatase (TRAP staining)

Tartrate-resistant acid phosphatase (TRAP) staining is a marker of hOCs and it is localized in the lysosomal compartment of the macrophages [212]. A positive relationship between TRAP-secretion in hOCs and the bone resorption activity has been described [213].
Human osteoclasts (hOCs) were treated with ZA/GGOH over a period of seven days, and culture media were changed twice per week. After one week of cell culture treatment, media were discarded, and cells were washed with PBS. Deionized water was prewarmed to 37°C and the fixative media were kept at a temperature of 18-26°C. Human osteoclasts (hOCs) were then fixed using a fixative solution for 30 seconds and rinsed with deionized water.
Tartrate-resistant acid phosphatase-staining material was prepared according to the

manufacturer's instructions, and cells were incubated for 1 h at 37°C. Nuclear counterstaining was then performed with hematoxylin and the cells were rinsed for several minutes in alkaline tap water to develop blue nuclei. After TRAP staining, TRAP-positive multinucleated cells were visualized under a contrast microscope (Zeiss, Oberkochen, Germany), and pictures were taken [17].

Western blot analyses of Rap 1A/B protein

Protein isolation

Human bone cells were cultured in six-well plates in the appropriate culture medium and then treated with ZA/GGOH for seven days. After one week, the medium was discarded, the cells were washed with PBS, and 500 µl ice-cold RIPA buffer with protease was added to each well. Cells were then scraped off the plate using a plastic cell-scraper. The cell suspensions were transferred into a microcentrifuge tube, which was incubated on ice for 30 min. The cells were homogenized by sonication (2 × 2 sec) and incubated again for 30 min on ice. After incubation, they were centrifuged at 10,000 × g for 10 min in a centrifuge pre-cooled to 4°C. The supernatant was transferred to a new microcentrifuge tube, and the pellets were discarded [17].

Micro BCA protein assay

The Thermo Scientific™ Micro BCA™ Protein Assay Kit was used for colorimetric measurement of total protein [214]. Bovine serum albumin (BSA) was diluted through a serial dilution in the vials A-H **(Figure 5)**. Protein samples were diluted in distilled water at a dilution of 1:10 and prepared for the measurement. The working reagent (WR) was made by mixture 25 parts of Micro BCA Reagent A (MA) and 24 parts Reagent B (MB) with one part of Reagent C (MC) [215].

Total volume of WR was determined by using the following formula:
(# Standards + # Unknowns) × (# Replicates) × (Volume of WR per sample) [216].
Each standard or unknown sample (100 µl) was pipetted into a 96-well microplate in duplicates, incubated at 37°C for 1 h and then measured using an ELISA Reader to measure the amount of protein in each well at 562 nm against a standard curve (Thermo Fisher Scientific, Munich, Germany).

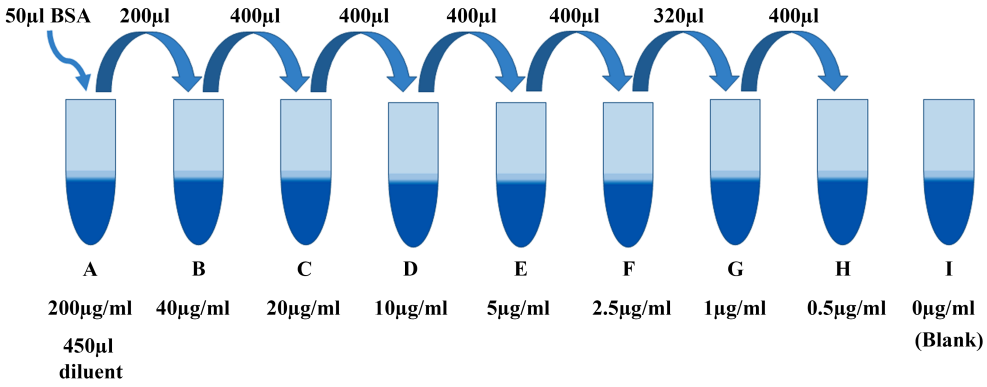


Figure 4: Preparation of diluted bovine serum albumin (BSA) standards

Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A separating gel with an optimal concentration of 15% and a thickness of 1.5 mm was prepared for western blotting. After preparing resolving and stacking gels, 10 µg of each protein sample was mixed with 5 µl of 4× Laemmli buffer/ß-mercapthol. The samples were then mixed in a screw-cap microcentrifuge tubes and boiled for 5 min at 100°C. Samples were loaded in the desired order, and 7 µl of PAGE Ruler Plus Prestained Protein Ladder was added to a separate column as a molecular weight marker. Samples were then run in 1× running buffer at a constant electric current of 50 mA for approximately 1 h, until the blue dye started to move to the end of the gel. Following SDS-PAGE, wet electroblotting was used to transfer the bands from the gel to the Polyvinylidene difluoride (PVDF) membrane. The gel, filter paper and fiber pads were equilibrated for 15 min in 1x blotting buffer. The PVDF membrane was charged for 5 seconds at RT in methanol and then rinsed with distilled water for one minute. After preparing the gel sandwich, the cassette was closed and run in 1× blotting buffer on a magnetic stirrer at 30 V overnight at 4°C.

Immunodetection

The membrane was rinsed in 5% skimmed milk as a blocking solution for one h at room temperature on an orbital shaker. Then, it was washed two times with washing buffer (tris-buffered saline and polysorbate 20 [TBST]). The primary antibody (Rap 1A/B antibody) was diluted in 5% skimmed milk at a dilution of 1:500, and the membrane was incubated with the

primary antibody at 4°C on an orbital shaker overnight. The following day, the membrane was rinsed twice with washing buffer for 15 min at room temperature and then it was incubated with the secondary antibody, goat anti-rabbit IgG, an HRP-linked antibody, which was diluted in 5% skimmed milk at a dilution of 1:4000 for one h at room temperature on an orbital shaker. After final washing, protein bands were detected with Luminata™ Forte Western HRP Substrate and with ImageQuant. The western blot bands were quantified using Image J software (https://imagej.nih.gov/ij/, version 1.52d, National Institutes of Health, Maryland, USA).

After detecting Rap 1A/B bands with ImageQuant, the PVDF membrane was washed and then stripped in stripping buffer at 50°C for 10 min in a hybridizer with constant rotation. It was then rinsed twice with washing buffer for 10 min at room temperature with moderate shaking. The membrane was then blocked for 60 min and incubated in the primary antibody (anti-glyceraldehyde 3-phosphate dehydrogenase [anti-GAPDH]), which was diluted in 5% skimmed milk at a dilution of 1: 2,000 at 4°C overnight. Afterwards, the membrane was washed again in washing buffer and incubated with the secondary antibody (anti-mouse (H&L) IgG antibody, diluted in 5% skimmed milk at a dilution of 1: 4,000) for one h at room temperature. The GAPDH bands were visualized using ImageQuant and quantified with Image J software.

Statistical analysis

All experiments were performed in triplicate with two different lots. Analysis of variance (ANOVA) was used to analyze the data. Data are presented as mean ± standard deviation. The significance level of the *p*-value was set at 0.05. The significance was expressed as $p<0.05$, $p<0.01$, $p<0.001$ and $p<0.0001$. The results were achieved using GraphPad Prism version 5.00 for windows (GraphPad Software, San Diego California USA (https://www.graphpad.com/) [17].

CHAPTER 3 RESULTS

Cell viability by WST-1 assay

Human bone cells (hOBs, hOCs) were treated with ZA, GGOH or a combination of ZA and GGOH for a duration of seven days. Bone cells cultured in normal culture media without any drug treatment served as a negative control. Bone cells treated with various concentrations of ZA/GGOH served as positive controls [17].

Effect of ZA on hOBs

The viability of the cells was 100% at negative control. The lowest concentration of ZA (0.1 μM) did not significantly affect cell viability; rather, it increased cell viability to about 120%. However, higher ZA concentrations, such as 25 μM and 100 μM, decreased the viability to around 80% and 20%, respectively, compared to the negative control.

Effect of GGOH on hOBs

Treatment of cells with low or moderate concentrations of GGOH (10-40 μM) increased viability to almost 140%. However, 80 μM GGOH suppressed the viability compared to other concentrations. These findings showed that GGOH increased the cell viability up to certain concentrations. However, a higher concentration of GGOH (80 μM) showed a dose-dependent cytotoxic effect on hOBs [17].

Effect of ZA and GGOH on hOBs

The negative effect of ZA was counteracted by co-treatment of hOBs with ZA and GGOH. After the addition of 10 μM and 20 μM GGOH, cell viability was increased at 0.1 μM ZA to around 140% and 120%, respectively. However, cell viability was increased at 25 μM treated with the same GGOH concentrations to around 220-230% and at 100 μM ZA to 120-130%, respectively. The treatment of the cells with 40 μM and 80 μM GGOH increased the viability at 0.1 μM ZA to about 120%, almost the same as the positive control.

Treatment of hOBs with 25 μM ZA and 40 μM GGOH led to an enhanced effect of cell viability. The viability was almost the same as when treated with 25 μM ZA and 20 μM GGOH. Treatment of hOBs with 80 μM GGOH and 100 μM ZA affected the viability in a positive manner in comparison with the negative control. These results demonstrate that GGOH had a dose-dependent positive effect on cell viability up to 40 μM. However, higher

concentrations of ZA/GGOH (100 µM ZA/40 µM GGOH) led to a coinhibitory and cytotoxic effect on bone cells [17].

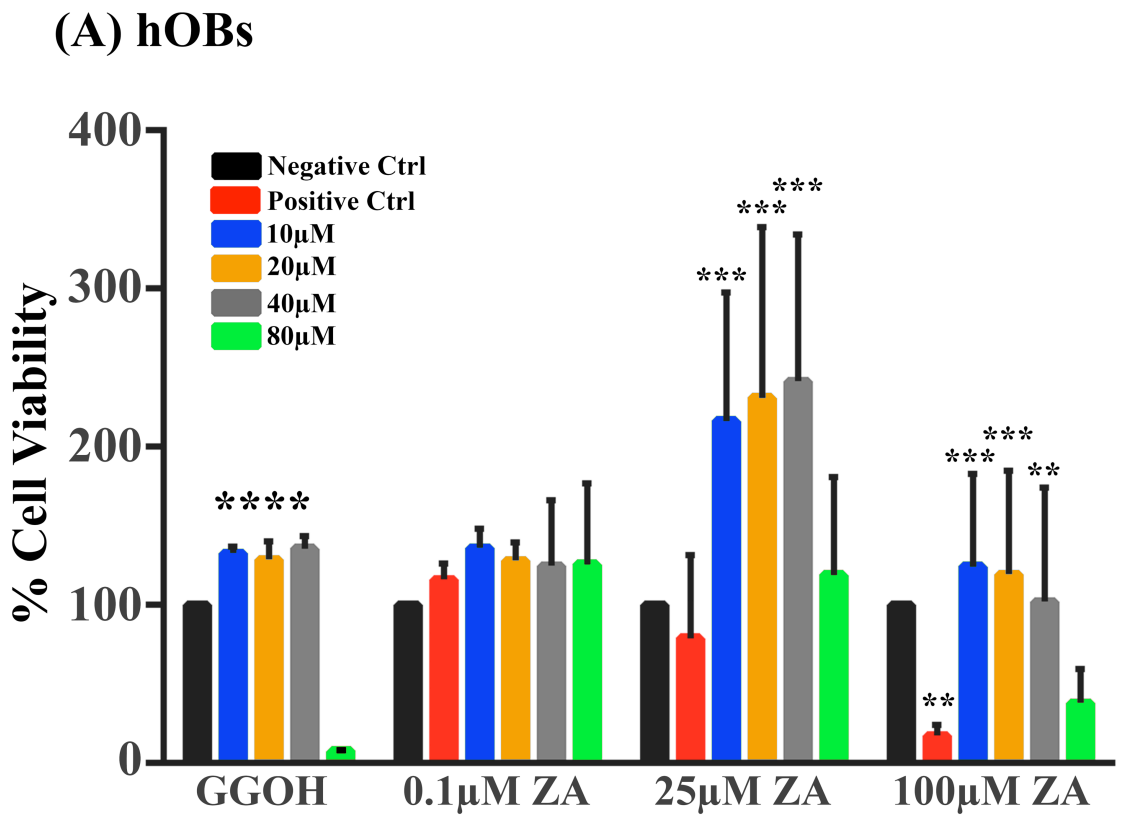


Figure 5: Water-soluble tetrazolium salt assay of hOB [17].

Human osteoblasts were cultured at different concentrations of GGOH (10-80 µM), ZA (0.1 µM, 25 µM, 100 µM) or combined ZA and GGOH for seven days. A two way ANOVA test was performed for multiple comparisons. Significant differences were observed between controls and test-groups:

* p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001

Effect of ZA on hOCs

The viability of the cells was considered to be 100% at negative control. The lowest concentration of ZA (0.1 μM) did not significantly affect cell viability. However, higher ZA concentrations, such as 25 μM and 100 μM, decreased viability to around 80% and 50% respectively, compared to the negative control [17].

Effect of GGOH on hOCs

Treatment of cells with low or moderate concentrations of GGOH (10-40 μM) increased the viability to around 120-135%. However, 80 μM GGOH strongly suppressed viability compared to other concentrations. These findings showed that GGOH alone increases viability up to certain concentrations. However, a higher concentration of GGOH (80 μM) has a dose-dependent cytotoxic effect on hOBs [17].

Effect of ZA and GGOH on hOCs

The negative effect of ZA was counteracted by co-treatment of hOCs with GGOH. After the addition of 10 μM, 20 μM and 40 μM GGOH, cell viability at 0.1 μM ZA was almost the same as in the positive control. However, viability was increased after addition of 20 μM and 40 μM GGOH to 25 μM ZA to around 150% ([25 μM ZA, 20 μM GGOH] and [25 μM ZA, 40 μM GGOH]). The addition of 10 μM, 20 μM, and 40 μM GGOH increased the viability at 100 μM ZA up to 100% in comparison to positive control (50%). 80 μM GGOH decreased the viability at 0.1 μM ZA to be about 40%.

The treatment of hOCs with 80 μM GGOH and 100μM ZA affected the viability in a negative manner. These results demonstrated that GGOH had a dose dependent positive effect on cell viability up to 40 μM. However, a higher concentration of ZA/GGOH led to a coinhibitory and cytotoxic effect on bone cells [17].

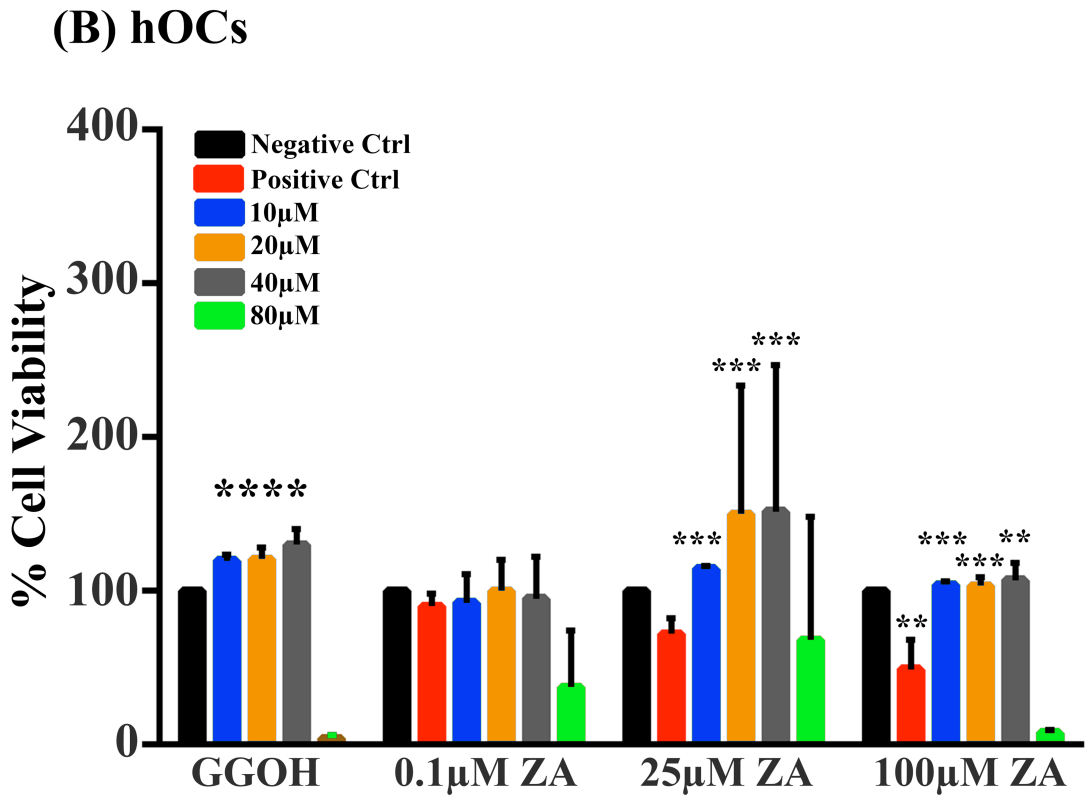


Figure 6: WST-1 assay of hOCs [17].

HOCs were cultured at different concentrations of GGOH (10-80 µM), ZA (0.1 µM, 25 µM, 100 µM) or combination of ZA/GGOH for seven days. Two way ANOVA test was done for multiple comparisons. Significant difference between controls and test-groups:

*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

Cell viability by Live/Dead staining

Live/dead staining with Calcein-Am and ethidium homodimer was used to examine cell viability under fluorescence microscope.

Live/dead assay of hOBs

Human osteoblasts (hOBs) cultured in normal osteoblast culture media without any drug treatment served as a negative control, and cells treated only with ZA served as a positive control [17]. The test group comprised cells treated with a combination of ZA and GGOH.
In contrast to 0.1 μM ZA, higher concentrations of ZA (25 μM and 100 μM) resulted in decreased cell survival, detected by decreases in cell numbers and density of living cells. This negative effect was reversed by the addition of GGOH up to 40 μM. However, at a concentration of 80 μM GGOH, lower cell density and lower fluorescence activity were detected. These results show that GGOH had a dose-dependent positive effect on rescue of hOBs treated with various concentrations of ZA. Lower concentrations of GGOH (10, 20, 40 μM) antagonized the negative effect of ZA. However, higher concentration of GGOH (80 μM) led to a negative effect on survival of hOBs [17].

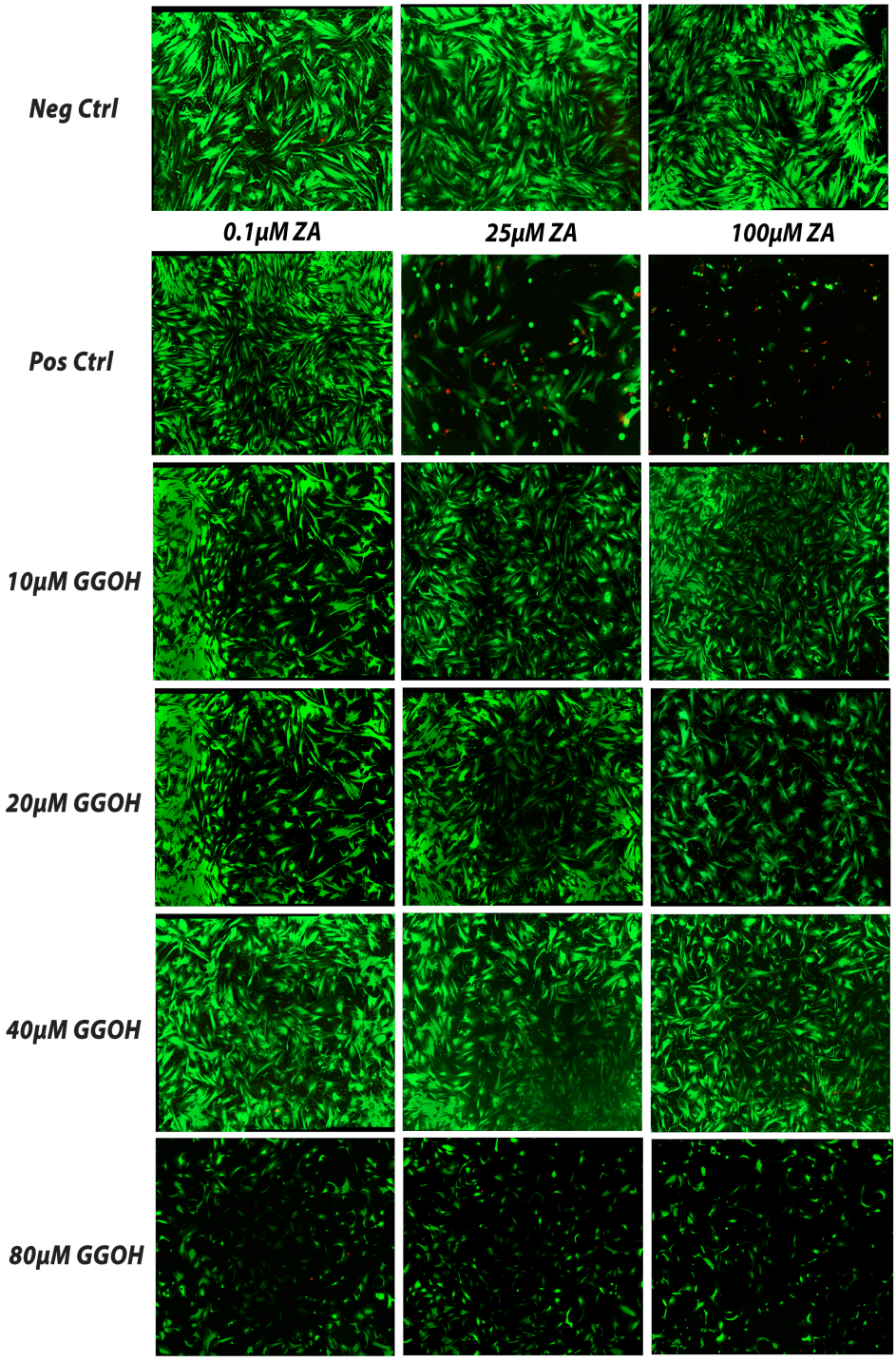


Figure 7: Live/dead fluorescence microscopy of hOBs treated with ZA/GGOH

Live/dead assay of hOCs

Human osteoclasts (hOCs) cultured in normal osteoclast culture media without any drug treatment served as a negative control, and cells treated only with ZA served as a positive control [17].

In contrast to 0.1 μM ZA, higher concentrations of ZA (25 μM and 100 μM) resulted in decreased cell viability, detected by decreases in cell number and density of living cells. This negative effect was reversed by the addition of GGOH up to 20 μM. However, at concentrations of 40 μM and 80 μM GGOH, lower cell density and fluorescence activity were detected. The results of this thesis showed that GGOH could rescue hOCs by reversing the negative effects of ZA in a dose-dependent manner.

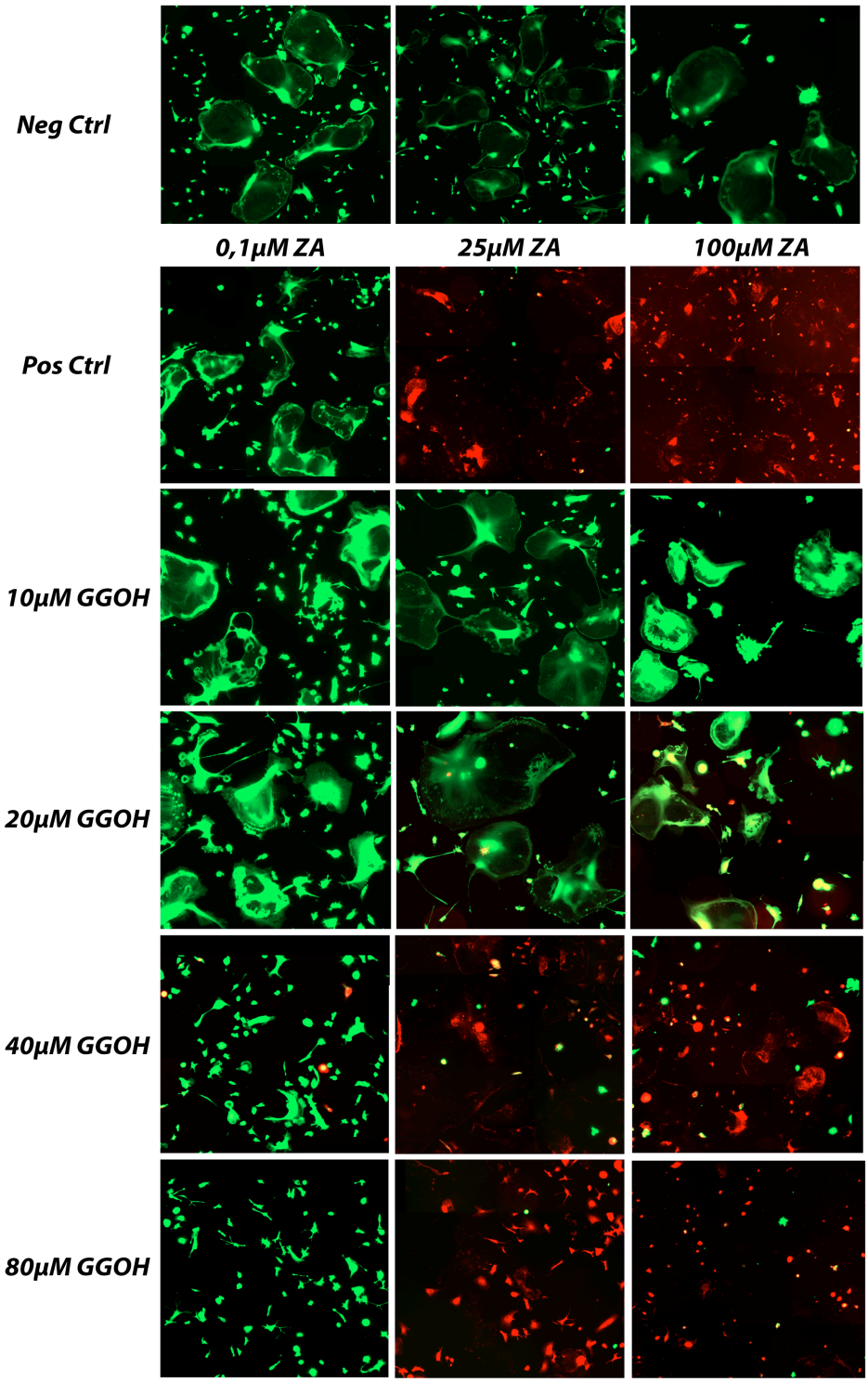


Figure 8: Live/dead fluorescens microscopy of hOCs treated with ZA/GGOH

Tartrate-resistant acid phosphatase staining (TRAP-staining)

Tartrate-resistant acid phosphatase is highly expressed in hOCs and therefore used as a histochemical marker for hOCs [217, 218]. In this experiment, the impact of ZA or ZA/GGOH on the morphology of the osteoclasts was examined by TRAP staining. As demonstrated in **Figure 9**, hOCs cultured in normal culture media without any drug treatment served as a negative control, and hOCs cultured at various concentrations of ZA served as a positive control. $TRAP^+$ multinucleated osteoclasts were visualized in great amounts in the negative control. The addition of 0.1 µM ZA to the culture media did not affect the osteoclast proliferation or morphology. However, a higher ZA concentration of 25 µM caused ruffled borders to be lost, the shape of the cells to change to small round forms, and the number of hOCs to decrease. The highest concentration of ZA (100 µM) showed the maximal inhibitory effect on osteoclasts proliferation, changing the shape of hOCs to small particles.

After the addition of GGOH at concentrations of 10-40 µM to 0.1 µM and 25 µM ZA, the cells maintained their shapes as large multinucleated cells. In contrast, the addition of 40 µM GGOH to 100 µM ZA led to a reduced number of $TRAP^+$ multinucleated cells. Human osteoclasts (hOCs) treated with the highest concentration of GGOH (80 µM) and various concentrations of ZA lost their morphology, changing their shape to small round forms with a reduced number of cells. These findings showed that GGOH had a positive effect on maintaining of osteoclasts morphology up to certain concentrations, while a higher concentration of GGOH (80 µM) with ZA has a dose-dependent and coinhibitory cytotoxic effect with ZA on hOCs.

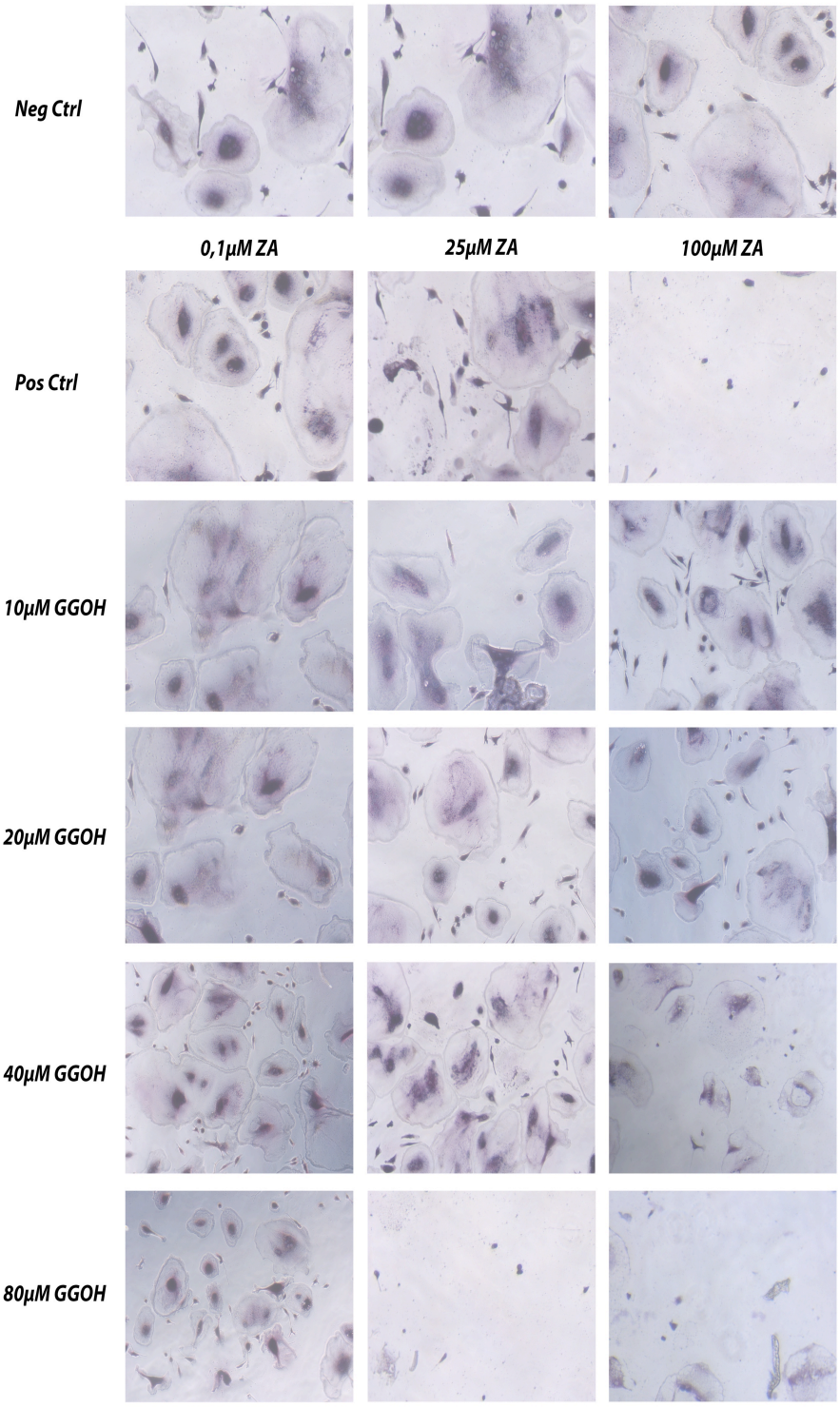


Figure 9: Tartrate-resistant acid phosphatase staining of hOCs differentiated in-vitro on polystyrene flasks

Western blot analyses of Rap 1A/B protein

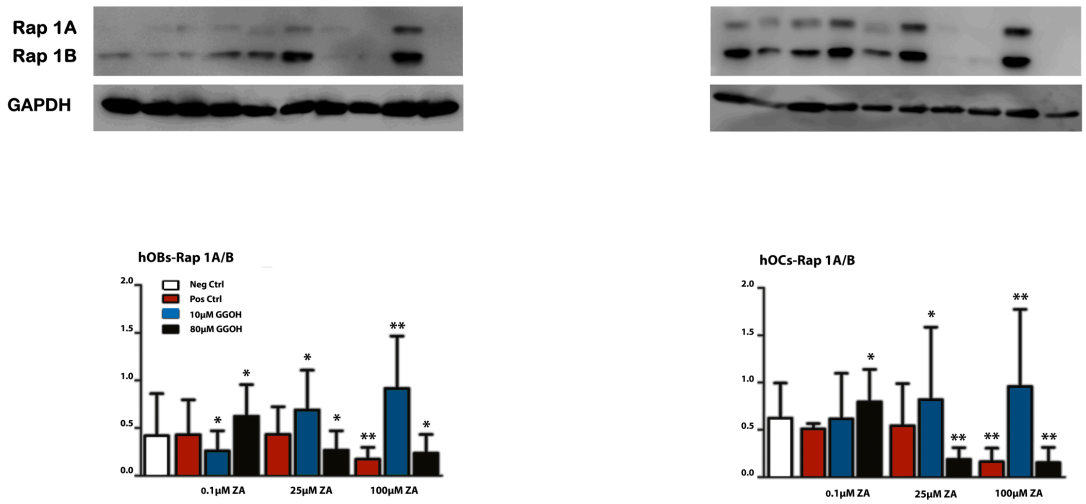
To investigate the effects of ZA and its antagonist GGOH on geranylgeranylation, western blot analyses were used to quantify the expression of small GTPase-protein Rap 1A/B after the treatment of bone cells with various concentrations of ZA and GGOH. The results were normalized to GAPDH.

Rap 1A/B expression in hOBs

As shown in **Figure 10**, hOBs treated with ZA at concentrations of 0.1 µM and 25 µM did not show a significant effect on Rap 1A/B expression compared to the negative control. At a concentration of 100 µM ZA, the expression of Rap 1A/B was strongly reduced to around 0.2-0.3 folds in comparison to the negative control. After treating the cells with 10 µM GGOH at 0.1 µM ZA, the Rap 1 A/B expression was almost half of that seen in positive control. The negative effect of ZA at concentrations of 25 µM and 100 µM was reversed after the addition of concentration of 10 µM GGOH; by 25 µM ZA it was increased to around 0.75 folds and by 100 µM ZA it was almost 1.0 folds.
In contrast, the treatment of hOBs with a higher concentration of GGOH (80µM) increased the Rap 1A/B expression at 0.1 µM ZA in comparison to the positive control. However, at 25 µM ZA, the expression was almost half the one in the positive control, and at 100 µM ZA it was nearly the same as in the positive control.

Rap 1A/B expression in hOCs

After treating hOCs with various concentrations of ZA, the expression level of Rap 1A/B was lower than in the negative control at 0.1 µM and 25 µM ZA. Additionally, at 100 µM ZA, Rap 1A/B was almost two times lower than in the negative control. There was no significant difference after the treatment of hOCs with 10 µM GGOH at 0.1 µM ZA. On the other hand, at higher concentrations of ZA (25 µM, 100 µM) and lower concentration of GGOH (10 µM), the Rap 1A/B expression was increased significantly to 0.75 and 1.0 folds, respectively.
At a higher concentration of GGOH (80 µM) and higher concentrations of ZA (25 µM, 100 µM), the expression of Rap 1A/B was strongly suppressed. These findings showed that higher concentrations of ZA and GGOH have a coinhibitory effect on the expression of Rap 1A/B, while lower concentrations of GGOH enhance the expression of this protein.



			0.1µM ZA			25µM ZA			100µM ZA	
	Neg Ctrl	Pos Ctrl	10µM GGOH	80µM GGOH	Pos Ctrl	10µM GGOH	80µM GGOH	Pos Ctrl	10µM GGOH	80µM GGOH
Rap1	++	++	+	+++	++	+++	+	+	++++	+

			0.1µM ZA			25µM ZA			100µM ZA	
	Neg Ctrl	Pos Ctrl	10µM GGOH	80µM GGOH	Pos Ctrl	10µM GGOH	80µM GGOH	Pos Ctrl	10µM GGOH	80µM GGOH
Rap1	+++	+++	+++	++++	+++	++++	+	+	+++++	+

Figure 10: Rap 1A/B expression in hOBs and hOCs [17].

Rap1A/B expression in hOBs and hOCs after treatment with various concentrations of ZA (0.1µM, 25µM, 100µM) and various concentrations of GGOH (10µM, 20µM, 40µM, 80µM) after seven days. GAPDH was used as the loading control. The results are presented as fold change of Rap 1A/B. Two-way ANOVA tests were used. P-value: * $p<0.05$, ** $p<0.01$.

CHAPTER 4 DISCUSSION

Discussion

Medication-related osteonecrosis of the jaw (MRONJ) is an uncommon, but serious side effect of antiresorptive medications and angiogenesis inhibitors [144, 219]. Of the antiresorptives, BPs inhibit a key enzyme in the MVP, and this causes impaired prenylation of small GTPases, which are important for cell function and survival [201, 220, 221]. The incidence of MRONJ in patients who have received oral BPs due to osteoporosis is estimated to be around 0.001-0.1% [53, 222, 223]. However, it is estimated to be around 1-11% in cancer patients with a history of IV treatment with BPs [165, 224-228]. According to the AAOMS (update 2014) [5], MRONJ occurs 50-100 times more often in patients with a history of ZA treatment than in patients without any exposure to antiresorptive medications [229-231].

The aim of this study was to investigate the role of GGOH in the MVP and in reversing one of the important side effects of ZA (N-BP). To evaluate this possible effect, we have investigated the effects of both ZA and GGOH on hOBs and hOCs, the main cells involved in bone turnover. Zoledronate (ZA) was used because it is the most potent IV BP and at the same time is associated with increased risk of MRONJ development [5, 17, 47, 74, 232]. It has been proven that the more potent N-BPs pose a higher risk compared to NN-BPs; IV administration also increases the risk of developing MRONJ, althogh patients who receive BPs orally may also be at risk if the duration of treatment is longer than four years [2, 223].

In previous studies, it has been shown that higher concentrations of ZA have negative effects on cell viability through inhibition of the MVP [233]. Consequently, the production of one important end-product of this pathway, GGPP, is impaired. This impairment leads to decreased prenylation (geranylgeranylation) of important proteins such as Ras, Rho, Rab and Cdc42, which are necessary in cell morphology [62], cytoskeleton arrangement, and cell migration, metabolism and survival [62, 234, 235]. The first cases of MRONJ were reported by Marx in 2003 [143]. Since then, many in vivo and in vitro studies have been performed to identify the conditions under which MRONJ occurs and to determine why the jaw bone is almost exclusively affected [236]. There are several theories related to the pathomechanism of MRONJ. However, none of them fully explains the mechanism by which MRONJ develops [198, 229-231, 237].

Studies have indicated that the main factors that increase the risk of MRONJ in patients with a history of BP uptake are surgical manipulations of the jaw, such as tooth extraction (44.4%), periodontitis (9.5%) and dental implant placement (3.2%) [157, 231, 238, 239]. Hasegawa et al. [231] have shown that the incidence of MRONJ in patients receiving IV BP treatment who have undergone tooth extraction is about 1.6-40%. Other risk factors for development of MRONJ are the type of drug, dosage, duration of treatment, and mode of administration [5].

Otto et al. [240] have shown a risk of MRONJ occurrence of 4.2% after tooth extraction in patients receiving BPs. They propose that the pre-existing infectious conditions may be a more important risk factor than tooth extraction for MRONJ development. Soutome et al. [241] have reported similar findings, stating that the presence of inflammation may be an important risk factor. Many studies have already underlined the importance of periimplantitis and periodontal diseases as risk factors for development of MRONJ [242, 243].

Due to the lack of clear data on the clinical setting of the disease, several cell culture studies have investigated MRONJ pathomechanisms. Walter et al. [244] have demonstrated that N-BPs have a negative effect on cell viability and migration of fibroblasts and induce apoptosis in hOBs, fibroblasts, and human umbilical vein endothelial cells (HUVECs). Acil et al. [245] demonstrated the cytotoxicity of ZA at concentrations of 0.15625, 0.3125, 0.625, 1.25, and 2,5 μM to hOBs and fibroblasts, which might increase the risk of MRONJ. Ravosa et al. [246] have evaluated the effect of ZA at concentrations of 5, 10, 30, 50, 75, 100 and 300 μM on oral epithelium and fibroblasts. They mention that BPs impair wound healing by inhibiting the growth and migratory capacity of oral fibroblasts, which are important for reepithelization [246].

There are data illustrating the important role of macrophages in allowing infection and inflammation to occur, resulting later in necrosis of the bone [247, 248]. Kaneko et al. [249] have demonstrated that ZA may cause inflammation by inducing M1 but not M2 macrophage polarization, resulting in the production of inflammatory cytokines in THP-1 cells.

Some studies have demonstrated the effect of BPs on cells at various pH levels. Otto et al. [250] have investigated the effect of different types of BPs, including two N-BPs (ZA and ibandronate) and one NN-BP (clodronate) at various pH-environments on mesenchymal stem cells. They suggest that higher concentrations of N-BPs, in combination with the acidic environment, common in inflammation, lead to a significant decrease in mesenchymal cell viability and activity. However, a similar concentration of clodronate did not show a

significant effect on the cells except for the acidic environment itself. Otto et al. [251] elucidated that the decreased pH value may lead to protonation of BPs and to their transformation to toxic levels. This may be an explanation of why inflammation could be a risk factor for development of MRONJ.

The AAOMS (update 2014) [5] suggests different therapy modalities for MRONJ based on its stage. Non-surgical therapies are suggested for stages 0 and I. However, surgical interventions are recommended for the more advanced stages of MRONJ (II and III).

Despite intensive research, no efficient, non-surgical methods of therapy exist for MRONJ treatment. Several innovative treatment modalities other than surgery have been explored in the literature based on the stage of the disease. It has been shown that ozone therapy induces cell proliferation and improves wound healing, reducing pain with promising results in stages I and II of MRONJ [252]. Low-intensity laser therapy (LILT) has been shown to have an antimicrobial effect and to improve healing of the wound as well. Several studies have simulated the biostimulating effect of LILT in MRONJ lesions [253, 254]. Pentoxifylline and α-tocopherol have been suggested to assist antimicrobial therapy in early stages of MRONJ [253, 255]. Longo et al. [256] have demonstrated that patients in stage II of MRONJ who are treated only with surgery, without PRP, show a success rate (17%), much lower than that of patients who have been treated with a combination of surgery and PRP group (63%). They find that thrombocyte concentrates may improve MRONJ treatment. Calvani et al. [257] have illustrated the efficacy of bovine lactoferrin after surgery, suggesting that greasy gauze soaked with lactoferrin induces earlier wound closure in comparison to classical surgical treatment. Zandi et al. [258] demonstrated that teriparatide therapy may improve MRONJ in both clinical and histopathological features in a dose-dependent manner. Future research should examine the efficiency of these treatment methods [5].

Some of the in vitro studies support the hypothesis that external supplementation of MVP mediators, which are strongly affected and reduced by N-BPs, might reverse the negative effect of N-BPs [19, 198]. It has been pointed out that one of the mediators that may play a important role in the pathomechanism of MRONJ is GGOH, which is downregulated by blocking the FPPS, an enzyme in the MVP. Geranylgeraniol (GGOH) is an acyclic diterpene alcohol (diterpenoid), which can be extracted from plants or produced synthetically. It is an important material for the synthesis of pharmacological mediators and hydrophobic vitamins such as vitamins A and E [74, 186].

Another possible mediator preventing development of MRONJ has been suggested by Camacho-Alonso et al. [259], who investigated the cytoprotective effects of melatonin at concentrations of 1, 10, 50, 100, and 200 μM on osteoblast viability previously treated with 1, 5, 10, 50, 100 and 300 μM ZA. At 24 h incubation with melatonin, the greatest osteoblast viability was observed at lower melatonin concentrations. However, 48 h and 72 h incubation showed greatest osteoblast viability at higher concentrations of melatonin (100, and 200 μM). This study demonstrates that melatonin could be promising as a means of prevention in patients at risk of BRONJ.

Several studies have mentioned the important role of GGOH as a useful mediator reversing the negative effect of N-BPs on various types of cells such as bone cell lines, macrophages, human oral keratinocytes (HOKs), fibroblasts, and HUVECs [19, 260].

Some studies have investigated the effects of different isoprenoids, including GGOH, on various cell lines treated with ZA. Hagelauer et al. [20] have investigated the effects of various isoprenoids, such as eugenol, farnesol and GGOH on the cell function of HUVECs, fibroblasts, and osteogenic cells treated with ZA. They treated the cells with different concentrations of GGOH (0, 10, 25, 50 and 100 μM) with or without ZA (50 μM) for 72 h. They found that only GGOH, as a natural isoprenoid, could reverse the negative effect of ZA on viability and wound healing capacity of the cells. However, Zafar et al. [22] have demonstrated that FOH can partially reverse the negative effect of ZA on cell viability of human gingival fibroblasts (HGFs). They treated the HGFs with two isoprenoids (GGOH [10, 50 μM] or FOH [10, 50 μM]) and ZA (30 μM) incubated for 72 h. They mention that GGOH at concentrations of 10 and 50 μM increases the HGFs viability. They also find, in contrast to Hagelauer et al., that not only GGOH but also FOH at concentrations of 30 and 50 μM can partially restore the viability of HGFs.

In another study, Zafar et al. [140] have also investigated also the effect of ZA (30, 50 μM), FOH (10, 50 μM), and GGOH (10, 50 μM) on primary hOBs isolated from human alveolar bone [140]. They found that the treatment of hOBs with various concentrations of ZA (30, 50 μM) resulted in a dose-dependent increase in expression of caspase-3/7, a marker for cell apoptosis, and in decreased cell viability. After administration of 50 μM GGOH to the hOBs treated with ZA, the cell viability was increased and the expression of caspase-3/7 was decreased. However, administration of 50 μM FOH did not have any significant effect on viability and apoptosis of hOBs. They have also mentioned the down-regulation of pathways

related to tissue repair such as angiogenic and osteogenic pathways in hOBs treated with ZA. These findings show that higher ZA concentrations (30, 50 μM) contribute to a decrease in angiogenic gene expression and consequently to impaired tissue regeneration. These negative effects were counteracted after the addition of GGOH at concentrations of 30 and 50 μM [140].

Cozin et al. [16] have mention that a combination of 10 ng/ml of human platelet-derived growth factor BB and 50 μM GGOH was able to increase cell proliferation and migration activity and to decrease the apoptosis in HGFs treated with various ZA and pamidronate concentrations (such as 0.003, 0.006, 0.008 and 0.1mM). They discovered that N-BPs caused a loss of cell adhesion and a reduction of F-actin bundles. These negative effects on HGFs treated with BPs were counteracted after the addition of platelet-derived growth factors and GGOH [16].

Pabst et al. [15] have investigated the positive effect of GGOH at a concentration of 10 μM on HOKs treated with various concentrations of different types of BPs (clodronate, ibandronate, pamidronate, and zoledronate). They analyzed viability, migration ability, and apoptosis of HOKs. Wound healing depends on the intact migration of HOKs from the stratum basale to the stratum corneum and on the renewal of the mucosal layer. The authors of the study found that BPs influenced the viability and the migration ability of HOKs negatively. They treated the HOKs with GGOH to reverse the negative effect of BPs. They found that GGOH did not have a significant positive influence on cells treated with clodronate (NN-BP). However, it increased the viability and migration ability of HOKs treated with ZA and reduced apoptosis of these cells [15].

A few studies have investigated the effect of MVP activator GGOH on bone cells that had previously been treated with ZA. Ziebart et al. [19] have demonstrated the positive effect of GGOH on the viability of hOBs treated with 5 μM and 50 μM ZA. They measured osteoblast viability at 5 μM ZA to be around 40% and at 50 μM ZA to be around 10%. After administration of 10 μM GGOH to the hOBs, which were previously treated with the aforementioned ZA concentrations, cell viability was increased at 5 μM ZA to nearly 55% and at 50 μM ZA to nearly 80% [19]. These results are similar to the findings of the present study, despite the different concentrations of ZA.

Only a few animal studies have investigated GGOH and its effect on the occurrence of MRONJ [19, 198]. Koneski et al. [198] have evaluated the effect of 5 mM GGOH in the form

of local solution on wound healing and development of osteonecrosis in rats treated intraperitoneally with ZA at a concentration of 0.06 mg/kg. After three weeks of treatment, the rats underwent first molar extraction on the right side of the mandible and were treated with drugs (ZA/GGOH) for two more weeks. The study found that 80% of rats treated only with ZA developed microscopical osteonecrosis. However, after daily administration of 5 mM GGOH to the extraction sockets in the form of local solution, the negative effect of ZA was reversed and only 22% of co-treated rats developed microscopical osteonecrosis in comparison to the groups treated only with ZA. This study suggests that GGOH could reverse the negative effect of ZA and improve wound healing and tissue proliferation.

Nagaoka et al. [179] have investigated the effect of systemic administration of GGOH/GGPP at concentration of 3 μM on osteoclast differentiation of bone marrow cells (isolated from mice), and on multinucleation and bone mineral deposition. They have demonstrated that the addition of 3 μM GGOH/GGPP reverses the negative effect of ZA (1-10 μM) and that it improved zoledronate-induced inhibition of osteoclast differentiation molecule TRAP. They also treated mice with intraperitoneally injected ZA (250 μg/kg) and GGOH (250 μg/kg). After two weeks of treatment, the right first molar in the maxilla were extracted and the mice were treated with ZA/GGOH for two more weeks. Micro-CT analysis of alveolar sockets showed a decreased alveolar bone mineral deposition in mice treated only with ZA. However, administration of GGOH could reverse the negative effect of ZA, and it could increase the alveolar mineral deposition [179].

In the present study, the effects of various ZA concentrations (0.1, 25, and 100 μM) and GGOH (10, 20, 40, and 80 μM) on bone cells (hOBs and hOCs) were investigated. The study finds that ZA has a dose-dependent negative effect on human bone cells. Higher concentrations of ZA, such as 25 μM and 100 μM, decrease the viability and metabolic activity of hOBs and hOCs. However, lower concentrations of ZA, such as 0.1 μM, seem to improve viability of these cells. Some other studies have reported similar results to ours [81, 261, 262]. Bellido et al. [81] have likewise reported that lower concentrations of BPs ranging from 0.001 μM to 1 μM ZA, increase osteoblast proliferation and growth. Thibaut et al. [262] have found that 0.1 μM ZA increases osteoblast proliferation but does not affect cell growth and morphology, and that a higher concentration of ZA (10 μM) induces a reduction of cell numbers and caused apoptosis of hOBs. Fromigue et al. [263] illustrated that a ZA concentration ranging from 10^{-8} μM to 10^{-5} μM could increase the hOB cell proliferation to

around 30%. These studies have also shown that higher ZA concentrations inhibit the MVP and result in impaired cell function.

Through our experiments, we have demonstrated that GGOH could reverse the negative effect of ZA in a dose-dependent manner. Geranylgeraniol may have beneficial effects on MRONJ through the prenylation of small GTPases, which are important for cell viability and survival.

In this study, WST-1 analysis demonstrated a successful replacement of cell viability, which is inhibited dose-dependent by ZA. Our results are similar to those of studies already mentioned. A lower concentration of ZA (0.1 μm) did not have a negative effect on cell viability. On the contrary, 0.1 μM ZA and a combination of ZA and GGOH (even at higher GGOH concentrations) resulted in an increase in hOBs and hOCs viability. Only hOCs at concentration of 0.1 μM ZA and 80 μM GGOH resulted in a decrease in cell viability (35-40%) [17]. First administration of higher ZA concentrations (25, 100 μM) decreased the viability of hOBs and hOCs. This negative effect was much more pronounced at higher concentrations of both ZA and GGOH.

Protein prenylation, first identified in fungi [264], is a post-translational modification of proteins in eukaryotic cells, and it includes protein farnesylation and geranylgeranylation. It is a necessary process for the activity of important proteins from the Ras family, small GTPases, and heterotrimeric G-proteins [265, 266]. The hydrophobic prenyl group is important for membrane targeting of proteins, and it enables multiple cell signaling pathways [265]. Inhibition of protein prenylation suppresses the activity of oncogenic Ras proteins to reach high antitumor activity [267]. However, the decreased or ineffective prenylation of Ras-related proteins (Rap 1), a group of small GTPases, may lead to the development of MRONJ [74]. Rap 1, which was discovered by Kitayama et al. [268], plays an important role in cell adhesion, including integrin- and cadherin-mediated adhesion. It activates and regulates integrin, which plays a key role in various cell processes [268, 269].

In our experiments, prenylated Rap 1 (a small GTPase) was investigated in hOBs and hOCs treated with ZA/GGOH for a period of one week. There are two isoforms of Rap 1 protein, Rap 1A and Rap 1B (Rap 1A/B). The expression level of Rap 1A/B was affected after the treatment of hOBs and hOCs with various concentrations of ZA/GGOH. As already mentioned, ZA inhibits the MVP, and protein prenylation is consequently blocked in a dose-dependent manner. In the present study, it is demonstrated that the level of prenylated protein (Rap 1A/B) increases not only in hOCs but also in hOBs when GGOH is added to the culture

of bone cell lines treated with N-BP (ZA). Rap 1 served as a biomarker for post-translational modification. This finding shows that GGOH could reverse the negative effect of N-BPs in a dose-dependent manner and that this may lead to unimpeded prenylation of the small GTPase Rap 1. A new finding of our experiments is that not only a combination of higher ZA/GGOH show cytotoxicity, but a higher concentration of GGOH alone also shows a cytotoxic effect on bone cells.

These findings may be important for the development of new therapy methods for the early stages of MRONJ. Apart from the dosage and duration of GGOH treatment, there are other open questions related to the clinical use of GGOH, such as the route of administration and the possible adverse effects associated with it. Systematically administered GGOH may be transferred faster to cells, especially to the basal mucosal layers, but this method is potentially dangerous because of the likely suppression of the antiresorptive activity of BPs. This may lead to neutralization of the effect of BPs, which is important in patients who receive them as a treatment for malignant diseases because of the risk of spreading the tumor cells [19, 74, 195, 196]. However, the local administration of GGOH in the form of mouth rinses may increase the concentration of medication in the wound, and this may improve wound healing [198]. Future studies should be directed toward developing appropriate local drug delivery systems, such as collagen membranes, which will effectively and safely transport the GGOH [20, 74]. Further research should also be done into the exact indications for the use of GGOH, its optimal concentration, and the duration of treatment. Because this study reveals that higher concentrations of GGOH may in fact be harmful to cells, the optimal concentration remains one of the most important questions. Future animal studies should be conducted to test these issues. Moreover, other possible side effects of GGOH should also be evaluated and taken into consideration.

The main limitation of this study is that it uses an in vitro model in regulated laboratory conditions; extrapolating the in vitro results to an in vivo situation may prove challenging. Another limitation is that the effects of drugs were investigated only on bone cells without any blood supply, in the absence of soft tissue, and without any possible inflammation factors or any of the complex immunological or other types of conditions that are present in the human body. Also, the interactions between different drugs and concentrations were evaluated only on hOBs and hOCs and not on broad spectrums of cells from different donors, which would have generated even more relevant data. However, the results of this study may

serve as a basis for further development of the possible use of GGOH as a preventive or therapeutic approach in the treatment of MRONJ.

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