

# Isoprenoid geranylgeraniol: the influence on cell characteristics of endothelial progenitor cells after bisphosphonate therapy *in vitro*

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## Abstract

**Objectives** Geranylgeraniol (GGOH) has been reported as a potential treatment option for bisphosphonate-associated osteonecrosis of the jaws (BP-ONJ). The aim of this study was to analyze the effects of GGOH on endothelial progenitor cells (EPC) after bisphosphonate treatment *in vitro*.

**Materials and methods** EPC were incubated with different nitrogen (N-BPs: ibandronate, pamidronate, zoledronate) and a non-nitrogen-containing bisphosphonates (NN-BP: clodronate) with and without GGOH. Cell viability was measured by MTT and PrestoBlue assay. Migration ability was analyzed with a Boyden and Scratch assay. Apoptosis rates were determined by colony-forming, Tunel and ToxiLight assays.

**Results** Negative effects of N-BPs on EPC were shown in all tests without GGOH. The substitution of GGOH demonstrated significantly increased cell viability (MTT:  $p$  each N-BP  $\leq 0.004$ ; PrestoBlue:  $p$  each N-BP  $< 0.001$ ) and migration ability (Boyden:  $p$  each N-BP  $< 0.001$ ; Scratch:  $p$  each N-BP  $< 0.001$ ). Concerning the apoptosis rates, increased EPC colony densities ( $p$  each N-BP  $\leq 0.009$ ), decreased numbers of apoptotic cells in the Tunel assay ( $p$  each N-BP  $< 0.001$ ), and a decreased adenylate kinase release in the ToxiLight assay ( $p$  each N-BP  $\leq 0.03$ ) were observed. For the clodronate-treated cells, no significant differences could be detected with or without GGOH in any assay ( $p$  each N-BP/NN-BP  $> 0.05$ ).

**Conclusions** GGOH cell treatment reversed the negative effects of bisphosphonates on EPC.

**Clinical relevance** These findings support the hypothesis that systemic or local GGOH treatment might lead to new therapeutic strategies for BP-ONJ.

**Keywords** Endothelial progenitor cells · EPC · Bisphosphonate · Geranylgeraniol · Bisphosphonate-associated osteonecrosis of the jaws

## Introduction

Bisphosphonate-associated osteonecrosis of the jaws (BP-ONJ) is a side effect of bisphosphonate therapy with an increasing incidence [1]. To date, the pathophysiology has not been clarified in detail, and there still is no effective causal therapy or prevention strategy.

Concerning the pathophysiology, several theories are being discussed in the literature. A multifactorial genesis is very likely. Typical theories describe reduced bone remodelling, an impact on the soft tissues and the immune system, and trigger factors such as periodontal disease and enoral wounds. Some research focuses on the strong negative influence of bisphosphonates on angiogenesis as a pathophysiological contributing factor since BP-ONJ might be an avascular necrosis [2–4]. This theory is supported by the fact that other recently developed antiangiogenic agents are also associated with the occurrence of osteonecroses of the jaws, e.g., anti-VEGF-antibodies and tyrosine-kinase inhibitors [5, 6]. Bisphosphonates' negative influence on mature endothelial cells *in vitro* has already been reported [7, 8], and it has been confirmed *in vivo* in mice treated with bisphosphonates [9]. This is reflected in *in vivo* investigations on BP-ONJ patients, showing a reduced density of circulating endothelial cells and

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decreased VEGF serum concentrations [10, 11]. In vitro studies showing bisphosphonates had a negative impact on endothelial progenitor cells (EPC) supported the in vivo results [12]. These findings, which are also reflected in in vivo investigations on BP-ONJ patients showing a reduced density of circulating EPC, led to the assumption that EPC might play a role in BP-ONJ development and therefore might be of scientific interest [10].

In addition to the unknown pathophysiology, there is no effective causal therapy for this disease to date.

Small necrotic areas can be treated by a conservative BP-ONJ management, while in advanced stages, surgical treatment should be preferred [13, 14]. Therapeutic options such as hyperbaric oxygen and soft laser therapy could not meet the expectations placed in them [15–18]. For these reasons, there was and still is a necessity for new therapy options.

Regarding this matter, a recent study focused on the molecular pharmacological point of action of nitrogen-containing bisphosphonates (N-BPs), which is decreasing the synthesis of the isoprenoid geranylgeraniol (GGOH) in the mevalonate pathway. It was demonstrated that the negative effects of N-BPs on cell characteristics of endothelial cells, fibroblasts, and osteoblasts could be reversed by GGOH substitution [18].

Therefore, with respect to the relevance of EPC and angiogenesis in BP-ONJ development, the aim of this study was to analyze the effects of GGOH substitution on EPC viability, migration ability and apoptosis rate after bisphosphonate treatment in vitro.

## Materials and methods

### Bisphosphonates

The three nitrogen-containing bisphosphonates (N-BPs) ibandronate (Bondronat®; Roche, Welwyn Garden City, UK), pamidronate (Pamifos®; Medac GmbH, Wedel, Germany), and zoledronate (Zometa®; Novartis, Nürnberg, Germany) as well as the non-nitrogen-containing bisphosphonate (NN-BP) clodronate (Bonefos®; Bayer, Leverkusen, Germany) were used. The concentrations used for the bisphosphonates (BP 0, 5, and 50 µM) and GGOH (10 µM; Sigma, Steinheim, Germany) have been evaluated in previous experiments [7, 8, 12, 16, 18, 19].

### EPC culture assay

Mononuclear cells (MNCs) were isolated by density gradient centrifugation from human peripheral blood with Biocoll (Biochrom KG, Berlin, Germany) like previously described by Ziebart et al. [12, 20]. After isolation, total MNCs ( $8 \times 10^6$

cells/ml medium) were seeded on 25-cm<sup>2</sup> culture flasks coated with human fibronectin (Sigma) and maintained in endothelium basal medium (EBM; Lonza, Basel Switzerland) supplemented with endothelium growth medium (EGM; Lonza) SingleQuots, 100 ng/ml vascular endothelium growth factor (VEGF; Sigma), and 20 % fetal calf serum (FCS; Sigma).

### Cell viability

#### MTT assay

For the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide assay (MTT assay), EPC were transferred into six-well plates (Sigma; EPC 250,000 cells/well). After 24 h, the cells were incubated with different bisphosphonate concentrations (0, 5, and 50 µM) and GGOH (10 µM). The control groups received no GGOH. After 72 h, the cell viability was evaluated using the MTT colorimetric assay (Sigma). Viable cells ferment tetrazolium bromide to formazan, which can be photometrically measured after cell lysis using a Synergy HT Multi-Mode Microplate Reader (corresponding setting: absorbance; BioTek Instruments, Winooski, USA).

#### PrestoBlue assay

The PrestoBlue assay (Invitrogen, Darmstadt, Germany) was used to analyze the EPC viability at different points of measurements and more frequently compared to the MTT assay. Six-well plates (Sigma) were prepared similar to the MTT assay (EPC 250,000 cells/well). After 24 h, the cells were incubated with the mentioned bisphosphonates in increasing amounts (0, 5, and 50 µM) and GGOH (10 µM). The control groups received no GGOH. At 0, 24, 48, and 72 h after applying 10 % PrestoBlue, the reduction induced color switchover of resazurin to resorufin was measured with a Synergy HT Multi-Mode Microplate Reader (corresponding setting: fluorescence; BioTek Instruments).

### Migration

#### Boyden migration assay

For the migration assay, a 24-well Boyden chamber assay system (Thin-Cert®; Greiner BioOne, Essen, Germany) was used according to the manual as previously described [2, 8]. EPC were cultured in 24-well plates (Greiner BioOne; EPC 100,000 cells/well). After 24 h, EPC were incubated with 50 µM of the different bisphosphonates for 72 h in two diverse test lines with and without GGOH (10 µM) and then transferred into the migration chambers (Greiner BioOne; EPC 12,500 cells/chamber). The cells were stained after 24 h with Calcein-AM fluorescent dye (Invitrogen). The inserts were

transferred to the wells of freshly prepared black 24-well plates (Sigma) containing 500  $\mu$ l Trypsin-EDTA (Invitrogen) per well and incubated for 10 min in a cell culture incubator at 37 °C and 5 % CO<sub>2</sub>. The inserts were discarded, and 250  $\mu$ l of each well of the Trypsin-EDTA solution, containing the detached migratory cells, was transferred to a well of flat-bottom black 24-well plates with transparent bottom (Sigma). A Synergy HT Multi-Mode Microplate Reader (corresponding setting: fluorescence; BioTek Instruments) was used for quantification.

#### *Scratch assay*

For the Scratch assay, EPC were transferred into six-well plates (Sigma; EPC 250,000 cells/well). After 24 h, a scratch wound using a sterile pipette tip (Sigma) was performed, and the size of the gap was measured and set to 100 %. The cells were incubated with the mentioned bisphosphonates (50  $\mu$ M) and GGOH (10  $\mu$ M). The control groups received no GGOH. The closing of the scratch wound was measured for 72 h or until the scratch wounds were closed.

#### Apoptosis

##### *Colony-forming assay*

To examine the effects on colony-forming (CF) ability, EPC were transferred into six-well plates (Sigma; EPC 250,000 cells/well). After 24 h, the cells were incubated with the bisphosphonates (0, 5, and 50  $\mu$ M) and GGOH (10  $\mu$ M). The control groups received no GGOH. After 72 h EPC were transferred and cultured in MethoCult<sup>®</sup> culture medium (STEMCELL Technologies, Vancouver, Canada) on six-well plates (Sigma) for 14 days, like described in the manufacturer's protocol. After 14 days, the culture plates were analyzed for colony formation.

##### *Tunel assay*

For the apoptosis assay, EPC were seeded in six-well plates (Sigma; EPC 250,000 cells/well). After 24 h, cells were incubated with the bisphosphonates (50  $\mu$ M) and GGOH (10  $\mu$ M) for 72 h. Controls received no GGOH. Next, cells were dissolved in trypsin-EDTA (Invitrogen) and centrifuged onto coverslips (Sigma) at 2000 rpm for 5 min. Then, cells were fixed with 3 % paraformaldehyde (Sigma) for 10 min at room temperature (RT), permeabilized with 0.1 % Triton X-100 (Sigma), 0.1 % sodium citrate (Sigma) for 2 min on ice, washed twice with phosphate-buffered saline (PBS; Invitrogen), pH 7.4, and incubated for 1 h at 37 °C in the dark with a dUTP nick end labeling (TUNEL)

reaction mixture (Boehringer, Indianapolis, USA) for in situ detection of cell death. After washing twice with PBS (Invitrogen), pH 7.4, the cells were incubated at RT with the Hoechst solution (Sigma) for 5 min. All Hoechst-positive nuclei as well as TUNEL-positive nuclei were visualized using a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Göttingen, Germany) as previously described [2].

##### *ToxiLight assay*

The ToxiLight BioAssay (Lonza) is a cytotoxicity assay that measures the release of adenylate kinase (AK) from damaged cells. Six-well plates (Sigma) were prepared similarly to the MTT assay (EPC 250,000 cells/well). After 24 h, the cells were incubated with the bisphosphonates (0, 5, and 50  $\mu$ M) and GGOH (10  $\mu$ M). The control groups received no GGOH. After 0, 24, 48, and 72 h, the supernatant was mixed with ToxiLight dissolution and the emitted light intensity was measured using a Synergy HT Multi-Mode Microplate Reader (corresponding setting: luminescence; BioTek Instruments).

#### Statistical analysis

Each experiment has been performed in triplicate. Comparisons were analyzed by ANOVA (post hoc test: Tukey) for experiments with more than two subgroups (SPSS version 17.0; IBM, Ehningen, Germany). Considered as statistically significant were  $p$  values  $\leq 0.05$ . The black bar in the middle of each box represents the median. The box includes all values between the 25th and the 75th percentile. Whiskers indicate values within 1.5 interquartile range (IQR). Outliers within 3 IQR are represented as circles. Outliers outside of 3 IQR are represented as asterisks.

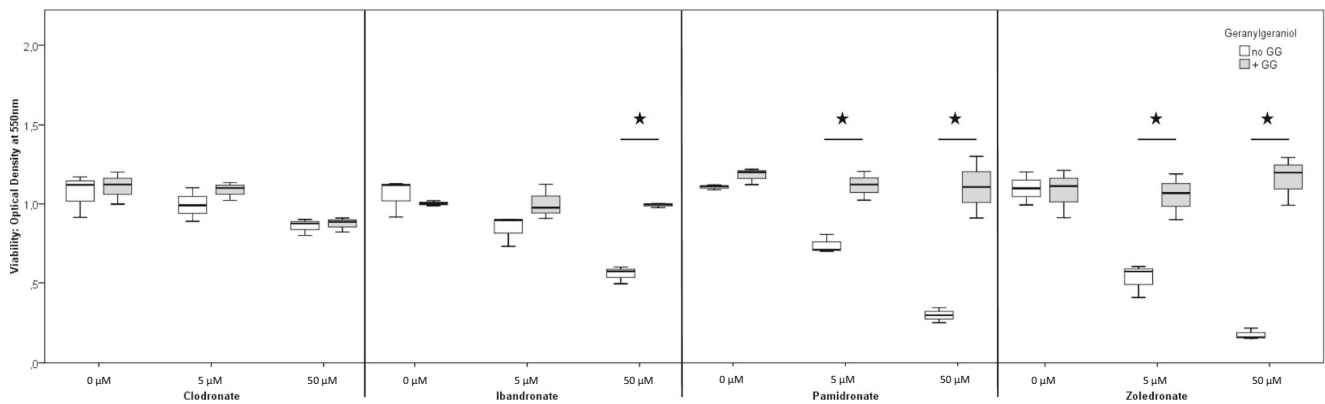
Asterisks demonstrate a statistically significant difference between the test line without GGOH and the test line with GGOH for the particular point of measurement ( $p \leq 0.05$ ).

## Results

### Cell viability

#### *MTT assay*

In the NN-BP clodronate group, no significantly decreased cell viability could be observed for any of the tested BP concentrations (5, 50  $\mu$ M;  $p$  each  $\geq 0.905$ ), and GGOH had no effect ( $p$  each  $\geq 0.808$ ).



**Fig. 1** MTT assay. *X-axis*=experimental groups and different bisphosphonate concentrations ( $\mu\text{M}$ =micromolar); *Y-axis*=cell viability, optical density at 550 nm (AU=absorbance units); *Asterisks* indicate

significant difference between the test line without GGOH (no GG) and the test line with GGOH (+ GG;  $p \leq 0.05$ )

For the N-BP ibandronate (50  $\mu\text{M}$ ), a significantly decreased cell viability was observed ( $p < 0.001$ ) and a significantly increased cell viability after GGOH substitution could be demonstrated ( $p < 0.001$ ). Incubation with pamidronate or zoledronate decreased the cell viability significantly for both concentrations (pamidronate 5, 50  $\mu\text{M}$ ;  $p$  each  $\leq 0.005$ ; zoledronate 5, 50  $\mu\text{M}$ ;  $p$  each  $\leq 0.001$ ). The substitution of GGOH increased cell viability (pamidronate:  $p$  each  $\leq 0.004$ ; zoledronate:  $p$  each  $< 0.002$ ; Fig. 1).

#### PrestoBlue assay

With clodronate no difference between the two test lines with and without GGOH could be demonstrated for any BP concentration at any point of time ( $p$  each  $\geq 0.991$ ).

For 50  $\mu\text{M}$  ibandronate, the substitution of GGOH increased the cell viability significantly after 48 and 72 h ( $p$  each  $< 0.001$ ). Between the two pamidronate test lines with and without GGOH, a significantly increased cell viability for the BP concentration of 5  $\mu\text{M}$  after 72 h ( $p < 0.001$ ) and for the BP concentration of 50  $\mu\text{M}$  after 48 and 72 h could be

detected ( $p < 0.001$  for each). For the zoledronate concentrations of 5 and 50  $\mu\text{M}$ , the substitution of GGOH revealed a significantly increased cell viability after 48 and 72 h ( $p$  each  $< 0.001$ ; Fig. 2).

#### Migration

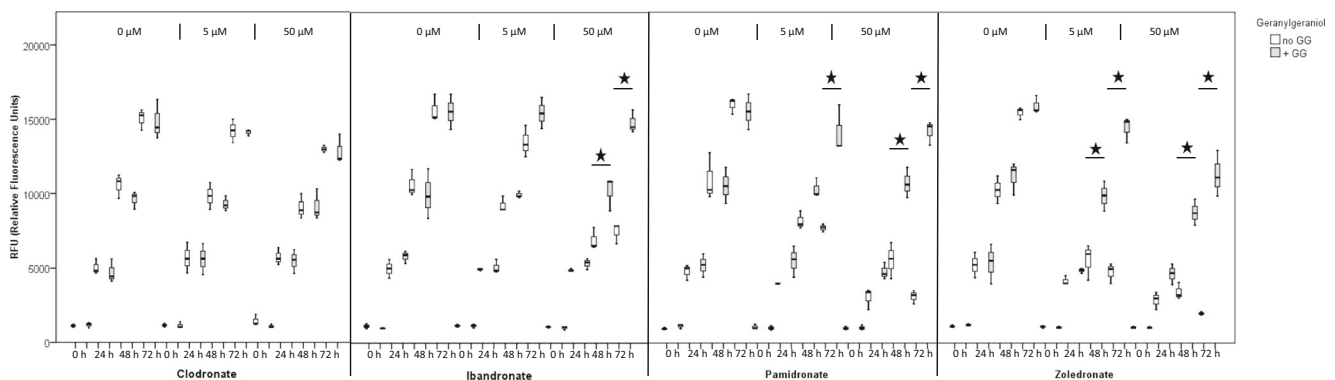
##### Boyden migration assay

Compared to the control group, the NN-BP clodronate showed no significantly decreased migration ability ( $p = 1.0$ ). GGOH had no influence ( $p = 0.977$ ).

The other BPs decreased the migration ability significantly in comparison to the control group ( $p < 0.001$  each), and the substitution of GGOH showed a positive effect ( $p < 0.001$  each; Fig. 3)

##### Scratch assay

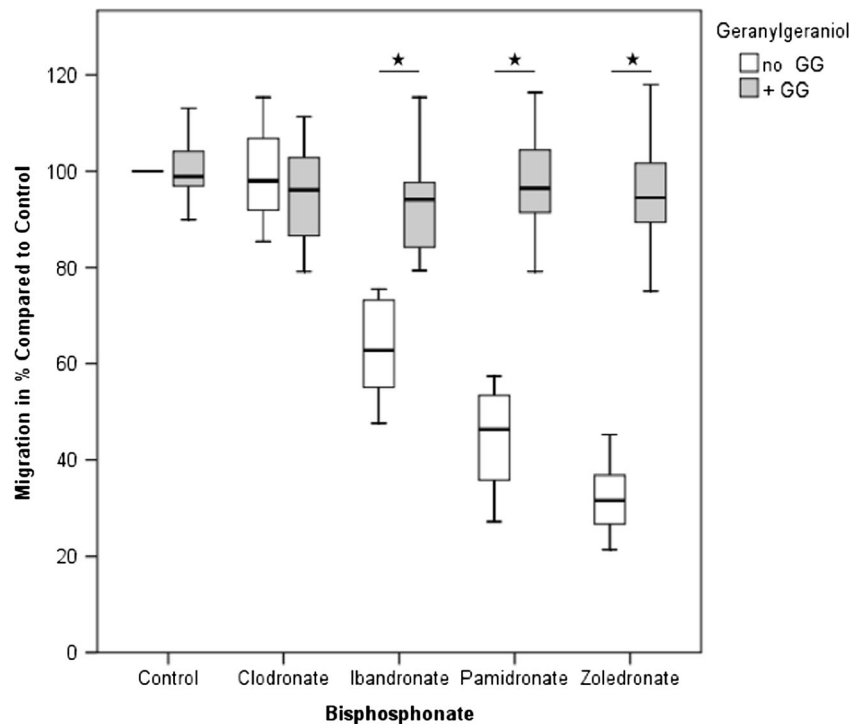
There were no statistical significant differences in the clodronate group with or without GGOH ( $p$  each  $\geq 0.922$ ).



**Fig. 2** PrestoBlue assay. *X-axis*=experimental groups, different points of measurement and different bisphosphonate concentrations ( $\mu\text{M}$ =micromolar); *Y-axis*=cell viability (RFU=relative fluorescence units);

*Asterisks* indicate significant difference between the test line without GGOH (no GG) and the test line with GGOH (+ GG;  $p \leq 0.05$ )

**Fig. 3** Boyden migration assay. *X-axis*=experimental groups; *Y-axis*=migration ability (in %) compared to the control group (set to 100 %); *Asterisks* indicate significant difference between the test line without GGOH (no GG) and the test line with GGOH (+ GG;  $p \leq 0.05$ )



For all N-BP, a significantly increased scratch wound closure after GGOH substitution could be demonstrated after 48 and 72 h ( $p$  each  $\leq 0.001$ ; Fig. 4).

**Apoptosis**

*Colony-forming assay*

The NN-BP clodronate did not decrease the colony density in the different concentrations (5, 50  $\mu\text{M}$ ;  $p$  each  $\geq 0.93$ ), and the substitution of GGOH had no effect ( $p$  each  $\geq 0.907$ ).

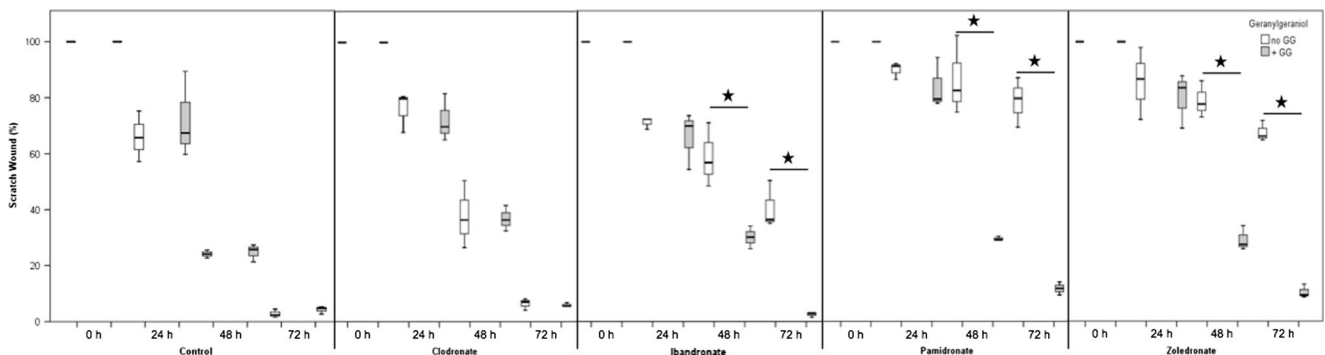
Ibandronate decreased the colony density significantly only at a concentration of 50  $\mu\text{M}$  ( $p < 0.001$ ), and the addition of GGOH for this BP concentration had a

reversing effect ( $p < 0.001$ ). Pamidronate and zoledronate reduced the colony density at 5 and 50  $\mu\text{M}$  ( $p$  each  $< 0.001$ ). The GGOH substitution had a positive effect for both BPs (ibandronate:  $p$  each  $\leq 0.009$ ; zoledronate:  $p$  each  $< 0.001$ ; Fig. 5).

*Tunel assay*

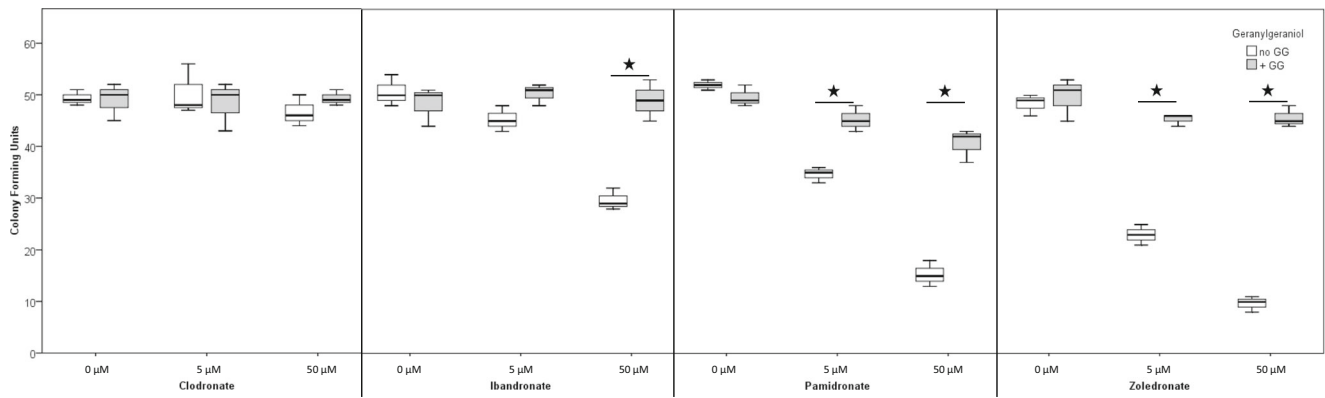
Compared to the control group, the NN-BP clodronate showed no significantly increased apoptosis rate ( $p = 0.631$ ) and no significant difference by adding GGOH ( $p = 1.0$ ).

For each of the tested N-BPs, a significantly increased apoptosis rate could be observed in comparison to the control group ( $p$  each  $< 0.001$ ) and a significantly reduced apoptosis



**Fig. 4** Scratch assay. *X-axis*=experimental groups and different points of measurement; *Y-axis*=open scratch wound area (in %) compared to the starting point after 0 h (set to 100 %); *Asterisks* indicate significant

difference between the test line without GGOH (no GG) and the test line with GGOH (+ GG;  $p \leq 0.05$ )



**Fig. 5** Colony-forming assay. *X-axis*=experimental groups and different bisphosphonate concentrations ( $\mu\text{M}$ =micromolar); *Y-axis*=colony-forming-units. *Asterisks* indicate significant difference between the test line without GGOH (no GG) and the test line with GGOH (+ GG;  $p \leq 0.05$ )

rate after GGOH substitution could be demonstrated ( $p$  each  $< 0.001$ ; Fig. 6).

#### ToxiLight assay

Neither clodronate nor the additional substitution of GGOH had any effect ( $p$  each  $\geq 0.315$ ).

The substitution of GGOH on EPC treated with ibandronate, pamidronate, or zoledronate reduced the release of adenylate kinase (AK) significantly for 5  $\mu\text{M}$  of BP after 48 and 72 h (ibandronate  $p \leq 0.03$  each; pamidronate  $p \leq 0.004$  each; zoledronate  $p \leq 0.001$  each) and for 50  $\mu\text{M}$  after 24, 48,

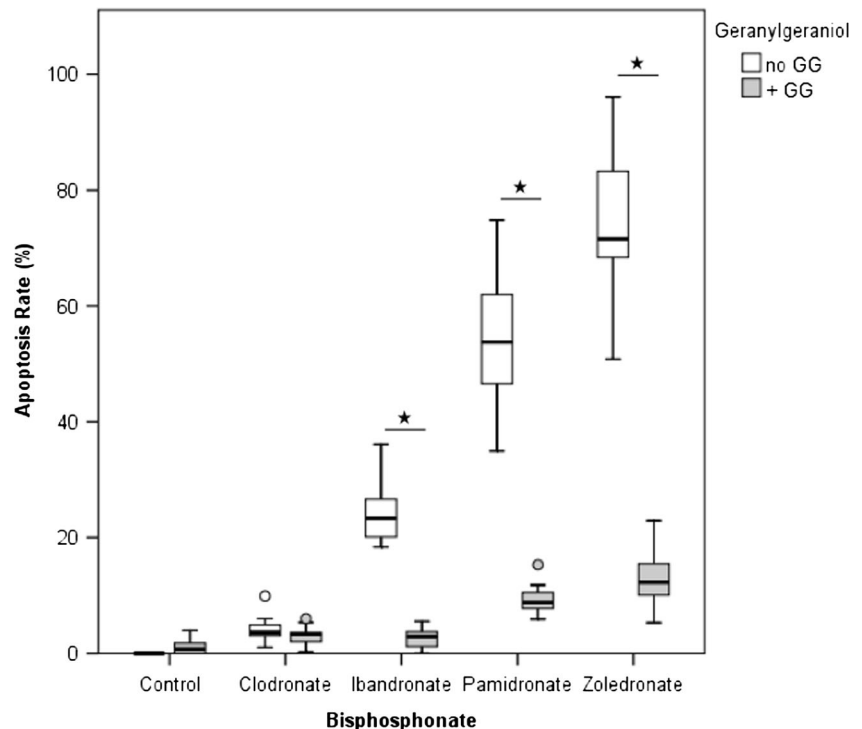
and 72 h (ibandronate:  $p < 0.001$  each; pamidronate and zoledronate:  $p < 0.001$  each; Fig. 7a, b).

#### Discussion

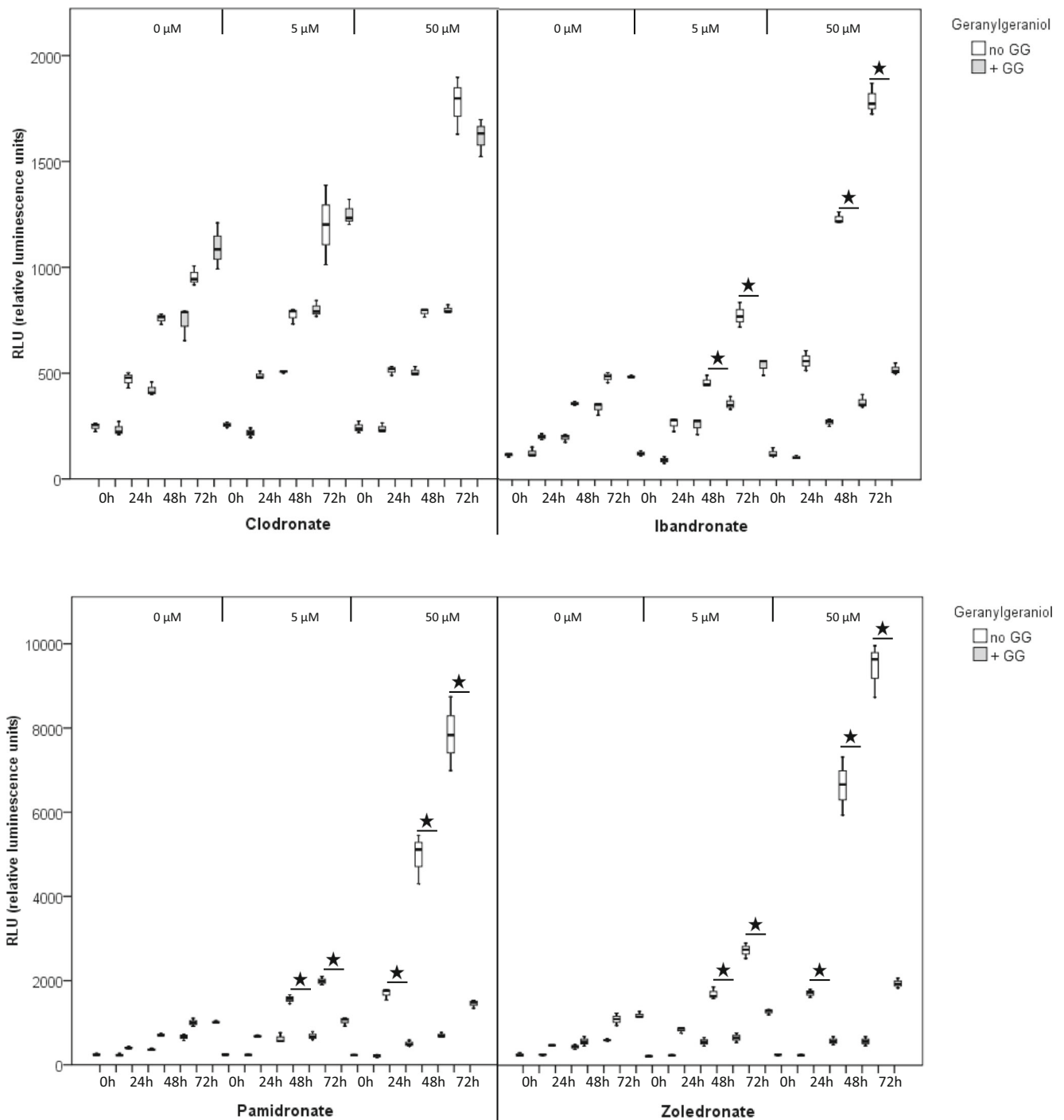
Overall, the results of this study demonstrated that the negative influences of nitrogen-containing bisphosphonates on EPC could be reversed by geranylgeraniol in vitro.

In this context, the following references will give a detailed overview over the exact mode of action of bisphosphonates in the mevalonate pathway and the function of geranylgeraniol [18, 21–24].

**Fig. 6** TUNEL assay. *X-axis*=experimental groups; *Y-axis*=number of living cells (in %) compared to the control group (set to 100 %); *Asterisks* indicate significant difference between the test line without GGOH (no GG) and the test line with GGOH (+ GG;  $p \leq 0.05$ )







**Fig. 7 a, b** ToxiLight assay. **a** clodronate, ibandronate; **b** pamidronate, zoledronate. *X-axis*=experimental groups, different points of measurement and different bisphosphonate concentrations ( $\mu\text{M}$ =micromolar); *Y-axis*=

adenylate kinase (AK) release (RLU=relative luminescence units); *Asterisks* indicate significant difference between the test line without GGOH (no GG) and the test line with GGOH (+GG;  $p \leq 0.05$ )

The strongest influence of GGOH substitution could be observed after N-BP pamidronate and zoledronate incubation, which are the bisphosphonates most frequently associated with BP-ONJ development [2, 4, 7–9]. There was no effect in the NN-BP treated EPC, since NN-BPs work as ATP-analogues and are not involved in the mevalonate pathway.

The improved EPC viability, migration ability, and decreased apoptosis rate after bisphosphonate incubation and GGOH treatment might be of special significance for BP-ONJ development and therapy with regard to the physiological functionality of EPC. After deriving from the bone marrow, EPC migrate to the vascular system, where they circulate and differentiate into mature endothelial cells [25–28].

Through the vessel system, the circulating EPC can reach the action sites, such as wounds or necrotic areas that need microvessel creation and revascularization. They then leave the vessel system by migration through the vascular wall and migrate into the surrounding tissues and the wound area, where they can stimulate the revascularization in two main ways: First, EPC stimulate the sprouting angiogenesis as well as the intussusceptive angiogenesis from preexisting vessels through the strong autocrine and paracrine effects of the production of several pro-angiogenic growth factors such as VEGF. Second, they can migrate into the surrounding tissues, inducing the development of completely new vessels through a process called neovasculogenesis [12, 18, 25–28]. Within this complex biochemical cascade, there are two points where bisphosphonates can interact and influence EPC: First, due to the accumulation of bisphosphonates in the entire skeleton and the long half-life of over 20 years depending on the specific bisphosphonate, they might interact with the EPC in the bone marrow and influence the release of EPC from the bone marrow niches through reduced cell viability and migration ability. Second, the local bisphosphonate concentrations in the hard and soft tissues of the oral cavity could interact with the stationary vessel cells and EPC in the mandibular bone, supporting the development and maintenance of BP-ONJ due to the strong negative influence on their viability and migration ability directly on site. It might be possible that at the above described points of action (bone marrow, oral tissues) bisphosphonates induce an EPC apoptosis, which automatically leads to a completely switched off EPC viability and migration ability. Overall, the associated impairment of microvessel creation might result in a significantly decreased wound healing of the oral hard and soft tissues, e.g., after surgical procedures, which could consequently result in the development of BP-ONJ.

Assuming BP-ONJ is caused in some way by the antiangiogenic properties of bisphosphonates on EPC, and therefore on the bone and soft tissue vascularization, GGOH might be an option for BP-ONJ prevention and therapy. For N-BPs, GGOH could restore the restriction of EPC viability, migration ability, and apoptosis rate back to regular conditions, which might result in normal revascularization.

An important question in this context is the potential mode of GGOH application in patients [18]. Since the effects of N-BPs on tumor cells can be at least partially neutralized by GGOH, a systematic GGOH application might decrease the pharmacological power of the applied bisphosphonates in the entire body and therefore also reduce the intended effects and the positive benefits of the bisphosphonates on the tumor growth in patients with malignant diseases [29–31]. In contrast, a topical application of GGOH with mouth rinses or local drug delivery systems could be performed to treat and prevent the development of BP-ONJ [18]. Therefore, further studies should focus on the optimal administration method,

the exact indication, and the adequate concentration and duration of GGOH treatment. In addition, a better understanding of GGOH pharmacokinetics is absolutely essential to prevent possible side effects of GGOH treatment like the abovementioned [18].

It is interesting that many other substances of the isoprenoid family, such as eugenol, farnesol, R-limonene, menthol, and squalene, are not able to have a positive effect on bisphosphonate-treated cells such as HUVEC, fibroblasts, and osteogenic cells. Only GGOH seems to harbor this effect [32]. In comparison to pro-angiogenic growth factors such as VEGF or prominin, GGOH offers the advantage of working directly on the N-BPs' pharmacological point of action, while VEGF or prominin would only indirectly stimulate angiogenesis. This study has also some limitations. Since this study has been designed as an *in vitro* study, it is debatable if the isolated EPCs demonstrate the same cell performance after bisphosphonate treatment *in vitro* as in an *in vivo* situation. Further, it is unknown if the bisphosphonate concentrations we used in the cell cultures can sufficiently imitate the bisphosphonate concentrations affecting on EPCs in the *in vivo* situation. Consequently, it is absolutely necessary to transfer the findings of this study in an *in vivo* model in order to overcome the mentioned limitations and to gather more information with reference to the outstanding questions.

**Conflict of interest** The authors declare that there is no conflict of interest. Christian Walter has received speaker fees from Roche and Riemser and funding from Novartis for a different study.

**Role of funding source** None

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