Journal of Bone Oncology 2 (2013) 110–115

Contents lists available at ScienceDirect

Journal of Bone Oncology

journal homepage: www.elsevier.com/locate/jbo

Research Article

# Regulation of VEGF by mevalonate pathway inhibition in breast cancer

Tilman D. Rachner [a,*], Andy Göbel [a], Maria Junker [a], Josefa Hötzel [a], Peggy Benad-Mehner [a], Peyman Hadji [b], Lorenz C. Hofbauer [a,c]

[a] Division of Endocrinology and Metabolic Bone Diseases, Department of Medicine III, Technical University, Dresden, Germany
[b] Department of Gynecology, Gynecological Endocrinology and Oncology, Philipps-University of Marburg, Marburg, Germany
[c] DFG Research Center and Cluster of Excellence for Regenerative Therapies, Technical University, Dresden, Germany

ARTICLE INFO

*Article history:*
Received 16 April 2013
Received in revised form
28 May 2013
Accepted 31 May 2013
Available online 7 June 2013

*Keywords:*
VEGF
Zoledronic acid
Atorvastatin
Mevalonate pathway
Breast cancer

ABSTRACT

Aminobisphosphonates are used for the treatment of benign and malignant bone disorders. As inhibitors of the mevalonate pathway they exert direct anti-tumor effects *in vitro* and in preclinical models of bone metastases. Bisphosphonates are thought to have an anti-angiogenic activity as decreased levels of VEGF have been reported in some, although not all patients, following treatment with bisphosphonates. Direct effects of bisphosphonates on tumor derived VEGF have not been examined in detail. We therefore investigated VEGF expression in breast cancer cell lines following mevalonate pathway inhibition. Treatment of cell lines with increasing doses of zoledronic acid and atorvastatin resulted in increased levels of VEGF production. Similar results were seen with the geranylgeranyltransferase I inhibitor GGTI-298. The induction of VEGF was reversed by the supplementation of geranylgeranyl pyrophosphate but not by farnesyl pyrophosphate indicating that this effect is mediated by inhibited geranylgeranylation. Previous reports have reported decreased VEGF levels in patients following BP treatment *in vivo*. We assessed VEGF levels in patients with non-metastatic breast cancer following repeated treatment with zoledronic acid. In contrast to our *in vitro* findings, VEGF serum levels decreased in all patients after 6–9 months of treatment (by an average of 41%) as assessed in a small pilot trial. These results indicate that tissues other than breast tumors contribute to the serum pool of circulating VEGF and may be responsible for the observed VEGF decreases. The increases of VEGF in the cancer cells may provide a rationale for the combined treatment with VEGF inhibitors.



## 1. Introduction

The occurrence of osteolytic lesions remains a feared long term complication in patients with breast cancer. Amino-bisphosphonates (BP) are a class of antiresorptive agents approved for the treatment of metastatic bone disease [1,2]. BP act via inhibition of the FPP-synthase, which is a key enzyme of the mevalonate pathway [3]. Aside from their potent antiresorptive properties, BP have been associated with a direct antitumor potential. *In vitro*, BP induce apoptosis and decrease proliferation and invasion of tumor cells [4]. While these results were confirmed in a number of preclinical *in vivo* models, results from two large clinical trials have yielded varying results regarding the adjuvant use of zoledronic acid in breast cancer patients [5,6]. One trial showed a significant reduction in the risk of disease progression in patients receiving zoledronic acid in addition to endocrine therapy compared to endocrine therapy alone in hormone-responsive breast cancer [5]. The other trial assessed the response of breast cancer patients with different hormone receptor expression and menopausal status to zoledronic acid in addition to their standard adjuvant treatment. This study failed to provide evidence to support the routine use of zoledronic acid in the adjuvant management of breast cancer [6]. One of the proposed antitumor effects of BP is their anti-angiogenic potential. A number of studies have shown that serum levels of VEGF decrease in tumor patients following treatment with bisphosphonates [7,8]. In one case, serum concentrations of VEGF were shown to be suppressed as early as 7 days after the first infusion of zoledronic acid and remained suppressed for the duration of the study, 84 days after the first infusion [7]. The finding of anti-angiogenic effects of these agents was further supported by the finding that bisphosphonates inhibited tumor vascularization in murine models of prostate carcinoma, melanoma and myeloma [9–11]. However, these results could not be confirmed in all studies and in some studies VEGF levels remained unaffected [12]. The molecular mechanisms responsible for these effects have not been looked at in detail. It remains unclear, if the observed regulation on VEGF is mediated via direct effects on cancer cells or by effects on other cells such as macrophages or endothelial cells.

*Abbreviations:* BP, amino-bisphosphonates; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate (FPP); GGPP, geranylgeranyl pyrophosphate; VEGF, vascular endothelial growth factor.

* Correspondence to: Division of Endocrinology, Diabetes and Bone Diseases, Department of Medicine III, Fetscherstraße 74, D-01307 Dresden, Germany. Tel.: +49 351 458 13731; fax: +49 351 458 5801.
*E-mail address:* tilman.rachner@uniklinikum-dresden.de (T.D. Rachner).

2212-1374/$ - see front matter © 2013 Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
http://dx.doi.org/10.1016/j.jbo.2013.05.003

This study is aimed at investigating the direct effects on mevalonate pathway inhibition using zoledronic acid, atorvastatin and specific inhibitors of farnesylation and geranylgeranylation in breast cancer cell lines with different metastatic properties and hormone receptor status.

## 2. Materials and methods

### 2.1. Cells and reagents

Human breast cancer cells were purchased from ATCC (Manassas, VA), except for the bone seeking MDA-MET cells (subclones of MDA-231 cells) which were a gift of Prof. L. Suva (Arkansas, USA). All cell lines were cultured in DMEM/Ham's F-12 (PAA, Pasching, Austria) with 10% fetal calf serum supreme (Lonza, Cologne, Germany) and 1% penicillin/streptomycin (PAA, Pasching, Austria). Cell line authenticity was determined by short tandem repeat profiling and by matching with the known profiles at DSMZ (German Collection of Microorganisms and Cell Culturs). Zoledronic acid, atorvastatin, mevalonate, geranylgeranyl-pyrophosphate (GGPP), farnesyl pyrophosphate (FPP), GGTI-298 and FTI-277 were obtained from Sigma-Aldrich (Munich, Germany). Zoledronic acid was solved in PBS. Mevalonate, FPP and GGPP were dissolved in methanol:$NH_4OH$ solution and atorvastatin was dissolved in DMSO. Appropriate controls were added to untreated control cells.

### 2.2. Cell culture and treatment

Breast cancer cell lines were treated with zoledronic acid (100 μM), atorvastatin (10 μM), FTI-277 (0.01, 0.1, 1 μM) and GGTI-298 (1, 5, 10 μM) for 24 h (unless otherwise indicated). Mevalonate substrates (GGPP, FPP and mevalonate) were supplemented together with atorvastatin and zoledronic acid at concentrations shown to reverse specific pathway inhibition.

### 2.3. RNA isolation, RT and real-time PCR

RNA from the cell lines was isolated using the HighPure RNA extraction kit from Roche according to the manufacturer's protocol. 500 ng RNA were reverse transcribed using Superscript II (Invitrogen, Darmstadt, Germany) and used for SYBR green-based real-time PCR reactions using a standard protocol (Applied Biosystems). Primer sequences for VEGFA were sense: GTGATGATTCTGCCCTCCTC and anti-sense: CCTTGCTGCTCTACCTCCAC; for GAPDH sense: CATCACCATCTTCCAGGAGCG and anti-sense: TGACCTTGCCCACAGCCTTG. PCR conditions were 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles with 95 °C for 15 s and 60 °C for 1 min. The melting curve as assessed in the following program: 95 °C for 15 s, 60 °C for 1 min and 95 °C for 30 s. The results were calculated applying the ΔΔCT method and are presented as relative expression to the house keeping gene (GAPDH) or as a percentage of control.

### 2.4. VEGF ELISA

VEGF ELISA was purchased from Thermo scientific (Waltham, MA) and conducted as proposed by the manufacturer. Briefly, cell culture supernatants were diluted 1:5 (as predefined by testing), serum samples were used undiluted. Samples were assayed in duplicates. 50 μl standard diluent was given to all wells and 50 μl of samples and diluted standards were pipetted to the appropriate wells and incubated for 2 h. Plates were washed and 100 ml of biotinylated Antibody Reagent was added to all wells, after which wells were incubated for another hour. After washing 100 ml of prepared Streptavidin–HRP Solution was given to all wells, incubated for an hour and washed thrice. Then 100 ml of TMB substrate solution was pipetted into all wells and after 30 min of incubation the reaction was stopped and wells were measured and values were calculated as proposed.

### 2.5. Western blot

Western blot analyses were performed as previously described [13]. Briefly, cells were washed and scraped in a lysis buffer and quantified. 20 μg of protein were loaded on a SDS–PAGE and transferred onto a 0.2 μm nitrocellulose membrane. After blocking for 1 h with 5% non-fat dry milk in Tris-buffered saline with 1% tween-20 (TBS-T), membranes were incubated with a primary antibody overnight. After washing, the membrane was incubated for 1 h with the HRP-conjugated secondary antibody. Membranes were washed 3 times with TBS-T again, and proteins were visualized with Super Signal (Pierce, Bonn, Germany) enhanced chemiluminescence. Antibody for RAP1A (sc-1482) was from Santa Cruz (Heidelberg, Germany) and the RAS (610001) antibody was from BD Biosciences (Heidelberg, Germany).

### 2.6. Serum samples

Serum samples of patients were obtained after informed consent and IRB approval. Patients with hormone receptor-negative, non-metastatic breast cancer were treated with infusions of 4 mg zoledronic acid every three months. All patients received 1000 mg Calcium and 1000 IE Vitamin D per day for the duration of the study. None of the patients receives any additional concomitant drugs known to influence bone turnover. None of the patients sustained a fracture during the study period nor were any other anti-resorptives given. Control serum was taken before the first administration of zoledronic acid and before each further administration. All serum samples were collected after an overnight fasting at the same time in the morning. Blood samples were immediately worked up and stored at −80 °C until the analyses were performed.

### 2.7. Statistical analyses

Results are presented as means ± standard deviation (SD). All experiments were repeated at least three times. Statistical evaluations were performed using a one-way ANOVA or a Student´s *T*-test. *P* values $< 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Zoledronic acid and atorvastatin increase VEGF expression in breast cancer

Three different breast cancer cell lines were treated with 100 μM zoledronic acid for 24 h and assessed for VEGF expression. Base line expression of VEGF varied greatly between the different cell lines. The hormone receptor positive MCF-7 cells had considerably lower levels of VEGF compared to MDA-231 and their metastatic subclones (data not shown). This is line with their lower aggressiveness and lower metastatic properties. Following exposure to zoledronic acid VEGF expression was significantly upregulated in all three tested cell lines (MCF-7, MDA-231 and MDA-MET) to 207%, 296% and 288% compared to PBS-treated cells, respectively (Fig. 1A). To test whether this effect was mediated via mevalonate pathway inhibition, the same cell lines were also treated with 10 μM of atorvastatin. Atorvastatin increased VEGF levels in all cell lines compared to control although the induction was smaller in MDA-231 and MDA-MET cells than previously seen with zoledronic acid (Fig. 1B). When treating MDA-231 cells for up to 48 h, VEGF increases were seen as early as 6 h after treatment with zoledronic acid and increased further after 24 h and 48 h