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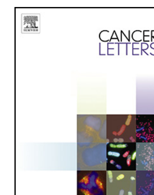

Original Articles

Combined inhibition of the mevalonate pathway with statins and zoledronic acid potentiates their anti-tumor effects in human breast cancer cells

Andy Göbel [a], Stefanie Thiele [a], Andrew J. Browne [a], Martina Rauner [a], Valentina M. Zinna [a], Lorenz C. Hofbauer [a,b], Tilman D. Rachner [a,*]

[a] Division of Endocrinology, Diabetes, and Bone Diseases, Department of Medicine III, Technische Universität Dresden, Dresden, Germany
[b] German Cancer Consortium (DKTK), Partner Site Dresden and German Cancer Research Center (DKFZ), Heidelberg, Germany

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ABSTRACT

Amino-bisphosphonates are antiresorptive drugs for the treatment of osteolytic bone metastases, which are frequently caused by breast and other solid tumors. Like statins, amino-bisphosphonates inhibit the mevalonate pathway. Direct anti-tumor effects of amino-bisphosphonates and statins have been proposed, although high concentrations are required to achieve these effects. Here, we demonstrate that the treatment of different human breast cancer cell lines (MDA-MB-231, MDA-BONE, and MDA-MET) by combined inhibition of the mevalonate pathway using statins and zoledronic acid at the same time significantly reduces the concentrations required to achieve a meaningful anti-tumor effect over a single agent approach (50% reduction of cell vitality and 4-fold increase of apoptosis; $p < 0.05$). The effects were mediated by suppressed protein geranylation that caused an accumulation of GTP-bound RhoA and CDC42. Importantly, the knockdown of both proteins prior to mevalonate pathway inhibition reduced apoptosis by up to 65% ($p < 0.01$), indicating the accumulation of the GTP-bound GTPases as the mediator of apoptosis. Our results point to effective anti-tumor effects in breast cancer by the combination of statins and zoledronic acid and warrant further validation in preclinical settings.

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Introduction

In European women, breast cancer remains the most frequent malignancy [1]. Breast cancer mortality is generally not caused by the primary tumor but rather by secondary metastasis to distant organs [2]. Here, the occurrence of metastatic bone lesions in up to 80% of patients with advanced disease remains a major long-term complication [3,4]. Bone metastases are characterized by a tumor-driven activation of osteoclasts that lead to an increased bone resorption and increase the risk of fractures, neurological complications and pain, all leading to a reduction in the quality of life of the patient [5,6].

Amino-bisphosphonates (N-BPs) are potent inhibitors of osteoclastic bone resorption and are a standard therapy for bone metastases [7]. N-PBs inhibit the farnesyl diphosphate synthase (FDPS), a key enzyme of the mevalonate pathway. This pathway plays an important role in the production of cholesterol, as well as in the posttranslational modification of signal proteins which is referred to as prenylation and comprises both farnesylation and geranylation [8]. Rho-family proteins that are small GTPases and include CDC42, Rac and Rho proteins represent a major family of proteins that undergo prenylation [9]. These are characterized by a cycle between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound status. In the normal cell Rho GTPases play a crucial role in migration, motility and adhesion and members of Rho signaling have been implicated in different types of cancer [10] including breast cancer [11].

Statins are another clinically approved class of drugs that inhibit the mevalonate pathway. They block the HMG-CoA-reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway, upstream of FDPS. Statins are used to reduce the production of cholesterol [12] and promote the internalization of plasma cholesterol by triggering a low density lipoprotein receptor (LDLR) feedback response [13].

Targeting the mevalonate pathway for the therapy of cancer emerged as a promising approach since cancer cells heavily rely on its various end products [14] and consume high levels of cholesterol to maintain their metabolism [15]. Moreover, an increased activity of the mevalonate pathway in breast cancer patients carrying a mutant p53 is associated with a poorer outcome of the disease [16]. Indeed, both N-BPs and statins have been shown to exert anti-tumor effects *in vitro* in a range of human tumor

* Corresponding author. Tel.: +49 351 458 4206; fax: +49 351 458 5821.
E-mail address: Tilman.Rachner@uniklinikum-dresden.de (T.D. Rachner).

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entities [13,17–22]. In addition, early clinical results suggested that bisphosphonates may reduce the occurrence of metastases to visceral and osseous sites [23]. In line with this observation, N-BPs were demonstrated to significantly reduce the occurrence of skeletal related events secondary to advanced breast cancer [7]. Following these results a large clinical trial program was initiated to assess the potential of zoledronic acid, the most potent bisphosphonate available to inhibit osteoclasts and bone loss [24], for the adjuvant treatment of breast cancer. Of note, in patients with early hormone receptor positive breast cancer, zoledronic acid improved disease-free survival and bone loss secondary to endocrine therapy, especially in postmenopausal women or those under hormone ablation therapy [25,26]. However, there were no effects on survival in women with hormone negative cancers [27] and the observed effects in the hormone receptor positive cohorts are currently considered to be most likely mediated by indirect effects on bone metabolism.

There are contradictory results from studies evaluating statins in cancer describing both reductions and no effects of the statins on the cancer risk as well [28].

A general finding from the *in vitro* studies was that high concentrations of statins and bisphosphonates, exceeding those generally achievable *in vivo*, are required for anti-tumor effects. Bisphosphonates in particular are rapidly cleared from the circulation [29], making it difficult to achieve and sustain high concentrations. In our study we tested the hypothesis that a combined mevalonate pathway inhibition by targeting FDPS and HMGCR at the same time has the ability to reduce the concentrations of the individual agents required for an effective anti-cancer therapy.

Materials and methods

Mevalonate pathway inhibitors

Cancer cells were treated with atorvastatin calcium salt trihydrate (ATO, Sigma-Aldrich, Hamburg, Germany), simvastatin (SIM, Sigma-Aldrich, Hamburg, Germany), rosuvastatin calcium (ROSU, SelleckChem, Munich, Germany) and/or zoledronic acid disodium salt (ZOL, Novartis, Basel, Switzerland). Stocks were prepared in DMSO (statins) or double distilled water (ZOL). The agents were administered individually or simultaneously in combination.

Cell culture

The human cancer cell lines MDA-MB-231 (breast), PC3 (prostate) and MDA-435s (melanoma) were obtained from ATCC (Manassas, VA, USA) and the MDA-BONE cells (also known as MB-231-TxSA cells) from the University of Texas (San Antonio, TX, USA). MDA-MET cells were kindly provided by Prof. L. Suva (Center for Orthopedic Research, University of Arkansas, AR, USA). MDA-MB-231, MDA-Bone and MDA-435s cell lines were cultured in DMEM/Ham's F12 (Gibco Life Technologies, Darmstadt, Germany) and PC3 cells in RPMI 1640 medium (Gibco Life Technologies) supplemented with 10% fetal bovine serum (FBS; Biochrome, Berlin, Germany) and 1% penicillin/streptomycin (Gibco Life Technologies). Cells were grown in a humidified atmosphere of 95% air and 5% CO_2. Short tandem repeat profiling of all used cell lines was performed at the DSMZ (German Collection of Microorganisms and Cell Cultures) to verify their genetic authenticity.

RNA isolation and real-time PCR

Gene expression analysis was performed as previously described [30]. The primer sequences (Sigma-Aldrich) for human genes were as follows: *GAPDH* (glyceraldehyde 3-phosphate-dehydrogenase): AGCCACATCGCTCAGACAC, GCCCAATACGACCAAATCC; *BCL-2* (B cell lymphoma 2): TGTGTGTGGAGAGCGTCAAC, GACAGCCAGGAGAAATCAAAC, *SVV* (Survivin): GAACTGGCCCTTCTTGGAG, AAGTCTGGCTCGTTCTCAGTG, *RAC1* (Ras-related C3 botulinum toxin substrate 1): AATCTGGGCTTATGGGATACAG, ATGGGAGTGTTGGGACAGTG; *RHOA* (Ras homolog gene family, member A): GGAGTGTTCAGCAAAGACCA, CAAGACAAGGCACCCAGATT; and *CDC42* (cell division cycle 42): ACGACCGCTGAGTTATCCAC, CCCAACAAGCAAGAAAGGAG. See Supplementary Materials for detailed description.

Transfection

MDA-MB-231 cells were grown until sub-confluence and transfected using DharmaFect and gene-specific siRNAs. SiRNAs and DharmaFect were separately mixed with FBS-free Opti-MEM and incubated for 5 min at room temperature (RT), pooled afterwards and incubated for 20 min at RT. Cells were washed once with Hank's balanced salt solution (HBSS) and 850 µl Opti-MEM without penicillin/streptomycin added. 150 µl of transfection mixtures were added dropwise to each well. The final concentration of the siRNAs was 50 nM. The medium was changed to normal DMEM/F-12 after 6 h to stop the transfection.

Metabolic and apoptosis analyses of cancer cells

Cell viability and the activity of caspases 3 and 7 were assessed using the CellTiterBlue® and Caspase 3/7 Glo® assays (Promega, Mannheim, Germany). The detection of fragmented DNA was performed by a Cell Death ELISA (Roche) according to the manufacturer's protocol. To normalize the caspase 3/7 activation to the total cell number in the rescue experiments, a crystal violet staining was performed. Here cells were washed with PBS and fixed using 10% paraformaldehyde (PFA) for 15 min. Cells were subsequently washed with double-distilled water (ddH_20) and stained with crystal violet working solution (0.02% in 2% ethanol) for 20 min. Stained cells were again washed with ddH_20, completely dried and the dye eluted with 10% SDS upon shaking. The absorbance was detected at 595 nm. The measurements were completed using the FluoStar Omega (BMG labtech, Jena, Germany).

Pull-down assay of activated (GTP-bound) RhoA and CDC42

For the analysis of the GTP-bound forms of CDC42, Rac1 and RhoA, a commercially available pull-down assay was used (Pierce, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, cells were washed with Tris buffered saline and proteins isolated with the provided Lysis/Binding/Wash Buffer. Afterwards at least 500 µg of total protein was added to a glutathione containing resin and incubated with the Rac/CDC42 binding domain of the human p21 activated kinase 1 protein or the Rho-binding domain of the human Rhotekin protein tagged with a glutathione-S-transferase for at least 1 h. After washing the resin for three times, GTP-bound forms of the Rho-GTPases were eluted using the SDS-Sample buffer containing β-mercaptoethanol (Sigma-Aldrich). Eluates were subsequently analyzed in a Western blot assay.

Immunohistochemistry

In order to analyze the cytoskeleton, cells were seeded on sterilized glass slides and treated for 48 h. Cells were washed once with PBS and fixed with 4% PFA at RT. Afterwards cells were permeabilized with 0.1% Triton-X-100 for 20 min. Using 1% BSA/0.05% Tween-20 unspecific binding sites were blocked for 1 h. The F-actin of the cytoskeleton was labeled by adding 2 U/ml (0.06 µM) Alexa-Fluor-488 conjugated Phalloidin (Thermo Fisher Scientific, Schwerte, Germany) for 1 h. After washing the cell nuclei were stained with 2.5 µg/ml DAPI (Sigma-Aldrich) for 5 min and washed with PBS several times. The cells were embedded in mounting medium (Dako, Hamburg, Germany) and morphological changes analyzed using the microscope Axiovert 40C (Carl Zeiss Jena, Jena, Germany).

Breast cancer cDNA array

The breast cancer cDNA array IV was purchased from Origene (Rockville, MD, USA) and the expression of HMGCR and FDPS was analyzed according to the supplier's protocol. GAPDH expression was assessed for normalization. The array contained 48 samples; 4 samples of normal breast tissue and 44 samples of breast cancer. The selected pathological grades ranged from I to IV. The average patient's age was 54 years.

Statistical analyses

Results are presented as means ± standard deviation. All experiments were repeated at least three times. Outliers were determined via Grubb's test. A student's t-test was used to perform single group comparisons and group analyzes were performed using one-way analysis of variance (ANOVA) by GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA). P-values < 0.05 were considered statistically significant.

Other methods

Please see Appendix: Supplementary Methods and Materials.

Results

FDPS and HMGCR expression positively correlate in clinical breast cancer samples

Baseline expression of the two target enzymes of statins and zoledronic acid, the HMGCR and FDPS, was assessed in clinical cDNA samples of human breast cancer. Thirteen of 24 (stage II, 54%) and 10 of 17 (stage III, 59%) samples showed an increase of HGMCR expression (Fig. 1a) compared to the average expression of the control

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