Zoledronic acid decreases mRNA six-transmembrane epithelial antigen of prostate protein expression in prostate cancer cells

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ABSTRACT. Background: Zoledronic acid (Zol) is used successfully to inhibit bone resorption in tumor bone disease of various human cancer. Zol inhibits the mevalonate pathway and other potential targets include the inhibition of tyrosine phosphatase activity, disruption of metalloproteinase secretion and down-regulation of the catalytic subunit of telomerase (hTERT). The six-transmembrane epithelial antigen of prostate protein (STEAP) is a new marker highly expressed at all phases of prostate cancer. Aim: Here, we analyzed for the first time the effect of Zol on STEAP gene expression in prostate cancer cells. Material and methods: We evaluated the effects of Zol in STEAP gene expression by RT real time PCR in androgen-sensitive (LNCaP) and androgen-non-sensitive (PC3 and DU145) cell lines. To confirm the pro-apoptotic effect of Zol, we also analyzed the caspase-3 gene expression, that resulted up-regulated in cancer cell apoptosis. Results: Zol strongly decreased cell viability and lowered STEAP gene expression in a dose-dependent manner. In addition, this effect was accompanied by an increase of apoptotic index and an up-regulation of caspase-3 gene expression. Conclusion: Zol may affect cancer cells also by targeting the gene expression of STEAP. (J. Endocrinol. Invest. 33: 244-249, 2010) ©2010, Editrice Kurtis

INTRODUCTION
Besides the use of bisphosphonates for bone diseases such as osteoporosis, zoledronic acid (Zol), a 3rd-generation bisphosphonate, has direct anti-tumor effects in vitro and can reduce tumor burden in a variety of animal models (1). Zol inhibits the mevalonate pathway (2) blocking the biosynthesis of precursors needed for the prenylation of small gtp-binding proteins and this reduces the activity of osteoclasts and also slows the progression of tumor cells (3). Other potential targets of bisphosphonates include inhibition of tyrosine phosphatase activity (4) and disruption of metalloproteinase secretion (5-7). Six-transmembrane epithelial antigen of the prostate (STEAP) is highly expressed at all steps of prostate cancer. Thus, we evaluated for the first time the effect of Zol on STEAP gene expression in prostate cancer cells. Material and methods: We evaluated the effects of Zol on STEAP gene expression by RT real time PCR in androgen-sensitive (LNCaP) and androgen-non-sensitive (PC3 and DU145) cell lines. To confirm the pro-apoptotic effect of Zol, we also analyzed the caspase-3 gene expression, that resulted up-regulated in cancer cell apoptosis. Results: Zol strongly decreased cell viability and lowered STEAP gene expression in a dose-dependent manner. In addition, this effect was accompanied by an increase of apoptotic index and an up-regulation of caspase-3 gene expression. Conclusion: Zol may affect cancer cells also by targeting the gene expression of STEAP. (J. Endocrinol. Invest. 33: 244-249, 2010) ©2010, Editrice Kurtis

Key-words: Prostate cancer, RT Real-time PCR, STEAP, zoledronic acid.
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MATERIALS AND METHODS

Cell culture
Androgen-non-sensitive human prostate cancer cell lines (PC-3 and DU-145) and androgen-sensitive human prostate cancer cell lines (LNCaP) were purchased from the American Type Culture Collection (ATTC Rockville, MD, USA). The DU-145 and PC3 cell lines were cultured and passaged in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 media containing 10% fetal bovine serum (FBS). The LNC-aP cell line was cultured in RPMI 1640 containing 10% FBS, supplemented with 100 nM testosterone.

Zol studies
Zol (1-hydroxy-2,1 imidazol-1-yl-ethylidene bisphosphonic acid) was kindly provided by Novartis. The neutralized sodium salts
of Zol was dissolved in sterile double distilled H2O at a final concentration of 100 mM. Stock solutions were aliquoted and kept at −20°C for long-term storage. Cells from 80% confluent cultures were washed with phosphate buffered saline and treated with trypsin/EDTA. Cells were plated at 1×10^6 in 25 cm² flask and incubated for 24 h at 37°C to allow cell adhesion to new culture plates. Cells were treated for 24, 48, and 72 h with Zol in DMEM/F12 containing 5% FBS. For each concentration, 3 separate flasks were treated.

Cell viability

Cell viability was evaluated by a colorimetric assay based on the reduction of the tetrazolium salt XTT (sodium 3-[1-phenylamino-carbonyl-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) by mitochondrial dehydrogenase of viable cells to a formazan dye (Cell proliferation kit II — XTT Roche). Briefly, 5×10^4 cell/well in 96 microtitre plates were cultivated in medium containing Zol ranging from 0.1 to 100 μM. After the treatment, 50 μl XTT labeling mixture was added to each well and incubated at 37°C for 4 h. The spectrophotometric absorbance of the samples was measured using a microtitre plate (enzyme-linked immunosorbent assay) reader at a wavelength of 450 nm.

RNA extraction and RT

Cells treated were trypsinized and the cell pellets were collected by centrifugation at 1000 g for 10 min at 4°C. Total RNA was extracted from each cell culture flask using the RNeasy minikit (Qiagen) with DNase I treatment. First-strand cDNA was generated using the High-Capacity cDNA Archive Kit, with random hexamers (Applied Biosystems PE) according to the manufacturer’s protocol. RT product was aliquoted in equal volumes and stored at −80°C.

Real time PCR

PCR was performed in a total volume of 50 μl containing 1×Taqman Universal PCR Master mix, no AmpErase UNG and 5 μl of cDNA; pre-designed, gene-specific primers and probe sets for each gene (STEAP Hs00185180_m1) (caspase 3 Hs00234387_m1) (B2M; Hs99999907) were obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems). Real time RT-PCR reactions were carried out in two-tube system and in multiplex. The real time amplifications included 10 min at 95°C (AmpliTaq Gold activation), followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min. Thermocycling and signal detection were performed with ABI Prism 7300 Sequence Detector (Applied Biosystems). Signals were detected according to the manufacturer’s instructions. This technique allows the identification of the cycling point where PCR product is detectable by means of fluorescence emission [threshold cycle (Ct) value]. As previously reported, the Ct value correlates to the starting quantity of target mRNA (15). Relative expression levels of STEAP and caspase 3 were calculated for each sample after normalization against the housekeeping gene B2M, using the ΔΔCt method for comparing relative fold expression differences (16). The data were expressed as mRNA fold.

Ct data

Ct values for each reaction were determined using TaqMan SDS analysis software. For each amount of RNA tested triplicate Ct values were averaged. Because Ct values vary linearly with the logarithm of the amount of RNA, this average represents a geometric mean.

Effect of zoledronate on STEAP gene expression

The apoptotic index was assessed in cell cultures treated with Zol ranging from 10 to 50 μM. The cell cultures were stained with hematoxylin and eosin as previously described (17). Ten high-power field (X 100) were randomly chosen within the slides. The total number of tumor cells and apoptotic cells were scored. At least 100 cells were counted. Briefly, cells showing pyknotic nuclei with nuclear condensation and condensed eosinophilic cytoplasm were scored as apoptotic cells. The apoptotic indices were defined as the percentage of apoptotic cells in relation to the total number cells.

Statistical analysis

Results are expressed as mean±SEM. The Wilcoxon test was used for non-parametric data. For analysis of dose responses, analysis of variance followed by Bonferroni as post-hoc analysis was performed. A p-value <0.05 was considered to be statistically significant. Analyses were applied to experiments carried out at least 3 times. Statistical analyses were performed using SPSS for Windows, version 16.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

Effects of Zol on cell viability

The cancer cell lines PC3, DU145, and LNCaP were treated with increasing concentration ranging from 0.1 μM to 100 μM of Zol. The XTT test assayed for vitality revealed that LNCaP are more sensitive to Zol with respect to androgen-independent prostate cancer cells. The levels of cell viability obtained are expressed in percentage (Fig. 1). Zol at 1, 10, 50, and 100 μM induced a significant decrease of tumor cell viability with respect to control in all cell lines after 48 h of treatment. In particular, at the concentration of 1 μM of Zol, the viability was 90%, 93%, and 80% in PC-3, DU145 and LNCaP (<0.05), respectively; at the concentration of 10 μM of Zol, the viability was 80%, 83%, and 74% in PC-3, DU145, and LNCaP (p<0.0001), respectively. At 50 μM and 100 μM Zol decreased the viability of cancer cells of about 52.6%, 48% (PC3), 54%, 50% (DU145), 48%, 46% (LNCaP), respectively (p<0.0001) (Fig. 1B). In addition, after 72 h, the treatment with Zol significantly decreased the cell viability (p<0.0001), even at the lowest concentrations tested (Fig. 1C).

Effect of Zol on STEAP and caspase-3 mRNA

STEAP is implicated in regulation of proliferation and invasiveness of prostate cancer cells (9), whereas caspase-3 expression is over-expressed during chemotherapeutic-induced-apoptosis (13). To investigate the effects of bisphosphonates on STEAP and caspase-3 mRNA expression in prostate cells, we performed a real time PCR. After 24 h of treatment, the difference between treated groups and controls did not reach the statistical significance (Fig. 2A and 3A). On the contrary, after 48 h of treatment, Zol decreased of about 0.68-0.55 (PC3), 0.7-0.49 (DU145), and 0.67-0.48 (LNCaP) fold at 10, 50 μM the STEAP expression, respectively (p<0.0001) (Fig. 2B). After 48 h of treatment, the expression of caspase 3 was increased in cells treated with Zol with respect to control. The caspase-3 gene expression increased significantly (p<0.0001) of about 1.32-, 1.44-, and 1.49-fold, respec-