Original Article
The anti-tumor activity of zoledronic acid and synergistic effect of zoledronic acid combination with docetaxel on prostate cancer cell line PC-3 in vitro at clinically achievable concentrations

Zhiyuan Shen, Shaojie Li, Weiqing Qian, Zhongquan Sun

Department of Urology, Huadong Hospital, Fudan University, 221 West Yan’an Road, Shanghai, China

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Abstract: To observe the effect of zoledronic acid alone or combination with docetaxel in different concentrations on prostate cancer PC-3 cells in vitro. Cell morphology, MTT, flow cytometry method was used to observe this effect. Above 200 μg/ml zoledronic acid significantly enhanced growth suppression (suppression ratio ≥44.5%, P<0.05), apoptosis (apoptosis ratio ≥5.93%, P<0.05). The combination of ZOL and DOC inhibited the proliferation of PC-3 cells significantly. The effect was dose-dependent by ZOL with a certain dose of DOC. When the concentration of DOC was 10 ng/ml, the inhibition rate was 49.3%, 61.4% respectively after the treat with 200 μg/ml ZOL or 400 μg/ml ZOL. The inhibition rate could rise to 59.7%, 74.2% with 200 μg/ml ZOL or 400 μg/ml ZOL exposure combined with 100 ng/ml DOC. Flow cytometry showed G2 and S phase of PC-3 cells in 400 μg/ml zoledronic acid were inhibited significantly more than the control group (P<0.05) and G1 phase was less inhibited (P<0.05). This research validates that ZOL has the effect of inhibiting proliferation and inducing apoptosis of hormone refractory prostate cancer PC-3 cells. ZOL arrests the cell cycle to S phase, G2/M phase. These effects are dose-dependent. The combination of ZOL and docetaxel inhibites proliferation more than each drug alone, the effect of which is also dose-dependent and the two drugs have synergia anti-tumor effect on prostate cancer PC-3 cells in vitro.

Keywords: Zoledronic acid, docetaxel, prostate cancer PC-3 cells, anti-tumor effect

Introduction

Bisphosphonates are synthetic compounds pyrophosphate, which are mainly used for prevention and treatment of senile osteoporosis for more than 20 years. Zoledronic acid (ZOL) is the third generation of nitrogen containing bisphosphonates (N-BPs). This medicine could inhibit osteoclast-mediated bone resorption, suppress skeletal metastases from cancer, and treat all kinds of osteoporosis, pathological fracture and hypercalcemia induced by bone metastases through inhibiting hydrolyase of osteocyte and impairing activity and proliferation of osteoclast by preferentially combining with abnormal sites of bone metabolism. In the prostate cancer setting, ZOL is a sort of bisphosphonates that was demonstrated to reduce skeletal related events and maintain endurable pain relief. Otherwise, recent studies showed that ZOL could directly inhibit growth of tumor cell, which was identified in tumor cells including breast cancer and myeloma in vitro [1]. ZOL administration may greatly contribute to increasing survival in patients with aggressive CRPC not only through action against development of bone metastases [2]. However, researches on ZOL directly inhibiting prostate cancer was seldom reported. Therefore, the aim of this study was to investigate the effects of ZOL on the hormone refractory prostate cancer cell line PC-3 at different levels of concentration, achieve to provide theoretical basis for clinical use of ZOL for prostate cancer.

Material and methods

Cell culture

The hormone refractory prostate cancer cell line PC-3, obtained from the Shanghai Institutes
For Biological Science Cell Resource Center, was routinely cultured in Ham’s F-12 media (GIBCO/BRL, New York, USA), supplemented with 10% foetal calf serum (HyClone, Utah, USA) and penicillin-streptomycin (100 u/ml penicillin and 100 μg/ml streptomycin). Cells were incubated in an atmosphere of 5% CO₂ at 37°C.

PC-3 cells at exponential growth phase of 2×10⁵/ml were seeded at a cell density of 4×10⁴ cells/well in 96-well plates. Cells were cultivated for 12 h to allow adherence to the plate.

**Drugs**

ZOL was purchased from Novartis (Basle, Switzerland). Docetaxel was purchased from Sanofi (Paris, France). A stock solution of ZOL was prepared and diluted in culture medium at appropriate concentrations at 4°C prior to use. Docetaxel was also diluted in culture medium at appropriate concentrations prior to use.

**Zoledronic acid exposure**

ZOL treatment consisted of continuous exposure of 6.25 μg/ml, 12.5 μg/ml, 25 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml concentrations for 2 h. Control groups were set.

**Drug combinations**

PC-3 cells were exposed to media contained with ZOL in various concentrations for 2 hours by the addition of DOC (10 ng/ml and 100 ng/ml) for a further 24 hours in vitro. Following exposure, cells were washed free of drug using PBS, 200 μl fresh media was added and the...
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Cells were incubated for an additional 48 hours before a MTT assay was performed.

**MTT assays**

Cells after treatment were added MTT for duration of 4 h. Then cells were solubilized with the solvent dimethylsulfoxide. Absorbance was tested in a 550 microplate reader (BIORAD, California, USA) at 540 nm and results are showed as the value of OD in the treated wells versus the controls. The inhibition ratio of tumor cell = (1- mean OD value of treatment group/mean OD value of controls group)×100%). Cell viability was assessed by an MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay.

**Flow cytometry**

PC-3 cells were cultured in the 6-wells dishes, treated with ZOL of different concentrations for 2 hours at first. Then the drugs were removed and the cells were incubated with fresh media for an additional 72 hours. At the end of the treatment, the cells were harvested and centrifuged. Then single cell suspension was made in the 70% frozen alcohol. The cell cycle and the apoptosis rate were analyzed by an EPISC-XL flow cytometer (Beckman Coulter, California, USA). 1×10⁵ cells were analyzed for each specimen by the software attached. Cells not exposed to drugs were served as a negative control. The experiment was carried out in triplicate.
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Statistical analysis

All statistical comparisons were made with Stata 9.0. Measurement data were reported as mean ± standard deviation. Differences between treatments were determined using the Student’s t test. The t test was used for the unequal variances. The level of significance was defined as P<0.05.

Results

Morphologic observation

The cell density and morphologic was observed through the light microscope. The density of PC-3 cells exposed with ZOL in different concentrations significantly decreased with unapparent morphologic change. Cytoplasm and nucleus was close to the control group, while the cell size went slightly smaller (Figure 1).

Effect of ZOL on cell viability

PC-3 cells were firstly exposed with media contained ZOL for 2 hours in different concentrations in vitro. Then, a MTT assay was performed to analysis the proliferation after further 72 hours incubation with fresh media. Results showed that, ZOL inhibited the proliferation of PC-3 cells significantly dose-dependently. If the concentration was below 100 μg/ml, the inhibition rate was unobvious [(19.0±4.6)%]. When the concentration of ZOL reached 200 μg/ml, the inhibition rate was remarkable [(44.5±1.3)%]. And it would rise to 57.9% with a 400 μg/ml ZOL exposure (Figure 2).

Figure 5. Proliferation of PC-3 cells treated with ZOL or the combination of ZOL and DOC for 72 h. A: Control, B: Treated with combination of ZOL and DOC.

Figure 6. MTT Assays of PC-3 cells treated with ZOL or the combination of ZOL and DOC for 72 h. A: Control, B: Treated with combination of ZOL and DOC.
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The cell cycle using FCM

Cell cycle and apoptosis rate was analyzed by FCM. The results showed that the apoptosis rate of PC-3 cells was 0.73%, and the proportion of the G1, S, G2 phase was 61.2%, 36.6%, 2.13% (Figure 3). The proportion of G1, S, G2 phase was 45.71%, 48.45%, 5.83% and the apoptosis rate was 5.93% after treated with 400 μg/ml ZOL. The cell cycle analysis showed an S phase arrest and a G2/M arrest, cell number of G1 phase reduced significantly while those of S phase and G2 phase increased significantly (Figure 4).

Effect of ZOL and docetaxel on cell viability

Cell density was certainly decreasing and shrinkage of cells was found through the light microscope (Figures 5, 6). The combination of ZOL and DOC inhibited the proliferation of PC-3 cells significantly. The effect was dose-dependent by ZOL with a certain dose of DOC (Figure 7). When the concentration of DOC was 10 ng/ml, the inhibition rate was 49.3%, 61.4% respectively after the treatment with 200 μg/ml ZOL and 400 μg/ml ZOL. It rose to 59.7%, 74.2% with a 200 μg/ml ZOL or 400 μg/ml ZOL exposure combined with a 100 ng/ml DOC (Figure 7).

Discussion

ZOL was demonstrated to reduce skeletal related events and maintain endurable pain relief [3]. However, there is a growing evidence, both from in vitro and in vivo models, showing that ZOL could also target tumour cells to increase apoptotic cell death and decrease proliferation, migration and invasion [4], and that this effect is significantly enhanced in combination with chemotherapy agents [5]. The mechanism of action is believed to be inhibition of the mevalonate pathway by preventing farnesylation and geranylgeranylation of small GTP binding proteins such as Ras, Rac and Rho, activation of caspase-3 and altering Bcl-2/Bax ratios [6, 7]. Zol decreased tumor associated macrophages infiltration into tumour stroma associating with decreasing levels of proMMP-9 and VEGF; and reduced TAM infiltration correlating to reduced metastatic spread, decreased tumour volume and angiogenesis and increased survival [8, 9]. Experiments in vitro have been confirmed ZOL played a role of tumor inhibition on breast cancer or myeloma [10, 11]. This study showed that ZOL at clinically achievable concentrations suppressed cell proliferation on PC-3 cells in vitro. The mechanism might be related to arresting the cell cycle and inducing apoptosis.

The half-lives of the ZOL was 0.23 h. After 1.75 hours, it got into long-term eliminating phase. The rapid postinfusion phase declined to 1% of peak plasma concentrations by 24 hours post-dose [12].

Through pre-test, we found that ZOL at low concentrations (1~100 μg/ml) persisted unchang-
ed inhibition on PC3 cells over time (24 h, 48 h, 72 h). Therefore, the value of peak concentration was picked and then diluted by the double-dilution method mimicking ZOL's pharmacokinetics in human body, which meant much more clinical significance. The exposure period to ZOL maintained just 2 hours. However, ZOL around peak concentrations showed a significant inhibition on PC-3 cells [inhibition rate = (57.9±1.3)\%], despite exposure for just 2 h. Therefore, we concluded that ZOL was demonstrated to inhibit prostate cancer cell line PC-3 in vitro at clinically achievable concentrations.

Cell cycle analysis by flow cytometry showed that the control group had a natural distribution of cells in the G0/G1, S and G2/M phases. ZOL treatment of PC3 cells resulted in S and G2/M phases arresting dose-dependently, which were consistent with other tumour cell treated by ZOL [13]. It indicated S and G2/M phases arresting were associated with inhibition of PC-3 cells by ZOL. Moreover, the inhibition could be enhanced through inducing PC-3 cells apoptosis.

Given that, this study combined ZOL with docetaxel, the first-line chemotherapy drug, to observe whether it could generate a synergia anti-tumor effect, which might support theory basis for ZOL's clinical use for advanced hormone independent prostate cancer.

Docetaxel is a taxoid analogue and a potent inhibitor of microtubular depolymerization. Some studies have shown synergistic action of ZOL and taxanes (both in vitro and in vivo) in prostate cancer [13-16]. The ability of ZOL to arrest cells in G2 and M or prolong the cell cycle progression raises the possibility of ZOL as a potential cell cycle chemosensitizer because G2 and M cells are more sensitive than cells within other phases of the cell cycle [17]. This synergia anti-tumor effect may also be due to downregulation of antiapoptotic protein Bcl-2 in PC-3 [18]. In this study, PC-3 cells were exposed to ZOL in various concentrations for 2 hours by the addition of DOC (10 ng/ml and 100 ng/ml) for a further 24 hours in vitro. The combination of ZOL and DOC inhibited the proliferation of PC-3 cells significantly. The effect was dose-dependent by ZOL with a certain dose of DOC.

**Disclosure of conflict of interest**

None.

**Address correspondence to:** Zhongquan Sun, Department of Urology, Huadong Hospital, Fudan University, 221 West Yan’an Road, Shanghai 200040, China. E-mail: drzhongquan@sina.cn

**References**


myeloma cell growth and secretion of IL-6 and MMP-1 by the tumoral environment. J Bone Miner Res 1999; 14: 2048-2056.


