Terazosin Suppress Human Prostatic Cancer PC3 Cell Viability via Proteasome Inhibition

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Abstract

Terazosin is one of classic quinazoline-based selective α1-adrenoreceptor antagonists, which is usually used for the treatment of benign prostate hyperplasia (BPH) patients. Several evidences suggest that terazosin can induce apoptosis of prostatic cancer cells in vitro and suppress prostatic tumor growth in vivo, but molecular mechanism contributing to these processes has not yet been fully elucidated. In this study, we report that the suppression of terazosin on prostatic cancer PC3 cells viability partially mediated by proteasome inhibition. We first examined cytotoxicity of terazosin in human prostatic cancer cell line PC3, including cell viability, cell cycle analysis and cell apoptosis analysis. Then the chymotrypsin-like proteasome activity, levels of ubiquitinated-proteins and selective protein substrate P27 in PC3 cells, which occurs prior to cell death. In view of these results, we conclude that terazosin suppress human prostatic cancer PC3 cell viability by cell cycle arrest and cell death induction, which is associated with its proteasome inhibitory activity.

Keywords: Terazosin; Prostatic cancer cell; Proteasome activity; Cell cycle; Cell death

Introduction

Prostatic cancer (PCa) is the most commonly diagnosed malignancy cancer in men worldwide. However, more than a half of patients are diagnosed with bone and lymph nodes metastasis predicated by pathologic or clinical evidence, or disturbed by progression from androgen-dependent cancer to androgen-independent cancer (CRPC) with more invasion and metastatic activity. Chemotherapy is still one of the main means for the treatment of CRPC, which is investigated to target prostatic cancer alone, or combine with other agents, to make CRPC cells undergo apoptosis effectively [1,2].

Terazosin is one of classic quinazoline-based selective α1-adrenoreceptor antagonists, which is usually used for the treatment of benign prostate hyperplasia (BPH) patients. Moreover, several evidences suggest that terazosin can induce apoptosis and suppress growth of both androgen-sensitive and -insensitive human prostatic cancer cells in vitro and in vivo [3]. But the molecular mechanism contributing to these processes has not yet been fully elucidated only with a few reports on antitumor potency of terazosin of apoptotic induction and antiangiogenic action [3,4], which need to be defined comprehensively.

Ubiquitin-proteasome pathway is one of important molecular targets of many chemotherapeutic agents, which has become a novel target for cancer therapy [5]. The proteasome is a primary multi-catalytic-protease-complex for selective degradation of more than 80% intracellular proteins. The degradation is considered to be a key step in intracellular-proteins-recycling, which is defined as “non-lysosomal recycling” [6]. Dysfunction of the ubiquitin proteasome system will lead intracellular equilibrium to an indiscriminate environment, followed by cell growth inhibition and cell death. Several native and synthetic compounds have been investigated with proteasome activity inhibition [7], and they have some classic structures [8-14]. C-terminal furanyl substitution is one of the classic structures, which have been identified to contribute to proteasome inhibition with unique mechanism [15], so exploitation of proteasome inhibitory activities of more furan-contained compounds may be a work worth paying attention to.

Since the chemical structure of terazosin is also a C-terminal furan-contained structure [16], which is considered to be one of classic structures of potential 20S proteasome inhibitors. In this study, we present that terazosin can also inhibit proteasome activity which is one of the molecular mechanisms partially mediate suppressive action of terazosin on CRPC PC3 cell lines.

Materials and Methods

Materials

Annexin-V-FITC/PI apoptosis detection kit was purchased from Keygen Company (Nanjing, China). PC3 cell lines were purchased from ATCC. Anti-PARP antibody was purchased from Cell Signaling.
Anti-Ubiquitin, anti-P27 and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology.

Cell culture

PC3 cells (purchased from ATCC) were cultured in F-12K medium (Gibco) with 10% (V/V) fetal bovine serum, and maintained at 37 with 95% humidified air and 5% CO₂.

Cell viability assay

8×10³ PC3 cells were plated in 96-well to incubated overnight, and treated with terazosin for the indicated periods. The plate was read in a microplate reader at 570/600 nm (VERSAmax; Molecular Devices) after staining with 10% Alamar blue solution for 4h. Analysis was performed on triplicate wells, and the data presented is representative of three independent experiments.

Cell cycle analysis

PC3 cells were treated with various concentrations of terazosin for the indicated periods. Cell cycle was measured by flow cytometry (FACScalibur, BD), and data was analyzed.

Cell death assay

Cells were plated at a density of 2×10⁵ cells/well in six-well plates and incubated overnight, and treated with 300 μM terazosin at indicated points. Then cells were collected and washed, re suspended in 0.5 ml of binding buffer, incubated with 2 μl Annexin V-FITC dye and 5 μl PI dye (Keygen, Nanjing, China) for 10 min in the dark. Cell death was detected using FACScalibur flow cytometry and Western blot analysis for cleaved-PARP fragment. Cell death was detected using FACScalibur flow cytometry and Western blot analysis for cleaved-PARP fragment.

Peptidase activity assay

Crude protein extracts (10 μg protein) from the cultured cells were added to a total volume of 180 μl reaction buffer (pH 7.4) containing HEPES (25 mmol/L), EDTA (0.05 mmol/L), NP-40 (0.05%), SDS (0.001% w/v) and the synthetic fluorogenic peptide Suc-LLVY-aminomethylcoumarin (AMC) (50 μmol/L) for the proteasomal chymotrypsin-like activity. The reaction mixture was then incubated at 37 for 90 min, and then fluorescence intensity of the free AMC was measured by a luminescence microplate reader (Varioskan Flash 3001, Thermo, USA). The excitation wavelength was 360 nm and the emission was 436 nm, respectively.

Western blot analysis

Cells were seeded in 6-cm diameter dishes and incubated overnight, then treated with various concentrations of terazosin for the indicated periods. The cells were collected and washed, and solubilized with lysis buffer. Protein samples (30-120 microgram) were separated by SDS/PAGE (10% gels) and transferred on to a nitrocellulose membrane (Millipore Corporation, Billerica). The membrane was incubated with anti-PARP (Cell Signaling), anti-ubiquitin, anti-P27 or anti-GAPDH (Santa Cruz Biotechnology) as primary antibodies, followed by incubation with horseradish peroxidase-conjugated IgG (Amersham Biosciences) as the secondary antibody. Then they were detected using the enhanced chemiluminescence (ECL) detection reagents (Thermo, USA) and exposed to X-ray films (Kodak).

Data Analysis

All the experiments were done in triplicate and quantitative data are presented as mean±SEM. Comparisons of the data among different treatments were evaluated by one-way ANOVA with Dunnett’s post hoc test using SPSS software version 13.0 and P values <0.05 were considered statistically significant.

Results

Terazosin suppresses viability of prostatic cancer cell PC3

PC3 cells were treated with various concentrations of terazosin for 48h, followed by staining with Alamar blue and measurement at indicated wavelength. The results showed that terazosin can potently decrease viability of PC3 cells in a dose-dependent manner, and the significant inhibition was identified when cells were treated with a dose higher than 50 μM terazosin (Figure 1).

Figure 1: Terazosin inhibits cell viability of PC3 cells in a dose-dependent manner. PC3 (8×10³ cells/well) cells were plated in 96-well plate and exposed to different concentrations of terazosin for 48h, followed by Alamar blue assay. N=3 independent experiments for each bar; *P<0.05 vs. control.

Terazosin induces cell cycle arrest at G0-G1 phase in PC3 cells

As cell cycle arrest is one of important influences of cell viability, we analyzed distribution of cell populations in different phases of cell cycle after terazosin treatment.

PC3 cells were treated with 0-300 μM terazosin for 24h, followed by PI staining and flow cytometer detection. The result of flow cytometry indicated that terazosin can induce G0-G1 phase arrest significantly in a dose-dependent manner (Figure 2A), and a time-dependent manner of cell cycle arrest was also shown after PC3 cells were exposed to 300 μM terazosin for indicated hours (Figure 2B), the percent of cells at G0-G1 phase increased from 54.86% to 74.72%.

activity using the synthetic fluorogenic peptide Suc-LLVY-
respectively (Figure 3A). In accordance with chymotrypsin-like
determine the certain sequence existed between proteasome inhibition
inhibition in PC3 cells, but kinetic experiments were still need to
determine that proteasome activity inhibition, polyubiquitinated-proteins, which
were tagged by polyubiquitins but not degraded by proteasome
an inhibitor, and cell death induced by terazosin.

Terazosin induces cell death in PC3 cells
Since most of cell viability inhibition is also dependent on cell death
induction, we next measured cell death in PC3 cells after terazosin
treatment, with annexin V-FITC/PI co-staining and flow
cytometry detection. Cell cycle was analyzed by cell cycle software.
N=3 independent experiments for each bar; *=p<0.05 vs. control.

Terazosin causes proteasome inhibition in PC3 cells
A furan-structure has been shown to be involved in some 20S
proteasome inhibitor, we next designed this study to investigate if
terazosin is able to inhibit proteasome activity and if proteasome
activity inhibition is one of molecule mechanisms mediate anti-cancer
cell activity of terazosin, and results of western blotting analysis revealed significant increased levels of cleaved-PARP fragment in terazosin (more than 200 μM)-treated cells compared with DMSO-treated cells (Figure 3B). Data above indicated that terazosin can sensitize PC3 cells to apoptosis, which is served to one
cause of cell viability suppression.

Proteasome activity inhibition precedes cell death induction
We performed kinetic experiments to judge whether proteasome activity inhibition precedes cell death induction. PC3 cells were treated with 300 μM terazosin for indicate hours up to 48h, then we detected cell death using flow cytometry, chymotrypsin-like proteasome activity using fluorescence measurement of proteasome substrate, and levels of ubiquitinated proteins, cleaved-PARP fragment, P27 (target substrate protein of proteasome) were detected by western blotting analysis, respectively. In PC3 cells, as early as 8h after 300 μM terazosin treatment, chymotrypsin-like proteasome activity was inhibited significantly (Figure 4A) meanwhile polyubiquitinated-proteins and P27 were also accumulated (Figure 5). Contrast to the previous proteasome activity inhibition, results of flow cytometric measurement and western blotting analysis showed that both of Annexin V-FITC-positive cells and cleaved-PARP fragments were obviously detected as late as 36h after 300 μM terazosin treatment (Figures 4B and 5).

These data revealed that proteasome activity inhibition precede induction of cell death in PC3 cells exposed to terazosin, suggesting that proteasome activity inhibition is one of molecular mechanisms of cell death induced by terazosin.

Discussion
Terazosin is a quinazoline-based α1-adrenoreceptor antagonist, which is widely used to relieve lower urinary tract symptoms [17]. Several further lines of evidence suggest that they possess the ability to suppress prostate tumor growth in vivo and their effects to decrease the viability of androgen-independent prostate cancer cells PC3 [18]. This apoptotic effect is α1-adrenoreceptor-independent, because transfection-mediated overexpression of α1-adrenoreceptor resulted in no significant change of prostate cancer cells sensitivity to apoptotic induction, and these actions have been identified to be specific for
quinazoline-based antagonists, other than other subtypes of α1-adrenoceptor antagonists such as the sulfonamide-based antagonist tamsulosin, an agent with a distinct chemical structure [19]. The signaling mechanisms of antitumor effect of doxazosin have been identified, involving increase in Bax expression, activation of TGF-β and IκB [20], reduction in focal adhesion kinase [21], inhibition of protein kinase B/Akt activation [22], and induction of death receptor-mediated apoptosis [23]. However, there are only a few reports on antitumor potency of terazosin except apoptotic induction and antiangiogenic action, the molecular mechanism mediate tumor cell cytotoxicity of terazosin is still not identified comprehensively. So we carried out this study to further discover molecular mechanisms underlying cell viability suppressing effect of terazosin.

![Figure 4: Proteasome inhibition mediated by terazosin occurs prior to induction of apoptosis. PC3 cells were treated with 300 μM terazosin for the indicated periods. Chymotrypsine-like proteasome activity was analyzed (A) and Cell death was analyzed by flow cytometry (B). N=3 independent experiments for each bar; *=P<0.05 vs. control.](image)

The abnormal ubiquitin-proteasome system plays a decisive role in cancer development is worldwide recognized [7]. Some classic structures of native and synthetic proteasome inhibitor have been identified, including α –keto carbonyl [8], boric acid [9], vinyl sulfone [10], vinyl α,β-unsaturated acyl peptidyl [11], epoxy ketone [12-14] et al. A recent report indicated that the C-terminal furanyl substitution may contribute to 20S proteasome inhibition with unique mechanism [15] Since terazosin also possess a furan-based structure, we carried out this study to investigate whether terazosin can inhibit 20S proteasome activity and whether this inhibitory effect mediate antitumor activity of terazosin against prostatic cancer cell or not.

We exposed CRPC PC3 cell lines to terazosin for indicated hours, and results of alamar blue assay showed that terazosin can inhibit cell viability with the IC50 value of about 130 μM (Figure 1), which is according to other previous reports [4,24]. Cell cycle progression are regulated by cyclins, CDKs (cyclin dependent kinases), CKIs (CDK inhibitors), and DNA repair enzymes etc. P27Kip1 is one of important CKIs, which are significant deficient in several cancer tissues [25]. P27Kip1–up-regulating could serve as a “weapon” to concentrate cells at G0-G1 phase and in turn decelerate cancer cells’ proliferation. Although some studies showed that terazosin could not affect prostatic cancer cell proliferation [3], many studies have shown that terazosin can arrest prostatic cancer cells at G0-G1 phase with increasing levels of p27KIP1[26,27]. Data obtained from our cell cycle analyze suggested that terazosin can significantly induce G0-G1 phase arrest in a dose- and time-dependent manner in PC3 cells.

![Figure 5: Accumulation of ubiquitinated-proteins and selective protein substrate occurs prior to appearance of cleaved-PARP fragment. PC3 cells were treated with 300 μM terazosin for the indicated periods, then rude proteins were extracted and followed by Western blotting analysis. The image is a representative immune blot from three independent experiments yielding similar results. GAPDH was used as loading control.](image)

Ubiquitin-proteasome-pathway (UPS) has been explored as a pivotal post-translational modification (PTM) for cell cycle regulatory proteins [28-31]. Therefore, proteasome inhibitor can restrict cell division through proteasome inhibition and subsequent weaken cell cycle regulatory proteins degradation in cancer cells [32,33]. In this study, we also observed up-regulation of P27Kip1, accompanied with accumulation of other ubiquitinated proteins, which were possibly ascribed to the milder protein degradation activity of UPS after terazosin exposure and took majority responsibility for cell cycle arrest. Although more crosstalks of cell cycle regulatory proteins and transcription factors with proteasome activity inhibition are still needed to investigate to consummate molecular mechanisms underlying cell cycle regulating of terazosin.

We also detected the down-regulation of proteasome activity, accumulation of ubiquitinated-proteins and selective protein substrate P27Kip1, and apoptosis in PC3 cells after terazosin treatment, and a kinetic study was carried out to clarify possible relationship between proteasome activity inhibition and cell apoptosis after terazosin treatment. Our data shown that terazosin can reveal inhibit proteasome activity as well as cell survival in a time-dependent manner, but these influences were not isochronous suggesting that proteasome activity inhibition caused by terazosin may partially mediated the subsequent cell apoptosis.
Our results here have revealed the inhibition characteristic of terazosin on proteasome activity. However, this study is restricted on account of the weak effects of terazosin on prostatic cancer with an IC50 of about 130 micro molar and only CRPC PC3 cell line was observed, further medicinal chemistry modifications and more in vivo experiments are needed to prove this molecular mechanism. Besides on many reports focus on combination of terazosin with other agents on LUT/BPH patients [34-37], Chang et al. also showed that combination of terazosin and genistein was more effective in inhibiting prostate cancer DU145 cells growth and inducing apoptosis than either alone [38]. Since proteasome activity inhibitory effect has been known to sensitize tumor cells to apoptosis [39-41] and autophagy [42], terazosin may prospectively be used in combination with lower-dosage of other anti-prostatic cancer drugs to improve therapeutic efficiency by proteasome activity inhibition.

Conflict of Interests
The authors declare that there is no conflict of interests.

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References


