Telmisartan is a potent target for prevention and treatment in human prostate cancer

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Abstract. Angiotensin II receptor blockers (ARBs) are widely used as hypertensive therapeutic agent. Recent studies have reported that ARBs have the potential to inhibit the growth of prostate cancer (PC) cells. Moreover, it was recently reported that Telmisartan (a kind of ARB) has peroxisome proliferator-activated receptor (PPAR)-γ activation. We previously reported that PPAR-γ ligand induces growth arrest of PC cells through apoptosis. In this study, we evaluated the effects of the Telmisartan and other ARBs on cell proliferation in several PC cell lines. We used normal prostate stromal cell (NPC), human hormone-refractory PC (PC3), androgen-independent PC (DU-145) and androgen-dependent PC (LNCaP) cell lines. Effects of Telmisartan and other ARBs (Candesartan, Valsartan, Irbesartan and Losartan) on PC cell growth were examined by MTT assay. Flow cytometry and Hoechst staining were used to determine whether or not ARBs induce apoptosis. Telmisartan caused marked inhibition of PC cells in concentration-dependent and time-dependent manner. PC cells with treatment of 100 μM Telmisartan induced early apoptosis and DNA fragmentation. However, NPC with treatment of 100 μM Telmisartan did not induce apoptosis or DNA fragmentation. Furthermore, other ARBs had no effect on cell proliferation in the PC cells and NPC. Telmisartan may mediate potent antiproliferative effects against PC cells through PPAR-γ. Thus, Telmisartan is a potent target for prevention and treatment in PC.

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Introduction

Prostate cancer (PC) comprises 32% of all cancers in American men and is on the increase worldwide. Because of increased screening, PC is frequently diagnosed at a clinically localized stage, making it amenable to therapy.

Nevertheless, it remains the second most common cause of cancer death in men. These patients generally respond to androgen deprivation therapy, but the vast majority eventually experience disease progression and become refractory to sustained hormonal manipulation. Typically, such patients progress with a rise in their serum prostate-specific antigen level. Unfortunately, standard therapeutic options at this stage of disease are limited, and while there has been some success with chemotherapy for hormone-refractory prostate cancer patients, the response is generally short-lived (1).

Angiotensin II (AII) is known as a key biological peptide in the renin-angiotensin system, which regulates blood pressure and renal hemodynamics, and AII receptor blockers (ARBs) are widely used as antihypertensive drugs (2). It is well known that angiogenesis is essential for tumor progression and metastasis (3,4). Several studies have shown that AII can induce neovascularization and ARBs inhibit vascular endothelial growth factor (VEGF) production (5,6). Benson *et al* discovered a structural resemblance between Telmisartan (a kind of ARB) and Pioglitazone, a peroxisome proliferator-activated receptor (PPAR)-γ ligand approved for the treatment of type II diabetes. They reported that Telmisartan has PPAR-γ modulating activity (7).

Peroxisome proliferator activator-receptor (PPAR)s are lipid-activated transcription factors that function as important regulars of lipid and glucose metabolism, adipocyte differen-tiation and energy homeostasis. PPAR subtypes (α, ß and γ) have been found. Both PPAR-α and -γ mediate the action of the hypolipidemic fibrates and anti-diabetic thiazolidinediones. PPARs therefore play a role in metabolic conditions such as dyslipidemia and type II diabetes, leading to atherosclerosis development (8). PPARs also have a regulatory role in inflammation. PPAR-γ provides a strong link between lipid
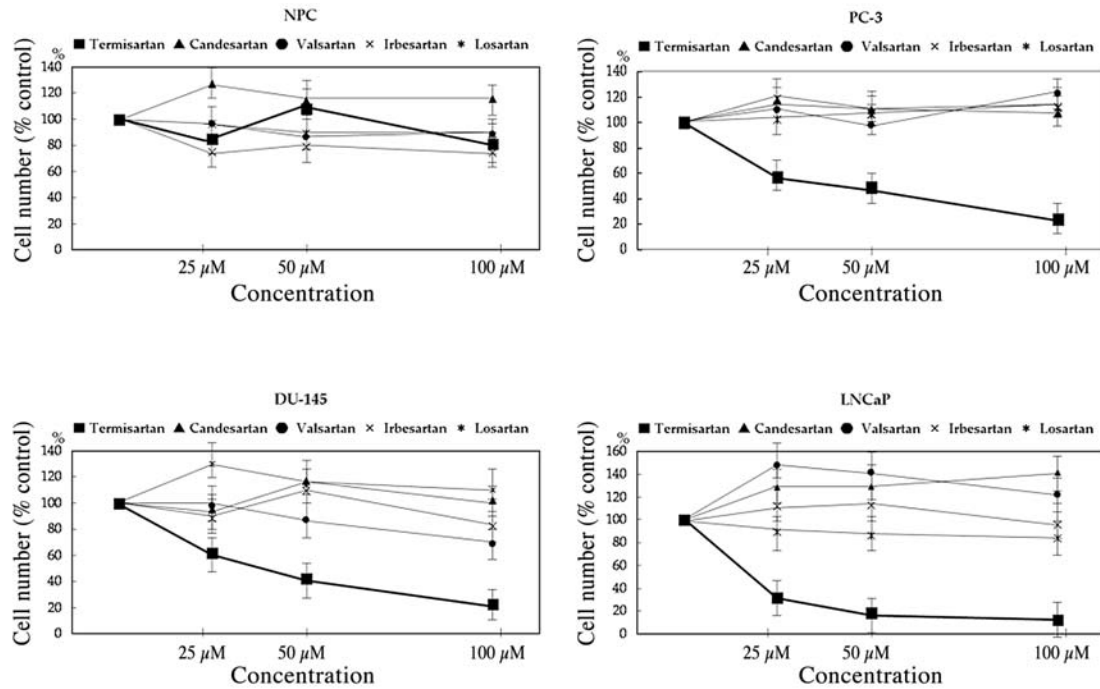
Figure 1. Effects of Telmisartan in a concentration-dependent manner. Termisartan induced a reduction of cell viability with half-maximal concentration of growth inhibition of prostate cancer (PC) cells in the range of 25-100 μM. Telmisartan stopped the growth of PC cells.

metabolism and regulation of gene transcription (9). PPAR-γ acts in adipose tissue and promotes lipogenesis under anabolic conditions. Recently, the receptor has also been implicated in inflammation and tumorigenesis. Significant evidence from many experimental systems suggests PPAR-γ is important in carcinogenesis.

PPAR-γ is up-regulated in malignant tissue, and PPAR-γ ligands induce terminal differentiation in human breast and colon cancer cells (10,11), and inhibit the growth of human lung and gastric cancer cells (12,13). In addition, PPAR-γ ligands induce growth arrest through apoptosis in macrophage, fibroblasts and endothelial cells (8,14,15). Our research elucidates the expression of PPARs in urological cancers and administration of PPAR-γ ligands as an anticancer therapy (16-20).

With this background, the purpose of our study was to evaluate the inhibitory effect of Telmisartan on human PC cell lines, and to determine whether or not Telmisartan induces apoptosis of such PC cells.

Materials and methods

Reagents and materials. RPMI-1640 was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS) and penicillin-streptomycin mixture were from Biowhitteker (Walkersville, MD, USA). Trypsin/EDTA was from Gibco BRL (Rockville, MD, USA). Telmisartan, Candesartan and Irbesartan were angiotensin II blockers (Toronto Research Chemicals, Inc., Canada). Losartan, one of the ARBs was from Cayman Chemical (Michigan, USA).

Cell cultures. The human PC cell lines LNCaP, PC-3, DU-145 and normal stromal prostate cell line (NPC) were obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan). Cells were grown in culture flask (Nunc,

Roskilde, Denmark) in RPMI-1640 supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin, in a humidified 5% CO_2 atmosphere at 37˚C. The media were changed every 3 days, and the cells were separated via trypsinization using trypsin/EDTA when they reached sub-confluence.

Cell-proliferative studies. Approximately 1.0×10^4 cells placed onto 8x8 mm diameter multichamber slides (Nunc, Copenhagen, Denmark) were treated with Telmisartan and other ARBs dissolved in ethanol. The final concentration of ethanol was 0.05%. Cell viability was measured at day 1 by a microplate reader using a modified 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay (WST-1 assay; Dojindo, Kumamoto, Japan), and presented as the percentage of control-culture conditions.

Flow cytometry
i) Annexin V and propidium iodide staining. The effects of Telmisartan and other ARBs (Candesartan, Valsartan, Irbesartan and Losartan) on PC cells were determined by dual staining with Annexin V-FITC and propidium iodide (PI) using Annexin V-FITC Apoptosis Detection Kit I (Biosciences Pharmingen). Annexin V-FITC and PI were added to the cellular suspension as in the manufacturer's instructions, and a sample fluorescence of 10,000 cells was analyzed by flow cytometry conducted with FACScan (Becton-Dickinson, Germany).

Cells that were Annexin V-FITC-positive and PI-negative were identified as early apoptotic. Cell that were Annexin V-FITC-positive and PI-positive were recorded as late apoptotic or necrotic.

ii) Identification of DNA fragmentation. The assay was performed using the TdT-mediated dUTP Nick End Labelling
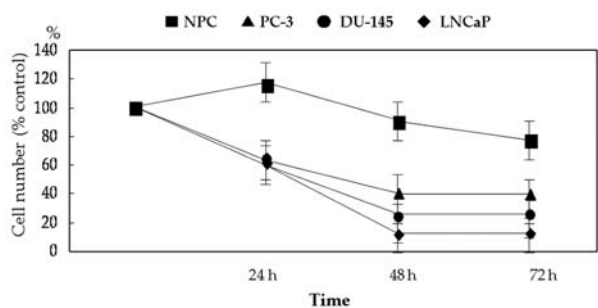
Figure 2. Effects of Telmisartan in a time-dependent manner. Counting cells at days 1, 2 and 3 clearly showed marked inhibition of cell proliferation using 100 μM of Telmisartan. Telmisartan stopped the growth of prostate cancer (PC) cells.

(TUNEL) method using APO-Direct™ kit (Becton-Dickinson). Following the experiments, PC cells in suspension (1x10^6/ml) were fixed with 1% PBS, washed in PBS and suspended in 70% (v/v) ice-cold ethanol. The cells were stored in ethanol at -20˚C until use. The positive and negative controls and the sample were stained with FITC-dUTP by incubation in terminal deoxynucleotidyl transferase buffer as per the manufacturer's instruction, and sample fluorescence of 10,000 cells was analyzed by flow cytometry (Becton-Dickinson). Results are expressed as the percentage (%) of TUNEL-positive cells.

Detection of apoptosis. DNA chromatin morphology was assessed using Hoechst staining. PC cells were incubated with 100 μM Telmisartan and other ARBs for 24 h. Cells were washed by RPMI-1640 and labeled with 8 mg/ml of Hoechst 33342 (Sigma-Aldrich Japan K.K. Tokyo, Japan) for 10 min; PI (Sigma-Aldrich Japan K.K.) was added (10 mg/ml final concentration), and the cells were examined by fluorescence microscopy.

Results

Telmisartan induces growth inhibition in PC cells as evaluated by MTT assay. To investigate the effects of Telmisartan and other ARBs on PC cell proliferation, we analyzed cell viability *in vitro* by modified MTT assay. As shown in Fig. 1, although Telmisartan and other ARBs had no effect on NPC proliferation, Telmisartan induced a reduction in cell viability with the half-maximal concentration of growth inhibition of all PC cell lines (Fig. 1) in the range of 25-100 μM. Counting cells at days 1, 2 and 3 clearly showed marked inhibition of cell proliferation using 100 μM of Telmisartan (Fig. 2). Telmisartan stopped the growth of all PC cells.
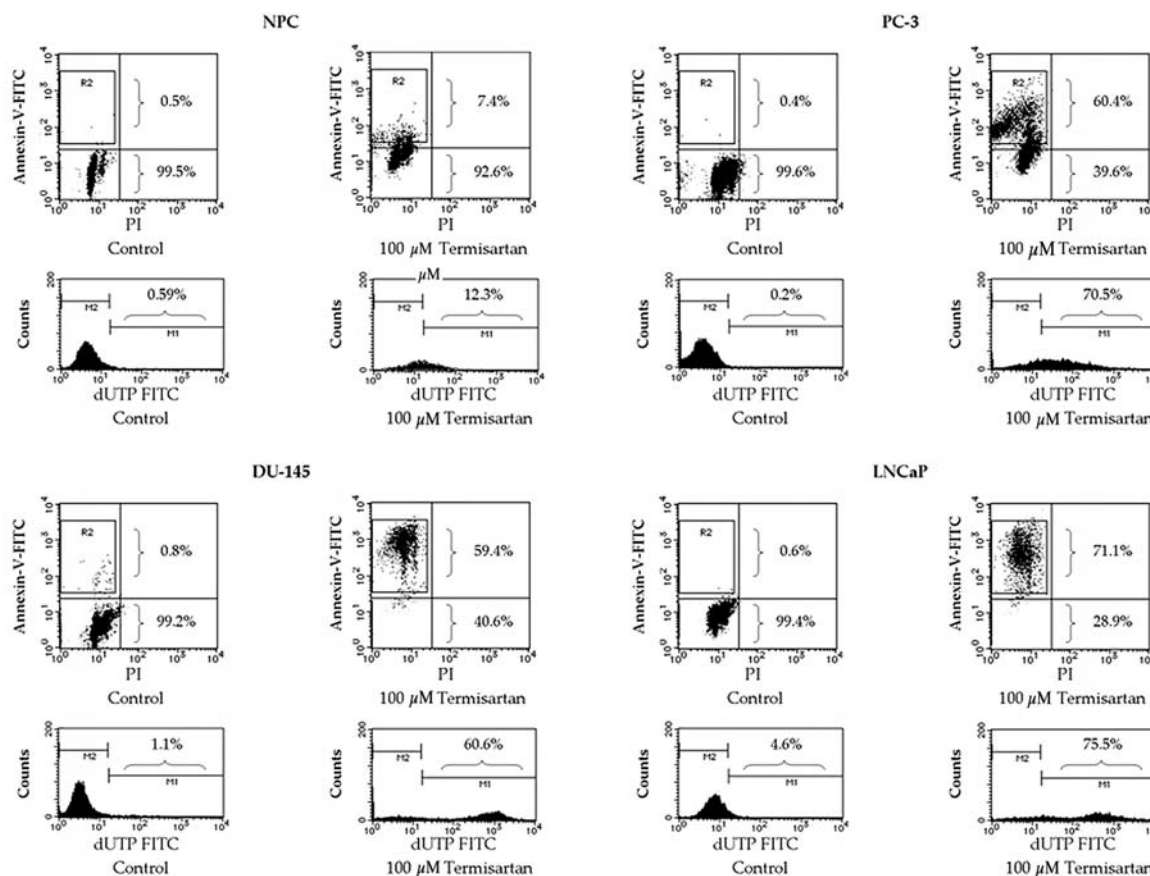

Figure 3. Effects of Telmisartan on early and late apoptosis as shown by flow cytometry. Treatment with 100 μM Telmisartan induced early apoptosis in almost the total percentage of prostate cancer (PC) cells. However, treatment with 100 μM Telmisartan did not induce apoptosis in NPC. The top left quadrants represent early apoptosis (Annexin V-FITC-positive cells and PI-negative cells). The top right quadrants represent late apoptosis and necrosis (Annexin V-FITC-positive cells and PI-positive cells). Diagrams of FITC-Annexin V/PI flow cytometry in a representative experiment are presented. Telmisartan (100 μM) induced DNA fragmentation in all PC cells. However, 100 μM Telmisartan did not induce DNA fragmentation in NPC. Typical flow cytometry analysis histograms in a representative experiment are presented.
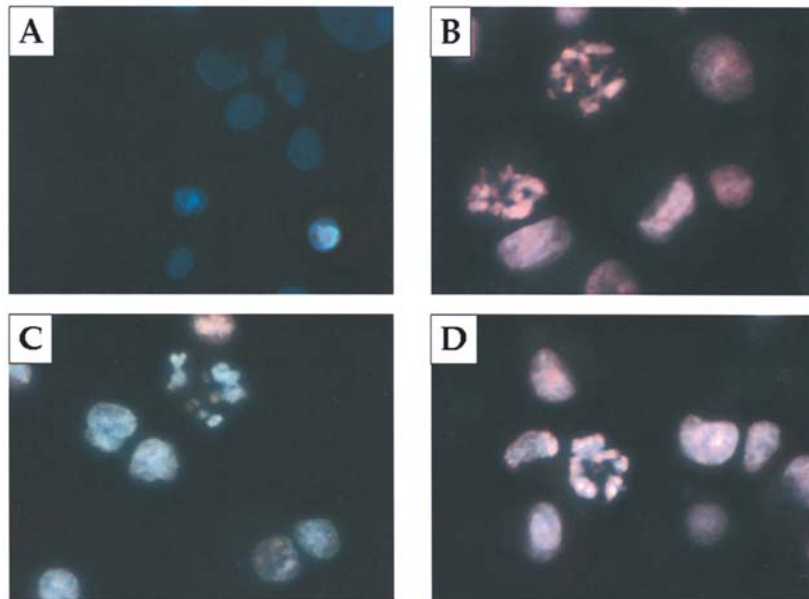
Figure 4. Effects of Telmisartan in induction of apoptosis on human prostate cancer cells. Cells treated with Termisartan showed significant chromatin conden-sation, cellular shrinkage, small membrane-bound bodies (apoptotic bodies), and cytoplasmic condensation. These cellular changes are typical characteristics of apoptosis (B), PC-3; (C), DU-145; (D), LNCaP). NPC treated with Telmisartan maintained normal chromatin patterns and cell size (A), NPC.

Telmisartan-induced apoptosis evaluated by flow cytometry. To confirm whether or not cell death induced by Telmisartan and other ARBs was achieved through apoptosis, we used flow cytometry. As shown in Fig. 3, the top left quadrants represent early apoptosis (Annexin V-FITC-positive cells and PI-negative cells). The top right quadrants represent late apoptosis and necrosis (Annexin V-FITC-positive cells and PI-positive cells). Treament with 100 μM Telmisartan induced early apoptosis in almost the total of PC cells. However, treatment of 100 μM Telmisartan antagonist did not induce apoptosis in NPC (Fig. 3).

Furthermore, 100 μM Telmisartan induced DNA fragmen-tation in all PC cells. However, 100 μM Termisartan did not induce DNA fragmentation in NPC (Fig. 3). On the other hand, other ARBs did not induce DNA fragmentation in all PC cells and NPC (data not shown).

Effect of Telmisartan in induction of apoptosis on human PC cells. To evaluate whether or not cell death induced by Telmisartan was through apoptosis, we evaluated the chromatin morphology of PC cells using Hoechst 33342 staining. Cells treated with Telmisartan showed significant chromatin condensation, cellular shrinkage, small membrane-bound bodies (apoptotic bodies), and cytoplasmic conden-sation. These cellular changes are typical characteristics of apoptosis (Fig. 4). All PC cell lines without Termisartan maintained normal chromatin patterns and cell size. On the contrary, cells treated with other ARBs did not show significant chromatin condensation, cellular shrinkage, apoptotic bodies or cytoplasmic condensation (data not shown).

Discussion

Anticancer and anti-angiogenesis effects of ARBs. Angiotensin II receptor blockers (ARBs) have been synthesized and available for the treatment of hypertension since the 1990s (21,22). Recently, angiotensin II has been reported to promote tumor growth and angiogenesis, ARBs have been considered a noteworthy anticancer and anti-angiogenesis therapeutic option (23).

Several tumor cell types, such as melanoma, pancreatic (24), renal (25,26), breast (27), bladder (28) and prostate cancer (29) have been reported to express angiotensin II receptor (24,25,28-31), and there have been several studies that investigated antitumor effects of ARBs throughout anti-angiogenesis. It was demonstrated that Candesartan inhibited vascular endothelial growth factor (VEGF) production, that is one of the most potent and specific angio-genic factor and decreased PC growth (29,32). Kosaka *et al* reported a specific ARB suppresses VEGF production, resulting in reduced tumor angiogenesis and slower progression of PC on a tumor xenograft model (29). Concerning other tumor types, Kosugi *et al* showed that Candesartan prevents pulmonary metastasis of renal cancer and bladder tumor by inhibiting tumor angiogenesis through the suppression of VEGF on a xenograft model (28). Uemura *et al* reported that they used Candesartan clinically on PC patients with hypertension, PSA declined and performance status improved (32). However, they also reported that Candesartan has no effect on tumor growth *in vitro* and they did not detect apoptosis. Based on their *in vitro* and *in vivo* experiments, they suggest that the antitumor effect of ARB is not a result of direct toxicity or apoptotic induction but of an anti-angiogenic effect (28,29).

Our experiments showed that Candesartan and other ARBs (except Telmisartan) did not induce a reduction of cell viability and early apoptosis of all PC cells. Only Telmisartan induced a reduction of cell viability with the half-maximal concentration of growth inhibition and early apoptosis and DNA fragmentation of all PC cells.

Telmisartan is a partial agonist of PPAR-γ. Benson *et al* discovered a structural resemblance between Telmisartan and Pioglitazone, a peroxisome proliferator-activated receptor (PPAR)-γ ligand approved for the treatment of type II diabetes. They found that Telmisartan not only blocks the angiotensin II receptor, but also activates PPAR-γ. Telmisartan functiond as a moderately potent, selective PPAR-γ, partial agonist, activating the receptor to 25-30% of the maximum level achieved by the full agonists Pioglitazon and Resiglitazone (7).

Anticancer effects of PPAR-γ. PPARs are members of the nuclear receptors super-family of ligand-activated trans-criptional factor such as steroids, thyroid hormone, vitamin D3 and retinoic acid. PPAR binds to peroxisome proliferator response element as a heterodimer with the retinoic receptor in the regulation of PPAR target genes. PPARs are considered important immunomodulatory factors as well as fatty acid regulators. PPARs modulate these activities in different immune cell types such as monocyte/macropharges, lympho-cytes and endothelial cells (33).

PPAR-γ is expressed at high level in adipose tissue and is a critical regulator of adipocyte differentiation. PPAR-γ is expressed in the immune system, in the spleen, monocytes bone-marrow precursors, and helper T-cell clones. PPAR-γ is also expressed in chondrocytes, synovial and bone tissues. Recent data have shown that PPAR-γ ligands lead to inhibition of phorbol ester-induced nitric oxide and macropharge-derived cytokines such as tumor necrosis factor-α, interleukin-1ß and interleukin-6, chemokines and adhesion molecules, in part by antagonizing the activities of transcriptional factors (12). Recently, it has been evidenced that thiazolidinedione, a new class of anti-diabetic as a specific ligand for PPAR-γ, and retinoic receptor agonists can regulate differentiation of cancer cells (34), and nuclear-acting prostanoids including 15-d-PGJ$_2$ are potent activators of the PPAR-γ receptor isoform (35,36). 15-d-PDJ$_2$ induces apoptosis in macro-phages, endothelial cells and choriocarcinoma cells (8,15,37) as well as thiazolidinediones-induced fibroblast apoptosis (9).

We previously reported that PPAR-γ was strongly expressed in PC tissues. The extent and intensity of PPAR-γ expression in PC tissues were greater in normal prostate tissues. PPAR-γ expression was higher in high group cancer than low group cancer. PPAR-γ ligands strongly induced early apoptosis in all PC cells by flow cytometry and Hoechst staining (16-20). In this study, only Telmisartan had direct toxicity throughout apoptosis. Thus, Teimisartan may mediate potent antiproliferative effects against PC cells through PPAR-γ. But in this study, that dose is not clinically achievable. Further studies are needed to extend the application of Telmisartan to a clinical trial of treatment for PC.

Acknowledgements

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