Clinically relevant doses of candesartan inhibit growth of prostate tumor xenografts in vivo through modulation of tumor angiogenesis

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ABBREVIATIONS: ARB, angiotensin 2 type 1 receptor blocker; VEGF, vascular endothelial growth factor; Ang II, angiotensin II; AT1, angiotensin II Type 1 receptor; AT2, angiotensin II Type 2 receptor; BrdU, bromodeoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Abstract

Angiotensin receptor blockers (ARBs), widely used anti-hypertensive drugs have also been investigated for their anti-cancer effects. The effect of ARBs on prostate cancer in experimental models compared to meta-analysis data from clinical trials is conflicting. Whereas this discrepancy might be due to the use of supra-therapeutic doses of ARBs in cellular and animal models as compared to the clinical doses used in human trials, it warrant further investigation on the effects of clinical dose of ARBs on prostate cancer in experimental models. In the current study, we sought to determine the effects of candesartan on prostate cancer cellular function in vitro and tumor growth in vivo, and characterize the underlying mechanisms. Our analysis indicated that clinically relevant doses of candesartan significantly inhibited growth of PC3 cell tumor xenografts in mice. Interestingly, the same concentrations of candesartan actually promoted prostate cancer cellular function in vitro, through a modest, but significant inhibition in apoptosis. Inhibition of tumor growth by candesartan was associated with a decrease in VEGF expression in tumors and inhibition of tumor angiogenesis, but normalization of tumor vasculature. While candesartan did not impair PC3 cell viability, it inhibited endothelial-barrier disruption by tumor-derived factors. Furthermore, candesartan significantly inhibited expression of VEGF in PC3 and DU145 cell lines independent of AT2 receptor, but potentially via AT1 receptor inhibition. Our findings clearly demonstrate the therapeutic potential of candesartan for prostate cancer and establish a link between ARBs, VEGF expression and prostate tumor angiogenesis.
Introduction

Prostate cancer is the most commonly diagnosed cancer among males according to the American Cancer Society (Siegel et al., 2012). About 68% of prostate cancer cases are diagnosed in the 55-74 year age group (Siegel et al., 2012) and this age group is also characterized by the high prevalence of comorbid conditions, most notably cardiovascular diseases (Roger et al., 2012).

Recently, a number of meta-analyses investigating a possible link between cancer incidence and cardiovascular disease drugs have been published (Sipahi et al., 2010; Mearns, 2011). One of the major targets of these analyses was the angiotensin II receptor blockers (ARBs), which are commonly prescribed for the management of cardiovascular diseases. The results of these analyses were controversial with some suggesting a causal link between cancer (Sipahi et al., 2010) and ARBs while others disputing such a link (Mearns, 2011). To further complicate the matter, there is a plethora of experimental evidence that suggest a possible beneficial role of ARBs in the management of multiple types of cancer, especially urogenital cancers (Miyajima et al., 2002; Kosaka et al., 2007; Takahashi et al., 2012). Experimental data demonstrated the ability of ARBs to inhibit progression and metastases in bladder, renal (Miyajima et al., 2002) and prostate cancer (Kosugi et al., 2006; Kosaka et al., 2007; Takahashi et al., 2012). This beneficial effect has been consistently reported in both monotherapy settings (Kosaka et al., 2007) and in combination with other antineoplastic agents. The antineoplastic effects of ARBs are believed to be due to their ability to inhibit cancer angiogenesis (Kosaka et al., 2007), which has been shown to be associated with severity and metastatic potential of prostate cancer (Kosaka et al., 2007).
Despite the solid experimental evidence supporting an antineoplastic effect of ARBs, the controversy between clinical and experimental data must be resolved. In the majority of experimental studies, the dose of ARBs used is supra-therapeutic and always in combinations with Angiotensin II (Ang II), which cannot be extrapolated to clinical practice (Uemura et al., 2003; Uemura et al., 2005a; Takahashi et al., 2012). This point has been critically reviewed and the importance of using clinically relevant doses of pharmacologic agents in preclinical studies has been noted (Reagan-Shaw et al., 2008). Another important consideration in investigating the effects of ARBs is the concomitant treatment with exogenous Angiotensin II (AngII) (Uemura et al., 2003; Kosaka et al., 2007; Chen et al., 2013), which only blunted AngII-mediated effects. This paradigm ignores AngII-independent effects of candesartan as well as the role of locally produced AngII, which has been well characterized in a variety of tissues and cell types (Reid et al., 2011; Angeli et al., 2013; Lu et al., 2013). Recently, candesartan was shown to be proangiogenic in cerebral microvascular endothelial cells (hCMECs) via activation of the AT2 receptor (Alhusban et al., 2013). This effect occurred even in the absence of exogenous AngII. These two observations highlight the importance of investigating the potential direct effects of ARBs on tumor cell function and angiogenesis in the absence of exogenous AngII to get better understanding of physiological responses. In the current study, the focus was to systematically investigate the effect of clinically relevant concentrations of ARBs on the progression of prostate cancer both in vivo and in vitro. In addition, we investigated the effect of ARBs on tumor angiogenesis and vascular normalization, and the molecular mechanisms leading to ARB action on prostate tumor cells and tumor vasculature.
Methods

In Vivo Nude Mouse Tumor Xenograft Model

All animal procedures listed in this article were performed as per the protocol approved by the Institutional Animal Care and Use Committee at the Charlie Norwood Veterans Affairs Medical Center, Augusta, GA (protocol # 12-06-049). PC3 cells were grown to confluence in 250-ml flasks. Next, cells were suspended in sterile saline to a concentration of 5\times 10^6/ml. Cell suspension (100 µl/mouse) was injected subcutaneously (SC) in 8-week-old male nude mice (athymic nude mice; Harlan, Indianapolis, IN) (Kochuparambil et al., 2011). Mice were divided into two groups. The groups were subjected to intra-peritoneal (i.p.) injections of candesartan (CV-11074 dissolved in 100 µL of 0.9% saline) at a dose of 6.5 mg/kg body weight every 24 h for 18 days (treatment started 6 days after SC tumor injection when tumors of equal size were clearly visible) and the respective controls were injected i.p. with 0.9% saline every 24 h. CV-11974 is an activated form of candesartan that does not need to be activated by the liver, and hence can be readily used for both in vitro and in vivo experiments. Tumor diameters were measured with digital calipers on day 6, 12, 18, and 24, and the tumor volume in mm³ were calculated by the modified ellipsoidal formula (Tumor volume = \( \frac{1}{2}[\text{length} \times \text{width}^2] \)) (Euhus et al., 1986). Mice were sacrificed on day 24 and tumors were dissected, weighed, and snap-frozen for further western blot and immunohistochemistry analysis.
Reagents, Cell Lines and Antibodies

Human metastatic PC3 and DU145 prostate cancer cell lines were obtained from ATCC (Manassas, VA) and maintained in DMEM-High Glucose (HyClone, Logan, UT) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO2 humidified atmosphere at 37 °C. Primary antibodies against p-Akt (S473), Total Akt, p-P38-MAPK, p-GSK3α/β, were purchased from Cell Signaling (Boston, MA). Anti-VEGF antibodies were purchased from Millipore (Billerica, MA). Primary antibodies against β-actin and laminin as well as AT2 receptor agonist (CGP-121141A) were purchased from Sigma (St Louis, MO). Primary antibodies against AT1 and AT2 receptors were purchased from Abcam (Cambridge, MA). Anti-mouse and anti-rabbit HRP conjugated secondary antibodies were obtained from Bio-Rad (Hercules, CA). Alexa-Fluor (488) secondary antibody was purchased from Invitrogen (Carlsbad, CA). Candesartan (CV-11074) was gifted to Dr. Fagan’s Laboratory from AstraZeneca Pharmaceuticals (Wilmington, DE). Clinically relevant concentrations for the treatment of prostate tumor cells in vitro and athymic nude mice in vivo were calculated using the previously published protocol (Reagan-Shaw et al., 2008).

Candesartan dose calculations for in vitro and in vivo experiments

Two important points were considered when calculating the drug concentrations/doses for in vitro and in vivo studies; 1) Dose calculations for i.p. administration of candesartan in vivo, as compared to i.v. drug administration in mice and 2) comparison of doses used in mice with that prescribed in humans. Therapeutic doses for mouse as compared to human was calculated using the equation: The human dose (mg/kg) = the
mouse dose x (mouse Km/Human Km) (Reagan-Shaw et al., 2008). According to the manufacturer's recommendation and from the literature (Gleiter and Morike, 2002), average daily maintenance doses of candesartan for adults who weigh >50 Kg is between 4-32 mg/day orally, and the bioavailability of candesartan is 15-40%. Since patients who are diagnosed with prostate cancer are elderly and given the maximum prescribed dose of 32 mg/day orally, expected bioavailability (the fraction of dose that reaches the circulation) of candesartan is 40%, which is: $32 \times 40\% = 12.8 \text{ mg/day}$ for patient weighing 50 kg or more, and a comparable i.v. dose will be: $12.8 \text{ mg/day}$ for 50 kg patient = 0.256 mg/Kg. In order to reach this concentration by i.p. administration, candesartan dose was doubled (0.512 mg/kg) considering the loss of drug through not being absorbed into circulation and the drug removal through the hepatic system entering through mesenteric arteries. By using the mouse formula (above) based for human doses, Dose of mouse (mg/kg) = dose of human/(km of mice/km of human): $0.512\text{mg/kg}/(3/37) = 6.4 \text{ mg/kg} (~6.5 \text{ mg/kg})$.

For in vitro experiments, candesartan concentrations ranging from 0.5 to 25 µM are considered therapeutic and anything above 25 µM was considered supra-therapeutic. Comparisons between therapeutic and supra-therapeutic concentrations of candesartan were included in in vitro studies.

**Western Blot Analysis**

PC3 cells were cultured in DMEM in six-well plates to reach a monolayer and were serum starved. The wells were treated with serum free-DMEM containing candesartan at concentrations 0.1 µM to 25 µM, or AT2 receptor agonist (CGP-121141A at
concentrations 100 nm to 1000 µM), and control cells were grown in DMEM alone. Whole-cell lysates were prepared using lysis buffer [50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM Na3VO4, and 1× complete protease inhibitors (Roche Applied Science, Indianapolis, IN)]. The protein concentration was measured by the D_L protein assay (Bio-Rad Laboratories, Hercules, and CA). Western blot analyses were performed as described previously (Goc et al., 2013).

**RT-PCR analysis of VEGF expression**

Both PC3 and DU145 cell lines were cultured to reach a monolayer in DMEM in six-well plates and serum starved. Wells were treated with serum free-DMEM containing candesartan, and control cells were grown in DMEM alone for 24 hrs. Total RNA was extracted using TRizol® reagent (Invitrogen, CA) following the manufacturer protocol. 1 µg of RNA was used to make cDNA using iScript cDNA synthesis kit (Bio-Rad, CA) in a total volume of 20 µl for each reaction following manufacturer protocol. GoTaq® green master mix 2x (Promega, WI) was used for PCR amplifications in a total volume of 50µl. RT-PCR amplification was done using Eppendorf thermal cycler (Eppendorf, NY) using following temperature and time periods: initial denaturation at 95 °C for 3 min, then was followed by 25 PCR cycles using 1) denaturation at 94°C for 30 seconds , 2) primer annealing at 55 °C for 30 second, 3) extension at 72 °C for 30 seconds and final extension for 72 °C for 5 min. 2% agarose gels were used to detect the RNA bands. The primers sequences used were as follow: VEGF forward, 5′-ctacctccaccatgccaagt-3′, and reverse, 5′-gcagtagctgcgtgataga-3′. GAPDH forward, 5′-acctgagaagactgtggatg-3′, and reverse, 5′-agtagagggcaggtgt-3′.
Cell Migration Assay

Cell migration assay (wound healing assay) was performed as described before (Goc et al., 2012b), (Alhusban et al., 2013). Briefly, PC3 cells were grown on 12-well plates to reach confluence (24 h) and then scratches were made in the cell monolayers using 1 ml pipette tips followed by treatment with different concentrations of candesartan (0.5, 5, 10, 25, and 200 µM) in serum-free DMEM. Control cells were incubated in DMEM alone. Images for scratches were taken at time zero and 24 h. The rate of migration was measured using the equation ([1-T24/T0] X 100), where T24 is the area at the end point (24 h) and T0 is the area at the start time (0 h).

Transendothelial Migration Assay

Transendothelial migration of prostate cancer (PC3) cells was measured using electric cell substrate impedance sensing (ECIS) equipment (Goc et al., 2012a) with human dermal microvascular endothelial cells (ATCC) plated on 8W10E+ array chips (Applied Biophysics, Troy, NY). Briefly, PC3 cells were plated in 6-well plates. After 24 h, the cells were incubated in serum-free DMEM containing candesartan (0.5, 25, and 200 µM), for 24 h. In order to avoid the direct effect of candesartan in the conditioned medium on endothelial cell monolayer, medium with candesartan along with control DMSO containing medium, was removed 1 hour after treatment and then supplemented with fresh serum-free medium and subsequent incubation for another 23 h (a total of 24 h post treatment). Control cells were incubated in DMEM alone, then conditioned media were collected and live cells were collected from the plate by using cell dissociation buffer (20 mM EDTA in PBS [pH7.4]) to avoid integrin/receptor loss due to trypsin
digestion. We then added either the cells or their conditioned media directly onto endothelial cell monolayers at a density of 5X10^4 cells/well in 50 µl medium (or 50 µl of conditioned media). Real-time measurements on the transendothelial migration of PC3 cells were recorded by ECIS up to 10 h.

**Cell Proliferation Assay**

Proliferation of the PC3 cell line was determined using the nonradioactive BrdU-based cell proliferation assay (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s protocol. Briefly, PC3 cells were plated in 96-well flat bottom plates at a density of 5 x 10^3 cells per well and allowed to grow for 24 h. Cells were then treated with candesartan (0.5, 5, 10, 25, and 200 µM) for an additional 24 h in serum free conditions. Control cells were incubated in DMEM alone. Cells were then subjected to 5-bromo-2-deoxyuridine assay using the BrdU Labeling and Detection Kit III as done previously (Goc et al., 2011; Goc et al., 2012a). BrdU incorporation into the DNA was determined by measuring the absorbance at both 450 and 690 nm on an ELISA plate reader.

**Cell Viability Assay**

Number of viable cells was assessed indirectly by means of MTT assay using tetrazolium salt conversion into formazan crystals (Al-Azayzih et al., 2012). Briefly, PC3 cells were plated in 96-well plates at 5 x 10^3 cells per well, and allowed to grow for 24 h. Medium was then replaced with fresh serum-free DMEM containing candesartan (0, 0.5, 5, 10, 25, and 200 µM), After 24 h treatment, cell viability was measured using the Cell Proliferation Kit I (MTT) (Roche Applied Science, Indianapolis, IN) according to the
manufacturer’s protocol and as standardized in the laboratory. The absorbance at 570 nm was measured using an ELISA reader and used to determine relative cell numbers in each well.

**Cell Apoptosis Assay**

Cytoplasmic histone-associated DNA fragments were quantified using Cell Death Detection ELISA PLUS kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s protocol (Al-Azayzih et al., 2012). Briefly, PC3 cells were plated in a 96-well plate at a density of $10^4$ cells per well. After 24 h, the cells were incubated in serum-free DMEM containing candesartan (0.5, 5, 10, 25, and 200 µM), for 24 h. Control cells were incubated in DMEM alone. Cells were lysed and centrifuged at 200g for 10 min, and the collected supernatants were subjected to ELISA. The absorbance was measured at 405 nm (reference wavelength, 492 nm).

**Immunohistochemistry**

Immunofluorescence staining of the tumor sections for laminin (blood vessels) was performed according to the standard protocol (Goc et al., 2012a). Briefly, formalin-fixed, frozen prostate (PC3) xenograft tumor sections from nude mice were subjected to the standard xylene-ethanol dehydration process and permeabilized with 0.3% Triton X-100 in 1X PBS. The nonspecific staining was blocked with 5% goat serum for 1 h at room temperature. The dehydrated, permeabilized, and blocked tissue sections were incubated with primary antibodies against laminin (dilution 1:750) overnight at 4 °C followed by washing with 1X PBS (4 times for 15 min each). Next, anti-mouse Alexa Fluor 488-labeled secondary antibodies (1:500) were applied for 1 h at room
temperature and washed four times for 15 min with 1X PBS. The slides were mounted with Vectashield (Vector Laboratories), and the images were taken by a Zeiss fluorescent microscope (Zeiss Axiovert100M, Carl Zeiss). Analysis of vascular lumen area and wall thickness was determined using NIH Image J software.

**Statistical Analysis**

All the data are presented as mean ± S.D. of 3-4 independent experiments. To determine significant differences between treatment and control values, we used Student’s two-tailed $t$ test. One way ANOVA was used for all the concentration-dependent analyses *in vitro*. The significance was set at 0.05 levels (marked with symbols wherever data are statistically significant).
Results

Clinically relevant dose of candesartan inhibited growth of prostate tumor xenograft in athymic nude mice

Our data indicated that candesartan inhibited prostate cancer (PC3) tumor xenograft progression as detected by the size and weight of the tumors after treatment with the clinically relevant dose of candesartan (Figure 1A). Although we observed a trend in inhibitory effect of candesartan on the growth of tumor xenograft starting from as early as 6 days post-treatment (12 days after PC3 cell administration), a significant anti-tumor effect was revealed from day 12 post-candesartan treatment (18 days after PC3 cells administration) (Figure 1B). Additionally, candesartan significantly reduced the weight of prostate tumor xenografts measured after tumor xenograft extraction on day 24 (Figure 1C). Furthermore, treatment with candesartan resulted in a significant reduction in the number of blood vessels approaching the tumor, suggesting an impairment of PC3 cell ability to attract host vasculature towards the tumor xenograft (Figure 1D).

Candesartan had a modest effect on the migration and apoptosis of PC3 cells

Since a clinical dose of candesartan was effective in inhibiting PC3 tumor growth and tumor neovascularization in vivo, we sought to determine if relevant concentrations of candesartan had any effect on PC3 tumor cell function in vitro. Our data showed that relevant concentrations of candesartan used in the in vivo tumor experiment induced a dose dependent anti-apoptotic effect in PC3 cells in vitro, which was observed even at supra-therapeutic doses (Figure 2A). Although modest, the effect of candesartan on tumor cell survival was significant and was in contrast to our data from in vivo analysis.
of tumor xenografts, which suggested that the effect of candesartan *in vivo* is much more complicated than its direct effect on tumor cells *in vitro*. Interestingly, although clinically relevant concentrations of candesartan did not elicit any effect on PC3 cell migration, supra-therapeutic doses of candesartan had a paradoxical inhibitory effect, as reported by many other laboratories, which probably might be due to its toxic effect at high concentrations (Figure 2B). Surprisingly, clinically relevant concentrations of candesartan did not have any effect on the proliferation or viability of PC3 cells in the short- (Figure 2 C and D) or in the long-term (72 hours) (Supplemental Figure 1).

*Candesartan did not affect the major survival pathways in PC3 cells*

In contrast to our *in vivo* data, *in vitro* data demonstrated a minimal effect of candesartan on the progression of cancer cells. To resolve this discrepancy we analyzed the major survival pathways in PC3 cells in response to candesartan treatment. Our data demonstrated that therapeutic concentrations of candesartan did not alter the activity of either Akt or GSK as determined by the changes in activity modulating phosphorylation (Figure 3A). Consistent with previous reports (Uemura et al., 2003; Uemura et al., 2005b; Uemura et al., 2006), candesartan inhibited p38MAPK signaling in a concentration dependent manner (Figure 3B), which might be responsible for the modest, but significant decrease in prostate cancer cell apoptosis *in vitro*. A recent study indicated modulation of AT1 and AT2 receptor expression by candesartan in endothelial cells (Alhusban et al., 2013). Hence, we determined if treatment with candesartan will modulate AT1 and AT2 receptor expression in prostate cancer cells. Our study indicated that while treatment with candesartan resulted in a significant and concentration-dependent decrease in AT1 receptor expression and increase in AT2
receptor expression in PC3 cells (Figures 3C and 3D, respectively), but not in DU145 cells (Supplemental Figure 2B and C), thus suggesting that effects of candesartan on AT1 and AT2 receptor expression, and probably the entire effect of candesartan on PC3 cells in vitro and tumor xenograft in vivo, may be purely limited to PC3 cells.

Candesartan inhibited cancer induced angiogenesis in prostate cancer xenograft

Since candesartan did not elicit a significant inhibitory effect on PC3 and DU145 prostate cancer cells in vitro, we postulated that the effect of candesartan in vivo could be a result of its ability to interfere with the tumor and stromal cell interactions. To assess the involvement of angiogenesis in candesartan-mediated inhibition of tumor growth in vivo, we determined the effect of clinically relevant dose of candesartan on the expression of the pro-angiogenic growth factor, VEGF and on tumor angiogenesis. Accordingly, PC3 tumor xenograft sections were stained with laminin, a marker of vascular density in xenografts and tumor lysates were subjected for Western analysis for VEGF expression. Candesartan significantly inhibited tumor angiogenesis in PC3 tumor xenografts (Figure 4A and B), and this was corroborated with a significant inhibition of VEGF expression (Figure 4C and D). Furthermore, our in vitro analysis indicated that treatment with clinically relevant concentrations of candesartan inhibit VEGF expression in both PC3 cells (Figure 4E) and DU145 cells (Supplemental Figure 2A) in a concentration-dependent manner. Together, these data indicated that the effect of candesartan on tumor cell VEGF expression, tumor angiogenesis and growth is indeed a global effect, and not cell-specific, and may be via an AT1/AT2 receptor independent mechanism.
Candesartan inhibits VEGF mRNA expression in prostate cancer cells

Next, we determined whether candesartan inhibits VEGF expression at the mRNA level or its effect is limited to protein translation. Our study revealed that in both PC3 and DU145 cells, candesartan treatment resulted in significant reduction in VEGF mRNA levels at both the 0.5 µM and 25 µM candesartan concentrations (Figure 5A-C), thus indicating the effect of candesartan on inhibition of VEGF expression in prostate cancer cells is at the transcriptional level.

Candesartan induces vascular normalization in tumor xenografts in vivo

Since the effect of candesartan in vivo was predominantly on the tumor vasculature, we analyzed the tumor blood vessels to determine the effect of candesartan on tumor vascular normalization, an important feature that determines the efficacy of anti-angiogenic and combinational therapies. According to our data, candesartan treatment resulted in significant reduction in tumor vascular lumen perimeter (Figure 6A) and lumen area (Figure 6B) associated with a significant reduction in AT1 receptor (Figure 6D) and increase in AT2 receptor (Figure 6E) expression levels indicating an overall vasodilatory effect of candesartan on tumor blood vessels. In addition, a significant increase in vascular wall thickness in candesartan treated tumor blood vessels compared to saline treated controls (Figure 6C), suggested the ability of candesartan to enhance endothelial-barrier integrity and activation in vivo. Together, these data indicated the effect of candesartan on tumor vascular normalization, potentially via inhibition of tumor vascular permeability, an important feature essential to promote neovascularization in tumors.
Conditioned media from candesartan treated PC3 cells did not compromise human microvascular endothelial-barrier function

The effect of candesartan on tumor vascular normalization prompted us to determine its effect on inhibition of vascular permeability by simulating the conditions in vitro using the ECIS technology. Our data showed that conditioned media collected from candesartan treated PC3 cells preserved endothelial-barrier function in a concentration-dependent manner (Figure 7A). This finding suggests that candesartan reduces the expression and release of mitogenic mediators such as VEGF by the prostate cancer cells and that these mediators primarily produce a paracrine rather than an autocrine effect. On the other hand, candesartan treated PC3 cells did not have a consistent effect on the endothelial-barrier function (Figure 7B). PC3 cells treated with low concentrations of candesartan induced a trivial breakdown in endothelial-barrier function that was rapidly restored. Interestingly PC3 cells treated with higher concentrations of candesartan failed to disrupt barrier function of endothelial cells.

Effect of candesartan on VEGF expression by tumor cells is independent of AT2 receptor activation

Finally, we determined whether the candesartan-mediated effect on VEGF expression by PC3 and DU145 cells was through activation of AT2 receptor. To do this, we treated PC3 and DU145 cells with various concentrations of the AT2 receptor agonist CGP-121141A. Our data indicated that AT2 receptor activation had no effect on VEGF expression in either PC3 or DU145 cells (Supplemental Figure 2).
Discussion

Our data demonstrate the ability of clinically relevant doses of candesartan to inhibit prostate cancer xenograft growth. Interestingly, candesartan-induced anti-tumorigenic effect was mediated indirectly through inhibiting cancer induced angiogenesis. This finding was confirmed by the lack of significant effect of candesartan on the behavior of prostate cancer cells in vitro. Additionally, whereas conditioned media from candesartan treated cells was able to maintain endothelial-barrier function in vitro, treatment with candesartan induced vascular normalization in tumor vasculature through inhibition of vascular permeability and thus impairing neovascularization and tumor perfusion.

Candesartan is an angiotensin II Type 1 (AT1) receptor antagonist and is widely prescribed for the management of hypertension and other cardiovascular disorders. It is extremely well-tolerated, as are all of the other angiotensin receptor blockers (ARBs), and has an addition advantage of being dosed orally once daily. The pleiotropic properties of the ARBs, beyond blood pressure lowering, include prevention of pathologic remodeling after myocardial infarction, vascular protection and the reduction of inflammation. The effects of ARBs on angiogenesis are debated and are likely to be dose, tissue and context dependent (Willis et al., 2011). Candesartan and other AT1 blockers have been demonstrated to inhibit the proliferation of cancer cells when used alone or in combination with other antineoplastic agents (Fujimoto et al., 2001; Miyajima et al., 2002; Kosugi et al., 2006; Kosaka et al., 2007; Chen et al., 2013). This anti-proliferative effect was observed with high doses of AT1 blockers that are not clinically relevant and may have serious side-effects such as hypotension and kidney failure. Recently published meta analyses had confusing results on the risk of cancer among...
AT1 blocker-treated patients (Sipahi et al., 2010; Mearns, 2011). Accordingly, we assessed the effect of clinically relevant doses of candesartan on the progression of prostate cancer using a xenograft model in nude mice. Similar to previously published data with the use of higher doses of candesartan (Uemura et al., 2006; Kosaka et al., 2007; Uemura et al., 2008), our data also demonstrated the ability of candesartan to inhibit the progression of prostate cancer in vivo. This finding has a highly significant translational impact as it provides the first experimental evidence on the effect of candesartan on prostate tumor growth at comparable doses with that used in clinical practice, and can help to resolve the recently raised concerns about the safety of ARBs with regard to the risk of prostate cancer.

The antiproliferative effects of ARBs have been extensively reviewed (Uemura et al., 2006). A common theme among the majority of published literature is the concomitant treatment of prostate cancer cells with AngII (Uemura et al., 2003; Uemura et al., 2005b; Uemura et al., 2006). In this context, the observed antiproliferative effects of ARBs are basically a reflection of AT1-mediated signaling in cancer cells. This approach does not take into account the possible role of locally produced AngII, which has been demonstrated to be of major importance in other cell types (Reid et al., 2011; Angeli et al., 2013; Lu et al., 2013). In addition, this approach also ignores the possibility of ARBs induced AngII-independent effects which have been demonstrated in other cell types (Alhusban et al., 2013). Another source of complexity in understanding ARB-induced anti-proliferative effects is the complex nature of the cancer microenvironment which has been shown to play a major role in the progression of prostate cancer (Uemura et al., 2005a). To account for all these variables, we were interested in assessing whether
ARB-induced anti-proliferative effects are due to the direct effects of the drug on prostate cancer cells. Accordingly, candesartan direct cytotoxicity was assessed by treating PC3 cells with different concentrations of candesartan in the absence of exogenous AngII. Apoptosis, proliferation, viability and migration of PC3 cells were assayed. Interestingly the anti-proliferative effect of candesartan was found to be unrelated to direct cytotoxicity against cancer cells. This finding was suggested by the relative lack of effect when candesartan was directly applied to prostate cancer cells. To our surprise, candesartan had an anti-apoptotic effect on PC3 cells, and this was observed at all concentrations tested, including the supra-therapeutic range. Candesartan-induced anti-apoptotic effect was not associated with an increase in either migration or proliferation of PC3 cells. In contrast, higher concentrations of candesartan inhibited the rate of PC3 cell migration. Taken collectively, these data suggest that clinical doses of candesartan do not have an appreciable direct effect on PC3 cells. This finding supports previously published data on the inability of candesartan to affect the progression of prostate cancer cells in vitro (Matsuyama et al., 2010).

Interestingly, candesartan inhibited the phosphorylation of P38MAPK in a concentration-dependent manner, and at all concentrations used. This finding reproduces previously published data on the effect of supra-therapeutic doses of ARBs on prostate cancer in the presence of exogenously added AngII (Uemura et al., 2003). This similarity supports the hypothesis that observed ARB-induced antiproliferative effects are mediated by antagonizing the effects of locally produced AngII. AngII is expected to mediate its pro-angiogenic effects through AT2 receptors as have recently been shown in our studies in brain endothelial cells (Alhusban et al., 2013). However, although candesartan
treatment resulted in significantly decreased AT1 and increased AT2 receptor expression in PC3 cells and tumor xenografts, this effect was not observed in DU145 cells, suggesting that the common candesartan effects seen may be due to its direct inhibitory effects on AT1, as reported in melanoma (Akhavan et al., 2011). An alternative explanation is the possible AngII independent effects of ARBs, which requires more detailed in depth analyses that goes beyond the scope of this investigation.

Cancer progression is a well-orchestrated interplay between cancer cells and their microenvironment (Uemura et al., 2005a). This interplay can be best demonstrated by cancer-induced angiogenesis (Hanahan and Folkman, 1996). Cancer cells have high metabolic rates and require higher rates of nutrient delivery. To match their metabolic requirements, cancer cells induce angiogenesis, vascular permeability and perfusion through the release of a variety of angiogenic mediators (Weidner et al., 1993). The lack of concordance between in vivo and in vitro effects of candesartan suggested possible involvement of other cell types in the observed anti-proliferative effect of candesartan in vivo. VEGF plays a major role in prostate cancer-induced angiogenesis (Feng et al., 2008; Mahabeleshwar et al., 2008). Our in vivo data demonstrated elevated levels of VEGF in prostate tumor xenografts from saline treated animals, and a significant inhibition of VEGF expression with a therapeutic dose of candesartan. The effect of candesartan on VEGF expression was also observed in both PC3 cells and DU145 cells. Interestingly, the AT2 agonist (CGP-121141A) had no significant effect on VEGF expression in either PC3 or DU145 cells, thus ruling out the role of AT2 receptor stimulation in candesartan-mediated inhibition of VEGF expression. Our previous
studies in a stroke model indicated increased VEGF expression by candesartan (Guan et al., 2011), probably through endothelial cells (Alhusban et al., 2013; Soliman et al., 2014), in the current model, where angiogenesis is mainly driven by the tumor cells, the mechanism leading to inhibition of VEGF expression is not via AT2 stimulation. As reviewed previously, the effects of ARBs on angiogenesis are dose, tissue and context-dependent (Willis et al., 2011)

Since no significant changes in survival pathways and tumor cell function with the clinical concentrations of candesartan was observed in the *in vitro* experiments, we wanted to rule out that the decrease in VEGF expression in candesartan treated tumors is due to the paracrine effect from prostate cancer cells. To do this and to confirm the antiangiogenic effect of candesartan, electrical cell substrate impedance sensing (ECIS) technique was employed. In this system, endothelial cells cultured to confluence were challenged by either candesartan-treated PC3 cells or their conditioned media and changes in electrical impedance were measured as a function of time to assess the changes in the endothelial-barrier resistance. ECIS findings demonstrated a protective effect of conditioned media from candesartan-treated PC3 cells on endothelial-barrier function compared to untreated control medium. This finding further supports the paracrine over the autocrine effect of VEGF in prostate cancer proliferation. In support of this, our immunohistochemistry analysis of tumor sections revealed that candesartan induced a vascular normalization effect on tumor vasculature as evidenced by reduced lumen size, increased vessel wall thickness and more rounded appearance as compared to saline treated controls. These characteristic features of tumor vasculature are associated with inhibition of tumor vascular permeability and tumor perfusion, thus
inhibiting neovascularization and depriving the tumor cells of necessary nutrients to grow, rather than a direct anti-proliferative effect of candesartan on tumor cells.

In conclusion, our data demonstrate the ability of candesartan to inhibit progression of prostate cancer through ablation of cancer-induced angiogenesis via inhibition of VEGF expression by prostate cancer cells. While a role for AT1 and AT2 in candesartan-mediated inhibition of VEGF expression and/or prostate tumor growth is ruled out, existence of an AT2 independent pathway by AngII in candesartan-mediated inhibition of prostate tumor growth and neovascularization needs to be investigated. Nevertheless, our study conclusively demonstrated that clinically relevant doses of candesartan, alone or in combination with other chemotherapeutic drugs can be developed into a potential therapeutic strategy for prostate cancer. Although monotherapy with candesartan induces vascular normalization and inhibits tumor angiogenesis in prostate tumor in our studies, it will be interesting to know the effect of candesartan in combination with other drugs on prostate tumors. Since vascular normalization is known to affect drug delivery in addition to its effects on tumor angiogenesis, result of combining candesartan with other drugs can be interpreted in both ways. Additional research will be necessary to address this discrepancy.

**Authorship Contributions**

*Participated in research design:* Alhusban, Al-Azayzih, Fagan, and Somanath

*Conducted experiments:* Alhusban, Al-Azayzih, Goc and Gao

*Performed data analysis:* Alhusban, Al-Azayzih, Goc, Gao, Fagan and Somanath

*Wrote or contributed to the writing of the manuscript:* Alhusban, Al-Azayzih, Fagan, and Somanath
References


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Legends for Figures

Figure 1: Candesartan inhibits the growth of prostate cancer xenograft *in vivo* at clinically relevant dose. (A) Representative pictures of PC3-cell prostate tumor xenografts showing reduced tumor size by treatment with clinically relevant dose of candesartan. (B) Graph showing the effect of clinical dose of candesartan on PC3-cell tumor xenograft volume as measured on days 12, 18 and 24 (6, 12 and 18 post-treatment). (C) Bar graph showing the effect of clinical dose of candesartan on PC3-cell tumor xenograft weight on day 24, after 18 day treatment with candesartan. (D) Bar graph showing quantified data on the number of blood vessels surrounding the PC3-cell tumor xenografts in the presence and absence of clinical dose of candesartan. Data presented as Mean ± SD.

Figure 2: Candesartan elicits modest effects on prostate cancer cellular function *in vitro*. *In vitro* effects of candesartan on prostate cancer cell migration, viability, proliferation, and apoptosis were examined. (A) Bar graph showing the effect of clinical doses of candesartan on PC3 cell apoptosis determined by the levels of cytoplasmic histone-associated DNA fragments. (B) Bar graph showing the effect of clinical doses of candesartan on PC3 cell migration. (C) Bar graph showing the effect of clinical doses of candesartan on PC3 cell proliferation determined by BrdU incorporation assay. (D) Bar graph showing the effect of clinical doses of candesartan on PC3 cell viability determined by MTT assay. Except with high concentrations; candesartan did not have any appreciable effects on the progression of prostate cancer cells. Data presented as Mean ± SD.
Figure 3: Candesartan inhibits phospho-P38 MAPK activation and modulates AT1/AT2 receptor expression in PC3 cells in vitro. PC3 cells were treated with a range of clinical doses of candesartan. (A) Western blot figures showing dose-dependent decrease in P38MAPK phosphorylation in PC3 cell lysates with no changes in the activity levels of Akt and GSK-3 (B) Bar graph showing quantification of the optical densities of Western blot protein bands showing significant and dose-dependent decrease in P38MAPK phosphorylation in PC3 cell lysates. (C) Bar graph showing significant dose-dependent inhibitory effect of candesartan on AT1 receptor expression in PC3 cells. (D) Bar graph showing significant dose-dependent effect of candesartan on enhancing AT2 receptor expression in PC3 cells. Data presented as Mean ± SD.

Figure 4: Candesartan elicits an antiangiogenic effect in prostate cancer xenografts in mice. Treatment of nude mice with clinically relevant dose of candesartan reduced vascular density via inhibition of VEGF expression. (A) Representative fluorescent images of tumor xenograft sections showing laminin positive blood vessels as indicated by arrows. (B) Bar graph showing reduced vascular area in tumor xenografts treated with clinically relevant dose of candesartan as compared to saline treated controls. (C and D) Representative Western blot picture (left) and bar graph (right) showing reduced expression of VEGF in tumor xenografts treated with clinically relevant dose of candesartan as compared to saline treated controls. (E) Representative Western blot picture (bottom) and bar graph (above) showing a dose-dependent inhibition of VEGF expression in PC3 tumor cells treated with clinically relevant dose of candesartan as compared to vehicle treated controls. Data presented as Mean ± SD.
Figure 5: Candesartan inhibits VEGF expression in PC3 and DU145 cells at the transcriptional level. (A) Representative image of PC3 and DU145 cell RT-PCR products for VEGF expression in the presence and absence of candesartan treatment as run on 2% agarose gels. (B and C) Bar graph showing densitometry analysis of VEGF mRNA levels in the presence and absence of candesartan treatment in PC3 and DU145 cells, respectively. Data presented as Mean ± SD.

Figure 6: Candesartan treatment helps with vascular normalization in tumor xenografts in vivo. (A) Bar graph showing reduced lumen perimeter in tumor xenografts treated with clinically relevant dose of candesartan as compared to saline treated controls. (B) Bar graph showing reduced vessel lumen area in tumor xenografts treated with clinically relevant dose of candesartan as compared to saline treated controls. (C) Bar graph showing increased vessel wall thickness in tumor xenografts treated with clinically relevant dose of candesartan as compared to saline treated controls. (D) Representative Western blot picture (insert) and bar graph showing significant inhibition of AT1 receptor expression in PC3 tumor xenografts with candesartan treatment. (E) Representative Western blot picture (insert) and bar graph showing significant increase in AT2 receptor expression in PC3 tumor xenografts with candesartan treatment. Data presented as Mean ± SD.

Figure 7: Candesartan preserves endothelial-barrier integrity via inhibiting vascular permeability. (A) Graph showing real-time changes in endothelial-barrier resistance in response to conditioned media collected from PC3 cell cultures treated with various doses of candesartan. Data shows that treatment with candesartan inhibits secretion of vascular permeability stimulating growth factors in a dose-dependent manner thereby
inhibiting the ability of the tumor cell conditioned media to induce endothelial-barrier fenestrations. (B) Graph showing real-time changes in endothelial-barrier resistance in response to topically introduced PC3 cells pre-treated with various doses of candesartan for 12 hours. Candesartan did not have an appreciable effect on the invasion potential of PC3 cells except at higher concentration. (C) Bar graph showing quantification of the endothelial-barrier resistance at 4, 6 and 8 hour post-addition of control and candesartan treated tumor cell culture conditioned medium. Data presented as Mean ± SD.
Figure 2

A

Apoptosis (Absorbance at 405 nm)

*C P < 0.05  
*n = 4

B

Migration (% of wound recovery)

C

Proliferation (Absorbance at 450 nm)

D

Viability (MTT Absorbance at 570 nm)

Candesartan

Candesartan

Candesartan

Candesartan

Candesartan
Figure 3

A

Candesartan

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B

*P < 0.05
n = 4

C

OD (p-p38 MAPK relative to β-Actin)

D

OD (AT1 receptor relative to β-Actin)

AT1

β-Actin

Candesartan

AT2

β-Actin

Candesartan
Figure 4

A Control

B

C

VEGF

β-actin

Control

Candesartan

D

E

*P < 0.05

**P < 0.01

n = 3

Candesartan

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Figure 5

A

PC3

DU145

VEGF

GAPDH

Control

0.5μM

25μM

Control

0.5μM

25μM

B

PC3

OD (VEGF: GAPDH)

C

DU145

OD (VEGF: GAPDH)

C

0.5mM

25mM

0.5mM

25mM

*P < 0.05

**P < 0.01

n = 3
Figure 6

Panel A: Vessel Lumen Perimeter (% of Control)

Panel B: Vessel Lumen Area (% of Control)

Panel C: Blood Vessel Wall Thickness (% of Control)

Panel D: OD (AT1 receptor relative to β-actin)

Panel E: OD (AT2 receptor relative to β-actin)

*P < 0.05
**P < 0.01
n = 6
Figure 7

(A) Normalized Resistance vs. Time (h) for PC3 cell conditioned medium with different concentrations of candesartan.

(B) Normalized Resistance vs. Time (h) for PC3 Cells with different concentrations of candesartan.

(C) Bar graph showing normalized resistance at 4, 6, and 8 hours for different concentrations of candesartan.

*P < 0.05
n= 3