Molecular and cellular effects of azilsartan: a new generation angiotensin II receptor blocker

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Background **Azilsartan medoxomil is a newly approved angiotensin receptor blocker (ARB) reported to lower 24-h blood pressure more effectively than maximally recommended doses of older ARBs. Although azilsartan is considered to be an unusually potent angiotensin II type 1 (AT_1) receptor antagonist, little is known about the potential pleiotropic effects of this molecule.**

Methods and results **We investigated pleiotropic features of azilsartan in cell-based assay systems independent of its effects on blood pressure. In cultured 3T3-L1 preadipocytes, azilsartan enhanced adipogenesis and exerted greater effects than valsartan on expression of genes encoding peroxisome proliferator-activated receptor-α (PPARα), PPARδ, leptin, adipsin, and adiponectin. The effects of azilsartan on adipocyte differentiation and gene expression were observed at concentrations of azilsartan that did not classically stimulate PPAR activity in cell-based transactivation assays. Azilsartan also potently inhibited vascular cell proliferation in the absence of exogenously supplemented angiotensin II. In aortic endothelial cells, azilsartan inhibited cell proliferation at concentrations as low as 1 μmol/l, whereas valsartan showed little or no antiproliferative effects at concentrations below 10 μmol/l. Antiproliferative effects of azilsartan were also observed in cells lacking AT_1 receptors. In addition, azilsartan, but not valsartan, blocked angiotensin II-induced activation of mitogen-activated protein kinase in vascular smooth muscle cells 4–8 h after washout of drug from the incubation media.**

Conclusion **These findings suggest that azilsartan can function as a pleiotropic ARB with potentially beneficial effects on cellular mechanisms of cardiometabolic disease through actions that could involve more than just blockade of AT_1 receptors and/or reduction in blood pressure.**
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Keywords: angiotensin II type 1 receptor blockers, angiotensins, azilsartan, azilsartan medoxomil, hypertension, TAK-491, TAK-536, valsartan

Abbreviations: ARB, angiotensin receptor blocker; AT_1, angiotensin II type 1; BAEC, bovine aortic endothelial cell; BP, blood pressure; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HAVSMC, human aortic vascular smooth muscle cells; IBMX, 1-methyl-3-isobutylxanthine; LBD, ligand-binding domain; MAPK, mitogen-activated protein kinase; PPAR, peroxisome proliferator-activated receptor

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Introduction

Although a variety of angiotensin II type 1 (AT_1) receptor antagonists are approved for the treatment of hypertension and related medical disorders, several companies are pursuing the development of new angiotensin receptor blockers (ARBs) with potential for greater therapeutic efficacy than the older generation molecules in clinical use today [1]. For example, recent phase 3 clinical trials have shown that a newly approved ARB, azilsartan medoxomil (TAK-491), exhibits a safety and tolerability profile similar to existing ARBs, but is significantly more effective in reducing 24-h SBP than maximally approved doses of other ARBs, including either valsartan or olmesartan medoxomil, one of the most effective blood pressure (BP)-lowering agents in the class [2,3]. Given increasing awareness about the impact of 24-h BP levels and morning BP surges on cardiovascular risk [4–6], the recent approval by the Food and Drug Administration (FDA) of a new ARB with improved efficacy in controlling BP throughout the entire 24-h period is of considerable therapeutic interest.

Azilsartan medoxomil (TAK-491) is an ester prodrug rapidly hydrolyzed to azilsartan (TAK-536), the bioactive moiety that selectively blocks AT_1 receptors [7]. Azilsartan is metabolized to major (M-II) and minor (M-I) metabolites that do not significantly contribute to AT_1 receptor blockade [3,7]. The 11-h plasma elimination half-life of azilsartan is similar to that of olmesartan and differences in simple clinical pharmacokinetics do not appear to readily explain the superior ability of azilsartan to control BP over a 24-h period (Table 1) [8]. Both azilsartan and olmesartan have very high affinity for AT_1 receptors and are characterized by half-maximal inhibitory concentration (IC_{50}) values in the very low nanomolar range for inhibiting specific

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Table 1 Plasma elimination half lives and half-maximal inhibitory concentration values

	Elimination half-life (h)	IC_{50} (nmol/l)	
		No drug washout	After drug washout
Azilsartan	11	2.6	7.4
Olmesartan	13	6.7	242.5
Valsartan	6	44.9	>10 000

Plasma elimination half-life data obtained from manufacturer's prescribing information for azilsartan medoxomil, olmesartan medoxomil, and valsartan. Half-maximal inhibitory concentration (IC_{50}) values obtained from the study of Ojima *et al.* [8] in which angiotensin II radioligand binding to human AT_1 receptors was tested in the presence of indicated drugs (no drug washout) and during a 5-h period after drug washout.

binding of angiotensin II to human AT_1 receptors [8]. However, detailed studies of receptor binding and vascular contractile responses to angiotensin II after drug washout have indicated that azilsartan may dissociate from AT_1 receptors much more slowly than olmesartan and other ARBs including valsartan [8]. In radioligand-binding studies conducted 5 h after drug washout, azilsartan showed a much more potent ability than other ARBs to persistently inhibit binding of angiotensin II to human AT_1 receptors (IC_{50} values being >30–1000-fold lower for azilsartan than other ARBs, e.g., Table 1) [8]. On the basis of these kinds of observations, Ojima *et al.* [8] have suggested that very tight binding of azilsartan to AT_1 receptors may contribute to unusually long-lasting angiotensin II antagonism *in vivo*, even after plasma drug levels have begun to significantly wane.

It is now well recognized that some ARBs are pleiotropic molecules that can also have cellular actions potentially relevant to cardiovascular protection that do not solely depend on the ability to block AT_1 receptors or lower BP [9]. For example, some ARBs can exert antiproliferative effects in cultured vascular cells that have not been stimulated with angiotensin II and also in cells that lack AT_1 receptors [10]. In addition, some ARBs are known to directly activate peroxisome proliferator-activated receptors (PPARs) including PPARγ, PPARα, and PPARδ, which are nuclear hormone receptors that regulate a variety of metabolic and inflammatory responses [11–14]. It is clear that azilsartan is an unusually strong AT_1 receptor antagonist; however, little is known about the potential pleiotropic nature of this molecule. Although azilsartan has been shown to have very strong inhibitory effects on vascular contractility [8], its effects on vascular cell proliferation have not been reported. In addition, although systemic treatment of diabetic mice with azilsartan can stimulate expression of the gene encoding PPARγ and of markers of adipocyte differentiation [15], it is unclear whether azilsartan can directly interact with the ligand-binding domains (LBDs) of the different PPAR isoforms. The ability of azilsartan to directly influence adipogenesis and adipocyte gene expression also remains to be defined. Therefore, we explored potential pleiotropic features of azilsartan by investigating the ability of this new ARB to directly activate different PPAR isoforms and to affect adipogenesis and adipocyte gene expression *in vitro*. Given the potential role of vascular cell proliferation in atherosclerosis, we also investigated antiproliferative effects of azilsartan and tested its ability to persistently inhibit angiotensin II-induced activation of p42/44 mitogen-activated protein kinase (MAPK).

Methods

Experimental compounds

Azilsartan was custom synthesized by MonomerChem Inc. (Research Triangle Park, North Carolina, USA) and the structure and purity was confirmed by liquid chromatography and mass spectrometry (Optimized Analytical Solutions, Durham, North Carolina, USA). Rosiglitazone and valsartan were obtained from the pharmacy and purified by HPLC. WY-14643 and GW0742 were obtained from Sigma-Aldrich (St Louis, Missouri, USA).

Peroxisome proliferator-activated receptor transactivation assays

Direct effects of azilsartan on PPAR activity were determined by transactivation assays as previously described in CV-1 cells transfected with 200 ng of the murine PPAR plasmid pGAL4-mPPAR LBD expressing the LBD of either the α, γ or δ PPAR isoform, 1 μg of the luciferase reporter plasmid pUAS-tk-luc, and 50 ng of pCMVSport β-gal as an internal control [11]. Twenty-four-hours post-transfection, cells were treated with varying concentrations of azilsartan and incubated for an additional 24 h. As positive controls, other cells were treated with either the PPARγ activator rosiglitazone, the PPARα activator WY-14643 or the PPARδ activator GW0742.

Adipocyte differentiation studies

Differentiation assays were performed on murine 3T3-L1 preadipocytes using techniques similar to those previously described [11]. After reaching confluence, cells were incubated in Dulbecco's modified Eagle medium (DMEM) containing adipocyte differentiation cocktail composed of 100-μmol/l dexamethasone, 5-μg/ml insulin, and 0.5-mmol/l 1-methyl-3-isobutylxanthine (IBMX) with 5% calf serum for 30 h, after which cells were washed with PBS and incubated for 7 days in medium containing either 10-μmol/l azilsartan, 10-μmol/l valsartan or vehicle control [dimethyl sulfoxide (DMSO)]. In the absence of exogenously added PPAR ligand, the 30-h period of exposure to dexamethasone, insulin, and IBMX induces little or no adipocyte differentiation, although a longer period of exposure to this cocktail is sufficient to trigger adipocyte differentiation. Seven days after drug treatment, cells were fixed and stained with Oil Red O. Quantitative evaluation of adipogenesis was performed using a modified version of the method of Ramirez-Zacarias *et al.* [16] by measuring absorbance at 510 nm.

Gene expression studies

On the basis of the studies by Iwai *et al.* [15] showing effects of orally administered azilsartan on adipose tissue gene expression *in vivo*, we tested for direct effects of azilsartan on expression of the following target genes in 3T3-L1 cells *in vitro*: *PPARγ*, *PPARα*, *PPARδ*, *adipsin*, *adiponectin*, *CD36*, *leptin*, and CCAAT/enhancer binding protein, alpha (*C/EBPα*). Gene expression was measured in 3T3-L1 cells that had been exposed to azilsartan or valsartan after prepriming with adipocyte differentiation cocktails, as in the adipogenesis studies. The 3T3-L1 cells were initially primed for adipocyte differentiation by incubation in the adipocyte differentiation cocktail. After 30 h, we washed the cells to remove the differentiation cocktail and added fresh media [DMEM + 5% calf serum + 1x antibiotic–antimycotic solution (Media-Tech Inc., Winchester, Virginia, USA)] with either azilsartan or valsartan at final concentrations of 10 μmol/l dissolved in DMSO. Control cells were treated with an equivalent volume of DMSO without addition of either azilsartan or valsartan ($n = 6$ samples per group with each sample run in duplicate). Treatment continued for 7 days. The cells were washed and RNA was extracted and cDNA was prepared for measurement of gene expression by real-time PCR using SYBR green reagents (Qiagen, Germantown, Maryland, USA) and methods similar to those previously described [11]. Expression levels of the target genes were normalized relative to the expression level of the gene for TATA-binding protein, TBP, which served as the internal control. The primer sequences used for each gene were as follows. *PPARγ* upstream, 5′-CACAGAG ATGCCATTCTGG and downstream, 5′-AGTGTGGA GCAGAAATGCTG; *PPARα* upstream, 5′-TCAGAAG AAGAACCGGAACA and downstream, 5′-CTTGCCC AGAGATTTGAGGT; *PPARδ* upstream, 5′-GAACCG CAACAAGTGTCAGT and downstream, 5′-GGTAG GCGTTGTAGATGTGC; *adipsin* upstream, 5′-CATG CTCGGCCCTACATGG and downstream, 5′-CACA GAGTCGTCATCCGTCAC; *adiponectin* upstream, 5′-TGTTCCTCTTAATCCTGCCCA and downstream, 5′-CCAACCTGCACAAGTTCCCTT; *CD36* upstream, 5′-AAGCTATTGCGACATGATT and downstream, 5′-GATCCGAACACAGCGTAGAT; *leptin* upstream, 5′-G AGACCCCTGTGTCGGTTC and downstream, 5′-CT GCGTGTGTGAAATGTCATTG; *C/EBPα* upstream, 5′-CAAGAACAGCAACGAGTACCG and downstream, 5′-GTCACTGGTCAACTCCAGCAC; and *TBP* upstream, 5′-ACCCTTCACCAATGACTCCTATG and downstream, 5′-TGACTGCAGCAAATCGCTTGG.

Cell proliferation and cell signaling studies

Bovine aortic endothelial cells (BAECs) were purchased from the UCSF cell culture facility and maintained in DMEM supplemented with 10% calf serum and 1x antibiotic–antimycotic solution. Human aortic vascular smooth muscle cells (HAVSMCs) were purchased from the American Type Culture Collection (Manassas, Virginia, USA) and maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1x antibiotic–antimycotic solution. Chinese hamster ovary cells (CHO-K1) lacking AT_1 receptors were obtained from Cell Culture Facility at UC San Francisco and CHO cells stably expressing human AT_1 receptors (CHO-AT_1) were obtained courtesy of Terry Elton, Ohio State University, Columbus, Ohio, USA [17]. HAVSMCs were studied at passage number 21. BAECs were studied at passage number 10.

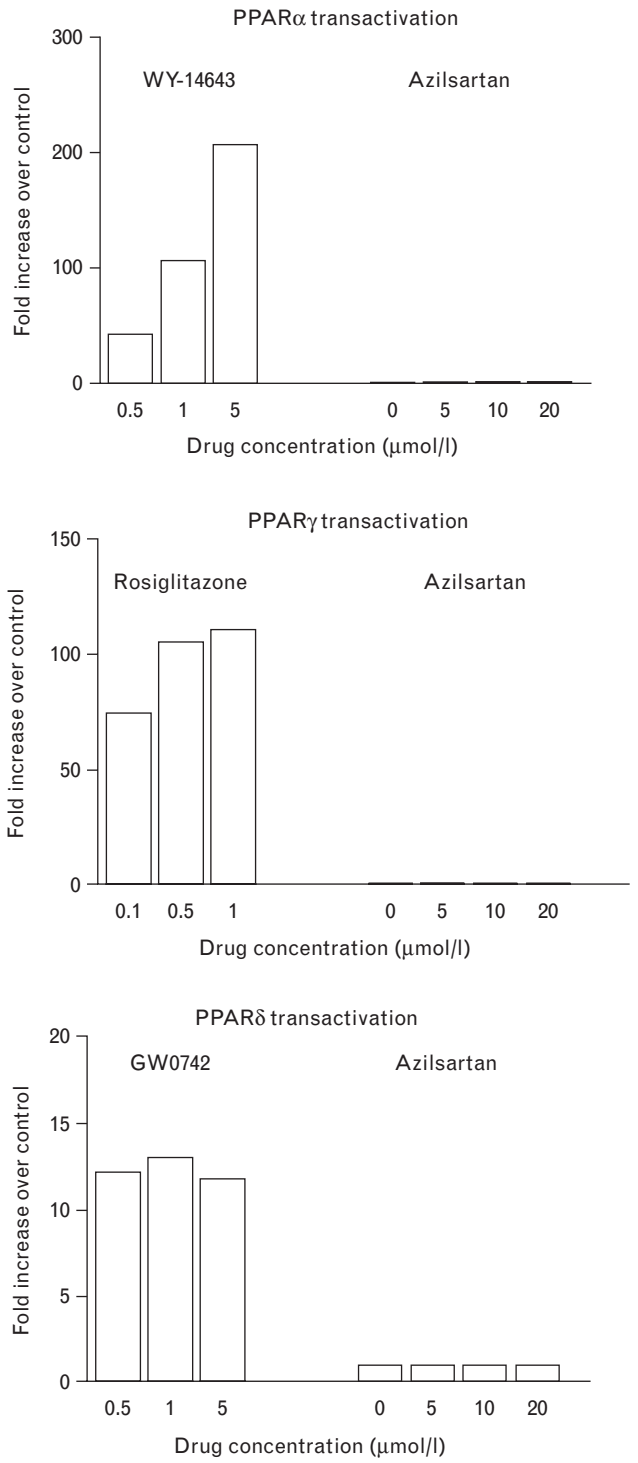
For proliferation studies, we plated 5×10^4 cells per 60-mm dish in the indicated medium. After attachment, cells were exposed to drug by adding the compounds to the growth medium. Untreated control cells not exposed to drugs were incubated with an equivalent volume of DMSO added to the medium. In studies in HAVSMCs and endothelial cells, treatment with the indicated drugs was carried out for 4 days, and in studies in CHO cells, drug treatments were carried out for 7 days. The media containing drugs was changed twice during the treatment periods. At the end of the treatment periods, cells were trypsinized and cell numbers were counted using a Cellometer Auto T4 (Nexcelom Bioscience, Lawrence, Massachusetts, USA).

For cell signaling studies, HAVSMCs at passage 23 were grown in DMEM/F12 with 10% FBS and 1x antibiotic–antimycotic solution. We then starved the cells in DMEM/F12 with 0.25% FBS and 1x antibiotic–antimycotic solution for 14 h. After 14 h, we began a 2-h incubation in DMEM/F12 with 0.25% FBS and 1x antibiotic–antimycotic solution with azilsartan or valsartan at a final concentration of 10 μmol/l. After the 2-h exposure to the drugs, we washed the cells to remove the drug from the media and continued incubation in DMEM/F12 with 0.25% FBS and 1x antibiotic–antimycotic solution. At 4 and 8 h after drug washout, we exposed the cells to 100-nmol/l angiotensin II for 5 min based on preliminary studies showing that this was the optimal time point for phosphorylation of p42/44 MAPK. We then measured phosphorylated p42/44 MAPK levels in protein extracts using the PathScan Phospho-p44/42 MAPK (Thr202/Tyr204) Sandwich ELISA kit (Cell Signaling Technology, Beverly, Massachusetts, USA), according to the manufacturer's protocol.

Statistical analysis

Data are expressed as mean ± SEM, except for relative gene expression ratios that are expressed as medians and interquartile (25 and 75 percentile) ranges. Statistical analysis of gene expression data was performed using the Relative Expression Software Tool (version REST 2009) that tests for significant differences by a randomization procedure (Qiagen, Germantown, MD, USA) [18]. Statistical analysis of other variables was performed by one-way analysis of variance with the Bonferroni

Fig. 1



Azilsartan does not directly activate peroxisome proliferator-activated receptors (PPARs) *in vitro*. Ability of azilsartan to directly activate PPARα, PPARδ, or PPARγ as measured in a cell-based transient transfection assay. As positive controls for the assay system, we measured the ability of WY-14643 to activate PPARα, GW0742 to activate PPARδ, and rosiglitazone to activate PPARγ. These known PPAR agonists caused dose-dependent activation of PPARα, PPARδ, and PPARγ, respectively, whereas azilsartan in concentrations ranging from 1 to 20 μmol/l had little or no effect on PPAR isoform activity.

adjustment for multiple comparisons of individual data groups.

Results

Peroxisome proliferator-activated receptor transactivation assays

Dose response testing in a cell-based transactivation assay showed that azilsartan in concentrations ranging from 0 to 20 μmol/l had little or no effect on transcriptional activity of PPARα, PPARγ, or PPARδ. In contrast, known ligands of these isoforms were clearly able to stimulate PPAR activity in the same assay system (Fig. 1).

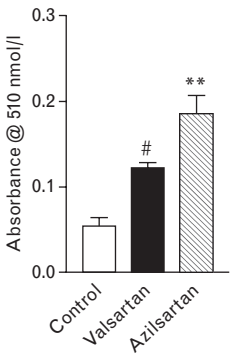
Effects on adipocyte differentiation

In 3T3-L1 cells pretreated with dexamethasone, insulin, and IBMX for 30 h, 7-day treatment with 10-μmol/l azilsartan dissolved in DMSO significantly stimulated adipogenesis compared with control treatment with DMSO alone ($P < 0.0001$, Fig. 2). The effect of azilsartan on adipocyte differentiation was also greater than that of valsartan, which caused a modest increase in adipogenesis compared with control ($P < 0.01$, Fig. 2).

Effects on adipocyte gene expression

We measured the effects of azilsartan on the expression of a selected set of target genes in 3T3-L1 cells treated with azilsartan after prepriming with adipocyte differentiation cocktail. The genes chosen for testing were based on previous studies by Iwai *et al.* [15] that examined the in-vivo effects of orally administered azilsartan on

Fig. 2



Azilsartan stimulates adipogenesis. Effects on adipogenesis and lipid accumulation in 3T3-L1 cells as measured by the absorbance at 510 nm of Oil Red O eluted from stained cells. In 3T3-L1 cells pretreated with dexamethasone, insulin, and 1-methyl-3-isobutylxanthine for 30 h, 7 day treatment with 10-μmol/l azilsartan stimulated adipocyte differentiation significantly more than control treatment with dimethyl sulfoxide or treatment with 10-μmol/l valsartan, which modestly increased adipogenesis relative to control. Results are expressed as means ± SEM with sample sizes of $n = 5–6$ per group. Statistical analysis performed by analysis of variance with the Bonferroni adjustment for multiple group comparisons. $^{**}P < 0.0001$ compared with control group and $P < 0.01$ compared with valsartan treatment; $^{\#}P < 0.01$ compared with control group.

adipose tissue gene expression in a mouse model of diabetes. We found that treatment of the 3T3-L1 cells with azilsartan upregulated expression of genes encoding PPARα, PPARδ, adipsin, adiponectin, and leptin compared with both control cells and valsartan-treated cells (Fig. 3) [18]. Valsartan had little or no effect on gene expression except for downregulation of the gene encoding PPARγ, compared with both control cells and azilsartan treated cells (Fig. 3). In 3T3-L1 cells not preprimed with adipocyte differentiation cocktail, treatment with azilsartan or valsartan had no discernible effect on expression of these genes (data not shown).

Effects on cell proliferation

Several ARBs have been reported to inhibit proliferation of vascular cells in the absence of exogenously angiotensin II and to also inhibit proliferation of cells that lack AT_1 receptors [10]. In the current studies, we found that azilsartan also dose dependently inhibited the proliferation of aortic endothelial cells in the absence of exogenously added angiotensin II (Fig. 4). Inhibitory effects of azilsartan on endothelial cell proliferation were observed at concentrations as low as 1 μmol/l. The antiproliferative effects of azilsartan were significantly greater than those of equimolar concentrations of valsartan (Fig. 4). We also observed antiproliferative effects of azilsartan in AVSMCs, although the inhibitory potency in SMCs was less than that in endothelial cells (data not shown).

To investigate whether the antiproliferative effects of azilsartan require the presence of AT_1 receptors, we tested the ability of azilsartan to inhibit the proliferation of CHO cells lacking AT_1 receptors as well as CHO cells engineered to express human AT_1 receptors [17]. Azilsartan treatment for 7 days inhibited cell proliferation to the same extent in CHO cells lacking AT_1 receptors (-26.6%, $P < 0.001$ compared with no drug treatment control) and in CHO cells engineered to express AT_1 receptors (-27.5%, $P < 0.001$ compared with no drug treatment control).

Inhibitory effects on angiotensin II-induced activation of mitogen-activated protein kinase

Activation of MAPK signaling by phosphorylation plays a key role in cell proliferation in response to growth stimuli, including angiotensin II [19]. Therefore, in HAVSMCs, we compared the ability of azilsartan and valsartan to inhibit angiotensin II-induced phosphorylation of p42/44 MAPK. On the basis of previous studies by Ojima *et al.* [8] and Buclin *et al.* [20] showing that the receptor blocking ability of azilsartan may persist for extended periods after drug elimination, we performed the current studies at 4 and 8 h after washout of drug from the incubation media. When tested after drug washout, the ability of angiotensin II to activate MAPK was inhibited by -30 to -80% in cells that had been pretreated with azilsartan ($P < 0.01$). In contrast, pretreatment of cells with valsartan had no inhibitory effect on the ability of angiotensin II to activate MAPK when tested at 4 and 8 h after drug washout (inhibitory effect of -1 to 8%, $P = \text{NS}$).

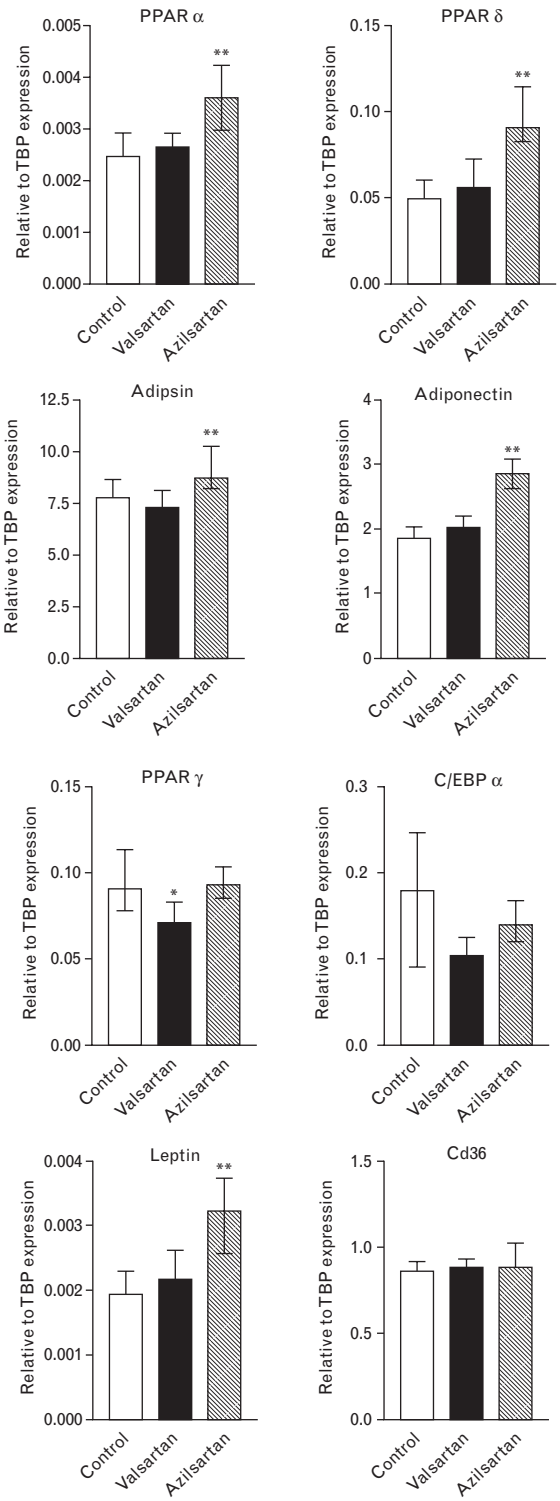
Discussion

Azilsartan is a recently approved ARB that has been reported to be significantly more effective in lowering 24-h BP than maximally approved doses of older ARBs including valsartan and olmesartan [2,3]. Apropos these clinical observations, one of the more interesting effects of azilsartan observed in cell-based laboratory studies is that it appears to remain bound to AT_1 receptors for unusually long periods after drug washout [8]. For example, in studies in isolated aortic strips pretreated with either azilsartan or olmesartan, Ojima *et al.* [8] observed that contractile responses to angiotensin II remained largely blocked even when tested several hours after removal of azilsartan from the incubation media. In contrast, contractile responses to angiotensin II were blocked to a much lower extent when tested several hours after removal of olmesartan from the incubation media. In washout studies designed to test the ability of different sartans to persistently inhibit binding of labeled angiotensin II to human AT_1 receptors, azilsartan was found to be superpotent and to dissociate from AT_1 receptors much more slowly than other ARBs including olmesartan, telmisartan, irbesartan, and valsartan [8].

In the current studies, we found that azilsartan persistently inhibited angiotensin II-induced activation of MAPK in VSMCs 4–8 h after washout of drug from the media. In contrast, valsartan showed little or no ability to block angiotensin II-induced MAPK activation after the same period of drug washout. These findings are consistent with those of Ojima *et al.* [8], indicating that azilsartan may bind more persistently to AT_1 receptors than other ARBs and that this may contribute to long-lasting functional effects of the molecule even after drug concentrations have significantly waned. Interestingly, azilsartan contains a 5-oxo-1, 2, 4-oxadiazole ring that is not found in any of the clinically approved ARBs and it has been proposed that this structural feature may contribute to its ability to block AT_1 receptors for an extended period (Fig. 5) [8]. The unusually persistent binding of azilsartan to AT_1 receptors may not only be relevant to its superior ability to maintain reductions in BP for long periods but also might contribute to sustained protection against the effects of angiotensin II on cell signaling pathways involved in the pathogenesis of cardiovascular disease.

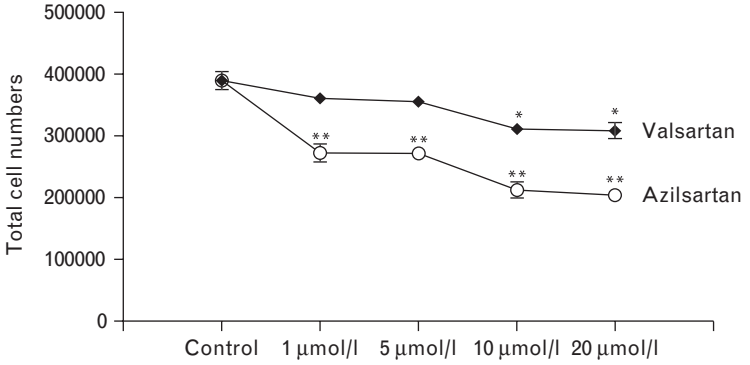
In the current studies, we also noted that azilsartan can potently inhibit vascular cell proliferation even in the absence of exogenously supplemented angiotensin II. In aortic endothelial cells that had not been deliberately stimulated with angiotensin II, we found that azilsartan was a much more potent inhibitor of cell proliferation than valsartan. Significant antiproliferative effects of

Fig. 3



Azilsartan increases gene expression in differentiating 3T3-L1 preadipocytes. Azilsartan treatment significantly increased expression of genes encoding peroxisome proliferator-activated receptor-α (PPARα), PPARδ, adipsin, adiponectin, and leptin, whereas valsartan treatment did not. The expression level of PPARγ was significantly lower in valsartan-treated cells than in azilsartan-treated cells or in untreated cells. Expression levels of the target genes are normalized relative to the expression level of the gene for TATA-binding protein, TBP, which served as an internal control. Results are expressed as medians ± interquartile ranges (25 and 75 percentile). Sample size, $n = 6$ per group with each sample tested in duplicate. Statistical analysis performed by the randomization test procedure of the Relative Expression Software Tool (version REST 2009) [18]. $^{**}P < 0.05$ compared with control cells and with valsartan-treated cells. $^{*}P < 0.05$ compared with control cells and with azilsartan treated cells.

Fig. 4

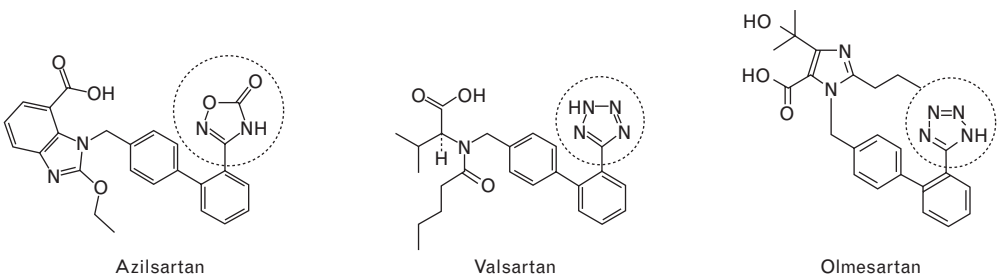


Effects of azilsartan and valsartan on proliferation of aortic endothelial cells in the absence of exogenously added angiotensin II. Azilsartan inhibited endothelial cell proliferation in a dose-dependent fashion to a greater extent than valsartan at all concentrations tested. Results (cell numbers) are expressed as means ± SEM with sample sizes of $n = 6$ per group. Statistical analysis performed by analysis of variance with the Bonferroni adjustment for multiple group comparisons. $^{**}P < 0.0001$ compared with control and with valsartan treatment. $^{*}P < 0.001$ compared with control group.

azilsartan were observed at concentrations as low as 1 μmol/l, which can be readily obtained in plasma with usual oral dosing in humans [21]. In contrast, valsartan showed little or no antiproliferative effects at concentrations below 10 μmol/l. In the current experiments, it is possible that these antiproliferative effects of azilsartan were due to inverse agonist properties of the drug [8,22] or to blockade of the growth effects of background amounts of angiotensin II that might have been present in the culture media. However, we also observed antiproliferative effects of azilsartan even in CHO cells that lack AT_1 receptors. This raises the possibility that azilsartan may exert inhibitory effects on cell proliferation at least in part through mechanisms that do not strictly depend on its ability to block AT_1 receptors.

In studies in a mouse model of diabetes, Iwai *et al.* [15] recently found that azilsartan is a much more potent stimulator of insulin sensitivity and tissue glucose uptake than candesartan. These investigators also found that oral administration of azilsartan reduced adipocyte size and stimulated adipose tissue expression of genes encoding PPARγ, aP2, C/EBPα, and adiponectin much more effectively than candesartan. However, little or no published data have been available regarding the potential ability of azilsartan to either directly increase activity of the various PPAR isoforms by interacting with the LBDs of these receptors and/or directly stimulate adipogenesis or gene expression in differentiating 3T3-L1 cells in culture. In the cell-based transactivation assays performed in the current studies, we found that azilsartan concentrations as high as 20 μmol/l had little or no effect on activity of either PPARγ, PPARα or PPARδ. However, we found that azilsartan can enhance adipogenesis of 3T3- L1 preadipocytes in culture and exert greater effects than valsartan on expression of genes encoding PPARα, PPARδ, adipsin, adiponectin, and leptin. Although azilsartan can affect expression of PPAR

Fig. 5



Chemical structure of azilsartan compared with valsartan and olmesartan. Note that azilsartan has a distinctive 5-oxo-1, 2, 4-oxadiazole ring in place of the tetrazole ring commonly found in other angiotensin receptor blockers such as valsartan and olmesartan shown here, as well as in losartan, irbesartan, and candesartan. The 5-oxo-1, 2, 4-oxadiazole ring in azilsartan and the tetrazole rings in valsartan and olmesartan are highlighted in the dashed circles.

isoform genes and known PPAR target genes including adiponectin, the results of our transactivation studies indicate that the effects of azilsartan on adipocyte gene expression and adipocyte differentiation do not involve conventional ligand-mediated activation of the PPAR isoforms themselves. Recently, studies by Choi *et al.* [23] have indicated that some molecules can have significant effects on PPARγ function by affecting receptor phosphorylation status independent of classical receptor transcriptional agonism. Thus, the current results and those of Choi *et al.* could serve to motivate future studies of the effects of azilsartan on phosphorylation status of PPARγ.

In summary, we have found that azilsartan is a pleiotropic ARB with antiproliferative effects in vascular cells that may not strictly depend on AT_1 receptor blockade. In addition, azilsartan, but not valsartan, blocked angiotensin II-induced activation of MAPK in VSMCs long after washout of drug from the incubation media. We also found that azilsartan promoted adipocyte differentiation to a greater extent than valsartan and exerted stimulatory effects on the expression of genes for PPARα, PPARδ, leptin, adipsin, and adiponectin, whereas valsartan did not. These effects on adipocyte differentiation and gene expression were observed at concentrations of azilsartan that did not classically activate PPAR activity in cell-based transactivation assays. The findings, first, are consistent with recent studies indicating that azilsartan may have an unusual capacity to maintain effective AT_1 receptor blockade for extended periods after drug concentrations have significantly waned and, second, suggest that azilsartan may have potentially beneficial effects on cellular mechanisms of cardiometabolic diseases through actions that involve more than just blockade of AT_1 receptors and/or reduction in BP.

Acknowledgement

Conflicts of interest

T.W.K. has received grant funding from the National Institutes of Health and lecture honoraria from GlaxoSmithKline, Boehringer-Ingelheim, Takeda Pharmaceuticals North America, Shionogi, Pfizer, and Merck; has served as a consultant for Theravance Inc., Takeda Pharmaceuticals North America, and Daiichi Sankyo Pharmaceuticals; and holds stock ownership interest in GlaxoSmithKline, Bethesda Pharmaceuticals, Ligand Pharmaceuticals, Novartis, and Pfizer. For the remaining authors, no potential conflicts of interest are declared.

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