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Angiotensin Type 1 Receptor Resistance To Blockade In The Opossum Proximal Tubule Cell Due To Variations In The Binding Pocket

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Running title: AT_1R binding pocket variations may explain ARB resistance

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Abstract

Blockade of the angiotensin (Ang) II receptor Type 1 (AT1R) with angiotensin receptor blockers (ARBs) is widely used in the treatment of hypertension. However, ARBs are variably effective in reducing blood pressure, likely due, in part, to polymorphisms in the ARB binding pocket of the AT1R. Therefore, we need a better understanding of variations/polymorphisms that alter binding of ARBs in heterogeneous patient populations. The opossum proximal tubule cell (OKP) line is commonly used in research to evaluate renal sodium handling and therefore blood pressure. Investigating this issue, we found natural sequence variations in the opossum AT1R paralleling those observed in the human AT1R. Therefore, we posited that these sequence variations may explain ARB resistance. We demonstrate that OKP cells express AT1R mRNA, bind 125I-Ang II, and exhibit Ang II-induced phosphorylation of Jak2. However, Jak2 phosphorylation is not inhibited by five different ARBs commonly used to treat hypertension. Additionally, non-radioactive Ang II competes 125I-Ang II efficiently, while a ten-fold molar excess of olmesartan and the AT2R blocker PD123319 are unable to block 125I-Ang II binding. In contrast, Ang II binding to OKP cells stably expressing rat AT1ARs, that have conserved AT1R binding pocket with human AT1R, is efficiently inhibited by olmesartan. A novel observation was that, resistance to ARB binding to opossum AT1Rs correlates with variations from the human receptor at positions 108, 163, 192 and 198 within the ARB binding pocket. These observations highlight the potential utility of evaluating AT1R polymorphisms within the ARB binding pocket in various hypertensive populations.

Keywords: hypertension, chronic kidney disease, polymorphisms, angiotensin II receptor Type 1, angiotensin receptor blockers

Introduction

The angiotensin (Ang) II receptor type 1 (AT_1R) is expressed in multiple kidney cell types including proximal tubule cells (PTCs) (20; 25). Actions of Ang II on the proximal tubule AT_1R contribute to the regulation of salt and fluid homeostasis and blood pressure (BP) (20). Increased activation of AT_1R in the proximal tubule can lead to excessive salt retention, oxidative stress, inflammation hypertension and ultimately chronic kidney disease (20; 25) . Hence, blockade with AT_1R blockers (ARBs) is an important treatment for hypertension and associated cardiovascular and kidney disease (9). However, antihypertensive responses to ARBs vary in different patient populations, suggesting that polymorphisms of the PTC AT_1R may play a role in differential BP responses to ARBs (2; 27; 34; 37)

There is mounting interest in the polymorphisms in the renin-angiotensin system (RAS) that contribute to race, sex and other demographic variations in BP responses to ARBs (12; 13; 16; 19; 22; 27; 34; 35; 37; 39). For example, AT_1R polymorphisms including A1166C (3'UTR) have been studied with respect to their propensity to confer increased risk for hypertension, cardiovascular and chronic kidney disease (35). However, polymorphisms in the AT_1R that alter the coding sequence that may alter ARB binding to PTCs and thus variably affect salt retention and BP have not been well established. Nevertheless, one polymorphism (T282M 7.35) has been reported on the NCBI website to be associated with renal tubular agenesis (27). Recently, another polymorphism in the human AT_1R (A163T 5.60) has been proposed to result in decreased binding of losartan to the AT_1R and was identified in a polymorphism screen to confer increased risk for chronic kidney disease in a Japanese cohort (2; 39). Although differences in the genetic makeup of individuals are well-recognized, systematic studies on human AT_1R polymorphisms and ARB resistance have not been undertaken (27).

ARBs bind to the AT_1R via a pocket that partially overlaps with that for Ang II, as expected for a competitive antagonist. Based on current evidence, the Ang II binding pocket is formed by: K102 (3.26 in the Ballesteros-Weinstein numbering system), H166 and R167 in the second extracellular loop (ECL2), E173 in the alpha helix within ECL2, K199 (5.42), W253 (6.47), F259 (6.54), T260 (6.55), D263 (6.58), and D281 (7.34) (Figure 1) (4; 21; 26; 38). ARBs (biphenyl compounds with tetrazole and imidazole rings) bind to AT_1Rs via residues shared with Ang II: K102, H166, R167, K199, and D263; as well as unique residues: V108 (3.32), N111 (3.35), A163 (4.60), and S252 (6.46); with minor or unclear contributions from other sites (Figure 1) (5; 21; 26; 38). Importantly,a single mutation, V108I (3.32), leads to a 40-fold reduction in binding for losartan, a prototype of the ARBs, but does not alter Ang II binding (17). Thus, single polymorphisms can substantially influence ARB binding and clinical responses.

During our investigation, we came across the genomic sequence of opossum AT_1R that has several variations in the ARB binding pocket when compared to the human AT_1R. AT_1R's are known to be expressed in abundance by PTCs on both the luminal brush border and the basolateral region (6; 8; 23; 28), suggesting their importance in renal sodium retention and blood pressure regulation. Therefore, we posited that exploring the effect of ARBs on Ang II binding characteristics and Ang II-mediated signaling in opossum PTCs would increase our understanding of the role of polymorphisms in modulating the efficacy of various ARBs in lowering BP in various populations.

Findings in the native OKP cell line are compared to an existing and well characterized stable rat $AT_{1A}R$-expressing OKP cell line (36). The rat $AT_{1A}R$ is remarkably well conserved when compared to the human AT_1R, especially with regards to Ang II and ARB binding pockets, deeming it a good substitute for human receptor studies when examining binding of Ang II and

ARBs. Evidence is presented for lack of effective ARB blockade of Ang II binding to, and signaling through, the opossum AT_1R despite functional expression of AT_1Rs in the native OKP cells. These observations are consistent with experimentally demonstrated variations/polymorphisms that inhibit ARB binding in humans and other species. These findings advance our understanding of opossum AT_1R, and support the continued use of OKP cells for studying Ang II-AT_1R signaling. Moreover, these data highlight the potential to ascertain amino acid variations in the ARB binding pocket of the human AT_1R as a genetic test to evaluate the therapeutic efficacy of ARBs.

Materials and Methods

PTC culture

Native OKP cells were a kind gift from Dr. D. Biemesderfer at Yale University. T35OK-AT_1R stably-expressing rat $AT_{1A}R$ in OKP cells (ATCC, Dr. John Raymond, MUSC, South Carolina) were generated in the laboratory of Dr. T. Thekkumkara at Texas Tech University (Amarillo, TX). Native OKP cells were grown in DMEM/F12 with 10%FBS and penicillin/streptomycin 100µg/ml. T35OK-AT_1R cell medium also contained G418 200µg/ml, transferrin, insulin, dexamethasone and epidermal growth factor. Both cell lines were starved in media containing 0.1%FBS, with the T35OK-AT_1R starvation medium additionally containing G418.

PCR and sequencing

Opossum specific PCR primers were designed based on AT_1R sequence (XM_001371246.1) published at the National Center for Biotechnology and Information (NCBI) website: Forward primer (F1) 5'- ATG GCC AAA GTG ACC TGC ATT-3' and reverse primer

(R1) 5'- TGA ATC TCA TAA GCC TTT TTC -3' are based entirely in exon 4 for the 248bp product. In addition, to confirm the sequence of AT_1R in native OKP cell line is similar to NCBI published sequences, primer sets were designed to flank the entire ORF for a total of ~1.2kb with the following primers: Forward primer (F2) 5'- CCCCCAAGATCATGCTGGCATAGC-3' and reverse primer (R2) 5'- TCCAAGGATGGAAACCCTTGCCAT -3'. Genomic DNA was extracted with "Blood and cell culture" DNA midi kit (Qiagen, Valencia CA) on previously frozen OKP cells, as per manufacturer's protocol. PTC genomic DNA was used as a template to PCR-amplify the putative AT_1R gene ORF by employing the "Long-Amp Taq" PCR kit (New England Bio-labs, Ipswich, MA). The thermo-cycling steps in the generation of OKP AT_1R gene product were optimized as per manufacturer's recommendations, except for addition of Taq polymerase after the "hot-start" step. The following thermo-cycling conditions were used: initial denaturing at 94ºC for 4 min, followed by 30 cycles at 94ºC for 30 sec, 55ºC for 1 min, and 65ºC for 2 min each for denaturing, annealing and synthesis respectively (the time for the latter cycle of synthesis at 65ºC for 2 min was increased by 15 sec every one cycle after the first cycle), followed by a final elongation step at 65ºC for 10 min. The ~1.2kb product was eluted from agarose gel and purified using "Qia-quick" gel-extraction kit (Qiagen, Valencia CA). Sequencing was done at DNA Core facility at the University of Missouri-Columbia using standard protocol. The genomic sequence was converted to protein sequence using the ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi). The ORF obtained thus was aligned to XP_001371283.1 using ClustalW.

AT_1R protein sequences

The NCBI database was used to search for published AT_1R protein sequences (Supplemental Table 1). The sequences were compared via ClustalW sequence alignment tool

and phylogenetic comparison was computed in Bioedit (14) and viewed in Treeview (29). The Ang II and ARB binding pockets were graphically analyzed utilizing WebLogo (11).

Jak2 phosphorylation by Western blot

Western blots were done using standard techniques. Briefly, monolayers were starved overnight and treated with the indicated ARBs for 1 hr prior to Ang II stimulation for 15 min. Whole cell lysates were prepared in lysis buffer containing 1% Triton-X100, 100mM NaCl, 20mM Tris pH 7.5, 2mM EDTA, 10mM $MgCl_2$, 10mM NaF, 40mM β-Glycerol Phosphate, 1mM PMSF, 2mM Na_3VO_4, 10mg/mL Aprotinin, 10mg/mL Leupeptin with additional protease inhibitors (Roche Applied Science, Indianapolis IN). Protein quantitation was done with BCA reagent (Pierce, Rockford IL) and 40 μg of protein was loaded in each well. Gels were transferred to nitrocellulose membranes and incubated with antibodies to Jak2 and phospho-Jak2 in 5%BSA with 0.1% TBST (Cell Signaling, Danvers MA). Bands were visualized with a Biorad phosphorimager after addition of ECL reagent (Pierce, Rockford IL) and quantified using Image lab (Biorad, Hercules CA).

Radioligand binding

Non-polarized PTCs

Native OKP cells and T35OK-AT_1R cells were grown to 70-95% confluence in T-150 flasks, washed with PBS, trypsinized in 0.05% Trypsin-EDTA, centrifuged at 600g for 5min and the cell pellet resuspended in DMEM containing 50% FBS. $2 \times 10^5 - 5 \times 10^5$ cells were used for radioligand binding. The binding assay was conducted as previously described (30), except that for the duration of the binding experiment the cells were maintained on ice and centrifuged at 4ºC. For the competition experiments, non-radioactive Ang II (10^{-11} M through 10^{-5} M) and PD123319 (10^{-11} M through 10^{-5} M) were added in increasing concentrations; olmesartan (10^{-6}

M) was used to verify binding to the AT_1R. Data was plotted and analyzed utilizing Graphpad Prism software.

Polarized PTCs

Native OKP cells were grown to 100% confluency on 6-well Transwell inserts (Corning, Tewksbury MA) with 0.4uM pore diameter for 1 week to form polarized layers. Polarization was confirmed using megalin as apical membrane marker. PTCs were starved overnight in DMEM/0.1% FBS and on the morning of the experiments, cells were washed in PBS and DMEM with no phenol red was used for the assays. Blockade of AT_1R was done with olmesartan for 1 hr followed by I^{125}-Ang II (50pM, Perkin Elmer, Waltham MA) treatment for 30min at RT to facilitate uptake into cells. I^{125}-Ang II binding to apical (AP) and basolateral (BL) receptors were assessed separately in individual wells. The inserts were washed 3 times with ice-cold 1X PBS and surface bound receptors removed with two 40-sec acid washes in ice-cold 5mM Trichloroacetic Acid in 150mM NaCl pH 2.5

For both sets of experiments the CPM's were measured on a 1480 Automatic Gamma Counter (Perkin Elmer).

Modeling of human AT_1R

Human AT_1R modeling was conducted via computerized prediction (for specifics see below) of the human AT_1R and β2-adrenergic receptor (β2-AR) secondary structures from their linear sequences, which resulted in remarkably similar predictions. The predicted β2-AR was then compared and refined to match the crystalized β2-AR (PBD ID: 3P0G) (32). The refined β2-AR prediction was then used to refine the AT_1R prediction, and when constructing the model in two dimensions (Figure 1) the crystalized β2-AR was used as a guide. CBS TMHMM and

193 TMpred (15) were used to predict transmembrane (TM) regions, and SCRATCH Protein
194 Predictor was used to run SSpro8 to predict the secondary structures.

195 **Statistics**

196 Results were analyzed using two-way ANOVA, or one-way ANOVA when appropriate,
197 with α set at 0.05 using NCSS 2007 (NCSS, LCC Kaysville, UT) and GraphPad Prism 5
198 (Graphpad Softward, San Diego, CA). Tukey-Kramer and Kruskal-Wallis Z Post-hoc tests were
199 applied for normal and ratiometric data, respectively, as indicated in the figures and were
200 considered significant only if $p < 0.05$. All data are reported as mean ± SEM.

201

202 **Results**

203 **Native OKP cells express the AT_1R**

204 Utilizing an opossum-specific PCR primer set based on published NCBI sequences, we
205 obtained a 250bp product in the native OKP cells as well as the T35OK-AT_1R cells, consistent
206 with the expression of AT_1R in this cell line (Figure 2). We sequenced the 250bp product as
207 well as 1.2kb of genomic DNA containing the entire AT_1R ORF to confirm that the clonal OKP
208 cells expressed an AT_1R consistent with published NCBI sequence (Supplemental Figure 1).
209 However, this sequence revealed multiple discrepancies with the human sequence; most notably,
210 there were alterations in the ARB, but not Ang II binding pockets (Figure 3).

211 **Amino acid variations in the OKP AT_1R compared to other species' AT_1Rs**

212 We aligned 28 vertebrate AT_1R protein sequences, utilizing ClustalW to identify areas
213 that are not conserved in the opossum (Supplement Figure 2, Supplement Table 1). All of the
214 Ang II-binding site amino acids in the opossum AT_1R are conserved when compared with the
215 human AT_1R (Figure 1**)** and they are relatively conserved across all species examined, and

especially well conserved in mammals, indicating the importance of Ang II in the physiological processes of evolution (Figure 3A). In contrast, variations within the ARB binding pocket are more pronounced (Figure 3B). In the opossum, position 108 and 163 amino acids differ from the human, each of which is critical for losartan binding (17).

Analysis of the genetic distance of the opossum AT_1R showed that it and the platypus AT_1R are quite distant from the rest of the mammalian AT_1Rs (Figure 4, Supplemental Table 1). Furthermore, the phylogenic tree shows that the AT_1R sequences follows the pattern of evolution in which fish are the most distant from the humans, followed by amphibians, birds, rodents, and then other mammals.

AT_1R-mediated signaling is present in OKP cells, but is not abrogated by olmesartan

To establish functional expression of the AT_1R in OKP cells, native OKP cells were treated with Ang II (10^{-7} M) for 15 min resulting in tyrosine phosphorylation of Jak2 (Figure 5). Jak2 is an established signaling pathway for AT_1Rs, but not AT_2Rs. However, olmesartan (10^{-6} M) did not inhibit Jak2 phosphorylation in native OKP cells, but ablated Ang II-mediated phosphorylation of Jak2 in the T35OK-AT_1R cells (Figure 5A). Recall the T35OK-AT_1R cells have a canonical ARB binding site. To further assess whether the lack of inhibition of Ang II-mediated activation of Jak2 is a class effect of ARBs and not specific to olmesartan, we used 4 additional ARBs (losartan, valsartan, irbesartan, and azilsartan at doses of 10^{-6}M) and could not inhibit Ang II-mediated phosphorylation of Jak2 in native OKP PTCs (Figure 5B). Ang II at very low concentration of 10^{-10} M did not stimulate Jak2 phosphorylation consistently in monolayer or polarized native OKP cells, but 10^{-9} M and 10^{-8} M Ang II resulted in mild phosphorylation of Jak2 that was not affected by 10^{-6} M of the five ARBs (Data not shown).

These data suggests that lack of suppression of Ang II signaling was not due to growing in a non-polarized state or insufficient doses of the ARBs.

Ang II binds to opossum AT_1R

Based on the functional and sequence data, we were curious to examine the binding of Ang II and olmesartan to the AT_1R in the native OKP cell line (Figure 6). A Bmax of 16,752 ± 1,550 and 110,859 ± 21,333 molecules Ang II/cell was calculated for the native OKP and T35OK-AT_1R cells, respectively, ($n = 4$, $p < 0.05$) indicating that the opossum AT_1R binds Ang II and that there are greater Ang II binding site on the T35OK-AT_1R cells as expected (Figure 6A). Next, to determine the affinity of the OKP AT_1R for Ang II, bound ^{125}I-Ang II was competed with nonradioactive Ang II (Figure 6B). The log Kd was -8.0 ± 0.1 and -7.7 ± 0.1 molar for the native OKP and T35OK-AT_1R cells, respectively. Thus, opossum Ang II receptors bind Ang II with similar affinity as the rat $AT_{1A}R$.

AT_1R and AT_2R specific antagonists cannot compete for OKP-specific AT_1R

Finally, we demonstrate that olmesartan (10^{-6} M) cannot compete with ^{125}I-Ang II for binding to the AT_1R in the native OKP cell line, but can compete for the rat $AT_{1A}R$ in the T35OK-AT_1R cell line (Figure 6C). Moreover, in both cell lines ^{125}I-Ang II binding was effectively competed by non-radioactive Ang II (10^{-7} M) (Figure 6C). We confirmed that ^{125}I-Ang II did not bind to an AT_2R via using increasing concentrations of PD123319 in both cell types (Figure 6D). To further assess whether binding to AT_1R is dependent on PTC polarization, AP and BL receptors were assayed for their ability to internalize ^{125}I-Ang II (Figure 7). AP AT_1R internalized ^{125}I-Ang II in slightly lower amounts (not significant) than BL AT_1R in native OKP PTCs confirming previous studies (36); however, olmesartan did not block uptake of either

AP or BL ^{125}I-Ang II confirming that the effects seen are not due to a lack of polarization of the PTCs.

Discussion

Variable human response to drug treatment is increasingly being attributed to polymorphisms in the genome for many widely used drugs including beta-blockers, other G-protein coupled receptor antagonists, statins and recently coumadin (33; 37). Although the concept of certain amino acid sites in AT_1R being central to ARB binding dates back ~15 years, translation of this concept to human ARB resistance has been delayed primarily due to absence of resources such as the SNP database, need to sample a larger cohort to get to significant differences (allele frequency is not known), and difficulty in selecting patient populations (17; 18). Availability of large-scale sequencing data from the Genome Project and other sister projects has enabled us to effectively utilize this information to test hypotheses about ARB resistance in novel ways. To put this in perspective, drug resistance attributable to RAS polymorphisms has spurred many investigations in the search to resolve human variations in blood pressure, chronic kidney disease and cardiovascular disease (12; 13; 16; 19; 22; 27; 35; 39). In this regard, the A1166C polymorphism in the AT_1R has been the focus of attention (many dozens of papers), and several humans with this polymorphism have been identified (13; 19). However, the A1166C polymorphism lies in an area of 3'- untranslated sequence of the AT_1R and its significance is still unclear (12). More recently, discovery of coding sequence polymorphisms via the SNP database has provided the potential to revolutionize the way we think about drug resistance. For example, the A163T (rs12721226) polymorphism is weakly associated with chronic kidney disease and a 10 mm Hg increase in systolic blood pressure (39).

283 More recent data from the Perindopril Genetic Association Study (PERGENE) shows that
284 carriers of ≥3 risk alleles of rs rs275651 (4230T>A) and rs5182 (Leu191) polymorphisms in
285 AT_1R were associated with poorer response to ACE inhibitors (37). The list of SNPs keeps
286 growing and the current list is documented at (http://www.ncbi.nlm.nih.gov/projects/SNP) and
287 summarized in Supplemental Table 2). Interestingly, at least 33 SNPs have been identified in the
288 coding region of agtr1, the human AT_1R gene. Of these, many alter the amino acids and the rest
289 are synonymous (http://www.ncbi.nlm.nih.gov/projects/SNP) (27).

290 In order to test our hypothesis about amino acid variations and ARB resistance, we chose
291 an opossum PTC line that has been well studied with respect to renal sodium transport (5; 36).
292 While here are many iterations of this cell line two of these; the OKP (Cole et al) and OK
293 (ATCC, John Raymond) have been primarily used by investigators (10; 36). To be a useful *in*
294 *vitro* model for the study of Ang II-mediated effects, AT_1Rs need to be functionally expressed
295 and pharmacologically blocked. Based on current literature, it is not clear to what extent
296 opossum PTC expresses AT_1Rs (5; 36). Moreover, pharmacological inhibition with an ARB
297 (losartan) has not conclusively established the presence of an AT_1R (7; 31).

298 Currently, identification of receptors is conducted via two methods: genetic and
299 pharmacological. Genetic identification is based solely on sequence homology, which in the
300 case of native OKP cells there is clearly an expressed AT_1R that is identical to the identified
301 opossum agtr1 (XP_001371283.1) on chromosome 7 (Figure 2 and Supplemental Figure 1).
302 Pharmacological identification utilizes two separate processes to identify receptors. First,
303 endogenous agonist binding is used to identify the receptor, which in the case of native OKP
304 cells femtomolar levels of Ang II binds to the cells (Figure 6A). Secondly, specific inhibitors are
305 used to further classify the receptor and denote receptor subtype, which in the case of native

OKP cells fails to identify the AT_1R due to a lack of interaction with ARBs (Figures 6C and 7). Due to the following data we conclude that native OKP cells express functional AT_1Rs that do not bind ARBs. : 1) the genetic identification of an AT_1R in native OKP cells; 2) the binding of Ang II to native OKP cells, and failure for PD123319 to compete for binding; and 3) Ang II-mediated phosphorylation of Jak2 in native OKP cells, which is known to occur through a conserved YIPP motif (18) on the AT_1R and not the AT_2R.

Previous studies have identified multiple residues on the AT_1R that interact with ARBs (18; 21; 24; 26; 38) (Figure 1 and 3). Systematic mutagenesis of these sites individually or in combination led to decreased binding affinity of ARBs. Furthermore, reversal of some of these mutations led to a return to normal binding characteristics of the AT_1R (17). However, as these are mutagenesis studies, some of the sites may not be directly involved in binding the ARB, yet may alter the structure of the receptor or its binding pocket. Specifically, L300 and F301 are probably not directly involved in ARB binding as they are at the cytosolic surface of the receptor; thus, we shade them yellow in Figure 1 and exclude them from Figure 3 to indicate their questionable significance (18). Similarly, H256 (6.51) appears to be partially involved in ARB binding, but only when substituted with Arg, not Gln, Ala and Glu (26). Both Arg and His are positively charged, and removing or altering the charge has no effect on binding. However, Arg is larger than His, and H256 (6.51) being near the pocket the alteration in binding is due to an altered shape of the pocket rather than direct interference with ARB binding.

The amino acid variation from the normal mammalian sequence at position 108 seen in the opossum AT_1R (Ile compared to the predominant Val) is identical to the experimentally established mutagenesis studies in rat $AT_{1A}R$ (V108I) that leads to an approximate 40-fold decrease in ARB binding affinity (24). These observations suggest that ARB binding to position

329 108 is dependent on the size of the R group; Ile (larger than Val) and Ala (smaller than Val), and
330 while both substitutions disrupt binding, they do so to different extents. Therefore, there is likely
331 a hydrophobic interaction between the ARB and AT_1R that is specifically supported by V108.
332 Moreover, replacement of I108 of *Xenopus* AT_1R with Val as well as other minor alterations
333 restores ARB binding to the receptor (18). As this difference in sequence exists in two of the
334 examined mammalian species [opossum and gerbil (Supplemental Figure 2, Supplemental Table
335 1)], this single amino acid is likely a key factor in the loss of ARB binding, and may be a single
336 polymorphism that can confer ARB resistance in humans.

337 A second prominent OKP variation is at position 163, which in the human AT_1R is
338 normally an Ala but in the opossum is a Val. In the rat $AT_{1A}R$, mutation of A163 to Thr, leads to
339 a 12-fold decrease in losartan inhibition of Ang II binding (17). Interestingly, this is also the site
340 where an amino acid change of a verified human agtr1 polymorphism results in a 7-fold
341 decreased binding of losartan (2). Similarly, T163 is the endogenous residue in bovine AT_1R,
342 and although bovine AT_1Rs are inhibited by ARBs, losartan (Dup 753) is 10-fold less potent at
343 the bovine AT_1R compared to the rat AT_1R (3). These data strongly indicate that position 163 is
344 important to ARB binding; however, it remains to be determined what magnitude change in
345 ARB binding would occur in an A163V mutant.

346 In addition to positions 108 and 163, the opossum differs from the human AT_1R at
347 positions 107, 192, and 198. S107 (3.25) in the rat $AT_{1A}R$, which is identical to the human, has
348 been shown to contribute weakly to ARB binding (17). In the opossum AT_1R, position 107 is a
349 Gly and thus may contribute to the lack of ARB binding. Positions 192 and 198 were previously
350 mutated to Met and Ala, respectively, in the rat $AT_{1A}R$, and each alteration resulted in
351 approximately two-fold reduction in ARB affinity for the receptor when singly mutated (17).

The human AT_1R contains P192 and T198, whereas the opossum contains L192 and S198. Substituting a Leu for Pro is not as dramatic as Met, but the loss of a Pro most likely changes the shape of the binding pocket. Substituting a Ser for a Thr likely has little effect on ARB binding. Thus, in the opossum the presence of L192 likely contributes to the effects of I108 and V163 on ARB affinity, while G107 and S198 are unlikely to greatly alter ARB binding.

Many investigators have modeled the AT_1R based on mutagenesis studies and/or the crystal structure of rhodopsin. We propose an alternative model that is largely based on the recent β2-AR crystal structure (32). Although two-dimensional modeling of the α-helixes fails to demonstrate the greater tertiary structure, it does provide structural insight into the general shape of the receptor. Four points are unique in this model: the broken transmembrane I (TMI) α-helix, the small α-helix in intracellular loop 2 (ICL2), the incomplete α-helix found in TMIII and TMVII, as well as the α-helix in ECL2. As this model was prepared with a view toward the β2-AR crystal structure, this approach has identified some of the structures that have been lost in standard models. Importantly, it is known that TMVII is adjacent to TMII, as is denoted by the disulfide bond, and this change in orientation also moves cysteines that form the second disulfide bond between the α-helix of TMIII and ECL2 into closer proximity. However, for this last disulfide bond to form, the ECL2 α-helix will have to lay across the receptor like a lid, as seen in the β2-AR. Lastly, TMV will likely transverse the membrane at an angle.

Collectively, the current and prior studies bring to light the importance of AT_1R polymorphisms in the human population that are not responsive to the therapeutic effects of ARBs. Most studies focus on unraveling a link between AT_1R polymorphisms and high blood pressure and other cardiovascular diseases, while there has been very little exploration for polymorphisms conferring resistance to ARBs. In this context, our finding that ARB resistance

can be traced to natural variations from the canonical mammalian sequence within the ARB binding pocket is particularly relevant. Hence, it is tempting to speculate that humans that are resistant to ARBs may harbor these or other variations in their AT_1R gene that may predispose ARB resistance (10; 37). A systematic analysis of the agtr1 gene should be conducted to determine: if there are polymorphisms that confer ARB resistance and clinically relevant, which amino acids are altered in the AT_1R, and what percentages of the population carry these polymorphisms (Supplemental Table 2).

ARBs have become the mainstay in treatment of high blood pressure, more so in the context of reduction in cardiovascular risk and slowing progression of chronic kidney disease. Importantly, the National Kidney Foundation and the American Heart Association have issued Grade A recommendation for the use of ARBs for reduction in proteinuria in type 2 diabetes, hypertension and cardiovascular disease (1). It has been well known over the past several years that ARBs are not equipotent in all patients; yet, ARBs have proven superior to other blood pressure lowering medications when it comes to cardiovascular risk reduction in the aforementioned patients. It follows then that ARB resistance can be a major problem in the effective therapeutic management of this group of patients.

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Figure Legends

Figure 1

Human AT_1R model. The alpha-helical regions are denoted by a twist in the structure and a green arrow denoting the direction of the helix; Cysteine disulfide bonds were modeled as presented previously (21) by blue lines with an orange shaded S. Red residues denote involvement in Ang II binding, blue residues denote involvement in ARB binding, orange residues indicate that the residue has minor contributions to Ang II binding, and yellow residues indicate that the residue has minor or questionable contributions to ARB binding. Overlapping yellow and orange shaded circles indicate both ARB and Ang II binding to a residue. White lettering depict notable differences between the human and opossum sequences, and the numbers indicate the most conserved residue according to the Ballesteros-Weinstein system (5).

Figure 2

AT_1R expression in native OKP cells. RT-PCR with opossum specific AT_1R primers generated a 250bp cDNA product as anticipated. RT+ indicates reverse transcriptase, RT- indicates the absence of reverse transcriptase, and water only indicates no template control.

Figure 3

Weblogo representation of Ang II and AT_1R binding pockets. A) The human and opossum Ang II binding pocket is compared to all vertebrates (top) and only mammals (bottom). The human and opossum sequences are listed above the weblogos. B) The human and opossum ARB binding pocket is compared to all vertebrates (top) and only mammals (bottom). For clarity, the scale for the Y-axis is hidden.

Figure 4

The phylogenetic tree for the AT_1R follows known patters of species evolution. As expected the opossum is distant from the human and related to the platypus. The cladogram was developed by using a neighborhood prodist tool with no root set.

Figure 5

Ang II signals in native OKP cells, but is not inhibited by ARBs. A) 24hr serum-starved native OKP and T35OK-AT_1R cells were treated with Ang II (10^{-7} M) or olmesartan (10^{-6} M) in the combinations shown and phosphorylation of Jak2 expressed as a ratio to total Jak2. Asterisk (*) indicates that the bar is significantly different than control by Kruskal-Wallis Z Post-hoc test, $n \leq 8$ for OKP, $n \leq 5$ for T35OK-AT_1R, $p<0.05$ For the picture, samples from native OKP and T35OK-AT_1R were run on the same gel but were cut and placed next to each other to mimic the sequence on the graph. Band intensities are as on original blot. B) Five different ARBs (losartan, valsartan, irbesartan, azilsartan and olmesartan) at 10^{-6} M fail to inhibit 10^{-7} M Ang II-mediated phosphorylation of Jak2. The asterisk (*) denotes the only group significantly different from Ang II by Kruskal-Wallis Z Post-hoc test, $n = 4$, $p<0.05$.

Figure 6

Examination of Ang II and ARB binding sites on non-polarized native OKP and T35OK-AT_1R PTCs. **A**) ^{125}I-Ang II saturation binding (Bmax) was calculated based on the number of cpm's per cell and converted to the number of molecules per cell, n=4. **B**) Non-radioactive Ang II competition of ^{125}I-Ang II binding to native OKP and T35OK- AT_1R cells. Native OKP cells are

depicted in black circles and T35OK- AT_1R cells are in gray squares, n =4. **C**) Olmesartan competition of ^{125}I-Ang II binding on native OKP and T35OK-AT_1R cells. H represents ^{125}I-Ang II alone (control), H+C represents ^{125}I-Ang II plus cold Ang II (positive control), and H+O represents ^{125}I-Ang II plus 10^{-6} M olmesartan (experimental). Bars with asterisk (*) are significantly different than ^{125}I-Ang II alone (H) as determined by a Tukey-Kramer post-hoc test, n = 4, $p<0.05$ **D**) The AT_2R antagonist PD123319 did not compete for ^{125}I-Ang II binding to OKP and T35OK- AT_1R cells. The graphical notation is the same as in A, $n \geq 3$.

Figure 7

Examination of Ang II and ARB binding sites on polarized native OKP cells. ^{125}I-Ang II (0.05 X 10^{-9} M) treatment of PTCs in transwell inserts (apical and basolateral) for 30min after blockade with olmesartan for 1 hr. CPM's were counted after removal of surface bound ^{125}I-Ang II. Bars with different symbols (Greek letters) are significantly different as determined by a Tukey-Kramer post-hoc test, n = 4, $p<0.05$ As in figure 6, H represents ^{125}I-Ang II alone (control) and H+O represents ^{125}I-Ang II plus 10^{-6} M olmesartan.

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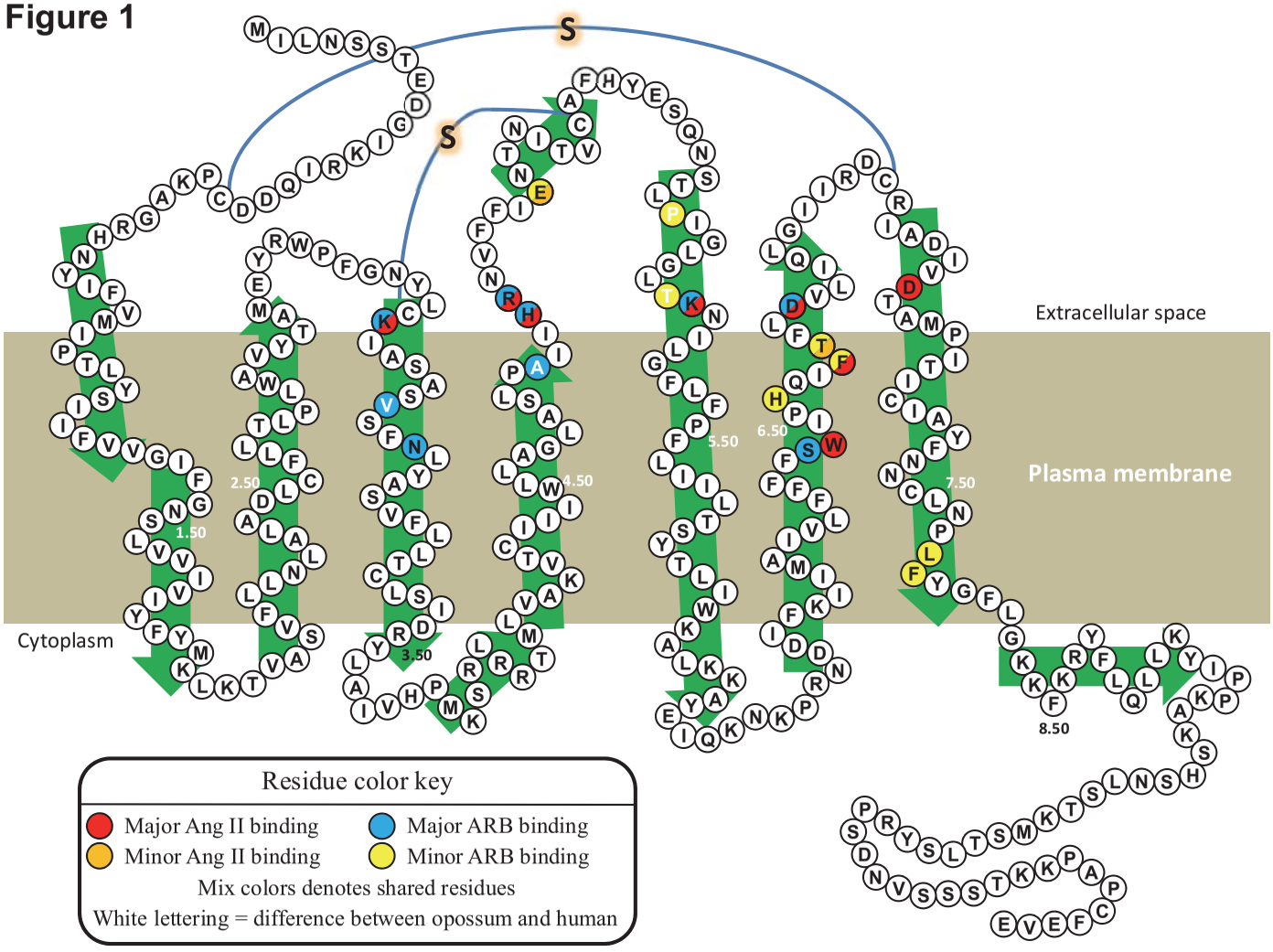
Figure 1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S
Extracellular space
Plasma membrane
Cytoplasm
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2.50
3.50
4.50
5.50
6.50
7.50
8.50
Residue color key
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Minor Ang II binding
Major ARB binding
Minor ARB binding
Mix colors denotes shared residues
White lettering = difference between opossum and human

Figure 2

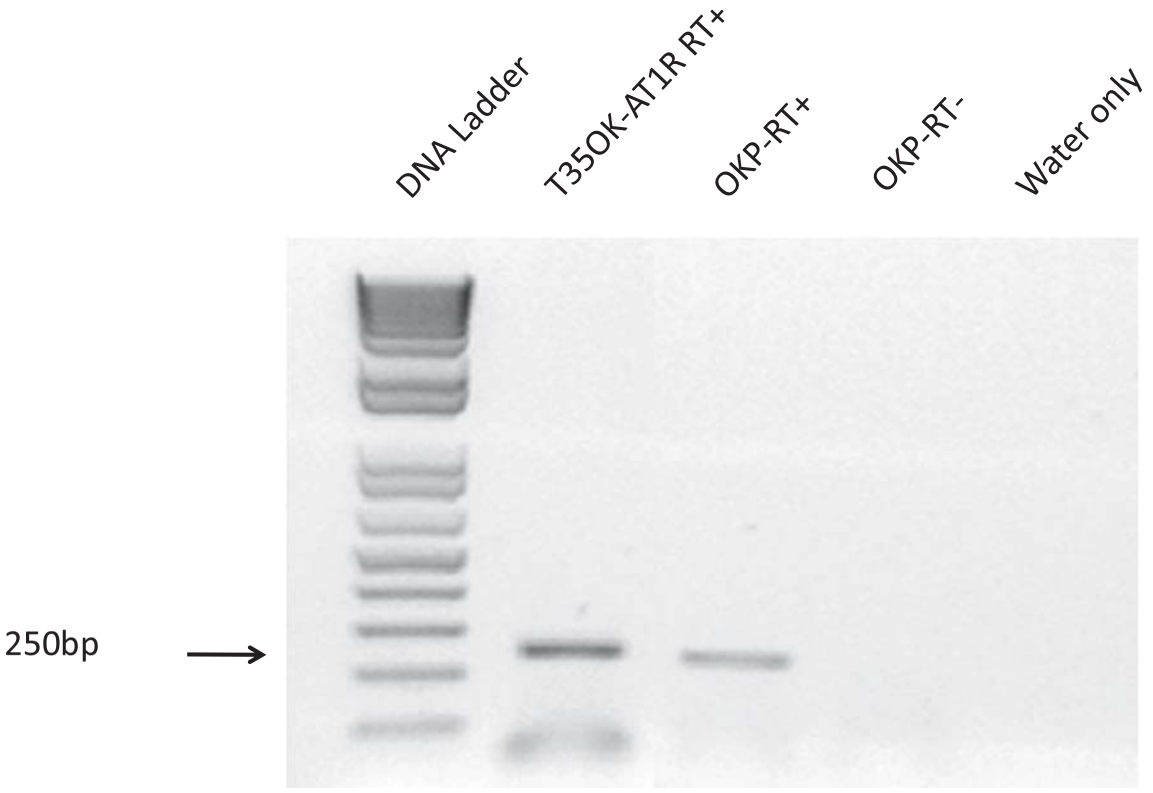
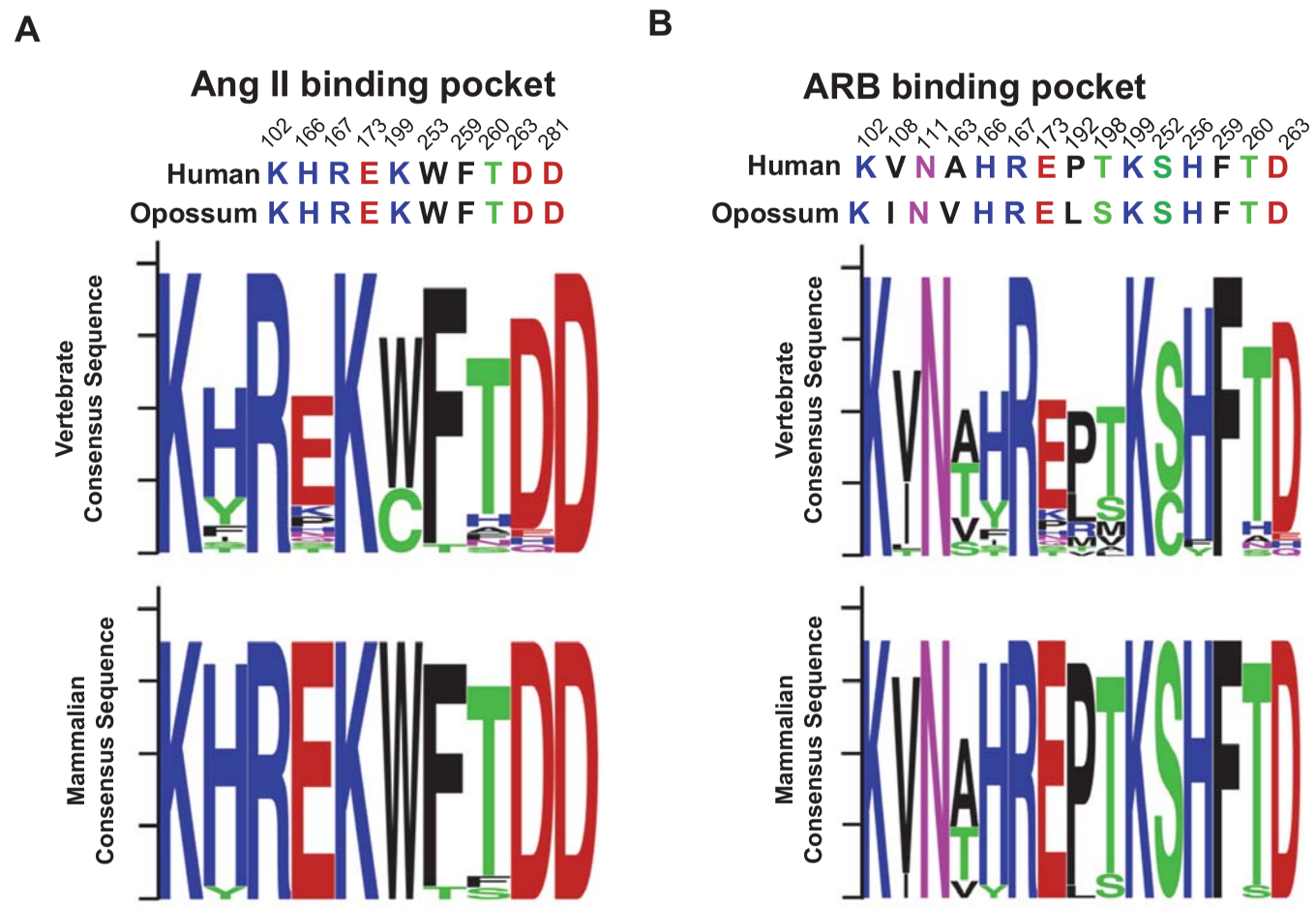


Figure 3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Ang II binding pocket
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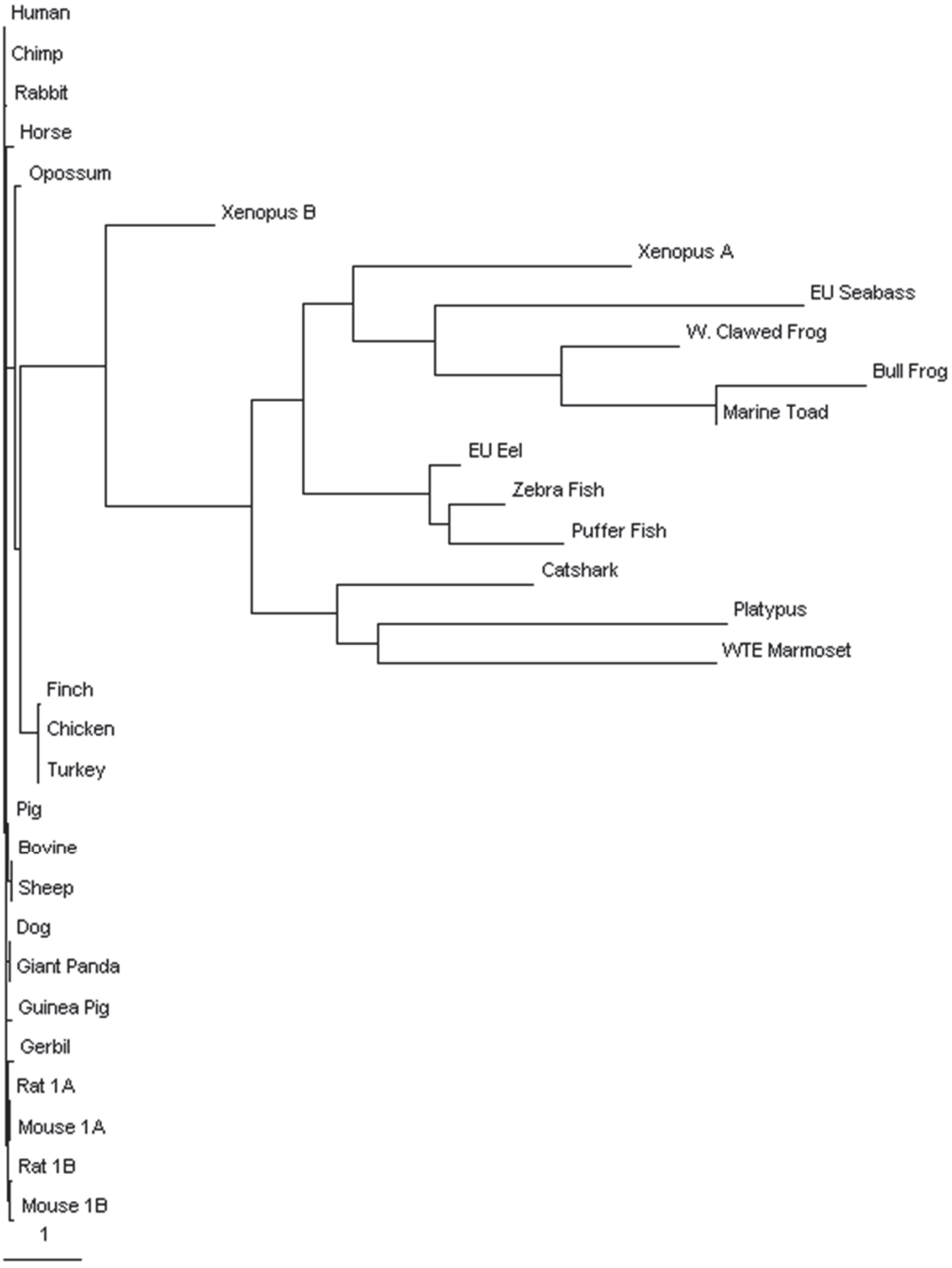
Opossum K H R E K W F T D D
Vertebrate Consensus Sequence
Mammalian Consensus Sequence
B
ARB binding pocket
102 108 111 163 166 167 173 192 198 199 252 256 259 260 263
Human K V N A H R E P T K S H F T D
Opossum K I N V H R E L S K S H F T D
Vertebrate Consensus Sequence
Mammalian Consensus Sequence

Figure 4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Rabbit
Horse
Opossum
Xenopus B
Xenopus A
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Bull Frog
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Puffer Fish
Catshark
Platypus
WTE Marmoset
Finch
Chicken
Turkey
Pig
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Dog
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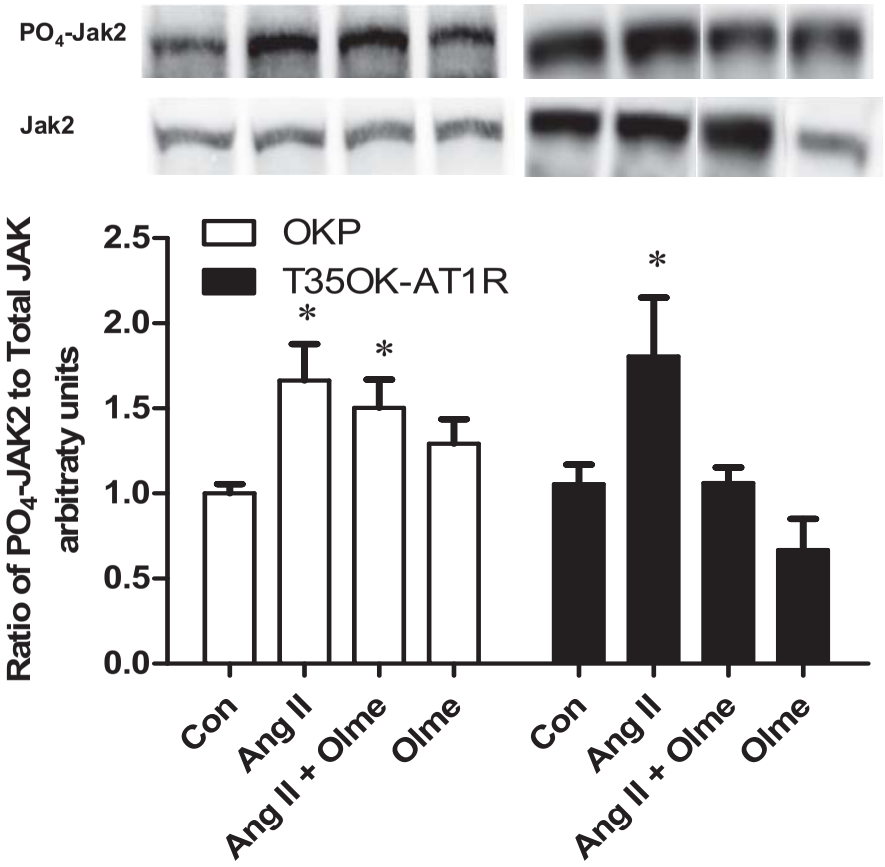
Guinea Pig
Gerbil
Rat 1A
Mouse 1A
Rat 1B
Mouse 1B
1

Figure 5A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OKP
T35OK-AT1R
Ratio of PO4-JAK2 to Total JAK
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2.0
1.5
1.0
0.5
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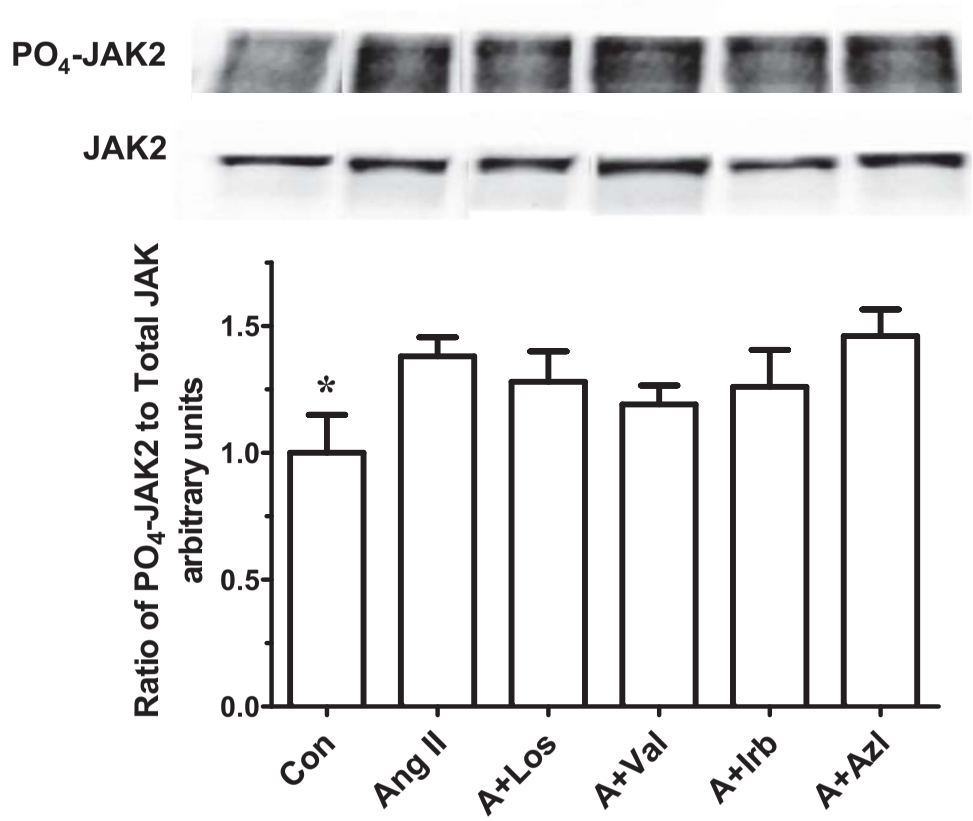
*
*
*
Con
Ang II
Ang II + Olme
Olme
Con
Ang II
Ang II + Olme
Olme

Figure 5B


PO4-JAK2
JAK2
Ratio of PO4-JAK2 to Total JAK
arbitrary units
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1.0
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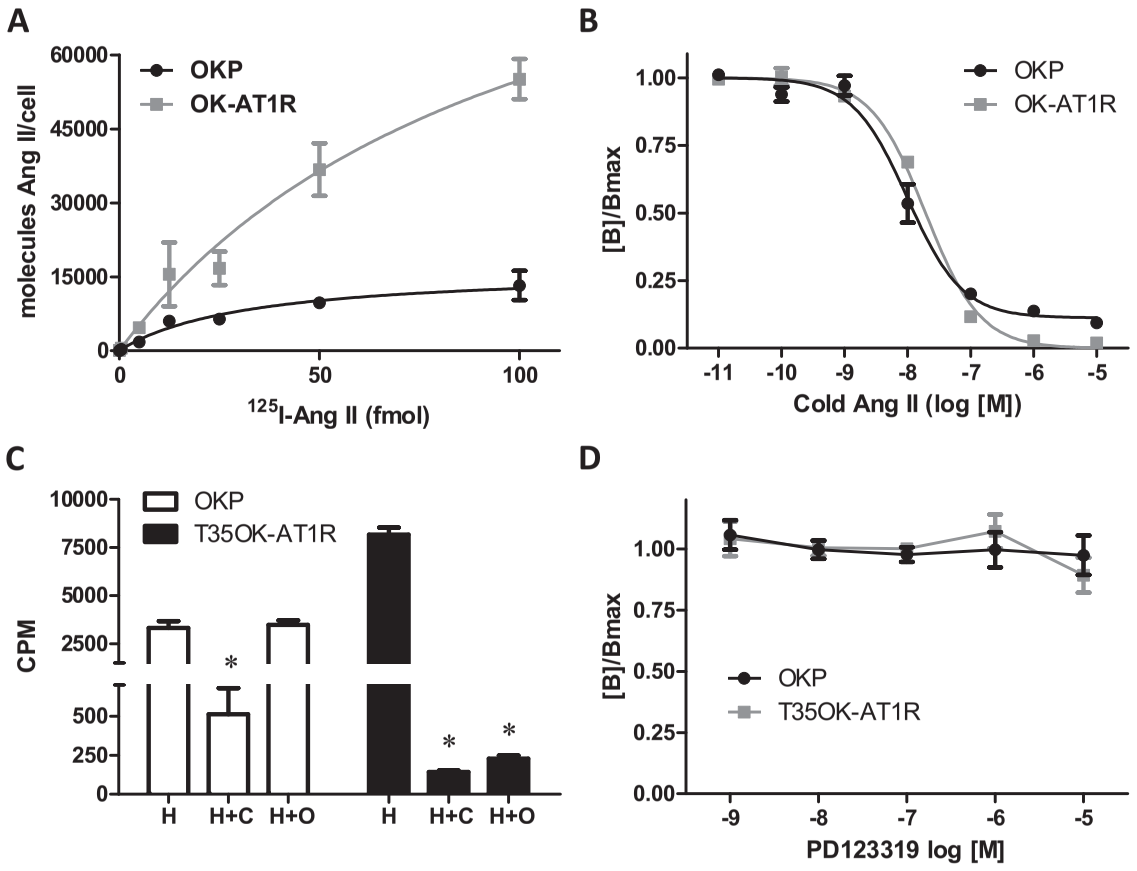
*
Con
Ang II
A+Los
A+Val
A+Irb
A+Azl

Figure 6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45000
30000
15000
0
molecules Ang II/cell
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OK-AT1R
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50
100
125I-Ang II (fmol)
B
1.00
0.75
0.50
0.25
0.00
[B]/Bmax
OKP
OK-AT1R
-11
-10
-9
-8
-7
-6
-5
Cold Ang II (log [M])
C
10000
7500
5000
2500
500
250
0
CPM
OKP
T35OK-AT1R
*
*
*
H
H+C
H+O
H
H+C
H+O
D
1.00
0.75
0.50
0.25
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[B]/Bmax
OKP
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PD123319 log [M]

Figure 7