Supplementary Discussion.

Mutagenesis of the ligand-binding pocket validates crystal structures

Point mutations were introduced in 17 residues of the AT_2R ligand-binding pocket and their effects on ligand affinity were assessed for [^{3}H]AngII peptide, as well as for the small molecule Cpds **1** and **2** in [^{3}H]AngII competition assays (Extended Data Fig. 8).

As expected from its anchor role in the pocket, Arg182^{ECL2} proved critical for both peptide and small molecule ligand binding. Mutation Arg182^{ECL2}Ala completely abolished binding of [^{3}H]AngII, precluding measurement of the affinities for Cpds **1** and **2**. However, mutation Arg182^{ECL2}Lys retained full binding of [^{3}H]AngII, but resulted in more than 20-fold reduced binding of either of the two small molecules. The difference between effects of this mutation on peptide and small molecule binding can be explained by the importance of the Arg182^{ECL2} guanidine moiety that forms both an ionic interaction with the tetrazole groups of Cpds **1** and **2**, as well as a stacking interaction with the benzene rings of these ligands. Mutation Arg182^{ECL2}Lys eliminates this stacking interaction, leading to a drastic drop in affinity of both Cpds **1** and **2**. Similarly, mutations of Lys215$^{5.42}$ to alanine or glutamine in AT_2R completely abolished binding of [^{3}H]AngII, precluding measurement of the Cpds **1** and **2** affinities. Interestingly, Lys199$^{5.42}$Ala in AT_1R also greatly affected AngII binding, but had only limited effect on olmesartan binding[25]. Structural analysis suggests that Lys$^{5.42}$ plays a more important role in ligand binding in AT_2R, because it is located closer to the binding pocket than in AT_1R, and forms an ionic interaction with the tetrazole moiety of both Cpds **1** and **2**.

Most mutations of the remaining 15 residues in the AT_2R pocket showed significantly reduced affinity (10-100 folds) for the small molecule Cpds **1** and **2**, while having modest, if any, effect on the AngII peptide affinity. Thus, Trp100$^{2.60}$Ala and Tyr51$^{1.39}$Ala mutations led to a 15-60 folds reduced affinity of Cpds **1** and **2**, while replacement of the corresponding residues in AT_1R with aliphatic amino acids completely abolished binding of olmesartan. Note that in AT_1R these two residues are critical for sartan binding

(Trp84$^{2.60}$ forms π-π interactions and Tyr35$^{1.39}$ forms hydrogen bonds with ligands), while in AT_2R their role is limited to van der Waals contacts. Residues Ile211$^{5.38}$, Met214$^{5.41}$ and Phe129$^{3.37}$ form hydrophobic interactions with the biphenyl moiety, and substitutions of each of them with alanine reduced binding affinity of Cpds **1** and **2** by greater than 10-fold. In the other part of the pocket, mutation Ile304$^{7.39}$Ala resulted in 28-100 folds reduced affinity of Cpds **1** and **2**, due to reduced interactions with the quinazolinone cores of the ligands. In the bottom part of the binding pocket, Phe308$^{7.43}$ forms hydrophobic interactions with the quinazolinone cores of Cpds **1** and **2**, and mutation of this residue to alanine decreased binding affinity by 15-100 folds. Interestingly, the corresponding mutation Tyr292$^{7.43}$Ala in AT_1R had a reverse effect leading to a 2-3 folds increase in affinity for AngII and olmesartan. Finally, mutations in residues Tyr103$^{2.63}$, Tyr104$^{2.64}$ and Tyr108^{ECL1}, which interact with the benzene and thiophene/furan rings of Cpds **1** and **2**, led to 3-15 folds decreased affinity, while, most sartans do not interact with the corresponding residues in AT_1R.

SAR provides further insights into receptor selectivity

To decipher the structural basis of SAR observations (Extended Data Table 2**)**, we performed unrestrained docking of all 14 members from the SAR dataset into both AT_2R and AT_1R crystal structures (Fig. 4). We found that the SAR ligand scaffolds consistently adopt binding poses almost identical to the scaffolds of the ligands in the crystal structures of the corresponding receptor. Interestingly, the distinct quinazolinone portion of the scaffold in AT_1R occupies a similar subpocket as the aromatic rings of olmesartan and ZD7155, also forming hydrogen bonds with Arg167^{ECL2} and Tyr35$^{1.39}$. Importantly, binding of the biphenyltetrazole-quinazolinone scaffold to AT_1R requires not only the reorientation of the biphenyltetrazole, but also a dramatic flip of the quinazolinone via a rotamer switch (Fig. 4). This conformational difference appears to stem from the different shapes of the AT_2R and AT_1R binding pockets. While AT_2R has a more compact pocket, located deeper in the receptor, the AT_1R binding site is more extended and shifted closer to ECL2.

Despite the dramatic differences in the ligand-binding poses between AT_2R and AT_1R, the docking results provided important insights into the structural basis of SAR observations. Thus, the n-propyl group in $\mathbf{R_1}$ position optimally fills hydrophobic sub-pockets in each receptor, even though these sub-pockets are formed by different residues. Interestingly, docking of the SAR ligands to AT_1R suggests that while the optimal n-propyl moiety helps to stabilize the ligand-binding position and to maintain quinazolinone hydrogen bonding with $Arg167^{ECL2}$ and $Tyr35^{1.39}$, smaller $\mathbf{R_1}$ substituents (ethyl and methyl) result in a ~1-2 Å lateral shift in the scaffold docking position, suboptimal hydrogen bonding and consequently in a reduced affinity. In contrast, in AT_2R, smaller $\mathbf{R_1}$ substituents do not affect the scaffold binding pose; likely because the quinazolinone core does not form hydrogen bonds with the AT_2R receptor and thus is more tolerant to minor shifts.

For the series of $\mathbf{R_2}$ substituents, their docking positions and interactions with AT_1R and AT_2R also explain the observed SAR. In AT_2R, each of the $\mathbf{R_2}$ aromatic substituents tightly binds to a distinct hydrophobic sub-pocket, adding significant contributions to the overall ligand binding score. This can explain a high sensitivity of the AT_2R affinity to the reduced size of any of the $\mathbf{R_2}$ substituents (Cpds **8**-**14**). Moreover, given that the secondary amide in Cpd **9** prefers a trans-conformation of the amide bond in the ligand-free state, and since a cis-conformation of the amide is required to fit in the binding pocket, the resulting strain likely contributes to the severely reduced affinity. In contrast, AT_1R does not have individual subpockets for the two $\mathbf{R_2}$ substituents, making it less sensitive to variations, and it can accommodate the bulky $\mathbf{R_2}$ substituents in the top solvent exposed part of the binding pocket.