The angiotensin II receptors AT$_1$R and AT$_2$R serve as key components of the renin–angiotensin–aldosterone system. AT$_1$R has a central role in the regulation of blood pressure, but the function of AT$_2$R is unclear and it has a variety of reported effects. To identify the mechanisms that underlie the differences in function and ligand selectivity between these receptors, we report crystal structures of human AT$_2$R bound to an AT$_2$R-selective ligand and to an AT$_1$R/AT$_2$R dual ligand, capturing the receptor in an active-like conformation. Unexpectedly, helix VIII was found in a non-canonical position, stabilizing the active-like state, but at the same time preventing the recruitment of G proteins or β3-arrestins, in agreement with the lack of signalling responses in standard cellular assays. Structure–activity relationship, docking and mutagenesis studies revealed the crucial interactions for ligand binding and selectivity. Our results thus provide insights into the structural basis of the distinct functions of the angiotensin receptors, and may guide the design of new selective ligands.

In humans, the effects of the octapeptide hormone angiotensin II are mediated by two types of receptor, AT$_1$R and AT$_2$R, which share approximately 34% amino acid sequence identity. AT$_1$R is mainly responsible for blood pressure regulation, with several antagonists and inverse agonists approved for clinical use as anti-hypertensive drugs. By contrast, the function of AT$_2$R is less well understood and remains controversial, with a growing number of studies suggesting that AT$_2$R signals primarily via non-canonical, G-protein- and β3-arrestin-independent pathways. In the cardiovascular system, AT$_2$R has been reported to counteract several of the effects mediated by AT$_1$R (refs 6, 7), conferring cardioprotection. For example, in the vascular system, AT$_2$R has been suggested to counter-balance blood pressure increases exerted by AT$_1$R (refs 8, 9). It has also been reported that activation of AT$_2$R in cardiomyocytes inhibits autophagy mediated by AT$_1$R (ref. 10). Moreover, in the central nervous system, AT$_2$R has been implicated in AT$_1$R-independent signalling pathways. It has been observed that activation of AT$_2$R in nociceptive neurons induces neurite outgrowth and elongation, and studies in sensory neurons selectively expressing AT$_1$R but not AT$_2$R also support the involvement of AT$_2$R in nociception. Further interest in AT$_2$R as a drug target has been sparked by the recent finding that the canonical AT$_2$R antagonist PD123319 blocks angiotensin-II-induced neuronal excitability, and exhibits oral bioavailability in several neuropathic pain models in rodents. Most excitingly, EMA401, an analogue of PD123319, demonstrated efficacy in phase II clinical trials in patients with post-herpetic neuralgia. Notably, activation of AT$_2$R by its small-molecule agonist compound 21 has also been reported to produce several beneficial in vivo effects, such as inducing pressure natriuresis, lowering blood pressure, conferring acute vasorelaxation, and exhibiting organ-protective effects.

We previously reported the crystal structures of AT$_1$R in complex with an antagonist ZD7155 (ref. 24) and with an inverse agonist olmesartan, and these provided insights into AT$_1$R-receptor–ligand interactions. The molecular mechanisms of functional diversity and ligand selectivity between AT$_1$R and AT$_2$R, however, remain elusive. With an increasing body of evidence indicating that selective targeting of AT$_1$R could be used for cardioprotection and for the treatment of neuropathic pain, there is an imminent need to determine the structural basis for the functional role of this receptor, and to develop type-selective ligands. In this study, we determined crystal structures of AT$_2$R bound to two high-affinity ligands, an AT$_2$R-selective ligand compound 1 (ref. 26) and an AT$_1$R/AT$_2$R dual ligand compound 2 (ref. 27). Both compounds are derivatives of a series of small-molecule antagonists of AT$_1$R, and thus could be assumed to exert the same function in AT$_2$R. However, classification of compounds using traditional G-protein-coupled receptor (GPCR) nomenclature into antagonists or agonists has proven difficult for AT$_1$R since it has not been reliably demonstrated to signal through any of the canonical GPCR signalling pathways involving G proteins or β3-arrestins. Furthermore, it has been observed numerous times that small changes to the structures of closely related compounds can lead to changes or bias in function. Therefore, we refer to compounds 1 and 2 using the neutral term ‘ligand’. In addition to a marked reshaping of the ligand-binding pocket between the two receptors, our AT$_2$R structures revealed an active-like conformation of the seven-transmembrane (7TM) helical bundle with a non-canonical positioning of the amphipathic helix VIII, potentially blocking recruitment of intracellular signalling partners.
Active–like conformation of 7TM bundle

The overall architecture of AT2R is comprised of a 7TM bundle (helices I–VII) and an intracellular amphipathic helix VIII (Fig. 1a). Similar to AT1R and other peptide-binding GPCRs, AT2R exhibits a β-hairpin conformation of the extracellular loop 2 (ECL2) and two pairs of disulfide bonds linking the N terminus with ECL3 (Cys35N-term–Cys290ECL3), and helix III with ECL2 (Cys117–Cys195ECL2); superscripts indicate residue numbers as per the Ballesteros–Weinstein nomenclature. A closer comparison of the AT1R and AT2R structures, however, revealed substantially different conformations (Fig. 1b–e). Although the previously determined AT1R structures captured the receptor in a classical inactive state, all three AT2R structures, obtained in this work, display an active-like conformation. Specifically, the intracellular end of helix VI shows an outward displacement by approximately 11.5 Å, whereas the intracellular end of helix VII exhibits an inward displacement by 4.9 Å, as compared to the structures of inactive AT1R (Fig. 1e). Similar large-scale shifts of helices VI and VII that open an intracellular cleft for the recruitment of G proteins and β-arrestins have been implicated in the activation of all class A GPCRs. Another major conformational rearrangement occurs at the extracellular end of helix V, which is shifted towards the ligand-binding pocket by around 4.8 Å, compared to AT1R (Fig. 1d). Similar shifts of the extracellular part of helix V, although typically with a smaller 2 Å amplitude, have been observed in structures of several activated GPCRs, such as the β2-adrenergic (β2AR) and serotonin 5-HT2A receptors. Such agonist-stabilized displacement of helix V is essential for triggering re-arrangements in the P3–P4 motif, leading to activation of these receptors.

The large-scale re-arrangements of helices during activation are accompanied by conformational changes in the conserved microswitches. We therefore compared the DR3.50Y, NP7.50XXY and P5.50-I3.40-P6.44 motifs of AT2R with those in the structures of inactive AT1R and of fully activated GPCRs, such as Gαq-protein-bound β2AR and arrestin-bound rhodopsin. The large-scale movement of helix VI upon activation is enabled by re-arrangements in the P5.50-I3.40-P6.44 motif (Pro223.50–Ile132.40–Phe265.44 in AT2R), as it was previously demonstrated in several structures of activated GPCRs (Fig. 1c). In the DR3.50Y motif, the Arg142.30 side chain of AT2R is rotated by around 90° compared to Arg126.30 in AT1R, adopting a similar conformation to other fully activated GPCRs. Additionally, in the NP7.50XXY motif, the side chain of Tyr318.53 is shifted by 6.5 Å and rotated by 45° from the corresponding position of Tyr302.53 in AT1R, following the inward movement of helix VII, as observed in other receptors upon activation. Finally, analysis of the conserved residue switch in the G-protein-binding pocket L[M]3.46–I[A]6.37–Y[Y]7.53 (AT1R residues are shown in brackets) confirms the rearrangement of interactions in this residue triad consistent with an active-like state of AT1R (Extended Data Fig. 4a, b). Notably, instead of a highly conserved large hydrophobic residue in position 6.37, AT2R has a rare alanine residue (~3% of class A GPCRs), which markedly reduces the hydrophobic contact area between helices III and VI in the inactive state, and probably facilitates the activation-related changes in AT2R. Therefore, all of the major conformational features indicate that the 7TM bundle of AT2R adopts an active-like conformation, similar to that observed in the crystal structures of other fully activated class A GPCRs bound to signalling partners or their mimics.

In addition to the conserved microswitches, a sodium-binding site consisting of 16 highly conserved residues is expected to undergo large-scale conformational changes upon activation of class A GPCRs. Superposition of AT2R with AT1R revealed that the putative sodium-binding pocket in AT2R is collapsed and rearranged, hindering sodium ion binding (Extended Data Fig. 4c, d), mainly owing to the inward shift of helix VII, which is consistent with the structures of other activated GPCRs. Only 2 out of 16 residues in the putative sodium pocket (Ile132.43 and Ser3117.46) are different from their counterparts in AT1R (Leu119.44 and Asn295.46). Notably,
mutation of Asn295 to Ala was implicated in the AT1R constitutive activation\textsuperscript{45,46}, potentially owing to the disruption of two hydrogen bonds between Asn295 and Asn11\textsuperscript{3,35} that stabilize the inactive conformation. By contrast, Ser311 in AT2R cannot engage in a similar interaction with Asn1273\textsuperscript{3,35}, potentially shifting the conformational equilibrium towards the active state. Indeed, AT2R has been reported to have high constitutive activity, and to induce apoptosis even in the absence of angiotensin II stimulation\textsuperscript{43,44}, potentially owing to the disruption of two hydrogen bonds between Asn295 and Asn11\textsuperscript{3,35} that stabilize the inactive conformation.

**Helix VIII blocks G protein and β-arrestin binding**

In most GPCR structures, helix VIII lies parallel to the membrane pointing outside of the 7TM bundle\textsuperscript{24}, regardless of the activation state of the receptor (Fig. 2a, b). Surprisingly, in the AT2R structures, helix VIII adopts a very different conformation by flipping over to interact with the intracellular ends of helices III, V and VI (Fig. 2c). This non-canonical conformation of helix VIII was found in all AT2R structures determined in this study, regardless of the ligand identity and different crystal packing environments (Extended Data Fig. 3c–e), suggesting that it is likely to be a genuine feature of AT2R, rather than an artefact of crystallization. Helix VIII forms a highly complementary interface with the intracellular cavity of the 7TM bundle, and is stabilized by extensive hydrophobic interactions mediated by Phe325\textsuperscript{8,50}, Leu329\textsuperscript{8,54}, Val332\textsuperscript{8,57} and Phe333\textsuperscript{8,58}, as well as by polar interactions between Arg324\textsuperscript{9,49}, Gln326\textsuperscript{8,51} and Lys328\textsuperscript{8,53}, and helices III, V and VI (Fig. 2d). Docking and molecular dynamics simulations suggest that helix VIII stabilizes the active-like conformation of the 7TM bundle of AT2R, while sterically blocking the binding of G proteins and β-arrestins (Fig. 2c), which is consistent with the lack of robust downstream signalling by AT2R as assessed by traditional G protein and β-arrestin assays\textsuperscript{45,46}. In molecular dynamics simulations, helix VIII not only remained in the range of positions within r.m.s.d. values of less than 4 Å to the crystallographic structure for a total of 4 μs of unbiased simulation (Extended Data Fig. 5a–c), but also quickly (<200 ns) and reproducibly (n = 4) returned to this conformation after consequent perturbations of its position (Extended Data Fig. 5d, e). Alternatively, when helix VIII was relocated to the position observed in the AT2R structures, it relaxed into a canonical membrane-bound conformation, and this motion was accompanied by an inward shift of the intracellular tip of helix VI towards its position in the inactive state AT3R structure in three out of six independent molecular dynamics runs (Extended Data Fig. 5f–h).

**Insights into ligand selectivity of AT1R and AT2R**

Many AT1R and AT2R ligands share a biphenyl-tetrazole scaffold, important for ligand binding affinity. However, the molecular mechanisms of ligand selectivity between the two types of angiotensin receptor remain elusive. Our radioligand competition binding studies obtained inhibition constant (K\textsubscript{i}) values of 3.7 nM and 0.35 nM for binding to AT1R and AT2R, respectively, indicating that this compound is a dual ligand with only about tenfold selectivity for AT1R over AT2R. By contrast, compound 1 exhibited an approximately 530-fold selectivity towards AT2R, with K\textsubscript{i} values of 180 nM and 0.34 nM for AT1R and AT2R, respectively (Extended Data Fig. 2c, e). In all three AT2R structures, strong electron density was observed in the orthosteric ligand-binding pocket, enabling accurate placement of both compounds 1 and 2 (Extended Data Fig. 6). We compared...
the positions and interactions of these compounds bound to AT1R with two ligands bound to AT1R (the AT1R-selective antagonist ZD7155, and the AT1R-selective inverse agonist olmesartan) (Fig. 3).

Surprisingly, despite the common scaffold, the ligands bind to the two receptor types very differently, with only the tetrazole moieties partially overlapping and forming crucial hydrogen bonds with an arginine in ECL2 (Arg167ECL2 in AT1R and Arg182ECL2 in AT2R). The overall biphenyl-tetrazole scaffold of the AT2R-bound ligands, however, is rotated about 45° as compared to its orientation in the AT1R-bound ligands, forming a distinct interaction pattern (Fig. 3a). Notably, ArgECL2, Tyr1.39 and Trp2.60, previously identified as key residues for ligand binding in AT1R, also provide crucial protein–ligand contacts in AT2R, although with different side-chain conformers. The side chain of Trp100.60 in AT1R is shifted about 3.1 Å compared to Trp84.60 in AT2R to form hydrophobic interactions with the thiophene ring of compound 1 in magenta, compound 2 in yellow, the AT2R ligands ZD7155 in orange, and olmesartan in light blue.

Ligand–binding pocket mutations validate structures

Point mutations were introduced in 17 residues of the AT1R ligand-binding pocket, and their effects on ligand affinity were assessed for [3H]angiotensin II peptide, as well as for the small-molecule compounds 1 and 2 in [3H]angiotensin II competition assays (Supplementary Discussion and Extended Data Fig. 8).

Overall, the effects of mutations in the binding pocket of AT1R are consistent with the receptor–ligand interactions observed in the co-crystal structures with compounds 1 and 2. Most of these effects are also distinct from the effects in AT1R–sartan complexes, corroborating the substantial differences in the ligand binding modes between AT2R and AT1R. Note that the effects of binding pocket mutations are practically identical for compounds 1 and 2, with the only notable exception of Trp269.48Phe modestly (around fivefold) affecting the affinity of compound 1, but not of compound 2.

SAR provides insights into receptor selectivity

The structure–activity relationship (SAR) of compound 1 analogues was studied using derivatization of the 3- (R1) and 7- (R2) positions of the quinazolinone core (Extended Data Table 2), and its structural basis was analysed by molecular docking to the AT1R and AT2R crystal structures (Fig. 4). The SAR dataset comprised a series of 14 compounds spanning a wide range of selectivity for the two receptor types. Two members of the series were found to be highly selective for AT1R (530-fold for compound 1 and 410-fold for compound 3), and two others showed a high degree of selectivity for AT1R (1,120-fold for compound 9 and 180-fold for compound 13). The remaining compounds exhibited moderate (<15-fold) to no selectivity.

The SAR data showed that the R1 substituent is crucial for high AT1R selectivity, whereas the R2 substituent mostly defines a high selectivity towards AT2R (Extended Data Table 2). Despite the notable differences in the pocket shapes and ligand-binding poses between AT1R and AT2R, the docking results provide important insights into the interaction and is shifted over together with a pronounced shift in the backbone of helix VII. This rearrangement opens a sub-pocket for the binding of the ethyl and propyl moieties of compounds 1 and 2. Overall, these changes result in different shapes of the ligand-binding pocket between the two angiotensin receptors and a deeper ligand binding in AT2R.

To gain additional insights into the selectivity of receptor types, we modelled an active-like state for AT1R and an inactive state for AT2R, and performed unrestrained cross-docking of ZD7155, olmesartan, and compounds 1 and 2 into the crystal structures and models of both receptors (Extended Data Fig. 7). The results suggest that although the conformational state of AT2R has little effect on the ligand binding affinity, the predicted active-like conformation of AT2R is not compatible with binding of any of the tested ligands, corroborating the status of these ligands as antagonists for AT1R. Furthermore, the binding modes of these ligands are imposed by the distinct shapes of the ligand binding pockets of the two receptors. Indeed, the biphenyl-tetrazole moieties of compounds 1 and 2 docked to the crystal structure of AT2R strongly preferred similar binding modes to those of olmesartan and ZD7155 (Extended Data Fig. 7a, b). The only differences were observed in the orientation of the benzene and heterocyclic rings of compounds 1 and 2, which occupy the top of the ligand-binding pocket, beyond the interaction site of olmesartan and ZD7155. Similarly, docking into the AT1R structure suggested that ZD7155 and olmesartan can fit in the AT1R pocket, with their biphenyl-tetrazole scaffolds closely following the scaffolds of compounds 1 and 2 in the corresponding AT2R crystal structures. Although docking supports binding of all four ligands in both the AT1R and AT2R crystal structures, the binding scores of the ligands vary considerably, reflecting different interactions of non-scaffold groups. The scores also qualitatively reflect differences in the affinities of these compounds, with the cognate ligands showing substantially better binding scores than the off-target binders.

Figure 3 | Ligand selectivity between AT1R and AT2R. a, Comparison of binding modes of different ligands in the binding pockets of AT1R (green) and AT2R (cyan). The side chains in contact with ligands are shown as sticks with labels representing AT1R and AT2R residues in the corresponding positions. b, c, Comparison of the ligand-binding pockets of AT1R (cyan) and AT2R (green). Receptors are shown in the same orientation of transmembrane helices with the conserved ArgECL2 (Arg167ECL2 in AT1R and Arg182ECL2 in AT2R) aligned. The ligands are shown as thick sticks, with carbon atoms of the AT1R ligands compound 1 in magenta, compound 2 in yellow, the AT2R ligands ZD7155 in orange, and olmesartan in light blue.
Extended Data Table 2 in the crystal structures of AT2R (a).

AT2R ligands share common scaffolds, the ligand-binding pockets of selectively bind to a specific receptor type is often challenging, but can and the treatment of several other conditions. Designing molecules that of AT2R could be useful for cardioprotection, neuropathic pain relief thus prolonging biological responses without desensitization 48,49.

The two types of the angiotensin II receptor are distinct in terms of their structural basis of SAR observations and directions for optimization of AT2R-selective ligands (see Supplementary Discussion).

Conclusions

The two types of the angiotensin II receptor are distinct in terms of their genetic variations, tissue-specific expression, signalling and regulation, as well as other physiological and pharmacological properties. 4,7,9

Although all of the conserved motifs of class A GPCRs are present in AT2R, it exhibits an atypical behaviour compared to other receptors. 1 Discerning the AT2R-specific signalling pathways has been challenging and remains unresolved to date. 3,5,9 The features revealed in the AT2R structures reported here might provide potential explanations for its poor coupling to G proteins and β-arrestins. On the basis of our results, we propose that helix VIII can play a dual role in the modulation of AT2R function. On the one hand, upon adopting a conformation captured in the crystal structures, helix VIII may stabilize an active-like receptor state, while repressing canonical AT2R activity in a self-inhibitory manner by sterically blocking the G protein and β-arrestin binding sites. On the other hand, upon switching to a membrane-bound conformation, helix VIII can support the recruitment of G proteins and β-arrestins for AT2R signalling. Therefore, helix VIII may work as a gatekeeper for either suppression or activation of the receptor depending on its post-translational modifications and interactions with various receptor partners and its environment. This hypothesis is consistent with the previously observed failure of AT2R to internalize, thus prolonging biological responses without desensitization. 48,49

Further investigation is needed to understand this phenomenon fully.

Both angiotensin II receptors are important drug targets, since the blockade of AT2R has anti-hypertensive effects, while the modulation of AT2R could be useful for cardioprotection, neuropathic pain relief and the treatment of several other conditions. Designing molecules that selectively bind to a specific receptor type is often challenging, but can be crucial for different therapeutic purposes. Although the AT2R and AT1R ligands share common scaffolds, the ligand-binding pockets of these two receptors are markedly different, and these differences could be exploited for designing selective ligands. The AT2R crystal structures determined in this study improve our understanding of the two types of the human angiotensin receptor and provide new insights into the structural basis for the binding and selectivity of small molecules of therapeutic significance. Our results are therefore expected to facilitate the rational structure-based drug design for improved selectivity.

Received 21 December 2016; accepted 3 March 2017. Published online 5 April 2017.

1. Karnik, S. S. et al. International Union of Basic and Clinical Pharmacology. XXIX. Angiotensin receptors: interpreters of pathophysiological angiotensinergic stimuli. Pharmacol. Rev. 67, 754–819 (2015).
2. Porrello, E. R., Delbridge, L. M. & Thomas, W. G. The angiotensin II type 2 receptor (AT2R): a challenging twin. Sci. STKE 2003, pe16 (2003).
3. Guimond, M. O. & Gallo-Payet, N. How does angiotensin AT2 receptor activation help neuronal differentiation and improve neuronal pathological situations? Front. Endocrinol. 3, 164 (2012).
4. Berk, B. C. Angiotensin type 2 receptor (AT2R): a challenging twin. Sci. STKE 2003, pe16 (2003).
5. Miura, S., Matsuo, Y., Kiyama, K., Naito, K. & Saku, K. Molecular mechanisms of the antagonistic action between AT1 and AT2 receptors. Biochem. Biophys. Res. Commun. 391, 85–90 (2010).
6. Hein, L., Barsht, G. S., Pratt, R. E., Dzau, V. J. & Kobilka, B. K. Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. Nature 377, 744–747 (1995).
7. Ichiki, T. et al. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. Nature 377, 748–750 (1995).
8. Porrello, E. R. et al. Angiotensin II type 2 receptor antagonizes angiotensin II type 1 receptor-mediated cardiomyocyte autophagy. Hypertension 53, 1032–1040 (2009).
9. Ruiz-Ortega, M. et al. Angiotensin II activates nuclear transcription factor kappaB through AT1 and AT2 in vascular smooth muscle cells: molecular mechanisms. Circ. Res. 86, 1266–1272 (2000).
10. Ruiz-Ortega, M., Lorenzo, O., Rupérez, M., Blanco, J. & Egido, J. Systemic infusion of angiotensin II into normal rats activates nuclear factor-kappaB and AP-1 in the kidney: role of AT1 and AT2 receptors. Am. J. Pathol. 158, 1743–1756 (2001).
11. Caballero, R. et al. Interaction of angiotensin II with the angiotensin type 2 receptor inhibits the cardiac transient outward potassium current. Cardiovasc. Res. 62, 86–95 (2004).
12. Zhao, Y. et al. Angiotensin II induces peroxisome proliferator-activated receptor gamma in PC12 cells via AT1 receptor type 2 activation receptor. J. Neurochem. 94, 1395–1401 (2005).
13. Guimond, M. O. & Gallo-Payet, N. Angiotensin II type 2 receptor in brain functions: an update. Int. J. Hypertens. 2012, 351758 (2012).
14. Anand, U. et al. Angiotensin II type 2 receptor (AT2R) localization and antagonist-mediated inhibition of capsaicin responses and neuropeptide outgrowth in human and rat sensory neurons. Eur. J. Pain 17, 1012–1026 (2013).
15. Smith, M. T., Woodruff, T. M., Wyse, B. D., Muralidharan, A. & Walther, T. A small molecule angiotensin II type 2 receptor (AT2R) antagonist produces analgesia in a rat model of neuropathic pain by inhibition of p38 mitogen-activated protein kinase (MAPK) and p44/42 MAPK activation in the dorsal root ganglia. Pain Med. 14, 1557–1568 (2013).
16. Smith, M. T., Wyse, B. D. & Edwards, S. R. Small molecule angiotensin II type 2 receptor (AT2R) antagonists as novel analgesics for neuropathic pain: comparative pharmacokinetics, radioligand binding, and efficacy in rats. Pain Med. 14, 692–703 (2015).
17. Smith, M. T., Lau, T., Wallace, V. C., Wyse, B. D. & Rice, A. S. Analgesic efficacy of small-molecule angiotensin II type 2 receptor antagonists in a rat model of antiretroviral toxic polyneuropathy. Behav. Pharmacol. 25, 137–146 (2014).
18. Rice, A. S. et al. EMA401, an orally administered highly selective angiotensin II type 2 receptor antagonist, as a novel treatment for postherpetic neuralgia: a randomised, double-blind, placebo-controlled phase 2 clinical trial. Lancet 383, 1637–1647 (2014).
19. Wang, Y. et al. Design, synthesis, and biological evaluation of the first selective nonpeptide AT2 receptor agonist. J. Med. Chem. 47, 5995–6008 (2004).
20. Kemp, B. A. et al. AT2 receptor activation induces natriuresis and lowers blood pressure. Circ. Res. 115, 388–399 (2014).
21. Larhed, M., Hallberg, M. & Hållberg, A. Nonpeptide AT2 receptor agonists. Med. Chem. Rev. 51, 69–82 (2016).
24. Zhang, H. et al. Structure of the angiotensin receptor revealed by serial femtosecond crystallography. Cell 161, 833–844 (2015).

25. Zhang, H. et al. Structural basis for ligand recognition and functional selectivity at angiotensin receptor. J. Biol. Chem. 290, 29127–29139 (2015).

26. Glinka, T. W. et al. L-161,638: a potent AT2 selective quinazolinone angiotensin II binding inhibitor. Bioorg. Med. Chem. Lett. 4, 1479–1484 (1994).

27. de Laszlo, S., Glinka, T., Greenlee, W., Chakravarty, P. & Patchett, A. Disubstituted 6-aminoquinazolinones. US patent 5,385,894 (1995).

28. Dosa, P. I. & Amin, E. A. Tactical approaches to interconverting GPCR agonists and antagonists. J. Med. Chem. 59, 810–840 (2016).

29. Murugasu, A. M. et al. From the first selective non-peptide AT3 receptor agonist to structurally related antagonists. J. Med. Chem. 55, 2265–2278 (2012).

30. Chun, E. et al. Fusion partner toolchest for the stabilization and crystallization of G protein-coupled receptors. Structure 20, 967–976 (2012).

31. Caffrey, M. & Cherezov, V. Crystallizing membrane proteins using lipidic mesophases. Nat. Protocols 4, 706–731 (2009).

32. Liu, W. et al. Serial femtosecond crystallography of G protein-coupled receptors. Science 342, 1521–1524 (2013).

33. Liu, W., Ischenko, A. & Cherezov, V. Preparation of microcrystals in lipidic cubic phase for serial femtosecond crystallography. Nat. Protocols 9, 2123–2134 (2014).

34. Weierstall, U. et al. Lipidic cubic phase injector facilitates membrane protein serial femtosecond crystallography. Nat. Commun. 5, 3309 (2014).

35. Ballesteros, J. A. & Weinstein, H. in Methods in Neurosciences Vol. 25 (ed. Selalton Stuart, C.) 366–428 (Academic Press, 1995).

36. Katritch, V., Cherezov, V. & Stevens, R. C. Structure-function of the G protein-coupled receptor superfamily. Annu. Rev. Pharmacol. Toxicol. 53, 531–556 (2013).

37. Venkatakrishnan, A. J. et al. Diverse activation pathways in class A GPCRs converge near the G-protein-coupling region. Nature 536, 484–487 (2016).

38. Rasmussen, S. G. et al. Crystal structure of the β2 adrenergic receptor–Gs protein complex. Nature 477, 549–555 (2011).

39. Wacker, D. et al. Structural features for functional selectivity at serotonin receptors. Science 340, 615–619 (2013).

40. Katritch, V. et al. Analysis of full and partial agonists binding to β2-adrenergic receptor suggests a role of transmembrane helix V in agonist-specific conformational changes. J. Mol. Recognit. 22, 307–318 (2009).

41. Kang, Y. et al. Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. Nature 529, 567–574 (2015).

42. Katritch, V. et al. AllostERIC sodium in class A GPCR signaling. Trends Biochem. Sci. 39, 233–244 (2014).

43. Balakumar, P. & Jagadeesh, G. Structural determinants for binding, activation, and functional selectivity of the angiotensin AT1 receptor. J. Mol. Endocrinol. 53, R71–R92 (2014).

44. Unal, H. & Karnik, S. S. Constitutive activity in the angiotensin II type 1 receptor: discovery and applications. Adv. Pharmacol. 70, 155–174 (2014).

45. Miura, S. & Karnik, S. S. Angiotensin II type 1 and type 2 receptors bind angiotensin II through different types of epitope recognition. J. Hypertens. 17, 397–404 (1999).

46. Miura, S. & Karnik, S. S. Ligand-independent signals from angiotensin II type 2 receptor induce apoptosis. EMBO J. 19, 4026–4035 (2000).

47. Akazawa, H., Yano, M., Yabumoto, C., Kudo-Sakamoto, Y. & Komuro, I. Angiotensin II type 1 and type 2 receptor-induced cell signaling. Curr. Pharm. Des. 19, 2988–2995 (2013).

48. Hein, L., Meinel, L., Pratt, R. E., Dzau, V. J. & Kobilka, B. K. Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. Mol. Endocrinol. 11, 1266–1277 (1997).

49. Widdop, R. E., Matrougui, K., Levy, B. I. & Henrion, D. AT2 receptor-mediated relaxation is preserved after long-term AT1 receptor blockade. Hypertension 40, 516–520 (2002).

Supplementary Information is available in the online version of the paper.

Acknowledgements This work was supported by the National Institutes of Health (NIH) grants R01 GM108635 (V.C.) and U54 GM094618 (V.K., V.C. and R.C.S.); the National Science Foundation (NSF) grant 1231306; (U.W. and W.L.); the Helmholtz Association through program oriented funds (T.A.W. and A.T.); A.T acknowledges financial support from ‘X-probe’ funded by the European Union’s 2020 Research and Innovation Program under the Marie Sklodowska-Curie grant agreement 637.295. Parts of this research were carried out at the Coherent X-ray Imaging (CXI) end station of the Linac Coherent Light Source (LCLS), SLAC National Accelerator Laboratory, operated by Stanford University on behalf of the US Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-76SF00515, and at the GM/CA CAT and IMCA-CAT of the Advanced Photon Source, Argonne National Laboratory. Parts of the sample delivery system used at LCLS for this research was funded by the NIH grant P41GM103393. Computational part of the study was supported by the University of Southern California Center for High-Performance Computing and Communications (https://hpc.usc.edu/). We thank J. Velasquez for help with molecular biology, M. Chu for help with baculovirus expression, M. Hanson for help with crystallographic data processing and A. Walker for assistance with manuscript preparation.

Author Contributions K.H., S.M.S., R.C.S., V.K. and V.C. conceived and managed the project. H.Z. designed, optimized, purified, and characterized receptor constructs for structural studies, crystallized the receptor in LCP. H.Z. and A.I. collected and processed synchrotron data. H.Z., A.B., A.I., M.S.H., U.W., W.L. and V.C. collected XFEL data. A.B., A.T. and T.A.W. processed XFEL data. G.W.H., H.Z. and A.B. solved and refined the structures. M.T.R., K.H., K.B., E.L.M., S.M.S. and S.S. interpreted the structure and designed experiments. R.D.K. and J.M.S. prepared VLPS for binding studies. P.S., M.G.-C. and B.Z. designed the binding experiments. B.Z. carried out radioligand-binding assays with VLPS. H.Z., B.Z., M.G.-C., A.S., N.P. and P.S. analysed the data and compiled the figures for the manuscript. N.P., A.S. and V.K. performed docking and molecular dynamics simulations. K.L.W. performed radioligand-binding experiments with receptor mutants. M.T.R., K.H. and K.B. selected compounds for SAR study and interpreted the data. H.Z., V.K. and V.C. wrote the manuscript with contributions from M.T.R. and K.H.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. Correspondence and requests for materials should be addressed to V.C. (cherezov@usc.edu) and V.K. (katritch@usc.edu).

Reviewer Information Nature thanks R. M. Carey, A. Hallberg and the other anonymous reviewer(s) for their contribution to the peer review of this work.
METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Protein engineering for structural studies. DNA encoding the human AT,R (UNIPROT P50052) was synthesized by GenScript with optimization for expression in insect cells. The construct has truncations of the AT,R residues 1–34 and 336–363. The thermostabilized apocytochrome b$_{59}$R (BRIL) from Escherichia coli was obtained as Met7Trp His102Ile Arg106Leu fusion protein with optimized coding for XFEL data collection and orthorhombic lattice parameters. Both datasets were merged separately using the standard CrystFEL (version 0.6.1+)定向优化 code and Ambiguity Trimmer without additional scaling step applying per-pattern resolution cutoff with ‘pushes 1.2’ option.

Structure determination. The structure was initially solved using molecular replacement with the monoclinic datasets collected at XFEL and synchrotron source. Molecular replacement models for AT,R were produced by alignment of AT,R sequence with sequences of previously solved GPCRs. The top 20 templates were further edited to preserve the conserved residues and trim non-conserved residues to alanines. Molecular replacement search with Phaser identified locations of two receptors in asymmetric unit with a TFE > 9. A further molecular replacement search with fixed positions of the two receptors using FDB code 1MT6 as the search model for BRIL found one BRIL molecule in the asymmetric unit. Refinement and model completion were performed by repetitive cycling between Refmac5 and autoBUSTER, followed by manual examination and rebuilding of the refined coordinates in Coot using both 2mFo – DF and mFo – DF maps, as well as omit maps calculated using Bhat's procedure. The second BRIL molecule was modelled manually in the available electron density when the Rfree value dropped below 0.33. The final data collection and refinement statistics are shown in Extended Data Table 1. The Ramachandran statistics determined by MolProbity are as follows: 96.8% in the favoured region, 3.2% allowed, 0 outlier for mononicl AT,R–compound 1, 97.6% in favoured region, 2.4% allowed, 0 outlier for orthorhombic AT,R–compound 1, 97.4% in favoured region, 2.6% allowed, 0 outlier for AT,R–compound 2.

Docking simulations. AT,R and AT,R selective and non-selective ligands were docked into the AT,R and AT,R crystal structures. AT,R active-like state model and AT,R inactive state model, respectively. Molecular models of the compounds were generated from two-dimensional representations and their three-dimensional geometry was optimized using MMFF94 force field. Molecular docking used biased probability Monte Carlo (BPMC) optimization of the ligand internal coordinates in the grid potentials of the receptor. To ensure exhaustive sampling of the ligand binding pose parameter thoroughness was set to 30 and at least 5 independent docking runs were performed for each ligand starting from a random conformation. The results of individual docking runs for each ligand were considered consistent if at least three of the five docking runs produced similar ligand conformations (r.m.s.d. < 2.0 Å) and binding score (−25.0 kJ mol$^{-1}$). The unbiased docking procedure did not use distance restraints or any other a priori derived information for the ligand–receptor interactions.

Molecular dynamics. The initial receptor coordinates were derived from the AT,R crystal structures in complexes with compounds 1 and 2. The N-terminal fusion partner BRIL was removed and the terminal amino acids Cys35 and Arg337 were acetylated and amidated, respectively. We used ICM-Pro package (http://www.molsoft.com) to construct the model in the regions of missing electron densities for side chains and especially for the intracellular loop (ICL) regions. The orientations of AT,R in the apo and the ligand bound models were aligned to AT,R orientation in the membrane taken from the OPM database (http://www.opm.phar.umich.edu/) on the basis of residues conserved in the orthorhombic BRIL structure. The MINCEFF force field (Molsoft) was used homogenously biased lipayer of 164 palmitoyloleoylphosphatidylcholine (POPC) lipids. TITSP water model was used for solvation of the lipids–receptor–ligand system with 150 mM salt concentration of Na$^+$ and Cl$^-$. The initial input files generated using CHARMM-GUI interface were simulated using GROMACS v.5.0.4 molecular simulation package compiled to run in parallel on a cluster of 4 nodes connected via InfiniBand, where a single node had 16 Intel Xeon 2.4 GHz processors and 2 NVIDIA Tesla K20 GPUs. Simulation parameters included a 1 fs time step for the initial phase of the equilibration, followed by a 2 fs time step for the rest of the equilibration and production simulations. During initial phase of NVT ensemble, temperature was set at 310 K using Berendsen per minute (120 Hz) with the 2.3 Megapixel Cornell–SLAC Pixel Array Detector (CSPAD). A total of 2,701,530 images were collected, 175,241 of which were identified as hits with the Cheetah program (6.5% hit rate). Surprisingly, indexing revealed two different lattices, monoclinic and orthorhombic, apparently belonging to different microcrystals of AT,R–compound 1 co-existing in the same crystalization batch. Therefore, 22,774 hits were successfully indexed in a 2/m Laue group and 15,804 hits in a mmm Laue group with different lattice parameters. Both datasets were merged separately using the standard CrystFEL (version 0.6.1+) code and Ambiguity Trimmer without additional scaling step applying per-pattern resolution cutoff with ‘pushes 1.2’ option.

Structure determination. The structure was initially solved using molecular replacement with the monoclinic datasets collected at XFEL and synchrotron source. Molecular replacement models for AT,R were produced by alignment of AT,R sequence with sequences of previously solved GPCRs. The top 20 templates were further edited to preserve the conserved residues and trim non-conserved residues to alanines. Molecular replacement search with Phaser identified locations of two receptors in asymmetric unit with a TFE > 9. A further molecular replacement search with fixed positions of the two receptors using FDB code 1MT6 as the search model for BRIL found one BRIL molecule in the asymmetric unit. Refinement and model completion were performed by repetitive cycling between Refmac5 and autoBUSTER, followed by manual examination and rebuilding of the refined coordinates in Coot using both 2mFo – DF and mFo – DF maps, as well as omit maps calculated using Bhat's procedure. The second BRIL molecule was modelled manually in the available electron density when the Rfree value dropped below 0.33. The final data collection and refinement statistics are shown in Extended Data Table 1. The Ramachandran statistics determined by MolProbity are as follows: 96.8% in the favoured region, 3.2% allowed, 0 outlier for mononicl AT,R–compound 1, 97.6% in favoured region, 2.4% allowed, 0 outlier for orthorhombic AT,R–compound 1, 97.4% in favoured region, 2.6% allowed, 0 outlier for AT,R–compound 2.

Docking simulations. AT,R and AT,R selective and non-selective ligands were docked into the AT,R and AT,R crystal structures. AT,R active-like state model and AT,R inactive state model, respectively. Molecular models of the compounds were generated from two-dimensional representations and their three-dimensional geometry was optimized using MMFF94 force field. Molecular docking used biased probability Monte Carlo (BPMC) optimization of the ligand internal coordinates in the grid potentials of the receptor. To ensure exhaustive sampling of the ligand binding pose parameter thoroughness was set to 30 and at least 5 independent docking runs were performed for each ligand starting from a random conformation. The results of individual docking runs for each ligand were considered consistent if at least three of the five docking runs produced similar ligand conformations (r.m.s.d. < 2.0 Å) and binding score (−25.0 kJ mol$^{-1}$). The unbiased docking procedure did not use distance restraints or any other a priori derived information for the ligand–receptor interactions.

Molecular dynamics. The initial receptor coordinates were derived from the AT,R crystal structures in complexes with compounds 1 and 2. The N-terminal fusion partner BRIL was removed and the terminal amino acids Cys35 and Arg337 were acetylated and amidated, respectively. We used ICM-Pro package (http://www.molsoft.com) to construct the model in the regions of missing electron densities for side chains and especially for the intracellular loop (ICL) regions. The orientations of AT,R in the apo and the ligand bound models were aligned to AT,R orientation in the membrane taken from the OPM database (http://www.opm.phar.umich.edu/) on the basis of residues conserved in the orthorhombic BRIL structure. The MINCEFF force field (Molsoft) was used homogenously biased lipayer of 164 palmitoyloleoylphosphatidylcholine (POPC) lipids. TITSP water model was used for solvation of the lipids–receptor–ligand system with 150 mM salt concentration of Na$^+$ and Cl$^-$. The initial input files generated using CHARMM-GUI interface were simulated using GROMACS v.5.0.4 molecular simulation package compiled to run in parallel on a cluster of 4 nodes connected via InfiniBand, where a single node had 16 Intel Xeon 2.4 GHz processors and 2 NVIDIA Tesla K20 GPUs. Simulation parameters included a 1 fs time step for the initial phase of the equilibration, followed by a 2 fs time step for the rest of the equilibration and production simulations. During initial phase of NVT ensemble, temperature was set at 310 K using Berendsen
Artificial search

polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester (Pierce, USA) for 60 min at room temperature. Membranes were harvested over 0.3% (w/v) bovine serum albumin (BSA) containing 1 nM [3H]angiotensin II (American Radiolabelled Chemicals, USA) for 15 min at 30,000× g. Aliquots were flash-frozen and stored at −80°C. Total protein concentration was determined by Bradford assay, and membrane pellets were frozen in liquid nitrogen and stored at −80°C. Binding reactions were carried out in 96-well microwells (Corning). The assay buffer (50 μl) consisted of 20 mM HEPES pH 7.4, 5 mM MgCl₂, 1 mM EDTA, and 0.005% Tween-20. Non-specific binding was determined in the presence of 1 μM angiotensin II. Final concentrations of [3H]angiotensin II (Perkin Elmer) were typically 0.05–5 nM in saturation and 0.3 nM in competition binding experiments. For both types of assay, 37.5 ng (wild-type AT₁R) or 13.5 ng (wild-type AT₂R) of VLPs were used per well. Competing ligands were added as DMSO solutions, resulting in a total organic solvent content of 1%. Reactions were incubated for 3 h at room temperature. Bound ligand was separated from free ligand by processing 25 μl of each reaction mixture on 96-well Zeba Spin desalting plates with a molecular mass cutoff of 40 kDa (ThermoScientific) according to the manufacturer’s instructions. Eluates were collected in 96-well isoplates by centrifugation (14,000×g, 10 min). All reagent transfers were conducted using a Hamilton liquid handler. Data were analysed by nonlinear curve-fitting using the program GraphPad Prism 6. Binding data are reported as mean ± s.d.

Radioligand-binding assays. Radioligand-binding studies were performed using mammalian virus-like particles (VLPs) containing wild-type AT₁R, wild-type AT₂R, or the engineered BRIL–AT₂R. VLPs were produced using the Expi293 MembranePro Expression System (ThermoFisher) per the manufacturer’s instructions. Total protein concentration was determined by Bradford assay. VLPs were re-suspended in cold Dulbecco’s PBS without calcium or magnesium (Gibco). Aliquots were flash-frozen and stored at −80°C. Radioligand binding assays were performed in 96-well isoplates by centrifugation (14,000×g, 10 min). All reagent transfers were conducted using a Hamilton liquid handler. Data were analysed by nonlinear curve-fitting using the program GraphPad Prism 6. Binding data are reported as mean ± s.d.

Mutagenesis of the ligand-binding pocket residues and radioligand binding assays. Mutagenesis of the ligand-binding pocket residues and radioligand binding assays were performed as previously described. In brief, ligand binding was measured using washed membranes from HEK 293 cells (FreeStyle 293 F; ThermoFisher, R79007) transiently expressing wild-type AT₁R or point-mutant AT₁R constructs. Cells were lysed in buffer (25 mM HEPES, pH 7.5) with protease inhibitor cocktail consisting of AEBSF (GoldBio), E-64, leupeptin, aprotinin (AGSScientific), and dounce homogenizer. Membranes were centrifuged for 15 min at 30,000×g. Total protein concentration was measured by Bradford assay, and membrane pellets were frozen in liquid nitrogen and stored at −80°C. Binding assays were carried out in a total volume of 0.25 ml in 96-well plates with a binding buffer (140 mM NaCl, 5 mM KCl, 1 mM EDTA, 25 mM HEPES, pH 7.4, 0.006% BSA) containing 1 nM [3H]angiotensin II (American Radiolabelled Chemicals, Inc.) for 60 min at room temperature. Membranes were harvested over 0.3% polyethyleneimine-treated, 96-well filter mats using a 96-well Filtermate harvester (Perkin Elmer) and washed three times with cold buffer (25 mM HEPES, pH 7.5). Filter mats were dried, waxed filter mats were melted onto each filter, and radioactivity was counted in a MicroBeta2 TriLux plate scintillation counter (Perkin Elmer). Angiotensin II dissociation constant (Kᵢ) values for wild-type and mutants were determined using homogeneous competition binding. Kᵢ values were determined using [1H]angiotensin II competition with 12 concentrations of unlabelled ligand (10,000 μM–0.01 nM). The data were analysed by Prism 6.05 (GraphPad Software) to give Kᵢ and Kᵢ values and reported as the mean ± s.d.

Compound preparation. All molecules presented in this manuscript were prepared according to the appropriate literature and/or patent publications: compounds 1 and 3 (ref. 26), compounds 2, 5, 6, 10, 12 and 14 (ref. 27), compounds 4 and 11 (ref. 64), compounds 7–9 (ref. 65) and compound 13 (ref. 66).

Cell lines. S9 cells were purchased from the American Type Culture Collection. HEK 293 cells were acquired from ThermoFisher. The cell lines have not been authenticated. All cell lines have been tested and shown to be free from mycoplasma.

Data availability. Atomic coordinates and structure factors have been deposited into the Protein Data Bank structures with accession codes 5UNF (AT₁R–compound 1, mononclonic), 5UNG (AT₁R–compound 1, orthorhombic), and 5UNH (AT₂R–compound 2). All other data are available from the corresponding authors upon reasonable request.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Extended Data Figure 1 | AT1R ‘snake’ diagram and protein engineering. Truncations are shown in grey, disulfide bonds in yellow, ligand-binding residues in red, and conserved motifs in green.
Extended Data Figure 2 | Radioligand-binding assays. 

a, b, Saturation binding of compound 1 and Sar1-ile8-angiotensin II. Specific binding of [3H]compound 1 (a) and [125I]Sar1-ile8-angiotensin II (b) to the wild-type (open circle) and engineered (closed triangle) AT2R, representative of two separate experiments. 

c–e, Competition binding of compound 1 (open circle), compound 2 (closed triangle) and angiotensin II (open square) to the wild-type AT2R (c), engineered AT2R (d) and wild-type AT1R (e) with [125I]Sar1-ile8-angiotensin II as a tracer; each point represents the mean ± s.d. of two separate experiments, performed in duplicate.
Extended Data Figure 3 | Crystallization of AT₂R and crystal packing.
a, AT₂R–compound 1 crystals grown in a syringe for XFEL data collection. b, AT₂R–compound 2 crystals grown in a glass sandwich plate for synchrotron data collection. c, Crystal packing in the monoclinic space group (AT₂R–compound 1 and AT₂R–compound 2 structures), side and top views (AT₂R in green and cyan; BRIL in orange and pink). d, Crystal packing in the orthorhombic space group (AT₂R–compound 1 structure), side and top views (AT₂R in cyan; BRIL in blue). e, Different BRIL orientations in the two BRIL–AT₂R molecules in the asymmetric unit of monoclinic AT₂R–compound 1 structure and AT₂R–compound 2 structure (pink and orange), and in the orthorhombic AT₂R–compound 1 structure (blue) with AT₂R in cyan, side and top views. Unit cell in c and d is outlined by the black line.
Extended Data Figure 4 | Conserved L[M]3.46-I[A]6.37-Y[Y]7.53 microswitch and sodium-binding pocket in AT1R and AT2R.

**a**, Comparison of the conserved residue triad between the AT1R (green, PDB code 4YAY) and AT2R (cyan) structures shows a rearrangement of interactions consistent with AT2R activation. **b**, Modelling of the AT2R in a hypothetical inactive state (cyan) based on the AT1R crystal structure template (green) shows that replacement of a large hydrophobic residue in position 6.37, which is conserved in most class A GPCRs, to a rare small Ala2586.37 in AT2R markedly reduces the hydrophobic contact in this region between helices III and VI in the inactive state. **c, d**, Sodium-binding pocket in AT2R (c) and AT1R (PDB code 4YAY) (d) is shown as a surface with hydrogen bonds between Asn7.46 and Asn3.35 as orange spheres. Putative sodium ion in the AT1R structure (d) is shown as a solid magenta sphere, while the same position in the AT2R structure (c) is marked as a dotted sphere. Potential sodium-coordinating residues are shown as sticks.
Extended Data Figure 5 | See next page for caption.
Extended Data Figure 5 | Summary of molecular dynamics simulations. 

a–c, Conformational stability of the AT2R structure is illustrated by representative conformations (c) from a total of 4 μs of molecular dynamics simulations (8 independent 500 ns runs), clustered by r.m.s.d. Traces of distances measured between different helices are shown for apo AT2R (a) and for the AT2R–compound 1 complex (b). Distances were calculated between the centres of mass of residues Ser792.39–Ile832.43 for helix II, Arg1423.50–Val1463.54 for helix III, Gln2536.32–Met2576.36 for helix VI, and Phe325-Lys328 for helix VIII. 

d, e, Conformational stability of helix VIII upon perturbations, using eight starting conformations of helix VIII (d) is revealed by r.m.s.d. traces (e), which all converge by ~250 ns of simulations. r.m.s.d. values are calculated for the centre of mass of Cα atoms of residues Phe325-Lys328 compared to the crystal structure of AT2R. Tick marks on the y axis show the starting frame r.m.s.d. values. Coloured lines are plotted using values averaged over a 500 ps window. 

f–h, Results of molecular dynamics simulations for a modified AT2R model with the backbone of helix VIII aligned with helix VIII from AT1R structure (PDB code 4YAY). Conformational snapshots of the AT2R model (f) are shown for every 100 ns (blue to red spectrum) from one of the six independent 700 ns molecular dynamics simulation runs (simulation 5). Green cartoon shows inactive-state conformation of CCR5 (PDB code 4MBS), helix VIII of which was found to be the closest to the final conformations of AT2R helix VIII in molecular dynamics simulations. Intracellular view (g) of snapshots from the same molecular dynamics simulation is shown, but at t = 0 and t = 700 ns. Traces of the distance between helices VI and II (h, top curves), calculated between the centres of mass of Cα atoms of residues Gln2536.32–Met2576.36 in helix VI and residues Ser792.39–Ile832.43 in helix II, show a change from 21 Å (active state) to under 16 Å (inactive state). Traces of the distance between helix VIII and the membrane (h, bottom curves), calculated between the centre of mass of Cα atoms of residues Arg330-Val332 and the closest phosphate atoms of lipid molecules, indicate a gradual shift of helix VIII towards the lipid bilayer, with the distance decreasing from ~10 Å to under 3 Å.
Extended Data Figure 6 | Electron density for compounds 1 and 2. 
a, b, Compound 1 can be modelled in two possible conformations (a and b), with alternative orientations of the benzene and thiophene rings. 
c, d, Compound 2 can be modelled in two possible conformations (c and d), with alternative orientations of the benzene and furan rings. 
$2mF_o - DF_c$ electron density (blue mesh) for compound 1 contoured at 1σ, and $mF_o - DF_c$ density (green mesh: positive; red mesh: negative) contoured at 3σ. The conformations shown in a and c were used in the final crystal structures because of a slightly better ligand fit and the absence of strong difference $mF_o - DF_c$ density. Both conformations for each ligand, however, are possible and indistinguishable by docking studies.
Extended Data Figure 7 | Ligand binding and cross-docking in AT$_2$R and AT$_1$R structures. a, b, Docking poses of compound 1 (magenta), compound 2 (yellow), olmesartan (blue) and ZD7155 (orange) in the crystal structures of AT$_2$R (a) and AT$_1$R (b). Receptors are shown in cartoon representation, ligands are shown as sticks, and hydrogen bonds/salt bridges are shown as dashed lines. 

| Compound | AT$_2$R $K_i$ nM | AT$_1$R $K_i$ nM | AT$_2$R inactive, docking score, kJ/mol | AT$_2$R active, docking score, kJ/mol | AT$_1$R active, docking score, kJ/mol |
|----------|-----------------|-----------------|----------------------------------|----------------------------------|----------------------------------|
| Cpd 1    | 180             | 0.34            | -30                              | -21                              | -44                              |
| Cpd 2    | 3.7             | 0.35            | -33                              | N/B                              | -43                              |
| Olmesartan | 5.3             | N/A             | -33                              | N/B                              | -27                              |
| ZD7155   | 3.0             | N/A             | -36                              | -19                              | -19                              |

N/A — data not available
N/B — no binding observed

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Extended Data Figure 8 | Mutagenesis of the AT$_2$R ligand-binding pocket.  

**a**, Ligand-binding pocket from the AT$_2$R–compound 1 crystal structure.  

**b**, Ligand-binding pocket from the AT$_1$R–olmesartan crystal structure.  

**c**, Schematics of interactions between compound 1 and AT$_2$R residues.  

**d**, Schematics of interactions between olmesartan and AT$_1$R residues. In all panels, residues are coloured according to their effect on affinity: more than 100-fold decrease in affinity (orange); 5–100-fold decrease in affinity (yellow); and less than 5-fold decrease in affinity (grey).  

**e**, Effects of single residue mutations in the AT$_2$R ligand-binding pocket on the ligand binding affinities. Values represent mean ± s.d. with the number of experiments shown in parenthesis.
## Extended Data Table 1 | Data collection and refinement statistics (molecular replacement)

|                          | AT$_2$R-Cpd 1 (XFEL) | AT$_2$R-Cpd 2 (Synchrotron) |
|--------------------------|-----------------------|-----------------------------|
| **Space group**          | P$_2_1$               | P$_2_1$                      |
| **Unit cell parameters** |                       |                             |
| a,b,c (Å)                | 77.4, 69.1, 90.1      | 70.3, 78.8, 93.4             |
| a,β,γ (°)                | 90.0, 104.3, 90.0     | 90.0, 90.0, 90.0             |
| **Data collection**      |                       |                             |
| Number of collected frames | 2,701,530           | 2,701,530                   |
| Number of hits / indexed images | 175,241 / 22,774   | 175,241 / 15,804             |
| Number of total / unique reflections | 1,412,692 / 22,934 | 1,139,069 / 13,330           |
| Resolution (Å)           | 30.2-8 (2.9-2.8)³    | 30.2-8 (2.9-2.8)             |
| Completeness (%)         | 100 (100)             | 100 (100)                   |
| Multiplicity             | 61.6 (16.3)           | 85.5 (27.2)                 |
| R$_{free}$               | 4.1 (0.8)             | 4.9 (1.0)                   |
| CC$^*$                   | 0.98 (0.24)           | 0.99 (0.36)                 |
| R$_{merge}$ or R$_{merge}$ (%) | 16.4 (172)        | 14.8 (124)                  |
| **Refinement**           |                       |                             |
| Resolution (Å)           | 29.57-2.80            | 28.96-2.80                  |
| Number of reflections / test set | 22,906/1,118       | 13,269/691                  |
| R$_{free}$ / R$_{free}$  | 0.227/0.256           | 0.241/0.262                 |
| **Number of atoms**      |                       |                             |
| Receptor / BRIL          | A: 3,103              | A: 2,983                     |
| Ligand                   | B: 3,048              | B: 2,800                     |
| Lipid and other          | 46                    | 46                           |
| Wilson B-factors (Å$^2$) | 90.8                  | 80.9                         |
| Mean overall B value (Å$^2$) | A: 131.5            | A: 72.0                      |
|                          | B: 84.6               | B: 114.2                     |
| Receptor                 | 141.7                 | 106.1                        |
| BRIL                     | 126.5                 | 62.1                         |
| Lipid and other          | -                     | -                            |
| R.m.s bonds (Å) / angles (°) | 0.010/0.90         | 0.009/0.92                   |

¹Data collected from AT crystals were used for the AT$_2$R-compound 2 structure determination.
²Numbers in parentheses represent values from the highest resolution shell.
# Extended Data Table 2 | SAR for quinazolinone-biphenyltetrazole derivatives in AT$_2$R and AT$_1$R

![Chemical structure](image)

| Compound | $R_1$ | $R_2$ | AT$_2$R $K_i$ (nM)* | AT$_1$R $K_i$ (nM)* | AT$_2$R fold selectivity |
|----------|-------|-------|----------------------|----------------------|-------------------------|
| 1        | Ethyl |       | 0.34 ± 0.06          | 184 ± 50             | 530x                    |
| 2        | $n$-Propyl |     | 0.35 ± 0.05          | 3.72 ± 0.03          | 11x                     |
| 3        | Methyl |       | 1.7 ± 0.4            | 700 ± 200            | 410x                    |
| 4        | $n$-Propyl |     | 0.65 ± 0.01          | 1.8 ± 0.2            | 2.8x                    |
| 5        |       |       | 11.5 ± 0.5           | 37 ± 3               | 3.2x                    |
| 6        |       |       | 120 ± 50             | 450 ± 20             | 3.8x                    |
| 7        | $n$-Propyl |     | 1.7 ± 0.5            | 10.4 ± 1.7           | 6.1x                    |
| 8        | $n$-Propyl |     | 10.9 ± 0.1           | 9.90 ± 0.01          | 0.9x                    |
| 9        | $n$-Propyl |     | 1.790 ± 150          | 1.6 ± 0.1            | 0.001x                  |
| 10       | $n$-Propyl |     | 4.9 ± 0.2            | 12.9 ± 2.8           | 2.6x                    |
| 11       | $n$-Propyl |     | 4.1 ± 0.8            | 6.7 ± 0.9            | 1.6x                    |
| 12       | $n$-Propyl |     | 18.8 ± 0.1           | 17.3 ± 4.3           | 0.9x                    |
| 13       | $n$-Propyl |     | 2.960 ± 80           | 16.7 ± 10.4          | 0.006x                  |
| 14       | Methyl |       | 5,300 ± 2,400        | 360 ± 80             | 0.07x                   |

*Competition binding assay with $[^{125}]$Sar$^4$-ile$^8$-angiotensin II as a tracer; each point represents the mean ± s.d. of two separate experiments, performed in duplicate.

© 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.