Crystal structures of human ET₉ receptor provide mechanistic insight into receptor activation and partial activation

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Endothelin receptors (ETₐ and ET₉) are class A GPCRs activated by vasoactive peptide endothelins, and are involved in blood pressure regulation. ET₉-selective signalling induces vasorelaxation, and thus selective ET₉ agonists are expected to be utilized for improved anti-tumour drug delivery and neuroprotection. Here, we report the crystal structures of human ET₉ receptor in complex with ET₉-selective agonist, endothelin-3 and an ET₉-selective endothelin analogue IRL1620. The structure of the endothelin-3-bound receptor reveals that the disruption of water-mediated interactions between W6.48 and D2.50 is critical for receptor activation, while these hydrogen-bonding interactions are partially preserved in the IRL1620-bound structure. Consistently, functional analysis reveals the partial agonistic effect of IRL1620. The current findings clarify the detailed molecular mechanism for the coupling between the orthosteric pocket and the G-protein binding, and the partial agonistic effect of IRL1620, thus paving the way for the design of improved agonistic drugs targeting ET₉.

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Endothelin receptors belong to the class A GPCRs, and are activated by endothelins, which are 21-amino acid peptide agonists. Both of the endothelin receptors (the ET_A and ET_B receptors) are widely expressed in the human body, including the vascular endothelium, brain, lung, kidney, and other circulatory organs. Three kinds of endothelins (ET-1, ET-2, and ET-3) activate the endothelin receptors (ETRs) with subnanomolar affinities. ET-1 and ET-2 show similar affinities to both of the endothelin receptors, while ET-3 shows two orders of magnitude lower affinity to ET_A. The stimulation of the ET_A receptor by ET-1 leads to potent and long-lasting vasoconstriction, whereas that of the ET_B receptor induces nitric oxide-mediated vasorelaxation. The human brain contains the highest density of endothelin receptors, with the ET_B receptor comprising about 90% in areas such as the cerebral cortex. The ET_B receptor in neurons and astrocytes has been implicated in the promotion of neuroprotection, including neuronal survival and reduced apoptosis. Moreover, the ET-3/ET_B signalling pathway has distinct physiological roles, as compared to the ET-1 pathway. In the brain, ET-3 is responsible for salt homeostasis, by enhancing the sensitivity of the brain sodium-level sensor Na_+ channel. The ET-3/ET_B signalling pathway is also related to the development of neural crest cells, and has an essential role in the formation of the enteric nervous system. Thus, mutations of the development of neural crest cells, and have an essential role in the establishment of the enteric nervous system.14. Thus, mutations of the ET-3 or ET_B genes cause Hirschsprung’s disease, a birth defect in which nerves are missing from parts of the intestine. Overall, the endothelin system participates in a wide range of physiological functions in the human body.

Since the activation of the ET_B receptor has a vasodilating effect, unlike the ET_A receptor, ET_B-selective agonists have been studied as vasodilator drugs for the improvement of tumour drug delivery, as well as for the treatment of hypertension. IRL1620 (N-Suc-[E9, A11, 15] ET-1_{21}) is a truncated peptide analogue of ET-1, is the smallest agonist that can selectively stimulate the ET_B receptor, and currently no non-peptidic ET_B-selective agonists have been developed. The affinity of IRL1620 to the ET_B receptor is comparable to that of ET-1, whereas it essentially does not activate the ET_A receptor, and thus it shows high ET_B selectivity of over 100,000-fold. Due to its large molecular weight, IRL1620 is not orally active and thus requires intravenous delivery. Despite its pharmacokinetic disadvantages, IRL1620 is an attractive candidate for the treatment of various diseases related to the ET_B receptor. Since the ET_B-selective signal improves blood flow, IRL1620 could be utilized for the improved efficacy of anti-cancer drugs by increasing the efficiency of drug delivery, as shown in rat models of prostate and breast cancer. Moreover, this strategy can also be applied to radiotherapy in the treatment of solid tumours, as the radiation-induced reduction in the tumour volume was enhanced by IRL1620. IRL1620 also has vasodilatation and neuroprotection effects in the brain. IRL1620 reduced neurological damage following permanent middle cerebral artery occlusion in a rat model of focal ischaemic stroke. Moreover, the stimulation of the ET_B receptor by IRL1620 reduces the cognitive impairment induced by beta amyloid (1-40), a pathological hallmark of Alzheimer’s disease, in rat experiments. These data suggest that ET_B selective agonists might offer new therapeutic strategies for neuroprotection and Alzheimer’s disease. The safety and maximal dose of IRL1620 were investigated in a phase I study. While a recent phase 2 study of IRL1620 in combination with docetaxel as the second-line drug reported no significant improvement in the treatment of advanced biliary tract cancer (ABTO), further trials for selected patients based on tumour types with various choices for the second-line drugs are still expected. Concurrently, the pharmacological properties of IRL1620 could also be improved for better clinical applications. However, little is known about the selectivity and activation mechanism of this artificially designed agonist peptide, although the ET_B structures in complex with ET-1 and antagonists have been determined.

In this study, we report the crystal structures of the ET_B receptor in complex with two ET_B-selective ET variant agonists, ET-3 and IRL1620. Together with their detailed pharmacological characterization, the structures reveal the different activation mechanisms of these agonists, especially for the partial activation by IRL1620.

Results

Functional characterization of ET-3 and IRL1620. We first investigated the biochemical activities of ET-3 and IRL1620 for the human endothelin receptors, by TGFAa shedding (G-protein activation, specifically the G_α_ and the G_β_2 families) and β-arrestin recruitment assays. The EC_{50} and E_{max} values of ET-3 for the ET_B receptor were similar to those of ET-1 in both assays, while the EC_{50} value for ET_A was about 5-fold lower (Fig. 1a, b, and Table 1, Table 2). These data indicate that ET-3 functions as a full agonist for the endothelin receptors, with moderate ET_B-selectivity. The EC_{50} values of IRL1620 for the ET_B receptor were almost the same as those of ET-1 in both assays. In contrast, a 320 nM concentration of IRL1620 did not activate ET_A in the TGFAa shedding assay (Fig. 1a). These data showed that IRL1620 is ET_B-selective by over 3000-fold, in excellent agreement with previous functional analyses. However, despite its subnanomolar affinity, the E_{max} values of IRL1620 for the ET_B receptor were 88% (TGFAa shedding assay) and 87% (β-arrestin recruitment assay) of the E_{max} value of ET-1 (Table 1 and 2), indicating that IRL1620 functions as a partial agonist for the ET_B receptor.

To obtain mechanistic insights into the different actions of these agonists, we performed X-ray crystal structural analyses of the human ET_B receptor in complex with ET-3 and IRL1620. For crystallization, we used the previously established, thermostabilized ET_B receptor (ET_B-Y5)27,30. IRL1620 also functions as a partial agonist for the thermostabilized receptor, as the E_{max} values for ET_B-Y5 were lower than those of ET-1 in both assays (84% and 85% in the TGFAa shedding assay and the β-arrestin recruitment assay, respectively), while the EC_{50} values of IRL1620 were increased for ET_B-Y5 by about 9- and 6-fold in the TGFAa shedding assay and β-arrestin recruitment assay, respectively, as compared to the wild type receptor (Fig. 1a, b middle and Tables 1, 2). To facilitate crystallization, we replaced the third intracellular loop (ICL3) of the receptor with T4 Lysozyme (ET_B-Y5-T4L), and using in meso crystallization, we obtained crystals of ET_B-Y5-T4L in complex with ET-3 and IRL1620 (Supplementary Figure 1a, b). In total, 757 and 68 datasets were collected for the ET-3- and IRL1620-bound receptors, respectively, and merged by the data processing system KAMO. Eventually, we determined the ET_B structures in complex with ET-3 and IRL1620 at 2.0 and 2.7 Å resolutions, respectively, by molecular replacement using the ET-1-bound receptor (PDB 5GLH) (Table 3). The datasets for the ET-3 bound receptor were mainly allowed the convenient collection of a large number of datasets. The electron densities for the agonists in both structures were clearly observed in the F_σ  − F_ω omit maps (Supplementary Figure 1c, d).

ET_B structure in complex with the full agonist ET-3. We first describe the ET_B structure in complex with ET-3. The overall structure consists of the canonical 7 transmembrane helices (TM), the amphipathic helix 8 at the C-terminus (H8), two antiparallel β-strands in the extracellular loop 2 (ECL2), and the

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Fig. 1 Pharmacological characterizations of ET-3 and IRL1620. a Concentration-response curves of AP-TGFα release in the ET-1, ET-3, and IRL1620 treatments of HEK293 cells expressing the indicated endothelin receptors. Symbols and error bars are means and s.e.m. (standard error of the mean) of five or seven independent experiments, each performed in triplicate. Note that the error bars are smaller than the symbols for most data points. b Concentration-response curves of β-arrestin recruitment in the ET-1, ET-3, and IRL1620 treatments of HEK293 cells expressing the endothelin receptors. Symbols and error bars are means and s.e.m. of four independent experiments, each performed in duplicate.

Table 1 TGFα shedding assay

| Ligand   | EC50, nM (pEC50 ± SEM) | Emax ± SEM, % | EC50, nM (pEC50 ± SEM) | Emax ± SEM, % | EC50, nM (pEC50 ± SEM) | Emax ± SEM, % |
|----------|------------------------|---------------|------------------------|---------------|------------------------|---------------|
| ETB (n = 7) |                        |               | ETB-Y5 (n = 7)          |               | ET A (n = 5)         |               |
| ET-1     | 0.11 (9.94 ± 0.04)     | 100           | 0.099 (10.01 ± 0.07)   | 100           | 0.13 (9.89 ± 0.04)   | 100           |
| ET-3     | 0.13 (9.89 ± 0.04)     | 100.5 ± 0.6 (NS) | 0.077 (10.12 ± 0.06)   | 100.9 ± 0.3 (NS) | 0.65 (9.19 ± 0.03)   | 101.2 ± 0.8 (NS) |
| IRL1620  | 0.11 (9.97 ± 0.03)     | 87.6 ± 1.0*** | 0.92 (9.04 ± 0.04)     | 83.6 ± 1.4*** | NA                     | NA            |

The EC50 and Emax values of the AP-TGFα release response for the endothelin receptors. The Emax value of the AP-TGFα release response in the ET-1 treatment was normalized to 100% for each experiment. Emax values that significantly differ from the wild-type are denoted by asterisks.

*** P < 0.001 as compared with ET-1, one-way ANOVA with Dunnett’s post hoc test

NS Not significant. NA Parameters not available owing to lack of responses

Table 2 β-Arrestin recruitment assay

| Ligand   | EC50, nM (pEC50 ± SEM) | Emax ± SEM, % | EC50, nM (pEC50 ± SEM) | Emax ± SEM, % | EC50, nM (pEC50 ± SEM) | Emax ± SEM, % |
|----------|------------------------|---------------|------------------------|---------------|------------------------|---------------|
| ETB (n = 4) |                        |               | ETB (n = 4)            |               | ET A (n = 4)         |               |
| ET-1     | 2.7 (8.56 ± 0.02)      | 100           | 3.3 (8.48 ± 0.02)      | 100           | 2.3 (8.63 ± 0.05)    | 100           |
| ET-3     | 3.3 (8.49 ± 0.03)      | 104.8 ± 0.5 (NS) | 3.5 (8.46 ± 0.02)      | 104.8 ± 0.5 (NS) | 10.8 (7.97 ± 0.05)   | 81.1 ± 0.7*** |
| IRL1620  | 3.1 (8.51 ± 0.05)      | 87.3 ± 2.7*** | 15.1 (7.82 ± 0.08)     | 85.4 ± 3.3*** | NA                     | NA            |

The EC50 and Emax values of the β-arrestin recruitment for the endothelin receptors. The Emax value of the β-arrestin recruitment in the ET-1 treatment was normalized to 100% for each experiment. Emax values that significantly differ from the wild-type are denoted by asterisks.

*** P < 0.001 as compared with ET-1, one-way ANOVA with Dunnett’s post hoc test

NS Not significant. NA Parameters not available owing to lack of responses
N-terminus that is anchored to TM7 by a disulfide bond (Fig. 2b), and is similar to the previous ET-1-bound structure27 (overall R.M.S.D of 1.0 Å for the Cα atoms) (Fig. 2c). Similar to ET-1, ET-3 adopts a bicyclic architecture comprising the N-terminal region (residues 1–7), the α-helical region (residues 8–17), and the C-terminal region (residues 18–21), and the N-terminal region is attached to the central α-helical region by the intrachain disulfide bonds (C1–C15 and C3–C31). The amino acid residues of the α-helical and C-terminal regions are highly conserved between ET-1 and ET-3 (Fig. 2a), and the agonist peptides superimposed well (Fig. 2c, d and Supplementary Figure 2a–d). Accordingly, these regions form similar interactions with the receptor in both structures (Supplementary Figure 3a, b). In contrast, all of the residues, except for the disulfide bond-forming C1 and C3, are replaced with bulkier residues in ET-3 (Fig. 2a). Despite these sequence differences, the N-terminal regions are similarly accommodated in the orthosteric pocket in both structures, because these bulky residues are exposed to the solvent and interact poorly with the receptor (Fig. 2d). These structural features explain the similar high affinity binding of ET-3 to the ETB receptor, as compared with ET-1.

Previous studies demonstrated that the N-terminal residues of the ETB receptor have a critical role in the virtually irreversible binding of the endothelins32,35. As in the ET-1-bound structure, the N-terminal tail is anchored to TM7 via a disulfide bond between C90 and C358 in the ET-3-bound structure, constituting a lid that prevents agonist dissociation. The high-resolution ET-3-bound structure allowed more accurate tracing of the elongated N-terminal residues (Fig. 2e, f, and Supplementary Figure 1e), as compared with the ET-1-bound structure, and revealed more extensive interactions with the agonist peptide. P88, I94, Y247ECL2, and K248ECL2 form a lid over ET-3, which is stabilized by a water-mediated hydrogen bonding network among the carbonyl oxygen of P93, the side chains of Y247ECL2 and K248ECL2, and D8 of ET-3 (Fig. 2e). In addition, three consecutive prolines (P87, 88, 89) stretch over the N-terminal region of ET-3, and two of them form van der Waals interactions with ET-3. Moreover, ECL1, 2 and the N-terminal residues form an extended water-mediated hydrogen bonding network over ET-3. These extensive interactions strongly prevent the agonist dissociation.

### Table 3 Data collection and refinement statistics

|         | ET-3   | IRL1620 |
|---------|--------|---------|
| Space group | C222_1 | C222_1 |
| Cell dimensions (Å) | 65.5, 172.3, 121.3 | 100.0, 303.9, 60.2 |
| α, β, γ (°) | 90, 90, 90 | 90, 90, 90 |
| Resolution (Å) | 50.0–2.00 (2.12–2.00) | 50.0–2.70 (2.80–2.70) |
| R-factor (all reflections) | 0.860 (18.057) | 0.499 (5.826) |
| R-free (15% of reflections) | 0.060 (1.727) | 0.108 (1.250) |
| CC1/2 | 0.973 (0.559) | 0.981 (0.382) |
| Completeness (%) | 100.0 (100.0) | 99.99 (100.0) |
| Redundancy | 204.6 (199.2) | 21.5 (21.5) |

*Values in parentheses are for highest-resolution shell*

**ETB structure in complex with the partial agonist IRL1620**

Next we describe the ETB structure in complex with the partial agonist IRL1620, a linear peptide analogue of ET-117 (Fig. 3a). Previous mutant and structural studies revealed that the N-terminal region contributes to the stability of the whole bicyclic structure, by the intramolecular disulfide bonds, and thus facilitates the receptor interaction17,27,33. IRL1620 completely lacks the N-terminal region, and consists of only the α-helical and C-terminal regions (Fig. 3b). Two cysteines in the α-helical region are replaced with alanines, and negative charges are introduced into the N-terminal end of the helix, by replacing lysine with glutamic acid (E9) and modifying the N-terminal amide group with a succinyl group (Fig. 3c). The consequent cluster of negative charges on the N-terminal end of IRL1620 (succinyl group, D8, E9, and E10) reportedly has an essential role in IRL1620 binding to the ETB receptor17. This cluster electrostatically complements the positively charged ETB receptor pocket, which includes K346ECL2 and R357ECL3 (Fig. 3d). Moreover, this negative cluster probably stabilizes the α-helical conformation of IRL1620, by forming a hydrogen-bonding cap at the N-terminally exposed amines34. Due to these effects, IRL1620 adopts a similar helical conformation, even without the intramolecular disulfide bonds (Supplementary Figure 2c, d), and forms essentially similar interactions with the receptor, as compared with the endogenous agonists, ET-1 and ET-3 (Supplementary Figure 3a–c). These structural features are consistent with the high affinity of IRL1620 to the ETB receptor, which is comparable to those of ET-1 and ET-3 (Fig. 1a, b). Nevertheless, the N-terminal side of the α-helical region of IRL1620 is less visualized in the electron density, suggesting its higher flexibility as compared to those of ET-1 and ET-3 (Supplementary Figure 1d), probably due to the lack of the N-terminal region. In contrast to the quasi-reversible binding of ET-1, IRL1620 binding is reportedly reversible35. Such structural differences may account for the different dissociation properties of these agonists.

IRL1620 does not bind to the ETA receptor at all in the same concentration range, confirming its high selectivity for the ETB receptor (Fig. 1a, b, and Table 1 and 2). To elucidate the mechanism of this selectivity, we compared the amino acid compositions of the IRL1620 binding sites between the ETB and ETA receptors (Fig. 4a and Supplementary Figure 4). While the transmembrane region is highly conserved, the residues in ECL2 are diverse. In particular, the hydrophobic residues L253 ECL2 and I254 ECL2 are replaced with the polar residues H236 and T238 in the ETA receptor, respectively (Fig. 4a, b). These residues form extensive hydrophobic interactions with the middle part of IRL1620. However, the double mutation of L252H and I254T only reduced the potency of IRL1620 by 2-fold in the TGFβ shedding assay (Fig. 4c and Table 4), suggesting that these residues are not the sole determinants for the receptor selectivity of IRL1620. Therefore, we focused on other residues of ECL2. In the ETB receptor, P259ECL2 and V260ECL2 generate a short kink
on the loop between the β-strand and TM5, but the ET_A receptor
has a truncated loop region and completely lacks these residues
(Fig. 4a). In addition, the ET_A receptor has a proline (P228) in the
first half of ECL2, which should disturb the β-strand formation as
in the ET_B receptor (Fig. 4b). These observations suggest that
ECL2 adopts completely different structures between the ET_A and
ET_B receptors. Moreover, ECL1 is also different between the two
receptors, as the ET_A receptor has a five amino-acid elongation as
compared to the ETβ receptor (Supplementary Figure 4). Since ECL1 interacts with the β-strands in ECL2 (Fig. 4a), this elongation could affect the orientation of the β-strands. Overall, the sequence divergences in the extracellular loops suggest that the ETA and ETB receptors have different extracellular architectures in these regions, which may account for their different selectivities for the isopeptides.

Receptor activation and partial activation. To elucidate the mechanism of the partial activation by IRL1620, we compared the IRL1620-bound structure with the full-agonist ET-3-bound structure (Fig. 5). IRL1620 forms essentially similar receptor interactions to those of the α-helical and C-terminal regions of ET-3 (Supplementary Figure 5a, b). The intracellular portions of the receptors are quite similar between the ET-3- and IRL1620-bound structures, in which TM7 and H8 adopt active conformations, while the remaining parts of the receptors still represent the inactive conformation of GPCRs (Fig. 5a, and Supplementary Figure 6). On the extracellular side, IRL1620 induces similar conformational changes to those observed in the ET-1- and ET-3-bound structures; namely, the large inward motions of TM2, 6, and 7, which are critical for receptor activation (Fig. 5b, c). However, the extent of the inward motion of TM6-7 is smaller by about 1 Å in the IRL1620-bound structure, as compared with that in the ET-3-bound structure, due to the different ligand architectures between IRL1620 and ET-3. Since IRL1620 lacks the N-terminal region, the orthosteric pocket of the receptor has more space, and consequently the α-helical region of IRL1620 is tilted differently toward TM6 (Fig. 5b). In addition, while the N-terminal region of ET-3 interacts with TM6 of the receptor, by forming a hydrogen bond between the carbonyl oxygen of T2 and K346 (superscripts indicate Ballesteros–Weinstein numbers), IRL1620 lacks this interaction, resulting in the different orientation of TM6-7. As TM6 has an especially important role for the cytoplasmic G-protein binding, this difference is probably related to the partial agonist activity of IRL1620.
A comparison of the intermembrane parts revealed further differences in the allosteric coupling between the orthosteric pocket and the intermembrane part. Previous studies have shown that the agonist binding induces the disruption of the hydrogen-bonding network around D1472.50, which connects TMs 2, 3, 6, and 7 and stabilizes the inactive conformation of the ET_{B} receptor28 (Supplementary Figure 7a). The present high-resolution ET-3-bound structure provides a precise mechanistic understanding of this rearrangement (Fig. 5d, and Supplementary Figure 7b). In particular, the water-mediated hydrogen bonds involving D1472.50, W3366.48, and N3787.45 in the inactive conformation collapse upon ET-3 binding, by the inward motions of TMs 2, 6, and 7. The W3366.48 side chain moves downward by about 2.5 Å, resulting in the disruption of the water-mediated hydrogen bond with D1472.50 and consequently, the D1472.50 side chain moves downward by about 3 Å and forms hydrogen bonds with the N3827.49 and N1191.50 side chains. The N3787.45 side chain also moves downward by about 1.5 Å and forms a hydrogen bond with the nitrogen atom of the W3366.48 side chain. The downward movements of the W3366.48 and N3787.45 side chains consequently induce the outward repositioning of the F3326.44 side chain and the middle part of TM6, by about 1 Å. W6.48 and F6.44 are considered to be the transmission switch of the class A GPCRs, which transmits the agonist-induced motions to the cytoplasmic G-protein coupling interface. Overall, our results show that the collapse of the water-mediated hydrogen-bonding network involving D147 2.50, W336 6.48, and N378 7.45 propagates as the structural change in the transmission switch, and probably induces the outward displacement of the cytoplasmic portion of TM6 upon G-protein activation (Supplementary Figure 6).

IRL1620 induces a similar but slightly different rearrangement of the hydrogen bonding network in the intermembrane part (Fig. 5e). Due to the smaller inward shift of the extracellular portion of TM6, the downward shift of the W3366.48 side chain is smaller in the IRL1620-bound structure, and it still forms a water-mediated hydrogen bond with the D1472.50 side chain. Consequently, the D1472.50 side chain forms a direct hydrogen bond with N3787.45, thereby preventing the downward motion of N3787.45 and the hydrogen bond formation between N3787.45 and W3366.48. Overall, the downward motions of the W3366.48 and N3787.45 side chains are only moderate, as compared to those in the ET-3-bound structure, and the hydrogen-bonding network involving D1472.50, W3366.48, and N3787.45 is partially preserved in the IRL1620-bound structure (Fig. 5e, and Supplementary Figure 7c). Accordingly, in the IRL1620-bound structure, the position of the “transmission switch” residue F3326.44 is in between those of the active (ET-3-bound) and inactive (K-8794-bound) structures (Fig. 5f). This intermediate position of F3326.44 should partly prevent the outward displacement of the cytoplasmic portion of TM6 that is required for G-protein

### Table 4 Pharmacological parameters for the L252H I254T double mutant

| Ligand | ET_{B}-WT (n = 6) | L252H/I254T (n = 6) |
|--------|------------------|---------------------|
| ET-1   | 0.15 (9.82 ± 0.11) | 0.14 (9.85 ± 0.11, NS) |
| IRL1620| 0.14 (9.85 ± 0.09) | 0.32 (9.50 ± 0.07*) |

*pEC_{50} value that significantly differs from the wild-type is denoted by an asterisk (P < 0.05 as compared with ET_{B}-WT, Sidak’s multiple comparisons test) NS Not significant
activation. Overall, the smaller inward shift of the extracellular portion of TM6 and the preserved interactions at the receptor core account for the partial agonistic activity of IRL1620 (Fig. 6).

**Discussion**

Previous studies have suggested that the α-helical and C-terminal regions of endothelins are critical elements for receptor activation, whereas the N-terminal region is only responsible for the ETR selectivity. Indeed, the N-terminal region-truncated analogue IRL1620 has similar EC50 values, as compared with ET-1. However, our pharmacological experiments for the first time proved that IRL1620 functions as a partial agonist for the ETB receptor, rather than a full agonist, suggesting the participation of the N-terminal region in the activation process of the ETB receptor. To clarify the receptor activation mechanism, we determined the crystal structures of the human ETB receptor in complex with ET-3 and IRL1620. The high-resolution structure of the ET-3-bound ETB receptor revealed that the large inward motions of the extracellular portions of TMs 2, 6, and 7 disrupt the water-mediated hydrogen bonding network at the receptor core (Fig. 5d, and Supplementary Figure 7a, b). The IRL1620-bound ETB structure revealed that the IRL1620-induced inward motions of TMs 6 and 7 are smaller by about 1 Å, as compared with those caused by ET-3 (Fig. 5b, c). Despite the lower resolution of the IRL1620-bound structure, the 2Fo – Fc map shows different rearrangement of water molecules and amino acid residues in the receptor core, in which the hydrogen-bonding network is partially preserved (Fig. 5e, and Supplementary Figure 7c). This preserved network, together with the smaller inward motion of TM6, may prevent cytoplasmic outward motion of TM6 that occurs upon G-protein binding. These observations suggest that the interactions between the N-terminal regions of endothelins and TM6 also participate in receptor activation, while the extensive interactions of the α-helical and C-terminal regions with the receptor primarily contribute to this process (Supplementary Figure 3c). This activation mechanism is different from that of the small-molecule activated GPCRs (e.g., β2 adrenaline and M2 muscarinic acetylcholine receptors), in which only a small number of hydrogen-bonding interactions between the agonist and the receptor induce receptor activation, by affecting the receptor dynamics.

D2.50 is one of the most conserved residues among the class A GPCRs (90%). Recent high-resolution structures have revealed that a sodium ion coordinates with D2.50 and forms a water-mediated hydrogen bonding network in the intermembrane region, which stabilizes the inactive conformation of the receptor, and its collapse leads to receptor activation. Our previous 2.2 Å resolution structure of the K-8794-bound ETB receptor revealed that a water molecule occupies this allosteric sodium site and participates in the extensive hydrogen-bonding network, instead of a sodium ion (Supplementary Figure 7a), and this hydrogen-bonding network is collapsed in the 2.8 Å resolution structure of the ET-1-bound ETB receptor, indicating its involvement in the receptor activation. Nevertheless, the precise

![Fig. 5](image_url)
rearrangement of this network still remained to be elucidated, due to the limited resolution. The current 2.0 Å resolution structure of the ET-3-bound ETβ receptor revealed that the collapse of the water-mediated interaction between W336.48 and D147.250 is critical for receptor activation (Fig. 5d). This network is still partly preserved in the IRL1620-bound structure (Fig. 5e), thus preventing the transition to the fully active conformation upon G-protein coupling (Fig. 6). W6.48 is also highly conserved among the class A GPCRs (71%)41, and the association between W6.48 and D2.50 has a critical role in the GPCR activation process, as shown in the previous nuclear magnetic resonance (NMR) study of the adenosine A2A receptor42. Given the importance of W3.36 and D2.50 in the activation of GPCRs, our proposed model of the partial receptor activation by IRL1620 is consistent with the previous functional analyses of GPCRs. To date, the β adrenergic receptor is the only receptor for which agonist- and partial agonist-bound structures were reported43. However, these structures are both stabilized in inactive conformations by the thermotabling mutations and thus revealed only slight differences (Supplementary Figure 8). Therefore, our study provides the first structural insights into the partial activation of class A GPCRs.

Our current study further suggests possible improvements in clinical studies using ETβ-selective agonists. IRL1620 is the smallest among the ETβ-selective agonists, and thus is expected to be useful for the treatment of cancers and other diseases18–22,24,25. While its effectiveness has been proven in rat experiments, a recent phase 2 study has failed26, and thus further improvement of IRL1620 is required for clinical applications. Our cell-based assays and structural analysis revealed the partial agonistic effect of IRL1620 on the ETβ receptor in the G-protein coupling and β-arrestin recruitment activities, suggesting the possible tuning of its efficacy. The development of ETβ-selective agonists by fine-tuning their G-protein activation and/or β-arrestin recruitment activities might be beneficial for clinical applications.

Methods

Expression and purification. We used the thermostabilized receptor ETβ-Y5-T4L, as previously established (Supplementary Table 1). In brief, the haemagglutinin signal peptide followed by the Flag epitope tag was added to the N-terminus, and a tobacco etch virus (TEV) protease recognition sequence was included between G37 and L66. The C-terminus was truncated, and three cysteine residues were mutated to alanine (C396A, C400A, and C405A). To facilitate crystallogenesis, T4 lysozyme (C54T and C97A) was introduced into intracellular loop 3. The ETβ-Y5-T4L was subcloned into a modified pFastBac vector, with the resulting construct encoding a TEV cleavage site followed by a GFP-His10 tag at the C-terminus. The recombinant baculovirus was prepared using the BacTo-Bac expression system (Invitrogen). Sf9 insect cells (Invitrogen) were infected with the virus at a cell density of 4.0 × 10^6 cells per millilitre in Sf900 II medium, and grown for 48 h at 27 °C. The cells were disrupted by sonication, in buffer containing 20 mM Tris--HCl, pH 7.5, 200 mM NaCl, 1% DDM, 0.2% cholesterol hemisuccinate, and 2 mg/ml iodoacetamide, for 1 h at 4 °C. The insoluble material was removed by ultracentrifugation at 180,000 × g for 20 min, and incubated with TALON resin (Clontech) for 30 min. The resin was washed with ten column volumes of buffer, containing 20 mM Tris--HCl, pH 7.5, 500 mM NaCl, 0.1% LMNG, 0.01% CHS, and 15 mM imidazole. The receptor was eluted in buffer, containing 20 mM Tris--HCl, pH 7.5, 500 mM NaCl, 0.01% LMNG, 0.001% CHS, and 200 mM imidazole. TEV protease was added to the eluate, and the mixture was dialysed against buffer (20 mM Tris--HCl, pH 7.5, 500 mM NaCl) containing 20 mM Tris--HCl, pH 7.5, 150 mM NaCl, 0.01% LMNG, and 0.001% CHS. Peak fractions were concentrated to 40 mg ml--1 using a centrifugal filter device (Millipore 50 kDa MW cutoff), and frozen until crystallization. ET-3 or IRL1620 was added to a final concentration of 100 μM, during the concentration.

Cryocrystallization. The purified receptors were reconstituted into molten lipid (monolein and cholesterol 10:1 by mass) at a weight ratio of 1:1.5 (protein:lipid). The protein-laden mesophase was dispersed into 96-well glass plates in 30 nl drops and overlaid with 800 nl precipitant solution, using an LCP dispensing robot.
LgBiT-βarr1, into HEK293A cells by the PEI method (1 μg ETR-SmBiT plasmid, 0.5 μg LgBiT-βarr1 plasmid, and 25 μl of 1 mg/ml PEI solution per 10 cm diameter dish). After a one-day culture, the transfected cells were harvested with EDTA containing Dulbecco’s phosphate-buffered saline and resuspended in 10 ml of HBSS containing 5 mM HEPES and 0.01% BSA (BSA-HBSS). The cell suspension was seeded in a 96-well white plate at a volume of 80 μl per well and loaded with 20 μl of 50 μM coelenterazine (Carbosynth), diluted in BSA-HBSS. After an incubation at room temperature for 2h, the background luminescence signals were measured using a luminescent microscope reader (SpectraMax L, Molecular Devices). The fold-change values were obtained above.

Data availability

The atomic coordinates and structure factors of the ETB receptor have been deposited in the Protein Data Bank (PDB) [www.pdb.org] with accession codes 6GK (ET3-bound) and 6GL (IRL1620-bound). The raw X-ray diffraction images are also available at SBGrid Data Bank (https://data.sbgdr.org/) with IDs 611 and 612, respectively. Other data are available from the corresponding authors upon reasonable request.

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