3 RESULTS

3.1 Ligand-Binding Studies at Endothelin-Receptor Subtypes

3.1.1 Structurally Different Entrances to the Binding-Cleft at ETA / ETB

As evidenced from studies involving a large number of other members of G protein-coupled receptors, the transmembrane regions of both endothelin-receptors subtypes are highly conserved, including the regions potentially involved in ligand binding.

In contrast, the extracellular regions reveal a high degree of amino acid variations, which could be responsible for the selectivity in binding various peptide ligands. The most striking difference is an insertion of a mostly hydrophilic stretch of 5 amino acid residues (Asp149-His150-Asn151-Asp152-Phe153 in human) into the first extracellular loop at ETA (Fig. 3-1).

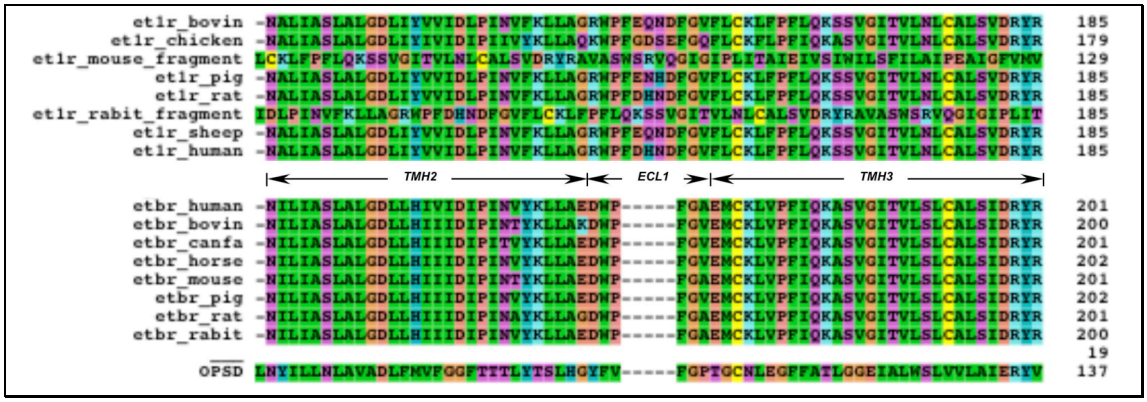

Figure 3-1: **Multiple sequence alignment of ETA and ETB focusing on the extracellular loop ECL1. While the transmembrane helices of ETA and ETB are highly conserved, their loop regions vary. Of particular importance in these variances is the insertion of five mostly hydrophilic residues into the extracellular loop ECL1 of ETA. It results in a differently shaped and functionalized loop structure in comparison to the shorter ECL1 of ETB.**

In addition, in ETA the sequence of the N-terminus in close proximity to ECL1 displays a mainly hydrophilic region (His66-Asn67-Tyr68-Cys69-Pro70-Gln71-Gln72-Thr73-Lys74-Ile75-Thr76-Ser77) compared to the mainly hydrophobic residues at the corresponding region in ETB (Pro87-Pro88-Pro89-Cys90-Gln91-Gly92-Pro93-Ile94-Glu95-Ile96-Lys97-Glu98). According to comparative models, the different interacting sequences between ECL1 and the N-terminal tail determine different conformations in this region in ETA and ETB.

A larger and more upright ECL1 in ETA could result in additional side chain interactions of ECL1 with the N-terminal part close to the membrane surface. In ECL2 a major difference between ETA and ETB may result from different locations of a proline residue, which is found either before (Pro228 in ETA) or after (Pro259 in ETB) the central cysteine (Cys239 in ETA and Cys255 in ETB). Additionally, two residues (Thr263-Ala264) are inserted in ECL2 in ETB, but not in ETA. As a result, the slight different ECL2 conformations seem to complement the differences in ECL1 at both receptor subtypes. Finally, the conformational

differences in the interactions between N-terminus and ECL1, flanked by slightly changed conformations of ECL2, result in different shapes of the entrances towards the ligand-binding clefts in between the transmembrane helices. Our models provide support for a somewhat narrower tunnel-shaped entrance in ETA and a widened funnel-formed entrance in ETB (scheme of model in Fig. 3-2, model in Fig. 3-4).

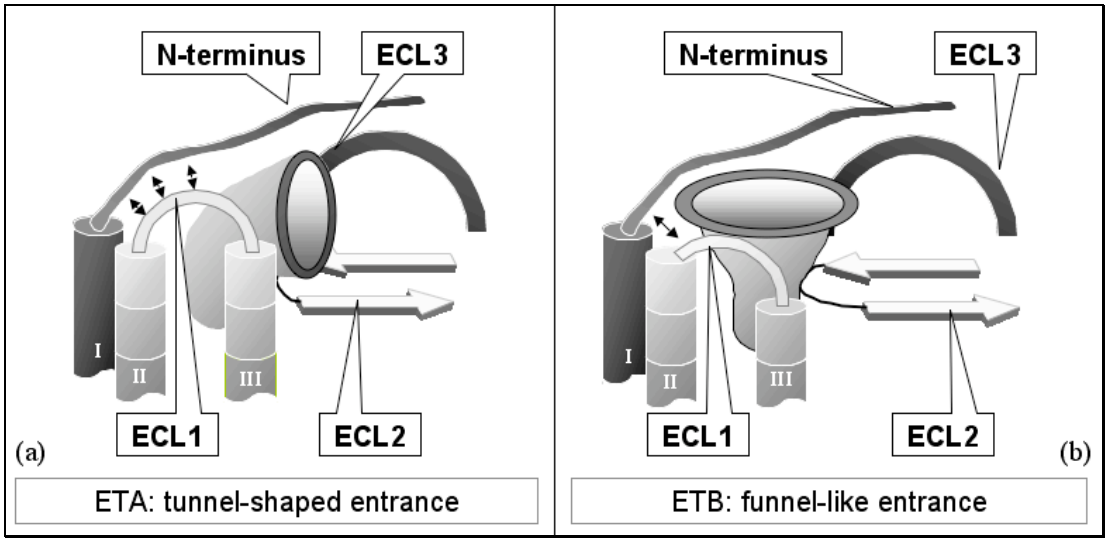

Figure 3-2: **Differences in the entrances to the ligand-binding clefts for ETA and ETB. (a) The more upright ECL1 in ETA leads to more interactions with the N-terminus resulting in a tunnel-shaped entrance. (b) Less interactions between the extracellular structure portions result in a rather widened funnel-like entrance to the ligand-binding cleft in ETB.**

3.1.2 Dissecting Ligands Into 4 Regions That Explain Binding and Selectivity

The peptide ligands of Tab. 3-1 were studied with respect to subtype-selectivity and binding affinity. In structure-function relationship studies the sequences and structures of peptide ligands were compared with their specificity for ETA and ETB as well as their effects on receptor activation (see also own preparatory work in Fig. 1-2). This lead to an initial separation into three different parts: an N-terminal addressor (residues 1-7), a hydrophilic region (8-10) and a C-terminal modulator region (11-21). Already short ligand constructs, containing residues 16 to 21, are sufficient to inhibit endothelin receptors (Rovero *et al.*, 1998), while the importance of residues 11-15 remained unclear. After MD simulations for ligand docking, the relevance of these five residues was recognized as essential for a hydrophobic core. It interacts with defined hydrophobic portions at the receptors (*e.g.* in ETA the aliphatic parts of Lys74 and Arg145, plus Trp146, Phe148, His150). As a result, we subdivided the peptide ligands into four regions: Addressor (1-7), hydrophilic hook (8-10), hydrophobic core (11-15), and modulator region (16-21) (Tab. 3-1, Fig. 3-3).

Table 3-1: Alignment of investigated endothelin-receptor peptide ligands, which stimulate strong (S), weak (s) or inhibit (i) the receptor-subtypes. The four regions that were assigned to the peptides by function are shown at the bottom of the table. The color codes (orange – addressor, magenta – hook, green – hydrophobic core, blue – modulator) are used for easier understanding of the figures of ligand binding at the receptors.

Ligand Name	Ligand Sequence (5 10 15 20)	Effect on ETA	Effect on ETB	References
ET-1	C S C S S L M D K E C V Y F C H L D I I W -OH	S	S	Yanagisawa *et al.*, 1988
ET-2	C S C S S W L D K E C V Y F C H L D I I W -OH	S	S	Yanagisawa & Masaki, 1989
Sfx6b	C S C K D M T D K E C L Y F C H Q D V I W -OH	S	S	Aimoto *et al.*, 1990
ET-3	C T C F T Y K D K E C V Y Y C H L D I I W -OH	s	S	Yanagisawa & Masaki, 1989
Sfx6c	C T C N D M T D E E C L N F C H Q D V I W -OH	s	S	Williams *et al.*, 1991
[Ala^{3,11}]ET-1	C S A S S L M D K E A V Y F C H L D I I W -OH	S	S	Randall *et al.*, 1989
[Ala^{1,15}]ET-1	A S C S S L M D K E C V Y F A H L D I I W -OH	s	S	Randall *et al.*, 1989
[Ala^{1,3,11,15}]ET-1	A S A S S L M D K E A V Y F A H L D I I W -OH		S	Saeki *et al.*, 1991
[Ala^{11,15}]ET-1(6-21)	L M D K E A V Y F A H L D I I W -OH		S	Saeki *et al.*, 1991
BQ-3020	Ac I M D K E A V Y F A H L D I I W -OH		S	Peter & Davenport, 1996
[Ala^{11,15}]ET-1(8-21)	D K E A V Y F A H L D I I W -OH		S	Saeki *et al.*, 1991
[Ala^{11,15}]ET-1(10-21)	E A V Y F A H L D I I W -OH		s	Saeki *et al.*, 1991
IRL-1620	Suc D E E A V Y F A H L D I I W -OH		S	Takai *et al.*, 1992
PD 142893	Ac *F L D I I W -OH	i	i	Doherty *et al.*, 1993
IRL-1038	A V Y F A H L D I I W -OH		I	Urade *et al.*, 1992

Addressor | Hook | Core | Modulator

*F = D-Diphenylalanine

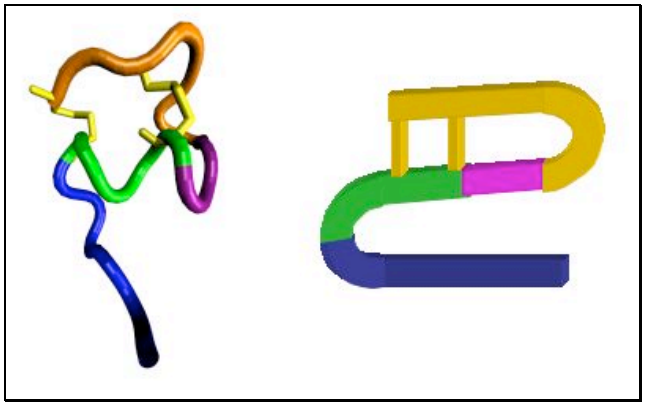

Figure 3-3: Assignment of the 4 regions of peptide ligand ET-1. Left side: The structure from PDB entry 1V6R. Right side: A cartoon of peptide ligands based on structural data. The colors code the addressor region (orange), hook region (magenta) and hydrophobic core (green) as well as the modulator region (blue).

3.1.3 Characterization of 4 Ligand-Complementary Regions at ETA and ETB

Analogous to peptide ligands, in both endothelin receptors four structural regions involved in ligand binding could be differentiated: an extracellular gateway, an edge, and a neck region forming the entrance to the transmembrane binding cleft (Fig. 3-4, 3-5). They are complementary to the four regions identified in the peptide ligands.

The gateway for ETA is composed of residues from the N-terminus (Cys69-Pro70-Gln71-Gln72), ECL1 (Phe148-Asp149), ECL2 (Gln235, Thr244-Ser245-Lys246) and ECL3 (Met336-Asp337, Arg340, Glu342, Leu347). Likewise, Cys90-Gln91-Gly93-Pro93-Ile94-

Glu95-Lys96 in the N-terminus, Glu165-Asp166-Trp167 in ECL1, Val260-Gln261 in ECL2 and Arg357-Cys358 in ECL3 construct the gateway in ETB.

The edge, formed by charged residues, is located in the inner rim of the tunnel in ETA (Asn151 in ECL1 and Glu230, Arg232, Gln235 in ECL2) and in the inner rim of the funnel in ETB (Glu95, Glu98 in the N-terminus, Glu165-Asp166 in ECL1 and Asp246, Lys248, Gln261 in ECL2).

The neck connects the entrance to the transmembrane binding cleft and is formed by mainly hydrophobic and aromatic residues in the N-terminus (Lys74), ECL1 (R145, Trp146, Phe148, His150), TMH3 (Phe153) and ECL2 (Arg232-Gly233, Thr244) in ETA. In ETB, residues in ECL1 (Trp167-Phe168), TMH3 (Phe169, Ala171) and ECL2 (Tyr247-Lys248, Tyr251-Leu252, Pro259-Val260) are involved.

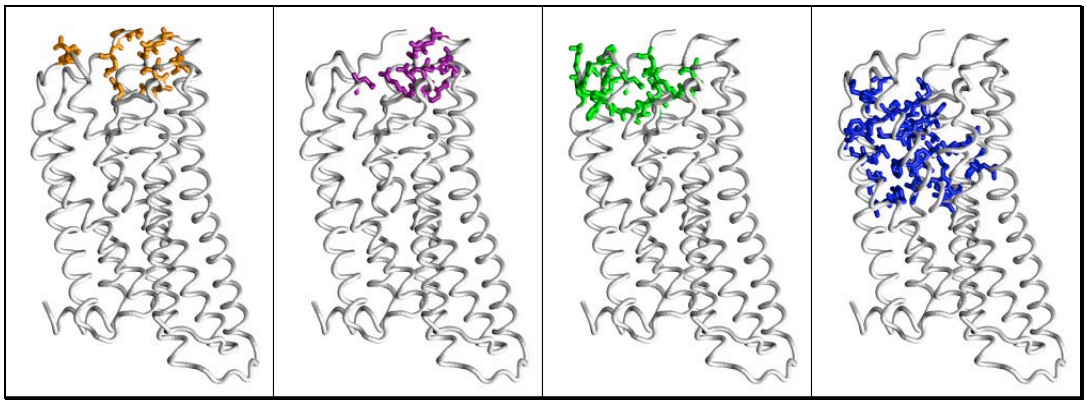

Figure 3-4: Ligand-complementary regions of the endothelin receptors. The gateway- (orange), edge- (magenta) and neck-region (green) as well as the transmembrane binding cleft (blue) are highlighted in ETA.

Docking studies, supported by constrained molecular dynamics (MD) simulations, allowed an assignment of the four regions of peptide ligands (addressor, hook, hydrophobic core and modulator) to the four corresponding structural portions at the receptor side (Fig. 3-4, 3-5). Thereby, evidence for ligand selectivity and for mechanisms of initial receptor activation/inhibition was provided as well.

3.1.4 Structure-Function Relationships of Endothelin Receptor-Ligand Interactions

Following the classical principle of receptor hormone interactions via `address` and `message` epitopes (Hechter and Calek, 1974) and based on dissected complementary regions at receptors and ligands as well, new structure-function-relationships for the interaction between peptide ligands of endothelin receptors are presented and separated for the interacting ligand-receptor regions.

Addressor – Gateway

The addressor region of peptide agonists determines their endothelin-receptor selectivity

The addressor region (ligand residues 1 through 7), which is the least conserved region in naturally occurring endothelin agonists, is involved in selective binding to endothelin-receptor subtypes, as well as the formation of the quasi-irreversible binding of ET-1 at ETB. The participation of selective binding to Endothelin receptors is highlighted by several line of evidence. For example, peptides lacking the addressor region bind to ETB with the same or only slightly reduced affinity, but with a 100- to 10,000-fold lower affinity to ETA. Structural data suggest that for binding of endothelins to ETA an anti-parallel orientation of the N-terminal stretch to the helix of the hydrophobic core is required. This is achieved by the formation of two disulfide bridges (Cys1-Cys15 and Cys3-Cys11, see structures in PDB entries: 1EDN, 1EDP, 1V6R, 1SRB) (Andersen *et al.*, 1992; Atkins *et al.*, 1995; Janes *et al.*, 1994; Takashima *et al.*, 2004a). The comparison of sequences of peptide ligands (see Tab. 3-1) with their endothelin receptor-selectivity shows that any difference of the three N-terminal residues from the ET-1 sequence results in decreased affinity for ETA and a more ETB-selective binding. Additionally, modifications of residues 4 to 7 within the addressor are further changing the extent of selectivity of peptide ligands most probably by changing the conformation of the addressor's loop.

An increasing reduction of the disulfide-linkage shifts the selectivity more and more to ETB. Peptides lacking the addressor are ETB-selective ligands. From that it follows, that an optimal addressor region for ETA-selective interactions is composed of (i) the 3 N-terminal residues Cys1-Ser2-Cys3 that (ii) have to be in an anti-parallel orientation closely connected to the hydrophobic core region, and (iii) the residues 4 through 7 have to connect this stretch to the hook region by a rather restrictive fold.

The gateway region of ETA is very restrictive in size and properties

The gateway at the narrow tunnel-shaped entrance of ETA functions as the selector for the addressor region of peptide ligands (Fig. 3-5a). Its narrow shape and the coated properties force the ligands into a defined orientation. The small hydrophilic side chains of the addressor region of ET-1 fit to this environment and point between the N-terminal tail, ECL2 and ECL3 of ETA (Fig. 3-5c). In contrast, the broad and funnel-like gateway at the entrance of ETB (Fig. 3-5b) accepts peptide ligands with a large N-terminal variety of sequence and structure (Fig. 3-5d).

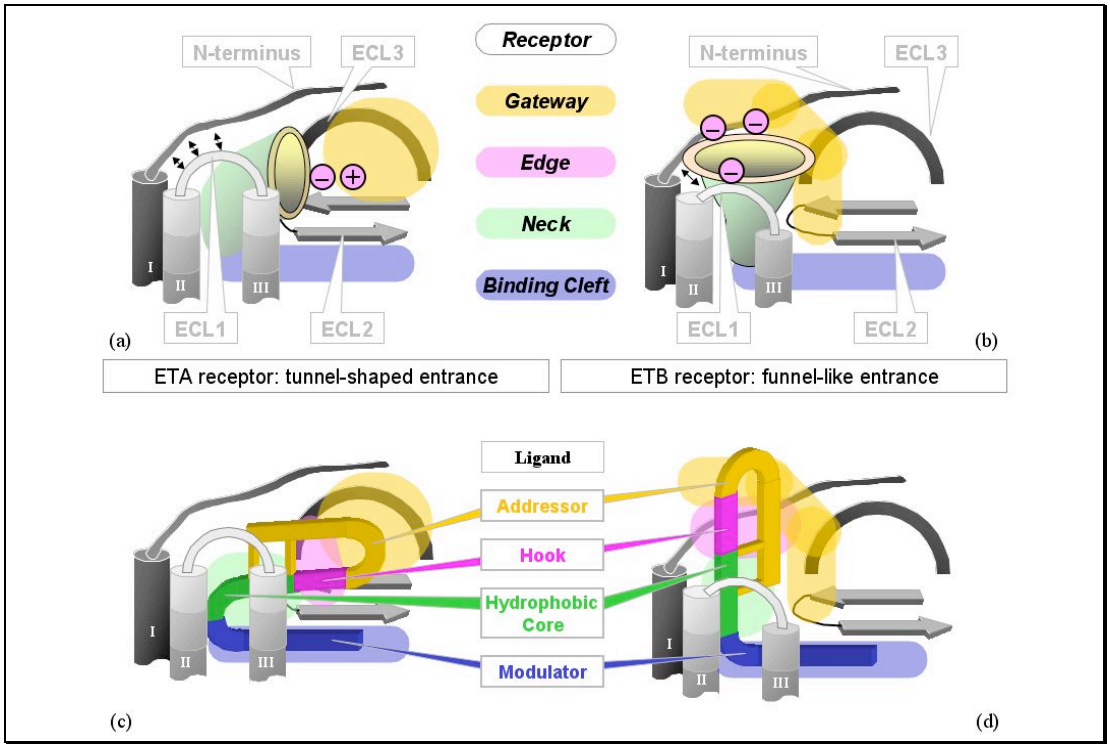

Figure 3-5: **Schematic view of the binding of peptide ligands at the endothelin-receptor subtypes A and B using the different entrances to their binding sites. The tunnel-like binding site of ETA accepts with its defined patterns of charges and shapes only a small variety of ligands. Because of this, the edge-region that separates peptide agonists from peptide antagonists is restricted in size and position to two contrarily charged residues on top of ECL2 (a). ETB, on the other hand, has a broad and funnel-like entry to its binding site. Separation of peptide agonists from peptide antagonists has to be more flexible. By using a common repulsive force of different negatively charged residues spread on N-terminus and ECL1 as well as ECL2 this occurs (b). At both receptor subtypes the binding sites are separated into 4 regions, which are complementary in shape and properties to 4 regions within the ligand peptides.**

More flexible side chains are responsible for quasi-irreversible binding of ET-1 at ETB.

Another phenomenon in binding to Endothelin receptors is the quasi-irreversible binding of ET-1 at ETB. Even in this case the addressor region has to be relevant, because ET-2 in ETB is more reversible in binding than ET-1 in ETB, but varies only in this region. For ETB, the gateway region includes an area below the N-terminus that is optimized for interaction with a small aliphatic side chain (*e.g.* Leu6 in ET-1). The modification of this ligand position to a residue with a larger, more bulky side chain (*e.g.* Trp6 in ET-2) results in somewhat disrupted interactions that could explain the less irreversible binding of ET-2 in ETB.

Hook – Edge

Three charged residues determine agonists but not antagonists

The hook region (ligand residues 8 to 10) is a hydrophilic pattern that is common to all native and synthetic peptide agonists, but it is not found in the antagonist IRL1038 (see Tab. 3-1). Three charged residues form the hook and have effects in binding to ETA and ETB. Modifications of each of the residues Asp8, Lys9 or Glu10 in ET-1 to amino acids with comparable length but altered electrostatic properties resulted in a loss of function

(Asp8→Asn, Asp8→Lys, Glu10→Gln, Glu10→Lys) or changed endothelin receptor-selectivity (Lys9→Glu) (Ergul *et al.*, 1995; Nakajima *et al.*, 1989b; Watanabe *et al.*, 1991). Asp8 is the most important residue in the hook region and crucial for ligand binding and receptor activation. The negative charge in position 8 appears to be involved in the activation of endothelin receptors, as replacement of Asp8 by Ala decreases its activity but not the binding affinity (Tam *et al.*, 1994). In contrast, the positive charge in position 9 is not essential for receptor binding and activation. This is demonstrated by the fact that modifications of the amino group of Lys9 by biotin- and photo-labels (*e.g.* Cy3) did not alter the binding to ETA and ETB (Tab. 3-2). However, position 9 may be involved in endothelin receptor-selectivity, because a negative charge is found in IRL1620 and Sf6c, the two most selective agonists for ETB. The replacement of Glu10 by alanine did not alter binding to ETA and ETB, which underlines the importance of the residues in position 8 and in position 9.

Table 3-2: Comparison of binding data on endothelin-receptor peptide ligands and their derivatives containing Cy3-photo label attached to Lys9 residue. Experimental data kindly provided by Alexander Oksche (Institut für Pharmakologie Charité - Campus Benjamin Franklin, 14195 Berlin, Germany).

Ligand	K_i for Binding at ETA	K_i for Binding at ETB
ET-1	0.230 nM	0.068 nM
Cy3-ET-1	0.045 nM	0.036 nM
BQ3020	> 40 nM	3 nM
Cy3-BQ3020	> 36 nM	7.9 nM

Taken together, the mutational data and sequence alignment observations suggest that the presence and absence of a negatively charged hook region distinguishes between the agonistic and antagonistic activity of the modulator regions. In both receptor subtypes, charged residues in the outer rims of the entrances act as an edge (Fig. 3-5a, 3-5b). The charged hook region, the most protruding part of the agonistic peptide structure located between the N-terminal addressor and the central helix of the hydrophobic core (Fig. 3-3), prevents agonists from sliding beyond the edge further into the receptor.

Resulting from the different gateway regions of the receptor subtypes, the ligands bind in different orientations. This is nicely accepted by the different orientations of the hook regions: in ETA the hook is positioned at ECL1 (Asn151) and ECL2 (Glu230, Arg232, Gln235) whereas in ETB the hook is surrounded by all extracellular regions of the neck region: the N-terminus (Glu95, Glu98), ECL1 (Glu165-Asp166) and ECL2 (Asp246, Lys248, Gln261). Although both receptors use a combination of positive and negative charges as well as hydrophilic residues, the amount of negative charges in usage at ETB is remarkably higher, allowing different binding modes for the peptide ligands at both receptors. During ligand binding the agonist's hook will be caught by charges of the receptor's edge. The negative

charges in the hook region of agonists, in particular Asp8, anchors the ligand at the edge region of the receptor and, thereby, gives restraints to the modulator region. The modulator region may then adopt a conformation that allows the activation of the endothelin receptors. In contrast, in the antagonist IRL1038, lacking the anchor region, the correct positioning and folding of the modulator region is not achieved, so that no activation occurs.

Hydrophobic Core – Neck

The naturally occurring Endothelin receptor agonists and the peptide derivatives both have the hydrophobic core (ligand residues 11-15) in common. Variations in the amino acid sequence in this region are very small and are only of conservative nature. However, it is much more likely that variation in the amino acid composition of the addressor region (N-terminal part of endothelins and sarafotoxins) account for the receptor selectivity (see Tab. 3-1). The hydrophobic core also comprises cysteines Cys11 and Cys15, which are involved in the formation of two disulfide bonds (Andersen *et al.*, 1992; Atkins *et al.*, 1995; Janes *et al.*, 1994; Takashima *et al.*, 2004a). While the replacement of these cysteine residues by alanine dramatically reduces the binding to ETA, it does not affect the binding to ETB. It is notable that native peptides and peptide derivatives, in which cysteine residues were replaced by alanine, display a helical structure (Atkins *et al.*, 1995; Katahira *et al.*, 1998). Thus, the residues 12, 13 and 14 (in ET-1: Val12–Tyr13–Phe14) are oriented in a way for optimal receptor interaction (in ETA: Lys74 (TMH1), Ala143 (ECL1), Gly144 (ECL1), Arg145 (ECL1), Trp146 (ECL1), Phe148 (ECL1), His150 (ECL1), in ETB Asp166 (ECL1), Trp167 (ECL1), Phe168 (ECL1), Gly170 (ECL1), Ala171 (ECL1)). As a consequence, modifications in this ligand region altering the hydrophobic environment lead to complete loss of ligand binding, as found for alanine replacements of Val12, Tyr13 or Phe14 (Tam *et al.*, 1994).

Modulator – Binding Cleft

The C-terminal modulator region (ligand residues 16 through 21) is highly conserved in all natural occurring agonists of the Endothelin receptor subtypes and also common in all peptide derivatives. Several residues (Asp18, Ile20, and Trp21) of this region were shown to affect the strength of binding (Tam *et al.*, 1994). Pivotal roles for signal transduction have the hydrophobic residues Ile20 and Trp21 as well as the negatively charged residue Asp18 and the carboxyl group at the C-terminus. Mutations and deletions of Trp21 abolish the interactions with the receptors (Nakajima *et al.*, 1989a; Tam *et al.*, 1994). Similarly, amidation of Trp21 abolishes ligand/receptor interactions (Rovero *et al.*, 1998). The likely

interactions of the modulator domain are difficult to assess, as no consistent structural data on the fold of the modulator region is available. In early NMR data of ET-1 (PDB entry: 1EDP) the structures of the C-terminal sequences could not be solved (Andersen *et al.*, 1992). X-ray crystallographic data of ET-1 (PDB entry: 1EDN) report a helical structure of the C-terminal chain, but since the crystal structure shows a parallel dimer along the C-terminal helices, the formation of this helix might be induced and an artifact of the particular crystal measured (Janes *et al.*, 1994).

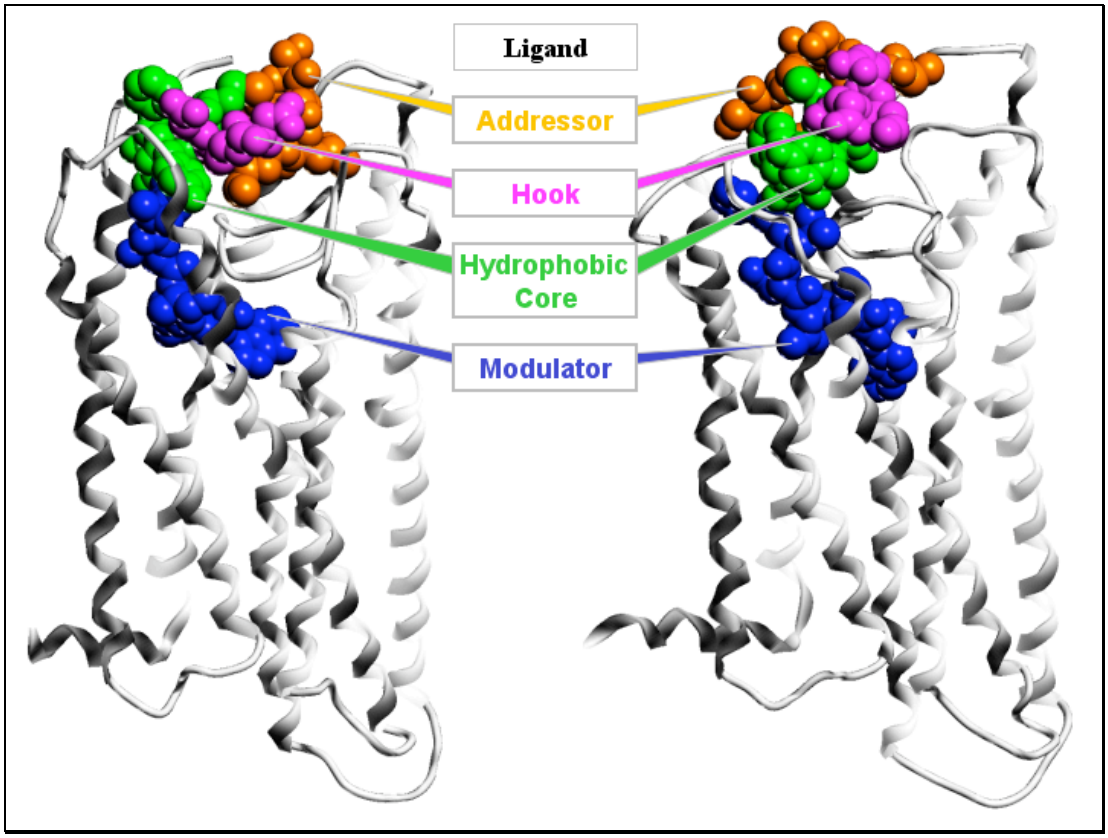

Figure 3-6: **The docked ET-1 ligand at both endothelin-receptor subtypes. At ETA (left) the addressor- and hook-region lay on top of ECL2; the hydrophobic core follows this parallel orientation regarding ECL2; the C-terminus – bent backwards below ECL2 – resembles somewhat the structure of endothelin ET-1 in PDB entry 1V6R. At ETB (right) addressor-, hook-, hydrophobic core- and modulator-region take a rather upright position into the receptor with a perpendicular orientation to ECL2. Thereby the structure of ET-1 resembles the structural data from sarafotoxin Sfx6b from PDB entry 1SRB.**

Later on, NMR structures of ET-1 (PDB entry: 1V6R) revealed an extended β-structure orienting the C-terminal residues Leu17 and Ile19 towards the central helix by forming a hydrophobic core around side chain Tyr13 (Takashima *et al.*, 2004a; Takashima *et al.*, 2004b). The NMR structure for Sfx6b (PDB entry: 1SRB) shows a rather high flexibility in the C-terminal residues representing the modulator region, which impedes the hydrophobic core around Tyr13 (Atkins *et al.*, 1995). Concluding, the C-terminal chain may adopt different spatial orientations, allowing the ligand to fit with its modulator region to the differentially shaped binding cleft in the two endothelin receptor subtypes (Fig. 1-2, 3-5, 3-6).

In our MD simulations, the binding of peptide ligands in endothelin receptors uses an induced-fit mechanism. Receptor and peptide ligand herein undergo slight changes in their conformations to find best match of their complementary regions. As a result, the conserved C-terminal modulator region of peptide ligands fits into the transmembrane binding cleft in the endothelin receptors and finds many conserved interaction partners. The potential interaction partners are tabulated in Tab. 3-3.

We found that this transmembrane binding cleft, which is located between TMH 2, 3, 5, 6 and 7, is large and contains an overlapping antagonistic and agonistic binding site. Although the difference between agonistic and antagonistic action of the ligands is determined by the presence and lack of the peptide's hook region, activation and inhibition transmit via the modulator.

Table 3-3: Interaction partners of the C-terminal modulator region of endothelin-receptor agonistic peptide-ligands at both receptor subtypes.

Peptide Residue	Interaction Partner at		Ballesteros' Numbering	ET-Subtype Specific Differences
	HETA	HETB		
His16	Asn83	Asn104	1.35	
	Tyr352	Tyr369	7.36	
Leu17	Gly154	Gly170	3.21	
	Val155	Ala171	3.22	◆
	Leu157	Met173	3.24	◆
	Cys158	Cys174	3.25	
Asp18	Lys80	Lys101	1.32	
	Lys140	Lys161	2.64	
Ile19	Phe161	Val177	3.28	◆
	Pro162	Pro178	3.29	
Ile20	Leu322	Leu339	6.51	
	Ile355	Ile372	7.39	
	Ala358	Ala375	7.42	
Trp21 (indole moiety)	Phe264	Phe282	5.47	
	Trp319	Trp336	6.48	
Trp21 (carboxyl group)	Lys166	Lys182	3.33	

We suggest that capturing of the hook region by the edge at the rim of the entrance prevents agonistic peptide ligands from sliding down deeper into the transmembrane binding cleft. Simultaneously, this restrains the C-terminal residues in a proper position for activation by forcing reorientations of receptor side chains, *e.g.* Lys3.33 and Trp6.48 (Fig. 3-7a).

On the other hand, peptidic antagonists such as IRL1038 that lack the hook region slide deeper into the binding cleft beyond the edge, and thus orientate the C-terminal residues Ile20 and Trp21 at an analogous position like the retinal in rhodopsin. At this location the peptide antagonists constrain the inactive state of the receptors by additional interactions such as with Phe5.47 and Ala7.42 (Fig. 3-7b). Thereby, the modulators orientation resembles those of 11-cis-retinal in rhodopsin (Fig. 3-7c).

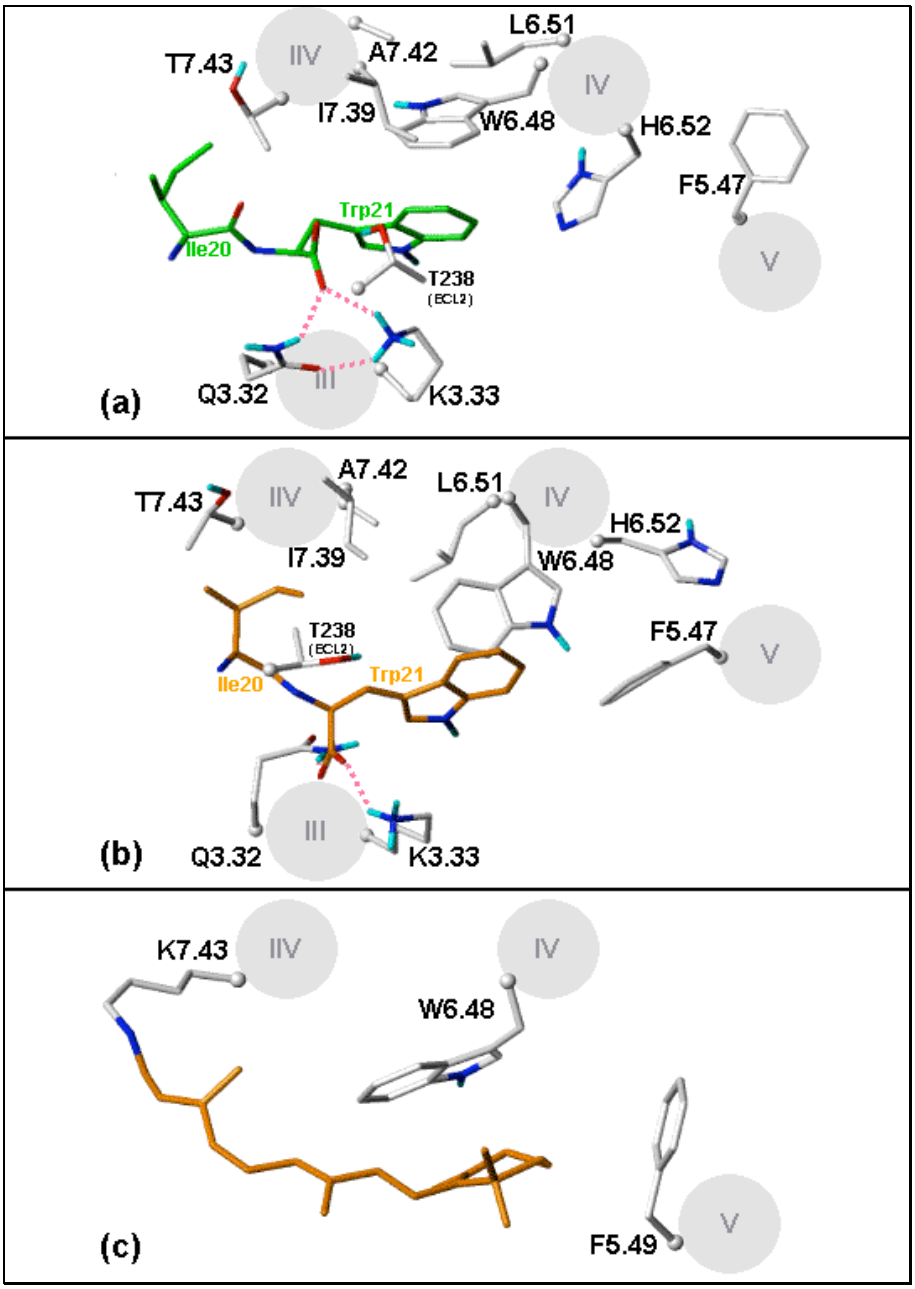

Figure 3-7: **Differences in binding mode of endothelin-receptor peptide ligands. The positions of the C-terminal residues Ile20 and Trp21 of agonists (a) and antagonists (b) are compared to the binding mode of 11-cis-retinal in bovine rhodopsin from PDB entry 1HZX (c). 11-cis-retinal – an inverse agonist restrains the inactive state of rhodopsin amongst others by interactions of its cyclohexene ring with Trp6.48 and Phe5.49. The C-terminus of Endothelin receptor peptide antagonists (b) remain in comparable position. It stabilizes the inactive state of the receptor among other things by interaction of the indol ring of Trp21 with Trp6.48 and Phe5.47. Induced by the hook-edge interactions agonists stay in a position into the binding cleft that is shifted to the left (a). Because of this the indol ring and free carboxylic group of Trp21 put pressure on Trp6.48 and Lys3.33, respectively, to reorient.**