4 DISCUSSION

4.1 Ligand Binding at Endothelin Receptor Subtypes

Although both endothelin receptor subtypes ETA and ETB bind the endothelin isopeptides ET-1, ET-2 and ET-3, their selectivity for these ligands (and other peptide ligands such as sarafotoxins) is different. ETA, which is also known as the ET-1 receptor, favors binding of ET-1 and ET-2, whereas its affinity for ET-3 is 100-fold lower. On the other hand, the ETB or unselective endothelin receptor, binds all endothelin isopeptides with similarly high affinities. Generally, native peptide ligands that interact with endothelin receptors are target for ETB but only few also for ETA. Since the first citation of ET-1 in 1988, several investigations of endothelins and the similar sarafotoxins, as well as their derivatives, were made (Itoh *et al.*, 1988). While N-terminal modifications (substitutions, deletions, changes of the disulfide linkage) severely affect the binding to ETA, modifications of the C-terminus reduce or abolish interactions to both receptor subtypes. Due to that, Menziani and colleagues concluded that the C-termini of peptide ligands are "highly important in receptor recognition and signal transduction" by interaction with the transmembrane binding cleft. On the other hand, the peptides' N-termini "might bind to residues located at the entrance of the binding cleft conferring high affinity and selectivity to the peptide" (Menziani *et al.*, 1995). But the molecular reasons of peptide ligand selectivity at ETA and ETB, as well as the lack of ETA selective peptide ligands, remained unclear.

An additional feature of many ETBs (*e.g.* human, bovine) are their quasi-irreversible binding of ET-1 by formation of a super-stable complex, which remains intact even in the late endosomes (Oksche *et al.*, 2000; Takasuka *et al.*, 1994). In other animals (such as rabbit) the interaction with ET-1 is reversible. In human ETB, mutational studies revealed an N-terminal region close to transmembrane helix TMH1, that is important in formation of this super-stable complex with ET-1, but the mechanism itself is unknown so far (Takasuka *et al.*, 1994).

Moreover, the binding affinities of peptide ligands such as ET-1 (0.01-0.06 nM) are remarkably higher than the binding affinities of small molecules such as Bosentan (4.7 nM) (Clozel *et al.*, 1994; Gregan *et al.*, 2004). These facts motivated us to research the molecular details of binding and initial activation of peptidic ligands such as ET-1 or IRL1620. This knowledge has a vital importance for designing small ligands. Such ligands, able to reach additional binding regions in endothelin receptors, may achieve binding affinities comparable to ET-1.

The modeled structures of endothelin receptor subtypes ETA and ETB are N-terminally, as well as C-terminally, shortened due to missing homology in these regions. Experimental results taken from the literature show that N-terminally and C-terminally truncated ETBs are

still effective in ligand binding and G protein-interaction (Grantcharova *et al.*, 2002; Horstmeyer *et al.*, 1996; Okamoto *et al.*, 1997). Due to that and the high sequence similarity between ETA and ETB, their model structures are a good basis for structural investigations.

Different entries into the binding site at ETA and ETB

At the extracellular entrance to the binding cleft, our data provides support for structural differences in ETA and ETB. The transmembrane regions of the endothelin receptor subtypes are highly conserved, whereas the extracellular areas show obvious differences between both receptor subtypes. The most remarkable difference in ETA, compared to its relative ETB, is the insertion of 5 additional hydrophilic residues into the C-terminal neighborhood of ECL1 (Fig. 3-2). This, and a more hydrophilic N-terminal stretch in close proximity to this extension, results in many interactions between the ETA's N-terminus and ECL1. In contrast, the shorter ECL1 in ETB, as well as a rather hydrophobic N-terminus in this region, have only few contacts. This hardly changes the extracellular structures of ETA and ETB. Slight changes in ECL2 conformation, by the use of different positions of proline residues, complete the different extracellular entrances to the binding clefts. This may explain the different results in ligand binding in both receptor subtypes. A narrow tunnel-shaped entry in ETA is functioning as a selector for the ligand's addressor region, whereas a broad funnel-formed entry at ETB is rather unselective for the addressor region of the ligands (Fig. 3-5).

Dissecting ligands and receptors into four regions

Our major finding is the dissection of four peptide ligand regions, and the delineation of four structural portions of the endothelin receptor binding sites, allowing their assignment by complementary interacting shapes and properties. Based on structure-function relationships we dissected the sequence of linear peptide ligands into four regions, namely the addressor-, hook-, hydrophobic core- and modulator-region (Tab. 3-1, Fig. 3-3, 3-6). Additionally, we found out that the receptor-binding site can be divided into four structural portions: extracellular gateway, edge- and neck-region and a transmembrane binding cleft (Fig. 3-4, 3-5). Moreover, our data delineate structural determinants that can explain (i) the peptide ligand selectivity of ETA and ETB, (ii) initial mechanisms of receptor activation/inhibition and (iii) the quasi-irreversible binding of ET-1 to ETB as well.

The Addressor of ET-1 makes it a specialist for interaction at ETA

The endothelin receptors' entrance to the binding site, composed of the gateway, edge and neck, allows separation of endothelin-selective ligands from other unrelated peptides, as well

as exclusion of several endothelin-isopeptides. In ETA, the tunnel-shaped entrance is restrictive to size, shape, orientation and electrostatic properties of potential ligands (Fig. 3-5, 3-6). In docking procedures, using constrained MD simulations with followed constrain-free relaxation, we found that in ETA the addressor of ET-1 favors an orientation between ECL2 and ECL3, below the N-terminus. This is possible due to its five N-terminal residues containing small sidechains. A more flexible N-terminus (*e.g.* in [1,15]Ala-ET-1, [3,11]Ala-ET-1) or residues with large side chains in this peptide region (*e.g.* Ser4→Phe in ET-3 or Ser4→Lys, Ser5→Asp in Sfx6b) lead in a slightly changed binding conformation and decreased affinity at ETA.

Based on NMR experimental data of the structure of IRL1620 (Katahira *et al.*, 1998), which is mainly α-helical, we hypothesized that any peptide ligand, without the disulfide-linkage, is structured this way. This structure, sterically hindered for the restrictive entrance in ETA, leads to reduced affinity in binding to ETA.

Poor recognition of ligands' addressor regions at ETB

In contrast, ETB has a funnel-formed entrance, allowing more N-terminal modifications in shape, size, orientation, electrostatic properties and even flexibility of potential peptide ligands (Fig. 3-5, 3-6). Due to that, ETB unselectively binds all endothelin-receptor selective peptide ligands. In addition, it explains why no selective peptide agonist is known for ETA.

ETB covers ET-1 in binding and holds it tight by offering a special hydrophobic spot

Also the quasi-irreversible interaction of ET-1 with many ETBs (*e.g.* human, bovine) is determined in this interaction of addressor and gateway. The sequence-specific differences of ETB's and ETA's N-termini may result in a ligand-induced lid-conformation that covers the binding site in ETB (not shown). Takasuka and co-workers demonstrated an involvement of Asp75 and Pro93 in the formation of this super-stable ligand-receptor complex (Takasuka *et al.*, 1994). We suggest an interaction of the free N-terminal charge of ET-1 with Asp75, which induces the lid-like conformation of the N-terminus, provided by the proline-rich sequence (Pro87, Pro88, Pro89 and Pro93) around Cys90– another typical feature of ETB. Thereby, the N-terminal lid forces ET-1 into an area below it, which is optimized for interaction with the small aliphatic side chain of 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 reduced irreversibility.

A hook, grasping at the edge, separates the agonists from the antagonists

Following the 7 residues of the peptide ligands' addressor, a small region consisting of charged residues exists. This hydrophilic hook region is common to all agonistic peptides, but not to the antagonistic peptides PD142893 or IRL1038 (Tab. 3-1). While ETA-selective agonists share a conserved Asp8-Lys9-Glu10 motif, ETB-selective ones also tolerate modifications of the Lys9 with acidic residues. Nevertheless, alanine-scans of the hook region in ET-1 have shown that the most necessary residue in this region is Asp8, located at the most protruding part of the ligand. ET-1 bearing an Asp8→Ala mutation binds with wild type affinity. However, it acts more as antagonists (with a stimulatory effect of $1/100^{th}$ of wild type peptide (Tam *et al.*, 1994)). We suggest a scenario, where the hook region of agonists specifically interacts with the receptor's edge epitope. The hook is the main anchor of agonists and prevents them from sliding further into the transmembrane binding cleft.

On receptor side, the edge, a region composed of charged and hydrophilic residues, is located in the inner rim of ETA's tunnel and ETB's funnel. The edge of ETA uses a combination of different hydrophilic properties, whereas ETB presents a high number of negative charges. Experimental data from literature on ET-1 mutant Lys9→Ala shows increased ETA affinity (about 500% of ET-1), but decreased activation to 60% of wild type peptide (Tam *et al.*, 1994). This is consistent to our ligand-receptor interaction model. The increased binding can be explained by the loss of bulk, which allows the mutant a better fit into the narrow tunnel of ETA. The partial agonistic effect can be explained with the loss of positive charge, which causes a weaker interaction of the peptide's hook with the negatively charged portion of the receptor's edge. This leads to an initial slight sliding down towards the binding cleft. Asp8, the major key player for agonistic effects, is still interacting with the edge, restricting this ligand in an agonistic way and preventing an antagonistic effect. Other mutants of ET-1, where the negative charge of Asp8 is removed, support this (Ergul et al., 1995; Nakajima et al., 1989b; Watanabe et al., 1991).

Mutation of Glu10→Ala, the third hydrophilic residue of the hook region in ET-1, did not show any significant effects for binding and activation, indicating that this position is not directly involved in hook/edge interaction.

ETB uses nonselective edge patterns because of a nonselective entrance

The answer to the question, why ETB's edge uses this high amount of negatively charged residues, is: The gateway of this receptor is accepting more flexibility in the ligands' addressor. This may also result in somehow differently oriented ligands. Due to this, a common repulsive force restricts agonists from sliding down to the transmembrane binding

cleft. Glu95 and Glu98 at the N-terminus as well as Glu165 and Asp166 at ECL1 are the respective portions. In addition, ETB also provides a common motif to the native endothelin ligands: Asp246, Lys248, Gln261 at ECL2. This motif is similarly used in ETA (Glu230, Arg232, Gln235 at ECL2).

Interactions of hydrophobic core and hydrophobic neck yield energy benefits, which explain the high ligand-affinity and allows more selectivity (if necessary)

Although the entrance to the transmembrane binding clefts is differently shaped in ETA and ETB, both include a common neck region. Several hydrophobic and aromatic residues in the extracellular junction of TMH2 and TMH3 crowd this region. Due to our structural investigations we hypothesize that this region, interacting with the ligand's hydrophobic core, leads to certain energy benefits for the ligand. This might partially explain the high interaction constants of peptide ligands at endothelin receptors. In addition, this might be necessary for the repulsive hook-edge interactions, described before.

Additionally, this region may address some selectivity to the receptors, which is easy to understand by comparing PD142893 and IRL1620. IRL1620 has a hydrophobic core and interacts selectively with ETB, while PD142893, missing this region, does not. Figure 3-3 shows general building plan of endothelin receptor-selective peptides and highlights the possible reason for the additional selectivity filter of this region. Because a correctly structured addressor is covalently linked to the core by the two conserved disulfide-bridges, the core region is somehow also interacting at the gateway. Due to this, the border between gateway and neck region has to have certain patterns giving additional selectivity to ligands with a conserved core region.

The binding cleft – a commonly used ligand interaction site at GPCRs

The transmembrane area – where the activation of the receptors takes place – is very conserved in both receptor subtypes. The peptide ligand's modulator region is also highly conserved in agonists and antagonists (Tab. 3-1). Additionally, only few mutations could be shown to be relevant for ligand interaction in this area. And some of them are difficult to interpret because of ambiguous data (Tab. 2-1). Nevertheless, we identified residues in the transmembrane area of both receptors (based on known mutations) necessary for ligand binding. These constraining residues allowed an orientation of the peptide ligands, where the C-terminal residues of the modulator region are placed into the transmembrane binding cleft. In comparison with the rhodopsin-retinal complex, it is obvious that the peptide ligand's last two C-terminal residues match the interaction partners of endothelin receptors around the

binding site of retinal in rhodopsin. Trp21 and Ile20, which have been shown to be crucially relevant (Tam *et al.*, 1994), find perfect interactions.

Agonists grasp at the edge with their hook, activating the receptors

The molecular differences of peptide ligand-induced receptor activation and inhibition can be explained, according to our model, by the existence and absence of negatively charged residues at the peptide ligand's hook region.

In our suggested scenario of receptor activation, the agonist's hook region interacts with the receptor's edge epitope. This way, the ligand's modulator region is restrained in a position, where its C-terminal residue Trp21 is oriented in between transmembrane helices TMH3, TMH6 and TMH7. This causes a rotamer change of the tryptophane at position 6.48 (Fig. 3-7a) as suggested for the rotamer toggle switch of this residue during the activation of the β2-adrenergic receptor (Swaminath et al., 2005). Additionally, a dispersing of transmembrane helices TMH3 and TMH6 very likely follows this event. The negative charge of the ligand's C-terminal carboxyl group anchors the peptide to the positively charged lysine at position 3.33 (Fig. 3-7a). This is consistent with previous findings, showing that removal of the C-terminal negative charge by amidation results in abolished ligand interactions (Rovero *et al.*, 1998). Furthermore, Lys182 (position 3.33) at ETB was shown to be involved in interaction of several endothelin peptides (Lee *et al.*, 1994).

Antagonists miss the edge – they bind and function like 11-cis-retinal in rhodopsin

In the case of inhibition, the ligand lacks the charged hook region and also lacks bulky moieties at the N-terminal portion. Such an antagonistic peptide is able to slide beyond the edge down into the transmembrane binding cleft. Thereby, it additionally constrains the interactions of residues in the transmembrane helices. This stabilization of the inactive state of the receptor works as inhibition. Our models showed an orientation of the antagonist's Trp21 towards the receptors tryptophane in position 6.48 of the binding cleft (Fig. 3-7b). This way Trp21 matches the location and orientation of the cyclohexene-moiety of 11-cis-retinal in the structure of the inactive dark state of bovine rhodopsin (Fig. 3-7c).

The C-terminal hexapeptide is the true ligand of both endothelin receptor subtypes

The retinal-like orientation of the C-terminal residues within the transmembrane binding cleft explains why the C-terminal hexapeptide interacts with the Endothelin receptors also alone. It is the true ligand for endothelin receptors but because of other missing regions it is only able to function as an antagonist (such as the inverse agonist 11-cis-retinal).

Why is this C-terminal hexapeptide the smallest linear peptide molecule that is interacting with endothelin receptors? A comparison with 11-cis-retinal of bovine rhodopsin might also lead to explanation. In this complex 11-cis-retinal is oriented in the right position by Schiff-base linkage to a lysine residue in rhodopsin. In case of endothelin receptors such a linkage does not exist. As a result, the basic ligand has to consist of a certain number of residues, allowing the optimal orientation of the most necessary part: the two C-terminal residues Ile20 and Trp21. This orientation is provided by specific interactions between the other four residues of the modulator region (ET-1: His16, Leu17, Asp18 and Ile19) within the transmembrane binding cleft (Tab. 3-3).

Excursus: Is the C-terminus of endothelin peptides flexible or not?

The existence of an intramolecular, hydrophobic interaction in ET-1 between Leu17 (modulator) and Tyr13 (hydrophobic core) has been fiercely discussed based on NMR structural data. In the structure of Sfx6b this contact could not be observed, most likely because of the exchange of Leu17 (in ET-1) to Gln17 (in Sfx6b) and a reduced tendency of interaction between residues 13 and 17 in Sf6b. Based on our studies it seems reasonable that the observed hydrophobic contact might be an artifact, and the C-terminus of endothelin receptor-selective peptide ligands is more or less unstructured (Fig. 1-2). It may become reoriented in binding to the Endothelin receptors (Fig. 3-5, 3-6). Nevertheless, the tendency of Tyr13 to get covered by C-terminal residues suggests a conserved mechanism to deflect water molecules from this residue (or the whole hydrophobic core region). This necessarily results in the removal of hydrophobic residues from the water environment, leading to high energy yields in protein interactions (or in this case in the binding of a peptidic ligand by a receptor protein). This might be one of the reasons for the high affinity in binding of endothelin receptor-selective peptides to their receptors.

Particularities of this study

Our delineation of the ligand binding mechanism of peptides in endothelin receptor subtypes ETA and ETB, using pairwise interacting complementary epitopes on ligand and receptor, is different to endothelin receptor models presented before (Bhatnagar and Rao, 2000; Orry and Wallace, 2000). Additionally, the dissection of the endothelin-receptor peptide ligands into four epitopes based on structure-function relationships, namely the ‘addressor’, ‘hook’, ‘hydrophobic core’ and ‘modulator’, represents the confirmation of the classical principle of receptor hormone interactions via `address` and `message` epitopes suggested earlier (Hechter and Calek, 1974).

4.2 Ligand Binding at Nicotinic Acid Receptors GPR109A/B

Identification of the binding pocket of nicotinic acid (Niacin)

Due to the remarkable clinical effects of nicotinic acid (niacin), its recently discovered GPCR represents one of the prime targets for the development of new antidyslipidemic drugs (Wise *et al.*, 2003). Major aim of new drug development in this field is, the improvement of the relatively low potency and unfavorable pharmacokinetic properties of nicotinic acid. In addition, a better ratio of wanted-to-unwanted effects would be desirable. However, the binding site of nicotinic acid was previously unknown. To understand more about the structural requirements in ligand binding to the nicotinic acid receptor, we have characterized the binding site on the human nicotinic acid receptor GPR109A. The major binding site of class A GPCRs for small molecule ligands, *e.g.* in the case of biogenic amine receptors, is located between TMH3, TMH5, and TMH6 (Gether, 2000; Kristiansen, 2004). We were surprised to find, that our data indicates that the binding site of nicotinic acid in GPR109A is different. Our major innovation: the binding crevice of GPR109A seems to be formed by the TMH2, TMH3, and TMH7 for the very small ligand nicotinic acid. Additionally, both the junction TMH2/ECL1 and ECL2 critically contribute to ligand binding. Chimera studies, combined with re-introduction of single amino acid changes, indicated that Asn86/Trp91 at the junction TMH2/ECL1 and Ser178 at ECL2 are essential determinants for nicotinic acid binding to GPR109A. The common feature, of all known ligands of GPR109A, is the presence of a carboxylic group, suggesting that this acidic residue is critically involved in binding. This is supported by the fact that any change or substitution at the carboxyl group of nicotinic acid, such as in nicotinamide, completely abrogates its pharmacological activity. Based on the assumption that the carboxylic acid group of nicotinic acid forms a salt bridge with a residue in one of the transmembrane helices of the receptors, we searched for positively charged residues, allowing an electrostatic interaction with the carboxyl oxygens. The recently discovered dicarboxylic acid receptor GPR91 has been suggested to require positively charged amino acids in TMH 6 and 7. In addition, in prostandoid receptors two positively charged residues in TMH3 and an arginine residue in TMH7 (He *et al.*, 2004) have been suggested to bind to the C1-carboxylate of prostanoids (Stitham *et al.*, 2003). Hence, four arginine residues in TMH3, TMH6, and TMH7 were predicted and validated by site-directed mutations (in cooperation with Sorin Tunaru and Stefan Offermanns, Heidelberg). Replacement of these positively charged residues in TMH6 and TMH7 did not interfere with the receptor's ability to bind nicotinic acid. In contrast, arginine Arg111 in TMH3 turned out to be absolutely essential for binding of nicotinic acid to GPR109A.

As already reported for other GPCRs (Balmforth *et al.*, 1997), in our homology model of GPR109A, the inactive state is also restrained by side-chain interactions between TMH3 and TMH6/TMH7. Arg111 (TMH3) is very likely involved in side-chain interactions with Ser247 (TMH6) and Thr283 (TMH7), stabilizing/constraining the inactive state conformation of the receptor. Arg111 is the pivotal residue for electrostatic recognition and binding, and it functions as a basic anchor point for the ligand's acidic group (Fig. 3-9). Our findings support a scenario, where the side chain of Arg111 is forced to delocalize from its inactive state orientation. The positively charged guanidine group of the arginine side chain moves toward the negatively charged acid group of the ligand upon binding. Thereby, the interaction between helices TMH3 and TMH6/TMH7 is weakened or lost. However, due to rotamer flexibility of the arginine side chain and the very small ligand, further interaction points of nicotinic acid in the binding crevice were difficult to predict.

Many class A-GPCRs for small molecule agonists bind their specific ligands via the extracellular half of the 7 TM-domain. Multiple mutagenesis experiments and molecular modeling structures clearly indicate that the binding sites for most small molecule agonists, such as biogenic amines, are located between TMH3, 4, 5, 6, and 7 (Bonini *et al.*, 2000; Ji *et al.*, 1998; Kristiansen, 2004; Shi and Javitch, 2002; Stenkamp *et al.*, 2002; Strader *et al.*, 1994). Although the major binding sites for small molecule ligands of GPCRs are localized in the transmembrane helices, there is evidence, from site-directed mutagenesis experiments, that certain residues in ECL2 can also particularly contribute to binding of small ligands to GPCRs (Kim *et al.*, 1996; Shi and Javitch, 2002; Zhao *et al.*, 1996). Similar to many other GPCRs, GPR109A also has a disulfide bond between ECL2 (Cys177) and TMH3 (Cys100). Because of the disulfide linkage, ECL2 is constrained over the ligand's binding pocket, and residues from ECL2 can take part in ligand binding. In our model, Ser178 of ECL2 reaches into the ligand's binding pocket and interacts with the nitrogen of the pyridine ring of nicotinic acid.

In several X-ray structures of nicotinic acid/protein complexes, nicotinic acid is surrounded by aromatic residues (Cheong *et al.*, 2001; Ellis *et al.*, 1997; Lovering *et al.*, 2001; Reddy *et al.*, 1996). In GPR109A, based on our docking poses of nicotinic acid in this receptor, the participation of aromatic residues as direct and indirect interaction partners was predicted. To distinguish between the direct and indirect aromatic interaction partners of the ligand, we suggested introducing strong (alanine) and weak (leucine) alterations of side chain-properties by mutations. Finally, mutants of Phe276 and Tyr284 showed identical strong effects for alanine and leucine, indicating a direct aromatic interaction of both positions in ligand binding. In contrast, the common differences between alanine and leucine mutants for Phe180

and Phe193 indicate rather indirect effects of the two residues on ligand binding. Leucine mutants are preserving the necessary hydrophobic properties needed for proper orientation of the neighbored side chains. The side chain's strong reduction in size and hydrophobicity, in case of alanine mutants, may lead to slightly altered assembly of the neighboring side chains and/or helices. Thus, it may indirectly affect the proper shape and size of the ligand's binding site.

Finally, all residues experimentally identified as essential direct interaction partners are located in very close spatial proximity. The binding site of nicotinic acid is most likely located between TMH3, junction TMH2/ECL1, TMH7, and the tip of ECL2 (Fig. 3-11). The experimental data combined with the computational model also suggest the possibility that some aromatic residues, such as Trp91 and Phe276, play a role in the formation of a gateway that allows nicotinic acid to access the binding pocket.

Therewith, our findings support a structural model for GPR109A, where nicotinic acid's binding pocket is localized between TMH2, 3, and 7. The ligands of various receptors, such as the adenosine A2a receptor (Jiang *et al.*, 1996; Kim *et al.*, 1996), the Ca^{2+}-sensing receptor (Miedlich *et al.*, 2004), the prostacyclin receptor (Stitham *et al.*, 2003), or the vasopressin V2 receptor (Wuller *et al.*, 2004), are also interacting within similar binding sites in their respective receptors.

Patterns for ligand selectivity between GPR109A and GPR109B

Nicotinic acid and Acipimox selectively interact on GPR109A, whose binding site was recently described by us (Tunaru *et al.*, 2005). Acifran binds to GPR109A and GPR109B. 1-Isopropyl-benztriazole-5-carboxylic acid has been recently characterized as GPR109B-selective ligand (Semple *et al.*, 2006). Utilizing commonalities and differences of structural and functional features of these GPR109A/B selective ligands, structure-activity relationships of the ligands' own selectivity was characterized. This way, we revealed the patterns discriminating the ligand binding between GPR109A and GPR109B (Fig. 3-13, 3-15), and we identified a new GPR109B-selective compound.

Compared to nicotinic acid (heterocyclic ring system and acidic group) Acifran contains another carbonyl oxygen, hydrophobic extensions by a methyl group, and a benzene ring at a chiral carbon (Fig. 3-16). These additional features obviously allow Acifran to bind in the binding sites of GPR109A and GPR109B. Thereby, the hydrogen-bond acceptor function of the aromatic nitrogen in position 3 of nicotinic acid is taken over by the carbonyl oxygen in meta-position at the furan ring of Acifran. The newly identified GPR109B-selective 2-oxo-octanoic acid also has a large hydrophobic extension, formed by an aliphatic chain, but it

lacks the additional hydrogen-bond acceptor function at the same spatial area. Instead, it provides such a functional group close to acidic group (Fig. 3-15). Taking the structure of recently published 1-Isopropyl-benztriazole-5-carboxylic acid into account (Semple *et al.*, 2006), the hydrogen bond acceptor found in Acifran and 2-oxo-octanoic acid, close to the carboxylic group, seems to be of less importance than a hydrophobic extension of eight carbons length (Fig. 3-16).

Although both receptors' binding sites seem to be similar, the binding modes of Acifran to GPR109B and GPR109A binding sites are somehow different, due to few varied residues. Since the functional group of Ser178 (ECL2) is missing in GPR109B-receptor, the necessary hydrogen bond to the carbonyl oxygen of Acifran is alternatively formed by the hydroxyl group of the GPR109B specific residue Tyr86 (at the junction of TMH2/ECL1). Replacement of Ser178 in GPR109A to Ile178 in GPR109B results in a less selective area atop of the binding site, which allows the binding of various ligands. Additionally, the side chain of Ile178 is larger than Ser178 (in GPR109A) and the δ-methyl group of Ile178 is flanking the hydrogen-substituted carbon. There is no need for a hydrogen-bond donor, such as Ser178 for the furan ring of Acifran in GPR109A. Thereby, the location of Acifran might be slightly shifted and subsequently either the methyl group of R-Acifran or the benzene group of L-Acifran in GPR109B is pointing closer to TMH2 than in GPR109A. This is nicely consistent with the fact that the potential counterpart residue Val83 in GPR109B provides more space with its shorter side chain than the longer side chain of Leu83 in GPR109A at the identical site (Fig. 3-13, 3-15, Tab. 4-1).

Different hydrophobic patterns of GPR109B and GPR109A

Analyzing the positions of different residues within GPR109B and GPR109A in the adequate region at TMH2 and TMH3 indicates that many exchanges of side chains preserve the hydrophobic properties, but result in different bulk and shape (Tab. 4-1).

The chimera GPR109B/GPR10A, consisting of the N-terminal part of GPR109B up to and including TMH3, followed by the C-terminal part of GPR109A, is still able to recognize 2-oxo-octanoic acid. We conclude that the binding site of 2-oxo-octanoic acid is just located in the first half of GPR109B receptor including the N-terminal tail, TMH1, TMH2, first extra cellular loop and TMH3. The majority of sequence differences between GPR109B and GPR109A is also located in this region (Tab. 4-1), which corresponds to our suggested binding site. Interestingly, the same chimera with two additionally introduced point mutations (GPR109B/Y86N/S91W//GPR109A) lost the binding ability for 2-oxo-octanoic acid. Since the counterpart of the aliphatic ligand chain very likely is hydrophobic, it seems that the loss

of the hydrophobic residue Tyr86 influences the result more than the introduction of the hydrophobic Trp91. This might result from a spatially closer position of Tyr86 to the hydrophobic pocket of GPR109B than Trp91. Additionally, it is very likely that the potential hydrogen-bond between Asn86 and Trp91 may influence the conformation of extra cellular loop ECL1 at GPR109A in a different way than the potential hydrogen bond between Tyr86 and Ser91 at GPR109B does.

Table 4-1: Residues within transmembrane helices TMH2 and TMH3 that could alter the shapes of the binding sites in GPR109A and GPR109B. To investigate their true influences on ligand binding, they are predicted for site-directed mutagenesis with following ligand-binding assays (no data available so far).

	GPR109A	GPR109B
Transmembrane Helix TMH1	Leu83	Val83
	Asn86	Tyr86
	Leu107	Phe107
Transmembrane Helix TMH2	Met103	Val103

In our model, the side chains of Tyr86 and the facing side chains Val83 and Val103 of GPR109B are able to form an additional hydrophobic cleft lower within the TMHs. Compared to GPR109A, Phe107 flanks the bottom of this cleft in GPR109B (Fig. 3-15). The corresponding residues of GPR109A are either hydrophilic (Asn86) or the two facing side chains (Leu83, Met103) are larger and provide less space for a hydrophobic cleft at this area. The main difference is that GPR109A misses the two aromatic residues forming the top (Tyr86) and the bottom (Phe107) of this hydrophobic cleft (Fig. 3-13). This construction of the binding site of GPR109A makes it impossible to bind all the ligands GPR109B interacts with.

As an example, the binding site of 2-oxo-octanoic acid is obviously determined by interaction partners in GPR109B that are not part of the binding site in GPR109A. To demonstrate this, Asn86 from the “upper” binding site of GPR109A, where nicotinic acid is interacting, was mutated to Tyrosine (as existing in GPR109B). Although, the binding crevice of GPR109A now resembles the “upper binding” pocket of GPR109B, binding of 2-oxo-octanoic acid was not improved. As a result, the binding pocket of this ligand in GPR109B is completely shifted downwards compared to the binding site of niacin in GPR109A. The “lower” binding site of GPR109B achieves its selectivity by a certain bulk and shape.

For the GPR109B receptor a certain ligand length is important

Variations of the ligands' aliphatic chain-length indicate that, for binding to GPR109B, obviously eight carbon atoms are optimal, six carbons bind weaker, while five carbons are not able to bind at all. Acifran has a similar length from the central carbon of its acidic group to the tip of its benzene group as 2-oxo-octanoic acid. Due to this, the benzene portion of (very likely the L-enantiomer of) Acifran may bind to the suggested hydrophobic cleft in GPR109B. Interestingly, the recently described GPR109B-selective ligand 1-Isopropyl-benztriazole-5-carboxylic acid contains a length of eight atoms as well (Semple *et al.*, 2006). This supports our hypothesis about orientation and size of the described binding pocket at GPR109B.

Apart from ligands' common acidic group anchoring to Arg111, nicotinic acid possesses the optimal hydrogen-bond patterns to bind to the rather hydrophilic patterns of the small binding site at GPR109A.

2-oxo-octanoic acid possesses optimal hydrophobic patterns to bind to the specific hydrophobic cleft of GPR109B.

Both receptor sites, of hydrophilic and hydrophobic patterns, are in spatial proximity. Acifran possesses the hydrophobic patterns (benzene, methyl) to bind to the hydrophobic binding cleft of GPR109B and also contains hydrophilic patterns (furan ring oxygen and carbonyl oxygen), allowing the binding to the hydrophilic site in the GPR109A as well.

4.3 Selective Interaction Patterns for G Protein-Subtypes

The exact nature of the selective molecular interaction mechanism of GPCR and G protein subtypes is currently unknown. The huge degrees of freedom concerning the number of sequences and the conformations at the intracellular loops of different GPCRs makes it difficult to identify selective patterns for G protein subtype selectivity.
This study is the first examination of charge distance patterns as features for selective G protein interaction. The data used was generated by theoretical and experimental investigations in respect of functional importance.
Based on our data we assume that negative charges or hydrogen bond acceptors of G proteins interact with positive charges positioned on the side of ligand molecules, if they have distinct comparable charge distances.

Mousli and colleagues have already discussed a peptidergic activation pathway for mast cells, achieved by cationic secretagogues including positively charged peptides, various amines and natural polyamines (Mousli *et al.*, 1990). Already in 1988 the tetradecapeptide mastoparan-X (MPX), which is a nonspecific secretagogue from wasp venom, was described to directly interact with G proteins (Higashijima *et al.*, 1988). Furthermore they showed that pertussis toxin (PTX)-dependent ADP-ribosylation, which uncouples receptors from some types of G proteins, also inhibits the G protein activation by MPX. So, they concluded that mastoparan-X interacts to G proteins in a receptor-similar mode. Various groups published structural investigations, giving information about Gαi selective MPX and its Gαs interacting derivative mastoparan-S, MPS (Kusunoki *et al.*, 1998; Sukumar and Higashijima, 1992; Sukumar *et al.*, 1997; Wakamatsu *et al.*, 1992). They showed the obvious selectivity of the two related receptor mimetic peptides to two different Gα protein-subtypes.
It was suggested that the adaptation of amphipathic α-helical conformations would allow a peptide to reach the cytosolic compartment. Furthermore, it was discussed that the interaction mode of these secretagogues is in a receptor-independent but membrane-assisted manner.
In the present study of the two mastoparan peptides, MPX from wasp venom and its derivative MPS were used for primary considerations regarding interactions of ligands at G proteins.

Specific charge distances at different secretagogues account for specific G proteins

For primary investigations about selective G protein-interactions we focused on mastoparan peptide MPX from wasp venom and its derivative MPS, because of the possible restriction of the G protein coupling to less complex patterns at these secretagogues.

Inspection of X-ray structures of mastoparans revealed a different helical conformation for MPS compared to MPX, and different placement and orientation of basic residues. By screening three available mastoparan structures of vesicle-bound MPX, G protein-interacting MPX and MPS, distances of positive charges of ligand molecules were measured and collected. These distances cluster in different ranges (6 to 12 Å for Gαi-interaction of MPX and 18 to 23 Å for Gαs-coupling of MPS, see Fig. 3-17). Comparing these clusters of charge distances regarding the known G protein interactions of these peptides (Bueb *et al.*, 1990; Mousli *et al.*, 1990), lead us to the hypothesis that these distances could be key features in the selectivity mechanisms of G proteins.

Specific and complementary charge-distances found at G protein α-subunits

To verify this theory, we used structural data of the Gα-subtypes Gαi (1GIA (Coleman *et al.*, 1994), 1GG2 (Wall *et al.*, 1995), 1GP2 (Wall *et al.*, 1995)) and Gαs (1AZT (Sunahara *et al.*, 1997), 1AZS (Tesmer *et al.*, 1997), 1CJK (Tesmer *et al.*, 1999)) to search for similar complementary distance patterns of negative charges on the G protein side. Thereby, we focused on common interaction sites at β6, β5 and the N-terminal helix, which are already described in the literature (Hamm *et al.*, 1988; Lambright *et al.*, 1996; Natochin *et al.*, 2000; Onrust *et al.*, 1997). At these sites enough negatively charged or at least hydrophilic amino acids could be identified for interactions with the positive charges of ligands. Interestingly, the amount and localization of acidic residues has been different between the compared Gα-subtypes Gαi, Gαq and Gαs. Thus, we measured distances of acid (and also hydrophilic) residues at these discussed interaction sites and additionally at nearby loops. These distances of around 10 Å at Gαi and above 17 Å at Gαs match with the mentioned distance ranges at G protein ligands above (Fig. 3-18).

Other G protein-modulators contain the same features like mastoparans

Based on these findings we looked for other G protein ligands to verify our hypothesis of G protein selectivity determining charge distance patterns. Alkyl-substituted amino acid derivatives seemed to be ideal models for this, because of a high amount of G protein selectivity and only few degrees of freedom.

In the literature these lipoamines are described to modulate G proteins (Breitweg-Lehmann *et al.*, 2002; Leschke *et al.*, 1997; Nurnberg *et al.*, 1999). Activation of Gα by lipoamines – in contrast to GPCRs – does not rely on the existence of the heterotrimeric complex Gαβγ. Breitweg-Lehmann and colleagues concluded that the way of action is more detergent-like. Nevertheless, it was shown that Gαi activating lipoamines are sensitive to pertussis toxin and, thereby, also interact at a site at Gα, which is close to the C-terminus and a common interaction site for GPCRs. The small structures consist of aliphatic tails (which interact most probably within membranes) and head groups mostly consisting of amino acid derivatives. These head groups contain a smaller number of functional groups than receptor mimetic peptides or GPCRs. In our description of interaction motifs at G protein-complex structures this was another important feature to us. We built models taking information and structural data (structural formulas) of lipoamines from literature, searching in their conformational spaces and recording distances of positive charges for every model.

The found distance patterns of positive charges are similar to those of the other G protein ligands and complementary to the G proteins they are selective to (Tab. 3-5). Analog to receptor mimetic peptides, we discuss an interaction site for lipoamines at Gα close to the C-terminal tail. This was experimentally verified for Gαi-interacting compounds of this series (such as FU132, FU244), using the pertussis toxin-sensitive ADP-ribosylation of the C-terminus, which abolished the interaction with these compounds and also their activating influence.

Using an iterative loop strategy between theoretical and experimental investigations it was possible to restrict the degrees of freedom (flexibility) and, thereby, the distances of positive charges of some compounds. These compounds showed only selectivity for Gαi but not for Gαs (FU244, FU245). Experimental proof therefore could be established (Fig. 3-20). Here again a distance of around 10 ± 3 Å between two positive charges is crucial for Gαi-interaction. Such compounds are unable to activate Gαs.

Transfer of these findings to GPCRs – The most complex system studied

After identifying complementary charges for G proteins and their small molecule ligands, the intracellular moieties of ETA and ETB were compared by sequence alignments and structural models, taking the described Gα-selectivity into account. The complementary charge patterns should by applied to G protein-selectivity of GPCRs

Smaller distances between positive charges could be identified within intracellular loops ICL1 and ICL2 of Gαi-selective ETB, whereas larger distances resulted from less positive charges

in this region at Gαq-selective ETA (Fig. 3-23, 3-24). The preliminary result matched with existing data from the literature. An ETA chimera including ICL2 and ICL3 of ETB was shown to interact with Gαi but not with Gαs (Takagi *et al.*, 1995). The *vice versa* receptor chimera of ETB including ICL2 and ICL3 of ETA showed selectivity for Gαs but not Gαi. It seemed reasonable to predict site-directed mutations that exchange single residues, thought to be necessary for Gα-selectivity, at one receptor subtype to the corresponding residue of the other receptor subtype.

After identification of residues within distance ranges, which were identified as Gα-subtype selective patterns before, several predictions were made for site-directed mutagenesis and experimental validation of existing hypothesis (Tab. 3-6).

Preliminary experimental results show a clear involvement of intracellular loop ICL2 in interaction with Gαi by both endothelin receptor subtypes (Arg192 at ETA and Lys216 at ETB). Although no complementary mutant in ETB has been developed so far for Arg192 in ETA, the complementary mutant to Lys216 in ETB (Leu200→Lys at ETA) showed no influence on interaction to Gαq and Gαi. This is an impressive result, suggesting differences in the way of selective G protein interaction.

Other methods, for prediction of Gα subtype-selectivity, commonly use patterns of similar sequences for identification. As an example of this strategy, Hidden Markov Model-based approaches such as PRED-COUPLE and PRED-COUPLE 2.0 give quite reasonable results (Sgourakis *et al.*, 2005). Our own tests of this prediction method for both endothelin receptor subtypes revealed selectivity for Gαi, Gαq and Gαs, being consistent with data from literature (Wong, 2003). Furthermore, PRED-COUPLE predictions also give information about the loops and residues involved in these interactions. So, compared to our results of Gαi interaction determinants within both receptors' ICL2, this method also predicts the second intracellular loop to be selective for interaction with Gαi. The areas predicted for interaction with Gαi are residues Val181-Asp182-Arg183-Tyr184-Arg185-Ala186-Val187-Ala188-Ser189 at ETA as well as corresponding sequence Ile197-Asp198-Arg199-Tyr200-Arg201-Ala202-Val203-Ala204-Ser205 at ETB. Both sequences vary only in the first shown residues and highlight a pattern identified for Gαi-selectivity before.

In contrast, we could experimentally verify that residues Arg192 at ETA and Lys216 in ETB are important for selective G protein-interaction with Gαi (cooperation with Alexander Oksche, Charite; Jens Furkert, FMP). Both residues are not included in the sequences, resulting from PRED-COUPLE, and, furthermore, at different places in the second intracellular loops. This also confirms our hypothesis of complementary charge patterns.

Additionally, Leu200→Lys, which is the ETA's complementary mutant to ETB's Lys216→Leu, does not show any influence in G protein selectivity. As a conclusion, within ICL2 the participating residues for G protein-selectivity in both endothelin receptor subtypes seem to be different and/or more complex than thought until now. It is to assume that this also is true for other intracellular regions. Most likely the interactions of the intracellular loops themselves are more intense than reflected by our models, restricting the resulting interface to G proteins to some exposed residues at these regions. Additionally, not all basic charges are equally participating in charge distance patterns. Prediction methods dealing with profiles based on sequence information alone have been keenly discussed in true prediction of G protein-interacting sites at GPCRs.

In general, differences between our suggestions and the experimental data of mutated positively charged residues might depend on both, wrong predictions and the unexpected interaction of ETA with Gαi, within the assays. A reason of ETA's behavior can be inappropriate cell lines that give ETA Gαi-selectivity (Cadwallader *et al.*, 1997; Hilal-Dandan *et al.*, 1994; Jones, 1996), while others do not (Gohla *et al.*, 1999; Horstmeyer *et al.*, 1996). ETA's behavior could also be based on the high amount of receptors by using transient expression (personal communication with Alexander Oksche). The increased Gαi-interaction for ETB triple mutant Lys128→Gln, Lys130→Ala, Lys210→Gln might also origin in expression-related problems.

Finally, it is worth mentioning that an interaction model of endothelin receptors and their G proteins is not predictable because of lack of information, based on (i) data from literature that is dealing with many different Gα-subtypes in many different cell lines, (ii) preliminary results by our co-operation partners that have to be proven, and (iii) experimental data suggesting differences in G protein-interaction at these homologous GPCR subtypes.

Excursus: G protein-activation is independent of GPCR-oligomers

In G protein-activation by GPCRs, a keenly discussed topic is the influence and/or necessity of GPCR dimers and tetramers. This topic came in focus after atomic force microscopy investigations on bovine rhodopsin in disc membranes (Fotiadis *et al.*, 2003). In ETA and ETB, heterodimerization was observed (Gregan *et al.*, 2004). Specific stimulation of ETB internalizes these dimmers, but a necessity for G protein-activation is not assumed.

Additionally, our G protein-GPCR interaction studies suggest a recognition/interaction mechanism between a GPCR and a trimeric G protein complex. Other GPCRs of possible oligomers seem to be unnecessary or only guide the G protein to the GPCR. It might be possible that the formation of GPCR oligomers has similar functions as the light-harvesting

complex of photosystem II, but evidence for this is still missing. Furthermore, oligomerization could be restricted to certain GPCRs or depend on high local concentrations as, for example, rhodopsin in disc membranes.

4.4 Summary

Using homology of GPCR subtypes as well as their differences in biological function, we characterized ligand binding for endothelin receptors ETA and ETB.

For the first time, endothelin receptor-selective peptide ligands were subdivided into 4 regions (addressor, hook, core, modulator), explaining all existing data in literature as well as data from our co-operation partners. This is based on interactions with 4 complementary regions (gateway, edge, neck, binding cleft), found at the endothelin receptors. The pairwise interactions of addressor and gateway, hook and edge, core and neck, as well as modulator and binding cleft accurately explain the peptide ligands' selectivity for endothelin receptors and endothelin receptor subtypes. We impressively demonstrated this by the design and experimental validation of a new ETB-selective peptide ligand. Additionally, for the first time we described the necessities and differences in activation and in inhibition of both endothelin receptors. According to our models the molecular differences of peptide ligand-induced receptor activation and inhibition can be explained by the existence and absence of negatively charged residues at the peptide ligand's hook region, as counterpart of the receptor's edge region. In the case of receptor activation we suggest a scenario where the ligand's hook region catches the receptor's edge region. In succession, the ligand's modulator region is restrained in a position where its C-terminus orients in between transmembrane helices TMH3, TMH6 and TMH7 and induces reorientation of several side chains, finally leading to receptor activation. In the case of inhibition, the ligands lack the charged hook region as well as bulky moieties at the N-terminal portion. Such antagonistic peptides slide beyond the edge down into the transmembrane binding cleft and additionally constrain the existing interactions between the transmembrane helices of the inactive state, leading to inhibition. One of the most impressive results is the orientation of the last two residues of the antagonist's C-terminal modulator region at the same site of the inverse agonist 11-cis-retinal in inactive rhodopsin, demonstrating conserved mechanisms in many GPCRs.

Additionally, our models explain the so far unclear formation of the super-stable complex between ET-1 and ETB occurring in many mammals. A lid-like mechanism on this receptor's N-terminus, which is different to ETA in sequence and structure, covers ET-1 and restrains it tight into the binding site.

In a second project, we utilized the high degree of sequence homology between the human nicotinic acid receptor GPR109A and its homologue GPR109B, which has only low

affinity for nicotinic acid. For binding of nicotinic acid to GPR109A we could identified crucial residues. The binding site is positioned between transmembrane helices TMH2, TMH3 and TMH7, and is therefore an interaction site different than most other rhodopsin-like GPCRs, where the binding site is located between TMH4, TMH5 and TMH6. Validated experimental data on predicted aromatic residues within TMH5 and TMH7, as well as at ECL2, clearly confirm this result. Combining mutagenesis data and comparative structural modeling allowed us to identify five residues located in close spatial proximity to the main interaction points for nicotinic acid. The characterization of the structural determinants and complementary pharmacophoric patterns for nicotinic acid binding in GPR109A is of general importance for understanding the binding mechanism of small molecule ligands to GPCRs. It is also important in the design and the development of new drugs, acting via GPR109A to treat dyslipidemic disorders. Comparisons of both binding sites explained the differences in ligand selectivity. Furthermore, they led to the proposal of 2-oxo-octanoic acid as a ligand selectively for GPR109B but not GPR109A, which was experimentally proven by our collaborators. Necessary interaction features, which were identified for 2-oxo-octanoic acid at GPR109B, could be unexceptionally applied to the recently published family of GPR109B-compounds based on benzotriazole-5-carboxylic acid.

Taken together, nicotinic acid possesses the optimal hydrogen-bond patterns to bind to the rather hydrophilic patterns of the small binding site in GPR109A, whereas 2-oxo-octanoic acid possesses optimal hydrophobic patterns to bind to the additional specific hydrophobic cleft of GPR109B. Both receptor sites consist of hydrophilic and hydrophobic patterns, and are in spatial proximity. The binding of Acifran, which is an unselective ligand for both receptors, possesses the hydrophobic patterns (benzene, methyl) to bind to the hydrophobic binding cleft of GPR109B. Acifran also contains hydrophilic patterns (furan ring oxygen and carbonyl oxygen), allowing the interaction with the hydrophilic site in the GPR109A.

In a third approach, investigations on interactions of G proteins with their small-, medium- and large-sized ligands identified a common pattern for recognition and/or interaction, namely the interaction of negative and positive charges on G proteins and their ligands. This pattern was clearly identified by studies of small-sized alkyl-substituted amino acid derivatives (so called lipoamines) as well as by medium-sized secretagogues mastoparan-X and mastoparan-S. It contains specific distances of positive charges within G protein-ligands, recognized by negatively charged residues of complementary distances in G

proteins. Investigations of four different G protein-subtypes (Gαi, Gαo, Gαq, Gαs) clearly showed different localizations and, therefore, distances of negatively charged residues in close proximity to the C-terminus (below 12 Å for Gαi, around 15Å for Gαq, and greater than 18 Å for Gαs). As a result, it was possible to design a new compound (FU244) that exclusively interacts with Gαi but not with Gαq or Gαs. The application of these patterns to the interaction interface of GPCRs and G proteins resulted in identification of two residues within ICL2 that are required for Gαi selectivity of ETA and ETB. This way, we demonstrated that also in GPCRs complementary charge patterns determine G protein-selectivity.

4.5 Conclusions & Outlook

The delineation of the ligand binding sites in endothelin receptor subtypes ETA and ETB – based on the dissection of the receptors and their native peptide ligands each into 4 regions – allows a deeper insight into their ligand binding mechanisms.
The molecular basis for selectivity of peptide ligands for endothelin receptors and endothelin receptor subtypes was identified. Based on a novel concept using repulsive charges in the binding of agonists to ETA and ETB, the mechanisms of receptor activation and receptor inhibition were defined. In addition, the molecular reasons of the formation of a super-stable complex between ET-1 and ETB could be described.
The suggested patterns for agonistic and antagonistic interactions of native peptides help to improve the affinity of small ligands regarding these receptors to the nanomolar range. In addition, the described selectivity patterns can be used in the future design of an ETA-selective peptide ligand.

The characterization of the binding site of nicotinic acid receptor GPR109A provides a basis for rational design of new leads for treatment in dyslipidemic disorders to avoid typical side effects of nicotinic acid (*e.g.* flushing). Additionally, the binding pocket of GPR109B was characterized. This receptor, currently termed low-affinity nicotinic acid receptor, has no described function or native ligand. Its identified binding pocket will help to de-orphanize this receptor by a structure based design strategy focusing on native compounds available in adipocytes.

Complementary charge distance patterns at the interaction interface of GPCRs and G proteins were identified, which are selective for G protein-subtypes. These selectivity patterns were also found in the interaction of lipoamines and secretagogues with G proteins. Further validation on other GPCRs will help to completely assign selectivity patterns for the multiplicity of GPCR-G protein interactions. In addition, the use of small membrane-penetrating molecules, presenting G protein-selective interaction patterns, may bypass the activation of G proteins by GPCRs.

This study demonstrated that the concept of sequence-structure-function analysis of homologous GPCRs with different biological functions allows the delineation of ligand-receptor interactions, the characterization of molecular mechanisms for receptor activation and inhibition, as well as the identification of selective patterns for G protein-selectivity.