3.2 Ligand-Binding at Nicotinic Acid Receptor Subtypes GPR109A/B

3.2.1 Characterization of the Ligand Binding Site at GPR109A Receptor

Ligands of GPR109A Receptor are Carboxylic Acids

Nicotinic acid (pyridine-3-carboxylic acid), Acipimox (5-methylpyrazine-carboxylic acid-4-oxide) and Acifran (4,5-dihydro-5-methyl-4-oxo-5-phenyl-2-furan-carboxylic acid) are GPR109A ligands. Preliminary comparisons revealed that all carry a common carboxyl group (Fig. 3-8).

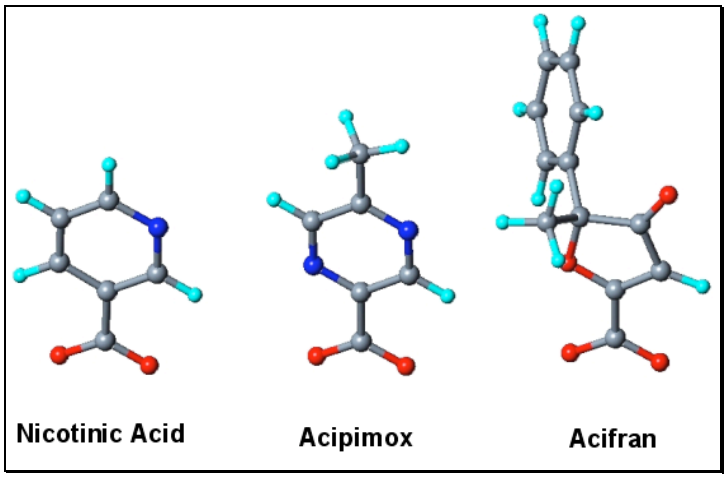

Figure 3-8: **Ligands of GPR109A receptor share a common carboxyl group that interacts with a positive charge within the receptor.**

Because of this, it is likely that a basic residue in GPR109A is important for binding of these ligands. Based on first computer models, three arginine residues in transmembrane helices were identified, which may provide a binding environment for the carboxyl groups of GPR109A receptor ligands. To test their potential involvement in nicotinic acid binding, arginine residues Arg111, Arg251 and Arg253 were selected for site-directed mutagenesis to alanine and binding experiments with known GPR109A ligands.

Experimental Validation of Predicted Arginine Mutations (in cooperation with Sorin Tunaru and Stefan Offermanns)

Binding of nicotinic acid and Acifran as well as expression and localization at the plasma membrane were investigated (Tunaru *et al.*, 2003). For the mutant Arg253→Ala, both nicotinic acid and Acifran behaved equally with regard to the wild-type receptor. The effects from the mutant Arg251→Ala were reduced. However, GPR109A receptor mutant Arg111→Ala could not be activated by addition of nicotinic acid and Acifran (Fig. 3-9).

Radioligand binding studies demonstrated that Arg111→Ala lost its ability to bind nicotinic acid, whereas Arg251→Ala showed reduced affinity for nicotinic acid. All three mutants were normally expressed and localized to the plasma membrane (Tunaru *et al.*, 2003). This suggests that Arg111, is crucial for ligand-dependent receptor activation by contributing to the binding pocket of the receptor. Additionally it was experimentally verified that ligands missing the carboxylic group (notably nicotinamide) are not able to activate the receptor, indicating the high importance of a salt-bridge contact to Arg111 (data not shown).

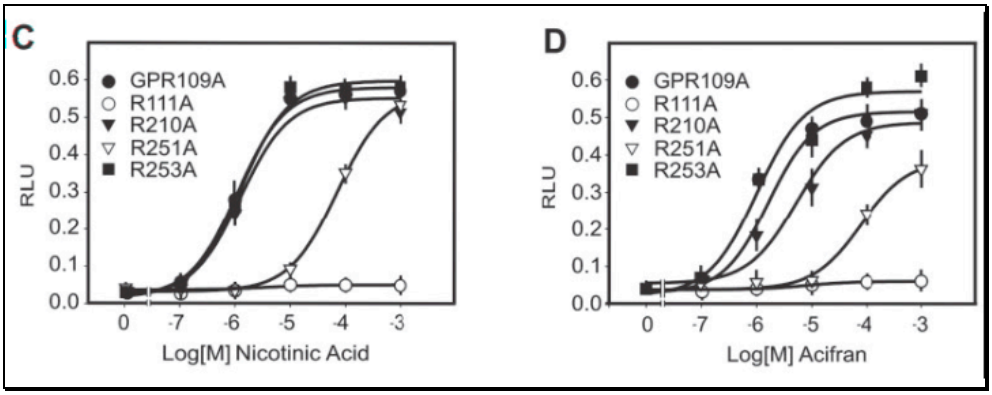

Figure 3-9: **Ligand binding assays of Arg→Ala mutants at GPR109A receptor. Binding for both nicotinic acid and acifran are totally abolished for Arg111→Ala indicating its role as the key player in receptor activation. The influences of Arg210, Arg251 and Arg253 on ligand binding are weaker and vary between both ligands, suggesting more general influences by formation of helix bundle and binding site. (Tunaru *et al.*, 2003)**

Delineation of the Binding Site

Taken together, residues Asn86 and Trp91 at the junction of TMH2 and ECL1, Arg111 at TMH3 and Ser178 are critically involved in ligand binding in the GPR109A receptor (Fig. 3-9; see also Fig. 1-7). Additionally, Arg251 at TMH6 may play a role in the increase of affinity for nicotinic acid and other GPR109A ligands. The experimental data indicated the activation of GPR109A by one ligand molecule per receptor. But the resulting binding site in initial GPR109A models based on known mutational data would be large enough to interact with two small molecules of the size of nicotinic acid or Acifran. Therefore, two hypothetical interaction sites are possible candidates as ligand binding sites (Fig. 3-10).

To delineate this large binding pocket into the true binding site of the GPR109A receptor, a focus on other proteins that interact with nicotinic acid was chosen. Analyzing the crystal structures of diverse prokaryotic proteins such as nicotinate mononucleotide dimethylbenzimidazole phosphoribosyltransferase (PDB entry 1D0V (Withers-Ward *et al.*, 2000)), dihydropteridine reductase (1ICR (Lovering *et al.*, 2001)), nicotinate nucleotide dimethylbenzimidazole phosphoribosyltransferase (1JHA (Cheong *et al.*, 2001)), dihydrodipicolinate reductase (1DRV (Reddy *et al.*, 1996)) and plant protein ferric soybean leghemoglobin (1FSL (Ellis *et al.*, 1997)), clearly demonstrated that the pyridine ring system

of the ligand is always bound in close proximity to aromatic side chains of the protein. This way, it is likely that the binding of nicotinic acid is supported by interactions with aromatic residues also in GPR109A. In addition to the already known aromatic residue Trp91 in ECL1, aromatic residues Phe180 (ECL2), Phe193 (TMH5), Phe276 (TMH7) and Tyr284 (TMH7) were predicted and suggested for site-directed mutagenesis to alanine and leucine residues to demonstrate, which of them are required for ligand induced signalling. Based on existing homology models of GPR109A Phe180 would be the only interaction partner for nicotinic acid in spatial area 1 (Fig. 3-10, blue circle), whereas the interaction partners of niacin in spatial area 2 (Fig. 3-10, red circle) would be Phe276 (direct), Tyr284 (direct) and Phe193 (indirect). Because of this, information about the sensitivity of ligand binding of these predicted mutations allows one to distinguish between the different spatial areas and to clearly localize the binding site of nicotinic acid at GPR109A as well as in ligand binding involved residues.

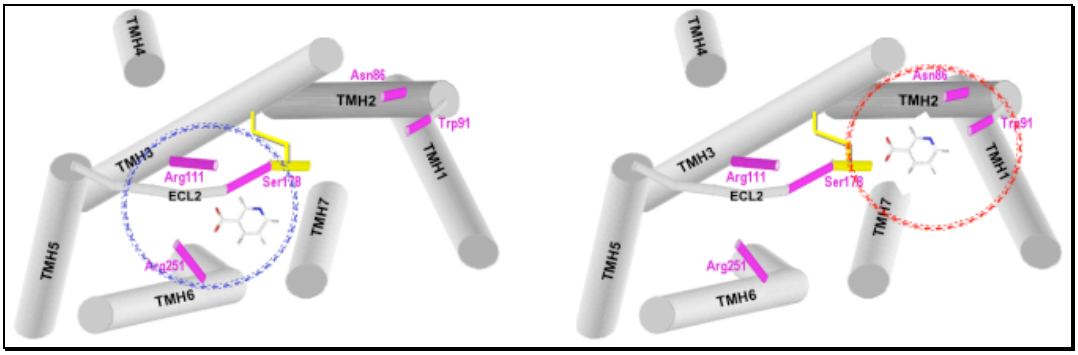

Figure 3-10: **Cartoon of GPR109A receptor and ligand-selective mutations Asn86, Trp91, Ser178, Arg111 and Arg251. Nicotinic acid is shown in adequate size to the receptor. Spatial area 1 (blue circle) is a typical GPCR binding site and would explain mutational data of Arg111, Ser178 and Arg251. Spatial area 2 (red circle) is supported by mutational data of Asn86, Trp91 and Arg111.**

Experimental Validation of Predicted Aromatic Mutants (in cooperation with Sorin Tunaru and Stefan Offermanns)

The drastic alterations of side chains by alanine mutants of all four aromatic residues revealed an identical phenotype, which showed no response for nicotinic acid nor for Acifran (Tab. 3-4) (Tunaru *et al.*, 2003). The binding profile of leucine mutants of the aromatic residues is different: Phe180→Leu and Phe193→Leu were only slightly affected (Tab. 3-4) indicating no interactions with the ligands. Leucine and alanine mutants of Phe276 as well as Tyr284 showed the same group phenotypes indicating direct involvement of these residues into binding of nicotinic acid and Acifran at GPR109A (Tab. 3-4). All receptor mutants are expressed and localized as the wild-type receptor (Tunaru *et al.*, 2003).

Table 3-4: Binding Studies of Nicotinic Acid and Acifran at GPR109A receptor mutants, where aromatic residues suggested as potential interaction partners were exchanged to Leucine (removal of aromaticity) and Alanine (removal of aromaticity and size). Mutants of Phe276 and Tyr284 indicate involvement of both residues in ligand binding (Tunaru *et al.*, 2003).

	EC_{50}		K_D (Binding of [^{3}H]-Nicotinic Acid)
	Nicotinic Acid	Acifran	
Phe180→Ala	> 100 µM	> 100 µM	> 500 nM
Phe180→Leu	1.8 ± 0.2 µM	5.4 ± 6 µM	
Phe193→Ala	> 100 µM	> 100 µM	> 500 nM
Phe193→Leu	4 ± 0.6 µM	8 ± 2 µM	
Phe276→Ala	> 100 µM	> 100 µM	> 500 nM
Phe276→Leu	> 100 µM	> 100 µM	> 500 nM
Tyr284→Ala	81 ± 17 µM	> 100 µM	300 ± 36 nM
Tyr284→Leu	153 ± 10 µM	> 100 µM	342 ± 40 nM

Refinement of the Binding Site of GPR109A Based on Known Mutational Data

Apart from the initial 4 residues, demonstrated to be crucially relevant for binding of nicotinic acid and Acifran in GPR109A: Asn86 (TMH2), Trp91 (ECL1), Arg111 (TMH3), Ser178 (ECL2), two additional aromatic residues were confirmed to be additionally involved in ligand binding: Phe276 (TMH7) and Trp284 (TMH7).

Arg251 (TMH6) may also be of interest.

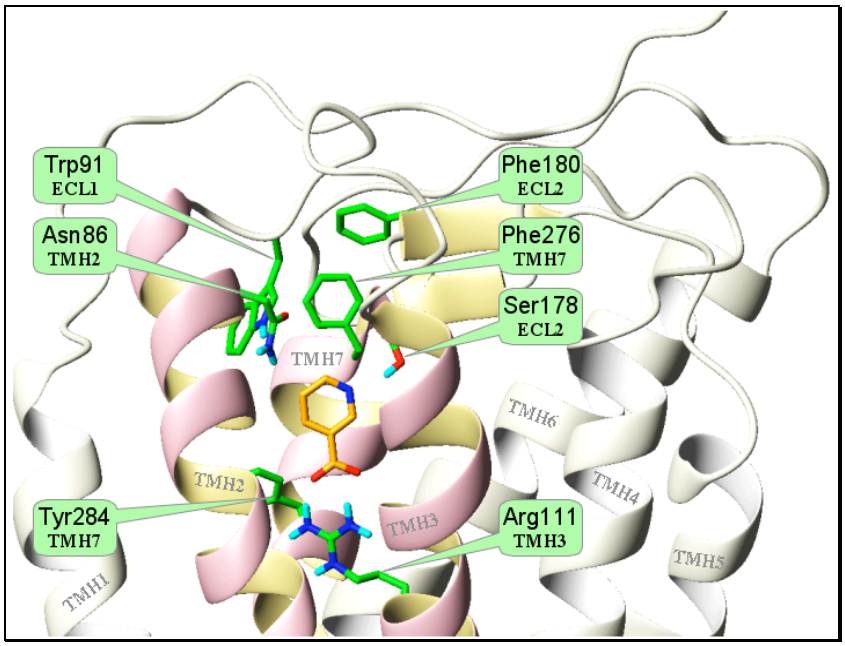

Figure 3-11: The final binding site (green residues) of nicotinic acid (orange) at GPR109A receptor is provided by transmembrane helices TMH2, TMH3 and TMH7 (pink/yellow ribbon).

In initial models of GPR109A, using the extracellular patterns of rhodopsin, 3 residues are clearly localized in the above-mentioned spatial area 1 (Arg111, Ser178, Arg251) but 4 residues are part of spatial area 2 (Asn86, Trp91, Arg111, Phe276, Trp284). This strongly suggests that the binding site of nicotinic acid and Acifran at GPR109A is provided by spatial area 2. The GPR109A mutants of residue Phe193 (TMH5) support this binding site. Phe193, which would be the only counterpart for the aromatic pyridine ring of nicotinic acid in spatial area 1, was demonstrated to be uninvolved in ligand binding (Tab. 3-4).

To include all residues, which have been shown to be crucial in ligand binding, new models were generated using a changed orientation of extracellular loop ECL2. In contrast to initial models with rhodopsin-like extracellular patterns, where ECL2 connects TMH4 and TMH5 by a counter-clockwise orientation, ECL2 in the final GPR109A model is clockwise oriented.
In the resulting binding site the pyridine-ring of nicotinic acid is embedded between Trp91 (ECL1), Phe276 (TMH7) and Tyr284 (TMH7), whereas its carboxyl group interacts with Arg111 (TMH3). The pyridine nitrogen interacts with Ser178 (ECL2) via a hydrogen bond. The main aromatic anchors Trp91 and Phe276 are additionally restrained by interaction with Asn86 and Phe180, respectively (Fig. 3-11).

3.2.2 Selectivity of Ligand Recognition at GPR109A and GPR109B Receptors

Docking of Nicotinic Acid and Acifran at GPR109A Receptor

For manual docking studies in the GPR109A receptor model, the structural information of nicotinic acid, Acipimox as well as R- and S-Acifran were used. Nicotinic acid and Acipimox are selective only for GPR109A, whereas Acifran is active in both GPR109 receptors.
Due to the very close structural similarity between nicotinic acid and Acipimox, the docking pose of Acifran is here only compared to the pose of nicotinic acid.
Both ligands interact at the defined binding site, using their aromatic rings for interaction with the main aromatic anchors Trp91 (ECL1) and Phe276 (TMH7) and using their carboxyl groups for salt-bridge contacts with Arg111 (Fig. 3-12).

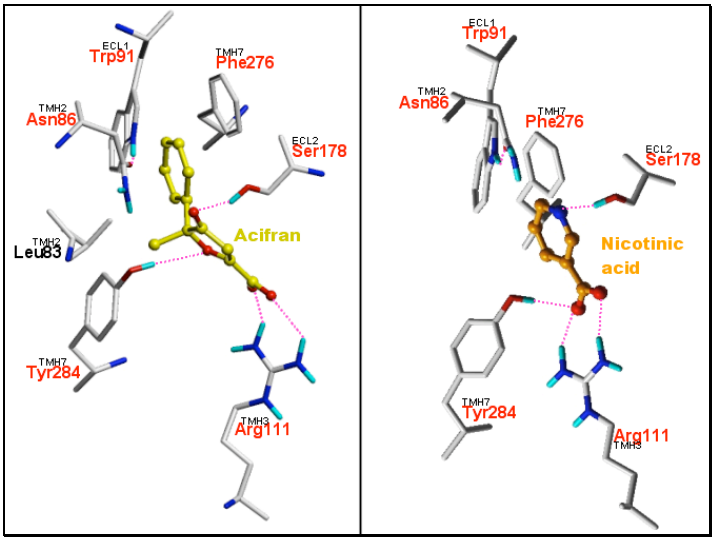

Figure 3-12: **Close-up view of the binding site of GPR109A with bound ligand Acifran (left side) or nicotinic acid (right side).**

Additionally, hydrogen bonds are formed in this ligand-receptor interaction. The hydrogen bonds from Ser178 are accepted in nicotinic acid by the pyridine-nitrogen and in Acifran by

the carbonyl-oxygen of the furan-ring. Another hydrogen bond may exist between Tyr284 (TMH7) and the furan-oxygen of Acifran and the carboxylic group of nicotinic acid. Based on the branch in Acifran its binding mode is somehow shifted to TMH2. At the corresponding position in TMH2 Leu83 restrains Acifran in optimal position in its binding site. Additional surrounding residues form a tall and slim binding site on the GPR109A receptor with the aromatic anchors Trp91 (ECL1) and Phe276 (TMH7) atop and the basic anchor Arg111 at the bottom (Fig. 3-13).

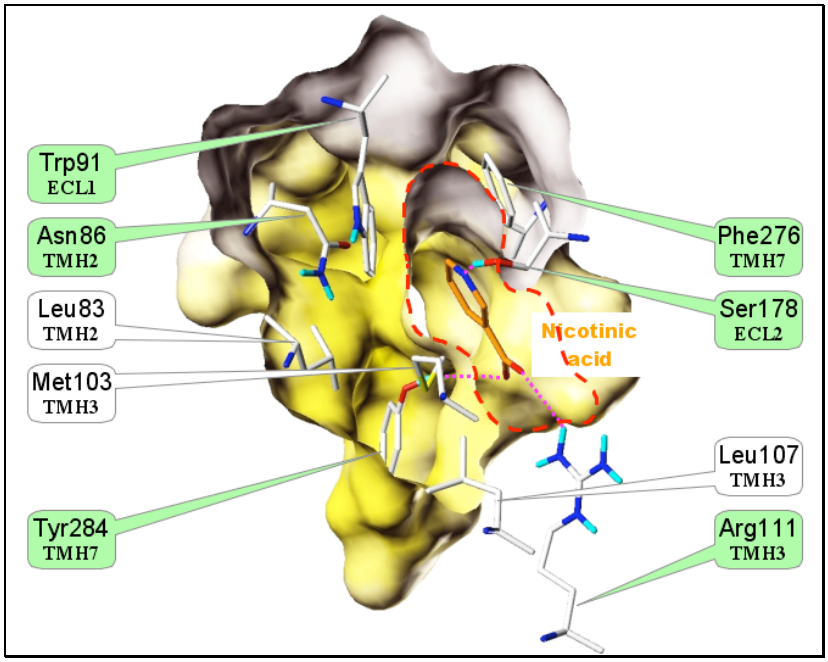

Figure 3-13: **van der Waals-surface of the binding site of GPR109A. Based on the aromatic residues atop of the binding site as well as Leu83 (TMH2) and Met103 (TMH3) the binding site is very narrow and tall. Size and shape are optimized for interaction with small aromatic ligands that include a carboxyl group attached to their aromatic ring (*e.g.* nicotinic acid).**

Different Binding Site at GPR109B Receptor

In comparison, the binding site of GPR109B has several variations based on sequence differences. One of the main aromatic anchors (Trp91) is exchanged by the small hydrophilic Ser91. Following, Asn86 is replaced by Tyr86, resulting in a lid that closes the upper part of the binding site and, thereby, reduces its height. Simultaneously, the patterns for hydrophobic contacts to ligands were lowered in the direction of the basic anchor residue Arg111. This is well supported by the introduction of Ile178 in GPR109B (instead of Ser178 in GPR109A).

The further exchange of residues Leu83 (TMH2) and Met103 (TMH3) in GPR109A by Val83 (TMH2) and Val103 (TMH3) in GPR109B results in a new hydrophobic binding-cavity, closely located to TMH1 and TMH2. An additional aromatic interaction between Phe107 (TMH3) and Tyr284 (TMH7), which is based on the exchange of Leu107 (GPR109A) to Phe107 (GPR109B), completes the lower part of the binding site close to Arg111.

Experimental investigations by Sorin Tunaru and Stefan Offermanns (University of Heidelberg) confirm these suggestions. Receptor chimeras of GPR109A and GPR109B receptors clearly suggest different binding areas for nicotinic acid and 2-oxo-octanoic acid (Fig. 3-14).

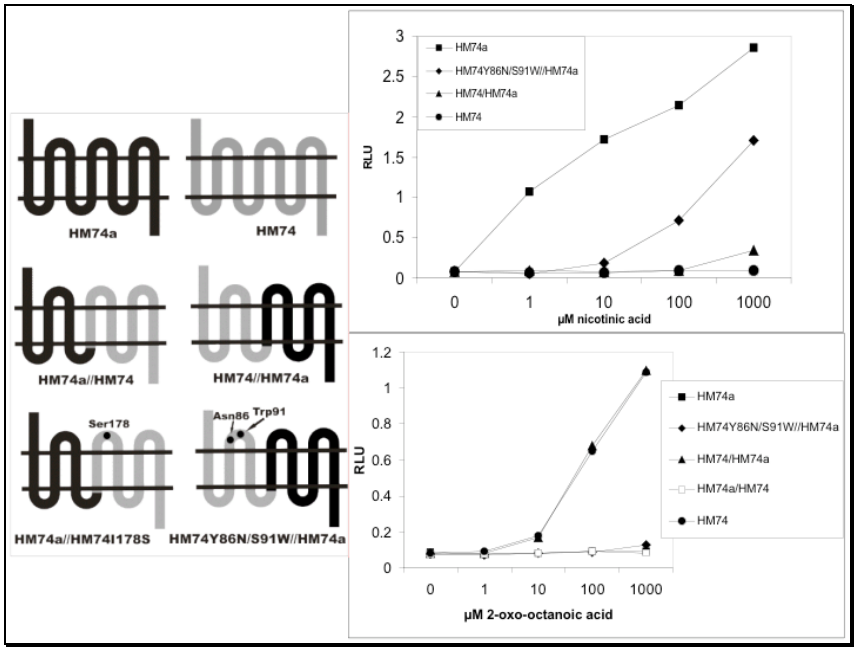

Figure 3-14: Binding of nicotinic acid and 2-oxo-octanoic acid to GPR109A (HM74A), GPR109B (HM74) and GPR109A-GPR109B-receptor chimeras. The left side shows a scheme of the building plan of the chimeras (middle row) based on the wild-type receptors (upper row) and furthermore also the constructed mutants of chimeras (lower row). Binding of nicotinic acid and 2-oxo-octanoic acid is shown in the upper right and lower right, respectively, and described in the text below.

The GPR109A (HM74A) receptor shows clear response to nicotinic acid, whereas GPR109B (HM74) does not. Chimera HM74/HM74A, which includes N-terminal portions of GPR109B and the C-terminal portions of GPR109A, shows binding of nicotinic acid similar to GPR109B receptor wild type because of the missing Asn86 and Trp91 residues in the upper part of the binding site in GPR109A. Re-introduction of these residues into HM74/HM74A – as shown in chimera mutant HM74/Y86N/S91W/HM74A – results in response to nicotinic acid.

In contrast, the binding of the GPR109B-selective ligand 2-oxo-octanoic acid is independent of this receptor region. GPR109B (HM74) and its N-terminal chimera HM74/HM74A respond to 2-oxo-octanoic acid, while GPR109A (HM74A) and its N-terminal chimera HM74A/HM74 do not. These data themselves would still suggest an inclusion of ECL1 into the binding site of 2-oxo-octanoic acid on the GPR109B receptor. But the data of chimera mutant HM74/Y86N/S91W/HM74A, which also not responds to 2-oxo-octanoic acid, clearly suggest another binding site based on differences between GPR109A and GPR109B in the transmembrane regions and thus exclude an interaction at ECL1.

The resulting binding site at GPR109B based on sequence dependent differences to GPR109A is, therefore, broad and small and prefers ligands with aliphatic chains attached to a carboxylic group (Fig. 3-15).

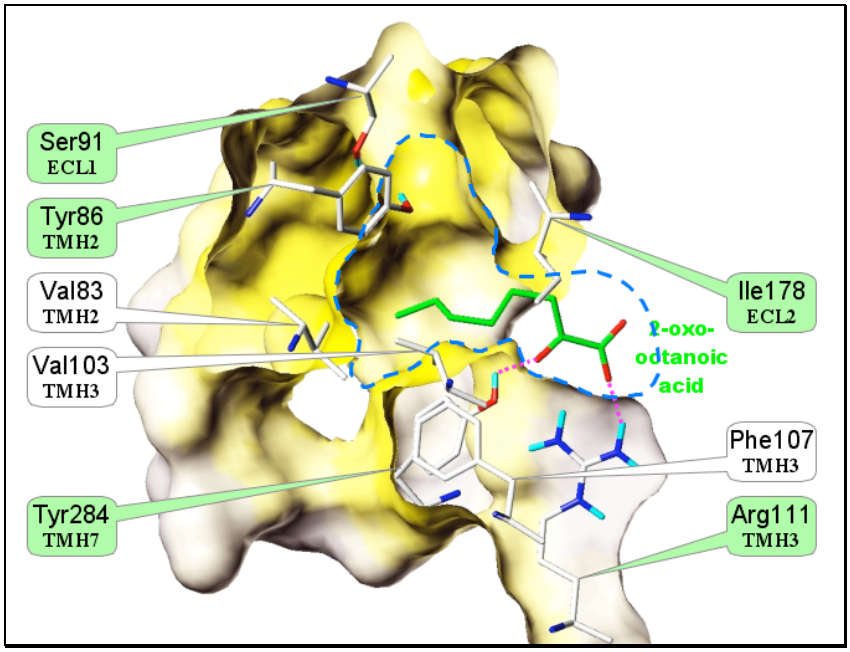

Figure 3-15: **van der Waals-surface of the binding site at GPR109B. Ser91 and Tyr86 atop of the binding site form a lid and close the upper binding site. Therefore, residues Val83 (TMH2) and Val103 (TMH3) open an interaction area in the direction of TMH2 and TMH1. The binding site is broad and small. Size and shape are optimized for interaction with ligands composed of an aliphatic chain of certain length with a attached carboxyl group (*e.g.* 2-oxo-octanoic acid).**

Conclusions About Ligand Selectivity on GPR109A and GPR109B Receptors

Based on binding site analysis, compared with the structural features of nicotinic acid, Acipimox, Acifran, 2-oxo-octanoic acid, as well as the recently published GPR109B-selective ligand 1-Isopropyl-benzotriazole-5-carboxylic acid (Semple *et al.*, 2006), the selectivity patterns for both nicotinic acid receptors were characterized.

GPR109A-selective ligands have to be based on aromatic moieties that include an attached short hydrogen bond acceptor in meta-position to the carboxylic group (Fig. 3-16). The aromatic ring and the hydrogen bond are restraining the ligand position, whereas the carboxylic group forces the side chain of Arg111 to reorient (Fig. 3-11, 3-12, 3-13).

The carboxylic acid group of GPR109B-selective ligands is used the same. Restraints of the ligand into the binding site are mainly applied by a hydrophobic succession of carbon atoms, where eight carbons seem to be optimal (Fig. 3-15). Additional hydrogen bond acceptors may increase the affinity, but their position is not as defined as in GPR109A selective ligands. In contrast to ligands for GPR109A, this hydrophobic extension need not be aromatic (Fig. 3-16).

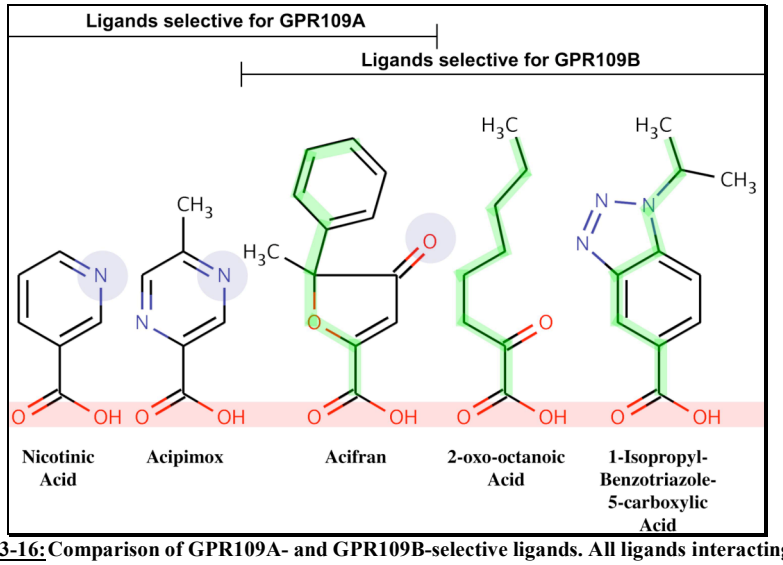

Figure 3-16: **Comparison of GPR109A- and GPR109B-selective ligands. All ligands interacting with GPR109 receptors share a common carboxylic group (red box). GPR109A-selective ligands are aromatic molecules with a common hydrogen bond acceptor (blue-filled circles). GPR109B-selective ligands are independent from an aromatic core but own a hydrophobic extension of ideally 8 carbons (green).**

Conclusions on the Activation Mechanism of GPR109A and GPR109B

Inspired by the common negative charge of the carboxylic group of all ligands selective for GPR109 receptors, positive charge counterparts were chosen for site-directed mutagenesis. Experimental data revealed that Arg111 is necessary for receptor activation by nicotinic acid of the GPR109A receptor (Tunaru *et al.*, 2003). Furthermore, the negative charge of GPR109 agonists is necessary for receptor activation. Nicotinamide, which differs from nicotinic acid only in the amidation of the carboxyl group, is unable to activate the GPR109A receptor. In summary, the activation of GPR109 receptors depends on a salt-bridge contact between the negatively charged carboxylic group of a ligand and the positively charged guanidino group of Arg111.

From the models one may conclude that in the inactive state Arg111 (TMH3) interacts with Ser247 (TMH6) and Thr283 (TMH7) stabilizing this state of the receptors. Upon ligand binding the orientation of that arginine side chain changes by releasing the interactions to these residues, which leads to activation of the GPR109 receptors.