Molecular Pharmacology Fast Forward. Published on August 11, 2005 as doi:10.1124/mol.105.015750
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MOLPHARM/2005/015750 (revised)

CHARACTERIZATION OF DETERMINANTS OF LIGAND BINDING TO THE NICOTINIC ACID RECEPTOR GPR109A (HM74A/PUMA-G)

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Molecular Pharmacology Fast Forward. Published on August 11, 2005 as DOI: 10.1124/mol.105.015750
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Running Title: Nicotinic acid receptor binding site

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Number of text pages: 28

Number of tables: 2

Number of figures: 4

Number of references: 40

Number of words in *Abstract*: 195

Number of words in *Introduction*: 501

Number of words in *Discussion*: 1146

List of non-standard abbreviations: ECL, extracellular loop; TMH, transmembrane helix; GPCR, G-protein coupled receptor

Support: This work was supported by the Deutsche Forschungsgemeinschaft (S.O.)

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ABSTRACT

The G-protein-coupled receptor GPR109A (HM74A/PUMA-G) has recently been shown to function as a receptor for nicotinic acid (niacin) and to mediate its antilipolytic effects. Nicotinic acid is able to strongly raise high-density lipoprotein (HDL) cholesterol levels, a property which distinguishes nicotinic acid from other lipid-lowering drugs. To investigate the structural determinants of GPR109A ligand binding, we performed site directed mutagenesis of putative ligand binding residues combined with generation of chimeric receptors consisting of GPR109A and its close relative GPR109B which does not bind nicotinic acid. We could identify N86/W91 (TMH2/ECL1), R111 (TMH3), S178 (ECL2), F276 (TMH7) and Y284 (TMH7) as amino acid residues critical for binding of nicotinic acid. Together with molecular modelling studies, our data suggest that the ligand binding pocket for nicotinic acid of GPR109A is distinct from that of most other group A receptors. While R111 at TMH3 serves as the basic anchor point for the carboxylate ligands, the ring system of nicotinic acid is embedded between W91 at the junction TMH2/ECL1 and F276/Y284 at TMH7. The heterocyclic ring is also bound to S178 at ECL2 *via* a H-bond. These data will facilitate the design of new antidyslipidemic drugs acting *via* GPR109A.

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INTRODUCTION

The water soluble B-complex vitamin nicotinic acid (niacin) has been shown to decrease plasma concentrations of total cholesterol, free fatty acids, and triglycerides when given at pharmacological doses to humans (Altschul et al., 1955; Carlson and Oro, 1962). Fore decades these effects are used to treat different dyslipidemic disorders (Knopp, 1999; Olsson, 1994). Remarkably, nicotinic acid is able to strongly raise high-density lipoprotein (HDL) cholesterol levels, a property which distinguishes nicotinic acid from other lipid-lowering drugs (Blum et al., 1977; Shepherd et al., 1979). However, relatively high doses of nicotinic acid have to be administered, and the beneficial effects on lipid metabolism are accompanied by mostly harmless but disturbing side effects like flushing, decreased glucose tolerance, or gastrointestinal effects (Olsson, 1994). Despite its long use in clinical practice, the exact mechanism of nicotinic acid-induced effects on lipid metabolism are still not completely understood. However, the inhibition of fat cell lipolysis *via* the activation of a G_i-coupled receptor and subsequent inhibition of cAMP formation (Aktories et al., 1980; Aktories et al., 1982) has been postulated to play an important role (Tornvall et al., 1990).

Recently, a G-protein-coupled receptor which binds nicotinic acid with the expected affinity has been identified (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003). The receptor termed GPR109A (HM74A in human and PUMA-G in mice) is expressed in adipocytes and immune cells and couples to G-proteins of the G_i family. Activation of the receptor by nicotinic acid decreases the activity of hormone-sensitive lipase *via* lowering of cAMP levels which results in a reduced hydrolysis of triglycerides to free fatty acids. In mice lacking the murine form of the nicotinic acid receptor, the anti-lipolytic effects of nicotinic acid, which result in a decrease in free fatty acid and triglyceride plasma levels, are abrogated (Tunaru et al., 2003).

Thus, GPR109A (HM74A/PUMA-G) is the receptor mediating the anti-lipolytic effects of nicotinic acid. Bioinformatic information indicates that GPR109A is a member of a subfamily of G-protein-coupled receptors which comprise GPR109A (HM74A/PUMA-G) and GPR81 of which both exist in humans and in rodent species. In addition, in humans but not in mice and rats, a third member of this receptor family, GPR109B (HM74), has been found (Soga et al., 2003; Wise et al., 2003). GPR109A, GPR81, and GPR109B are colocalized on human chromosome 12q24.31 and are most likely the result of gene duplications (Zellner et al., 2005). Interestingly, nicotinic acid and related compounds like acipimox (5-methylpyrazine carboxylic acid 4-oxide) are only able to bind to GPR109A (Soga et al., 2003; Wise et al., 2003). In contrast, acifran (4,5-dihydro-5-methyl-4-oxo-5-phenyl-2-furancarboxylic acid) which also induces changes in lipoprotein profile similar to those induced by nicotinic acid (Cayen et al., 1982; LaRosa et al., 1987) is an agonist on both GPR109A and GPR109B, indicating that GPR109B is a functional G-protein-coupled receptor (Wise et al., 2003). No ligand has been described for GPR81 so far.

Since the nicotinic acid receptor is of great interest as a target for new anti-dyslipidemic drugs, we have aimed in the present study to characterize the structural requirements of GPR109A for binding of nicotinic acid. Based on the high degree of homology between the human high-affinity nicotinic acid receptor GPR109A and the receptor GPR109B, which has only very low affinity for nicotinic acid, we used an iterative approach combining site-directed mutagenesis, analysis of GPR109A/GPR109B chimeras and of structural modelling to characterize the nicotinic acid-binding pocket on GPR109A.

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MATERIALS AND METHODS

Materials - Nicotinic acid (pyridine-3-carboxylic acid) was from Sigma, acifran (4,5-dihydro-5-methyl-4-oxo-5-phenyl-2-furancarboxylic acid) was from Tocris.

Calcium mobilization - CHO-K1 cells stably transfected with a calcium sensitive bioluminescent fusion protein consisting of aequorin and GFP (Baubet et al., 2000) were seeded in 96 well plates and were transfected with indicated cDNAs or control DNA (50 ng/well) using FuGENE6 reagent (Roche). Two days after transfection, cells were loaded with 5µM of coelenterazine *h* (Biotium) in calcium-free Hank's Balanced Salt Solution (HBSS) containing 10 mM HEPES (pH 7.4) for 3.5 h at 37°C. 45 min prior to experiments the buffer was replaced with HBSS containing 1,8 mM $CaCl_2$. Measurements were performed by using a luminometer plate reader (Luminoskan Ascent, Labsystems).

Radioligand binding - Equilibrium binding of ^{3}H-labelled nicotinic acid (American Radiolabeled Chemicals; 50 Ci/mmol) was performed on 30 µg of membranes from HEK293T cells expressing wild-type or mutant receptors in a total volume of 250 µl binding buffer [50 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, 0,02 % (v/v) CHAPS] as described (Tunaru et al., 2003). After 4 h of incubation at 25°C unbound and membrane-bound radioactivity were separated by filtration of the samples through nitrocellulose filters, followed by two washing steps with 4 ml ice-cold binding buffer. Non-specific binding was determined in the presence of 200 µM unlabelled nicotinic acid.

Site-directed mutagenesis, generation of receptor chimeras - Specific mutations introduced in GPR109A receptors were carried out based on QuickChange site directed mutagenesis procedure (Stratagene) using *Pfu Turbo* as a proof reading DNA polymerase. A receptor version carrying a

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FLAG-tag at the N-terminus was used as a template for mutagenesis. Correct mutagenesis was confirmed by DNA sequencing. For generation of receptor chimeras, GPR109A and GPR109B receptor cDNAs were cut with BglII endonuclease. BglII has a recognition site within both receptors cDNAs which corresponds to a location between amino acids I139 and S140 of GPR109A. Additional point mutations were introduced in chimeras by site directed mutagenesis as described above.

Determination of receptor expression and localization - CHO/G5A cells grown on glass coverslides in 6 well plates were transfected with the indicated N-terminally FLAG tagged receptors using FuGENE (Roche). 24 hours later, cells were transferred onto ice, and the medium was removed. Cells were washed two times with ice-cold PBS and were then fixed for 30 min in 4 % PFA in PBS at room temperature. To reduce the unspecific binding of the antibody, cells were blocked for 30 min in 4 % FBS in PBS at room temperature, followed by incubation for 2 h at room temperature with anti-Flag antibody (Sigma). After three washing steps with 2 % BSA in PBS, cells were labeled with anti-mouse TRITC conjugated secondary antibody (Jackson ImmunoResearch) for 30 min at room temperature. Cells were washed six times with Tris buffer (50 mM Tris, 150 mM NaCl, pH 7,5) and stained cells were visualized using a confocal imaging system (Leica DM IRE2*).*

Cell surface expression of wild type and mutant FLAG-tagged receptors was assessed by enzyme-linked immuno-sorbent assay (ELISA) using Anti-FLAG antibody and horse-radish peroxidase (HRP) conjugated secondary antibody (Amersham). Light generated by incubation with HRP substrate (Roche) was measured by using an Ascent Luminometer (Labsystems).

Homology modelling and ligand docking - To generate a structural model for GPR109A/B we adopted the X-ray structure of rhodopsin (Teller et al., 2001) from entry 1hzx of PDB (Berman et

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al., 2000) as a template. Several receptor specific corrections were made based on sequence alignement investigations (SeqLab, Wisconsin Package Version 10.2, Accelrys Inc. San Diego, USA). At the N-terminal tail the two consecutive cysteines C18 and C19 are forming two additional disulfide bridges towards ECL2 (C18-ECL2:C183) and ECL3 (C19-ECL3:C266), respectively. For TMH2, a structural ‚bulge' of Rhodopsin caused by side chain/backbone interaction of three consecutive threonines in the rhodopsin structure would localize D85, N86 at the membrane-oriented phase of the helix. New construction of the TMH2/ECL1 junction avoids the ‚bulge' structure of rhodopsin and considers a proline kink from other TM structures (Sansom and Weinstein, 2000). The new conformation is similar to chemokine receptor models (Proline is located on the same sequence-position like in GPR109A/B). Also at TMH5 a minor change of orientation (10 to 15 degrees twist) of the N-terminal half of TMH5 due to different residues compared to rhodopsin prior the proline kink at Pro200 was generated. The length of ECL3 was extended by an additional helix turn at TMH6 because of more residues in GPR109A/B than in the rhodopsin template. Gaps of missing residues in the intracellular loops of the template structure were closed by the 'Loop Search' tool implemented in Sybyl6.8 (Tripos Inc., 1699 South Hanley Rd., St. Louis, Missouri, 63144, USA) using GPR109A/B sequence. Concerning the orientation of ECL2 we started with two different models: One with the original rhodopsin fold in anticlockwise order of residues around C177 and a second model with reversed, clockwise order of ECL2 residues around C177 by preserving the β-strand motifs. This results in a suitable geometry for pairing the additional disulfide bridges. After model generation the structures were minimized using Amber4.1 force field and Amber95_Protein_ALL charges. In a first step the ligands are manually docked according to at that time known sensitive mutants as potential interaction points. In a second step the stability of the ligand in the binding site was studied by molecular dynamics runs in a water-vacuum-water box system (ter Laak and Kuhne,

1999) without any restrains (1.0 ns, periodic boundary box, charges neutralized by adding chlorine ions) using AMBER 7.0 (Case et al., 2002). The quality of the model and stability is validated by checking the geometry by PROCHECK (Laskowski et al., 1996) and the stability during the MD run (overall backbone RMSD 1.7 Å).

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RESULTS

Both, the human nicotinic acid receptor GPR109A and the paralogous receptor GPR109B are functional G-protein-coupled receptors which can be activated by the furan-carboxylic acid derivate acifran (Wise et al., 2003). In contrast, pyridine-3-carboxylic acid (nicotinic acid) as well as pyrazine-carboxylic acid derivatives like acipimox function as high-affinity agonists only on GPR109A but not on GPR109B (Soga et al., 2003; Wise et al., 2003). In addition to a shorter C-terminus, GPR109A differs from GPR109B in only 17 amino acid residues, which cluster around extracellular loops (ECL) 1 and 2 (Fig. 1). It is likely that these amino acid residues, of which 14 are conserved in human, mouse and rat versions of the receptor, are critically involved in ligand binding. We have therefore systematically mutated each of these amino acid residues in GPR109A into the corresponding residue of GPR109B. Nicotinic acid-induced activation of mutant receptors was tested in cells coexpressing receptor mutants and the promiscuous G-protein α-subunit $G\alpha_{15}$ in a Ca^{2+} reporter assay as described previously (Tunaru et al., 2003). All receptor mutants were N-terminally tagged with the FLAG epitope, and expression as well as membrane localization was verified by confocal microscopy and ELISA (see methods). Of the 14 mutants tested, only N86Y, W91S, and S178I showed a severely reduced ability to respond to nicotinic acid (Fig. 2a; Tab. 1). All other single amino acid mutants showed unaltered or only very slightly reduced EC_{50} values for nicotinic acid-induced Ca^{2+}-mobilization. The three mutants which only weakly responded to nicotinic acid were well expressed and showed membraneous localization (Fig. 2g, Tab. 1). In addition, they still responded to acifran. Interestingly, the potency of acifran to act via these three mutants was reduced to the level observed for GPR109B (Fig. 2b; Tab. 1). Collectively, these data indicate that the mutants were functionally active. We then performed radioligand binding assays using ^{3}H-labelled nicotinic

acid (see Tab. 1). All mutants (N86Y, W91S and S178I) showed severely reduced binding affinities for nicotinic acid. Thus, asparagine 86, tryptophan 91, and serine 178 of GPR109A are required for high affinity binding of nicotinic acid but are not necessary for acifran-induced receptor activation.

Since all known ligands for GPR109A carry a carboxylate group, it appeared likely that a basic residue in GPR109A is important for binding of carboxylic acid ligands. This is supported by the fact that any change or substitution at the carboxylic acid moiety of nicotinic acid such as in nicotinamide completely abrogates its pharmacological activity (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003). We identified four arginine residues in transmembrane helices (TMH) as candidates which may provide a binding environment for the carboxylate groups of GPR109A receptor ligands (Fig. 1). To test their potential involvement in nicotinic acid binding, we mutated arginine residues 111, 210, 251, and 253 into alanine. While the effects of nicotinic acid and acifran on the R253A and R210A mutants were indistinguishable from those on wildtype receptor, the potencies of both agonists were reduced when tested on the GPR109A mutant R251A. However, no receptor activation at all could be observed when nicotinic acid and acifran were tested on the GPR109A mutant R111A (Fig. 2c/d; Tab. 1). Subsequent radioligand binding studies showed that the R251A mutant had a somewhat reduced affinity for nicotinic acid, while R111A lost its ability to bind nicotinic acid (Tab. 1). The R111A mutant was normally expressed and localized to the plasma membrane as shown by confocal microscopy and ELISA (Tab. 1, Fig. 2g). This suggests that arginine 111, which is localized in TMH3 of GPR109A and GPR109B, is crucial for ligand-dependent receptor activation by contributing to the binding pocket of the receptor. The corresponding position 3.33 (Ballesteros and Weinstein, 1992) in many GPCRs has been shown to be involved in ligand binding (Gether, 2000).

As is typical for class A G-protein-coupled receptors, also GPR109A has a cysteine residue at the extracellular end of TMH3 (C3.25), which forms a disulfide bond with a cysteine residue in the ECL2. Removal of the disulfide bond by mutagenesis has been shown to severely interfere with the function of GPCRs (Noda et al., 1994; Savarese et al., 1992). GPR109A has two cysteine residues in the 2nd extracellular loop, C177 and C183 (Fig. 1). A C183A mutation in GPR109A rendered the receptor still responsive to nicotinic acid, although the potency was severely reduced, while C100A and C177A mutations resulted in a receptor which was completely unable to respond to nicotinic acid or acifran (Tab. 1; Fig. 2e, f). Analysis of receptor expression revealed that both the C100A and the C177A mutants of GPR109A were only poorly expressed. This indicates that the disulfide bond conserved in class A GPCRs (Gether, 2000) is formed between cysteine residues 100 and 177 of GPR109A. The reduced potency of nicotinic acid at the C183A mutant of GPR109A can be explained by an additional disulfide bond established between ECL2 and one of the cysteines at the N-terminal tail which may stabilize the conformation of ECL2.

The three residues differing between GPR109A and GPR109B which were found to be essential for binding of nicotinic acid to GPR109A are either at the junction of TMH2 and the first ECL (N86 and W91), or in the second ECL (S178). This suggests that both, junction TMH2/ECL1 and ECL2, are part of the binding site for nicotinic acid in GPR109A. To gain further insight into the structural requirements of GPR109A for binding of nicotinic acid, we generated chimeras of GPR109A and GPR109B. The chimeras were constructed in a manner that the first three TM regions including the junction TMH2/ECL1 were from one receptor, while the C-terminal four TM regions including the second ECL were from the corresponding homologous receptor (see Methods; Fig. 3a). The unselective agonist acifran was still able to activate both chimeras, although the potency of acifran towards the GPR109B/GPR109A chimera was

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somewhat reduced (Fig. 3b,d). In contrast, both receptor chimeras were completely unresponsive to nicotinic acid (Fig. 3b,c). Radioligand binding assays showed that none of the chimeras bound nicotinic acid (Fig. 3b). To further characterize the involvement of the junction TMH2/ECL1 and of ECL2 in binding of the receptor to nicotinic acid, we mutationally reintroduced the GPR109A-specific extracellular residues required for nicotinic acid binding (N86, W91, and S178) into the GPR109B portion of the GPR109A/GPR109B chimeras (see Fig. 3a). The GPR109B(Y86N,S91W)/GPR109A chimera was now again able to bind and respond to nicotinic acid. Similarly, the GPR109A/GPR109B(I178S) chimera gained responsiveness and binding towards nicotinic acid (Fig. 3b,c). B_{max} values were 3.98 ± 0.34 pmoles/mg for GPR109B(Y86N,S91W)/GPR109A and 4.01 ± 0.23 pmoles/mg for GPR109A/GPR109B(I178S). All mutants were normally expressed (s. Fig. 2g, data not shown). These data clearly support the notion, that N86, W91, and S178 in the junction of TMH2/ECL1 and in ECL2 of GPR109A are critically involved in nicotinic acid binding.

Our modified homology model of GPR109A including the reversed order of ECL2 was most consistent with the experimental data, where the residues N86, W91 and S178 are located in close spatial proximity between the junction TMH2/ECL1 and ECL2. The mutation-sensitive basic side chain R111 (TMH3) is located in the interior centre of the classical binding area for the majority of GPCR ligands. Docking studies by anchoring the acidic group of nicotinic acid towards the most sensitive basic residue R111 at TMH3 in the centre of the receptor allows to define two potential binding areas for the very small ligand. In binding site I the pyridine ring of nicotinic acid is embedded between the aromatic rings of W91 (junction TMH2/ECL1), F276 (TMH7) and Y284 (TMH7) whereas the ring nitrogen is simultaneously bound to S178 at ECL2 *via* a H-bond. The suitable side chain orientation of the two aromatic side chains W91 and F276 are in case of W91 (TMH2/ECL1) restrained by N86 (TMH2) *via* H-bond and in case of F276

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(TMH7) by F180 (ECL2) *via* aromatic interactions (Fig. 4 a, b). Binding site II would be localized at the interior receptor cleft between TMH3, TMH6 and TMH5 at the extracellular half of the transmembrane region, a common ligand binding region for the majority of small ligands like the biogenic amine receptors of class A GPCRs. The locations of nearly all sensitive mutants for binding nicotinic acid (Tab. 1) are spatially distributed at site I (R111, N86, W91, S178) of the GPR109A model. The mutation of R251 which is localized at site II had only a weak effect on ligand binding and ligand-induced signalling might be based on an indirect effect. (Tab. 1; Fig. 2).

From analyses of crystal structures of nicotinic acid bound at diverse prokaryotic proteins such as nicotinate mononucleotide dimethylbenzimidazole phosphoribosyltransferase (PDB entry code: 1D0V) (Cheong et al., 1999), dihydropteridine reductase (1ICR) (Lovering et al., 2001), nicotinate nucleotide dimethylbenzimidazole phosphoribosyltransferase (1JHA) (Cheong et al., 2001), dihydrodipicolinate reductase (1DRV) (Reddy et al., 1996) and the plant protein ferric soybean leghemoglobin (1FSL) (Ellis et al., 1997), it is evident, that the pyridine ring system of the ligand is always bound in close proximity to aromatic side chains of the protein. Following the structural homology paradigm we assumed that the binding pocket of GPR109A is also coated by aromatic side chain(s) as additional binding partner for nicotinic acid. According to our ligand/receptor interaction model, in addition to W91, also F276 (TMH7) and Y284 (TMH7) were predicted to be direct interaction partners. Residue F180 (ECL2), was predicted to be an indirect aromatic interaction partner at binding site I (Fig 4b), whereas residue F193 (TMH5) would be a possible partner at a potential binding site II. To study whether the aromatic residues are required for ligand induced signalling and to distinguish between binding site I and II, leucine and alanine mutants of these aromatic residues were generated. Indeed, mutation of the aromatic residue F276 (TMH7) and Y284 (TMH7) at binding site I to alanine or leucine had

the strongest effects on binding of nicotinic acid and acifran, indicating a direct effect on the ligand binding site (Tab. 2) *via* aromatic interactions. The alanine mutants of F180 and F193 had the same phenotypes, whereas F180L and F193L mutants were only slightly affected in their ability to bind ligand. This indicates indirect effects of alanine mutants on the ligand binding site and provides additional support for binding site I, because F193 is at TMH5 located in far distance to binding site I and would be the only residue located at a hypothetical binding site II (Fig 4a). All mutants have expression levels similar to wild type (not shown). The suggested additional mutations of aromatic residues further support the binding site I and confirmed the direct and indirect effects of aromatic residues at binding site I derived from the structural model.

Taken together, an iterative approach combining site-directed mutagenesis and comparative modelling of the binding pocket of nicotinic acid at GPR109A identified five residues as main interaction points. The binding pocket is located between the basic anchor site at TMH3 (R111) for the acidic group of the ligand and the interaction site for the ring system of the ligands formed by the junction TMH2/ECL1 (N86, W91), TMH7 (F276, Y284) and the tip of ECL2 (S178).

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DISCUSSION

Based on the remarkable clinical effects of nicotinic acid, its recently discovered G-protein-coupled receptor represents one of the prime targets for the development of new anti-dyslipidemic drugs (Pike and Wise, 2004). Major aims of new drug development in this field are e.g. the improvement of the relatively low potency as well as of the unfavourable pharmacokinetic properties of nicotinic acid. In addition, a better ratio of wanted and unwanted effects would be desirable. To understand more about the structural requirements for binding of ligands to the nicotinic acid receptor we have characterized the binding site on the human nicotinic acid receptor GPR109A. Surprisingly, our data indicate that the binding site of GPR109A for its known pharmacological small molecule ligands differs from the classical binding site of class A G-protein-coupled receptors for small molecule ligands, which in the case of biogenic amine receptors is located between TMH3, TMH5 and TMH6 (Gether, 2000; Kristiansen, 2004; Strader et al., 1994). Our major new finding is that the binding crevice of GPR109A for nicotinic acid appears to be formed by the TMH2, TMH3, and TMH7, and 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 N86, W91 at the junction TMH2/ECL1 and S178 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 group is critically involved in binding. This is supported by the fact that any change or substitution at the carboxylic acid moiety of nicotinic acid such as in nicotinamide completely abrogates its pharmacological activity. Based on the assumption that the carboxylic acid group forms a salt bridge with a residue in one of the TM regions of the

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receptors, we searched for positively charged residues, which would allow for an electrostatic interaction with the carboxyl oxygens. Of four candidate arginine residues in TMHs 3, 6, and 7, only arginine 111 in TMH3 was absolutely required for binding of nicotinic acid to GPR109A. While in contrast mutational deletion of positively charged residues in TMH6 and 7 did not interfere with the ability of the receptor to bind nicotinic acid. Interestingly, the recently discovered dicarboxylic acid receptor GPR91 has been suggested to require positively charged amino acid residues in TMH 6 and 7, and two residues in TMH3 (He et al., 2004), and an arginine residue in TMH7 of prostanoid receptors has been suggested to bind to the C1-carboxylate of prostanoids (Stitham et al., 2003).

Class A GPCRs for small molecule agonists bind their specific ligands *via* the extracellular half of their 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 TMH 3, 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). Our findings support a structural model for GPR109A in which the nicotinic acid binding pocket is localized between TM helices 2, 3, and 7. The ligands of various receptors such as the C3a receptor (Kim et al., 1999), the Ca^{2+}-sensing receptor (Miedlich et al., 2004), the prostacyclin receptor (Stitham et al., 2003) or the vasopressin V_2 receptor (Wuller et al., 2004) are also interacting with a similar binding site on their respective receptors.

While the major binding sites for small molecule ligands of GPCRs are localized in the TM helices, there is evidence from site-directed mutagenesis experiments that especially certain residues in ECL2 can also contribute to binding of small ligands to GPCRs (Kim et al., 1996; Shi and Javitch, 2004; Zhao et al., 1996). Similar to many other GPCRs, also GPR109A has a disulfide bond between ECL2 (C177) and TMH3 (C100). Due to the disulfide linkage ECL2 is

constrained over the ligand binding pocket, and residues from ECL2 can take part in ligand binding. In our model, S178 of ECL2 reaches into the ligand binding pocket and interacts with the nitrogen of the pyridine ring of nicotinic acid.

As already reported for other GPCRs (Balmforth et al., 1997; Groblewski et al., 1997), in our homology model of GPR109A the inactive state is also restrained by side chain interactions between TMH3 and TMH6/TMH7, where R111 (TMH3) is very likely involved in side chain interactions with S247 (TMH6) and T283 (TMH7), stabilising/constraining the inactive conformation. Arginine 111 at TMH3 is the pivotal residue for electrostatic recognition and binding by functioning as a basic anchor point for the acidic group of the ligands. Our findings support a scenario in which the arginine side chain is forced to delocalise 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, and the interaction between the helices TMH3 and TMH6/TMH7 is weakened or lost.

According to several X-ray structures of nicotinic acid/protein complexes, in which nicotinic acid is surrounded by aromatic residues (Cheong et al., 1999; Cheong et al., 2001; Ellis et al., 1997; Lovering et al., 2001; Reddy et al., 1996) and based on our docking model of nicotinic acid/GPR109A, the participation of aromatic residues as direct and indirect partners in the binding site was predicted. To distinguish between direct and indirect aromatic interaction partners of the ligand we introduced strong (alanine) and weak (leucine) alterations of side chain properties by mutations. Mutants of F276 and Y284 showed identical strong effects for alanine and leucine as well, indicating a direct aromatic interaction of both positions on ligand binding. Whereas the common differences between alanine and leucine mutants for F180 and F193 indicate rather indirect effects of the two residues on ligand binding (see above). Leucine mutants are preserving the necessary hydrophobic properties needed for proper orientation of the

neighboured side chains. The strong side chain reduction in size and hydrophobicity in case of alanine mutants may lead to slightly altered assembly of the neighbouring side chains and/or helices and thus may affect indirectly the proper shape and size of the ligand binding site. Finally all residues experimentally identified as essential direct interaction partners are located in the GPR109A model in very close spatial proximity and provide support that the binding site of nicotinic acid is located between TMH3, junction TMH2/ECL1, TMH7 and the tip of ECL2. The data combined with the model suggest also the possibility that some aromatic residues such as W91 and F276 may play a role in the formation of a gateway which allows nicotinic acid to access the binding pocket.

Taking advantage of the high degree of sequence homology between the human high-affinity nicotinic acid receptor GPR109A and the receptor GPR109B, which has only very low affinity for nicotinic acid, we have identified critical residues for the binding of nicotinic acid to GPR109A. By combining mutagenesis data and comparative structural modelling we were able to identify five residues located in close spatial proximity as the main interaction points for nicotinic acid. The characterization of the structural determinants and complementary pharmacophoric patterns for binding of nicotinic acid at GPR109A is of general importance for understanding the binding mechanism of small molecule ligands to GPCRs as well as for the design and development of new drugs acting *via* GPR109A to treat dyslipidemic disorders.

Molecular Pharmacology Fast Forward. Published on August 11, 2005 as DOI: 10.1124/mol.105.015750
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ACKNOWLEDGMENTS

The authors wish to thank R. LeFaucheur for help with the manuscript.

Molecular Pharmacology Fast Forward. Published on August 11, 2005 as DOI: 10.1124/mol.105.015750
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FIGURE LEGENDS

Figure 1. Secondary structure of the human nicotinic acid receptor GPR109A. White amino acid symbols on black circles and squares indicate residues in GPR109A which differ from GPR109B. The arrows point to the corresponding amino acid in GPR109B. Grey circles and squares indicate amino acid residues of GPR109A which, in addition, have been mutated in this study. Shown in squares are those residues which showed a significant difference in nicotinic acid binding when mutated in GPR109A. Extracellular, transmembrane, and cytoplasmic regions are based on the structure of rhodopsin. The disulfide bond found in the extracellular part of the receptor is indicated by a straight line. GPR109B has a C-terminus which is extended by 24 amino acids.

Figure 2. Concentration-dependent changes in $[Ca^{2+}]_i$ evoked in CHO-K1 cells expressing GPR109B, GPR109A or the indicated mutants of GPR109A by nicotinic acid (A, C, E) or acifran (B, D, F). RLU, relative light units. Shown are mean values ± S.D. of at least three independently performed experiments. G, confocal images of CHO-K1 cells transfected with the indicated wildtype and mutant receptors. Staining was performed using an anti-FLAG antibody recognizing the N-terminal FLAG tag of the receptors.

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Figure 3. (A) Structure of the chimeric GPR109B/GPR109A receptors. Black indicates sequences and residues from GPR109A, grey indicates sequences from GPR109B. (B) Nicotinic acid and acifran evoked Ca^{2+} responses (EC_{50}) and nicotinic acid binding Kd) for chimeric GPR109A and GPR109B receptors. (C,D) Effect of increasing concentrations of nicotinic acid (C) or acifran (D) on the free intracellular Ca^{2+}-concentration in CHO-K1 cells expressing the promiscuous G-protein $G\alpha_{15}$ together with GPR109B, GPR109A or the indicated chimeric receptors. Shown are mean values ± S.D. of at least three independently performed experiments.

Figure 4. Interaction model of nicotinic acid (orange) at the binding site (green residues) of GPR109A receptor. Stable conformation after a 1.0 ns molecular dynamics run. (A) The binding site of nicotinic acid is located between transmembrane helices TMH 2, 3 and 7 (pink/yellow ribbon). Our data do not support an alternative binding site between TMH 3, 4, 5 and 6 (e.g. magenta residue F193) (B) Close up view of the binding site. Acidic group of nicotinic acid (orange) interacts with the basic anchor point R111 at TMH3, whereas the pyridine ring is embedded between W91 at the junction TMH2/ECL1, F276 and Y284 at TMH7. The pyridine nitrogen is also bound to S178 at ECL2 *via* a H-bond. N86 (TMH2) restrains the orientation of W91 by hydrogen bond and F180 (ECL2) restrains the orientation of F276 by aromatic interactions leading to a suitable and rigid binding cleft.

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TABLES

Tab. 1. Nicotinic acid and acifran evoked Ca^{2+} responses (EC_{50}) and nicotinic acid binding affinity (K_d) for GPR109A mutants.

	EC_{50} (μM)		[^{3}H] Nicotinic acid binding		Surface Expression (RLU)
	Nicotinic acid	Acifran	K_d (nM)	B_{max} (pmoles/mg)	
WT receptors					
GPR109A	0.7± 0.2	1.9± 0.4	60 ± 8	3.36± 0.45	39 ± 2.21
GPR109B	inactive	90 ± 12	>500		42.4 ± 5.09
GPR109A mutants					
L83V	3 ± 0.5	2 ± 0.3			
N86Y	>100	88 ± 20	>500		45.27 ± 1.91
W91S	>100	96.8± 10	>500		51.24 ± 4.9
K94N	1.4± 0.8	4 ± 0.7			
M103V	5.3± 1.1	2.2± 0.5			
L107F	3.1± 0.5	2.9± 0.3			
R142W	1 ± 0.3	4.3± 0.5			
I156V	0.5± 0.1	2.8± 0.4			
M167L	2.3± 0.3	5.6± 0.2			
P168L	2.3± 0.4	4.7± 0.6			
G173P	1.1± 0.2	2.4± 0.3			
L176V	3 ± 0.4	18.7± 2			
S178I	>100	93.1± 15	>500		49 ± 2.8
F198L	0.7± 0.1	2 ± 0.1			
R111A	inactive	inactive	>500		39.27 ± 3.01
R210A	0.8± 0.2	6 ± 1.5			
R251A	70 ± 12	80 ± 14	351± 22	4.1 ± 0.21	40.2 ± 3.4
R253A	0.9± 0.1	1.9± 0.4			
C100A	low expression	low expression	low expression		3.5 ± 0.27
C177A	low expression	low expression	low expression		0.51 ± 0.02
C183A	>100	>100	>500		20.37 ± 1.7
C266A	20 ± 3	40± 9	160 ± 13	3.03± 0.25	39.87 ± 3.1

Shown are data for GPR109A and GPR109B wildtype receptors as well as for the indicated GPR109A mutants. The EC_{50} values of nicotinic acid-induced increases in $[Ca^{2+}]_i$ were determined in CHO-K1 cells expressing $G\alpha_{15}$ and the indicated wildtype or mutant receptors. Saturation binding results are the mean +/- SD of at least three experiments, shown are the K_D and B_{max} values determined by Scatchard analysis of ^{3}H-nicotinic acid binding saturation isotherms. Relative cell surface expression levels of wild-type and GPR109A receptor mutants were determined by ELISA assay in non-permeabilized CHO-K1 cells (see Methods). RLU-relative light units.

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Tab. 2. Nicotinic acid and acifran evoked Ca^{2+} responses (EC_{50}) and nicotinic acid binding affinity (K_d) for aromatic residue mutants of GPR109A

	EC_{50} (μM)		[^{3}H] Nicotinic acid binding		Surface Expression (RLU)
	Nicotinic acid	**Acifran**	**K_d** (nM)	**B_{max}** (pmoles/mg)	
GPR109A mutants					
F180A	>100	>100	>500		40.78 ± 2.5
F180L	1.8 ± 0.2	5.4 ± 6			
F193A	>100	>100	>500		42.2 ± 3.9
F193L	4 ± 0.6	8 ± 2			
F276A	>100	>100	>500		39.96 ± 4.1
F276L	>100	>100	>500		37.88 ± 2.4
Y284A	81 ± 17	>100	300 ±36	3.60 ± 0.42	39.91 ± 3.7
Y284L	153 ± 10	>100	342 ±40	3.75 ± 0.39	39.08 ± 4.5

Shown are data for the indicated GPR109A mutants. The EC_{50} values of nicotinic acid-induced increases in $[Ca^{2+}]_i$ were determined in CHO-K1 cells expressing $G\alpha_{15}$ and the indicated wildtype or mutant receptors. The K_D and B_{max} values were determined by Scatchard analysis of ^{3}H-nicotinic acid binding saturation isotherms and represent the mean ± SD of at least three independent experiments. Relative cell surface expression levels of wild-type and GPR109A receptor mutants were determined by ELISA assay in non-permeabilized CHO-K1 cells (see Methods). RLU, relative light units.

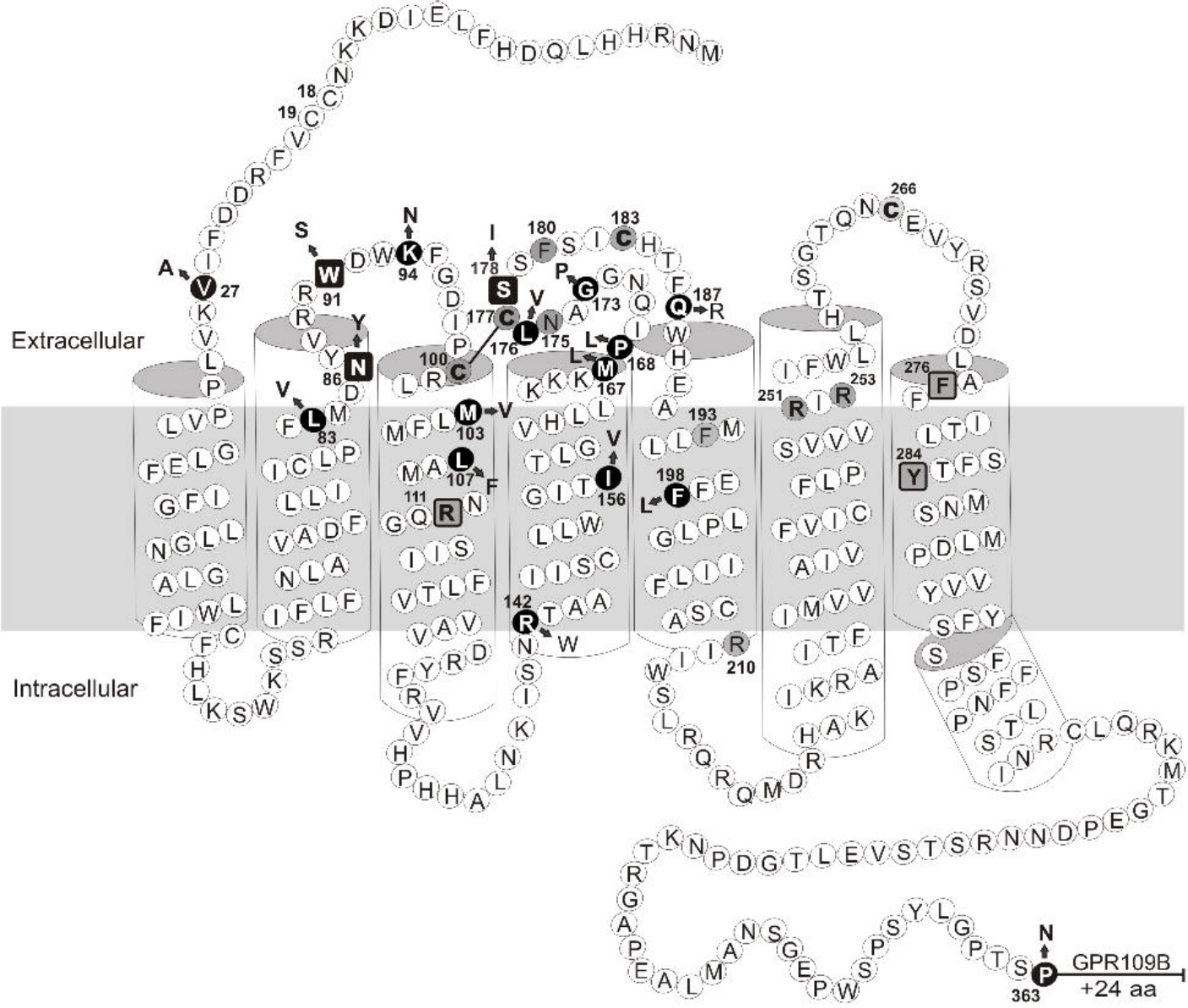


Figure 1

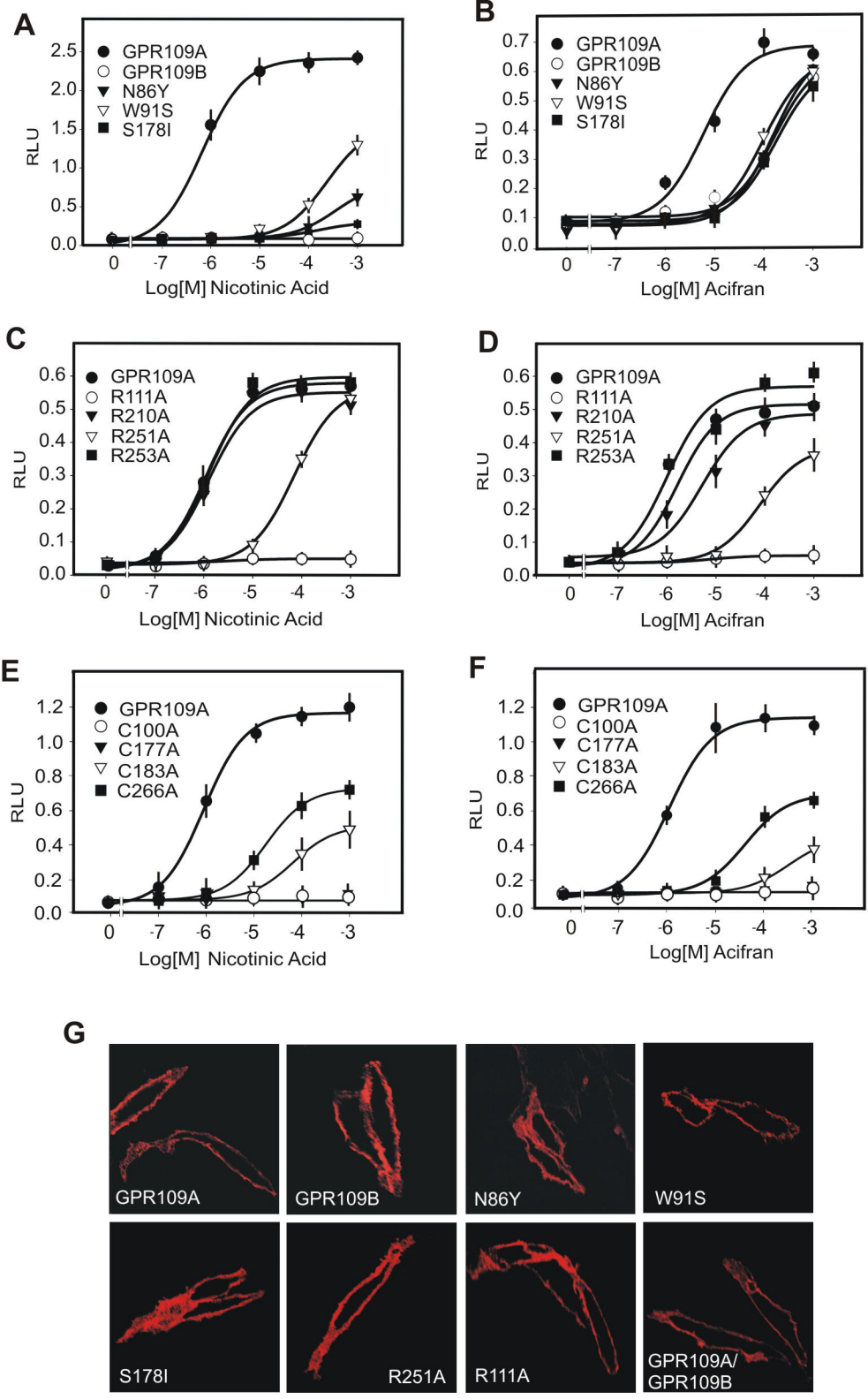
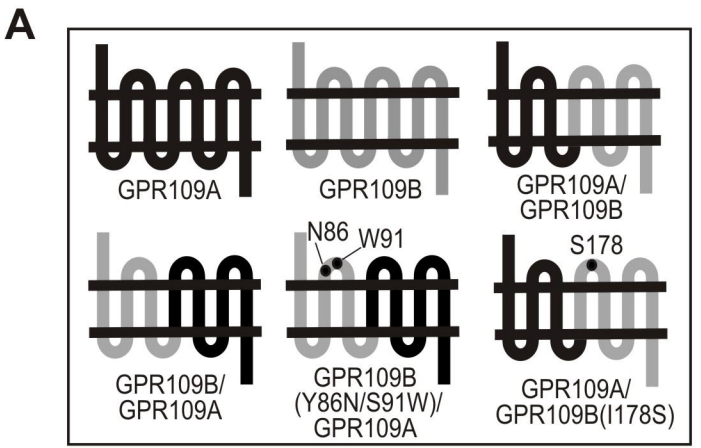
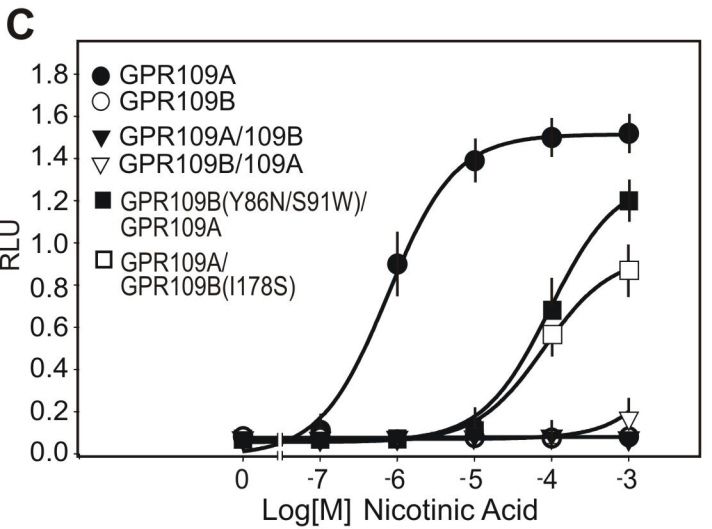

A
GPR109A
GPR109B
N86Y
W91S
S178I
RLU
Log[M] Nicotinic Acid
B
GPR109A
GPR109B
N86Y
W91S
S178I
RLU
Log[M] Acifran
C
GPR109A
R111A
R210A
R251A
R253A
RLU
Log[M] Nicotinic Acid
D
GPR109A
R111A
R210A
R251A
R253A
RLU
Log[M] Acifran
E
GPR109A
C100A
C177A
C183A
C266A
RLU
Log[M] Nicotinic Acid
F
GPR109A
C100A
C177A
C183A
C266A
RLU
Log[M] Acifran
G
GPR109A
GPR109B
N86Y
W91S
S178I
R251A
R111A
GPR109A/
GPR109B

Figure 2



B

Chimeric receptor	Ec_{50} (μM)		K_d (nM)
	Nicotinic acid	Acifran	Nicotinic acid
GPR109A	0.76±0.2	1.9±0.4	60±9
GPR109B	>100	90±14	>500
GPR109A/GPR109B	Inactive	>100	>500
GPR109B/GPR109A	>100	2±0.3	>500
GPR109B(Y86N/S91W)/ GPR109A	93±10	2±0.19	471±51
GPR109A/ GPR109B(I178S)	100±12	20±3	490±39



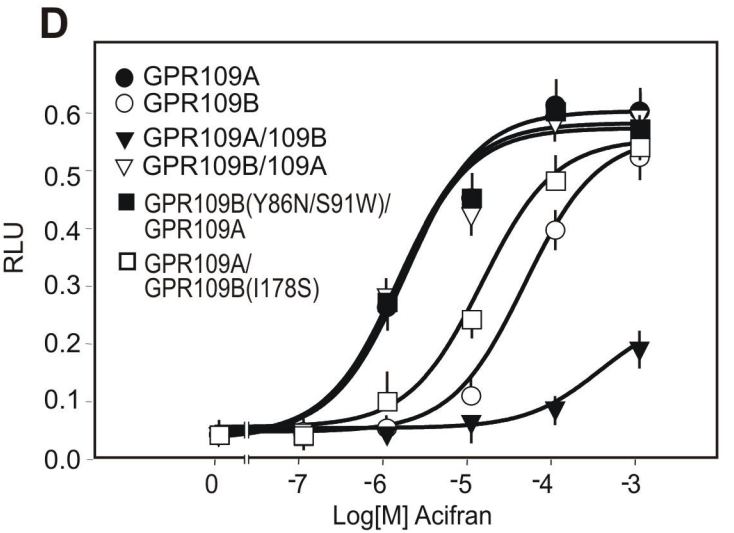


Figure 3

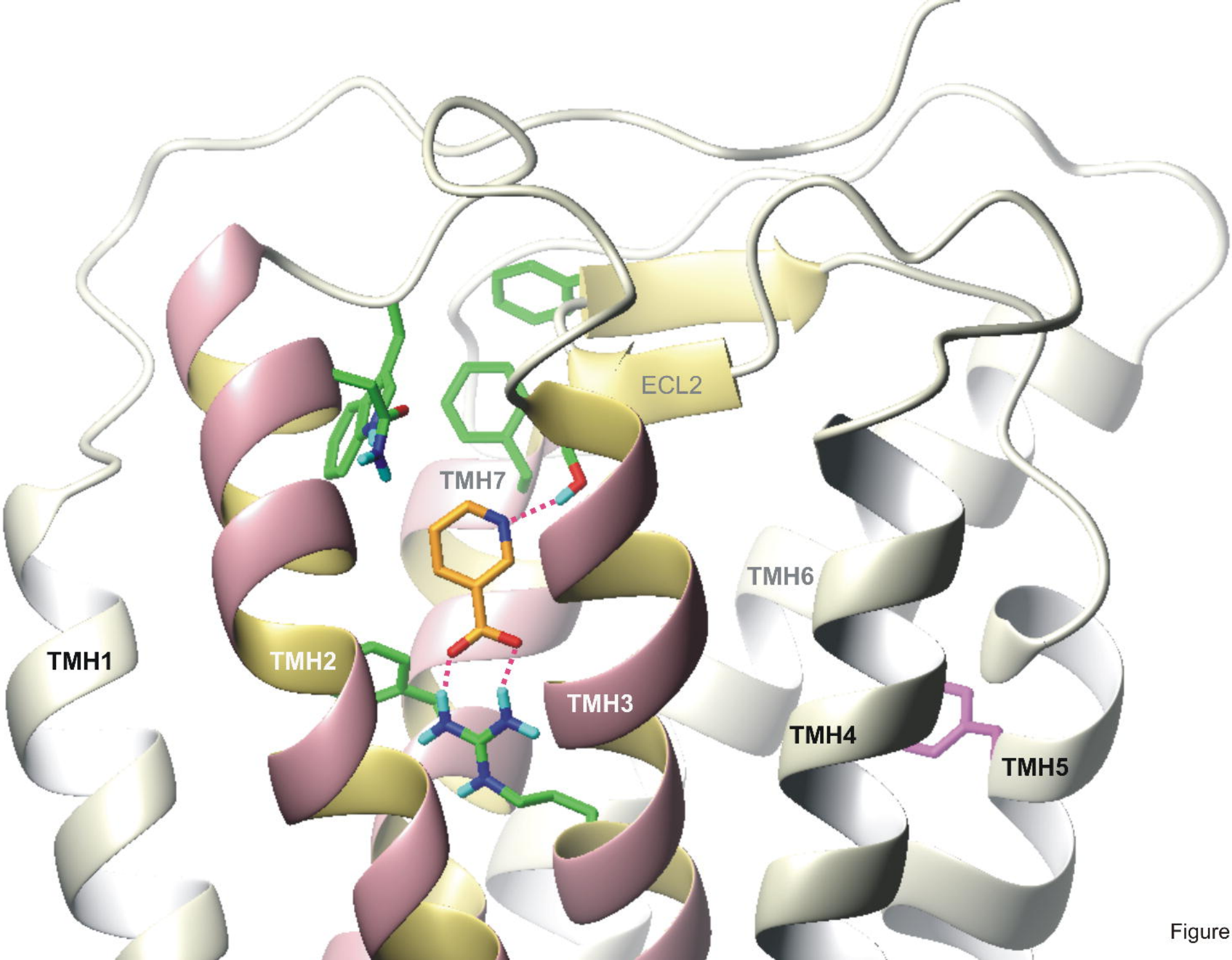


Figure 4a

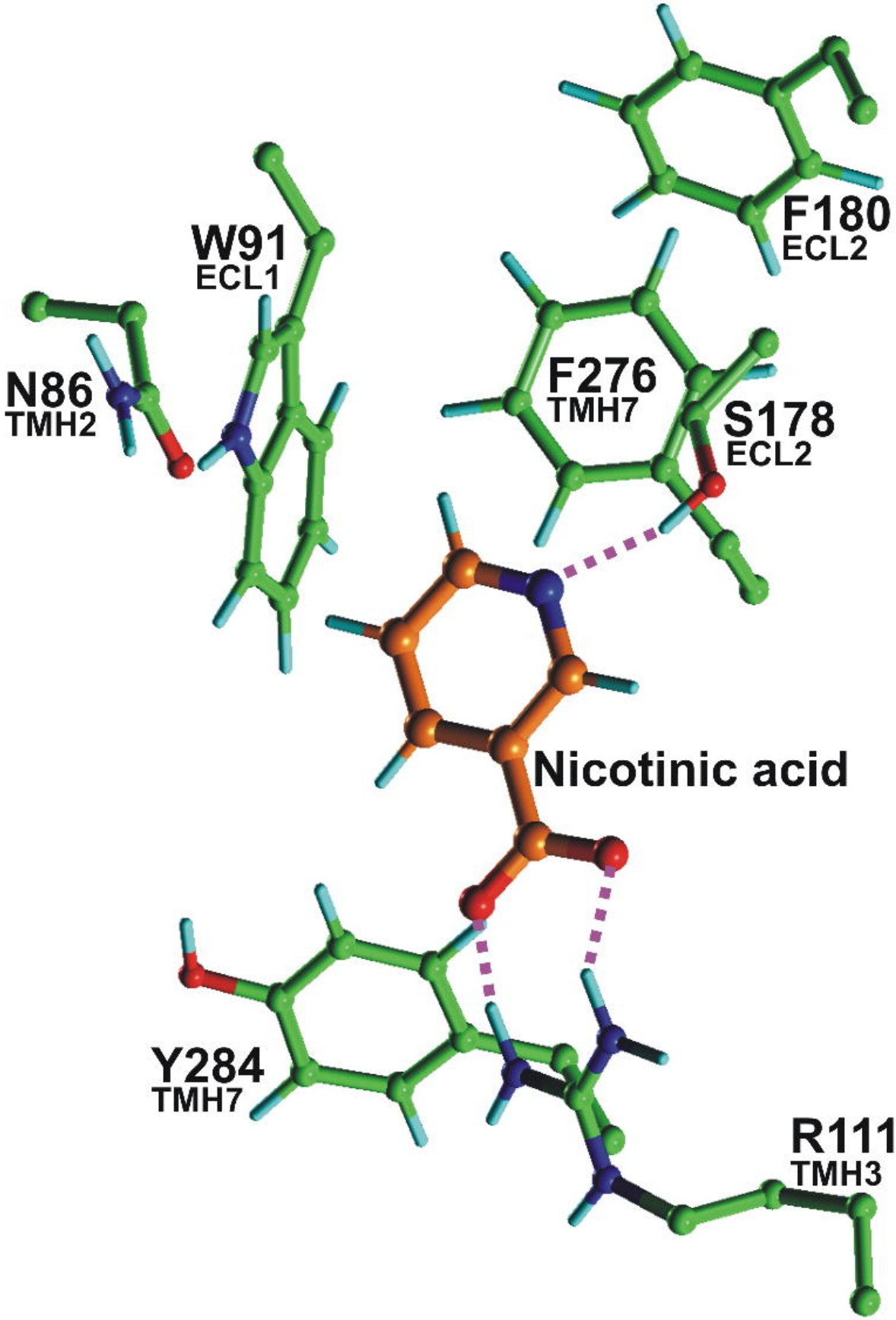

W91
ECL1
F180
ECL2
N86
TMH2
F276
TMH7
S178
ECL2
Nicotinic acid
Y284
TMH7
R111
TMH3

Figure 4b