## Molecular Determinants of Ligand Binding to the Short-Chain Fatty Acid Receptor FFA2

The G protein-coupled Free Fatty Acid Receptor FFA2 is an important signalling target of short-chain fatty acids, which are produced by the gut microbiota through fermentation of non-digestible carbohydrates. Due to its involvement in metabolic and inflammatory processes, this receptor is a potential therapeutic target for conditions such as inflammatory bowel disease and obesity-related disorders (1). Understanding the molecular determinants of ligand binding to FFA2 would greatly contribute to drug discovery efforts. Initial studies into ligand binding at FFA2 employed functional assays in combination with mutagenesis (2). Two arginines, R180 in TMD V and R255 in TMD VII, were critical for agonist function and are thought to anchor the negatively charged carboxylate moiety in FFA2 ligands. Furthermore, histidine residue H242 in TMD VI was also necessary for agonist activity. In order to extend these studies, we have employed a radiolabelled FFA2 antagonist to probe ligand binding to FFA2 and in combination with mutagenesis and molecular modelling studies define how agonist and antagonist ligands interact with the receptor (3).

Saturation equilibrium binding assays carried out in cell membranes were employed to determine total binding of radioligand [$^3$H]GLPG0974 to the receptor. Non-specific binding was determined in the presence of 10 μM synthetic FFA2 antagonist CATPB (4). Displacement assays were performed to calculate ligand affinities in terms of $K_i$ from $pIC_{50}$ values. All data are shown as mean ± SE (n = 3) and statistical analysis was carried out by fitting data from triplicate experiments and comparing the resulting values to WT by one-way ANOVA followed by Dunnett's test.

Saturation binding experiments suggest that [$^3$H]GLPG0974 binds human FFA2 with a relatively high $K_d$ of 7.5 ± 0.4 nM. Interestingly alanine replacement of R180 or R255 affected radioligand binding only modestly with $K_d$ values of 21.8 ± 1.3 and 13.0 ± 0.5 nM (***$p$ < 0.001), respectively. Only the combined R180A-R255A mutation abolished specific binding. In contrast, radioligand affinity was increased at H242A ($K_d$ = 3.7 ± 0.3, **$p$ < 0.01). Displacement assays at respective mutants showed that both C3 and synthetic agonist Cmp 1 (5) lost their ability to displace the radioligand at any of the binding pocket mutants. In contrast antagonists CATPB and GLPG0974 show a small but mostly significant reduction in affinity at the arginine mutants, while at FFA2-H242A affinity was not significantly affected (see table 1).

**Table 1.** Determined pKi values of FFA2 ligands (***$p \leq 0.001$ vs. WT)

| Receptor | CATPB (4) | GLPG0974 |
|---|---|---|
| WT | 7.87 ± 0.08 | 7.88 ± 0.08 |
| R180A | 7.32 ± 0.06*** | 7.14 ± 0.06*** |
| R255A | 6.98 ± 0.06*** | 7.59 ± 0.09 |
| H242A | 7.63 ± 0.07 | 8.04 ± 0.04 |

In summary, there seem to be clear differences between the mode of interaction of agonists and antagonists. Agonists require both arginine residues to interact with the receptor, while antagonists only require interaction with one of the two arginine residues. A homology model rationalising these observations has been developed and will greatly contribute to developing new and improved FFA2 agonists and antagonists to define the function of this receptor.

(1) Tremaroli V and Backhed F (2012). *Nature* **489:** 242–249.
(2) Stoddart LA *et al*. (2008). *J Biol Chem* **283:** 32913–32924.
(3) Sergeev E *et al.* (2016). J Biol Chem. **291:** 303–17.
(4) Hudson BD *et al*. (2012). *J Biol Chem* **287:** 41195–41209.
(5) Hudson BD *et al*. (2013). *J Biol Chem* **288:** 17296–17312.