Extracellular Loop 2 Of The Free Fatty Acid Receptor 2 Mediates Allosterism Of A Phenylacetamide Ago-Allosteric Modulator


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Non-standard abbreviations: 4-CMTB, 4-chloro-α-(1-methylethyl)-N-2-thiazolylbenzeneacetamide; ECL2, 2nd extracellular loop; eYFP, enhanced yellow fluorescent protein; FFA1/2/3, free fatty acid receptor 1, 2 or 3; GPCR, G protein-coupled receptor; [35S]GTPγS, guanosine 5′-O-(3-[35S]thio)triphosphate; HACC, hydrogen bond acceptor; HDON, hydrogen bond donor; SAR, structure-activity relationship; SCFAs, short chain fatty acids.
ABSTRACT

Allosteric agonists are powerful tools for exploring the pharmacology of closely related G protein-coupled receptors that have non-selective endogenous ligands, such as the short chain fatty acids at Free Fatty Acid receptors 2 and 3 (FFA2/GPR43 and FFA3/GPR41, respectively). We explored the molecular mechanisms mediating the activity of 4-CMTB, a recently described phenylacetamide allosteric agonist and allosteric modulator of endogenous ligand function at human FFA2, by combining our previous knowledge of the orthosteric binding site with targeted examination of 4-CMTB structure-activity relationships and mutagenesis and chimeric receptor generation. Here we show that 4-CMTB is a selective agonist for FFA2 that binds to a site distinct from the orthosteric site of the receptor. Ligand structure-activity relationship studies indicated that the N-thiazolyl amide likely provides hydrogen bond donor/acceptor interactions with the receptor. Substitution at Leu<sup>173</sup> and the exchange of the entire extracellular loop 2 of FFA2 with that of FFA3 was sufficient to respectively reduce, or ablate, allosteric communication between the endogenous and allosteric agonists. Thus, we conclude that extracellular loop 2 of human FFA2 is required for transduction of co-operative signaling between the orthosteric and an as yet undefined allosteric binding site of the FFA2 receptor that is occupied by 4-CMTB.
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INTRODUCTION

Many G protein-coupled receptors (GPCRs) share either the same endogenous ligand or respond to a group of ligands with overlapping selectivity, making efforts to ascribe individual biology particularly challenging. This is especially problematic when the ‘orthosteric’ binding pocket for endogenous ligands is very highly conserved between receptor subtypes, thereby limiting selectivity at these GPCRs. Recently, selectivity issues at related GPCRs have been circumvented by the use of ‘allosteric’ ligands which, as their name suggests, bind to a distinct or ‘other’ site on the receptor (Christopoulos, 2002; Kenakin, 2009; Smith et al., 2010, 2011). Thought to have been under less evolutionary pressure to remain conserved (Soudijn et al., 2004), allosteric binding sites provide a novel means of selectively regulating and therefore functionally characterizing related GPCRs.

In cases where selectivity has been achieved through allosteric binding sites, e.g. for the muscarinic acetylcholine receptors (Antony et al., 2009; Christopoulos and Kenakin, 2002; Conn et al., 2009; Eglen, 2005), efforts have been aided by the vast array of molecular and pharmacological tools available to researchers. This is not the case, however, for many recently de-orphanized GPCRs that are paired with endogenous ligands possessing only low or moderate potency for their cognate receptor. Because of their poor potency, radiolabeling these ligands to examine receptor binding has not been possible, and maxima of concentration-response curves are often not clearly defined within the concentration range practical to employ. Thus, identification of selective synthetic ligands at these receptors is imperative before characterization of receptor activation is possible.

Of a number of recently de-orphanized GPCRs, a family of receptors attracting interest is the Free Fatty Acid receptors 1, FFA1, FFA2 and FFA3 (Stoddart et al., 2008b, Milligan et al., 2009), historically named GPR40, GPR43 and GPR41, respectively (Brown et al., 2003; Kotarsky et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). In particular, FFA2 and FFA3 are both activated by short chain fatty acids (SCFAs) of chain length C1-C5 and
we have recently demonstrated a high degree of similarity in the orthosteric binding pockets of these closely-related receptors (Stoddart et al., 2008a). Although little is known about the physiological and pathological roles of these receptors, FFA2 knock-out mice have implicated the receptor in the regulation of inflammation in models of colitis, arthritis and asthma (Maslowski et al., 2009; Sina et al., 2009). Furthermore, colonic effects of SCFAs generated via fermentation processes may play a role in maintaining energy homeostasis, particularly via FFA2 (Sleeth et al., 2010).

Recently, Lee et al., (2008) reported a phenylacetamide (S)-4-chloro-α-(1-methylethyl)-N-2-thiazolylbenzeneacetamide (S-4-CMTB) as the first selective ligand for FFA2. S-4-CMTB was shown to be an ‘ago-allosteric’ modulator in that it was both a direct agonist at FFA2 and also a positive allosteric modulator of the actions of SCFAs at the receptor (Lee et al., 2008). Given the potential utility of a selective ligand at FFA2, herein we have examined the molecular determinants for allosterism and agonism of this and related compounds at FFA2. We report that allosteric communication between 4-CMTB and the SCFA propionate was dependent upon the nature of the 2nd extracellular loop (ECL2), whereby replacement of this region of FFA2 with the equivalent region of FFA3 entirely eliminated allosteric communication at the receptor without limiting direct agonism by either ligand, and even mutation of the single amino acid Leu\textsuperscript{173} was sufficient to disrupt allosterism. Thus, ECL2 of FFA2 acts as a molecular switch to transmit conformational changes between orthosteric and allosteric binding sites of the FFA2 SCFA receptor.
MATERIALS AND METHODS

Materials: Tissue culture reagents were from Invitrogen (Paisley, Strathclyde, U.K.). Experimental reagents were from Sigma-Aldrich (Dorset, U.K.). Ligands HWD001–HWD009, HWD011–HWD013, S-4-CMTB and R-4-CMTB were prepared in our laboratories as described in Supplemental File 1. Commercially-obtained ligands were from Enamine (Kiev, Ukraine) (HWD014–HWD018, S-HWD020, R-HWD020) and Pharmeks (Moscow, Russia) (HWD019). Absence of major secondary products and purity (83-99%) of purchased ligands was confirmed by HPLC-PDA, HPLC-MS (ESI) and 1H-NMR spectroscopy by Laia Miret Casals and Fernando Albericio (Universitat de Barcelona, Spain). The radiochemical [35S]GTPγS was from PerkinElmer Life Sciences (Buckinghamshire, U.K.).

Site-directed mutagenesis and generation of ECL2 swap: Human (h)FFA2 or FFA3 was fused via the C terminus to eYFP and subcloned into pcDNA5/FRT/TO (Invitrogen), as previously described (Stoddart et al., 2008a). Primers for PCR of FFA2 (FFA3 ECL2)-eYFP chimeric receptor were designed around the conserved regions within transmembrane domains 4 and 5 (see FIGURE 8). Receptor cDNA was amplified using primers annealing at the N-terminal and conserved region for one receptor and the conserved region and C-terminal for the other. These fragments were then combined in a single PCR reaction where they were allowed to anneal at the conserved region and act as “primer-templates” to synthesize the complete chimera. This was then amplified using the N-terminal primer from the first receptor and the C-terminal primer from the second. Restriction sites built into the primers were used to subclone the chimera into pcDNA5/FRT/TO with eYFP as described above.

Cell culture and generation of stable Flip-In T-REx 293 cells: Cells were maintained in Dulbecco’s Modified Eagle’s Medium without sodium pyruvate (Invitrogen, cat#41965) supplemented with 10% (v/v) dialyzed fetal bovine serum, 1% penicillin/streptomycin
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mixture, and 10 µg/ml blasticidin at 37 °C in a humidified atmosphere of air/CO₂ (19:1). Inducible Flp-In T-REx 293 cells were generated for each of hFFA2-eYFP (Stoddart et al., 2008a) and the various receptor mutants and hFFA3-eYFP, as described previously (Smith et al., 2009; Stoddart et al., 2008a). Antibiotic-resistant clones were screened for receptor expression by fluorescence imaging and eYFP measurement in membranes using a PHERAStar FS (BMG Labtech, UK). Cells were treated with 0.5 µg/ml doxycycline 24 hours before harvesting or imaging to induce receptor expression.

[^35S]GTP\(\gamma\)S incorporation assays: Membranes were prepared from induced stable cell lines as described elsewhere (Stoddart et al., 2007).[^35S]GTP\(\gamma\)S binding experiments were performed in duplicate according to the method of Liu et al. (Liu et al., 2009). Briefly, 5 µg of cell membranes were added to assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1 µM GDP and 0.5% fatty acid-free BSA) containing indicated concentrations of ligands and pre-incubated for 15 minutes at 25°C. To initiate the assay, 50 nCi[^35S]GTP\(\gamma\)S was added to each tube and the reaction terminated by rapid filtration through GF/C glass filters using a 24 well Brandel cell harvester (Alpha Biotech, Glasgow, UK) after 1 hour incubation. Unbound radioligand was washed from filters by three washes with ice-cold wash buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂) and[^35S]GTP\(\gamma\)S binding determined by liquid scintillation spectrometry. Mutant FFA2 data was normalized to eYFP fluorescence to account for differences in receptor expression.

Data analysis: All data were quantified, grouped and analyzed using GraphPad Prism 5.02 and are expressed as mean ± S.E.M.. Data were fit to both three parameter (fixed Hill slope) and four parameter non-linear regression isotherms and in all cases the three parameter curve was statistically appropriate. Experimental data from[^35S]GTP\(\gamma\)S binding studies investigating the interaction of 4-CMTB with propionate in wild type and mutant FFA2 receptors were analysed according to the operational model of allosteric modulation according to Keov et al., (2011) using the following equation:
in which \( E \) indicates the effect, and \( A \) denotes the orthosteric and \( B \) the allosteric ligand, respectively. \( K_A \) and \( K_B \) are the corresponding equilibrium dissociation constants of ligand binding to otherwise unliganded receptors. The cooperativity factor \( \alpha \) denotes the allosteric modulation of binding affinity, whereas the empirical parameter \( \beta \) quantifies the allosteric modulation of orthosteric ligand efficacy; its values may range between zero and infinity and it describes the extent by which the allosteric agent changes the efficacy of the orthosteric agonist on the ARB ternary complex. The ability of the orthosteric and allosteric ligands, respectively, to favor receptor activation is described by the parameters \( \tau_A \) and \( \tau_B \); furthermore they incorporate the intrinsic efficacy of each ligand, the total density of receptors and the efficiency of stimulus-response coupling. \( E_m \) denotes the maximal possible system response and \( n \) the slope factor of the transducer function by which occupancy is linked to response. Limitations of the operational model of allosteric modulation are related to the correlation between parameters and the dependence of some of these parameters on system properties (Keov et al., 2011). Therefore, in the current study its main use was the determination of semi-quantitative estimates of modulator affinity and overall cooperativity \((\alpha \beta)\).

Global nonlinear curve fitting of \( E \) as the dependent variable with \( A \) and \( B \) as the independent variables yielded estimates for \( K_A, K_B, \alpha, \beta, \tau_A \) and \( \tau_B \), except for the data shown in Fig. 7B and Fig. 8D, in which \( K_A \) and \( K_B \) were constrained to numerical estimates which had been obtained from the corresponding global fits of the data sets illustrated in Fig. 7C and Fig. 8E, respectively, because they were ambiguously resolved if left unconstrained.

The results of global nonlinear curve fitting were compared between reciprocally performed
sets of curves by subjecting the numerical values of selected parameters to a t-test; p<0.05 was considered as the level of significance. All other statistical analyses were performed as detailed in the text.

RESULTS

4-CMTB is a selective ago-allosteric modulator at FFA2. The phenylacetamide (S)-4-chloro-α-(1-methylethyl)-N-2-thiazolylbenzeneacetamide (hereafter referred to as S-4-CMTB) has previously been described as an ago-allosteric ligand at FFA2, acting as both a direct agonist and a positive allosteric modulator of the action of SCFAs that are the endogenous activators of this receptor (Lee et al., 2008). To confirm these findings, we employed a filtration-based [35S]GTPγS binding assay to examine the ability of racemic 4-CMTB to activate members of the Pertussis toxin-sensitive Gαi/o family of G proteins, as we and others have previously demonstrated that FFA2 is both a Gαi/o and Gαq-coupled GPCR (Brown et al., 2003; Kimura et al., 2001; Le Poul et al., 2003; Stoddart et al., 2008a). In membranes from Flp-In T-REx 293 cells induced to express wild type hFFA2 linked to enhanced yellow fluorescent protein (eYFP; hFFA2-eYFP)(Stoddart et al., 2008a), 4-CMTB was a relatively potent agonist (pEC50 = 6.38 ± 0.12, mean ± S.E.M.) that produced similar maximal responses as the endogenous, orthosteric FFA2 agonist propionate (pEC50 = 4.12 ± 0.22) for stimulating [35S]GTPγS binding and, therefore, G protein activation (FIGURE 1A).

4-CMTB appeared to be specific for hFFA2 as it failed to stimulate [35S]GTPγS binding via the closely-related Gαq-coupled hFFA3 receptor (FIGURE 1B).

A hallmark of allosterism is the ability of a modulator to alter the potency and/or efficacy of an orthosteric ligand and such effects should occur in a reciprocal fashion (Christopoulos and Kenakin, 2002; Smith et al., 2011). 4-CMTB was also a positive allosteric modulator of the effects of propionate at hFFA2 (FIGURE 1C). The potency of propionate
was increased in the presence of increasing concentrations of 4-CMTB (pEC$_{50}$ range: 3.90 ± 0.09 in the absence of 4-CMTB to 5.16 ± 0.33 in the presence of 1 x 10$^{-5}$ M 4-CMTB, p<0.05 according to one-way ANOVA). Critically, this effect was reciprocal as the potency of 4-CMTB was also increased in the presence of increasing concentrations of propionate (FIGURE 1D; pEC$_{50}$ range: 6.31 ± 0.08 in the absence of propionate to 7.20 ± 0.31 in the presence of 1 x 10$^{-3}$ M propionate, p<0.05 according to one-way ANOVA). Global analyses of the data were performed using the operational model of allosteric modulation as described (Keov et al., 2011), which led to the estimation of strongly positive overall co-operativity (αβ) of 4-CMTB and propionate at wild type FFA2. Such analyses also yielded estimates of the affinity of propionate (pK$_A$ = 3.20 ± 0.29) and 4-CMTB (pK$_B$ = 5.26 ± 0.43). Furthermore, the αβ values obtained for curves in FIGURE 1C and FIGURE 1D were not significantly different from each other, supporting reciprocity of effect (average αβ=194). 4-CMTB was also selective for hFFA2 with respect to allosterism as it was not able to positively or negatively allosterically modulate the effect of propionate at hFFA3-eYFP (FIGURE 1B).

4-CMTB is a partial agonist at ERK1/2 and is specific for FFA2. We also examined the actions of 4-CMTB at the mitogen-activated protein kinases ERK1/2 pathway and found it to be a reasonably potent (pEC$_{50}$ = 6.59 ± 0.23) but partial (p<0.05) agonist with respect to propionate (pEC$_{50}$ = 4.03 ± 0.21) (FIGURE 2A). As for the [$^{35}$S]GTP$_{γ}$S assay, 4-CMTB was neither an agonist nor an antagonist at hFFA3-eYFP or hFFA1-eYFP (FIGURE 2B and 2C) when ERK1/2 phosphorylation was recorded as the signal, further supporting 4-CMTB selectivity.

4-CMTB does not bind within the orthosteric binding site of hFFA2. Allosteric modulators, by definition, bind at a site(s) distinct from orthosteric ligands. We have previously used site-directed mutagenesis, in concert with SCFA-mediated phosphorylation of ERK1/2 and the elevation of [Ca$^{2+}$], to define critical orthosteric residues of hFFA2 (Stoddart et al., 2008a). Herein, membranes produced from Flp-In T-REx 293 cells induced
to express Arg(5.39)Ala (residue 180) or Arg(7.35)Ala (residue 255) hFFA2-eYFP (numbered according to the system of Ballesteros and Weinstein (Ballesteros and Weinstein, 1995)) did not promote $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding in response to propionate, presumably because the arginines act to coordinate the carboxylate head group of the SCFA, as for FFA1 and FFA3 (Smith et al., 2009; Sum et al., 2007). For His(4.56)Ala (residue 140) there was a trend towards reduced potency whilst for His(6.55)Ala (residue 242) hFFA2-eYFP, propionate potency was reduced (p<0.01 according to a one-way ANOVA) although function was not eliminated (FIGURE 3A). In contrast, 4-CMTB stimulated $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding in each case with unaltered potency (p > 0.05) and with limited effects on maximal signal (FIGURE 3B), and this was also the case in membranes containing a double Arg(5.39)Ala + Arg(7.35)Ala mutation of hFFA2-eYFP (FIGURE 3B), indicating that these key residues of the orthosteric binding pocket of hFFA2 are not involved directly in 4-CMTB binding and receptor activation. Notably, radioligands are still not available for FFA2, thus intensity of eYFP was measured as a surrogate for hFFA2 expression, even though this approach cannot clearly define cell surface expression (TABLE 1). Although we found that expression was lower than wild type for each of these 5 mutants, in no case was the difference greater than 2-fold.

4-CMTB structure-activity relationships at FFA2. To elucidate the structural features of 4-CMTB that contribute to its binding and activity, we undertook a concise structure-activity relationship (SAR) survey with a series of 4-CMTB analogs (FIGURE 4) using hFFA2-eYFP in the $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ assay. The $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ assay was deemed ideal for our SAR studies because it is the most receptor-proximal assay available and we found propionate and 4-CMTB to have equal efficacy in this assay (unlike for ERK1/2 in FIGURE 2A and $[\text{Ca}^{2+}]$, data not shown). For our targeted SAR, we focused primarily on isopropyl replacement variants (compounds HWD001-HWD008, HWD018, HWD020) and amide N-substituent variants (HWD009, HWD011-HWD017).
Isopropyl replacement variants displayed a chain length-dependence for stimulating \[^{35}\text{S}]\text{GTP} \gamma \text{S} \] binding at a maximally effective concentration (1 x 10\(^{-5}\) M), FIGURE 5A. The greatest maximal effect with n-alkyl chains from C\(_1\) to C\(_5\) was seen with ethyl (HWD003), a chain length equivalent to the isopropyl group of the parent ligand, though all of the compounds exhibited enhanced activity over the \(\alpha\)-unsubstituted analog (HWD001). Disubstitution at the \(\alpha\)-carbon, whether by methyl or bulkier butyl groups, compromised activity (HWD007 and HWD008). Whilst none of the ligands displayed enhanced, or even equivalent, potency compared to the parent 4-CMTB, potency values were generally within a log unit of 4-CMTB (TABLE 2). A compound (HWD018) with a phenyl replacement for the 4-CMTB isopropyl group was commercially available but lacked the parent ligand’s chlorine atom. We therefore extended the SAR survey to include this compound and 3-methyl-2-phenyl-\(N\)-(thiazol-2-yl)butanamide (HWD019), also commercially available, to allow incremental analysis of the effects of removing the 4-CMTB chlorine and replacing the isopropyl group by phenyl. HWD018 was active as an agonist at FFA2, albeit with significantly reduced potency when compared to 4-CMTB (TABLE 2). This reduced potency and efficacy is directly attributable to the phenyl replacement of the isopropyl group rather than removal of the chlorine, however, as HWD019 largely retained the efficacy and potency of 4-CMTB (TABLE 2). As 4-CMTB is an ago-allosteric modulator of hFFA2 (FIGURE 1), we also examined whether the relatively conservative replacement of the isopropyl group by butyl in HWD004 affected allosterism. Shown in FIGURE 5B, addition of a fixed concentration of HWD004 also produced a significant increase in potency of propionate, consistent with positive allosteric modulation. Thus, a single moderately bulky alkyl group at the \(\alpha\)-carbon favors agonist activity, with optimal activity conferred on 4-CMTB itself by the \(\beta\)-branched isopropyl group. An ethyl replacement had little impact on efficacy but caused a 6-fold reduction in potency, while longer/larger groups and smaller groups compromise both
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potency and efficacy. The allosteric action of the ligands appears to be tolerant of isopropyl replacement, at least in the case of a butyl group.

The effect of amide N-substituent alteration was more variable. Introduction of a methyl group at the 5-position of the thiazole (HWD017) or replacement of the thiazole by 4,5-dihydrothiazole (HWD014) were well tolerated (TABLE 2), the latter substitution indicating that a (hetero)aromatic N-substituent is not absolutely required for activity. In contrast, more radical changes to the amide N-substituent profoundly influenced activity, and replacement of the thiazolyl ring by cyclooctyl (HWD015) or acetylaminoethyl (HWD016) completely abolished agonism (FIGURE 5C). As the N-thiazolyl amide might provide key hydrogen bonding features in binding to FFA2, we examined the impact of N-methylation on 4-CMTB and replacement of the thiazole by pyridyl groups. Significantly, N-methylation (HWD013) ablated activity, potentially consistent with the amide NH binding to FFA2 as a hydrogen bond donor (HDON). The analog (HWD011) with an N-(2-pyridyl)- group, a thiazole replacement of slightly increased size but preserving the endocyclic nitrogen as a potential hydrogen bond acceptor (HACC) site adjacent to the amide NH, possessed similar activity to 4-CMTB itself. In contrast, the isosteric N-(3-pyridyl)- and N-(4-pyridyl)-substituted ligands (HWD009 and HWD012), in which the endocyclic nitrogen is moved progressively around the ring, showed substantially reduced (HWD009) or lack (HWD012) of activity (TABLE 2). These results are consistent with a hydrogen bond acceptor role for the endocyclic nitrogen of 4-CMTB that is maintained in HWD011. In principle, this type of HACC/HDON combination might form a complementary binding motif for amide functionality in the protein (i.e. Asn/Gln side chains or the protein backbone as seen in co-crystal structures of other proteins with N-(thiazolyl)amide-containing ligands (Jadhav et al., 1997; Kamata et al., 2004)).

We next examined HWD015, HWD016 and the lower efficacy isopropyl replacement analogs, HWD001 and HWD008, for evidence of allosteric activity in the presence of
propionate (FIGURE 5D). Consistent with the previous findings, single concentrations (10 µM) of HWD015 and HWD016 displayed no direct agonism while HWD001 and HWD008 caused small increases in G protein activation. However, none of these compounds altered propionate potency at hFFA2-eYFP, indicating either an absence of allosterism or, as is most likely the case for the low efficacy compounds HWD001 and HWD008, an effect on propionate binding and function that is beyond the sensitivity of this assay. Finally, to determine whether HWD015 and HWD016 were in fact binding to hFFA2 and acting therefore as simple antagonists of 4-CMTB, each of the ligands was co-incubated with increasing concentrations of 4-CMTB. Shown in FIGURE 5E, neither amide N-substituent variant was able to modulate 4-CMTB agonism, indicating that these ligands are not able to bind to the receptor with significant affinity. As anticipated, counter-screening of the SAR ligands at hFFA3-eYFP revealed all but one compound to be inactive, with only HWD017 able to partially stimulate [35S]GTPγS at the very highest concentration used (3 x 10⁻⁵ M) (not shown).

The S-isomer is required for maximal biological activity of 4-CMTB. In the study that identified 4-CMTB as an agonist at FFA2, the authors described only the use of the S-stereoisomer of 4-CMTB (Lee et al., 2008). To further characterize 4-CMTB structure and function, and in light of the reasonable tolerance of racemic mixes of isopropyl group substitutions described in FIGURE 5, we prepared both R- and S-4-CMTB and examined their activity alone and in combination at hFFA2-eYFP. S-4-CMTB was slightly more potent and efficacious at hFFA2 than the racemate (FIGURE 6A) and produced similar maximal responses as the orthosteric agonist propionate. Interestingly R-4-CMTB was also able to stimulate G protein activity with greater potency than propionate but significantly lower efficacy than either S- and racemic 4-CMTB or propionate. No signal was observed for racemic, S- or R-4-CMTB at FFA3 (FIGURE 6B), indicating that the stereochemistry of 4-CMTB was not responsible for differences in selectivity at hFFA2 versus hFFA3. Attempts to
perform Schild analysis to determine the apparent affinity of 4-CMTB for hFFA2 using the stereoisomers were unsuccessful, as we were unable to add sufficient $R\text{-}4\text{-CMTB}$ to compete with $S\text{-}4\text{-CMTB}$ (**FIGURE 6C**). Finally, given that an ethyl replacement for the bulkier isopropyl group in racemic 4-CMTB retained agonist efficacy in HWD003, we examined separate enantiomers for an $\alpha\text{-}ethyl$ substituted compound. Conveniently, both antipodes of the unchlorinated $\alpha\text{-}ethyl$ analogue, HWD020, were commercially available. Only $S\text{-}HWD020$ promoted [$^{35}\text{S}$]GTP$\gamma$S binding within the concentration range that could be employed (**FIGURE 6D**) and was not inhibited by increasing concentrations of $R\text{-}HWD020$ (**FIGURE 6D inset**) indicating that only $S\text{-}HWD020$ is able to bind to the receptor within concentration ranges practical to test. Thus, although enantiopure compounds are not critical for function, 4-CMTB-related ligands with an $S\text{-}configured$ stereogenic centre at the $\alpha\text{-}position$ are the preferred agonists for G protein activation.

**Allosterism is impaired at Leu$^{173}\text{Ala hFFA2-eYFP.** One of the key residues suggested by Lee et al. (2008) to contribute to recognition of 4-CMTB by FFA2 was Leu$^{173}$, located in ECL2 of hFFA2. Their preferred homology model predicted an H-bond interaction between Arg$^{255}$ (7.35) and the carbonyl backbone of Leu$^{173}$. Given this link between the possible site of binding of the orthosteric and allosteric ligands and the requirement that there must be communication between such sites to produce reciprocal modulation of ligand function, we explored allosterism at a Leu$^{173}\text{Ala}$ mutant of hFFA2-eYFP. Both propionate and 4-CMTB were, on their own, effective agonists at Leu$^{173}\text{Ala-hFFA2-eYFP}$ with potencies similar to wild type hFFA2-eYFP (**FIGURE 7A**). Global analysis of allosteric interactions (Keov et al., 2011) in **FIGURES 7B and 7C** as per Figure 1 revealed that overall allosterism was both reciprocal and significantly reduced for this mutant (**TABLE 3**) compared to wild type FFA2 (average $\alpha\beta$=21, $p<0.01$ according to one-way ANOVA with Newman-Keuls multiple comparison test). Thus, the overall structure of FFA2 ECL2 was considered for involvement in the transmission of allosterism between propionate and 4-CMTB.
Allosterism, but not agonism, is abolished by an ECL2 swap between hFFA2 and hFFA3. Given the appreciated difficulties in trying to predict specific ECL conformations (Peeters et al., 2011), we decided to further explore the role of ECL2 in 4-CMTB allosterism and agonism by taking advantage of the observation that 4-CMTB selectively activates hFFA2 but not the closely-related hFFA3 receptor (as demonstrated in FIGURE 1). Thus, we generated a hFFA2(ECL2 hFFA3)-eYFP chimera where ECL2 of hFFA3 replaced the equivalent sequence of hFFA2 (FIGURE 8A). Again a stable Flp-In T-REx 293 cell line able to induce expression of this construct was generated and tested initially for signaling to propionate as a measure of correct folding of the receptor (FIGURE 8B). Critically, hFFA2(ECL2 hFFA3)-eYFP retained propionate responsiveness. Furthermore, 4-CMTB potency was equivalent to wild type hFFA2-eYFP, indicating that the binding of 4-CMTB is largely unperturbed by the differences in both amino acid sequence and potentially broader conformational dissimilarities between hFFA2 and hFFA3 in this region (FIGURE 8C). However, maximal efficacy was reduced for both ligands, either reflecting the significantly reduced receptor expression of this chimera or subtle structural differences affecting receptor activation. Despite retention of agonism, however, allosteric communication between propionate and 4-CMTB was now completely abolished (FIGURE 8D and 8E and TABLE 3; average αβ=1.2). Thus, it appears that either the non-conserved residues in ECL2 or the overall conformation of this region is required for allosterism between 4-CMTB and an orthosteric agonist at hFFA2. The importance of ECL2 in receptor activation has been shown for several GPCRs (Bokoch et al., 2010; Sum et al., 2009). Since it is impossible to accurately predict the conformation of ECL2 using comparative modeling, it is difficult, therefore, to provide a mechanistic basis for the influence of ECL2 on 4-CMTB allosterism.
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DISCUSSION

The free fatty acid receptor FFA2 is attracting considerable interest due to its potential role in the regulation of inflammation and the importance of gut microflora in generating SCFAs that activate this receptor. However, selectivity between FFA2 and the closely related receptor FFA3 remains problematic (Brown et al., 2003; Le Poul et al., 2003; Stoddart et al., 2008a; Stoddart et al., 2008b). Although selective orthosteric ligands have not yet been reported for either FFA2 or FFA3, a pair of recent studies has identified a series of ligands, based on the phenylacetamide S-4-CMTB, that are thought to circumvent this issue by binding to an allosteric site on FFA2 (Lee et al., 2008; Wang et al., 2010). Using the predominantly Gaαi/o-specific [35S]GTPγS binding assay to examine both wild type and mutated versions of hFFA2, we confirmed both the direct agonism and positive allosteric effects on the function of the SCFA propionate by 4-CMTB and related ligands. In previous studies we had identified key basic residues in FFA2, based on alignments of FFA2 with both FFA3 and FFA1 and the recognition that corresponding fatty acid amides are not agonists at these receptors (Stoddart et al., 2008a), that following mutation either eliminated or substantially reduced the potency of SCFAs at FFA2. This allowed us, in part, to define the mode of binding of the orthosteric SCFAs at both FFA2 and FFA3 (Stoddart et al., 2008a). Importantly, mutation of these key residues, His4.56, Arg5.39, His6.55 and Arg7.35, did not alter the measured potency of 4-CMTB, confirming that 4-CMTB does not share a common binding site with the SCFAs.

These studies were combined with a targeted SAR survey of 4-CMTB analogs to investigate the possible mode of binding of the phenylacetamides. The SAR survey focused on variations in the ligand’s α-substitution and N-(thiazolyl)amide substructure, the latter because we envisaged that it might constitute a key hydrogen bonding motif for binding the receptor. Our SAR data are in close agreement with those recently disclosed by Wang et al. (Wang et al., 2010) during the course of our work. Jointly, the results of these two studies
are consistent with a receptor-bound state for the ligand that exhibits a near co-planar conformation for the N-(thiazolyl)amide functionality and in which the amide NH and thiazole nitrogen present, respectively, HDON and HACC sites for engagement of the protein. Precisely this conformational organization and functional role is seen in the co-crystal structures of two unrelated N-(2-thiazolyl)amide-containing compounds bound to human glucokinase and HIV protease (Jadhav et al., 1997; Kamata et al., 2004). In both instances the N-(thiazolyl)amide engages two adjacent peptide linkages in the protein backbone, and a corresponding engagement in FFA2 would therefore implicate interaction of the motif with a loop region rather than the core transmembrane helices, wherein the backbone hydrogen bonding sites are sequestered in intra-helix interactions. At the present stage, however, we cannot discount other engagement modes for the N-(thiazolyl)amide such as hydrogen bonding to side chain amide functionality in Asn or Gln residues. From the compounds with α-alkyl group variations it appears that a C₂ chain length is optimal, though branching at the β carbon, as in the isopropyl group of 4-CMTB itself, is preferred for ligand potency. Interestingly, Wang et al. (Wang et al., 2010) reported that only the (S)-enantiomer of 4-CMTB possessed biological activity when measuring regulation of cAMP, although they also demonstrated that a requirement for chiral structures was not absolutely necessary for ligand function by showing that ligands having the α-carbon as part of a cycloalkyl group can retain a good measure of the activity of 4-CMTB. In our [³⁵S]GTPγS binding assay, however, we found R-4-CMTB to be only 6 fold less potent than the racemic material or separate (S)-enantiomer of 4-CMTB, suggesting that the absolute configuration of the ligand may be less critical for activity than originally indicated.

A key finding of our study was that apparent allosteric communication between orthosteric and allosteric sites within hFFA2 was reduced by a single point mutation within ECL2 (Leu¹⁷³Ala) and abolished by replacement of ECL2 from FFA2 with that of FFA3. This finding was confirmed both by loss of significant shifts in potency with increasing
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centrations of modulating ligand and also by significant reduction of global co-operativity estimates (TABLE 3). In both cases this occurred without any change in propionate or 4-CMTB potency as direct agonists. Although a truly striking set of observations, such interactions remain impossible to model structurally. The available atomic level structures of GPCRs show ECL2 to adopt very different conformations (Peeters et al., 2011) and it is simply impractical to estimate this de novo with any expectation of accuracy. However, these data demonstrate clearly the importance of this region in communication between the two binding sites and are consistent with the appreciated role of ECL2 in receptor activation (Ahn et al., 2009; Baneres et al., 2005; Bokoch et al., 2010; Conner et al., 2007; Huber et al., 2008; Peeters et al., 2011; Scarselli et al., 2007;; Unal et al., 2010). To date, the best attempt to delineate the molecular mechanisms of allosterism, efficacy and co-operativity have been at the M4 muscarinic acetylcholine receptor, although these required a series of well defined and selective probes, including dualsteric ligands, and existing knowledge of allosteric and orthosteric binding sites (Nawaratne et al., 2010). Clearly, much remains to be determined with respect to the mechanism of allosteric signal propagation at FFA2 and will depend upon the development of more potent and selective ligands and greater understanding of ligand binding at both orthosteric and allosteric sites on the receptor.
ACKNOWLEDGEMENTS

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Smith, Tikhonova, Adams and Milligan

Conducted experiments: Smith, Ward, Hudson

Contributed new reagents or analytical tools: Stoddart, Morris, Ulven and Kostenis

Performed data analysis: Smith, Tränkle, Hudson, Adams and Milligan

Wrote or contributed to the writing of the manuscript: Smith, Tikhonova, Adams and Milligan

Other:
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REFERENCES


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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: 4-CMTB is a selective ago-allosteric modulator at FFA2

Flp-In TREx 293 cells stably expressing inducible hFFA2-eYFP (A, C and D) or hFFA3-eYFP (B) were induced with 0.5 µg/ml doxycycline for 24 hours before harvesting and membrane preparation for subsequent [35S]GTPγS incorporation assays. A. Both 4-CMTB and propionate are full agonists in this assay. Inset: representative example of absolute values (DPM) for basal (-) and propionate (C3; 10 mM) and 4-CMTB (10 µM) from a single hFFA2-eYFP membrane preparation. B. 4-CMTB is not an agonist, antagonist, or allosteric modulator of propionate at the closely-related hFFA3-eYFP receptor. Inset: representative experiment for hFFA3-eYFP, as per A inset. C. 4-CMTB and propionate interact allosterically, as increasing fixed concentrations of 4-CMTB result in enhanced potency of propionate. The shift in potency is indicated by the diagonal arrow, *p<0.05 according to one-way ANOVA with Dunnett’s post-hoc analysis. D. The allosteric relationship is reciprocal. Again, the shift in potency is indicated by the diagonal arrow, *p<0.05 according to one-way ANOVA with Dunnett’s post-hoc test. For each panel, data are mean ± S.E.M (n=3).

Figure 2: 4-CMTB is a partial agonist at ERK1/2 and is selective for FFA2 compared to FFA3 and FFA1

Flp-In TREx 293 cells stably expressing FFA2-eYFP were stimulated for 10 min with varying concentrations of propionate (C3) or 4-CMTB and processed for ERK1/2 activation using the AlphaScreen Surefire ERK1/2 assay (Perkin Elmer). *p<0.05 for 4-CMTB E_max being less that for propionate according to Student’s t-test. Data are mean ± S.E.M (n=3). B. Equivalent experiments were performed using hFFA3-eYFP cells (n=3), where only propionate was an agonist in the ERK1/2 pathway. C. Western blots of hFFA3-eYFP cell lysates treated as for
B. Foetal bovine serum (FBS) (10% v/v) was included as a positive control for ERK1/2 activation. D. Western blot as per C. except cells expressing FFA1-eYFP were stimulated with 100 μM lauric acid. PT = pre-treatment.

**Figure 3: 4-CMTB does not require a functional orthosteric ligand binding site for signaling**

Flp-In TREx 293 cells stably expressing inducible wild type or orthosteric binding site mutants of hFFA2-eYFP (Stoddart et al., 2008a) were induced with 0.5 µg/ml doxycycline for 24 hours before harvesting and membrane preparation for subsequent \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) incorporation assays. A. Mutation of either Arg(5.39)Ala, Arg(7.35)Ala or both residues (RARA) completely abolishes propionate activity, whilst propionate potency is markedly impaired at the His(6.55)Ala mutation, ** p<0.01 according to a one-way ANOVA with Dunnett’s post-hoc test. B. 4-CMTB potency is unaltered at any of the orthosteric binding site mutants of hFFA2-eYFP. Efficacy was only significantly impaired for Arg(7.35)Ala, *p<0.05 according to one-way ANOVA with Dunnett’s post-hoc analysis. Data are mean ± S.E.M with experiment repeats indicated in parentheses.

**Figure 4: Analogs used for 4-CMTB SAR survey**

**Figure 5: Function of selected 4-CMTB SAR ligands at hFFA2-eYFP**

A. 4-CMTB isopropyl replacement variants (replacement area on 4-CMTB indicated on the structure by a circle) in the \[^{35}\text{S}]\text{GTP}\gamma\text{S}\ incorporation assay demonstrate chain length and bulk dependence for maximal efficacy. Significant differences from 4-CMTB maximum are indicated, where *p<0.05, **p<0.01 and n.s. is not significant, according to one-way ANOVA with Dunnett’s post-hoc analysis (n=4). B. HWD004 is also a positive allosteric modulator at
10 µM in the presence of increasing propionate concentrations. Arrow indicates shift in potency where *p<0.05 according to Student’s t-test. C. Not all 4-CMTB analogs displayed agonist activity at hFFA2-eYFP. D. Inactive (HWD015 and HWD016) or weakly efficacious (HWD001, HWD008) ligands were tested for allosterism (10 µM fixed concentration of HWD ligands) but failed to alter the potency of propionate. n.s.: no significant difference in potencies according to one-way ANOVA. E. Neither HWD015 nor HWD016 could compete at 10 µM with 4-CMTB at hFFA2-eYFP. Data represent mean ± S.E.M. of three independent experiments.

Figure 6: S-4-CMTB is more potent than R-4-CMTB at hFFA2-eYFP

A. Racemic 4-CMTB and individual stereoisomers were examined for their ability to promote[^GTPγS] incorporation. R-4-CMTB was significantly less potent and efficacious than both racemic 4-CMTB and S-4-CMTB, according to one-way ANOVA with Dunnett’s post-hoc analysis. *p<0.05, **p<0.01. B. 4-CMTB stereoisomers are not active at the closely-related hFFA3-eYFP. C. R-4-CMTB and S-4-CMTB are not additive when co-incubated, suggesting binding to the same site on hFFA2-eYFP. D. Only the S-isomer of the structurally-related ligand, HWD020, is able to activate hFFA2-eYFP. Inset: R-HWD020 is unable to inhibit signaling in response to 30 µM S-HWD020. Data are mean ± S.E.M (n=3) for all panels.

Figure 7: Leu^{173} in hFFA2-eYFP is required for 4-CMTB allosterism but not agonism

A Leu^{173}Ala mutation was introduced into the 2nd extracellular loop of hFFA2-eYFP and this inducible construct was expressed stably in Flp-In TREx 293 cells. A.[^GTPγS] incorporation in response to either propionate (pEC_{50} = 3.95 ± 0.15) or 4-CMTB (pEC_{50} = 6.14 ± 0.06) is unaffected by Leu^{173}Ala mutation within hFFA2-eYFP. B. Co-incubation of Leu^{173}Ala hFFA2-eYFP with fixed concentrations of 4-CMTB and varying amounts of propionate no longer resulted in a significant shift in the potency of propionate, indicated by
the arrow. n.s.: not significant according to one-way ANOVA. C. The loss of allosterism was reciprocal, with no significant difference in pEC$_{50}$ range indicated by the arrow. n.s.: not significant according to one-way ANOVA. Data are mean ± S.E.M (n=3) in each panel.

**Figure 8: 4-CMTB requires the 2nd extracellular loop of hFFA2 for allosteric modulation of propionate.**

The 2nd extracellular loop of hFFA2 (Val$^{144}$ to Glu$^{182}$) was replaced with ECL2 from the closely-related hFFA3 receptor (Val$^{150}$ to Glu$^{187}$) to generate an hFFA2(ECL2 FFA3)-eYFP chimeric protein and stable Flp-In TREx 293 cells generated as before. A. Extracellular loop 2 alignment between hFFA2 and hFFA3 using ClustalX (Accession numbers: hFFA2: NP005297; hFFA3: NP005295). B. Propionate activates hFFA2(ECL2 FFA3)-eYFP with equivalent potency to hFFA2-eYFP WT (pEC$_{50}$ = 3.96 ± 0.09 and 4.03 ± 0.14, respectively; n=4) but with marginally reduced efficacy; p=0.0501 according to an unpaired t-test. C. 4-CMTB has significantly reduced efficacy at hFFA2(ECL2 FFA3) when compared to hFFA2-eYFP WT; p<0.001 according to an unpaired t-test. Potency is equivalent between WT and chimeric receptors according to a t-test (pEC$_{50}$ = 6.50 ± 0.15 and 6.75 ± 0.11, respectively; n=4). D. Allosterism between 4-CMTB and propionate is lost at the hFFA2(ECL2 FFA3)-eYFP chimera, where the arrow indicates potency and n.s. represents no significant difference in potency values according to one-way ANOVA (n=3). E. Reciprocal loss of allosterism at hFFA2(ECL2 FFA3)-eYFP according to one-way ANOVA and indicated by the arrow (n=3).
### Table 1: FFA2 mutant receptor expression per 5 micrograms protein as determined by eYFP fluorescence.

<table>
<thead>
<tr>
<th>FFA2 receptor</th>
<th>eYFP (RLU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
</tr>
<tr>
<td>HEK293 no receptor</td>
<td>556 ± 25.9</td>
</tr>
<tr>
<td>Wild type</td>
<td>4193 ± 405</td>
</tr>
<tr>
<td>His^{140}Ala (4.56)</td>
<td>2110 ± 320</td>
</tr>
<tr>
<td>Leu^{173}Ala (ECL2)</td>
<td>2041 ± 431</td>
</tr>
<tr>
<td>Arg^{180}Ala (5.39)</td>
<td>1798 ± 187</td>
</tr>
<tr>
<td>His^{242}Ala (6.55)</td>
<td>2655 ± 101</td>
</tr>
<tr>
<td>Arg^{255}Ala (7.35)</td>
<td>3024 ± 201</td>
</tr>
<tr>
<td>Arg^{180}Ala/Arg^{255}Ala (5.39/7.35)</td>
<td>2072 ± 101</td>
</tr>
<tr>
<td>FFA2 (ECL2 FFA3)</td>
<td>1886 ± 112</td>
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Table 2: Structure-activity relationship of 4-CMTB-based ligands at $[^{35}\text{S}]$-GTPγS assay of G protein activation

<table>
<thead>
<tr>
<th>Ligand</th>
<th>FFA2-eYFP</th>
<th>pEC50 mean ± S.E.M</th>
<th>Emax$^\dagger$ mean ± S.E.M</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td>Propionate</td>
<td>4.39 ± 0.26</td>
<td>100.0 ± 4.0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4-CMTB</td>
<td>6.18 ± 0.18</td>
<td>105.2 ± 3.8</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>S-4-CMTB</td>
<td>6.52 ± 0.12</td>
<td>118.9 ± 5.2</td>
<td>3</td>
<td></td>
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<tr>
<td>R-4-CMTB</td>
<td>5.74 ± 0.09</td>
<td>77.7 ± 4.0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD001</td>
<td>5.24 ± 0.56</td>
<td>69.6 ± 3.0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD002</td>
<td>5.50 ± 0.30</td>
<td>81.3 ± 3.5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD003</td>
<td>5.38 ± 0.13</td>
<td>103.4 ± 3.5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD004</td>
<td>5.64 ± 0.17</td>
<td>90.0 ± 2.6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD005</td>
<td>5.50 ± 0.19</td>
<td>92.6 ± 3.3</td>
<td>3</td>
<td></td>
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<tr>
<td>HWD006</td>
<td>5.67 ± 0.38</td>
<td>80.1 ± 4.4</td>
<td>3</td>
<td></td>
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<tr>
<td>HWD007</td>
<td>4.99 ± 0.25</td>
<td>88.8 ± 6.0</td>
<td>3</td>
<td></td>
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<tr>
<td>HWD008</td>
<td>5.04 ± 0.48</td>
<td>80.9 ± 9.0</td>
<td>3</td>
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<tr>
<td>HWD009</td>
<td>5.05 ± 0.30</td>
<td>37.1 ± 7.3</td>
<td>3</td>
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<tr>
<td>HWD011</td>
<td>6.58 ± 0.11</td>
<td>128.7 ± 6.4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD012</td>
<td>inactive$^c$</td>
<td>inactive</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD013</td>
<td>inactive</td>
<td>inactive</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD014</td>
<td>5.39 ± 0.26</td>
<td>90.0 ± 4.4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD015</td>
<td>inactive</td>
<td>inactive</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HWD016</td>
<td>inactive</td>
<td>inactive</td>
<td>3</td>
<td></td>
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<tr>
<td>HWD017</td>
<td>6.11 ± 0.20</td>
<td>100.7 ± 3.2</td>
<td>3</td>
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<tr>
<td>HWD018</td>
<td>4.72 ± 0.33</td>
<td>77.5 ± 6.6</td>
<td>3</td>
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<tr>
<td>HWD019</td>
<td>5.84 ± 0.25</td>
<td>96.6 ± 3.4</td>
<td>3</td>
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<tr>
<td>S-HWD020</td>
<td>5.27 ± 0.16</td>
<td>95.2 ± 13.1</td>
<td>3</td>
<td></td>
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</table>
Emax is expressed as a percentage of propionate signal

Inactive was defined as <10% propionate maximum signal at the highest concentration tested (10 µM).
Table 3: Parameters obtained from the operational model of allosteric modulation where A is propionate (fixed concentrations) and B is 4-CMTB (variable concentrations).

<table>
<thead>
<tr>
<th></th>
<th>FFA2 WT mean</th>
<th>S.E.M.</th>
<th>FFA2 L173A mean</th>
<th>S.E.M.</th>
<th>FFA2 (ECL2 FFA3) mean</th>
<th>S.E.M.</th>
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<tr>
<td>logK_A</td>
<td>-3.20</td>
<td>0.29</td>
<td>-3.13</td>
<td>0.30</td>
<td>-3.82</td>
<td>0.27</td>
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<tr>
<td>logK_B</td>
<td>-5.26</td>
<td>0.43</td>
<td>-5.27</td>
<td>0.37</td>
<td>-6.43</td>
<td>0.26</td>
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<tr>
<td>logα</td>
<td>1.88</td>
<td>0.59</td>
<td>0.82</td>
<td>0.56</td>
<td>0.13</td>
<td>0.42</td>
</tr>
<tr>
<td>logβ</td>
<td>0.31</td>
<td>0.11</td>
<td>0.66</td>
<td>0.21</td>
<td>0.23</td>
<td>0.11</td>
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<tr>
<td>logαβ</td>
<td>2.19</td>
<td>0.71</td>
<td>1.48</td>
<td>0.78</td>
<td>0.36</td>
<td>0.53</td>
</tr>
<tr>
<td>αβ</td>
<td>155</td>
<td></td>
<td>30*</td>
<td></td>
<td>2.3**</td>
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</tr>
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</table>

*p<0.05 and **p<0.01 according to one-way ANOVA with Newman-Keuls multiple comparisons post-hoc analysis.
Figure 4

HWD001 $R^1 = H$ $R^2 = H$
HWD002 $R^1 = H$ $R^2 = $ methyl
HWD003 $R^1 = H$ $R^2 = $ ethyl
HWD004 $R^1 = H$ $R^2 = $ 1-propyl
HWD005 $R^1 = H$ $R^2 = $ 1-butyl
HWD006 $R^1 = H$ $R^2 = $ 1-pentyl
HWD007 $R^1 = $ methyl $R^2 = $ methyl
HWD008 $R^1 = $ butyl $R^2 = $ butyl

HWD013

HWD014

HWD015

HWD016

HWD017

R-HWD020 $R^1 = $ ethyl $R^2 = H$
S-HWD020 $R^1 = H$ $R^2 = $ ethyl