Complex pharmacology of novel allosteric Free Fatty Acid 3 Receptor ligands

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FFA2, Free Fatty Acid 2 receptor; FFA3, Free Fatty Acid 3 receptor; eYFP, enhanced Yellow Fluorescent Protein; GPCR, G protein-coupled receptor; NAM, negative allosteric modulator; PAM, positive allosteric modulator; SCFA, short chain fatty acid.
Abstract

Analysis of the roles of the short chain fatty acid receptor FFA3 has been severely limited by the low potency of its endogenous ligands, the crossover of function of these on the closely related FFA2 receptor and a dearth of FFA3-selective synthetic ligands. From a series of hexahydroquinolone-3-carboxamides we demonstrate that 4-(furan-2-yl)-2-methyl-5-oxo-N-(o-tolyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide is a selective and moderately potent positive allosteric modular (PAM)-agonist of the FFA3 receptor. Modest chemical variations within this series resulted in compounds completely lacking activity, acting as FFA3 PAMs, or appearing to act as FFA3 negative allosteric modulators (NAMs). However, the pharmacology of this series was further complicated in that certain analogs displaying overall antagonism of FFA3 function actually appeared to generate their effects via a combined positive allosteric binding cooperativity, and negative allosteric effect on orthosteric ligand maximal signalling response. These studies show that various PAM-agonist and allosteric modulators of FFA3 can be identified and characterized. However, within the current chemical series considerable care must be taken to define the pharmacological characteristics of specific compounds before useful predictions of their activity and their use in defining specific roles of FFA3 in either in vitro and in vivo settings can be made.
Introduction

A pair of closely related G protein-coupled receptors (GPCRs) recognize and are activated by short chain fatty acids (SCFAs) produced in the body predominantly through the fermentation of poorly digestible carbohydrates by the gut microbiota (Brown et al., 2003, Le Poul et al., 2003, Stoddart et al., 2008b, Cani et al., 2013). In recent times, these receptors, FFA2 (previously designated GPR43) and FFA3 (previously GPR41), have attracted considerable attention, not least because understanding of the role of the microbiota in the regulation of health has developed and deepened (Tan et al., 2014). Indeed, broad appreciation of the role of the microbiota in areas including metabolic health and the regulation of inflammatory processes has encouraged detailed analysis of the SCFA receptors and resulted in suggestions that they might be novel and effective therapeutic targets (Ulven, 2012, Hara et al., 2013, Yonezawa et al., 2013, Tan et al., 2014). To date, understanding of the specific roles of FFA2 and FFA3 has been derived mainly from studies of receptor ‘knock-out’ lines of mice (Maslowski et al., 2009, Sina et al., 2009, Zaibi et al., 2010, Bjursell et al., 2011, Tolhurst et al., 2012, Bellahcene et al., 2013, Kim et al., 2013, Kimura et al., 2013). The reliance on such mouse models to define the individual functions of FFA2 and FFA3 reflects, at least in substantial part, that both receptors are activated by the same group of SCFAs (Brown et al. 2003, Stoddart et al., 2008a,b), that the expression patterns of the two receptors can overlap (Nøhr et al., 2013) and that synthetic ligands capable of selectively activating or inhibiting FFA2 and FFA3 have been limited in availability and detailed characterization (Hudson et al., 2011). In recent times this situation has improved somewhat for FFA2, with the description and use of both orthosteric agonists and antagonists (Schmidt et al., 2011, Hudson et al., 2012a, 2013a) as well as a group of phenylacetamide-based ‘ago-allosteric’ modulators (Lee et al., 2008, Wang et al., 2010, Smith et al., 2011). These compounds have subsequently been used to define the contribution of FFA2 in SCFA-
mediated inhibition of lipolysis (Lee et al., 2008; Hudson et al., 2013a), release of the incretin GLP-1 from enteroendocrine cells (Hudson et al., 2013a) and in neutrophil chemotaxis (Vinolo et al., 2011). In contrast, only two reports to date that have examined the action of an FFA3 selective agonist, AR420626 (N-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydro-quinoline-3-carboxamide), indicating this compound produces a modest but significant stimulation of GLP-1 release from murine colonic crypt cultures (Nøhr et al., 2013) and inhibits ghrelin secretion from murine gastric ghrelin cells (Engelstoft et al., 2013). However, recent suggestions of a key role for FFA3 in mediating the effects of the SCFA propionate (C3) on allergic inflammation (Trompette et al., 2014) has further heightened the need to identify and characterize FFA3-selective compounds.

We here describe the characterization of ligands reported in the patent literature to have activity at FFA3 (Leonard et al., 2006), and of some analogs of these. A number of moderately potent ligands were identified that either activate this receptor as allosteric agonists, act as positive allosteric modulators (PAMs) of the effect of SCFAs at the receptor without detectable direct agonism, or inhibit the effects of SCFAs in a non-competitive manner as negative allosteric modulators (NAMs). Although the detailed pharmacology of this group of ligands is shown to be complex, careful selection may provide useful tool compounds to further define the role of FFA3 in both human and rodent cells and tissues.
Materials and Methods

Materials and compounds

Tissue culture reagents were from Life Technologies Inc. (Paisley, UK). Compounds 1-8 were synthesized as described in the supplementary information (Supplemental Methods). The radiochemical $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ was from PerkinElmer Life and Analytical Sciences (Beaconsfield, Buckinghamshire, UK). All other experimental reagents were from Sigma Aldrich (Poole, UK).

Cell culture and transfection

All cells used in these experiments were derived from Flp-In$^{\text{TM}}$ T-REx$^{\text{TM}}$ 293 cells designed to express the desired receptor on demand following induction with the antibiotic doxycycline. All cells used in these studies were previously described and designed to express either human, mouse or rat FFA3 (Hudson et al., 2012a); human FFA2 (Stoddart et al., 2008a); or mutated forms of human FFA3 (Stoddart et al., 2008a). In all cases the receptor construct expressed was fused in frame to enhanced Yellow Fluorescent Protein (eYFP) at its carboxy terminal. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) without sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin mixture (Sigma Aldrich) in a humidified atmosphere containing 5% CO$_2$. To induce receptor expression cells were incubated overnight with 100 ng.ml$^{-1}$ doxycycline.

$[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ incorporation assay

Cell membrane preparations were first generated as described previously (Stoddart et al., 2008a). $[^{35}\text{S}]\text{GTP}^\gamma\text{S}$ binding experiments were then performed using a described method (Smith et al., 2011). Briefly, cell membrane preparations 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 0.1% fatty acid-free bovine serum albumin) containing the appropriate concentrations of ligand and allowed to reach equilibrium by pre-incubating for 15 min at 25°C. [³⁵S]GTPγS was then added to initiate the assay, before incubation for 1 h at 25°C. The reaction was terminated by filtration through GF/C glassfiber filters and unbound [³⁵S]GTPγS was washed from the filters with ice-cold wash buffer (50 mM Tris-HCl, pH 7.4, and 10 mM MgCl₂) before the remaining [³⁵S]GTPγS was quantified by liquid scintillation spectrometry.

**Extracellular Signal Regulated Kinase 1/2 (ERK1/2) phosphorylation assay**

Phosphorylation of ERK1/2 was assessed using a previously described protocol (Hudson et al., 2012b). Briefly, 80000 cells were seeded per well in a 96 well plate, allowed to attached, before incubating overnight with doxycycline (100 ng.ml⁻¹) to induce receptor expression. Cells were then incubated in serum free DMEM for 5-6 h prior to the assay. Test compounds were added to the cells and incubated for 5 min at 37°C before cells were lysed and assayed for phospho-ERK1/2 using an Alphascreen-based detection kit according to the manufacturer’s protocol (Perkin Elmer, Beaconsfield, Buckinghamshire, UK).

**cAMP assay**

cAMP experiments were performed using a homogenous time-resolved FRET-based detection kit (CisBio Bioassays, Codolet, France) according to the manufacturers protocol. Cells already induced to express the desired receptor were plated in low volume 384 well plates and the inhibition of 5 μM forskolin-stimulated cAMP production was assessed following a 30 min co-incubation with test compounds.
**Data analysis and curve fitting**

All data presented represent mean ± standard error of at least three independent experiments. Data analysis and curve fitting were carried out using the Graphpad Prism software package v5.0b. Concentration-response data were fit to three parameter sigmoidal concentration-response curves. Global curve fitting of allostery data was carried out using the following operational model equation described previously (Keov et al. 2011; Smith et al., 2011):

\[
E = \frac{E_m(\tau_A[A](K_B + \alpha \beta[B]) + \tau_B[B]K_A)^n}{([A]K_B + K_AK_B + [B]K_A + \alpha[A][B])^n + (\tau_A[A](K_B + \alpha \beta[B]) + \tau_B[B]K_A)^n}
\]

where \( E \) is the measured response, and \( A \) and \( B \) represent the orthosteric and allosteric ligands respectively. In this equation, \( E_m \) is the maximal system response, \( \alpha \) is a measure of the allosteric cooperativity on ligand binding affinity, and \( \beta \) is an empirical measure of the allosteric effect on efficacy. \( K_A \) and \( K_B \) are measures of the binding affinities of the orthosteric and allosteric ligands respectively. The value \( n \) represents the slope factor of the transduction function, while the abilities of the orthosteric and allosteric ligands to directly activate the receptor are incorporated through the values \( \tau_A \) and \( \tau_B \). In order to fit experimental data to this equation, in all cases the system maximum (\( E_m \)) and slope (\( n \)) functions were constrained, allowing for estimations of \( \alpha, \beta, \tau_A, \tau_B, K_A, \) and \( K_B \). In order to fit the data presented in Figures 7B, 8B 9B and 9C, the \( K_A \) value for C3 was constrained to the average value obtained in the experiments presented in Figure 4 (summarized in Table 1). Finally, it was also required that the value for \( \tau_B \) be constrained to a value of effectively 0 in order to fit data for allosteric modulators that did not produce any direct agonism on their own (Compounds 4 and 6).
Results

Based on a patent disclosing synthetic ligands as regulators of the FFA3 receptor we initially synthesized the representative compound 1 (4-(furan-2-yl)-2-methyl-5-oxo-N-(o-tolyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide) (Figure 1). FFA3 is known to couple to the Gi-family of heterotrimeric G proteins (Brown et al., 2003, Le Poul et al., 2003, Stoddart et al., 2008a,b). In [35S]GTPγS binding assays performed on membranes of Flp-InTM TRExTM 293 cells that had been induced to express a C-terminally eYFP-tagged form of human (h)FFA3, compound 1, increased incorporation of this radionucleotide in a concentration-dependent fashion with pEC₅₀ of 5.65 ± 0.07 (Figure 2A). In this assay 1 produced a similar maximal response as the endogenous SCFA propionate (C3) but was approximately 100 fold more potent (pEC₅₀ for C3 = 3.47 ± 0.09). These effects of both 1 and C3 reflected interactions with hFFA3 as no significant response to either ligand was observed using membranes derived from the cells that had not been pre-treated with doxycycline to induce hFFA3-eYFP expression (Figure 2A). Furthermore, 1 was highly selective for hFFA3 over the closely related hFFA2 receptor because this compound did not increase [35S]GTPγS incorporation in membranes of Flp-InTM TRExTM 293 cells induced to express hFFA2-eYFP, although C3 did generate the expected response (pEC₅₀ = 4.18 ± 0.16) (Figure 2B). In addition to stimulating [35S]GTPγS incorporation, compound 1 also produced similar signalling responses to those of C3 in other endpoint measures of hFFA3 function in cells induced to express hFFA3-eYFP. These included inhibition of forskolin-stimulated cAMP production (Figure 2C), with pEC₅₀ values of 5.77 ± 0.05 and 4.58 ± 0.09 for 1 and C3 respectively, and phosphorylation of the extracellular signal-regulated kinases 1 and 2 (pERK1/2) (Figure 2D): pEC₅₀ values of 5.27 ± 0.16 for compound 1 and 3.68 ± 0.13 for C3. As in the [35S]GTPγS incorporation assay, in both cAMP and pERK1/2 assays 1 produced similar maximal responses to that of C3.
It has previously been established that the carboxylic acid functional group of C3 and other SCFAs are integral to their function at FFA3, forming key ionic interactions with arginine residues at positions 5.39 and 7.35 (numbering system of Ballesteros and Weinstein (1995)) (Stoddart et al., 2008a). However, compound 1 does not contain a carboxylic acid functional group, nor a negatively charged carboxylic acid bioisostere. We assessed, therefore, if 1 functioned as an orthosteric ligand for hFFA3. It did not: while mutation to Ala of either of Arg5.39 or Arg7.35 completely eliminated response to C3 in the \( ^{35}\text{S} \)GTP\( \gamma \)S binding assay (Figure 3A), at both of these mutants the function of 1 was essentially unaltered (Figure 3B). It is also important to note that both the R5.39A and R7.35A mutants of hFFA3-eYFP were expressed effectively from the inducible locus of stable Flp-In\textsuperscript{TM} TREx\textsuperscript{TM} 293 lines, yielding 137 ± 5 and 179 ± 4 % of wild type hFFA3-eYFP levels respectively as measured by eYFP fluorescence (Figure 3C).

The above results suggested that 1 likely functioned as an ‘allosteric agonist’ of hFFA3, binding to a site distinct from that occupied by the endogenous SCFA agonists. Such allosteric agonists are often also found to act as allosteric modulators, exhibiting binding cooperativity or altering the maximal response of orthosteric agonists (Smith et al., 2011, Hudson et al., 2013b), and such compounds are often described as PAM-agonists. As measured in functional assays, these allosteric effects will manifest in alterations in the measured potency or maximal signalling response to the orthosteric ligand when the allosteric modulator is present. Indeed, co-addition of concentrations of 1 ranging from 100 nM to 10 \( \mu \)M resulted in observed increases in both the potency and maximal signalling response for C3 in the \( ^{35}\text{S} \)GTP\( \gamma \)S binding assay (Figure 4A), suggesting that 1 is a PAM-agonist of hFFA3. Reciprocal studies showed that increasing concentrations of C3 (30 \( \mu \)M to 3 mM) also enhanced the measured potency and maximal signalling response of 1 (Figure 4B). Global analyses of the data were performed using an operational model of allosteric
modulation as described previously (Keov et al., 2011, Smith et al., 2011). This led to estimations of allosteric effects on binding cooperativity (log(α)) and modulation of the maximal signalling response (log(β)) between C3 and 1; estimates of the ability of C3 and 1 to directly activate hFFA3 (log(τ_A) and log(τ_B)); as well as estimates of the affinities of these two ligands for the receptor (pK_A and pK_B) (Table 1). These analyses demonstrated reciprocal allosteric modulation between C3 and 1 that is primarily attributed to a positive binding cooperativity (α values of 20 and 16 for the reciprocal experiments), although some positive modulation of the maximal response was also indicated (β values of 1.7 and 2.8 for the reciprocal experiments). It is important to note that similar values were obtained for α in each data set regardless of whether C3 or 1 was treated as modulator, as this value is expected to be conserved due to the reciprocal nature of allosterism (Keov et al., 2011). In contrast, the values for β need not be conserved, in large part because this value is expected to depend on the intrinsic efficacy of the modulator ligand, and therefore is predicted to track with changes in the τ value of the modulator (Keov et al., 2011). However, in this case given that the τ_A and τ_B values were similar (average τ_A= 2.3 and τ_B=1.8), it is not surprising that similar values were obtained for β in the reciprocal experiments.

In order to extend these studies, comparable reciprocal allosterism experiments were also performed in the cAMP (Figures 4C and 4D) and pERK1/2 (Figures 4E and 4F) assays. In these experiments similar response patterns were observed, and global fitting of these data indicated that again C3 and 1 exhibited positive binding cooperativity and, to a lesser degree, enhancement of each others maximal responses (Table 1). In both cases there were no statistical differences between the α values in reciprocal experiments, as would be predicted. Interestingly, although the largest β value was observed for the modulation of 1 on C3 function in the cAMP assay (β = 5.6), this does appear to track with the observed τ values.
as the largest average $\tau$ value was indeed observed for 1 in the cAMP assay (average $\tau_B = 2.8$). Similarly, the smallest average $\tau$ value was obtained for C3 in the pERK1/2 assay (average $\tau = 1.6$) and, as would be predicted from this, C3 modulation of 1 in this assay also yielded the lowest value for $\beta$ (1.4). A more complete analysis suggested that the average $\tau$ value obtained within an assay for the compound used as a modulator did correlate extremely well with the corresponding estimated $\beta$ values in the same assay (Supplemental Figure 1). Together these results suggest that although some differences are observed between ligands and across assays in the values for $\beta$, these differences can be attributed to differences in the intrinsic efficacy of the ligands and do not appear to represent bias in the allosteric modulation of FFA3. Combined $\alpha\beta$ values are often reported as a means to measure the overall cooperativity between orthosteric and allosteric ligands, taking into account both the allosteric effects on binding cooperativity and maximal response (Smith et al., 2012). Whilst analysis of these values with C3 and 1 did show some differences between assays (Table 1), again, given that statistically similar values were obtained for $\alpha$, and $\beta$ values were found to track with modulator $\tau$ value, the assay differences observed in $\alpha\beta$ cannot be attributed to bias modulation.

In addition to estimates of the degree of allosterism and agonist activity of C3 and 1, global fitting of the allosterism experiments also provided estimates of the affinities of these two ligands (Table 1). As would be expected, these values were generally very similar across each assay and agonist/modulator combination, yielding an average $pK_A$ for C3 of $3.27 \pm 0.15$ and an average $pK_B$ for 1 of $5.01 \pm 0.16$. Given the difficulties in developing binding assays for the FFA class of receptors (Hudson et al., 2011), such estimations may be particularly useful in predicting the affinity of ligands for the FFA3 receptor.
We have previously noted marked differences in effectiveness of a number of synthetic FFA2 ligands between species orthologs (Hudson et al., 2012a, 2012b, Hudson et al., 2013a) and next, therefore, assessed the activity of 1 at both rat (r) and mouse (m) orthologs of FFA3. In the \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding assay, as anticipated from our previous work (Hudson et al., 2012a), C3 was significantly more potent at both rFFA3 (pEC\(_{50} = 4.82 \pm 0.13\)) and mFFA3 (pEC\(_{50} = 5.25 \pm 0.10\)) than at hFFA3 (3.47 ± 0.09) (Figure 5A). In contrast, 1 displayed modestly reduced potency at the rodent orthologs (pEC\(_{50} 5.27 \pm 0.08\) for rat, and 5.20 ± 0.07 for mouse) compared to its potency at the human receptor (5.65 ± 0.07) (Figure 5B). Given that 1 was found to be a PAM-agonist of hFFA3, we next assessed whether this allosteric modulation of C3 by 1 was also observed at mFFA3. For this we generated concentration-response curves to C3 in the presence of increasing concentrations of 1 (Figure 5C), as well as reciprocal experiments generating concentration-response curves to 1 in the presence of increasing concentrations of C3 (Figure 5D). Global curve fit analysis of these data were performed and the resulting estimated parameters are shown in Table 2. Indeed, through these reciprocal experiments positive binding cooperativity was observed and with nearly identical values for \(\alpha\) (Table 2). However, the values for \(\alpha\) obtained were lower than those observed for hFFA3, suggesting the allosteric cooperativity is not as strong at mFFA3 as it is at hFFA3. However, as was the case with hFFA3, some positive allosteric modulation of the maximum signalling response was also observed with mFFA3, and again the magnitude of this correlated with the observed \(\tau\) value for the modulator. Specifically, 1 displayed a lower average \(\tau\) value (average \(\tau_B = 1.3\)) than C3 (average \(\tau_A = 1.9\)) in the cAMP assay at mFFA3 and, as would be predicted from this, also yielded a lower \(\beta\) value when used as the modulator (\(\beta = 2.1\)) than did C3 (\(\beta = 4.2\)). It was also interesting to note that the average affinities between these reciprocal experiments at mFFA3 suggest a pK\(_A\) of 4.41 for C3 and pK\(_B\) of 4.74 for 1. Comparison of these values with those obtained for hFFA3 (Figure
demonstrated that although 1 does show slightly reduced affinity for mFFA3 compared with hFFA3, C3 appears to have significantly higher affinity for the mouse ortholog than it has for the human. Both of these assessments are in good agreement with the relative potency of these compounds in functional assays at mFFA3 compared with hFFA3 (Figures 5A and 5B).

The Arena Pharmaceuticals patent (Leonard et al., 2006) lists 14 ligands designated as either agonists or antagonists of FFA3. To explore the structure-activity relationship (SAR) and extend the pharmacology of compound 1 we next explored the functions of an additional reported agonist 2 and an antagonist 3 (See Figure 1 for compound structures) at hFFA3-eYFP in the [35S]GTPγS binding assay (Figure 6A and Table 3). The 3-furyl analog 2 showed increased maximal signalling response but reduced potency, while 3, carrying a 4-phenoxyphenyl in the place of the furyl, did not exhibit significant activity on its own, in agreement with its purported antagonist activity. To further examine structure-activity relationships, 2-bromophenyl (4), 3-biphenyl (5), 3-phenoxyphenyl (6), and 4-biphenyl (7) analogs were assessed (Figure 6B and Table 3). Among this series, while the 4-biphenyl substituted 7 retained full agonist activity, although with lower potency, the 3-biphenyl compound 5 showed reduced maximal signalling response, and the two phenoxyphenyl compounds (3, 6) and the 2-bromophenyl 4 were essentially inactive. We also synthesized AR420626 (8), which is identical to 1 apart from a 2,5-dichlorophenyl terminal ring and is reported to have pEC₅₀ values of 6.57 and 6.92 in inositol phosphate accumulation and cAMP assays, respectively (Nøhr et al, 2013; Engelstoft et al, 2013). We found 8 to be a full agonist with potency resembling 1 (pEC₅₀ = 5.74 ± 0.11) in the [35S]GTPγS binding assay (Figure 6C).
Analysis of the analogs tested failed to identify any compounds with significantly improved potency compared with 1. Indeed, across the series there was only relatively modest differences in potency among compounds that displayed agonism (Table 3). There were, however, compounds producing varying maximal responses, including two that appeared to be super agonists (2, 7), and another that was a partial agonist of hFFA3 (5).

Considering this partial agonist, we next assessed whether 5 also allosterically modulated C3 function at hFFA3, as this might suggest the compound series could also be used to identify pure allosteric modulators of FFA3 that do not possess intrinsic efficacy. For this, we first confirmed the function of 5 in the pERK1/2 assay, where, as in the \([35S]GTP\gamma S\) assay, it acted as a weak partial agonist (pEC$_{50}$ = 5.08 ± 0.40; E$_{\text{Max}}$ = 24 ± 8%) (Figure 7A). Interestingly, 5 was also a strong PAM of C3 function in this assay. However, unlike compound 1 that primarily increased the potency of C3 (Figure 4E), 5 significantly increased the maximal response of C3 with little apparent effect on potency (Figure 7B). Global curve fitting of these data confirmed this observation, indicating a log$\alpha$ value of only 0.11 ± 0.29, but a log$\beta$ of 1.33 ± 0.21 for the allosteric effect of 5 on C3, suggesting that 5 has little positive binding cooperativity with C3, but does positively enhance the C3 signalling response in this assay. These analyses also were used to estimate the affinity of 5, indicating a pK$_B$ value of 3.95 ± 0.18.

The strong allosteric modulation of C3 by 5, despite this compound displaying limited agonist activity, suggested that compounds appearing to be inactive when tested as agonists might, in fact, be allosteric modulators if they still bind to the allosteric site of hFFA3. To explore this, we first examined the effect of a single high concentration (100 $\mu$M) of the reported antagonist 3 as well as the two additional inactive compounds, 4 and 6, on the concentration-response to C3 in the \([35S]GTP\gamma S\) assay (Figure 8A). In these experiments, 3 largely eliminated response to C3, as did 6, suggesting that the compounds were likely
negative allosteric modulators (NAMs) of hFFA3. By contrast 4 enhanced the potency of the C3 response without altering maximal response and, therefore, appeared to act as a PAM of C3. To explore this in further detail, increasing concentrations of 4 were used in combination with C3 concentration-response curves (Figure 8B). Global fitting of these data to the operational model of allosterism indicated that compound 4 has a pKB of 5.43 ± 0.17. Further, these analyses confirmed a modest but positive binding cooperativity between C3 and 4 (logα = 0.79 ± 0.10), with effectively no modulation of the maximal C3 signalling response (logβ = 0.08 ± 0.05). Although this represents only modest cooperativity between compound 4 and C3, it does suggest that this compound may well be a promising lead to develop further and more effective PAMs of hFFA3.

Next we explored further details of the two compounds that in the initial studies appeared to be FFA3 NAMs, 3 and 6. We generated C3 concentration-response curves in the presence of increasing fixed concentrations of either 3 (Figure 9A) or 6 (Figure 9B) in the [35S]GTPγS binding assay. In each case there was a clear concentration-dependent decrease in the C3 maximal response (but not potency), suggesting that compounds 3 and 6 are indeed NAMs of C3. Global curve fitting of the data for compound 6 yielded a pKB for this compound of 5.46 ± 0.38. Interestingly, the α and β values generated from these analyses suggested that whilst a negative effect on the signalling response was observed (logβ = -1.92 ± 0.20), a positive binding cooperativity (logα = 1.20 ± 0.39) was also observed between C3 and compound 6. As this was a somewhat surprising result, we also examined the allosteric effects of 6 on C3 in the pERK1/2 assay, which yielded a similar pattern of responses to C3 in the presence of compound 6 (Figure 9C). Once more, while the most striking observation was a clear inhibition of C3 maximal response by 6, the global curve fit analyses demonstrated that this resulted from a combination of a negative allosteric β (logβ = -1.88 ±
0.25) and a positive allosteric $\alpha$ ($\log \alpha = 0.98 \pm 0.07$). As a means to depict the apparently divergent effects on C3 maximal signalling response and potency produced by compound 6 in the pERK1/2 assay measures of these two parameters ($E_{\text{Max}}$ and $pEC_{50}$) obtained from individual 3-parameter concentration-response curve fits to C3 in the presence of increasing concentrations of 6 were plotted (Figure 9D). This clearly demonstrated opposing effects on C3 maximal response and potency by this compound, and that both effects were produced with near identical $pEC_{50}$ values ($5.77 \pm 0.10$ and $5.75 \pm 0.24$ respectively). As would be predicted, these are similar to the $pK_B$ value obtained from the global curve fit analysis of the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ data with this compound (Figure 9B). Together, these findings indicate that although 6 may have initially appeared to be a hFFA3 NAM, this compound may be better described as a PAM-antagonist, showing positive binding cooperativity with the orthosteric ligand, whilst also negatively modulating orthosteric ligand signalling responses.

Finally, as these represent the first compounds described that could be used as functional antagonists of FFA3, we also wished to establish whether the negative allosteric effect on C3 signalling of compound 6 was also observed at the mouse ortholog of FFA3. To do so, concentration response curves were generated to C3 in the pERK1/2 assay in the absence or presence of a 100 $\mu$M concentration of 6 (Figure 9E). The presence of 6 at this concentration substantially reduced C3 efficacy to $23 \pm 7\%$ of the control, suggesting that this compound may be useful as a functional FFA3 antagonist in murine systems as well as human.

**Discussion**

The SCFA receptors FFA2 and FFA3 have generated increasing interest in recent years, particularly for their roles linking the microfloral composition of the gut to health (Tan...
et al., 2014). Involvement of these receptors has now been demonstrated in this respect both in relation to metabolism (Kimura et al., 2013) and inflammation (Trompette et al., 2014), and this has stimulated interest in the receptors as novel therapeutic targets (Ulven, 2012, Yonezawa et al., 2013, Dranse et al., 2013). However, clearly defining the specific roles of FFA2 versus FFA3, and importantly, which would ultimately be a more effective therapeutic target in distinct pathological conditions, has been challenging in the absence of selective pharmacological tool compounds. This has been particularly problematic for FFA3 where, until recently, only a single publically available patent describes a series of FFA3 selective, but poorly characterized, compounds (Leonard et al., 2006). Recently, a representative compound from this series, designated AR420626, was used to demonstrate a contribution of FFA3 to SCFA-mediated GLP-1 (Nøhr er al., 2013), and ghrelin (Engelstoft et al., 2013) secretion. However, the detailed pharmacology of this compound series had remained largely unknown. In the present study we have begun to explore this by demonstrating that compounds from within this series act as selective ligands for FFA3 but with varied pharmacological properties.

Initially we synthesized and studied 4-(furan-2-yl)-2-methyl-5-oxo-N-(o-tolyl)-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (compound 1) as an exemplar. Although able to selectively regulate a series of signalling endpoints via hFFA3 with similar maximal responses to the endogenous SCFA C3, the structure of 1 suggested it was unlikely to be an orthosteric agonist. Indeed, this was confirmed as 1 still acted as an effective agonist at forms of hFFA3 in which either of the two arginine residues known to be key in co-ordination of the carboxylate function of SCFAs (Stoddart et al., 2008b) were mutated. Although 1 was entirely selective for FFA3 over FFA2, this compound displayed only modest potency for FFA3; particularly when compared with what had been described recently for the similar compound AR420626 (Nøhr er al., 2013; Engelstoft et al., 2013). To establish whether this
was related to intrinsic differences in potency between these two compounds, or instead due to the use of distinct assay systems, we synthesized AR420626 (compound 8) and found it to have similar potency to 1 in the [35S]GTPγS assay. This suggests that, despite lower measured potency in this assay, 1 is likely to be an equally useful selective FFA3 ligand as AR420626.

By extending the SAR of compound 1 we also identified a number of compounds with activity at FFA3 but with diverse pharmacological properties. Interestingly, relatively minor chemical modifications had substantial impact on ligand function, switching compounds from PAM-agonists, to PAMs lacking intrinsic agonism, or to PAM-antagonists having divergent effects on orthosteric ligand potency and maximal signalling response. Similar observations have been reported previously, particularly for allosteric modulators of the metabotropic glutamate receptors, where these chemical modifications have been described as 'molecular switches' capable of altering the function of the allosteric ligands (Wood et al., 2011). The SAR of the FFA3 ligand series suggests that such switches may also be present among these compounds. In particular, the presence of either a 2-bromophenyl (4) or a 3- or 4-phenoxyphenyl (3, 6) substituent on the hexahydroquinolone scaffold appears to eliminate intrinsic agonism of the ligand. In the case of the phenoxyphenyl variants this also appears to result in the gain of a negative modulatory effect on endogenous agonist maximal signalling response. Whilst such 'molecular switches' result in a series of compounds with diverse and interesting pharmacology, they clearly also complicate ligand optimization and, potentially, present a significant problem in selecting compounds that might be used in vivo (Wood et al. 2011).

Another consideration that may complicate the use of these compounds is that the two compounds identified with antagonistic properties, 3 and 6, displayed further complexity in
their pharmacology. Specifically, while they did negatively modulate C3 maximal signalling response, at the same time they exhibited positive binding cooperativity with C3. Similar pharmacology has been observed for allosteric modulators of other GPCRs, with certain modulators of the CB1 cannabinoid receptor being particularly notable examples (Price et al., 2005, Baillie et al., 2013). Indeed, this class of compound might be termed PAM-antagonists and possess the particularly interesting property that they should increase the potency of their antagonism with increasing amounts of orthosteric agonist present. While this complex pharmacology may complicate the use of these compounds as tools, as long as they are used in sufficient concentrations, and under carefully planned experimental conditions, they should still act effectively as functional antagonists that will help define further the role of FFA3.

A further key set of observations within these studies is that compounds including 1 and 6 are effective regulators of the mouse ortholog of FFA3 as well as the human receptor. This is important given that allosteric ligand binding sites have been predicted to be under less evolutionary pressure than orthosteric sites and, therefore, less likely to be conserved across species (Hudson et al., 2013b). Interestingly, the opposite may be true among the SCFA receptors, where significant species differences have been described for both FFA2 and FFA3 orthosteric ligands (Hudson et al., 2012a; Hudson et al., 2012b; Hudson et al., 2013a), while the function of the allosteric ligands described here appears more similar across species. This may result from the nature of the SCFA receptors as nutritional sensors (Dransse et al., 2013; Milligan et al., 2014) that respond to ligands derived from fiber fermented by the gut microbiota. Specifically, as different species rely on markedly different amounts of fiber in their diet, they are exposed to significantly different concentrations of SCFAs (Bergman, 1990). It might then be predicted that the affinity of these receptors for the endogenous SCFA ligands will also differ significantly between species (Hudson et al., 2012a). Indeed, as noted herein, C3 is substantially more potent at mouse and rat FFA3 than at the human ortholog and
we have previously reported on significant variation in SCFA potency at human, rodent and ruminant orthologs of FFA2 (Hudson et al., 2012a, 2012b).

Despite some potential challenges, as described above, members of this series of FFA3 allosteric ligands represents the best currently available options to selectively target this receptor pharmacologically. The diverse and complex pharmacology observed, even within the relatively small number of analogs explored in detail to date, suggests that further development of this series may yet provide allosteric agonists, PAM-agonists, PAMs, NAMs, or PAM-antagonists for the FFA3 receptor with even more useful pharmacological properties.

Authorship Contributions

\textit{Participated in research design}; Hudson, Murdoch, Ulven, Milligan

\textit{Conducted experiments}; Hudson, Murdoch, Jenkins

\textit{Contributed new reagents or analytic tools}; Madsen, Hansen, Christiansen, Ulven

\textit{Performed data analysis}; Hudson, Murdoch, Jenkins, Milligan, Ulven

\textit{Wrote or contributed to writing of manuscript}, Hudson, Milligan, Ulven
References


enteroendocrine cells vs FFAR3 in enteric neurons and FFAR2 in enteric leukocytes.

*Endocrinology* **154**: 3552-3564.


Footnotes

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Figure Legends

Figure 1. Chemical structure of compounds used in this study.

Figure 2. Compound 1 is a selective activator of hFFA3.

The structure of the endogenous SCFA propionate (C3) and compound 1 are shown (A). The concentration response of 1 to stimulate [35S]GTP\gamma S incorporation into membranes from cells either induced, or not (-Dox) to express hFFA3-eYFP is shown in B. The effects of C3 and 1 in the [35S]GTP\gamma S assay on membranes from cells expressing hFFA2-eYFP is in C. Concentration responses for C3 and 1 showing inhibition of forskolin-stimulated cAMP production (D) and phosphorylation of ERK1/2 (E) were generated from cells induced to express hFFA3-eYFP.

Figure 3. Compound 1 is an allosteric agonist of hFFA3.

The capacity of C3 (A) and compound 1 (B) to promote binding of [35S]GTP\gamma S to each of wild type (circles), R5.39A (triangles) and R7.35A (inverted triangles) hFFA3-eYFP is shown. C. As defined by levels of eYFP fluorescence both R5.39A and R7.35A hFFA3-eYFP were expressed more highly than the wild type receptor.

Figure 4. Compound 1 is a PAM-agonist of hFFA3.

Various fixed concentrations of 1 were added to concentration-response assays for C3 in each of [35S]GTP\gamma S binding (A), cAMP inhibition (C) and pERK1/2 activation (E) studies. Reciprocal experiments in which various fixed concentration of C3 were added to concentration-response curves for compound 1 are shown for [35S]GTP\gamma S binding (B), cAMP inhibition (D) and pERK1/2 (F). See text and Tables for details of the curve fit analyses.
Figure 5. Compound 1 displays similar function at human and rodent orthologs of FFA3.

The orthosteric agonist C3 is more potent at rat (r) and mouse (m) orthologs of FFA3 than at the human receptor (A). By contrast compound 1 displays similar potency at these species orthologs (B). The effects of adding various fixed concentrations of 1 to the concentration response of C3 were assessed in the cAMP assay (C). Reciprocal experiments using fixed concentrations of C3 are also shown (D).

Figure 6. Analogs of compound 1 display diverse activity and maximal signalling response.

The concentration response curves as direct agonists at hFFA3 in $[^{35}S]$GTPγS binding studies of various ligands are shown in (A-C). Response curves to C3 (A and B) or 1 (C), are shown for comparison as dashed lines.

Figure 7. The weak partial agonist compound 5 acts as a PAM of C3 activity.

The effect of compound 5 as a direct agonist in the pERK1/2 assay was compared to C3 (A). Although a weak partial agonist, 5 acted as a positive allosteric modulator of the efficacy, and potency of C3 (B).

Figure 8. Analogs of compound 1 display diverse pharmacology with compound 4 acting as an hFFA3 PAM.

In A, the effects of single concentrations of compounds 4, 3 and 6 (100 μM) as allosteric modulators of C3 function at hFFA3-eYFP in the $[^{35}S]$GTPγS assay are shown. Testing the effects of additional concentrations of 4 in this assay (B), indicates that although lacking direct agonist activity, 4 is a positive allosteric modulator of the potency of C3.
Figure 9. Compounds 3 and 6 are PAM-antagonists of the function of C3 at FFA3.

Fixed concentrations of compounds 3 (A) and 6 (B, C) were added to concentration-response curves of propionate (C3) in either [35S]GTP\gammaS binding (A, B) or pERK1/2 (C) assays. In D, effects of 6 on both the potency (right Y axis) and measured maximal response (left Y axis) of C3 in pERK1/2 assays are plotted separately to highlight the separate and distinct allosteric effects of 6 on the potency and maximal response of C3. Concentration- response curves to C3 at mouse FFA3-eYFP in the absence and presence of 100 μM 6 are shown in E.
Table 1. Operational model analysis of C3 and compound 1 at hFFA3

<table>
<thead>
<tr>
<th></th>
<th>[35S]GTPγS</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Agonist</td>
<td>C3</td>
<td>1</td>
<td>C3</td>
<td>1</td>
<td>C3</td>
<td>1</td>
</tr>
<tr>
<td>Modulator b</td>
<td>1</td>
<td>C3</td>
<td>1</td>
<td>C3</td>
<td>1</td>
<td>C3</td>
</tr>
<tr>
<td>log α</td>
<td>1.30 ±0.16</td>
<td>1.21 ±0.13</td>
<td>1.11 ±0.31</td>
<td>1.70 ±0.27</td>
<td>1.34 ±0.16</td>
<td>1.40 ±0.19</td>
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<tr>
<td>log β</td>
<td>0.23 ±0.07</td>
<td>0.44 ±0.04</td>
<td>0.75 ±0.12</td>
<td>0.28 ±0.09</td>
<td>0.31 ±0.06</td>
<td>0.16 ±0.07</td>
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<td>log τ A c</td>
<td>0.30 ±0.05</td>
<td>0.42 ±0.05</td>
<td>0.05 ±0.05</td>
<td>0.34 ±0.09</td>
<td>0.31 ±0.04</td>
<td>0.03 ±0.07</td>
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<tr>
<td>log τ B d</td>
<td>0.06 ±0.02</td>
<td>0.40 ±0.05</td>
<td>0.45 ± 0.16</td>
<td>0.44 ±0.10</td>
<td>0.10 ±0.05</td>
<td>0.33 ±0.05</td>
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<td>pK A e</td>
<td>3.16 ±0.11</td>
<td>3.18 ±0.09</td>
<td>3.96 ±0.16</td>
<td>3.41 ±0.18</td>
<td>2.96 ±0.08</td>
<td>2.97 ±0.09</td>
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<tr>
<td>pK B d</td>
<td>5.86 ±0.07</td>
<td>4.92 ± 0.08</td>
<td>5.15 ±0.17</td>
<td>4.85 ±0.18</td>
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<td>4.84 ±0.13</td>
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<td>αβ</td>
<td>34</td>
<td>44</td>
<td>72</td>
<td>95</td>
<td>44</td>
<td>50</td>
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a. Agonist is the compound used to generate concentration-response information
b. Modulator is the compound used in fixed concentrations
c. τ A and pK A are values estimated for C3
d. τ B and pK B are values estimated for 1
Table 2. Operational model analysis of C3 and compound 1 at mFFA3

<table>
<thead>
<tr>
<th>Ago</th>
<th>Mod</th>
<th>logα</th>
<th>logβ</th>
<th>τ_A</th>
<th>τ_B</th>
<th>pK_A</th>
<th>pK_B</th>
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<tr>
<td>C3</td>
<td>1</td>
<td>0.64 ±0.36</td>
<td>0.32 ±0.14</td>
<td>0.31 ±0.05</td>
<td>0.29 ±0.16</td>
<td>4.40 ±0.11</td>
<td>4.96 ±0.24</td>
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<tr>
<td>1</td>
<td>C3</td>
<td>0.65 ±0.27</td>
<td>0.62 ±0.14</td>
<td>0.24 ±0.06</td>
<td>0.18 ±0.11</td>
<td>4.42 ±0.21</td>
<td>4.51 ±0.10</td>
</tr>
</tbody>
</table>

a. Ago is the compound used to generate concentration-response information
b. Mod is the compound used in fixed concentrations
c. τ_A and pK_A are values estimated for C3
d. τ_B and pK_B are values estimated for 1
Table 3. Potency and efficacy of chemical variants of 4-(furan-2-yl)-5-oxo-N-(o-tolyl)-5,6,7,8-tetrahydroquinoline-3-carboxamide (compound 1)

<table>
<thead>
<tr>
<th>Compound</th>
<th>hFFA3 pEC\textsubscript{50}\textsuperscript{a}</th>
<th>hFFA3 efficacy\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>3.47 ± 0.09</td>
<td>100%</td>
</tr>
<tr>
<td>1</td>
<td>5.65 ± 0.07</td>
<td>101 ± 4%</td>
</tr>
<tr>
<td>2</td>
<td>5.24 ± 0.08</td>
<td>135 ± 8%</td>
</tr>
<tr>
<td>3</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>NR</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.70 ± 0.43</td>
<td>42 ± 8%</td>
</tr>
<tr>
<td>6</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.74 ± 0.20</td>
<td>132 ± 4%</td>
</tr>
<tr>
<td>8 (AR420626)</td>
<td>5.74 ± 0.11</td>
<td>120 ± 4%</td>
</tr>
</tbody>
</table>

NR- No response

\textsuperscript{a} pEC\textsubscript{50} values using hFFA3-eYFP in a \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding assay.

\textsuperscript{b} Percentage of C3 response using hFFA3-eYFP in a \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding assay.
Figure 1
Figure 2

A, B, C, and D are graphs showing concentration-response relationships for different conditions.
Figure 3

A

B

C

[Image of the figures showing the data for different conditions and a bar graph for eYFP expression]
Figure 4
Figure 5
Figure 6

A

B

C

[\text{\textsuperscript{35}S\textsc{GTP}}_\text{S} \text{ Incorporation}] (\% of C3)

\begin{align*}
\text{Log [ligand] (M)} &= -10, -9, -8, -7, -6, -5, -4, -3, -2 \\
\text{Log [ligand] (M)} &= -10, -9, -8, -7, -6, -5, -4, -3, -2 \\
\text{Log [ligand] (M)} &= -10, -9, -8, -7, -6, -5, -4, -3, -2
\end{align*}
Figure 7

A

B