

Complex Pharmacology of Novel Allosteric Free Fatty Acid 3 Receptor Ligands[§]

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ABSTRACT

Analysis of the roles of the short chain fatty acid receptor, free fatty acid 3 receptor (FFA3), has been severely limited by the low potency of its endogenous ligands, the crossover of function of these on the closely related free fatty acid 2 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. 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 signaling 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 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, free fatty acid 2 receptor (FFA2) (previously designated GPR43) and free fatty acid 3 receptor (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 knockout 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 have examined the action of a FFA3-selective agonist, AR420626 [N-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,

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ABBREVIATIONS: eYFP, enhanced yellow fluorescent protein; FFA2, free fatty acid 2 receptor; FFA3, free fatty acid 3 receptor; hFFA, human FFA; mFFA, mouse FFA; NAM, negative allosteric modulator; PAM, positive allosteric modulator; pERK, phosphorylation of extracellular signal-regulated kinase; SAR, structure-activity relationship; SCFA, short chain fatty acid.

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) have further heightened the need to identify and characterize FFA3-selective compounds.

In this study, we 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 noncompetitive 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 (Paisley, UK). Compounds 1–8 were synthesized as described in the supplemental information (Supplemental Methods). The radiochemical [³⁵S]GTPγS 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 T-Rex 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 (h)FFA2 (Stoddart et al., 2008a); or mutated forms of hFFA3 (Stoddart et al., 2008a). In all cases, the receptor construct expressed was fused in frame to enhanced yellow fluorescent protein (eYFP) at its C terminal. The cells were maintained in Dulbecco's modified Eagle's medium without sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin mixture (Sigma-Aldrich) in a humidified atmosphere containing 5% CO₂. To induce receptor expression, cells were incubated overnight with 100 ng/ml⁻¹ doxycycline.

[³⁵S]GTPγS Incorporation Assay. Cell membrane preparations were first generated as described previously (Stoddart et al., 2008a). [³⁵S]GTPγS-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 preincubating for 15 minutes at 25°C. [³⁵S]GTPγS was then added to initiate the assay, before incubation for 1 hour at 25°C. The reaction was terminated by filtration through GF/C glass-fiber 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 Phosphorylation Assay. Phosphorylation of extracellular signal-regulated kinase (pERK)1/2 was assessed using a previously described protocol (Hudson et al., 2012b). Briefly, 80,000 cells were seeded per well in a 96-well plate, allowed to attach, before incubating overnight with

doxycycline (100 ng/ml⁻¹) to induce receptor expression. Cells were then incubated in serum-free Dulbecco's modified Eagle's medium for 5–6 hours prior to the assay. Test compounds were added to the cells and incubated for 5 minutes at 37°C before cells were lysed and assayed for pERK1/2 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 homogeneous time-resolved fluorescence resonance energy transfer-based detection kit (CisBio Bioassays, Codolet, France), according to the manufacturer's 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-minute incubation with test compounds.

Data Analysis and Curve Fitting. All data presented represent mean ± S.E. 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 allosterism 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, α is a measure of the allosteric cooperativity on ligand-binding affinity, and β 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, whereas the abilities of the orthosteric and allosteric ligands to directly activate the receptor are incorporated through the values τ_A and τ_B. 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 α, β, τ_A, τ_B, K_A, and K_B. To fit the data presented in Figs. 7B, 8B, 9B, and 9C, the K_A value for C3 was constrained to the average value obtained in the experiments presented in Fig. 4 (summarized in Table 1). Finally, it was also required that the value for τ_B be constrained to a value of effectively 0 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) (Fig. 1). FFA3 is known to couple to the G_i 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-In T-Rex 293 cells that had been induced to express a C-terminally eYFP-tagged form of hFFA3, compound 1 increased incorporation of this radionucleotide in a concentration-dependent fashion with pEC₅₀ of 5.65 ± 0.07 (Fig. 2A). In this assay, 1 produced a maximal response similar to 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 pretreated with doxycycline to induce hFFA3-eYFP

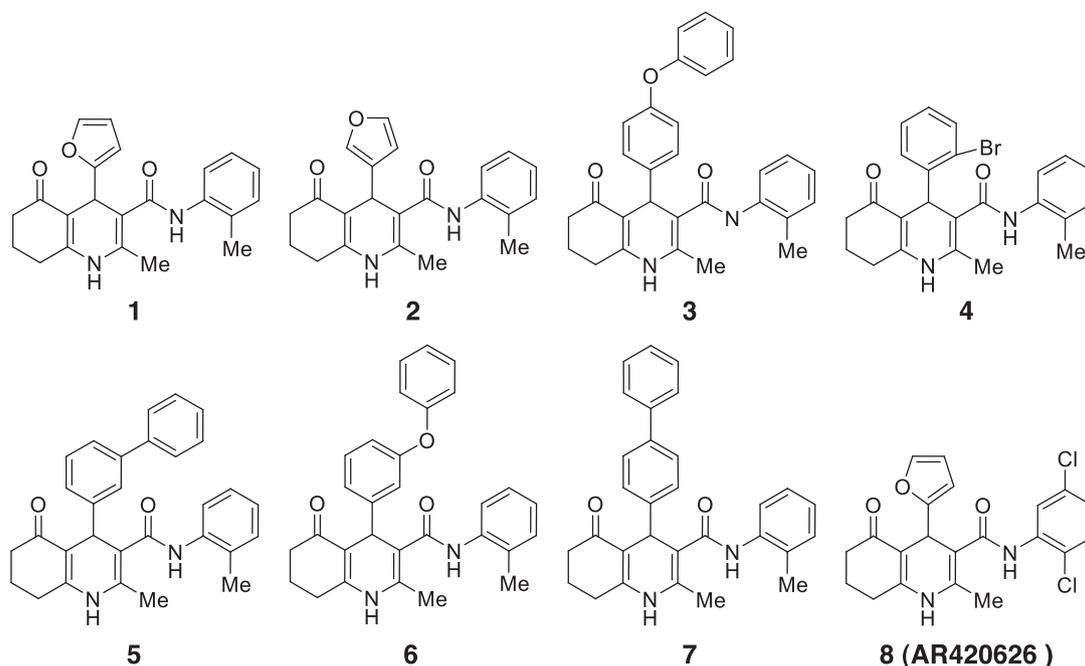


Fig. 1. Chemical structure of compounds used in this study.

expression (Fig. 2A). Furthermore, 1 was highly selective for hFFA3 over the closely related hFFA2 receptor because this compound did not increase [35 S]GTP γ S incorporation in membranes of Flp-In TReX 293 cells induced to express hFFA2-eYFP, although C3 did generate the expected response ($pEC_{50} = 4.18 \pm 0.16$) (Fig. 2B). In addition to stimulating [35 S]GTP γ S incorporation, compound 1 also produced similar signaling 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 (Fig. 2C), with pEC_{50} values of 5.77 ± 0.05 and 4.58 ± 0.09 for 1 and C3, respectively, and pERK1/2 (Fig. 2D): pEC_{50} values of 5.27 ± 0.16 for compound 1 and 3.68 ± 0.13 for C3. As in the [35 S]GTP γ S 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, whether 1 functioned as an orthosteric ligand for hFFA3. It did not: although mutation to Ala of either Arg^{5.39} or Arg^{7.35} completely eliminated response to C3 in the [35 S]GTP γ S-binding assay (Fig. 3A), at both of these mutants the function of 1 was essentially unaltered (Fig. 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 TReX 293 lines, yielding 137 ± 5 and $179 \pm 4\%$ of wild-type hFFA3-eYFP levels, respectively, as measured by eYFP fluorescence (Fig. 3C).

The above results suggested that 1 most 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 signaling response to the orthosteric ligand when the allosteric modulator is present. Indeed, coaddition of concentrations of 1 ranging from 100 nM to 10 μ M resulted in observed increases in both the potency and maximal signaling response for C3 in the [35 S]GTP γ S-binding assay (Fig. 4A), suggesting that 1 is a PAM-agonist of hFFA3. Reciprocal studies showed that increasing concentrations of C3 (30 μ M to 3 mM) also enhanced the measured potency and maximal signaling response of 1 (Fig. 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 ($\log\alpha$) and modulation of the maximal signaling response ($\log\beta$) between C3 and 1; estimates of the ability of C3 and 1 to directly activate hFFA3 ($\log\tau_A$ and $\log\tau_B$); 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

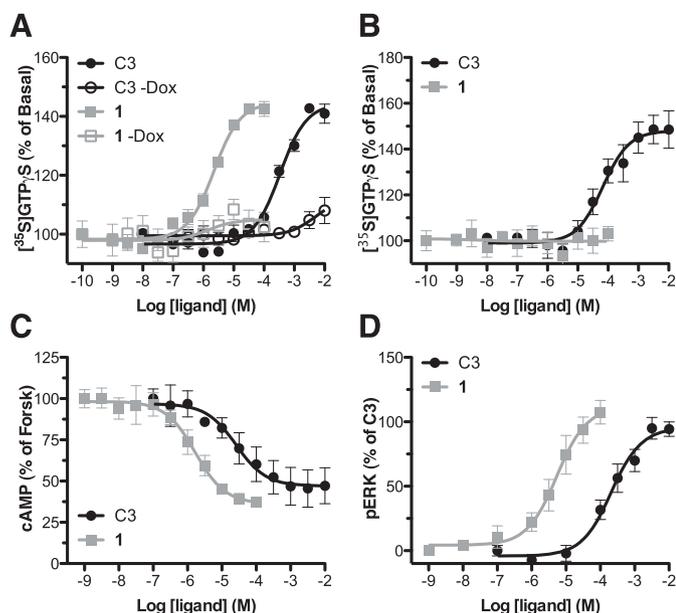


Fig. 2. Compound 1 is a selective activator of hFFA3. The concentration response of 1 to stimulate $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ incorporation into membranes from cells either induced, or not (-Dox) to express hFFA3-eYFP is shown in (A). The effects of C3 and 1 in the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ assay on membranes from cells expressing hFFA2-eYFP are in (B). Concentration responses for C3 and 1 showing inhibition of forskolin-stimulated cAMP production (C) and pERK1/2 (D) were generated from cells induced to express hFFA3-eYFP.

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 $\tau_A = 2.3$ and $\tau_B = 1.8$), it is not surprising that similar values were obtained for β in the reciprocal experiments.

To extend these studies, comparable reciprocal allosterism experiments were also performed in the cAMP (Fig. 4, C and D) and pERK1/2 (Fig. 4, E and F) 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 other's 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 ($\beta = 5.6$), this does appear to track with the observed τ values as the largest average τ value was indeed observed for 1 in the cAMP assay (average $\tau_B = 2.8$). Similarly, the smallest average τ 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 β (1.4). A more complete analysis suggested that the average τ value obtained within an assay for the compound used as a modulator did correlate extremely well with the corresponding estimated β values in the same assay (Supplemental Fig. 1). Together, these results suggest that, although some differences are observed between ligands and across assays in the values for β , 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.

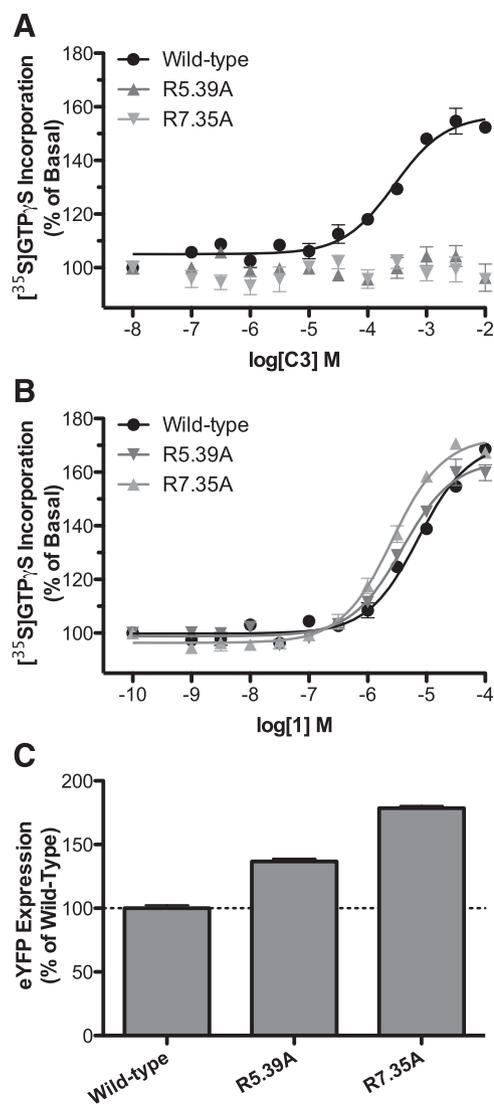


Fig. 3. Compound 1 is an allosteric agonist of hFFA3. The capacity of C3 (A) and compound 1 (B) to promote binding of $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ to each of wild-type, R5.39A, and R7.35A hFFA3-eYFP is shown. (C) As defined by levels of eYFP fluorescence, both R5.39A and R7.35A hFFA3-eYFP were expressed more highly than the wild-type receptor.

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., 2011). Whereas 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 α , and β values were found to track with modulator τ 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 ± 0.15 and an average pK_B for 1 of 5.01 ± 0.16 . Given the difficulties in developing binding assays for the FFA class of receptors (Hudson et al., 2011), such estimations may be

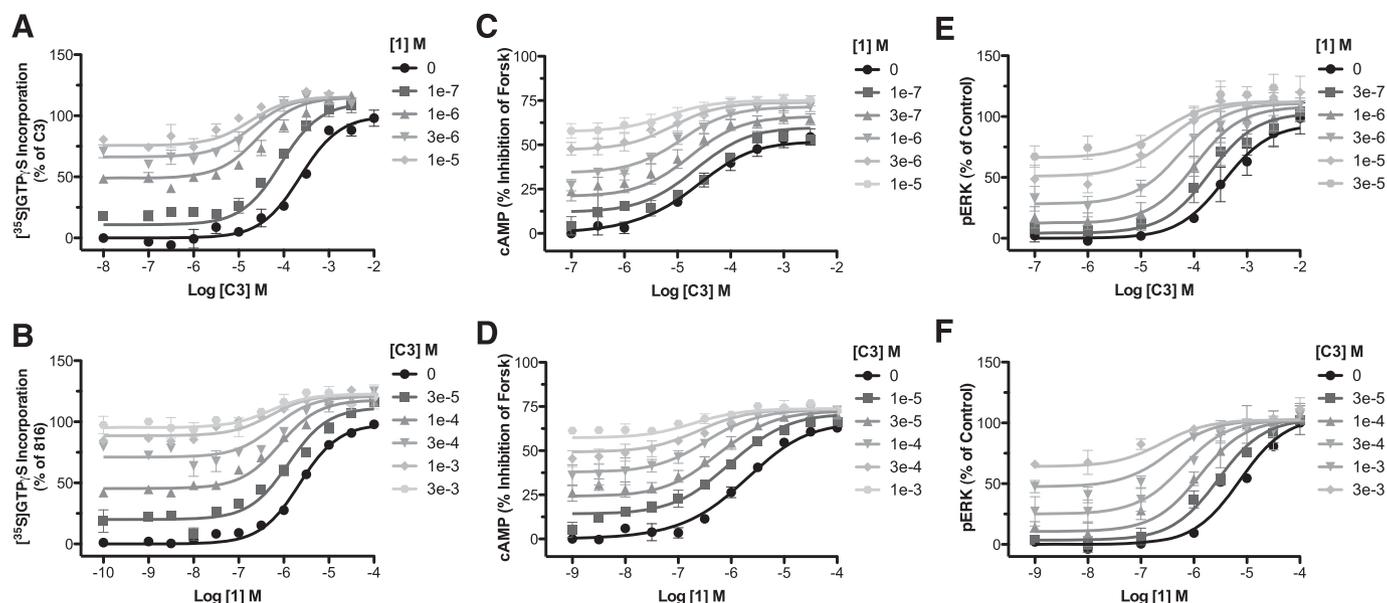


Fig. 4. Compound 1 is a PAM-agonist of hFFA3. Various fixed concentrations of 1 were added to concentration-response assays for C3 in each of [^{35}S]GTP γ S binding (A), cAMP inhibition (C), and pERK1/2 activation (E) studies. Reciprocal experiments in which various fixed concentration of C3 was added to concentration-response curves for compound 1 are shown for [^{35}S]GTP γ S binding (B), cAMP inhibition (D), and pERK1/2 (F). See text and tables for details of the curve fit analyses.

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,b; Hudson et al., 2013a) and next, therefore, assessed the activity of 1 at both rat and mouse (m) orthologs of FFA3 (mFFA3). In the [^{35}S]GTP γ S-binding assay, as anticipated from our previous work (Hudson et al., 2012a), C3 was significantly more potent at both rat FFA3 ($\text{pEC}_{50} = 4.82 \pm 0.13$) and mFFA3 ($\text{pEC}_{50} = 5.25 \pm 0.10$) than at hFFA3 (3.47 ± 0.09) (Fig. 5A). In contrast, 1 displayed modestly reduced potency at the rodent orthologs (pEC_{50} 5.27 ± 0.08 for rat, and 5.20 ± 0.07 for mouse) compared with its potency at the human receptor (5.65 ± 0.07) (Fig. 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 (Fig. 5C), as well as reciprocal experiments generating concentration-response curves to 1 in

the presence of increasing concentrations of C3 (Fig. 5D). Global curve fit analysis of these data was 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 α (Table 2). However, the values for α 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 signaling response was also observed with mFFA3, and again the magnitude of this correlated with the observed τ value for the modulator. Specifically, 1 displayed a lower average τ 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 β 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 (Fig. 4; Table 1) demonstrated that,

TABLE 1
Operational model analysis of C3 and compound 1 at hFFA3

Agonist ^a Modulator ^b	[^{35}S]GTP γ S		cAMP		pERK1/2	
	C3 1	1 C3	C3 1	1 C3	C3 1	1 C3
$\log\alpha$	1.30 ± 0.16	1.21 ± 0.13	1.11 ± 0.31	1.70 ± 0.27	1.34 ± 0.16	1.40 ± 0.19
$\log\beta$	0.23 ± 0.07	0.44 ± 0.04	0.75 ± 0.12	0.28 ± 0.09	0.31 ± 0.06	0.16 ± 0.07
$\log\tau_A^c$	0.30 ± 0.05	0.42 ± 0.05	0.05 ± 0.05	0.34 ± 0.09	0.31 ± 0.04	0.03 ± 0.07
$\log\tau_B^d$	0.06 ± 0.02	0.40 ± 0.05	0.45 ± 0.16	0.44 ± 0.10	0.10 ± 0.05	0.33 ± 0.05
pK_A^c	3.16 ± 0.11	3.18 ± 0.09	3.96 ± 0.16	3.41 ± 0.18	2.96 ± 0.08	2.97 ± 0.09
pK_B^d	5.86 ± 0.07	4.92 ± 0.08	5.15 ± 0.17	4.85 ± 0.18	4.92 ± 0.10	4.84 ± 0.13
$\alpha\beta$	34	44	72	95	44	50

^aAgonist is the compound used to generate concentration-response information.

^bModulator 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.

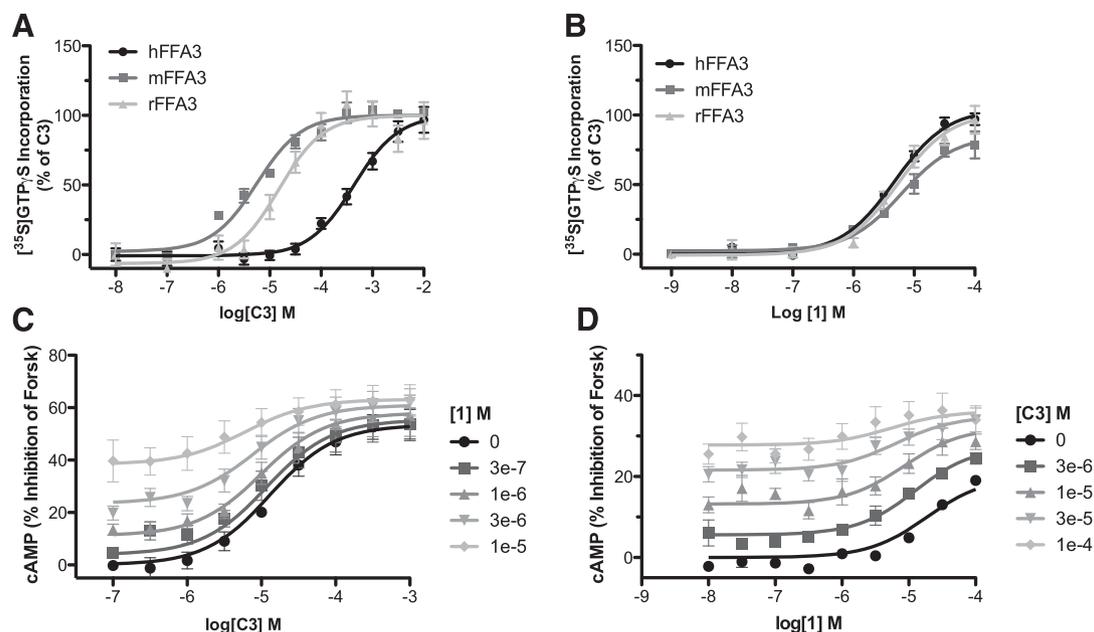


Fig. 5. Compound 1 displays similar function at human and rodent orthologs of FFA3. The orthosteric agonist C3 is more potent at rat (r) and mouse (m) orthologs of FFA3 than at the human receptor (A). By contrast, compound 1 displays similar potency at these species orthologs (B). The effects of adding 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).

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 (Fig. 5, A and B).

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 Fig. 1 for compound structures) at hFFA3-eYFP in the [³⁵S]GTPγS-binding assay (Fig. 6A; Table 3). The 3-furyl analog 2 showed increased maximal signaling response but reduced potency, whereas 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 the SAR, 2-bromophenyl (4), 3-biphenyl (5), 3-phenoxyphenyl (6), and 4-biphenyl (7) analogs were assessed (Fig. 6B; Table 3). Among this series, whereas the 4-biphenyl substituted 7 retained full agonist activity, although with lower potency, the 3-biphenyl compound 5 showed reduced maximal signaling 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 (Engelstoft et al., 2013; Nøhr 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 (Fig. 6C).

Analysis of the analogs tested failed to identify any compounds with significantly improved potency compared with 1. Indeed, across the series there were 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 superagonists (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, in which, as in the [³⁵S]GTPγS assay, it acted as a weak partial agonist (pEC₅₀ = 5.08 ± 0.40; E_{Max} = 24 ± 8%) (Fig. 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 (Fig. 4E), 5 significantly increased the maximal response of C3 with little apparent effect on potency (Fig. 7B). Global curve fitting of these data confirmed this observation, indicating a logα value of only 0.11 ± 0.29,

TABLE 2

Operational model analysis of C3 and compound 1 at mFFA3

Ago ^a	Mod ^b	logα	logβ	τ _A ^c	τ _B ^d	pK _A ^c	pK _B ^d
C3	1	0.64 ± 0.36	0.32 ± 0.14	0.31 ± 0.05	0.29 ± 0.16	4.40 ± 0.11	4.96 ± 0.24
1	C3	0.65 ± 0.27	0.62 ± 0.14	0.24 ± 0.06	-0.18 ± 0.11	4.42 ± 0.21	4.51 ± 0.10

^aAgo is the compound used to generate concentration-response information.

^bMod is the compound used in fixed concentrations.

^cτ_A and pK_A are values estimated for C3.

^dτ_B and pK_B are values estimated for 1.

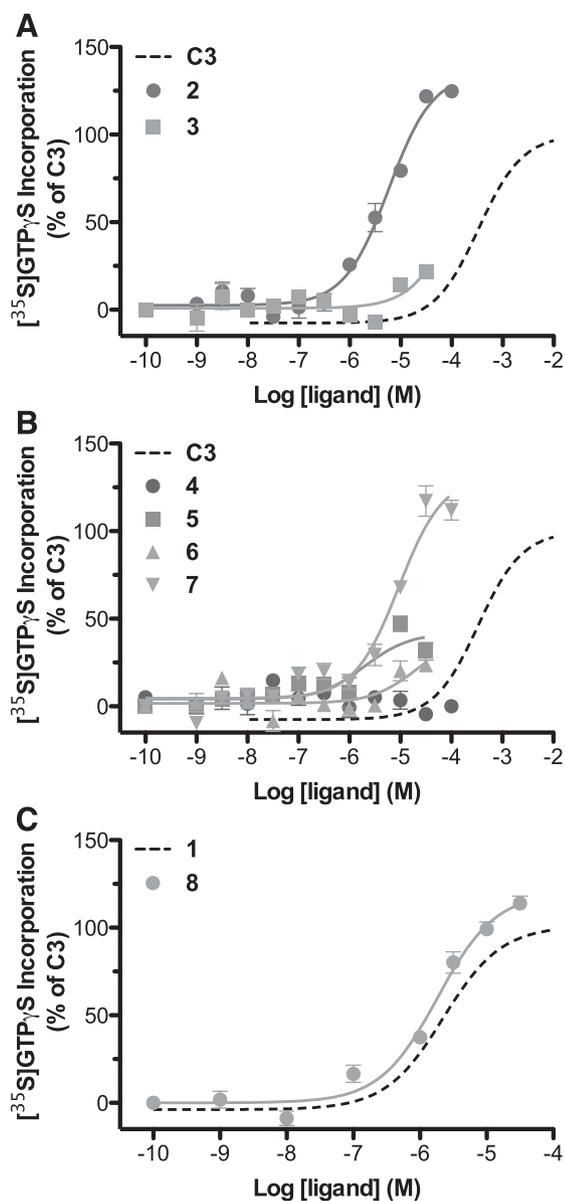


Fig. 6. Analogs of compound 1 display diverse activity and maximal signaling response. The concentration-response curves as direct agonists at hFFA3 in [³⁵S]GTP γ S-binding studies of various ligands are shown in (A–C). Response curves to C3 (A and B) or 1 (C) are shown for comparison as dashed lines.

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 signaling 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 μ 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 γ S assay (Fig. 8A). In these experiments, 3 largely eliminated response to C3, as did 6, suggesting that the

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)

Compound	hFFA3 pEC_{50}^a	hFFA3 Efficacy ^b
C3	3.47 ± 0.09	100%
1	5.65 ± 0.07	$101 \pm 4\%$
2	5.24 ± 0.08	$135 \pm 8\%$
3	<4	
4	NR	
5	5.70 ± 0.43	$42 \pm 8\%$
6	<4	
7	4.74 ± 0.20	$132 \pm 4\%$
8 (AR420626)	5.74 ± 0.11	$120 \pm 4\%$

NR, no response.

^a pEC_{50} values using hFFA3-eYFP in a [³⁵S]GTP γ S-binding assay.

^bPercentage of C3 response using hFFA3-eYFP in a [³⁵S]GTP γ S-binding assay.

compounds were most likely 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 (Fig. 8B). Global fitting of these data to the operational model of allosterism indicated that compound 4 has a pK_B of 5.43 ± 0.17 . Furthermore, these analyses confirmed a modest but positive binding cooperativity between C3 and 4 ($\log\alpha = 0.79 \pm 0.10$), with effectively no modulation of the maximal C3 signaling response ($\log\beta = 0.08 \pm 0.05$). 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 (Fig. 9A) or 6 (Fig. 9B) in the [³⁵S]GTP γ S-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 pK_B for this compound of 5.46 ± 0.38 . Interestingly, the α and β values generated from these analyses suggested that, whereas a negative effect on the signaling response was observed ($\log\beta = -1.92 \pm 0.20$), a positive binding cooperativity ($\log\alpha = 1.20 \pm 0.39$) 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 (Fig. 9C). Once more, whereas 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 ($\log\beta = -1.88 \pm 0.25$) and a positive allosteric α ($\log\alpha = 0.98 \pm 0.07$). As a means to depict the apparently divergent effects on C3 maximal signaling response and potency produced by compound 6 in the pERK1/2 assay, measures of these two parameters (E_{Max} and pEC_{50}) obtained from individual three-parameter concentration-response curve fits to C3 in the presence of increasing concentrations of 6 were plotted (Fig. 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

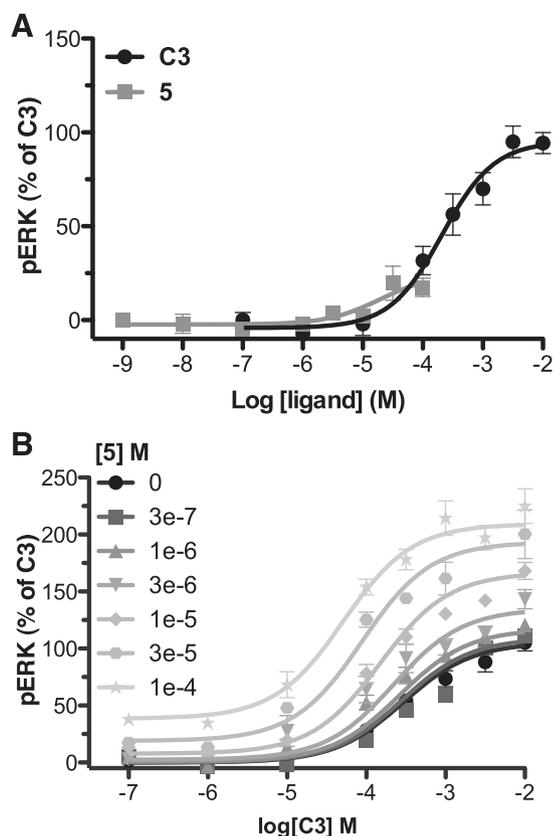


Fig. 7. The weak partial agonist compound 5 acts as a PAM of C3 activity. The effect of compound 5 as a direct agonist in the pERK1/2 assay was compared with C3 (A). Although a weak partial agonist, 5 acted as a positive allosteric modulator of the efficacy and potency of C3 (B).

(5.77 ± 0.10 and 5.75 ± 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 S]GTP γ S data with this compound (Fig. 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, while also negatively modulating orthosteric ligand-signaling 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 signaling 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 μ M concentration of 6 (Fig. 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

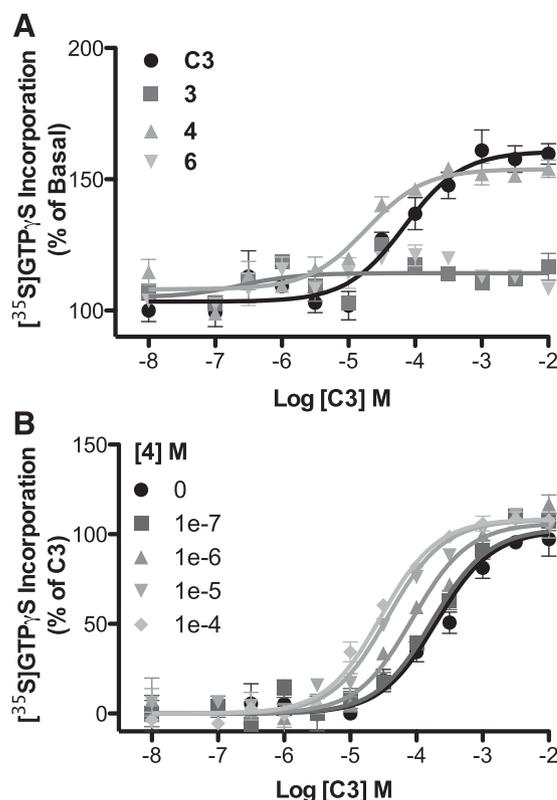


Fig. 8. Analogs of compound 1 display diverse pharmacology, with compound 4 acting as a hFFA3 PAM. In (A), the effects of single concentrations of compounds 4, 3, and 6 (100 μ M) as allosteric modulators of C3 function at hFFA3-eYFP in the [35 S]GTP γ S assay are shown. Testing the effects of additional 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.

demonstrated in this respect in relation to both 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; Dranse et al., 2013; Yonezawa 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 pathologic conditions, has been challenging in the absence of selective pharmacological tool compounds. This has been particularly problematic for FFA3, for which, until recently, only a single publicly 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 et 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 signaling endpoints via hFFA3 with similar maximal responses to the endogenous SCFA C3, the structure of 1 suggested it was unlikely to be an orthosteric

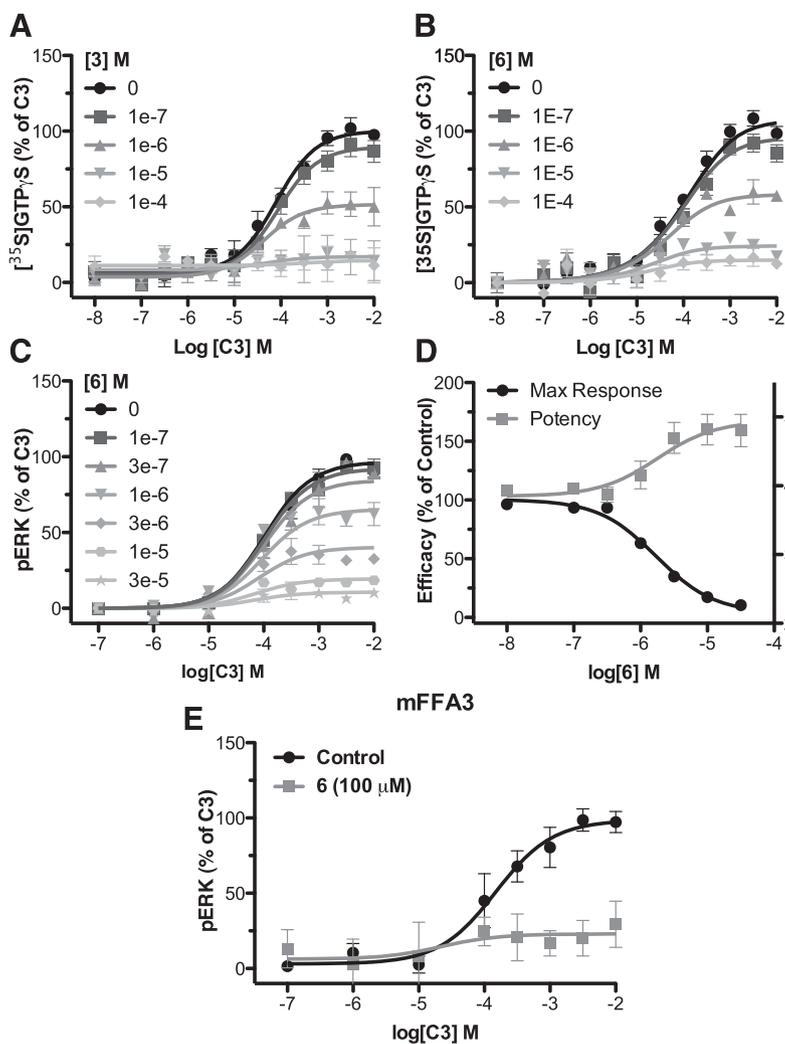


Fig. 9. Compounds 3 and 6 are PAM-antagonists of the function of C3 at FFA3. Fixed concentrations of compounds 3 (A) and 6 (B, C) were added to concentration-response curves of propionate (C3) in either $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ -binding (A and B) or pERK1/2 (C) assays. In (D), effects 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).

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 coordination of the carboxylate function of SCFAs (Stoddart et al., 2008b) was 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 (Engelstoft et al., 2013; Nøhr 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 $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ 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 signaling response. Similar observations have been reported previously, particularly for allosteric modulators of

the metabotropic glutamate receptors, in which 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 signaling response. Although 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, whereas they did negatively modulate C3 maximal signaling response, at the same time they exhibited positive binding cooperativity with C3. Similar pharmacology has been observed for allosteric modulators of other G protein-coupled receptors, 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. Whereas 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, in which significant species differences have been described for both FFA2 and FFA3 orthosteric ligands (Hudson et al., 2012a,b, 2013a), whereas the function of the allosteric ligands described in this study appears more similar across species. This may result from the nature of the SCFA receptors as nutritional sensors (Dranse 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 in this study, 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,b).

Despite some potential challenges, as described above, members of this series of FFA3 allosteric ligands represent 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

Participated in research design: Hudson, Murdoch, Ulven, Milligan.

Conducted experiments: Hudson, Murdoch, Jenkins.

Contributed new reagents or analytic tools: Madsen, Hansen, Christiansen, Ulven.

Performed data analysis: Hudson, Murdoch, Jenkins, Milligan, Ulven.

Wrote or contributed to the writing of the manuscript: Hudson, Milligan, Ulven.

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