MINIREVIEW

The Pharmacology and Function of Receptors for Short-Chain Fatty Acids

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ABSTRACT

Despite some blockbuster G protein-coupled receptor (GPCR) drugs, only a small fraction (~15%) of the more than 390 nonodorant GPCRs have been successfully targeted by the pharmaceutical industry. One way that this issue might be addressed is via translation of recent deorphanization programs that have opened the prospect of extending the reach of new medicine design to novel receptor types with potential therapeutic value. Prominent among these receptors are those that respond to short-chain free fatty acids of carbon chain length 2–6. These receptors, FFA2 (GPR43) and FFA3 (GPR41), are each predominantly activated by the short-chain fatty acids acetate, propionate, and butyrate, ligands that originate largely as fermentation by-products of anaerobic bacteria in the gut. However, the presence of FFA2 and FFA3 on pancreatic β-cells, FFA3 on neurons, and FFA2 on leukocytes and adipocytes means that the biologic role of these receptors likely extends beyond the widely accepted role of regulating peptide hormone release from enteroendocrine cells in the gut. Here, we review the physiologic roles of FFA2 and FFA3, the recent development and use of receptor-selective pharmacological tool compounds and genetic models available to study these receptors, and present evidence of the potential therapeutic value of targeting this emerging receptor pair.

Introduction

Short-chain fatty acids (SCFAs) are saturated aliphatic organic acids containing 2–6 carbon atoms. Within the body, they are predominantly the by-product of the fermentation of nondigestible carbohydrates (fibers) through the action of intestinal anaerobic bacteria (den Besten et al., 2013). The amount of SCFAs released in the intestine is influenced by several factors, among which are the strain and quantity of microbiota in the colon, substrate source, and intestinal transit time (Wong et al., 2006). In general, SCFAs reach an intestinal concentration in the millimolar region, in which acetate (C2), propionate (C3), and butyrate (C4) represent the most abundant metabolite species (~95%) (Topping and Clifton, 2001). In 2003, the previously designated orphan seven transmembrane (TM) domain polypeptides GPR41 and GPR43 were identified as G protein-coupled receptors (GPCRs) that are activated by SCFAs (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). Following this discovery, interest surrounding SCFAs and their receptors has risen dramatically, in relation to both the discovery of selective ligands and the physiologic role of these receptors. GPR41 and GPR43 were subsequently renamed FFA3 and FFA2, respectively (Stoddart et al., 2008b), based on their responsiveness to SCFAs.

FFA2/FFA3 Receptor Structure and Signal Transduction

The genes encoding FFA2 and FFA3, together with the medium-/long-chain fatty acid receptor GPR40 (FFA1;...
Stoddart et al., 2008b), cluster as a group of intronless sequences located in humans at chromosome 19q13.1. They were first identified during a search for novel human galanin receptor subtypes (Sawzdargo et al., 1997). A further gene that shares 98% identity with FFA3 was also identified within this region, and is designated GPR42. This latter potential receptor is now classified as a functional polymorph of FFA3, although its physiologic role and expression in humans is yet to be fully clarified (Liaw and Connolly, 2009; Puhl et al., 2015). FFA2 and FFA3 are closely related, with 43% amino acid identity (Stoddart et al., 2008b). This translates into poor ligand selectivity between the two receptors. Nonetheless, there is a rank order of potency for SCFAs in activating human FFA2 and FFA3 receptors, where FFA2 is activated more potently by shorter-chain fatty acids, whereas, in general, the opposite is the case for FFA3. Specifically, the rank order of potency for human FFA2 is reported as C2 > C3 > C4 > C5 > C1, whereas for human FFA3, it is C3 = C4 = C5 > C2 > C1 (Milligan et al., 2009) (Fig. 1). Although this results in acetate being significantly more potent at human FFA2 than at human FFA3 (Schmidt et al., 2011) and is sometimes, therefore, used as a selective activator of FFA2, there is great need to identify more selective synthetic ligands that would allow improved discrimination between the biologic functions of FFA2 versus FFA3 both in vitro and in vivo.

The deorphanization of GPR41 and GPR43 led to the observation that the carboxylic acid group of SCFAs is the key element in the activity of these endogenous molecules at both receptors. Previous studies had shown that positively charged amino acids within the TM regions are essential for the binding and function of other GPCRs whose ligands contain a carboxylic acid group (Stitham et al., 2003; He et al., 2004; Tunaru et al., 2005; Sabirsh et al., 2006). This, together with the observation that uncharged ester derivatives of SCFAs are inactive at FFA2 and FFA3 (Le Poul et al., 2003), led Milligan’s group to hypothesize that basic residues might also play a crucial role in the binding of SCFAs to their receptors (Stoddart et al., 2008a). Sequence alignment of
FFA2 and FFA3 with FFA1 revealed that five positively charged amino acids were conserved across these fatty acid receptors. Generation of homology models, linked to a mutagenic strategy, was then used to identify the key polar amino acids for ligand recognition contained in the water-filled cavity within the TM domains of FFA2 and FFA3 (Stoddart et al., 2008a). From this, four positively charged amino acid residues were identified: histidine (His) in TM IV (residue position 4.56), arginine (Arg) in TM V (5.39), His in TM VI (6.55), and Arg in TM VII (7.35) (Stoddart et al., 2008a) (for a more detailed consideration of the position and significance of these amino acids in FFA2 and FFA3, see Ulven, 2012). Studies in cells expressing alanine-substituted forms of these residues in FFA2 and FFA3 led to the conclusion that both Arg 5.39 and 7.35, as well as His 6.55, were essential for coordinating the recognition and functionality of SCFAs at both FFA2 and FFA3. Indeed, in each case, these alterations completely abrogated response to SCFAs (Stoddart et al., 2008a). By contrast, mutation of His 4.56 displayed a more diverse outcome between the two receptors, suggesting that this amino acid may not be directly involved in the binding of the carboxylate group but, rather, plays a role in fatty acid chain length selectivity (Stoddart et al., 2008a). The identification of these key residues involved in the orthosteric binding site paved the way for the study of other, and potentially selective, small-molecule ligands. Schmidt and colleagues (2011) established structure-activity relationships (SARs) of a group of small, nonfatty acid, carboxylic acids (SCAs) at FFA2. Although this work identified some molecules that were relatively selective for FFA2 over FFA3—for example, 2,2-dimethylacrylic acid has approximately 800 times higher potency at FFA2 compared with FFA3—the potency of such molecules was still very modest and too low to be useful as pharmacological tools for in vitro and, in particular, in vivo studies. Given the small size of these molecules, the low potency of both SCFAs and SCAs is hardly surprising. However, they did display high ligand efficiency (LE). LE is a measure of ligand binding free energy per heavy atom count and is mathematically expressed by δg (Hopkins et al., 2014). This concept has been widely used for the selection and optimization of fragments or small ligands at specific pharmacological targets. Schmidt et al. (2011) indicated that the ligand efficiency for C2, C3, and SCAs was approaching the maximal possible δg value, indicating that it would be unlikely that potency at FFA2 and FFA3 could be improved without increasing ligand size substantially.

An additional binding pocket in FFA2, distinct from that for the endogenously produced SCFAs, was first hypothesized by researchers at Amgen. This was based on outcomes from a high-throughput screen campaign to identify FFA2 activators (Lee et al., 2008). This resulted in the characterization of the first moderately potent FFA2 selective, synthetic ligand, (S)-2-(4-chlorophenyl)-3-methyl-N-(thiazol-2-yl)butanamide (4-CMTB) (Fig. 1). This ligand was shown to cause activation of both Goq10 and Goq11-mediated pathways via FFA2 with no effect at FFA3 (Lee et al., 2008). Despite this, some subsequent studies have indicated very limited ability of this ligand to produce elevation of Ca2+ levels in transfected cells (discussed later). Moreover, further analyses of the pharmacological properties of this ligand revealed an ability to exert positive cooperativity with both C3 and C2, indicating that 4-CMTB behaves as an allosteric agonist at FFA2 (Lee et al., 2008; Wang et al., 2010; Smith et al., 2011). Mutagenic studies have revealed that extracellular loop 2 plays an important role in the allosteric effect of 4-CMTB. Replacement of extracellular loop 2 in FFA2 with the equivalent region from FFA3 completely abolished the observed positive cooperativity between 4-CMTB and C3 (Smith et al., 2011). Despite this, the details of the allosteric binding pocket for 4-CMTB remain to be fully defined.

As noted earlier, in terms of signal transduction, FFA2 is a promiscuous receptor that has the ability to couple to pathways transduced by both Goq10 and Goq11 proteins (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). Moreover, screens performed in strains of the yeast Saccharomyces cerevisiae containing different yeast/mammalian Ga subunit chimeras also indicated an ability of FFA2 to interact with Goq12, Goq13, and Goq14 (Brown et al., 2003). However, interaction of FFA2 with these G proteins has yet to be validated in cells that express native, full-length mammalian G proteins. By contrast, the activation of FFA3 appears to induce only Goq11-mediated signaling, as receptor effects are generally attenuated by pertussis toxin (PTX) treatment of cells (Brown et al., 2003; Le Poul et al., 2003). In addition, SCFA occupancy of FFA2 has been reported to recruit both β-arrestin-1 and, in particular, β-arrestin-2 to the receptor, and these are responsible for FFA2 internalization from the cell surface and the initiation of G protein–independent signal transduction (Hudson et al., 2012b, 2013a; Lee et al., 2013). To date, there are no published data regarding the ability of FFA3 to recruit arrestin isoforms.

**Physiologic Roles of FFA2/FFA3**

**Roles in Immune Cells.** When the tissue expression of FFA2 was first described, its most notable presence was in cells of the innate immune system, both polymorphic nucleocytes (PMNs) and peripheral blood mononuclear cells (Lee et al., 2003) (Fig. 2). Recent studies have confirmed the presence of FFA2 in neutrophils, eosinophils (Maslowski et al., 2009), and in leukocytes of the lamina propria (Nehr et al., 2013). The presence of FFA3 remains contentious (Brown et al., 2003; Maslowski et al., 2009). Naturally, given that SCFAs are a by-product of anaerobic fermentation by gut bacteria, it is to be expected that immune cells should be able to respond to such ligands.

One area of particular interest is inflammatory conditions of the lower gut, such as ulcerative colitis and Crohn’s disease (Xavier and Podolsky, 2007). Butyrate enemas have been used to attempt to treat colitis, with mixed results (Bocker et al., 2003), and a high-fiber diet can result in improvement in ulcer and colitis scores, with matching reduction in neutrophil infiltration (Kataoka et al., 2008). Despite these promising results, two different studies examining the role of FFA2 in rodent models of colitis have generated conflicting effects. Maslowski et al. (2009) found that colitis could be rescued in germ-free mice by FFA2 agonism with acetate, whereas Sina et al. (2009) found that SCFAs recruit PMNs via FFA2 to worsen tissue damage. Moreover, a “first in man” clinical trial with the FFA2 antagonist -[(R)-1-(benzo[b]thiophene-3-carbonyl)-2-methyl-azetidine-2-carbonyl]-(3-chloro-benzyl)-amino)-butyric acid (GLPG0974; discussed later) for the treatment of ulcerative colitis did not provide any immediate beneficial effects, resulting in this study being terminated.
Clearly, FFA2 signaling in leukocytes is a complicated scenario that deserves further dissection and analysis. Most reports suggest that SCFAs mediate a shift from proinflammatory to anti-inflammatory cytokine release from leukocytes. C2 and C4 can inhibit tumor necrosis factor-α release (Säemann et al., 2000; Maslowski et al., 2009; Ohira et al., 2013), and C4 in particular can mediate a switch from a Th1 (which is exaggerated in Crohn’s disease) to a Th2 profile of cytokine production (Säemann et al., 2000; Cavaglieri et al., 2003). Neither FFA2 nor FFA3 knockout mice recruit Th1 cells in a rectal inflammation model (Kim et al., 2013). Finally, C3 can trigger the release of the anti-inflammatory interleukin-10 from regulatory T cells, and this happens in an FFA2-specific manner (Smith et al., 2013).

In terms of cell signaling, C2 and C3 (Le Poul et al., 2003; Maslowski et al., 2009), but not C4 (Nakao et al., 1992), increase intracellular Ca\(^{2+}\) in PMNs in a manner that has been described as partially (Nakao et al., 1992) or fully PTX-sensitive and intracellular store-dependent (Le Poul et al., 2003), raising the possibility that this effect may be mediated via FFA2 and G\(_{q/11}\). PMNs require calcium for chemotaxis toward N-formylmethionyl-leucyl-phenylalanine, a peptide which is often used as a model of bacteria-stimulated chemotaxis (Chen and Jan, 2001). PMNs migrate along a C3 or C4 gradient, in an FFA2-, p38-, extracellular signal-regulated kinase 1/2-, and phosphorylated Akt gradient, in an FFA2-, p38-, extracellular signal-regulated kinase 1/2- system (Chen and Jan, 2001). This may reflect opposing signaling via FFA2 and G\(_{q/11}\) and FFA3 and G\(_{i/o}\), a process which helps limit the extent of inflammation (Nordenfelt and Tapper, 2011)—and C2 increases apoptosis (Maslowski et al., 2009), perhaps again via FFA2. Broadly speaking, it would appear that C2 and C3 stimulate, whereas C4 inhibits, the various functions of innate immune cells. This may explain conflicting results using mixes of SCFAs, although nonreceptor mediated effects cannot be ruled out. In terms of cytokine release, all SCFAs appear to have anti-inflammatory effects. There are not yet many data demonstrating the signaling pathways upstream of cytokine release, or indeed whether both FFA2 and FFA3 are involved. As with analysis of function of FFA2 and FFA3 in other tissues (discussed later), the low potency and pleiotropic effects of the SCFAs mean that, without access to new, well characterized, and highly selective synthetic ligands, unraveling the specific roles of FFA2 and FFA3 in immune cells and in immune cell–mediated disease processes will remain challenging.

Roles in the Intestine. C4 is an important energy source for enterocytes, and SCFAs have a multitude of positive effects in the colon, such as helping maintain the intestinal barrier and decreasing the risk of cancer (Canani et al., 2011) (Fig. 2). Indeed, model animals on total parenteral nutrition fare better when supplemented with SCFAs, showing less mucosal atrophy, an improvement that correlates with increased expression of proglucagon (Gee et al., 1996; Pratt et al., 1996; Tappenden and McBurney, 1998). Studies using fluorescently labeled L-cells show FFA2 to be highly expressed in colonic L-cells, and FFA3 in small intestinal L-cells (Tolhurst et al., 2012). FFA3 partially colocalizes with gastrin and ghrelin in the stomach, and completely colocalizes with cholecystokinin, glucose-dependent insulinotropic polypeptide (GIP), and secretin in the proximal small intestine and with peptide tyrosine tyrosine, neurotensin, and glucagonlike peptide-1 (GLP-1) in the distal small intestine. A gradient of expression of FFA3 increases distally in D-cells and enterochromaffin cells. Conversely, FFA2 is only observed sparsely in a subpopulation of enteroendocrine cells of the small intestine, although the lack of fluorescence in the model used cannot be taken to be conclusive proof of lack of expression.
(Nøhr et al., 2013). In accordance with the expression profile of FFAR2 and FFAR3, stimulation of enteroendocrine cells with SCFAs can trigger GLP-1 (Reimer and McBurney, 1996; Lin et al., 2012; Tolhurst et al., 2012; Nøhr et al., 2013; Psichas et al., 2015), GLP-2 (Akiba et al., 2015), peptide tyrosine tyrosine (Lin et al., 2012; Psichas et al., 2015), and GIP (Lin et al., 2012) release. However, reports as to whether oral SCFAs can increase enteroendocrine hormones in vivo are mixed. One study found oral SCFAs had no effect on GLP-1, but decreased the plasma GIP response to glucose challenge (Tang et al., 2015). FFAR2 agonists may also act on enterochromaffin cells to trigger 5-hydroxytryptamine release, which together with GLP-2 would help prevent mucosal injury (Akiba et al., 2015), suggesting a mechanism for the benefits to adding SCFAs to total parenteral nutrition. Finally, there is a link between SCFAs and decreased intestinal motility, which is FFAR3- and neuroendocrine-independent (Dass et al., 2007). Overall, results show that FFAR2 and FFAR3 are involved in enteroendocrine hormone production and intestinal functions, but further studies with selective synthetic ligands and new animal models are needed to better define the roles of these receptors in the intestine.

Roles in the Pancreas. Early reports showed that C2 improves glucose clearance in rats (Shah et al., 1977), and improves glucose-stimulated insulin secretion (GSIS) in isolated rat islets (Patel and Singh, 1979). More recently, it has been demonstrated that islets express both FFAR2 and FFAR3 (Brown et al., 2003; Leonard et al., 2006; Regard et al., 2007) (Fig. 2), and crucially, they are found in beta cells of the islet (Tang et al., 2011). Interestingly, FFAR2 is upregulated in pregnancy in the mouse, suggesting that it may aid in this insulin-resistant state (Layden et al., 2010). This suggests a simple relationship of FFAR2/FFAR3 agonism leading to insulin secretion, but the reality is likely to be more complicated. One study reported that, whereas C2 increases insulin secretion via FFAR2 (Priyadarshini et al., 2015), C3 inhibits insulin secretion via FFAR3 (Priyadarshini and Layden, 2015). Other results indicate that C2 has an autocrine role, suggesting it inhibits insulin secretion via Gαi/o coupled to both FFAR2 and FFAR3 in beta cells (Tang et al., 2015). The latter appears more logical, given the importance of CAMP in boosting GSIS (Yajima et al., 1999). Interpretation must remain cautious at this point, however, because it is possible that different SCFAs display ligand bias (discussed later) at FFAR2/FFAR3. For example, FFAR2 agonist–mediated enhancement of GSIS activation is reported to occur via phospholipase C-beta (Priyadarshini et al., 2015). Perhaps FFAR2-dependant effects of C2 in the islet are biased to Gαi over Gαq. Studies in FFAR2 knockout mice showed a depressed level of plasma insulin in response to an oral glucose load, but it is difficult to draw firm conclusions from these data given that FFAR2 also plays a role in GLP-1 secretion (Tolhurst et al., 2012) and insulin sensitivity (Bjursell et al., 2011), both of which would be anticipated to have a knockon effect on insulin levels. To describe separate, potentially opposing roles of FFAR2 and FFAR3 in the islet, selective ligands for each receptor are sorely needed.

Roles in Adipose Tissue. There is strong evidence for the presence of FFAR2 in adipocytes (Fig 2). Two of the original deorphanization studies noted this (Brown et al., 2003; Le Poul et al., 2003), and subsequent studies have agreed (Ge et al., 2008; Al-Lahham et al., 2010; Lemor et al., 2010; Kimura et al., 2013). The presence of FFAR3, however, remains a subject of debate. FFAR3 mRNA is reported to be in adipose tissue and adipocyte cell lines (Le Poul et al., 2003; Xiong et al., 2004; Mielenz et al., 2008; Al-Lahham et al., 2010) but, equally, many groups report that adipocytes themselves do not possess the receptor (Brown et al., 2003; Hong et al., 2005; Zaibi et al., 2010; Bellahcene et al., 2013). Knocking out FFAR2 expression has not provided clarity as to its possible function in adipocytes. FFAR2 receptor knockout lines have been reported to show increase adiposity (Kimura et al., 2013) and protect against diet-induced obesity (Bjursell et al., 2011). In addition, lean and obese humans have similar expression levels of FFAR2 (Dewulf et al., 2013). Still, it is clear that SCFAs act on adipocytes. C2 derived from alcohol metabolism reduces circulating FFAs (Crouse et al., 1968), and resistant starch supplementation inhibits lipolysis and hormone-sensitive lipase to the same effect (Robertson et al., 2005; Ge et al., 2008). Exogenous C2, C3, and C4 all inhibit lipolysis (Hong et al., 2005; Ge et al., 2008; Zaibi et al., 2010). Importantly this effect is PTX-sensitive (Ohira et al., 2013) and absent in FFAR2 knockout mice (Ge et al., 2008). Reports suggest that FFAR2 activation reduces insulin sensitivity in the adipocyte by Gαi/o-mediated inhibition of Akt phosphorylation downstream of the insulin receptor (Kimura et al., 2013).

SCFAs can drive adipocyte differentiation from stem cells (adipogenesis). C2 and C3 trigger the preadipocyte cell line 3T3-L1 to differentiate, and C3 increases expression of the adipogenesis markers peroxisome proliferator-activated receptor γ-2 and CCAAT/enhancer binding protein α. When these cells are treated with small interfering RNA against FFAR2, expression of peroxisome proliferator-activated receptor γ-2 and aP2 (another adipocyte marker) decreases (Hong et al., 2005). FFAR2 knockout mice have fewer adipocytes (Bjursell et al., 2011), but curiously, these findings from rodent models may not translate to humans. Adipocytes cultured from the omentum did not show any increase in aP2 after treatment with FFAR2 agonists (Dewulf et al., 2013). A lack of difference in white adipose tissue between FFAR2 knockout and wild-type pups in the embryonic stage has even led one group to conclude that the effect of FFAR2 in adipogenesis may be an in vitro artifact (Kimura et al., 2013).

Finally, the adipocyte does not function as merely an inert energy store; it is also an endocrine cell, secreting metabolic hormones, including adiponectin and leptin (Sethi and Vidal-Puig, 2007). Higher levels of leptin have been reported in both FFAR2 (Bjursell et al., 2011) and FFAR3 knockout mice fed a high-fat diet (Bellaiche et al., 2013), although a different FFAR2 knockout line had plasma leptin levels comparable to the wild type (Kimura et al., 2013). In vitro, SCFAs also increase leptin mRNA in bovine adipocytes (Soliman et al., 2007) and increases leptin secretion from primary murine adipocytes (Xiong et al., 2004) in a PTX-sensitive manner. As all these somewhat contradictory studies demonstrate, the true role of the FFAR2 in the adipocyte is far from fully defined.

Roles in Neurons. The autonomic nervous system regulates energy output, and in particular, the sympathetic nervous system acts to decrease energy use during periods of starvation (Kimura et al., 2011). It has been proposed that SCFAs can act at FFAR3 as a switch to modulate sympathetic regulation of energy. In times of plenty, plasma levels of C4 will increase (especially if diets are high in fiber) (Nilsson et al., 2010), whereas during starvation, levels of ketone bodies, such as β-hydroxybutyrate (BHB; a putative FFAR3
antagonist (Inoue et al., 2012) (or possibly agonist) (Won et al., 2013), will increase.

FFA3 is expressed by both prevertebral and paravertebral ganglia (Kimura et al., 2011; Won et al., 2013; Nøhr et al., 2015) (Fig. 2). FFA3 knockout mice have a lower resting heart rate and less sympathetic innervation of the heart, whereas C3 given to wild-type mice can raise heart rate. Coculturing cardiomyocytes and neurons together reveals an FFA3-dependent relationship where C3 can increase beat rate. This effect is PTX-sensitive, and involves G_{i/o}, phospholipase C-beta, and extracellular signal-regulated kinase 1/2 MAP kinases. In this system, BHB can antagonize the effect of C3 (Kimura et al., 2011). C3 causes the release of noradrenaline from the superior cervical ganglion via the same signaling cascade and synapsin 2. Again, this is inhibited by BHB (Inoue et al., 2012). However, there is a conflicting report that describes a different mechanism of FFA3 action. Won et al. (2013) showed inhibition of N-type Ca^{2+} channels by G_{i/o}, complex generated by activation of FFA3, an effect that was elicited by each of C2, C3, and BHB, and would presumably decrease catecholamine release from neurons (Won et al., 2013). How these data fit within the sympathetic modulation theory remains to be defined.

FFA3 is also expressed in ganglia of the enteric and sensory nervous systems, i.e., submucosal ganglia, myenteric ganglia, nodose ganglion, dorsal root ganglia, and trigeminal sensory nervous systems, i.e., submucosal ganglia, myenteric ganglia (Nøhr et al., 2013, 2015). The physiologic function of FFA3 in these ganglia has yet to be described. Finally, FFA3 colocalizes with a neuronal marker in the portal vein wall, where it is proposed that C3 can act to form a gut-brain axis regulating intestinal gluconeogenesis (De Vadder et al., 2014).

**Experimental Challenges and Current Perspectives for the Validation of FFA2/FFA3 as Therapeutic Target(s)**

Target validation is an essential step in drug-development studies and assists in defining the physiologic role(s) of a GPCR and its importance in pathophysiologic conditions, with the aim of developing a pipeline of potential therapeutic medicines (Smith, 2003). Among several approaches, validation of a GPCR can be achieved by using ligands that selectively perturb the target of interest in vitro and/or in vivo.

**Synthetic Ligands for SCFA Receptors.** There is a general paucity of selective ligands for FFA2 and FFA3, and the coexpression of these two GPCRs in several tissues (discussed earlier) presents a major obstacle in understanding the pathophysiologic role of each receptor. This is further complicated by differences between the pharmacology of species orthologs of FFA2 and FFA3 (Fig. 1). For example, although C2 has been used in a number of in vivo studies both because it is the most abundant SCFA in the body (McOrist et al., 2008) and because of its reported selectivity for FFA2 over FFA3 (Schmidt et al., 2011), this selectivity of C2 is most pronounced at the human SCFA receptors. By contrast, Hudson et al. (2012b) demonstrated that this is not the case for the murine orthologs of FFA2 and FFA3 (Fig. 1). Indeed, for the mouse receptors, C2 is equipotent in activating FFA2 and FFA3. Moreover, no endogenous SCFA is sufficiently selective to define a role for FFA2 over FFA3, or vice versa, in murine cells and tissues (Hudson et al., 2012b). This highlights the need for more potent and markedly more selective ligands for the two receptors.

**Orthosteric Agonist Ligands.** Pleiotropic nonreceptor-mediated effects, as well as the low potency and lack of selectivity of the SCFAs between FFA2 and FFA3, have certainly become a major obstacle to the study of the function of these receptors, both in vitro and in vivo. The work of Schmidt et al. (2011) highlighted that, although small molecules containing a carboxylic acid group can achieve close to-optimal LE and a reasonable degree of selectivity at FFA2 versus FFA3, their modest potency and a lack of knowledge of potential “off-target” effects, means they remain unsuitable for ex-vivo and/or in vivo study of these receptors, although they have been used in a limited number of cases (e.g., Priyadarshini et al., 2015).

The first class of more potent synthetic ligands of FFA2 were initially described and patented by Euroscreen SA (Gosselies, Belgium) (Hoveyda et al., 2010). From this series of ligands, Hudson et al., (2013a) synthesized and characterized “compound 1” (3-benzyl-4-(cyclopropyl-(4-(2,5-dichlorophenyl)thiazol-2-yl)amino)-4-oxobutanoic acid) as a selective orthosteric agonist at human FFA2 (Fig. 1). In a range of assays, including those reflecting FFA2-mediated G_{i/o}, Go_{q/11} and β-arrestin pathways, this ligand is able to activate human FFA2 with potency in the high nanomolar range. The mode of binding of this ligand has not yet been completely elucidated. Compound 1 and related molecules contain a carboxylic acid pharmacophore, and this is required for function because replacement by a methyl or tert-butyl ester eliminates activity (Hudson et al., 2013a). Moreover, it is clearly orthosteric in action as it also lacks function at mutants of the key positively charged residues of the orthosteric binding site of human FFA2 (Hudson et al., 2013a). Moreover, function of compound 1 is inhibited by the orthosteric antagonist (S)-3-(2-(3-chlorophenyl)acetamido)-4-(4-((trifluoromethyl)phenyl)butanoic acid (CATPB) (Hudson et al., 2013a) in a competitive and surmountable fashion. However, compound 1 also contains the N-thiazoylamide pharmacophore, as found in the allosteric agonist 4-CMTB. Despite this, compound 1 does not appear to act as a “bitopic” ligand (Lane et al., 2013), because in β-arrestin-2 recruitment assays, compound 1 did not also display competitive interactions with 4-CMTB (Hudson et al., 2013a). Studies with related compounds, designated 9, 14, 101, and 105, containing both carboxylate and N-thiazoylamide moieties (Brown et al., 2015), indicated these ligands were also able to interact with the orthosteric binding site of FFA2, but not with the allosteric binding site.

In cells endogenously expressing FFA2, compound 1 has been reported to reduce lipolysis in both the human and mouse immortalized adipocyte cell lines, SW872 and 3T3-L1, respectively, with a mechanism that has been shown to be G_{i/o}-dependent. Moreover, compound 1 has also been reported to induce GLP-1 release from the murine STC-1 enteroendocrine cell line (Hudson et al., 2013a). Importantly, although compound 1 displays reasonable potency at rodent orthologs of FFA2 (Hudson et al., 2013a), certain other compounds from this chemical series show markedly lower potency in rodents compared with humans (Hudson et al., 2013a). Although a good deal might be learned from a systematic SAR analysis of this compound series, such studies have not been reported to date. Docking studies of compound 1 to a homology model of human FFA2 revealed that the phenyl substituent in this ligand may interact with residue position 3.29 of the receptor (Hudson et al., 2013a). Interestingly, this residue differs
between human (serine) and rodent (glycine) orthologs. More recently, a further homology model of this receptor, based on the X-ray structure of the related receptor FFA1, has indicated that tyrosine 90 (residue position 3.33) is also in close proximity to this phenyl ring (Sergeev et al., 2016), and mutation of this residue has also previously been shown to markedly reduce the potency of compound 1 (Hudson et al., 2013a).

Orthosteric Antagonist Ligands. Receptor antagonists, by blocking the action of either endogenous or synthetic agonists, routinely provide powerful tools to help define biologic roles of a receptor.

Interest in the biologic actions and potential therapeutic application of FFA2 antagonists emerged with the discovery that FFA2 is expressed by neutrophils and can dictate their migration in inflammatory states, including those of the lower intestine (Maslowski et al., 2009; Sina et al., 2009). Moreover, recent studies have shown that FFA2 and FFA3 activation in beta cells of the pancreas can block insulin secretion (Tang et al., 2015), suggesting that antagonists at either (or both) of these receptors could be beneficial for the treatment of type II diabetes.

In recent years, two series of FFA2 antagonists have been described. The first series of antagonists was reported by Euroscreen SA (Brantis et al., 2011). Among those compounds, CATPB (Fig. 1) inhibited effects of C3 in cells expressing human FFA2 in both guanosine 5′-O-(3-[35S]thio)triphosphate binding and calcium-based assays, with reported pIC50 values of 7.70 and 8.00, respectively (Brantis et al., 2011). Lack of effect in cells expressing FFA3 indicated CATPB to be specific for FFA2. Experiments using [3H]CATPB showed that C3 was able to fully displace the radiolabeled ligand, consistent with, although not defining, CATPB binding to the orthosteric site of human FFA2 (Brantis et al., 2011). Subsequent experiments using guanosine 5′-O-(3-[35S]thio)triphosphate binding revealed that CATPB was also able to decrease the constitutive activity of human FFA2 expressed in HEK293 cells, indicating that CATPB acts as an inverse agonist at this receptor (Hudson et al., 2012b).

The second series of FFA2 receptor antagonists contain an azetidine pharmacophore and were reported by Galapagos NV (Mechelen, Belgium) as potentially of interest for their effects in metabolic and inflammatory disorders, based on an ability to block SCFA-induced neutrophil migration (Sanière et al., 2012). The most studied compound from this series is GLPG0974 (Fig. 1). This ligand showed high potency to antagonize acetate-mediated Ca2+ elevation, with a reported pIC50 of 8.04 (Pizzonero et al., 2014). Using human neutrophils, this compound blocked both acetate-induced migration and expression of the neutrophil activation marker CD11b [AE], consistent with a potential capacity of GLPG0974 to decrease inflammatory processes (Pizzonero et al., 2014). Good pharmacodynamic properties and the pharmacokinetic profile of this ligand resulted in first-in-man trials of GLPG0974 in a phase 2, randomized, double-blind, placebo-controlled clinical trial in 2013. During this study, the safety and efficacy of the compound were tested in patients affected by mild to moderate ulcerative colitis. However, no improvement in the clinical profile of patients over a short-term treatment resulted in termination of this program.

FFA2 antagonists could represent an extremely useful tool for preclinical drug development and proof-of-concept studies. Unfortunately, neither of the FFA2 antagonists that have been described to date in primary peer-reviewed publications (CATPB and GLPG0974) appear able to interact with rodent orthologs of FFA2 (Fig. 1). Recently, Sergeev and colleagues (2016) analyzed the binding interaction of [3H]GLPG0974 at hFFA2. From this study it emerged that the orthosteric antagonists GLPG0974 and CATPB do not require interaction with both arginine residues, Arg 5.39 and 7.35, in the orthosteric binding pocket to engage with the receptor (Sergeev et al., 2016). In addition, it was found that these different classes of antagonists displayed preferential interaction with different arginine residues (Sergeev et al., 2016). The characterization of ligand-receptor interactions is likely to be important for the design of ligands that also display antagonism at rodent orthologs of the receptor.

Allosteric Ligands. Allosteric modulators are defined as ligands that interact at a site of a receptor that is distinct from and does not overlap with the orthosteric binding site. The interaction of a ligand with an allosteric binding site can generate a conformational change in the receptor that is transduced to the orthosteric site and/or directly to the intracellular effector (Kenakin and Miller, 2010; Wootten et al., 2013). Allosteric ligands can regulate the affinity and/or the potency of orthosteric ligands in a positive [positive allosteric modulators (PAMs)] or negative [negative allosteric modulators (NAMs)] manner (Kenakin and Miller, 2010; Wootten et al., 2013). Molecules may also bind to the allosteric site but have no effect on receptor activity, in which case they are called neutral allosteric ligands. In addition to potentially modulating the activity of orthosteric ligands, allosteric modulators may also possess agonist activity, or intrinsic activity, in their own right (Kenakin and Miller, 2010; Wootten et al., 2013). This is often only evident at high levels of occupancy of the allosteric site and in highly sensitive signal transduction assays.

Therapeutically, allosteric ligands potentially offer certain advantages over orthosteric ligands. This includes improved selectivity due to the fact that allosteric sites are often in nonconserved regions of the receptor, and that the cooperativity of allosteric ligands can be receptor subtype–specific. Furthermore, the effects of allosteric modulators are saturable, and this can limit possible side effects, including overdose. Moreover, PAMs and NAMs have the advantage of maintaining both temporal and spatial properties of endogenous ligand function, without altering or disrupting the physiologic system (Kenakin and Miller, 2010; Wootten et al., 2013).

Currently, phenylacetonamides, of which 4-CMTB (Fig. 1) is by far the most studied example, represent the only described class of allosteric modulators at FFA2. 4-CMTB is an allosteric agonist at FFA2 in that it has the ability to activate Gq11 and Gαq11, and β-arrestin–mediated FFA2 pathways directly as well as behaving as a PAM of the potency of SCFAs (Lee et al., 2008; Wang et al., 2010; Smith et al., 2011). However, some studies suggest that 4-CMTB might affect various FFA2-mediated signaling responses somewhat differently than SCFAs (Smith et al., 2011). Whether 4-CMTB displays “functional selectivity” (Hudson et al., 2013b) at FFA2 thus deserves further investigation.

In vitro, 4-CMTB has been found to produce inhibition of lipolysis in both mouse and human adipocytes (Lee et al., 2008; Wang et al., 2010; Brown et al., 2015), to induce GLP-1 release from enteroendocrine cells (Brown et al., 2015), and to...
promote chemotaxis of neutrophils (Vinolo et al., 2011). Unfortunately, 4-CMTB has poor pharmacokinetic properties (Wang et al., 2010), and consequently, it is not suitable for in vivo validation of FFA2.

There is an even greater paucity of available FFA3-selective ligands. The only currently described class of synthetic ligands was identified by Arena Pharmaceuticals (San Diego, CA) (Leonard et al., 2006). This series of ligands was later shown to contain each of allosteric agonists, PAMs, NAMs, and PAM antagonists (Hudson et al., 2014), although the basis for the SAR of such effects remains uncharted. One compound derived from this series, \(N\)-(2,5-dichlorophenyl)-4-(furan-2-yl)-2-methyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (AR420626) (Fig. 1), has been used to demonstrate the involvement of SCFAs in both GLP-1 secretion from colonic crypts (Nøhr et al., 2013) and ghrelin secretion from gastric mucosa cells (Engelstoft et al., 2013). Although this class of compounds needs to be further developed and characterized, the diverse and rather complex pharmacology of this series of ligands could provide new insight into the biologic functions of FFA3.

Interestingly, FFA2 and FFA3 allosteric modulators show similar function at human and rodent orthologs of those receptors, where they retain both their potency and allosteric properties (Lee et al., 2008; Hudson et al., 2012b, 2014). This is in contrast to the behavior of the synthetic orthosteric ligands discussed earlier. Although it has been reasoned that allosteric binding sites should be under less evolutionary pressure to be maintained than orthosteric sites because endogenously produced regulators do not bind to these regions (May et al., 2007; Hudson et al., 2013b), it is helpful that the allosteric ligands described earlier do display function at rodent orthologs of FFA2 and FFA3. Considering that different species ingest different amounts of fiber, and they are consequently exposed to varying concentrations of SCFAs (Dranse et al., 2013; Milligan et al., 2014), it is reasonable to imagine that this may have driven alterations in the orthosteric binding site between species (Hudson et al., 2014). This could be extremely important in terms of drug development programs. As already introduced, as well as showing activity at the human receptor, ligands preferably should show activity in different species as preclinical studies are performed in animal models. In this regard, allosteric modulators at FFA2 could be a useful approach to validate FFA2/FFA3 in vitro and in vivo.

**Biased Ligands.** In the last 20 years, it has become clear that some ligands have the ability to preferentially activate specific receptor-mediated intracellular signaling pathways over others. This phenomenon is defined as “biased signaling” or “functional selectivity,” and is believed to reflect the capacity of a receptor to adopt multiple activated states and/or the ability of a ligand to preferentially induce specific receptor active states (Kenakin, 2013; Kenakin and Christopoulos, 2013). It has been suggested that such “biased” ligands may have clinical benefit if they can facilitate beneficial physiologic processes without simultaneously driving signals that may be contraindicated. This has generated many ideas in ligand design and, although still to be shown directly to result in clinical benefit, highlights the need to pharmacologically characterize ligands in an array of functional assays (Kenakin and Christopoulos, 2013; Kenakin, 2015). FFA2 in particular has been reported to be a promiscuous receptor due to its ability to interact with various G proteins and to recruit arrestins (Brown et al., 2003; Stoddart et al., 2008a). Signal bias is certainly not restricted to synthetic ligands. For example, endogenous ligands for chemokine receptors clearly exert bias (Zweemer et al., 2014), and receptors, such as FFA2, that respond to multiple endogenously generated ligands may have the potential to display variation in signal flux.

At present, it is unclear whether different SCFAs display bias, or indeed, whether such bias at SCFA receptors could have a therapeutic application. Certainly, however, biased agonists at FFA2 or FFA3 would be invaluable tools to understand the physiologic implications of distinct signaling pathways to the biologic effect of these receptors. For example, the role of FFA2 in pancreatic beta cells remains controversial (Priyadarshini et al., 2015; Tang et al., 2015). Nonetheless,

![Diagram](https://example.com/diagram.png)

**Fig. 3.** DREADDs strategy for FFA2. In wild-type animals, FFA2 and FFA3 are both activated by SCFAs, i.e., propionate (C3). In tissues coexpressing both SCFA receptors, the physiologic response of C3 results from the activation of both FFA2 and FFA3. In mice engineered with the humanized FFA2-DREADD, the mutated FFA2 is solely activated by the administration of the nonendogenous ligand sorbic acid and inert to the endogenous ligand C3. Hence, the physiologic responses of C3 result only from FFA3 activation, whereas the responses of sorbic acid are uniquely mediated by FFA2-DREADD activation. Moreover, the FFA2-DREADD retains high affinity for the human-specific antagonist ligands CATPB and GLPG0974. As such, on-target FFA2-DREADD-mediated responses of sorbic acid will be blocked by these antagonists but not potential off-target effects.
Extensively to define G protein mutants have been termed designer receptors exclusively inert (Armbruster et al., 2007; Dong et al., 2010; Alvarez-to the natural ligand, and instead allows the receptor to be orthosteric binding site of receptors result in a loss of activity family has provided the framework for the development of a cated genetic approaches can be adopted that can provide a clinical potential of targeting these receptors, more sophisti-

**Limits and Extensions to FFA2 and FFA3 Knockout Studies.** As highlighted earlier, a useful technique for discovering the function of GPCRs is to create transgenic mouse knockout models, and characterize the resulting phenotype(s). In the case of FFA2 and FFA3, however, the results of knocking out either receptor have proven to be conflicting. For example, a disparity is seen in adiposity between the different knockout models. The FFA2 knockout mouse produced by Kimura et al. (2013) is heavier than the wild type, and has more adipose tissue, but another model shows no difference in weight gain between the wild type and knockout (Tang et al., 2015), whereas yet another actually shows the knockout is protective against weight gain on a high fat diet (Priyadarshini et al., 2015). Likewise, FFA3 knockout models have both increased adiposity (Bellahcene et al., 2013) and the same amount of adipose tissue as the wild type (Samuel et al., 2008). Model-dependent differences have also been observed in glucose tolerance and insulin sensitivity in both FFA2 (Bjursell et al., 2011; Tolhurst et al., 2012; Priyadarshini et al., 2015; Tang et al., 2015) and FFA3 knockout lines (Kimura et al., 2011; Tolhurst et al., 2012; Tang et al., 2015).

These discrepancies may be due to one of two reasons. First, as the endogenous ligands for FFA2 for FFA3 overlap between the two receptors, knockout of one may simply result in compensation by the other. Second, FFA2 and FFA3 in mice are located adjacent to each other on chromosome 7. Attempts to alter the transcript for one gene may affect the transcription of the other. One group has indeed reported that FFA2 was downregulated in their FFA3 knockout model (Zaibi et al., 2015), whereas others have not checked for this issue.

**Chemogenetic Approaches to Determining the Physiologic Function and Drug Responses of FFA2 and FFA3.** Whereas gene knockout studies provide an approach for understanding the physiologic role of FFA2 and FFA3 and provide important indications of the physiologic impact and clinical potential of targeting these receptors, more sophisticated genetic approaches can be adopted that can provide a direct measure of the impact of pharmacologically selective ligands. Work centered largely on the muscarinic receptor family has provided the framework for the development of a chemogenetic approach where mutations introduced into the orthosteric binding site of receptors result in a loss of activity to the natural ligand, and instead allows the receptor to be activated by a synthetic chemical ligand that is otherwise inert (Armbruster et al., 2007; Dong et al., 2010; Alvarez-Curto et al., 2011; Urban and Roth, 2015). Such receptor mutants have been termed designer receptors exclusively activated by designer drugs (DREADDs) and have been used extensively to define G protein–dependent in vivo responses (Urban and Roth, 2015).

Using the distinct endogenous ligand selectivity of bovine and human FFA2, two mutations introduced into the orthosteric binding site of human FFA2 reduced the response of the receptor to endogenous SCFAs by >100-fold. This receptor mutant was instead activated by sorbic acid, a naturally produced but not endogenously generated ligand, which activates bovine FFA2 but not human FFA2, as well as a series of small synthetic compounds (Hudson et al., 2012a) (Fig. 3). Hence, these studies generated the first genuine FFA2-DREADD receptor, which possessed the following properties: 1) the FFA2-DREADD was no longer activated by endogenous SCFAs; 2) instead, this receptor mutant was activated by a synthetic chemical ligand (e.g., in this case, sorbic acid); and 3) the wild-type receptor (i.e., human FFA2) was not activated by the synthetic ligand (Hudson et al., 2012a) (Fig. 3).

This FFA2-DREADD opens up the possibility of using a chemogenetic approach akin to that successfully used by the muscarinic-DREADDs. Hence, by using gene-targeting techniques that replace the mouse FFA2 gene with the coding sequence for the FFA2-DREADD, the mutant receptor will be expressed at physiologically relevant levels and in the same cell types as the wild-type FFA2. By administration of sorbic acid to these FFA2-DREADD mutant mice, not only will researchers be able to define the physiologic role of FFA2, but also this approach will provide the first insights into the potential therapeutic response that can be expected of a drug that selectively targets FFA2 (Fig. 3). Moreover, the FFA2-DREADD retains high affinity for the human-specific antagonist ligands CATPB and GLPG0974. As such, on-target, FFA2-DREADD–mediated effects of sorbic acid will block such effects but not potential off-target effects (Fig. 3). It will be fascinating to see the results of such studies, not least as FFA3 will still be responsive to the endogenously generated SCFAs.

**Conclusions**

SCFA receptors are relatively newly discovered GPCRs. Emerging evidence suggests that these receptors are implicated in a variety of physiologic functions, and their pharmacological modulation could represent invaluable therapeutic targets. However, translational pharmacology has been limited by a paucity of selective ligands and by receptor species ortholog differences. Moreover, animal genetic knockout approaches have resulted in challenging data interpretation, perhaps due to compensatory effects.

Alternative and more refined strategies, such as the DREADD chemogenetic approach, may hold great potential to unravel the impact of pharmacologically selective ligands at FFA2 and define the physiologic importance of FFA2 versus FFA3.

**Authorship contributions**

Wrote or contributed to the writing of the manuscript: Bolognini, Moss, Tobin, Milligan.

**References**


