Complex Pharmacology of Free Fatty Acid Receptors

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CHEMICAL REVIEWS

Review

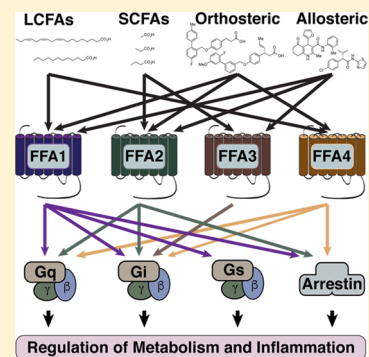# Complex Pharmacology of Free Fatty Acid Receptors

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ABSTRACT: G protein-coupled receptors (GPCRs) are historically the most successful family of drug targets. In recent times it has become clear that the pharmacology of these receptors is far more complex than previously imagined. Understanding of the pharmacological regulation of GPCRs now extends beyond simple competitive agonism or antagonism by ligands interacting with the orthosteric binding site of the receptor to incorporate concepts of allosteric agonism, allosteric modulation, signaling bias, constitutive activity, and inverse agonism. Herein, we consider how evolving concepts of GPCR pharmacology have shaped understanding of the complex pharmacology of receptors that recognize and are activated by nonesterified or "free" fatty acids (FFAs). The FFA family of receptors is a recently deorphanized set of GPCRs, the members of which are now receiving substantial interest as novel targets for the treatment of metabolic and inflammatory diseases. Further understanding of the complex pharmacology of these receptors will be critical to unlocking their ultimate therapeutic potential.

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1. INTRODUCTION

Alongside the multitude of hormones, neurotransmitters, and other regulatory factors that are generated by cells and tissues of multicellular organisms to allow integration of communication, recent years have seen an explosion of information about the capacity of molecules contained within or derived from food sources to also regulate and control cellular function and maintain homeostasis. Key among these are the nonesterified or "free" fatty acids, which have long been known to have diverse effects on many biological processes, including those related to cardiovascular health, metabolism, and inflammation. Although the fatty acids were traditionally believed to produce their effects through intracellular targets, it is now clear that they also activate several cell surface G protein-coupled receptors (GPCRs). Targeting GPCRs has long been a mainstay of drug development programs; however, there is growing appreciation that ligand regulation of these receptors is much more complex than originally thought, going far beyond simple competitive ligands binding to the same site as the endogenous ligand(s). The current review centers on a family of GPCRs activated by free fatty acids and in particular will examine the complex pharmacology of both the fatty acid and the synthetic molecules that regulate the function of these receptors, in particular as this relates to the ability of these receptors to regulate metabolism and inflammation. The members of this receptor family have garnered substantial interest as potential therapeutic targets,[1−7] and we will consider how the current state of knowledge impacts the future potential of developing ligands targeting these receptors as therapeutics. Finally, consideration will be given to key areas of both biological knowledge and pharmacological tools that are still lacking that would further expand our understanding of this important family of receptors.

1.1. Fatty Acids

Fatty acids consist of a carboxylic acid linked to an aliphatic tail of varying chain length. Fatty acids are, therefore, typically classified and defined on the basis of their aliphatic chain. Initially this includes the length of the chain, with short chain fatty acids (SCFAs) considered those with 6 or less carbon chains, medium chain fatty acids (MCFAs) having 7−12 carbon chains, and long chain fatty acids (LCFAs) ≥13 carbon chains. In addition to chain length, fatty acids also vary in the number of unsaturations, often broadly classified into the saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids. The position of unsaturation is also important and typically is defined by how far from the terminal carbon the first unsaturation occurs, where, for example, an omega $(n)-3$ fatty acid has its first unsaturation three carbon atoms from the end of the chain. Generally unsaturations in naturally occurring fatty acids are in the cis conformation; however, trans fatty acids also occur naturally in the gut of

ruminants, as well as being generated via hydrogenation of unsaturated fats during food production processes.[8,9] Together, the various combinations of chain length as well as position, location, and conformation of unsaturations greatly increase the number of possible unique fatty acids. The field of lipidomics focuses on characterizing the full range of lipid species, including the fatty acids, in biological samples and how the amounts and proportions of these may be altered in response to diet, disease, or other manipulations of the environment.[10−12]

The MCFAs and LCFAs are derived either from fat obtained in the diet or through de novo synthesis, occurring primarily in the liver.[13] While humans are able to synthesize SFAs and MUFAs, we lack the enzymes required to incorporate unsaturations at the $n-3$ and $n-6$ positions, limiting de novo synthesis of the various PUFAs required for health.[14] Instead, interconversion of linoleic acid (LA; 18:2$n-6$) and α-linolenic acid (aLA; 18:3$n-3$) obtained through the diet does allow synthesis of these other PUFAs; hence, LA and aLA are often described as "essential" (Figure 1). In the body LCFAs are
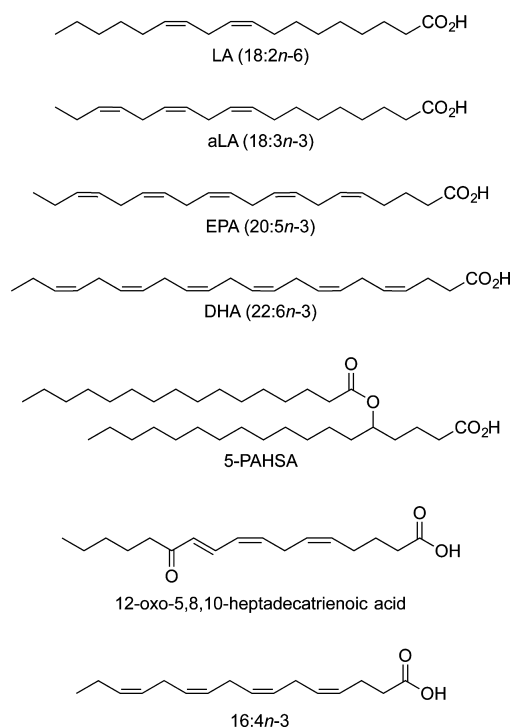
Figure 1. Chemical structures of LCFAs.

utilized, through β-oxidation, as a source of energy, serve as key components of the phospholipids forming cellular membranes, and are incorporated into triglycerides for energy storage. Beyond these roles, nonesterified, i.e., "free" fatty acids, also have further widespread and pleiotropic roles in the body. These include acting as the precursors of many signaling molecules, including resolvins, prostaglandins, and leukotrienes,[15−17] and acting directly as ligands of various cell surface and intracellular receptors.[18−20] Although quantitatively relatively minor in amount, PUFAs, such as the $n-3$ fatty acids, DHA (22:6$n-3$) and EPA (20:5$n-3$) (Figure 1) that are found in high levels in oily fish are often linked to positive metabolic and cardiovascular health outcomes[21,22] and hence the exhortations to include fish oil in the diet.[23] In contrast, trans-fatty acids are generally considered to be detrimental to

cardiovascular and metabolic health,[8,24] and recommendations are routinely to avoid or limit consumption of such fatty acids.[23] Detailed lipidomic studies have also identified further modified fatty acids with unique biological activity, for example, fatty acid esters of hydroxy fatty acids (FAHFAs), such as 5-PAHSA (Figure 1), which despite being present in very low levels in biological systems has been reported to have anti-inflammatory and antidiabetic activity.[25] It is likely that further specific but relatively uncommon fatty acids, including the so-called "platinum-induced fatty acids" (PIFAs), 12-oxo-5,8,10-heptadecatrienoic acid and hexadeca-4,7,10,13-tetraenoic acid (16:4n−3) that induce chemotherapeutic resistance[26,27] (Figure 1), will be shown to have dramatic effects on cell function and health. A major challenge going forward will be to define the molecular targets for these molecules that account for their apparently potent and specific effects and to understand how they might function in the presence of quantitatively much higher levels of other fatty acids. A substantial discussion of the biochemistry of synthesis, interconversion, and generation of MCFAs and LCFAs and their metabolites is beyond the scope of this review, but a series of recent reviews provides excellent primers for those wishing to read further.[13,15,16]

In contrast to the LCFAs, the SCFAs are produced primarily via bacterial fermentation of fiber in the gut, although production of acetate through the metabolism of ethanol may also provide a significant source of SCFAs in certain circumstances (Figure 2). Variation in the composition of gut
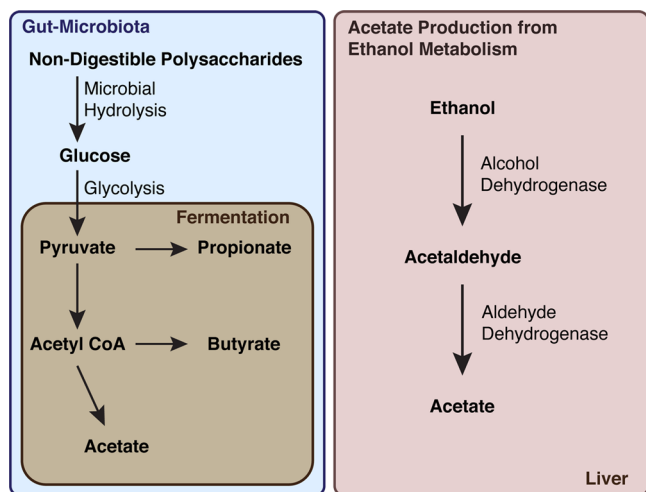
Figure 2. Biosynthesis pathways for SCFAs. SCFAs are synthesized primarily by microbiota present in the gut (left). Nondigestible polysaccharides are converted into monosaccharides such as glucose, which undergo glycolysis to produce pyruvate. Fermentation processes (marked by brown box) convert pyruvate to acetate, propionate, or butyrate. Under certain circumstances, the SCFA acetate is also produced in substantial quantities through ethanol metabolism in the liver (right).

microflora responsible for SCFA production is now frequently linked to health and disease,[28−30] and many studies are demonstrating that SCFAs are the key signaling molecules underlying this link. Therefore, there is considerable interest in manipulating the microfloral balance in the gut as a means to alter SCFA levels and profile. For example, as certain species of bacteria are known to favor production of different SCFAs,[31,32] manipulating bacterial composition to, for example, increase acetate (**C2**) production over propionate (**C3**) and butyrate

(**C4**), may have substantial effects on health and disease. This is an area receiving expanding research focus in recent times, not least because of advances in technologies capable of high-throughput gene sequencing to define the diversity of the microbiota.[29]

1.2. G Protein-Coupled Receptors

It is frequently noted that GPCRs are the largest family of transmembrane signaling polypeptides encoded within the genomes of eukaryotic species.[33] Given the now clear-cut evidence that signal transduction initiated by activation of GPCRs is not limited to events transduced by subunits of heterotrimeric G proteins,[34,35] the alternative term 7-trans-membrane domain (7-TMD) receptor is growing in popularity. This terminology highlights the key architectural feature of this family of proteins, i.e., that the corresponding gene encodes a single polypeptide in which an N-terminal domain is located on the opposite side of the plasma membrane from the C-terminus. This results in serpentine organization in which the 7-transmembrane domains are linked by three extracellular and three intracellular loops (Figure 3). Informatic analysis, based
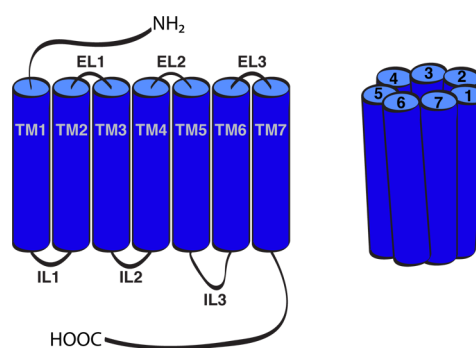
Figure 3. Topology of a GPCR. A GPCR has an extracellular N-terminus and intracellular C-terminus with 7 membrane spanning helical domains linked through three extracellular and three intracellular loops (left). Viewed from outside the cell, these TM domains are arranged in a counterclockwise fashion (right).

on the predicted presence of seven hydrophobic domains of sufficient reach to span the plasma membrane and a series of amino acid signatures highly conserved throughout the predominant subfamily, allowed prediction that the human genome would likely contain in the region of 800 distinct genes encoding GPCRs. Initial drafts and subsequent more detailed follow-up results have confirmed such predictions.[33] The ubiquity of the 7-TMD design means that GPCRs have evolved to recognize ligands, chemicals, and stimuli as diverse as photons, odorants, various ions, aminergic neurotransmitters, peptide hormones, and small proteins such as chemokines. Consistent with this, GPCRs control or modulate a vast range of physiological functions and, based on this, have been targeted to mask, treat, or ameliorate a broad range of diseases.[36−38]

Recent years have seen tremendous breakthroughs in structural insights into the organization of GPCRs and the chemical basis of their selective recognition of both natural and synthetic ligands.[39−42] The substantial amounts of the photon-recognition receptor rhodopsin in rod outer membrane segments of the eye and its ease of purification allowed crystallization of the inactive state of this GPCR almost a decade ahead of other GPCRs.[43,44] However, development of

methods including incorporation of well-understood proteins, such as T4-lysozyme, into the sequence of GPCRs and more suitable detergents to allow for stability of protein solubilized from membranes of expression systems has resulted in a large number of both inactive and active atomic level structures being defined in recent years.[45−47] In general, many of the predictions derived from work on rhodopsin have been validated across the broader family of "rhodopsin-like" or "class A" GPCRs. In essence the 7-TMD helices are organized (as viewed from the outside of the cell) in a counterclockwise orientation (Figure 3), providing a potential central, predominantly hydrophilic, cavity at the cell surface that water-soluble ligands may enter to make specific chemical interactions that help define the ligand binding pocket. The organization of the extracellular loops, particularly the second extracellular loop (EL2) connecting transmembrane domains IV and V, is more variable and appears to play a role in ligand binding and entry to the classical ligand binding pocket.[48,49]

Unsurprisingly, given the widespread utilization of this topological framework, the basic 7-TMD design of the GPCR superfamily has been modified in a number of cases to provide further specialization. For example, in cases such as the group of protease-activated receptors the ligand for the receptor is contained and constrained within the N-terminal domain of the receptor polypeptide, and this is released by cleavage by an appropriate protease.[50] A distinct means to provide signaling diversity from a single 7-TMD polypeptide is exemplified by the calcitonin receptor-like receptor which interacts selectively with either of two peptide hormones, calcitonin gene-related peptide and adrenomedullin, involved in control of cardiovascular function.[51] This selectivity is defined by the interaction of the 7-TMD element of the receptor with different members of a small group of single TMD receptor activity modulating polypeptides (RAMPs).[52] The basis for the peptide binding specificity has recently been explored by X-ray crystallography.[53] An alternative way to increase diversity still further is exemplified by cases in which multiple copies of either the same GPCR sequence or copies of distinct family members can interact directly to form, respectively, homomers and heteromers.[54] In many cases such interactions alter either the detailed patterns of recognition of ligands or the regulation of the receptor polypeptides.[55−57]

1.3. Complex Pharmacology of GPCRs

As flexible and dynamic proteins with predominantly hydrophobic external surfaces, studies on the basis of their recognition by synthetic chemicals have, in recent years, uncovered a hitherto unappreciated diversity in the basis and sites of such interactions. Although classic pharmacological analysis focused heavily on chemical ligands in which the mode of interaction could be described mathematically by substitution for (orthosteric agonism) or prevention of access to (orthosteric antagonism) the same binding pocket as the endogenously generated regulators of a GPCR, it is now clear that the diversity of chemical space can allow a much broader set of opportunities for interaction. Interactions that occur at a site or sites distinct from that filled by the endogenous ligand are generically described as "allosteric" and such chemicals as "allosteric ligands". The location of such allosteric sites can be varied and, indeed, spatially rather distant from the orthosteric pocket. For example, pepducins are synthetic peptides corresponding or closely related to sequences from one of the intracellular loops of a GPCR[58] and interact at the

intracellular side of the receptor.[59] By contrast, many other allosteric chemical ligands bind in close proximity to the orthosteric binding site but in a nonoverlapping manner.[60] Given situations in which binding pockets for orthosteric and allosteric ligands are in close proximity, considerable efforts have been expended to identify ligands where binding occurs across these two locations. Such ligands are frequently termed "bitopic"[61,62] or "dualsteric".[63,64] This has been a particularly fruitful approach in identifying ligands that are selective between GPCR subtypes, such as the five subtypes of muscarinic receptors, which share acetylcholine as a common endogenous orthosteric ligand. Allosteric effects of ligands can also reflect interactions between either multiple copies of the same GPCR[65] or interactions of a GPCR with either a second, molecularly distinct GPCR[66] or with other non-GPCR polypeptides.[67]

1.3.1. Orthosteric Ligands.
As noted above, the binding pocket for the endogenously produced regulators of GPCRs is defined as the "orthosteric" site. Although synthetic chemicals that interact in a noncovalent manner with the receptor and act to either activate the receptor or block the effects of an endogenous agonist do not inherently have to bind within the same region, they frequently do so. The actions of orthosteric ligands on a GPCR are defined by two key fundamental properties, their affinity for the receptor and their intrinsic efficacy, or ability to activate the receptor. In a practical sense, agonist ligands are those that both have affinity to bind the receptor and have efficacy to activate the receptor, while antagonist ligands have affinity but lack intrinsic efficacy. As it is often not possible to measure these properties directly, the pharmacology of ligands is instead often characterized through functional assays, where a concentration−response curve is generated to establish a potency (EC_{50}), and maximal response for a ligand. While potency is related to affinity and maximal response related to intrinsic efficacy, due to the complexities of functional systems both potency and maximal response vary depending on the specific assay system being used. It is therefore not possible to directly compare the potency of two ligands at the same receptor unless they have been tested under exactly the same conditions. Due to this limitation, within the current review, we will refrain from comparing potency values of compounds, except in cases where they have been tested as part of the same study under identical assay conditions.

When studying the pharmacology of orthosteric GPCR ligands in functional systems there are clear predictions for the observed response when either an agonist or an antagonist is coadded. In the case of an orthosteric agonist, this will depend on the efficacy or maximal response of the synthetic agonist. When fixed concentrations of a "full" synthetic agonist with efficacy equal to the endogenous ligand are coapplied with the endogenous ligand, no shift in the maximal response or potency will be observed (Figure 4A). In contrast, if a "partial" agonist with reduced efficacy is used, an increase in the concentration of endogenous ligand will be needed to displace the competing partial agonist, resulting in a dextral or "right" shift in the endogenous agonist concentration response (Figure 4B). Likewise, if a "super"agonist with enhanced efficacy is used, again the concentration−response to the endogenous agonist will be right shifted, but in this case it will manifest as a decrease in maximal response as the higher efficacy superagonist is displaced by the lower efficacy endogenous agonist (Figure 4C). A fixed concentration of a competitive orthosteric antagonist is anticipated to result in a requirement for higher
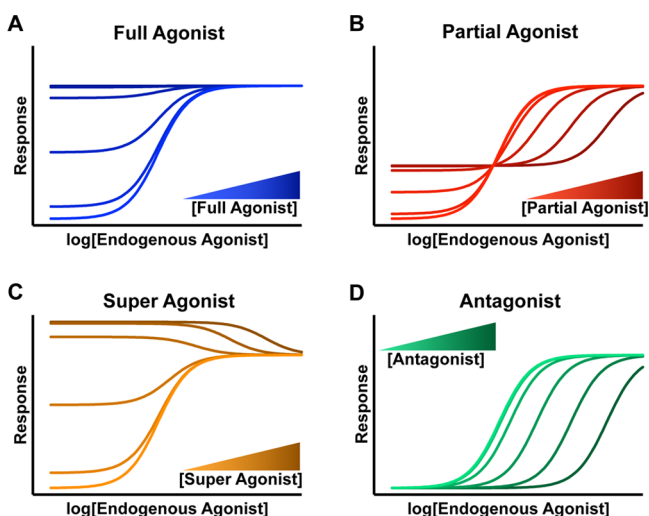
Figure 4. Pharmacological characterization of orthosteric ligands. Simulated concentration response data is shown for an endogenous agonist coappled with increasing concentrations (represented by darker shades) of a full agonist (A), partial agonist (B), superagonist (C), and antagonist (D). While the full agonist results in no shift in observed potency for endogenous agonist, each of the others results in a dextral shift (i.e., to higher concentrations) in its concentration–response curve.

Figure 5. Pharmacological characterization of allosteric ligands. Simulated concentration response data are shown for an endogenous agonist coappled with increasing concentrations (represented by darker shades) of a PAM (A), agonist-PAM (B), NAM of affinity (C), and NAM of efficacy (D). The PAM and agonist-PAM result in left shifts (i.e., requiring lower concentrations) in the response to the endogenous agonist, while only the agonist-PAM produces a response in the absence of an endogenous agonist. The NAM of affinity produces a dextral shift in potency (i.e., requiring higher concentrations), while the NAM of efficacy reduces the maximal response generated by the endogenous agonist. In each case the effect of the allosteric modulator reaches a maximal level, representative of full occupancy of the allosteric binding site.

concentrations of the endogenous agonist to achieve the same level of effect, again resulting in a dextral shift in the agonist concentration–response (Figure 4D). As sufficiently high concentrations of agonist should fully displace and overcome the antagonist effect, this form of competitive antagonism is often described as being "surmountable". Although historically competitive antagonists were viewed as neutral blockers that simply prevented agonist access to the binding pocket, it is apparent that many of these compounds are also able to inhibit ligand-independent activity of a receptor, and such ligands are now descried as "inverse agonists" (see below in section 1.3.4). There are well-established mathematical models that can be used to describe each of these ligand behaviors,[68] and indeed, they remain a mainstay in identifying and describing novel orthosteric ligands for GPCRs.

1.3.2. Allosteric Ligands. Many synthetic compounds have affinity to bind a site(s) distinct from the "othosteric" binding pocket of a GPCR. Such compounds are defined as being "allosteric". These may possess intrinsic efficacy, acting as agonists when added alone (allosteric agonists); they may apparently have no detectable effects when added alone but modulate the potency and/or efficacy of the endogenous ligand when they are both present (allosteric modulator) or, indeed, they may show both allosteric agonist and modulator behaviors (Figure 5). Allosteric modulation of orthosteric ligand function is generally classified based on two properties, modulation of orthosteric ligand affinity and modulation of orthosteric ligand efficacy.[69] A modulator may enhance either of these parameters, acting as a so-called positive allosteric modulator (PAM), or may decrease them, acting as a negative allosteric modulator (NAM). Each of the properties of an allosteric ligand, affinity for the allosteric site, intrinsic efficacy, modulation of orthosteric ligand efficacy, and modulation of orthosteric ligand affinity, are independent of each other, and thus, the pharmacology of these compounds can often be very complex. As such, a number of operational mathematical models have

been developed to quantitatively describe allosterism for GPCR ligands. This is a topic that has been reviewed and analyzed in considerable detail and at length in recent years, and the reader is directed to excellent reviews that cover the basis and means of analysis of such effects.[70,71]

Developing allosteric ligands as either therapeutic or tool compounds may provide a number of advantages over orthosteric ligands.[4,72−74] Most notably, there are many examples of receptor families with multiple receptor subtypes, all binding the same orthosteric ligand. In these cases, evolutionary pressure to maintain binding of the same endogenous ligand often necessitates structurally similar orthosteric binding pockets, and as a result, it can be very difficult to develop synthetic ligands targeting the orthosteric binding sites that are markedly selective for one subtype over another. As allosteric sites are not believed to be under the same evolutionary pressures, targeting these sites has proven to be a very useful approach to developing selective compounds for individual family member subtypes.[75]

A key advantage of allosteric modulators, particularly related to their potential as therapeutics, is that by nature their effects will be saturable and also require the presence of an endogenous ligand to be manifest. In cases where antagonism is desired, unlike a competitive orthosteric antagonist, which will continue to have a greater ability to inhibit a response with infinitely increasing concentrations, a NAM will only continue to enhance the level of inhibition until the allosteric site is fully occupied. In theory, this provides a clear potential to develop therapeutics with improved safety profiles. In cases where agonism is the desired outcome, developing a modulator that will enhance the effects of an endogenous ligand will have the obvious advantage of maintaining the spatial and temporal

activation of the endogenous ligand. This may be particularly relevant for the development of modulators targeting neurotransmitter or hormone receptors where the specific time and location of activity will be critical.

In addition to the concept that ligands binding to allosteric sites on the same GPCR may modulate orthosteric ligand function, it is also now appreciated that ligands binding to the orthosteric sites of two distinct GPCRs may also modulate each other in an allosteric manner through receptor heteromers.[66] This has been shown to modulate all aspects of receptor function, including orthosteric ligand affinity, efficacy, and downstream signaling.[54,55] More recent studies have even now demonstrated that two ligands binding to the same orthosteric site on a GPCR may allosterically modulate each other through GPCR homomers.[76,77]

Adding a further level of complexity, with the increase in GPCR structural information now available, it is apparent that in at least some cases the orthosteric and allosteric binding pockets are in close proximity.[60] This has led to the development of bitopic ligands, engineered to interact simultaneously with both orthosteric and allosteric binding sites of the receptor. Such approaches have typically led to the identification of both highly selective compounds and ones often with unique pharmacology in their ability to favor or bias receptor activation toward certain signaling pathways over others.

1.3.3. Bias Ligands. Conformational flexibility of GPCRs indicates that these proteins exist in either multiple distinct conformations or, potentially, as a continuum of states.[78−80] As binding of a ligand to a GPCR generally stabilizes the protein, this implies that different ligands could selectively stabilize a particular conformational state or states and, therefore, enrich a subset of these at the expense of others. It has become increasingly clear that the previous view that individual GPCRs interacted exclusively with one specific G protein partner is an oversimplification. Indeed, recent studies employing sensors that report interactions between a GPCR and different G protein α subunits provide evidence, at least in transfected cell lines, for a very complex pattern of interactions.[81] This also implies that different ligands may stabilize receptor conformations that interact selectively with different G proteins and, by so doing, initiate different signaling cascades (Figure 6A). Beyond this feature of G protein bias, as noted in section 1.2, GPCRs can also promote signals that occur independently of G protein activation. Although such signals may derive from interactions with a variety of cellular proteins, focus has been predominantly on interactions with arrestins.[82] Ligands able to selectively promote interactions of GPCRs with either an arrestin or a G protein are therefore biased between these pathways (Figure 6B) and have been identified for many GPCRs.[83] Moreover, in at least a number of cases it has been suggested that in a therapeutic context some of the panoply of signals that can be generated by ligand activation of a GPCR may be harmful rather than beneficial.[84] Such hypotheses have resulted in the development and ongoing clinical assessment of biased ligands at both the angiotensin AT1 receptor and the μ-opioid receptor.[85]

1.3.4. Ligand-Independent Constitutive Activity. In the least complex situation (the so-called "two-state" model) GPCRs can be viewed as ligand sensors that transit from inactive to active states upon binding of an agonist chemical. It follows, therefore, that GPCRs should be able, at least to some degree, to interconvert between these states spontaneously, and
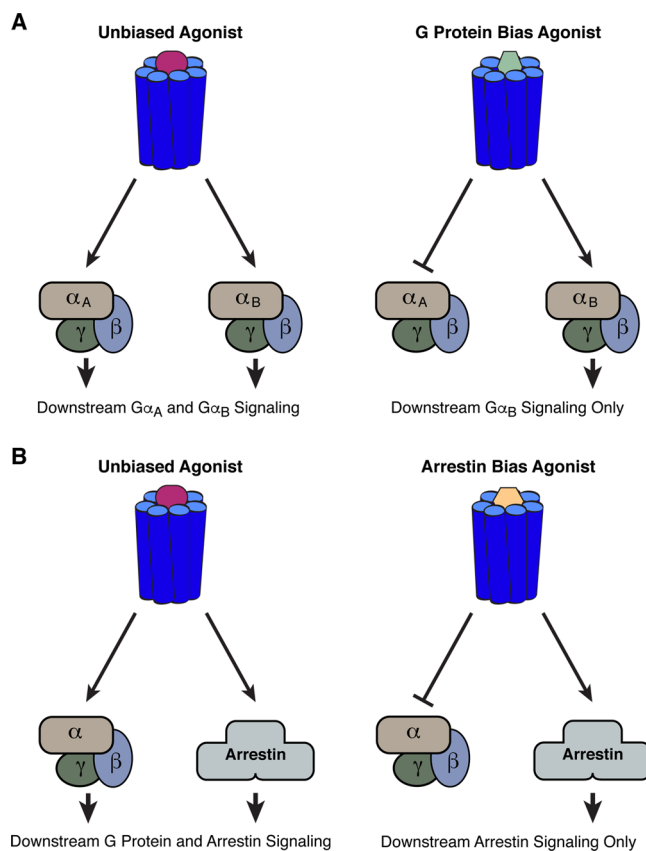
Figure 6. GPCR biased agonism. GPCR bias agonism may manifest either as bias signaling through one specific Gα subunit over another (A) or as bias signaling toward G protein vs arrestin pathways (B).

the degree of agonist-independent signaling observed will reflect the equilibrium between these two states (Figure 7A). Activity that can be measured in the absence of ligand interaction with a receptor is thus described as ligand-independent or constitutive activity. This varies markedly between different GPCRs[86,87] and can be modified markedly by mutation or within polymorphic variants of the same receptor.[88] Although antagonists were defined initially simply as ligands that could block agonist activation of a GPCR, nowadays, in molecular terms an (neutral) antagonist is defined as a chemical that binds with equal affinity to distinct conformational states of a receptor and does not favor the GPCR adopting one state over another. However, it was recognized that a number of "antagonist" ligands were able to reduce the level of agonist-independent signal transduction and therefore were interpreted to selectively stabilize the inactive ground state of the receptor.[89,90] Ligands with this capability are, therefore, described as inverse agonists (Figure 7B and 7C).

1.4. "Orphan" GPCRs and Receptor Deorphanization

Despite early successes in pairing cloned and expressed GPCRs with endogenously generated ligands that produced receptor activation and downstream signaling, the use of "homology cloning", in which cDNA generated from tissue extracts was PCR amplified using primers based on sequence from the most highly conserved TMDs of previously cloned 7-TMD proteins, resulted in the identification of a large number of potential GPCRs for which the activating ligands were unknown. Such sequences are defined as orphan receptors. A workflow of
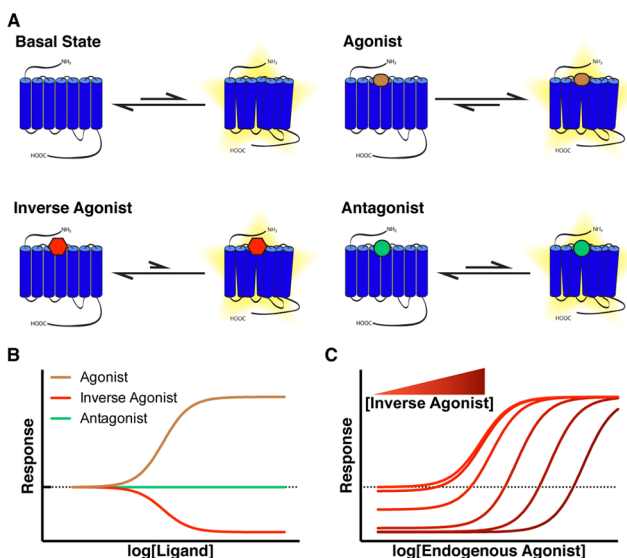
Figure 7. Constitutive activity and inverse agonism of GPCRs. In the simplest model of activation, a GPCR can be viewed to be in equilibrium between an inactive and an active conformation (A). In the absence of ligand if this equilibrium favors a proportion of the receptor adopting the active conformation, this is termed ligand-independent "constitutive" activity. Addition of an agonist ligand shifts this equilibrium to favor the active conformation, while an inverse agonist shifts the equilibrium further toward the inactive conformation. A neutral antagonist binds equally to both conformations, and thus although able to competitively inhibit agonist signaling, it will produce no effect on its own. (B) Simulated concentration–responses for each ligand type at a constitutively active GPCR. Assuming the inverse agonist is competitive, increasing concentrations of inverse agonist will both decrease the basal and right shift (i.e., requiring higher concentrations) the potency of the endogenous agonist (C).

strategies to identify endogenously generated chemicals that activate such orphan GPCRs is often described as "reverse pharmacology".[91,92] This might be initiated using sequence alignment of the orphan polypeptide with other previously characterized GPCR family members and informatic analysis to predict potential chemical ligands for the orphan. For example, sequence alignment of an orphan receptor sequence designated AXOR35 showed it to be closely related to the previously characterized histamine H_3 receptor. Histamine also potently activated AXOR35 leading to deorphanization as the histamine H_4 receptor.[93] Similarly, knowledge of the physiological actions of chemical ligands and of the tissues in which they generate responses has also been effective in deorphanization programs. For example, nicotinic acid was known for many years to interact with and activate G_i-family G proteins in white adipocytes,[94] although the receptor mediating this process was not. Therefore, testing the responsiveness to nicotinic acid of a number of previously uncharacterized orphan GPCRs that were known to be expressed by adipocytes resulted in appreciation that both HM74 and the closely related HM74A (also known as GPR109A) could be activated by varying concentrations of nicotinic acid, with HM74A requiring substantially lower concentrations to generate responses.[91] These receptors have subsequently been named systematically as hydroxycarboxylic acid (HCA) receptors,[95] based on the accepted true endogenously produced regulators of these receptors (whereas nicotinic acid, also known as niacin or vitamin B_3, is obtained from the diet as an essential nutrient). A third receptor, previously named GPR81, and now designated HCA_3, was

shown initially to have very weak potency for nicotinic acid[91] but has subsequently been deorphanized as a receptor for lactic acid.[95,96]

The recognition that a number of GPCRs were activated by very simple chemical structures derived from intermediary metabolism considerably altered the mindset of researchers working on aspects of metabolism and metabolic diseases. It was now clear that molecules present in high levels could function as homeostatic regulators via GPCR activation and that foodstuffs would be likely to contain molecules that either directly activated one or more GPCRs or did so following conversion via intermediary metabolism. Moreover, although substantial focus had been on chemicals with high affinity for GPCRs as being the likely endogenous regulators, this was potentially only the case for ligands, e.g., peptide hormones, in which substantial energy commitment was required from the host to generate and control levels of the receptor regulator. By contrast, as long as metabolic intermediates were present at levels commensurate with fractional occupancy of a GPCR and were regulated in a physiologically relevant manner then there was every reason to assume they might act to integrate metabolic processes via one or more orphan GPCR.

This resulted in deorphanization programs, particularly within the pharmaceutical industry, constructing chemical libraries of naturally occurring and endogenously produced ligands for screening as well as the more normal synthetic chemical libraries. Moreover, a number of deorphanization efforts turned to the use of tissue extracts,[97,98] with the prediction that unappreciated ligands for GPCRs must exist within such extracts because of the high number of orphan receptors. Metabolomic analysis is now a central component of such efforts. An obvious extension of these efforts was that detailed expression profiling was performed to attempt to predict orphan GPCRs that might be relevant disease targets and, therefore, worthy of significant effort to study and find ligands for. An obvious example of this was GPR40, the first deorphanized receptor for free fatty acids. This is now defined systematically as the FFA1 free fatty acid receptor.[99]

1.5. GPCRs Activated by Free Fatty Acids

It is now recognized that several GPCRs are activated by various free fatty acids. This includes four receptors that have been officially classified as members of a free fatty acid receptor family: FFA1–FFA4.[99,100] Two of these receptors, FFA1 (previously designated as GPR40) and FFA4 (previously GPR120), are activated by MCFAs and LCFAs, while the remaining two, FFA2 (previously GPR43) and FFA3 (previously GPR41), are activated by the SCFAs. FFA1–FFA3 are all structurally related, sharing between 30% and 40% sequence identity with each other and are encoded in tandem on chromosome 19 in man.[101] In contrast, FFA4 shows very little sequence identity with the other family members. In addition to the current FFA family members, there are several additional GPCRs reported to be activated by free fatty acids. GPR84 is an orphan receptor that recognizes MCFAs, while the mouse olfactory receptor Olfr78 (OR51E2 in human) appears to be activated by the SCFAs. Finally, although the HCA_2 receptor is most commonly associated with β-hydroxybutyrate as its endogenous agonist, the SCFA butyrate has also been found to activate this receptor, albeit only at high concentrations. The following sections of this review will examine the complex pharmacology of both endogenous fatty acids and synthetic ligands at each of these receptors focusing,

in particular, on how these receptors regulate aspects of metabolism and inflammation.

2. FFA1

FFA1 was the first GPCR to be deorphanized as being activated by free fatty acids. Initially reported simply as an uncharacterized 7-TMD sequence, located in man at chromosome 19q13.1,[101] three separate studies in 2003 all demonstrated that the receptor was activated by MCFAs and LCFAs.[102−104] Clearly a member of the predominant rhodopsin-like or class A group of GPCRs, various fatty acids with greater than 6 carbon chain length caused elevations in intracellular $[Ca^{2+}]$ through FFA1 and included a broad range of both saturated and unsaturated fatty acids.[102]

Signals from activation of FFA1 (Figure 8) are transduced predominantly via $G_{q/11}$-family G proteins, and the pharmaco-
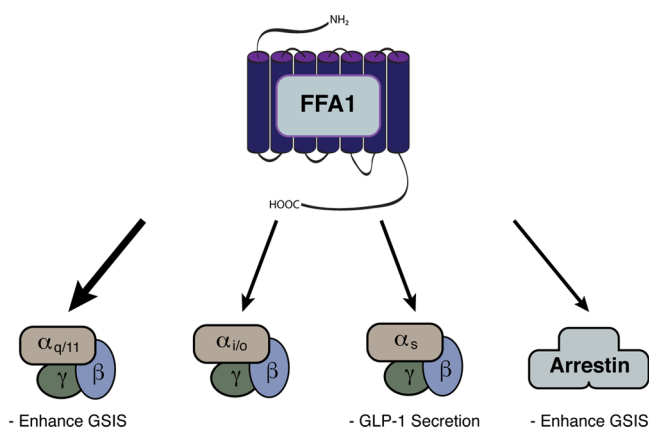

Figure 8. Signaling pathways and their associated biological outcomes for the FFA1 receptor.

logical $G_{q/11}$ inhibitor YM-254890[105] has been used in several studies to confirm this observation.[106−109] Recently, a second, closely related depsipeptide $G_{q/11}$ inhibitor, FR900359, has also been used to confirm such observations.[110] Although less common, some level of G_i-mediated FFA1 coupling has also been described for FFA1.[103,104] This may be dependent on the specific cell type involved, with G_i-coupling particularly reported in several breast cancer cell lines[111,112] and in keratinocytes.[113] A limited number of studies have also suggested G_s signaling through FFA1.[114,115] Finally, although G protein-independent signaling has not been described extensively for FFA1, arrestin-2 and arrestin-3 (also known as β-arrestins 1 and 2) recruitment to this receptor has been demonstrated,[116−118] and there are some initial indications that this might play a role in G protein-independent FFA1 signaling for certain ligands.[116]

2.1. Expression of FFA1

Early studies on FFA1 quickly identified pancreatic islets and, in particular, the β-cells as having particularly high levels of the receptor,[102−104,119] while subsequent studies have also found FFA1 expression in the α cells of the islets.[120−122] FFA1 is expressed by various enteroendocrine cell types, including the L cells that secrete glucagon like peptide-1 (GLP-1) and peptide hormone YY (PYY),[123] the I cells that secrete cholecystokinin (CCK),[124,125] and the K cells that secrete gastric inhibitory peptide (GIP).[126] Additional tissues with reported FFA1 expression include skeletal muscle, heart, liver, bone, brain,

and monocytes,[102,104,127] although the functional roles of FFA1 in most of these tissues have received less attention. A number of studies on potential roles of FFA1 in the brain on pain perception, neurodevelopment, and neurogenesis have been carried out, and although they are outside of the scope of the current article interested readers should consult[128] for more details.

2.2. LCFAs at FFA1

In the initial studies that deorphanized FFA1 a wide range of individual MCFAs and LCFAs were shown to activate the receptor.[102−104] Although each study found many different individual fatty acids produced effects at FFA1, all displayed modest potency, with EC_{50} values all greater than 1 μM. There was also relatively little discernible structure−activity relationship (SAR) apparent when comparing the activity of the various fatty acids, and indeed, each study found a different fatty acid or related molecule to be the most potent: 5,8,11-eicosatriynoic acid,[102] DHA (22:6n−3),[103] or stearidonic acid (18:4n−3).[104] A more recent study addressed the SAR of various MCFAs and LCFAs at FFA1 in a systematic manner,[129] and a survey of this and other studies in the area noted at least some trends in the SAR of fatty acid ligands at FFA1.[13] Notably, this included that the long chain PUFAs typically show the highest potency at FFA1. In addition, the activity of SFAs at FFA1 is strictly dependent on chain length with examples shorter than 10 carbon showing little activity, while activity also drops off for chain lengths longer than 14 carbon. Also of particular note, several studies have indicated that the trans-unsaturated fatty acids tend to be poor agonists at FFA1,[102,103,129] which may be of particular interest given that the trans-fatty acids are often reported to be detrimental to health.[9,24]

2.2.1. LCFAs, FFA1, and Pancreatic Islets. As the initial studies on FFA1 identified high-level expression of the receptor in pancreatic islets and particularly the insulin secreting β-cells,[102,103] the majority of studies examining biological effects of LCFAs mediated by FFA1 have focused on this tissue. LCFAs have diverse effects on pancreatic islets. Most notably, while acute LCFA treatment enhances glucose-dependent insulin secretion (GSIS), chronic exposure to LCFAs inhibits insulin secretion through lipotoxicity and associated loss of β-cell mass. A role for FFA1 in LCFA-mediated enhancement of GSIS was quickly established,[102,103] and this has been confirmed in many studies utilizing combinations of in vitro and in vivo models of β-cell function with pharmacological inhibition of FFA1 or receptor knockdown or knockout approaches.[103,107,109,130−133] Mechanistic studies have indicated that the FFA1-mediated effect on GSIS is through a $G_{q/11}$-phospholipase C pathway,[106,109] leading to phosphorylation of protein kinase D and modulation of intracellular granule transport.[134] It is important to keep in mind, however, that although a role for FFA1 in LCFA-mediated enhancement of GSIS is now very well accepted, FFA1 appears to contribute only some 50% of the total effect of LCFAs on GSIS.[109]

By contrast, a potential involvement of FFA1 in the chronic lipotoxic effects of LCFAs on β-cells has been much more contentious.[2,135,136] Despite early work suggesting FFA1 did contribute to LCFA-mediated lipotoxicity,[137] the vast majority of subsequent studies has found these effects are independent of FFA1.[109,121,131] Indeed, some studies have even indicated that FFA1 activation may in fact be protective against lipotoxicity and β-cell death.[138,139] Although the current consensus appears to be that FFA1 is not responsible for the

detrimental chronic effects of LCFA exposure, recent studies have continued to suggest FFA1 may play some role in lipotoxic effects of LCFAs.[140−142] One possible explanation could be, given the diversity of LCFAs known to activate FFA1, that efficacy differences reported among LCFAs,[129] or perhaps even signaling bias in their effects at FFA1, could account for these discrepancies. The concept of signaling bias in GPCRs with multiple endogenous ligands is not unusual and, indeed, has been widely reported within the chemokine receptor family.[143,144] Although currently no studies have directly assessed LCFA-mediated signaling bias at FFA1, one recent review has suggested that abnormal activation of FFA1 by conjugated linoleic acids may account for the particularly strong lipotoxic effects of this series of LCFA.[145] Clearly further work is needed in this area to fully elucidate involvement of FFA1 in the effects of different LCFAs on β-cells and insulin secretion.

2.2.2. LCFAs, FFA1, and Enteroendocrine Cells.

Away from the pancreas, the area that has received the most attention for FFA1-mediated metabolic effects of LCFA has been the enteroendocrine cells of the gut. FFA1 is expressed in several enteroendocrine cell types including L, K, and I cells and has been directly implicated in LCFA-mediated secretion of GLP-1, GIP, and CCK.[123,124] The role of FFA1 in the gut has contributed to the concept that FFA1 functions as a nutrient-sensing receptor detecting dietary fats as they are converted to fatty acids and that, therefore, it may be possible to target the receptor to improve metabolic health through dietary manipulations.[2,13] Interestingly, however, a recent study found that while LCFAs do stimulate GLP-1 release from the rat small intestine, this was only observed when the LCFA was administered via the vasculature and not when administered via the intestinal lumen.[146] Although a limited observation from a single study, this surprising result suggests a need to absorb the LCFA in order to produce the effect on GLP-1 release. If confirmed, this is likely to have significant implications as to how FFA1 functions as a nutrient sensor and, indeed, if and how it will be possible to manipulate FFA1 function through diet.

2.3. FFA1 Ligand-Independent Constitutive Activity

In one of the early studies that deorphanized FFA1 a measurable level of ligand-independent activation of the receptor was described in the reporter assay used,[102] and a subsequent study confirmed this finding in a [^{35}S]GTPγS-based assay utilizing a FFA1-G$_q$ fusion protein.[108] However, more detailed examination in the latter study found that the level of apparent constitutive activity was reduced by the addition of bovine serum albumin (BSA) to the assay.[108] LCFAs are known to bind to albumin, and it is therefore not surprising that coaddition of BSA with LCFA prevents LCFA-mediated signaling at FFA1.[103] Taken together, in part because the GTPγS studies were performed on membranes derived from cells expressing FFA1, it has been suggested that the observed high level of constitutive activity is at least consistent with the concept that an unidentified fatty acid(s) was present in the preparation and this acted to partially activate the receptor. The added albumin was then able to outcompete the receptor to bind this ligand(s).[147] A later molecular modeling study suggested that constitutive activity (or lack thereof) of FFA1 was regulated by an ionic lock formed between two glutamate residues present in EL2 and two arginine residues within the ligand binding region.[148] Finally, a more recent study has revisited ligand-independent activity of FFA1 in the context of

receptor internalization.[117] In this work, FFA1 was shown to undergo both ligand-dependent and -independent internalization but through distinct pathways.[117] Importantly, in this study, the addition of BSA to the assay did not affect the level of constitutive FFA1 internalization, indicating that, at least in this system, the effect was not due to LCFAs present in the assay.

2.4. Synthetic Ligands for FFA1

FFA1 is the free fatty acid-responsive GPCR that has attracted the greatest level of interest to date in terms of therapeutic and translational applications.[149−151] It is not surprising, therefore, that it also has the largest described and characterized group of chemical ligands that act as either agonists or antagonists. However, despite the early controversy around the possible role of FFA1 in mediating lipotoxic effects in β-cells raising questions about whether agonism or antagonism would be preferred in a therapeutic context,[152] the general consensus now strongly favors agonism as the desired mode of action in targeting this receptor. As a result, although there are many different chemical series with detailed pharmacological characterization as FFA1 agonists, only a very limited number of compounds have been described as FFA1 antagonists.

2.4.1. FFA1 Agonists. The first reported synthetic agonists of FFA1 were based on an *N*-substituted 3-(4-aminophenyl)-propanoic acid template.[153] These compounds had many structural similarities to the endogenous fatty acids, and **1**, subsequently designated GW9508 (Figure 9), was first reported
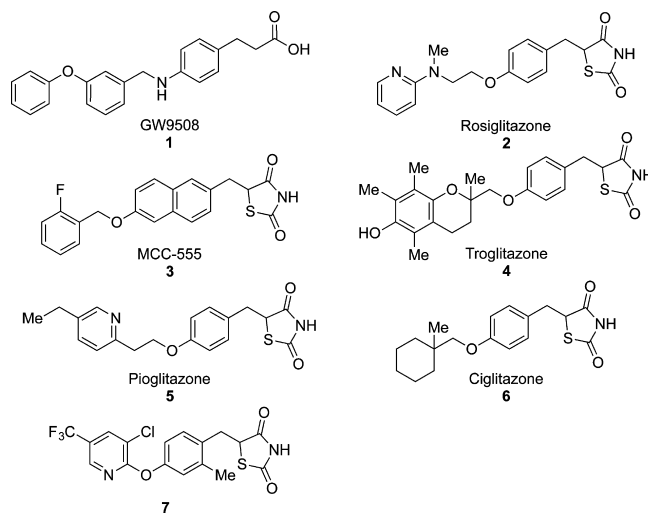
Figure 9. FFA1 agonists (1).

within this series. Interestingly, in relation to much later developments on whether there might be multiple ligand binding pockets in this receptor, these studies noted that a carboxylate was not required for agonist function, although carboxamides were less effective.[153] GW9508 was characterized subsequently in greater detail as a FFA1 agonist by Briscoe et al. and displays good potency to activate the receptor.[130] However, GW9508 was also shown to activate FFA4, although displaying some 70-fold lower potency to do so (see section 4.5.1). Importantly, the studies of Briscoe et al. also introduced GW1100 as the first FFA1 antagonist.[130] Over time, GW9508 and GW1100 have been by far the most widely used FFA1 ligands for both in vitro and preclinical studies. GW9508

promoted GSIS from rodent MIN6 insulinoma cells, and this was also blocked by GW1100.[130]

In one of the initial FFA1 deorphanization studies the peroxisome proliferator−activated receptor-γ (PPARγ) agonist and antidiabetic thiazolidinedione drug, rosiglitazone (2), and the related molecule MCC-555 (3; netoglitazone) were noted to activate FFA1,[104] and this was later extended to the related thiazolidinedione molecules: troglitazone (4)[108] and both pioglitazone (5) and ciglitazone (6) (Figure 9).[154] Indeed, several studies have now implicated FFA1 in various biological effects of the thiazolidinediones, including proliferation of bronchial epithelial cells,[155] apoptosis in osteocytes,[156] and differentiation of osteoblasts.[157] Adding a level of complexity to the situation, the thiazolidinediones also regulate the expression level of FFA1 through their actions at PPARγ.[158] Indeed, although studies have found FFA1 to play a role in thiazolidinedione effects on β-cell survival and insulin secretion, these effects appear to result from PPARγ-mediated alterations in FFA1 expression rather than direct effects on FFA1 itself.[158,159] Moreover, a recent study demonstrated that effects of rosiglitazone to upregulate gene targets through PPARγ involved an initial activation of FFA1, suggesting a complex, dual-receptor signaling pathway between PPARγ and FFA1.[160] While these findings suggest a complex pharmacology of the thiazolidinediones, it must be noted that the potency of these ligands at FFA1 is rather modest and potentially at the limits of clinically relevant concentrations.[154] Therefore, it seems unlikely that significant elements of the clinical efficacy of these drugs in diabetes reflect direct activation of FFA1.

In efforts to identify novel FFA1 synthetic agonists, derivatives of the thiazolidinedione core have been identified that bind to and activate FFA1 but lack activity at PPARγ.[161] One key example of these, 7 (Figure 9), was able to enhance GSIS from islets isolated from wild-type mice but not from those lacking FFA1.[161] The compound also reduced blood glucose excursion in in vivo glucose tolerance tests (GTT) in wild-type but not FFA1^{−/−} mice. Further variations on this core have also been reported to display FFA1 agonism, although none were found to have significantly better potency than 7.[162]

As mentioned earlier, even though a handful of thiazolidinediones with activity on FFA1 were reported concomitantly with the deorphanization of the receptor,[104] the first potent FFA1 agonists to appear in the literature were the fatty acid mimicking series represented by GW9508.[102,153] Soon thereafter, Song et al. disclosed a 3-aryl-3-(4-alkoxyphenyl)propionic acid series identified in a high-throughput screening campaign conducted by Johnson & Johnson with 8 (Figure 10) as the most potent representative.[163] By the use of an NMR-based method for GPCR screening, Bartoschek and co-workers were able to identify novel submicromolar FFA1 agonists such as 9.[164] Using free fatty acids as the starting point and inspiration from the early thiazolidinediones in the design of small drug-like free fatty acid-mimicking compounds, Christiansen et al. identified a phenoxyacetic acid hit containing a central alkyne that was optimized to the potent and selective TUG-424 (10), which was confirmed to enhance insulin secretion at high but not low glucose levels in a FFA1-dependent manner.[165] This compound, however, displayed lower than ideal in vitro metabolic stability and short plasma half-life, suspected to reflect metabolism of the ortho-methyl group and/or beta-oxidation. To address these issues, focus was placed on lowering the lipophilicity of the compounds while preserving or increasing potency. Replacement of the terminal benzene ring
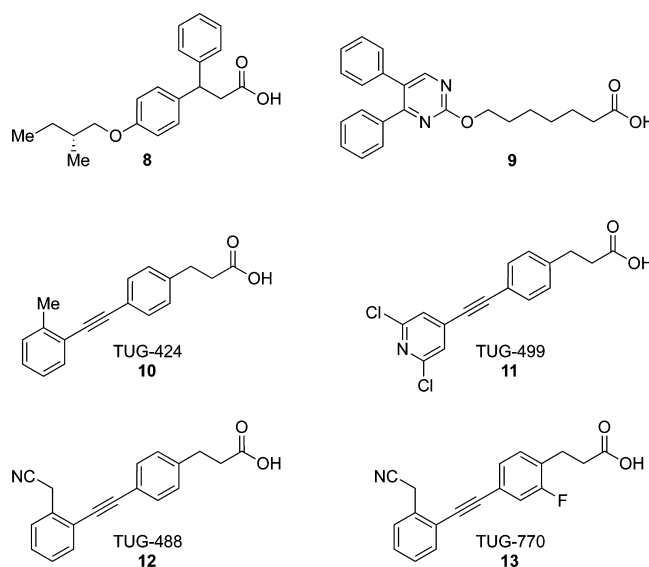
Figure 10. FFA1 agonists (2).

by nitrogen-containing heterocycles led to structures such as TUG-499 (11).[166] Replacement of the central ring by pyridine resulted in similar effects on potency and lipophilicity as for the terminal position.[166] Appending polar substituents resulted in the significantly less lipophilic and more polar compound TUG-488 (12).[167] Investigations of their ability to permeate mucus producing membranes indicated better penetration for 10 and 12 than for 11.[168] Indeed, both 10 and 12 appear to be fully bioavailable in mice.[167] Further optimization led to TUG-770 (13), a highly potent agonist with lipophilicity in the ideal range that exhibited robust lowering of plasma glucose in rodents, an effect that was fully preserved after 1 month of chronic treatment.[166]

Structure−activity relationship exploration around other hits from the process above combined with the simultaneously published GW9508 series resulted in the observations that methyleneoxy was a preferred central linker for small western substituents whereas methyleneamino was preferred for larger compounds and led to the identification of TUG-469 (14, Figure 11) as a potent FFA1 agonist with ability to enhance insulin secretion in a glucose concentration-dependent fashion.[169] The compound was subsequently shown to protect the rodent insulinoma-derived cell line INS-1E against palmitate-induced toxicity through activation of FFA1.[139] Since high lipophilicity was recognized as a potential problem for TUG-469, combination with the hydrophilic 3-mesylpropoxy appendage of the simultaneously disclosed TAK-875 (15) was explored and resulted in TUG-905 (16), an agonist with low lipophilicity and high potency on both human and murine FFA1 orthologs.[170]

Takeda pioneered understanding of the FFA1 field by discovering the potential of this receptor to enhance insulin secretion in a glucose-dependent fashion[103] and was also the first company to initiate clinical development and trials with a selective FFA1 agonist. The clinical candidate TAK-875 (15) was disclosed in 2010.[171] Originally inspired by free fatty acids, the bicyclic system was installed to resolve issues related to β-oxidation of the 3-phenylproponic acid moiety, leading to investigation of bicyclic systems that could block beta-oxidation and the identification of the dihydrobenzofurane-3-acetic acid moiety such as in 17.[171−173] With the high lipophilicity of the
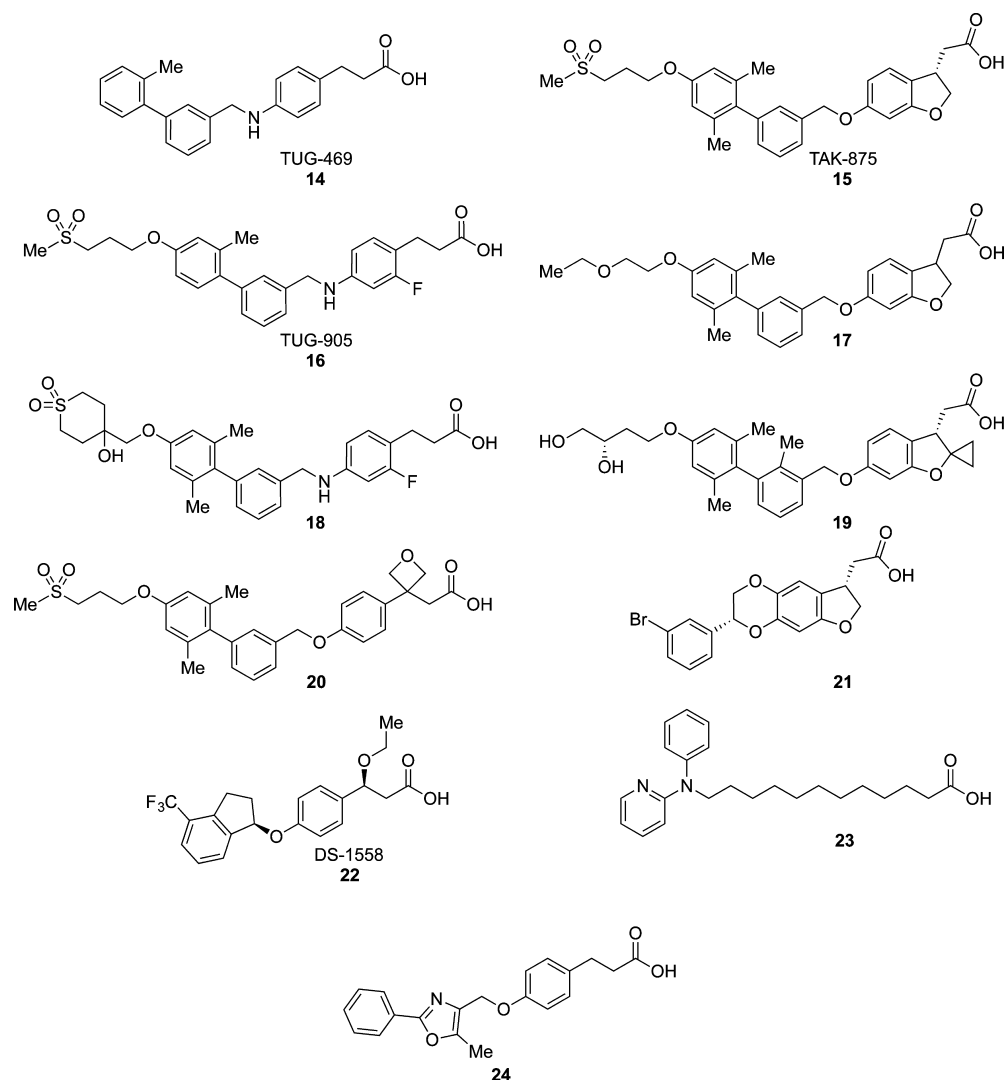
Figure 11. Synthetic FFA1 agonists (3).

compound series representing an obvious problem for a clinical candidate molecule, hydrophilic moieties were attached in the para position of the biphenyl system that allowed accommodation of diverse modifications. This lead to structures with improved properties such as **18** and TAK-875 (Figure 11).[172]

The structure of TAK-875 has influenced the development programs in several companies. For example, Astellas disclosed the relatively close TAK-875 analog **19** and demonstrated its ability to also reduce plasma glucose and increase insulin levels.[174] Moreover, this compound has been reported to act synergistically with the dipeptidyl peptidase-4 inhibitor and clinically approved drug sitagliptin to normalize glucose and increase insulin and GLP-1 levels, although the compound did not induce GLP-1 secretion when used alone.[174] Other companies, including Piramal and Hengrui, have also developed FFA1 agonists based on TAK-875, for example, **20** and **21**,[175,176] while additional structural scaffolds including those of **22**, **23**, and **24** have also recently been reported (Figure 11).[177−179]

TAK-875 has undoubtedly had the greatest impact in understanding the therapeutic potential for FFA1 in the treatment of type 2 diabetics.[171] Experiments carried out in vitro using TAK-875 (subsequently designated as Fasiglifam) found this molecule was able to acutely enhance GSIS through

FFA1,[180,181] importantly without the detrimental chronic effects that are observed with the LCFAs themselves.[140,180] Interestingly, TAK-875 does not appear able to reproduce the FFA1-mediated effects on gut hormone secretion reported for LCFAs.[115,182] Regardless, TAK-875 is orally bioavailable, and in vivo studies found TAK-875 to improve glucose tolerance in healthy rats[171] as well as to improve hyperglycemia in type 2 diabetic rats.[180] On this basis, TAK-875 progressed into clinical trials as an antihypoglycaemic/antidiabetic treatment. After passing initial safety trials,[183,184] the compound proceeded into phase II and, indeed, III trials. Unfortunately, these trials were terminated because of potential liver toxicity,[185,186] but both the phase II data[187,188] and the initial results of the phase III studies demonstrated clear beneficial effects of TAK-875 on glycaemia and HbA1c levels in diabetic patients while appearing to reduce the risk of hypoglycemia.[185] Given that FFA1 is not expressed highly in the liver, the current consensus is that the failure of TAK-875 is most likely a specific compound issue and not a target issue. Indeed, a recent study indicates that TAK-875 can inhibit hepatobiliary transporters and suggested that this off-target effect of TAK-875 in the liver may account for its withdrawal from clinical trials.[189] Considering this and that all in vivo studies in mice and clinical studies in man have routinely demonstrated a capacity for FFA1 agonists to regulate

Figure 12. Unique binding pose of TAK-875 revealed in a FFA1 crystal structure. The cocrystal structure of TAK-875 bound to FFA1 revealed that TAK-875 extends out of the central binding pocket between TMIII and TMIV and into the cell membrane (A).[190] Top (B) and side (C) views of the TAK-875 binding pocket. Key residues in FFA1 involved in coordinating the carboxylate of TAK-875 are shown and marked in red, while the surface of the binding pocket is marked in green.
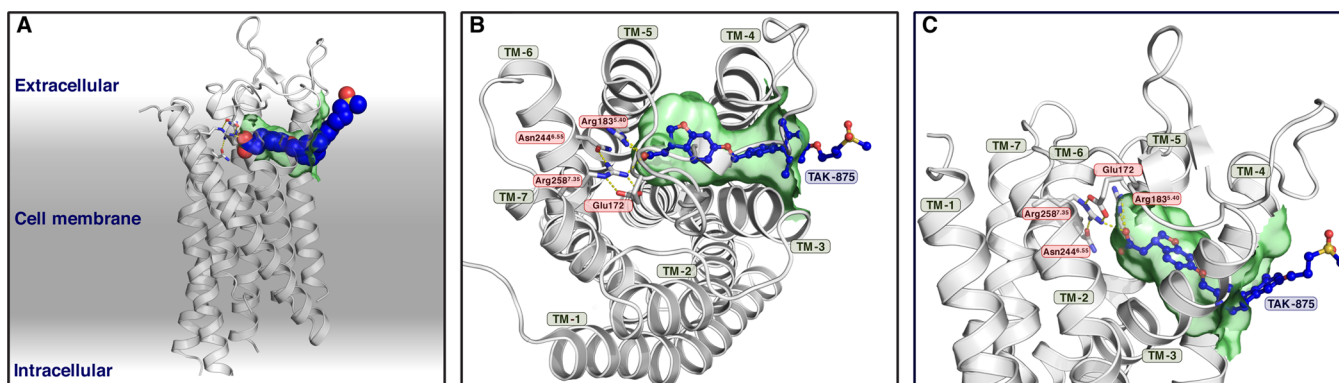
glucose homeostasis, there is every expectation that further trials of other FFA1 agonists will take place.

2.4.2. Mode(s) of Agonist Interaction with FFA1. Initial efforts to predict the mode of agonist ligand binding to FFA1 were based on a homology model constructed from the available X-ray structure of bovine rhodopsin.[190,191] These studies identified a group of key residues, Arg183[5.39], Arg258[7.35], and Asn244[6.55] (Ballesteros and Weinstein numbering in superscript[192]), near the top of the TMD helical bundle that were predicted to coordinate the carboxylate present in both linoleic acid (18:2n−6) and GW9508. Alanine mutations of these residues significantly reduced GW9508 and, to a significantly lesser degree, linoleic acid potency at FFA1, thus appearing to confirm their predicted role in ligand binding.[190] Although appreciated from early studies not to be a requirement for FFA1 agonism[153] the vast majority of synthetic FFA1 agonists contain a similar carboxylate[175,193] and thus have typically been predicted to form such interactions. A notable early exception to this was the thiazolidinediones ligands. However, as these ligands also lost potency at alanine mutations of Arg183[5.39], Arg258[7.35], and Asn244[6.55] it was confirmed that the thiazolidinedione moiety acted as a carboxylic acid bioisostere and maintained a similar mode of interaction to other FFA1 agonists.[154] The recent publication of an atomic level X-ray structure for a thermally stabilized form of FFA1 interacting with TAK-875 has further confirmed the importance of these three residues.[194] However, in the crystal structure it was only Arg183[5.39] and Arg258[7.35] that directly interacted with the carboxylate of TAK-875, whereas Asn244[6.55] formed a hydrogen-bond interaction with Arg258[7.35] that acted to position this residue within the binding pocket.[194]

While the FFA1 crystal structure demonstrated that the early homology models predicted interactions between the ligand carboxylate and receptor relatively well, predictions from these models of the mode of interaction for the rest of the molecule proved to be far from correct (Figure 12). Although the homology models had docked FFA1 agonists within the classic GPCR binding pocket within the TMD core of the receptor, remarkably in the crystal structure much of TAK-875 protruded outside the core GPCR helical structure and presumably into the lipid environment of the plasma membrane.[194] In particular, this occurred through a gap between TMIII and TMIV of the receptor. A similar gap was observed in the crystal structure of another lipid-binding GPCR, the S1P-1 receptor, although in

this case it was between TMVII and TMI and predicted to provide ligand access to a binding pocket that remained within the central TM core.[195] The implications of the unique mode of TAK-875 binding revealed in the FFA1 crystal structure on the basis for binding of other FFA1 ligands are not fully clear. However, at least one recent modeling study has examined the binding of several key ligands to a model based on the FFA1 crystal structure.[196] This work found that most of the FFA1 ligands assessed could adopt a binding pose similar to TAK-875, extending into the space between TMIII and TMIV, but that they could also potentially adopt an alternate binding pose where they extended into a gap between TMIV and TMV.[196]

A further surprising feature of agonist ligand interactions with FFA1 that has developed is the recent view that FFA1 appears to have multiple, allosteric binding pockets.[197] Given that most synthetic FFA1 agonists contain a carboxylate head and an extended hydrophobic tail, these typically have been viewed as akin to synthetic fatty acids (see earlier). It was, therefore, assumed routinely if not always directly tested that these ligands would all interact with FFA1 in a similar manner, with the carboxylate anchored to Arg183[5.39] and Arg258[7.35]. This, however, now requires reassessment. In large part this is based on a study using the FFA1 agonist AMG 837 (**25**; Figure 13).[197,198] This study first noted that AMG-837 displayed reduced efficacy at FFA1 compared with the PUFA DHA as well as two other FFA1 synthetic agonists, AM 8182 (**26**) and AM 1638 (**27**). Surprisingly, when competition binding experiments were conducted using [³H]AMG 837 it was found that AM 1638 enhanced rather than decreased [³H]AMG 837 binding, while DHA and AM 8182 decreased [³H]AMG 837 binding but in a manner that was not consistent with a competitive interaction.[197] Such observations are clearly incompatible with a model where each of these ligands binds to the same site on FFA1. Furthermore, binding of [³H]AM 1638 was increased substantially and in a concentration-dependent fashion by addition of both AMG 837 and DHA.[197] Various cooperative effects, consistent with positive allosteric modulation, between AM-1638, AMG-837, and DHA, were also observed in functional studies (Figure 13).[197] Finally, when specifically assessing AMG 837, AM 1638, AM 8182, and DHA at R183A[5.39] and R258A[7.35] FFA1 mutants it was apparent that although activity of AMG-837 was completely lost at these mutants some activity remained for DHA and AM 8182, while the activity of AM-1638 was largely unaffected.[197] Together,
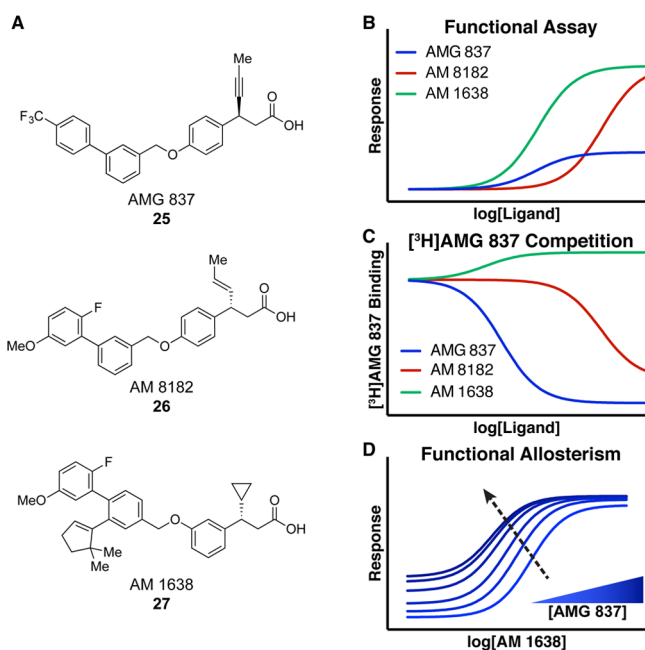
Figure 13. Evidence of allosterism among structurally related FFA1 agonists. (A) Chemical structures of the three key compounds used to demonstrate multiple allosteric binding sites on FFA1.[193] When tested in functional experiments, all three compounds were agonists, although while AM 8182 and AM 1638 were full agonists, AMG 837 was only a partial agonist (simulated data shown in B). Despite similar chemical structures, when tested in a competition binding assay using [³H]AMG 837, AM 8182 was unable to fully displace [³H]AMG 837 while AM 1638 enhanced [³H]AMG 837 binding (simulated data shown in C). Such observations are inconsistent with competitive binding for these compounds. Functional experiments showed that the AM 1638 concentration response is shifted to the left by AMG-837 (simulated data in D, effect on AM 1638 potency shown by dashed line), indicative of allosteric modulation.

these data suggest a very complex picture of ligand binding to FFA1 and that this receptor must have at least three relatively similar binding sites.

A key implication of the demonstration that similar ligands must bind to distinct sites on FFA1 is that it became unclear which binding site many of the previously described synthetic agonists, including TAK-875, actually interact with. Through a combination of functional and competition binding experiments, it is now apparent that TAK-875 and perhaps most other reported synthetic compounds interact with the same binding site as AMG 837.[115] This suggests TAK 875 is in fact an allosteric agonist of FFA1, and studies have now demonstrated positive allosteric modulation of LCFA responses at FFA1 by TAK-875.[199] Xiong et al. extended this concept further to suggest that positive allosteric modulation between synthetic and endogenous agonists might contribute to the therapeutic benefit of these compounds in improving postprandial glucose homeostasis.[200] Attempts to model the mode of binding of ligands that appear to bind to distinct sites have generated mixed results as, typically, most synthetic ligands appear able to dock into the same TAK-875 binding site identified in the crystal structure.[115] However, a recent molecular modeling study found that the more bulky AM 1638 did not fit into this site and, indeed, did not form interactions with the key Arg183[5.39] and Arg258[7.35] residues.[196] Instead, it was suggested that this molecule might interact at a

distinct allosteric site anchored by the positively charged Lys62[2.60] residue. However, this prediction has not yet been tested experimentally. Although the evidence now strongly points to multiple binding sites within FFA1, many issues remain to be answered on this topic. Most importantly, it remains unclear where the MCFAs and LCFAs, which are presumably the endogenous ligands of FFA1, bind to this receptor.

2.4.3. Partial Agonism and Ligand Bias at FFA1. Connecting, at least partially, to the observation that different ligands bind to different sites on FFA1 is the question of whether different ligands are able to produce different signaling responses via FFA1. First, relating to this is the understanding that various synthetic agonist ligands at FFA1 display markedly different levels of efficacy.[182,197,201] This may seem obvious, but reliance in many studies on assessing ligand potency and efficacy in transfected cell lines that probably express markedly higher levels of the receptor than native tissues can result in both overestimates of likely ligand potency and efficacy in native tissues (due to the presence of receptor reserve). It was thus important that the studies of Brown et al. highlighted that although AMG 837 appeared to have high efficacy, akin to the $n-3$ fatty acid DHA in cells expressing high levels of the receptor, in cells with substantially lower levels of the receptor AMG 837 displayed only modest, partial agonism, compared to DHA.[201] By comparison, AM 1638 displayed full agonism and was more effective in regulating blood glucose and insulin levels than AMG 837.[201] In an extension to these studies, Luo et al. showed that full agonists, such as AM 1638, are more efficacious in promoting secretion of the incretins GLP-1 and GIP from mouse intestinal enteroendocrine cells as well as promoting insulin secretion from both mouse and human islets.[182] Importantly, these observations were extended to in vivo studies where robust effects on GLP-1, GIP, and insulin levels were seen only with the full agonist AM 1638.[182] The fact that full vs partial agonism of FFA1 also correlates with the specific binding site on FFA1 the ligand interacts with[197] may suggest that activation of FFA1 through distinct binding sites could lead to different active conformations of the receptor and potentially result in signalling bias. Hauge et al. were the first to directly assess this when they demonstrated that full agonist ligands such as AM 1638, which bind to distinct sites from TAK-875 and AMG 837, are able to engage and transduce signals via both G_s as well as $G_{q/11}$-family G proteins.[115] By contrast, TAK-875, AMG 837, and the LCFAs engaged only G_q-mediated signaling pathways.[115] Whether this is truly a reflection of receptor bias through unique active signaling conformations or simply an issue of efficacy remains to be established. It is also noteworthy that alongside potential bias in G protein activation profiles, certain ligands may also display bias between G protein and arrestin-mediated signaling pathways. In the case of FFA1, a recent study reported that TAK-875 was more effective than several LCFAs in recruiting arrestin 2 and arrestin 3 to the receptor, whereas the LCFAs were more efficacious than TAK-875 in a $G_{q/11}$-dependent Ca^{2+} assay.[116] Moreover, this may have functional and therapeutic consequences because although the insulinotropic activity of the LCFAs was ablated by pharmacological inhibition of $G_{q/11}$, the insulinotropic effect of TAK-875 was reduced in islets from arrestin 3$^{-/-}$ mice.[116]

2.4.4. FFA1 Antagonists. The FFA1 antagonist GW1100 (**28**, Figure 14) has been a key tool compound used extensively in in vitro efforts to explore the biology of this
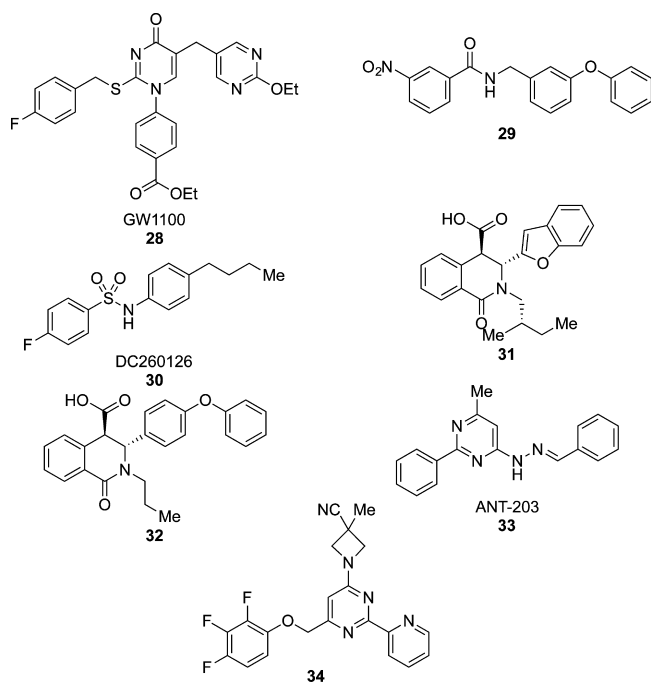
Figure 14. FFA1 antagonists.

receptor.[108,130,202−204] However, use in vivo has been much more limited, mostly restricted to studies assessing roles of FFA1 in the CNS where either intrathecal or intracerebroventricular delivery has been used.[205−207] In addition to GW1100, several other FFA1 antagonists have been described (Figure 14). The first of these, 29, was identified through virtual screening of FFA1 homology models and was a ligand where the carboxylic acid headgroup present in all FFA1 agonists known at that time was replaced with a nitro group.[208] Although informative, the IC$_{50}$ of this compound was >1 μM, and therefore, it has not found use in further studies. More recent FFA1 antagonist ligands have included sulfonamide derivatives such as DC260126 (30),[209] which has been reported to improve insulin sensitivity but not glucose handling in Zuker rats[210] and to protect pancreatic β-cells from apoptosis in diabetic mice.[211] A further 1,2,3,4-tetrahydroisoquinolin-1-one FFA1 antagonist series, originally generated by Pfizer and including, for example, 31,[212] has also received some attention in characterizing FFA1 function. In particular, a representative molecule from this series, 32, has been used to demonstrate the FFA1 dependence of insulin release by conjugated linoleic acids[213] as well as to examine the role of FFA1 in lipotoxicity.[139] Kristinson et al. identified the pyrimidinylhydrazone ANT-203 (33) in a high-throughput screen and demonstrated the usefulness of the compound in studies with isolated islets and pancreatic-derived cell lines.[140] Waring et al. described a related series of 2-(pyridinyl)pyrimidines, exemplified by 34, as antagonists of FFA1 capable of reducing overload of β-cells (Figure 14).[214] Little has been done to detail the pharmacology of the FFA1 antagonists and how their binding might be related to the multiple agonist binding sites noted to be present in the receptor. In the initial study identifying GW1100, this ligand produced antagonism of GW9508 that would not be considered consistent with competitive antagonism, while the first study reporting ANT-203 suggested, in passing, that this compound was competitive with elaidic acid.[140] Unlike the situation with antagonists of FFA2 (see

section 3.5.2), the FFA1 antagonists described have typically been found to function across species, albeit with substantially different IC$_{50}$'s.[215] This indicates that considerable care should be taken when selecting an appropriate antagonist and effective concentration to use in studies on FFA1 in nonhuman cells and tissues.

2.4.5. FFA1 Fluorescent Tracers. In recent times, fluorescently labeled GPCR ligands have proven to be extremely useful tools to visualize and quantify ligand binding to GPCRs in real time.[216] In the case of FFA1, very early efforts were made to generate fluorescent ligands for this receptor by incorporating a bodipy fluorophore into a saturated fatty acid, 35 (Figure 15).[217] Although this approach was moderately
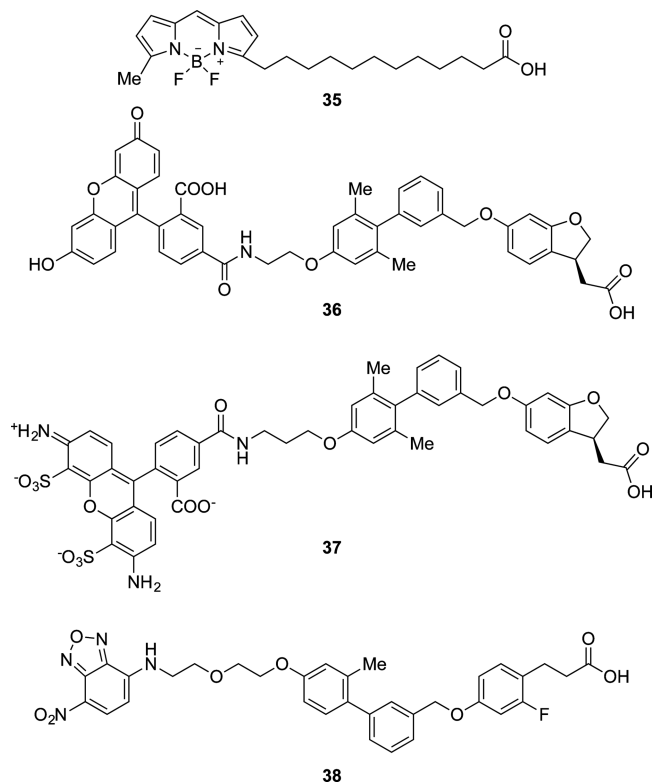
Figure 15. FFA1 fluorescent ligands.

successful and used in a flow cytometry-based assay to assess the affinity of various LCFAs, the low affinity and high lipophilicity of 35 significantly limited its utility. More recently, based largely on insights gained through the TAK-875/FFA1 cocrystal structure, significantly higher affinity FFA1 fluorescent ligands have been described based on either TAK-875 (36 and 37)[218,219] or TUG-905 (38).[220] Although these ligands display high affinity, they remain highly lipophilic or amphiphilic, which has made quantifying and/or visualizing their binding by conventional fluorescence methodologies challenging. To overcome this, Bertrand et al. utilized an approach in which cells were first labeled with 37 and then the signal was boosted using an antibody directed against the Alexa488 fluorophore present in 37.[218] By contrast, Christiansen et al. developed a bioluminescence resonance energy transfer (BRET)-based assay using 38.[220] For this, the small, bright, Nanoluciferase, which can serve as an energy donor to the 4-amino-7-nitrobenzofurazan fluorophore in 38, was introduced into the N-terminal domain of FFA1. In this format the nonspecific signal was expected to be greatly reduced, as only molecules of

ligand in close proximity to the Nanoluciferase-tagged receptor are detected.[221] Indeed, despite being highly lipophilic, **38** displayed high affinity and very good specific to nonspecific binding signals using the BRET assay format. Taken together, it is likely that these and additional fluorescent FFA1 ligands will be important tools in assessing ligand interaction with this receptor.

3. FFA2 AND FFA3

In a similar time frame as the deorphanization of FFA1, two of the other 7-TMD sequences, GPR43 and GPR41 noted by Sawzdargo et al. to also be located in human at chromosome 19q13.1,[101] were paired with SCFAs as endogenous ligands.[222−224] Subsequent systematic nomenclature has redefined GPR43 as FFA2 and GPR41 as FFA3.[225] However, as with FFA1, the initial designations as GPR43 and GPR41 remain firmly entrenched in the literature.

Examination of the signaling pathways of FFA2 and FFA3 (Figure 16) has revealed that FFA3 couples predominantly if
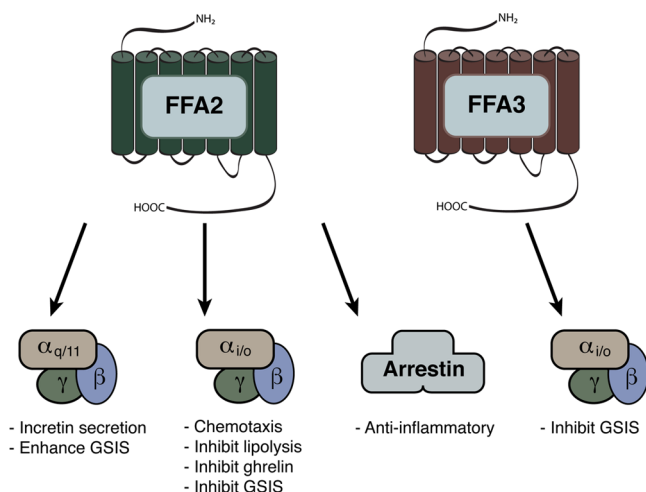
Figure 16. Signaling pathways and their associated biological outcomes for the FFA2 and FFA3 receptors.

not exclusively to "G$_{i/o}$"-family G proteins and hence is best appreciated as being able to mediate inhibition of adenylyl cyclase and reduce levels of intracellular cAMP.[222−225] By contrast, as well as interacting with "G$_{q/11}$"-family G proteins, FFA2 is also able to transduce signals via G$_{q/11}$/G$_{11}$-linked pathways.[222] Few studies have examined G protein-independent signaling of FFA2 and FFA3; however, FFA2 does recruit arrestin-3,[226,227] and at least one study has linked anti-inflammatory actions of FFA2 to this pathway.[228] By contrast, FFA3 interactions with arrestins have not typically been reported.[5,136]

3.1. Expression of FFA2 and FFA3

Early studies identified various immune cells as expressing both of the SCFA receptors, with FFA2 particularly highly expressed in monocytes and polymorphonuclear cells.[222−224] Given that the SCFAs are generated primarily in the gut through fermentation of dietary fiber (Figure 2), it is perhaps not surprising that both FFA2 and FFA3 are expressed in the gut, primarily by the enteroendocrine cells.[229−234] Expression of both FFA2 and FFA3 has also been described in pancreatic β-cells,[235] and interest has been increasing recently in the role of these receptors in regulating insulin secretion.[236−238] Ex-

pression of both FFA2 and FFA3 in adipose tissue has also been reported,[239,240] although the expression of FFA3 has been contentious, with many studies finding only FFA2.[227,241,242] FFA3 only appears to be expressed highly in the neurons present in both sympathetic ganglia and the enteric nervous system.[243−247] Finally, FFA2 and FFA3 expression has been described in a number of cancers, including breast, colon, and liver.[248−250] Although few studies have assessed the function of these receptors in cancer, there is some evidence that FFA2 in particular has tumor-suppressive actions.[248,249]

3.2. GPR42

In addition to FFA1, FFA2, and FFA3, in primates there is a fourth GPCR encoded in tandem within the same linkage group on chromosome 19q13.1 in human.[101] This GPCR, known as GPR42, although closely related to the FFA receptors, has received relatively little attention due to it being described as a potential pseudogene in the original studies of Brown et al. that deorphanized FFA2 and FFA3.[222] GPR42 is most closely related to FFA3, and indeed, there are only six amino acid variations between human FFA3 and GPR42 (Figure 17).[222] One of these is the replacement by
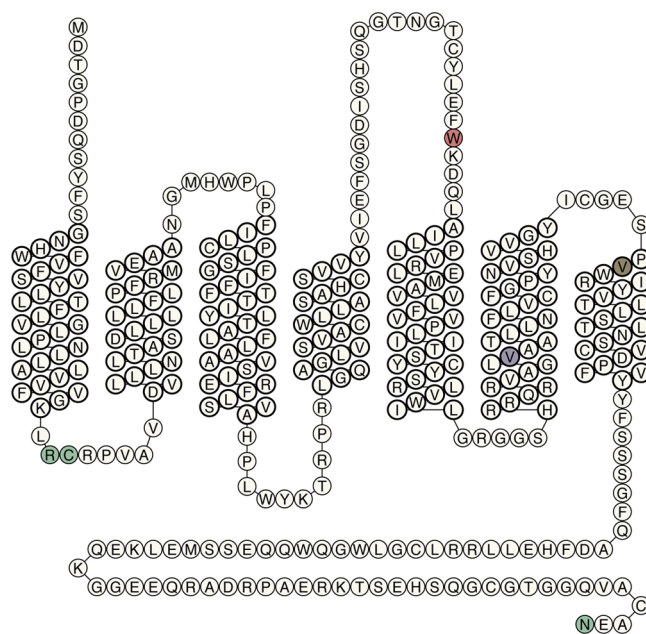
Figure 17. Position and function of residues that differ between human GPR42 and human FFA3. A snake diagram shows the amino acid sequence and position of residues in GPR42. Residues highlighted are those that differ between GPR42 and FFA3. When the equivalent residues were mutated in FFA3 to match those in GPR42 those marked in green did not affect function, those in red abolished function, those in blue increased constitutive activity, and those in brown had only a modest effect on function of the endogenous SCFAs.[212]

tryptophan of an arginine residue in FFA3 (Arg174^{EL2}), which appears to be integral for agonist function.[222] However, Liaw and Connolly indicated GPR42 to be markedly polymorphic, and when over 200 GPR42 alleles were analyzed, more than 60% had arginine at this position,[251] and thus were likely to respond to SCFAs. Another recent study has further demonstrated a number of functional GPR42 variants with the capacity to be expressed in man.[252] In this study, despite the very similar sequences of GPR42 and FFA3, the pharmacology

of the functional GPR42 variants differed in terms of potency of the SCFA propionate.[252] There is also some indication of polymorphism within the copy number for GPR42, with a relatively high 18.5% of alleles containing a deletion of the GPR42 sequence, while other alleles are likely to have multiple copies.[252,253] It should also be noted that as most expression studies on FFA3 have only analyzed mRNA levels through RT-PCR, given the sequence similarity between GPR42 and FFA3, unless primers were designed to specifically exclude detection of the GPR42 transcript it is likely that they would fail to differentiate between the two. Indeed, in many studies utilizing RT-PCR the primers used would detect both FFA3 and GPR42 expression.[223,227,250] Taken together, it appears likely that GPR42 does represent more than simply a pseudogene, but the broader implications in terms of tissue distribution and whether functional effects of SCFAs via GPR42 are restricted to "gene-dosage" or truly distinct pharmacology compared with FFA3 remains essentially unexplored.

3.3. SCFAs at FFA2 and FFA3

SCFAs with chain length between C1 and C6 are agonists at both FFA2 and FFA3. However, unlike FFA1, where there is limited discernible SAR among the various LCFAs, clear trends based on carbon chain length can be observed in relation to the potency of SCFAs at these receptors.[99,136] In the case of FFA2, by incorporating data from multiple studies a general consensus for the rank order of potency at the human receptor of C2 ≈ C3 ≈ C4 > C5 > C6 ≈ C1 has been established.[136,254] In contrast, the rank order established for human FFA3 is C3 ≈ C4 ≈ C5 > C6 > C2 > C1. Importantly, these differences in rank order of agonist potency between FFA2 and FFA3 mean that certain SCFAs show greater potency at one receptor over the other. Most notably C2 is consistently reported to be approximately 10 times more potent at human FFA2 across multiple assay end points.[223,255] This has resulted in some researchers using C2 as a selective activator of FFA2.[234,242] Despite the relatively modest potency difference for C2 between these two receptors, this can have value when combined with selective knockout or knockdown of one or other of the receptors, particularly because the range and availability of high-affinity selective ligands for these two GPCRs remains limited (see sections 3.4 and 3.5). However, further issues exist in terms of using the SCFAs as selective agonists for FFA2 or FFA3 when employing rodent models and cell lines derived from rodents. Hudson et al. demonstrated substantial differences in SCFA potency and selectivity between species orthologs of these receptors.[256] Thus, while confirming the higher potency of C2 for human FFA2 than FFA3, this SCFA was shown to be equipotent at the mouse orthologs. Moreover, although C3 is equipotent at human FFA2 and FFA3 it is markedly selective for FFA3 over FFA2 in mouse.[256] Molecular analysis showed these variations to be linked to the extent of constitutive activity displayed by the species orthologs, and both features could be switched by judicious mutagenesis of residues in EL2 of the receptors.[256]

To establish the mode of SCFA binding to FFA2 and FFA3, Stoddart et al. aligned the sequences of FFA1, FFA2, and FFA3 and noted that the two key arginine residues implicated in ligand binding to FFA1 at positions 5.39 and 7.35 were conserved in both FFA2 and FFA3, while the key asparagine residue at 6.55 was a semiconserved histidine in the two SCFA receptors.[225] When each of these three residues was mutated to alanine in FFA2 or FFA3 a complete loss in activity for the

SCFAs was observed.[225] Thus, it appears that the carboxylate of the SCFAs interacts with the same conserved residues of FFA2 and FFA3 as have been shown to interact with the carboxylate of TAK-875 in the FFA1 crystal structure. Recently, a more detailed map of the mode of binding of the SCFA propionate was generated based on a homology model constructed from the FFA1 crystal structure. While confirming the direct roles of arginine residues 5.39 and 7.35 in coordinating the carboxylate this model suggests that the key contribution of the histidine at position 6.55 is to organize the binding pocket by making a direct interaction with the arginine at 7.35 to effectively position this residue.[257]

Assessing the biological functions of the SCFAs at FFA2 and FFA3 has proven particularly challenging.[136] This is in large part due to similar expression profiles, the very low potencies of the SCFAs at these receptors, and the relatively similar pharmacology between them. Together these factors have made it highly challenging to separate FFA2 from FFA3 function based on pharmacology of the endogenous ligands. Further complicating the issue is that expression of FFA2 has been reported to be reduced in at least one line of FFA3$^{-/-}$ mice,[242] potentially hindering the interpretation of knockout mouse studies with these receptors. Despite these challenges, a growing number of studies have now implicated FFA2 and FFA3 in SCFA effects on both metabolism and inflammation.

3.3.1. SCFAs, FFA2, and FFA3 in the Gastrointestinal Tract. As the SCFAs are generated primarily through the fermentation of fiber by gut microbiota, the highest levels of SCFAs are within the GI tract. It is, therefore, not surprising that this is one of the tissues that has received substantial attention in terms of the functions of the SCFA receptors. As was the case with FFA1, the ability of FFA2 and/or FFA3 to regulate gut hormone secretion has received particular attention. Although many early studies had demonstrated expression of both FFA2 and FFA3 in enteroendocrine cells,[230−233] it was the work of Tolhurst et al. that first established a clear functional role.[234] These studies demonstrated that C2 and C3 both stimulate GLP-1 release from primary murine colonic cultures and that this was not blocked by treatment of the cells with pertussis toxin, indicating a non-G$_{i/o}$-mediated pathway. The effect was lost in cultures derived from FFA2$^{-/-}$ but not FFA3$^{-/-}$ mice, and as it was also shown that C2 and C3 increased intracellular [Ca^{2+}], it was concluded that the SCFAs increased GLP-1 via a FFA2-G$_{q/11}$-mediated pathway.[234] In these studies, although it was found that GLP-1 levels and associated glycaemic control were altered in FFA2$^{-/-}$ mice, it was not tested whether the SCFAs stimulated GLP-1 release in vivo. It was not until a more recent study demonstrated that intracolonic infusion of C3 increased secretion of both GLP-1 and PYY in vivo and that these effects were absent in FFA2$^{-/-}$ mice that the ability of SCFAs to stimulate gut hormone secretion via FFA2 in vivo was confirmed.[258]

In addition to enteroendocrine cells, FFA2 and FFA3 are also expressed in the stomach, and in particular, they appear to be expressed by ghrelin-containing cells.[259] Interestingly, SCFAs regulate ghrelin secretion from ex vivo gastric tissue, but unlike GLP-1 and PYY, C2, and C3 both appear to inhibit ghrelin secretion.[259] Pharmacological characterization indicates that this is mediated by a G$_{i/o}$-mediated pathway, as it was blocked by treatment with pertussis toxin, and although these cells express both FFA2 and FFA3, tissue from knockout mice

indicates that FFA2 is the primary receptor regulating ghrelin secretion by SCFAs.[259]

3.3.2. SCFAs, FFA2, FFA3, and Adipose Tissue. The SCFAs have long been known to have diverse effects on adipose tissue, influencing processes such as adipogenesis, lipolysis, and leptin secretion.[260] Since the identification of FFA2 and FFA3 as receptors for SCFAs, significant efforts have been made to establish which effects are mediated by each receptor. Most notably, the ability of SCFAs to inhibit lipolysis in adipocytes had long been established.[260] This was quickly linked to actions of FFA2.[241,261] In the key studies of Ge et al. it was found that C2 and C3 inhibited lipolysis in both a cell line model (differentiated 3T3-L1 cells) and in primary murine adipocytes and, importantly, that this effect was absent in adipocytes from FFA2$^{-/-}$ mice.[261] As would be expected, the pathway was linked to FFA2-G$_{i/o}$ signaling as the antilipolytic effect of C2 was eliminated by pertussis toxin pretreatment.

The influence of FFA2 on adipogenesis has also been explored. Hong et al. demonstrated that C2 and C3 SCFAs enhance lipid content during differentiation of 3T3-L1 adipocytes.[241] It was also found that FFA2 transcript expression was substantially upregulated during the differentiation process and that transfection with FFA2 siRNA inhibited differentiation.[241] Together these in vitro findings suggest a role for SCFAs and FFA2 in adipogenesis. An initial in vivo study using FFA2$^{-/-}$ mice perhaps supports such a role in that the knockout mice had reduced body fat mass when fed a high-fat diet.[262] By contrast, a study using a different strain of FFA2$^{-/-}$ mice found that these animals are obese and that mice overexpressing FFA2 in adipose tissue are lean.[263] This study found that in vivo adipogenesis was not different across these transgenic mice, suggesting little role for FFA2 and SCFAs in adipogenesis in vivo. Similarly, a study on primary human adipocyte differentiation also found no evidence for a relationship between SCFAs, FFA2, and adipogenesis.[264] It appears clear from these studies that there is significant species and strain variation in the effects of the SCFAs and FFA2 in particular on adipocyte function. As the microbiota play such a central role in production of the SCFAs it will be of great interest to understand how this may vary between locations and if this might contribute to variation in experimental observations.

The relationship between SCFAs and leptin secretion has also resulted in some controversy.[136] Notably, while an early study suggested SCFA increased leptin secretion via activation of FFA3,[240] subsequent work has generally not found FFA3 to be expressed in adipocytes.[227,241,242] Interestingly, a study using adipocytes from FFA3$^{-/-}$ mice did find that C2 and C3 lost their ability to stimulate leptin secretion when FFA3 was not present.[242] However, as noted earlier, the authors observed decreased FFA2 expression in the cells from these FFA3$^{-/-}$ mice, and because they were unable to detect FFA3 expression in the wild-type adipocytes, it was concluded that most likely FFA2, and not FFA3, mediated SCFA-stimulated leptin secretion.[242] A later study using FFA2$^{-/-}$ mice found that circulating leptin levels were not affected by FFA2 knockout but, surprisingly, were decreased by adipocyte overexpression of FFA2.[263] In terms of signaling, early studies indicated that the SCFA effect on leptin secretion was eliminated by pertussis toxin treatment, implicating a G$_{i/o}$-mediated pathway,[240] and therefore, possibly either FFA2 and/or FFA3 could be involved. Finally, SCFAs have also been reported to regulate glucose uptake in adipocytes, in particular, enhancing insulin-stimulated

glucose uptake.[239,265] One study has found that these effects are reduced by transfection of FFA3 siRNA, suggesting a role for the FFA3 receptor.[239] However, this study did not assess the effects of FFA2. It has, however, also been suggested in a non-peer-reviewed patent application that FFA2 activation may modestly stimulate glucose uptake.[266] While the study on FFA3 suggested that its actions were to enhance insulin activity on adipocytes, this is less clear for FFA2. Indeed, in vivo C2 administration was found to suppress insulin signaling in adipocytes of wild-type but not FFA2$^{-/-}$ mice.[263] Using adipocytes generated from these FFA2$^{-/-}$ mice it was shown that C2 inhibited insulin-stimulated glucose uptake in wild-type but not the knockout animals and that this was blocked by pertussis toxin treatment but not by a G$_q$-targeted siRNA.[263] Together, these data suggest that FFA2-G$_{i/o}$ pathways inhibit insulin signaling and glucose uptake in adipocytes. Most interestingly, this study also indicated that this was not the case in either muscle or liver, with the authors suggesting the resulting shift in insulin-stimulated metabolism from adipose to other tissues accounted for the beneficial effects of FFA2 on energy utilization and metabolic homeostasis.[263]

3.3.3. SCFAs, FFA2, FFA3, and Insulin Secretion. Unlike FFA1, where focus has been predominantly on function in pancreatic islets, less work has examined potential roles of FFA2 and FFA3 in this tissue. Although not highlighted to express FFA2 or FFA3 in the early deorphanization studies, both of these receptors were found in various rodent insulinoma β-cell lines.[235] Several recent studies have now begun to assess directly roles of FFA2 and FFA3 on insulin secretion, although they have yielded somewhat different results. Two studies have found that SCFAs activate FFA2 to enhance GSIS from mouse islets both in vivo and in vitro and that this occurs through a G$_{q/11}$-mediated pathway.[236,237] By contrast, a separate study found instead that SCFAs inhibit GSIS through actions at both FFA2 and FFA3 and that this occurs through a G$_{i/o}$-mediated pathway.[238] While these results appear to be contradictory, it has been found consistently in mouse islets that G$_{q/11}$ pathways via FFA2 enhance while G$_{i/o}$ pathways via FFA2 and FFA3 inhibit GSIS.[236−238] Therefore, it is most likely that the differences between studies on the role of FFA2 represent other factors, including potentially the strain of mice used, which may influence whether FFA2-G$_{q/11}$ or -G$_{i/o}$ signaling pathways predominate. Studies on human islets, however, have not found any effect of SCFAs on GSIS, despite the fact these ligands did activate both G$_{q/11}$ and G$_{i/o}$ signaling.[237] This has been taken to suggest that in human islets the combination of activating G$_{q/11}$ and inhibiting G$_{i/o}$ pathways results in no net change in GSIS.[237] More work will be needed to determine which factors determine whether FFA2 G$_{q/11}$ vs G$_{i/o}$ signaling predominates and whether SCFAs will stimulate vs inhibit GSIS in different settings.

3.3.4. SCFAs, FFA2, FFA3, and Inflammation. Each of the SCFAs receptors is expressed on certain immune cells. Functionally, FFA2 appears to be important in promoting SCFA-mediated chemotaxis of neutrophils.[267−269] As might be expected, the FFA2-chemotactic response was inhibited by pertussis toxin treatment and thus is G$_{i/o}$ mediated, presumably via the release of $\beta\gamma$ complexes.[268] A few studies have also explored whether FFA2 regulates cytokine production with, for example, acetate being found to inhibit secretion of tumor necrosis factor (TNF) from mononuclear cells: an effect that was reported to be inhibited by an anti-FFA2 antibody.[270] A further study carried out on intestinal epithelial cells indicated
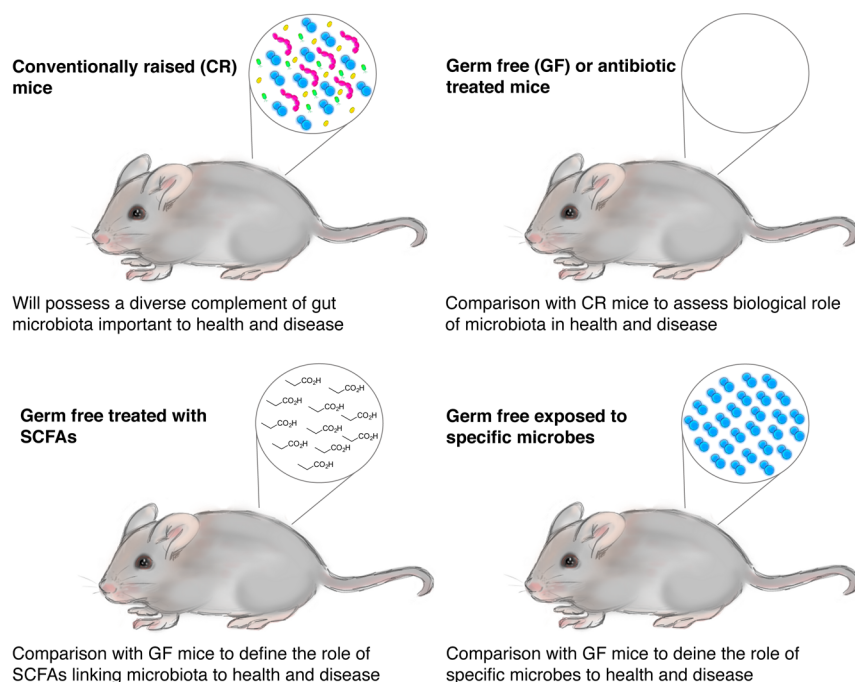
Figure 18. Experimental approaches to study the links between gut microbiota and SCFAs in health and disease. Several experimental approaches have been used to link the microbiota and the SCFAs they produce to health and disease. Most notably, mice can be raised under germ-free conditions or treated with antibiotics to establish what effects on health the microbiota have. Treating such mice with exogenous SCFAs can then be used to determine which beneficial effects of the microbiota are recovered. Alternatively, mice may be exposed to specific microbes, for example, those that produce higher levels of acetate in order to establish the importance of individual microorganisms. Finally, FFA2$^{-/-}$ and/or FFA3$^{-/-}$ mice can be used in combination with these approaches to determine the contribution of these receptors to the biological effects of the microbiota.

that SCFAs enhance the protective secretion of cytokines and chemokines from such cells, in this case via activation of both FFA2 and FFA3.[271]

3.4. FFA2, FFA3, and the Gut Microbiota

In large part due to the very low potency of SCFAs at FFA2 and FFA3, establishing specific functions of these receptors in vivo has been challenging. As described above, the primary source of SCFAs in the body is through fermentation of dietary fiber by the gut microbiota. There are strong links established between the gut microbiota, health, and disease.[28−31,272,273] Therefore, it is not surprising that a number of studies have aimed to assess whether the SCFAs, acting through FFA2 and/or FFA3, provide key links between the gut microbiota and health. A number of experimental approaches have been employed in this regard, but most commonly either germ-free (GF) or antibiotic-treated mice lacking gut bacteria have been studied in combination with exogenous SCFAs administration, exposure to specific microbiota, and/or the use of receptor knockout animals (Figure 18).[263,267,274−278]

The studies on FFA2 have demonstrated how this receptor in particular links gut microbiota SCFA production to inflammation. The studies of Maslowski et al. were the first in this area, demonstrating that FFA2$^{-/-}$ mice had dysregulated immune responses to induced colitis and that similar dysregulation was observed in wild-type GF mice.[267] Importantly, acetate treatment ameliorated the detrimental effects of GF mice, suggesting but not proving a mechanistic link to FFA2. The later studies of Smith et al. were able to provide a more direct link by examining how the gut microbiota regulate the population of regulatory T cells (T$_{Regs}$) in the intestine.[276] Here, it was found that GF conditions reduced the population of T$_{Regs}$ in the intestine and that SCFAs restored

these populations. Critically, Smith et al. demonstrated that the effects of SCFAs on GF mice were lacking in FFA2$^{-/-}$ animals, providing strong evidence to link the gut microbiota through SCFAs to biological functions mediated by FFA2.[276] Studies have also demonstrated that the effects of gut-derived SCFAs on inflammation through FFA2 extend beyond the intestine, where, for example, IL-1β and CXCL1 production are decreased in response to C2 and C3 in a murine model of gout in GF, antibiotic-treated, and FFA2$^{-/-}$ mice. Once again, the effects of raising the mice GF were reversed by exogenous administration of SCFAs.[278] Finally, GF mice have also been used to link the gut microbiota to the function of FFA2 in adipose tissue, where in one study FFA2$^{-/-}$ mice were found to be obese compared to wild-type mice when raised under conventional conditions but were not different from wild type when raised GF.[263]

A number of studies have also assessed the function of FFA3 using similar approaches. Samuel et al. found that conventionally raised FFA3$^{-/-}$ mice had decreased adiposity compared to their wild-type controls, but this was not apparent in GF mice.[275] In particular, this appeared to relate to PYY levels, which were decreased in FFA3$^{-/-}$ mice when gut microbiota were present but were not in GF mice. One study has also linked dietary fiber, the microbiota, SCFAs, and FFA3 to allergic airway disease.[277] In this study a high-fiber diet altered microbiota composition in both the gut and the lung, increasing SCFA levels, and protected against allergic airway disease. Exogenous C3 administration was found to produce similar effects to fiber, but such effects of C3 were absent in FFA3$^{-/-}$ but not FFA2$^{-/-}$ mice.

These studies highlight only the beginning of what is likely to be a key area studying the endogenous functions of FFA2 and FFA3 in coming years. While the studies using antibiotics and

GF mice are clearly informative, more work is needed to examine how altering the makeup of the microbiota in more subtle ways, such as altering the specific species present, affects health and disease via the SCFA receptors. This is particularly relevant given that it is recognized that certain species of microbiota produce higher amounts of C2 and C3 while others will produce more C4[2] and that these SCFAs are known to have different potency at FFA2 and FFA3. Indeed, in terms of human health this may have significant implications, particularly with the probiotic industry aiming to colonize the gut with beneficial bacteria.

3.5. Synthetic Ligands for FFA2

Despite initial challenges, a growing number of synthetic ligands have now been reported for FFA2. These include orthosteric and allosteric examples and both agonists and antagonists. A particular challenge within this area has been identifying compounds with good selectivity for FFA2 over FFA3, and although this has improved in recent years, it is not yet fully resolved. Species differences between human and rodent orthologs of FFA2 have also proven challenging to overcome, and particularly this relates to the pharmacology of FFA2 antagonist ligands.

3.5.1. Orthosteric FFA2 Agonists. Initial studies highlighted the ability of SCFAs of varying chain length to activate FFA2. While the potency of formic acid (C1) at FFA2 is extremely low, the fact that it does act as an agonist indicates that a carboxylate is all that is required to both bind and activate this receptor. However, the limit in acceptance of aliphatic chain length to C5−C6 suggested that the pocket for orthosteric ligands is small and constrained. Indeed, apart from the endogenous SCFAs, in early years the only other orthosteric ligands reported in the primary literature to activate FFA2 were a group of synthetic small carboxylic acids (SCAs),[255] although several useful ligand series and interesting data on these had appeared in the patent literature.[279] Chemicals within the SCA group that activated FFA2 in preference to FFA3 included trans-2-methylcrotonic acid and cyclopent-1-enecarboxylic acid and, in general, possessed sp²- or sp-hybridized α-carbons. However, even though markedly selective for FFA2 these SCAs were not more potent than the SCFAs.[255] Despite this, at least two of these sp-hybridized α carbon SCAs, propiolic acid and 2-butynoic acid, were found to potentiate GSIS in wild-type but not FFA2^{−/−} mice.[237] Molecular modeling has been conducted to identify potential binding poses of the FFA2-selective SCAs and has suggested additional residues, including Tyr90[3.33], Glu166[EL2], Phe168[EL2], Leu183[5.42], Cys184[5.43], and Tyr238[6.51], beyond the arginine and histidine residues previously implicated in SCFA binding as likely to contribute to the orthosteric binding pocket.[255] Over time the bulk of these have been mutated and analyzed for function.[227,256]

One alternate approach that has been utilized to selectively activate a specific member within a receptor family that contains very similar orthosteric binding pockets is to modify the receptor instead of the ligand to generate selectivity. Typically this involves the generation of a receptor activated solely by synthetic ligand (RASSL) or designer receptor exclusively activated by designer drugs (DREADD) form of the receptor, which will be activated by a synthetic ligand but not by the receptor's endogenous ligand(s).[280,281] In order to develop such a version of FFA2, Hudson et al. took the novel approach of utilizing species differences in the pharmacology of

FFA2 (Figure 19).[226] Given the importance of nondigestible carbohydrates to the diet of ruminants, the activity of SCFAs at

C3 but not sorbic acid stimulates all FFA2-downstream signaling pathways

Only sorbic acid will activate FFA2 signaling comparable to C3 at wild type
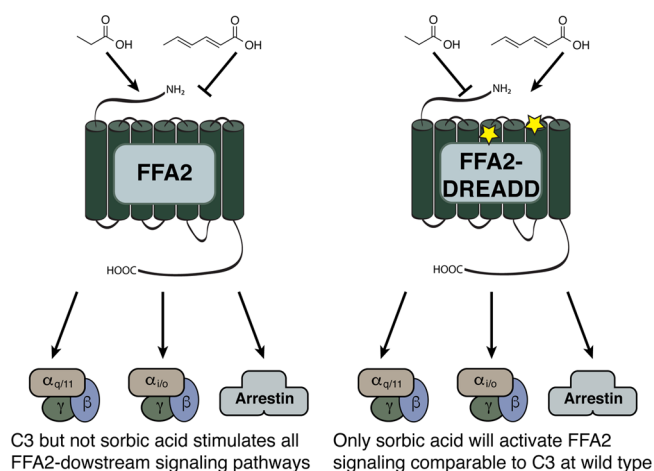
Figure 19. Characteristics of an FFA2-DREADD. A designer receptor exclusively activated by designer drugs (DREADD) form of FFA2 was generated based on species differences between the bovine and the human orthologs of this receptor, where two mutations were incorporated, one in TMV and the other in TMVI.[216] At wild-type FFA2 C3 but not sorbic acid activates the receptor to stimulate various downstream signaling pathways. In contrast, at the DREADD, C3 has no effect while sorbic acid is able to activate equivalent pathways to those activated by C3 at wild-type FFA2.

the bovine ortholog of FFA2 was examined and indicated that this ortholog, where C6 was the optimal chain length, had a strong preference for longer chain lengths than human FFA2.[226] Moreover, when tested at the bovine receptor, within the group of SCAs described by Schmidt et al.,[255] a number were markedly more potent than at human FFA2. Most notably sorbic acid (2,4-hexadienoic acid) (Figure 19) was more than 1000-fold selective for bovine FFA2 over human.[226] Molecular modeling and mutational studies were undertaken to identify residues that were different in the bovine ortholog and that could account for this effect. A single C141G[4.57] mutation in human FFA2 resulted in a substantial gain in potency for sorbic acid.[226] Although this form of the receptor now responded to sorbic acid, it maintained function for the SCFAs, and therefore, in order for it to be useful as a RASSL/DREADD it was critical to further mutate the receptor to eliminate SCFA binding. Alteration of His242[6.55] to glutamine achieved this, resulting in a form of the receptor that still displayed strong responsiveness to sorbic acid but now with virtually no response to any endogenous SCFA.[226] Interestingly, earlier studies had indicated that mutation of this residue to alanine allowed a level of activation of human FFA2 by both caproic acid (C6) and even caprylic (C8).[225] Importantly, as FFA2 can interact with both $G_{i/o}$ and $G_{q/11}$-family G proteins, the modified receptor showed good responses to sorbic acid across the full range of assays usually employed to assess FFA2 signaling, including inhibition of forskolin-stimulated cAMP levels, elevation of intracellular $[Ca^{2+}]$, phosphorylation of ERK1/2, and global cellular changes assessed through dynamic mass redistribution.[226] This suggests that this RASSL/DREADD version, if incorporated into a knock-in animal in which this form replaces the wild-type receptor, could be a powerful tool to dissociate FFA2 from FFA3-mediated

biological effects in vivo. This idea has recently been discussed further by Bolognini et al.[282]

Given the interest in the contribution of SCFAs and the microbiota to health and disease noted earlier[273,283,284] there is a remarkable paucity of larger and more potent synthetic FFA2 orthosteric agonists described to date. Indeed, only a few series of chemical ligands with these features have been reported in the primary scientific literature.[279] Of these, a series of compounds based on a 4-oxobutanoic acid backbone is best characterized in terms of their basic pharmacology. On the basis of an international patent application[266] Hudson et al. synthesized **39** and the related **40** (Figure 20) and
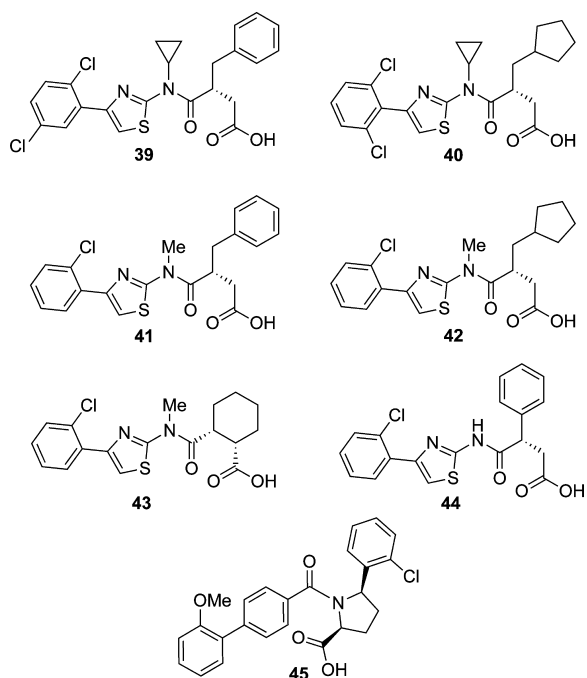

Figure 20. FFA2 orthosteric agonists.

demonstrated that they were both able to activate human FFA2 with submicromolar potency and were highly selective over FFA3 as well as the other free fatty acid receptors.[227] These compounds were clearly orthosteric in action because, like the SCFAs and SCAs, agonist function was lacking at R180A$^{5.39}$, R255A$^{7.35}$, and H242A$^{6.55}$ mutants of FFA2. Moreover, the carboxylate moiety of these compounds was also integral to function as methyl and *tert*-butylesters of **40** were found to be inactive.[227] Modeling of the potential mode of binding of **39** into the FFA2 homology model described by Sergeev et al. illustrated interactions with not only the key orthosteric residues that bind the SCFA but also with further residues including tyrosine 3.33, valine 5.38, tyrosine 6.51, as well as a further tyrosine in EL2.[257] Each of these residues had previously been shown to be likely to contribute to interaction with this ligand as mutation of each reduced the potency of **39**.[227]

Perhaps as might be generally expected for an orthosteric ligand, FFA2 signaling elicited by **39** was very similar to that by the SCFA C3, as assessed across multiple signaling end points.[227] Interestingly, a recent study by Brown et al. generated four additional FFA2 agonists from this chemical series, **41−44** (Figure 20), finding markedly different signaling responses among them.[285] Notably, while each of these

compounds acted as a potent agonist in a [^{35}S]GTPγS assay, only **41** and **44** showed equivalent FFA2 agonism in a cAMP assay, while **42**, **43**, and **44** all appeared rather to act as inverse agonists in a yeast-based assay designed to assess G_i coupling to FFA2.[285] These differences perhaps suggest some level of bias in the actions of these compounds at FFA2. GPCR signaling bias is often associated with bitopic ligands that interact with the orthosteric site as well as additional allosteric sites outside of the orthosteric binding pocket, and Brown et al. considered whether this might be the case for this series of ligands. Although these studies did not find clear support for a bitopic mode of action within this series,[285] it should be noted that, given the very small size of the SCFAs, which display very high ligand efficiency, any synthetic agonist with markedly higher potency will be required to extend outside the direct binding pocket of the SCFAs. Indeed, this means that such synthetic agonist ligands must make additional contacts with FFA2 compared to the SCFAs. Whether this results in "bitopic-like" properties of such synthetic compounds will need to be more fully addressed in the future.

Synthetic agonists from this series have also now been used to confirm several of the biological functions of this receptor in vitro. Both Hudson et al. and Brown et al. confirmed FFA2-mediated secretion of GLP-1 and inhibition of lipolysis using these compounds.[227,285] Brown et al. also demonstrated that the compounds elicited a Ca^{2+} response in neutrophils. Importantly, the effects on lipolysis were absent in adipocytes from FFA2$^{-/-}$ mice,[285] confirming that the ligand effects were via FFA2. Finally, within this series, Hudson et al. also noted some species variation in activity finding, in particular, that although **39** was active at both human and mouse FFA2, **40** appeared to act as a potent agonist only at the human ortholog.[227] This indicates that considerable care must be taken when selecting compounds from this series for in vivo or other rodent-based studies.

In addition to representatives from the chemical series discussed above a recent study has employed an example from a separate chemical series of FFA2 agonists that was also described initially in the patent literature.[286] Forbes et al. synthesized **45** (Figure 20), demonstrated it to be a potent and selective FFA2 agonist, and assessed the in vivo pharmacology of this compound.[287] Although the molecular basis for interaction of **45** with FFA2 was not assessed in detail, given that it contains a carboxylate and was not allosterically modulated by C2,[287] this compound is likely to be an orthosteric FFA2 agonist. Testing the compound in vivo demonstrated that it stimulated GLP-1 secretion, but this was only detected when the compound was coadministered with a dipeptidyl peptidase-4 inhibitor to prevent GLP-1 degradation.[287] This suggests that efficacy to promote GLP-1 release may be modest. Instead, Forbes et al. suggested the primary effect of **45** was on PYY secretion, finding that gut transport time was increased by the compound and that both food intake and body weight were decreased in a diet-induced obesity mouse model. Together, these results present encouraging evidence for targeting FFA2 agonism to limit obesity, but substantially more work will be needed to characterize the function of this and other FFA2 agonists in vivo.

3.5.2. Orthosteric Antagonists. An alternate approach to the identification and use of FFA2 orthosteric agonists to define pathophysiological roles of FFA2 is the development of orthosteric antagonists. Such chemicals would be predicted to block effects of SCFAs in a receptor-dependent fashion. The

first reported example was **46** (described by Hudson et al. as "CATPB") (Figure 21). On the basis of a chemical series
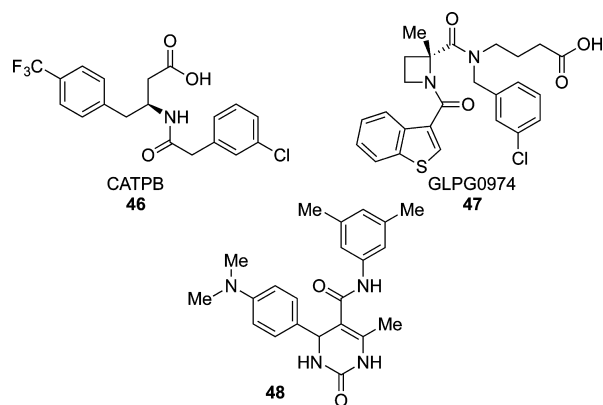

Figure 21. FFA2 orthosteric antagonists.

described in a patent from Euroscreen,[288] **46** both antagonized the ability of propionate to stimulate human FFA2 in a concentration-dependent manner and clearly acted as an inverse agonist, as it was similarly able to inhibit constitutive activity of the receptor.[256] Although not explored in detail at this point, these studies were also the first to note the major limitation on the use of **46** (and all other currently known FFA2 antagonists): that it is specific for human FFA2 and has no useful affinity for the rodent orthologs of FFA2. Despite this, **46** is a very useful orthosteric antagonist at human FFA2, moving the concentration–response curves for each of the orthosteric agonists, propionate, **39**, and **40**, to higher concentrations and in a surmountable manner, suggesting it likely binds to the orthosteric site.[227] Compound **46** has also been useful in confirming that reduction in glycerol release from SW872 human adipocytes in response to an FFA2 agonist is indeed a reflection of FFA2 activation.[227]

Recently, a second class of highly selective FFA2 antagonists has been described.[289] A group of azetidines, of which **47** (GLPG0974) (Figure 21) is the best characterized, is also a human-specific FFA2 antagonists. GLPG0974 is able to block human neutrophil migration induced by acetate.[289] Importantly for potential clinical development, GLPG0974 was also shown to block acetate-induced expression of CD11b activation-specific epitope, a marker of neutrophil activation, in a concentration-dependent manner in blood.[289] This, therefore, provided a biomarker to ensure target engagement of the compound in vivo. This compound entered clinical trials in patients displaying mild to moderate ulcerative colitis. Although well tolerated, safe, and found to reduce neutrophil activation and influx, this was insufficient to induce a measurable clinical difference between GLPG0974 and placebo-treated patients within 4 weeks.[290]

Like **46**, GLPG0974 appears to be an orthosteric antagonist of human FFA2 in that it blocks the function of a synthetic orthosteric agonist in a surmountable and concentration-dependent manner.[257] GLPG0974 has subsequently been radiolabeled and used to study the importance of various amino acids of the orthosteric binding pocket to the recognition and activity of both agonist and antagonist ligands. This chemical displayed high affinity (K_d 7.5 ± 0.4 nM) binding to wild-type human FFA2 and lost affinity to the extent that specific binding of up to 100 nM ligand could no longer be detected in a double R180A$^{5.39}$/R255A7$^{.35}$ mutant of the

receptor.[257] Surprisingly, however, unlike all known FFA2 orthosteric agonists, which completely lose activity at single mutations of these two residues, [^{3}H]GLPG0974 maintained high affinity at the single mutants, indeed losing affinity by only 2–3-fold.[257] Competition binding experiments demonstrated similar modest losses in affinity for **46** at these two mutants as well. These studies indicated that these antagonists only require interaction with one or the other of the two arginine residues to maintain high-affinity binding to FFA2. Perhaps supporting this is the observation that methyl esters of both **46** and of a compound related to GLPG0974 retained high affinity for FFA2.[257]

A recent study has disclosed a third, chemically distinct, series of FFA2 antagonists that lack a carboxylate, including, for example, **48** (Figure 21).[291] These compounds appear to be relatively low-potency, inverse agonists of human FFA2, producing such effects on FFA2 with 10–30-fold lower potency than **46**.[291] Limited studies suggest these compounds are likely to also be orthosteric, despite lacking a carboxylate. Surprisingly, several of the compounds were reported to enhance GLP-1 secretion from NCI-H716 cells. However, it must be noted that rather high concentrations of the ligands were used in these studies; thus, potential off-target effects cannot be ruled out at this stage.[291] Perhaps the most interesting finding from this work is that like the other series of FFA2 antagonists described above these compounds also completely lack activity at rodent orthologs of FFA2. The molecular basis for this currently remains unknown, but it will be critical for future studies to assess this and identify FFA2 antagonists suitable for use in rodents and tissues and cells derived from them.

3.5.3. Allosteric Agonists. The first synthetic chemical described as a selective activator of FFA2 was **49** (Figure 22).[292] In various publications **49** has been designated
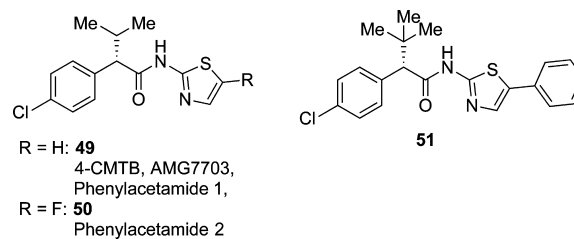
Figure 22. FFA2 allosteric agonists.

"phenylacetamide 1",[292] "4-CMTB",[293] or "AMG7703". Identified in high-throughput screens for FFA2 agonism, **49** is a moderately potent activator of the receptor. Moreover, in the original publication the activity of this chemical to regulate both $G_{i/o}$- and $G_{q/11}$-mediated end points and to activate mouse FFA2 as well as at the human ortholog was highlighted.[292] Activity at rodent orthologs allowed demonstration of the role of FFA2 in regulating lipolysis in differentiated rat 3T3-L1 adipocytes.[292] Although clearly able to function as a direct agonist, **49** retains the capacity to activate a variety of orthosteric binding site mutants of FFA2[293] and, therefore, acts as an allosteric agonist. Moreover, this compound acts as a PAM of the SCFAs,[292,293] increasing the observed potency of the endogenous agonists (Figure 23). It is often noted that allosteric ligands can display "probe dependence", i.e., that the detection of an allosteric effect of a chemical can vary depending on which orthosteric agonist is used.[69] This, indeed,
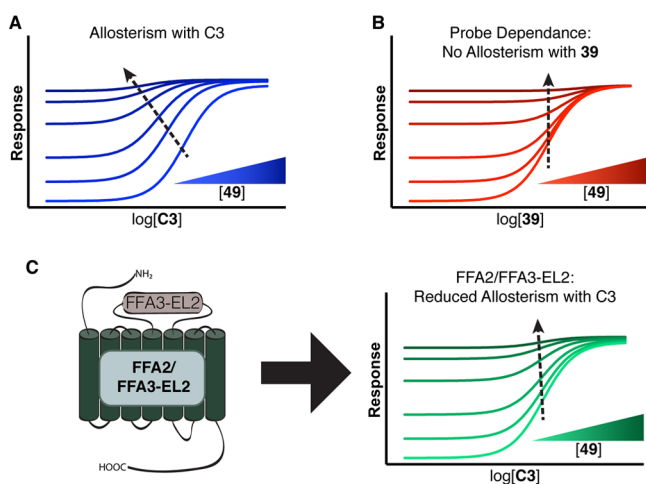
Figure 23. Pharmacology of FFA2 PAM-agonists. Functional studies with **49** at FFA2 demonstrated that in addition to acting as an agonist of this receptor, this ligand also enhanced the potency of C3.[281,282] Simulated data demonstrating this is shown in A, where the effect on C3 potency is indicated by the dashed line. Probe dependence of the allosteric effect was found where this ligand did not allosterically modulate the potency of the orthosteric synthetic agonist **39** (simulated data shown in B).[217] The molecular basis for allosteric communication between C3 and **49** was explored, where it was found that replacing EL2 of FFA2 with EL2 from FFA3 resulted in a near complete loss of allosteric effect (simulated data in C).[282]

is the case for **49** as no allosteric effect of this compound on the behavior of the synthetic orthosteric agonist **39** was observed (Figure 23).[227]

Subsequent studies explored the SAR of the phenylacetamide series, but despite substantial effort very little improvement in potency over the originally reported compound has been noted.[293,294] However, Wang and colleagues did note that **49** displayed a poor pharmacokinetic profile in male Sprague–Dawley rats and, therefore, used **50** ("phenylacetamide 2") for in vivo studies to demonstrate an FFA2-mediated reduction in plasma nonesterified fatty acids in wild-type mice following intraperitoneal injection of the ligand that was lacking in FFA2$^{-/-}$ animals.[294] The authors did, however, report effects of this compound in FFA2$^{-/-}$ mice when using higher doses, and this may limit more extensive use in vivo.[294] Although also failing to uncover compounds with significantly better potency than the parental **49**, Smith et al. highlighted the contribution of EL2 of the receptor and particularly of Leu173^{EL2} to effective allosteric communication with the SCFAs (Figure 23).[293]

The commercial availability of **49** has resulted in its use in a number of functional studies. For example, Vinolo et al. used this ligand in conjunction with SCFAs and a receptor knockout model to record the role of FFA2 in control of chemotaxis of mouse bone marrow-derived neutrophils.[269] Equally, Nohr et al. used a related compound **51** to examine the contribution of FFA2 to SCFA-mediated release of GLP-1 from mouse colonic crypts,[229] while **51** was also shown to reduce ghrelin secretion from primary gastric mucosal cells, implicating FFA2 as the relevant receptor.[259]

As noted in section 3.3.3, the SCFAs appear to have diverse effects on insulin secretion depending on whether the FFA2 signaling couples to G$_{q/11}$- or G$_{i/o}$-mediated pathways. The work of Priyadarshini et al. indicated that although SCFAs stimulate GSIS in mouse islets, **49** had the opposite effect.[237] In line with other studies on FFA2 and insulin release, this

appeared to relate to differences in the ability of the SCFAs and **49** to couple to G$_{i/o}$ vs G$_{q/11}$ signaling pathways.[237] In particular, although the authors measured robust intracellular [Ca^{2+}] and cAMP responses to C2, they could not measure cAMP responses to **49**, suggesting that the divergent effects on GSIS of these compounds are likely down to G$_{i/o}$ vs G$_{q/11}$ bias of these compounds. It was, however, noted that **49** retained a level of effect in islets from FFA2 knockout mice, suggesting some off-target effects. Interestingly, it had previously been noted that **49** is only a partial agonist for activation of the ERK-1/2 MAP kinases when compared to the SCFA C3.[293] It is thus clear that **49** should not be considered to be equivalent to orthosteric agonists of FFA2. Indeed, a recent study aiming to more fully characterize the function of **49** used label-free biosensors to demonstrate that this compound displayed unique signaling kinetics compared to orthosteric FFA2 agonists.[295] Surprisingly, detailed analysis of the kinetics of **49** responses in such label-free systems suggested that this compound activates FFA2 initially via the orthosteric binding site, before producing a prolonged effect through an allosteric site.[295] Grundmann et al. suggest this may represent a novel pharmacological approach to regulate GPCR function;[295] however, the broader importance of ligands able to sequentially interact with different binding sites on FFA2 and indeed the wider GPCR family remains to be elucidated. Taken together, although there are now important tools available with which to study FFA2, it is obvious that further novel ligands are required to fully explore the biology of this receptor. In particular, identification of each of orthosteric antagonists with broad cross-species affinity and higher affinity selective orthosteric agonists would be of major benefit.

3.6. Synthetic Ligands for FFA3

Developing potent and selective ligands for FFA3 has proven particularly challenging. While targeting allosteric binding sites has proven successful in identifying one series of ligands selective for FFA3 over FFA2, the potency of these compounds remains relatively low. Moreover, unlike FFA2, where potent orthosteric ligands have been described, to date there are no examples of such compounds for FFA3. Despite this, some FFA3-selective compounds based on the SCFAs themselves have been reported.

3.6.1. Orthosteric Ligands. To date, the identification of synthetic orthosteric ligands for FFA3 lags considerably behind that of FFA2. Although responding to the same group of SCFAs, FFA3 displays a distinct SAR for the SCFAs, and as was the case with FFA2, this led to the examination of SCAs in early efforts to identify FFA3-selective compounds.[255] Although a number of chemicals, largely those with substituted sp^3-hybridized α-carbons, were identified that showed somewhat higher potency at FFA3, in no case was the potency and level of selectivity sufficient to recommend use as an FFA3 ligand. Despite this, at least one study has employed the SCA, cyclopropanecarboxylic acid, as a "partially FFA3-selective" ligand.[263] Also, β-hydroxybutyrate, a metabolite produced during ketosis, has also been described as an FFA3 ligand. However, in different publications it has been reported as either an agonist[247] or an endogenous antagonist[244] of this receptor. This remains to be clarified, as does whether this plays a significant role in a physiological setting. It should be noted that hydroxycarboxylic acid 2 (HCA$_2$) receptor is generally considered the key target for β-hydroxybutyrate.[296]

3.6.2. Allosteric Ligands. Although still very limited in scope and potency, a series of compounds has been described that act as allosteric FFA3 ligands. These all derive from a series of hexahydroquinolone-3-carboxamides disclosed originally in a patent from Arena Pharmaceuticals.[297] One compound from this series, designated AR420626, was used as an FFA3-selective agonist and found to promote GLP-1 release from murine colonic crypt cultures.[229] The same compound was also used to show that FFA3 was not involved in the inhibition of ghrelin secretion by SCFAs.[259] However, these studies did not extensively examine the pharmacology of the compound. Hudson et al. demonstrated that another representative of this series, **52** (Figure 24A), was able to directly activate human
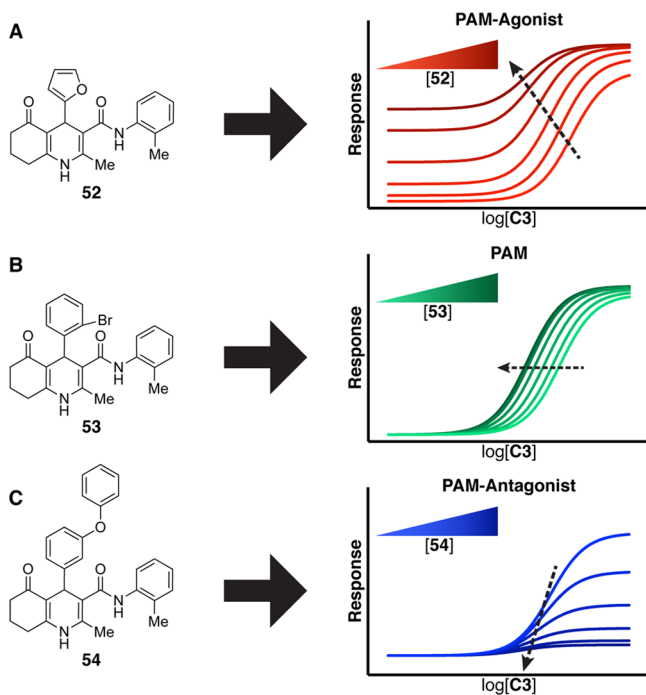

Figure 24. Modest structural changes to FFA3 allosteric ligands have profound effects on function. Structural variants of hexahydroquinolone-3-carboxamide FFA3 allosteric ligands were found to have diverse effects modulating the concentration−response to C3 at FFA3.[286] While **52** was a PAM-agonist both enhancing C3 potency and acting as an agonist on its own (simulated data in A), **53** was a pure PAM enhancing potency of C3 with no intrinsic efficacy of its own (simulated data in B) and **54** was a PAM-antagonist, enhancing potency of C3 while at the same time decreasing C3 efficacy (simulated data in C).

FFA3 with modest potency while lacking activity at FFA2.[298] Importantly, this study also characterized the pharmacology of this compound in much more detail, demonstrating that it maintained activity after mutation of the key arginine residues, Arg185[5.39] and Arg258[7.35], involved in orthosteric SCFA binding to FFA3, suggesting that the compound must be allosteric. Moreover, in addition to showing direct agonism, the compound also acted as a PAM, enhancing the potency of the SCFA C3 in functional assays (Figure 24A).[298]

Interestingly, modifications to the hexahydroquinolone scaffold of **52** had profound effects on its activity at FFA3. Such profound differences in allosteric ligand function are not uncommon and have at times been described as "chemical switches" to indicate that small modifications in certain parts of

a molecule will switch a compound from agonism to antagonism or from a PAM to a NAM.[299] In the case of the FFA3 ligands, inclusion of a 2-bromophenyl group as in **53** resulted in a compound that maintained its PAM activity but lost all intrinsic agonism; by contrast, 3- or 4-phenoxyphenyl compounds, like **54**, displayed NAM effects on C3 efficacy while still maintaining their PAM effect on C3 affinity (Figure 24).[298] As a result, **54** was described as a FFA3 PAM antagonist, having the interesting property of becoming a higher affinity antagonist with increasing concentrations of orthosteric agonist. Engelstoft et al. used one of these compounds as a FFA3 antagonist to confirm that FFA3 was not involved in SCFA-mediated inhibition of ghrelin secretion.[259] Although this series of compounds represents the best synthetic FFA3 ligands currently available, the utility of these compounds in vivo remains highly questionable due to their modest potency. Further, given that small chemical modifications have profound effects on activity, it would be very difficult to predict, therefore, the biological effects of metabolic products of these compounds. Considering these factors there is a clear need to develop novel and better pharmacological tool compounds to assess the functions of FFA3.

4. FFA4

In 2005 Hirasawa and colleagues reported that the orphan GPCR, GPR120, was a second GPCR responsive to LCFAs.[300] This receptor was activated by a wide range of LCFAs and, in particular, by the PUFAs. The identification of GPR120 as a receptor for free fatty acids was, perhaps, somewhat unexpected, however, as it is only distantly related in terms of sequence identity to the other FFA-responsive GPCRs. Regardless, many studies have now clearly demonstrated that GPR120 is responsive to LCFAs, and as such, it is now officially designated FFA4 and as a member of the FFA family of receptors.[100]

Much like FFA1, the primary G protein-mediated signal transduction pathway of FFA4 is reported to be $G_{q/11}$ (Figure 25). Activation of FFA4 has been widely reported to link to
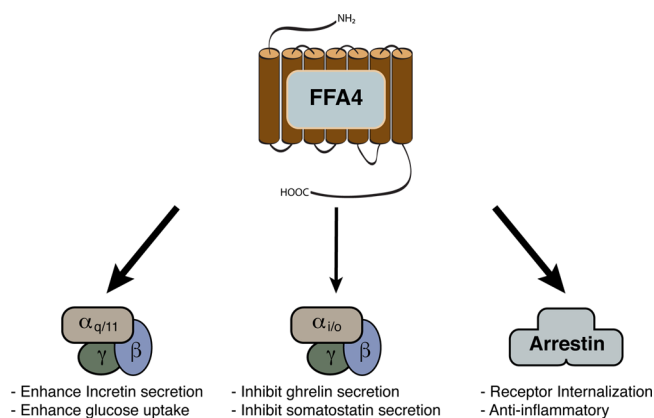
Figure 25. Signaling pathways and their associated biological outcomes for the FFA4 receptor.

increases in intracellular $[Ca^{2+}]$,[130,203,300−303] and this is blocked by the $G_{q/11}$-selective inhibitor YM-254890.[203] As with FFA1 there are also reports that certain signals relevant to the physiological role of FFA4 reflect activation of "$G_{i/o}$"-family G proteins. For example, FFA4-mediated inhibition of ghrelin secretion from ghrelin-containing mouse gastric cells[259] as well

as inhibition of somatostatin release from pancreatic islet delta cells[304] are blocked by pertussis toxin treatment, implicating such a pathway.

In the initial studies of Hirasawa et al. a key assay used to identify LCFAs as agonists of FFA4 was internalization of the receptor from the surface of transfected cells.[300] Indeed, FFA4 internalizes rapidly and dramatically upon agonist binding.[203,303] This is correlated with robust, agonist-induced interaction of the receptor with arrestins 2 and 3, a process that is defined largely by the phosphorylation status of a series of serine and threonine residues within the intracellular C-terminal tail of the receptor.[203,305−309] These modifications can involve contributions from various kinases, including both G protein-receptor kinases (GRKs) and protein kinase C.[307] The agonist-induced interaction between FFA4 and arrestin 3 is sufficiently robust that this is also used routinely as a means to assess chemical ligands for agonist activity at the receptor.[118,129,203,303,310,311] As scaffolding of arrestins and other cellular adaptor proteins can generate noncanonical signals that are distinct and separate from those that reflect G protein activation, Prihandoko et al. explored which signals persisted and which were ablated when various G proteins were blocked in CHO cells transfected to express murine FFA4.[309] Phosphorylation of the kinase Akt was unaffected by these manipulations, while in equivalent cells expressing a mutant form of FFA4 that is unable to be phosphorylated in response to agonist activation, signaling to Akt was partially blocked.[309] More importantly, there are also indications that arrestin 3-mediated signaling of FFA4 plays a key role in the function of this receptor. Specifically the effects of FFA4 activation in macrophages, acting to regulate the production of inflammatory mediators, are sensitive to knockdown of arrestin 3, while knockdown of $G_{q/11}$ did not produce this effect.[312]

4.1. Expression of FFA4

Initial studies exploring the expression of FFA4 in human, mouse, and rat found the highest levels in the lower intestine, lung, spleen, and adipose tissue.[300,313,314] Detailed examination of FFA4 expression in the intestine indicated that FFA4 is expressed by the enteroendocrine cells on each of the L, K, and I cells.[125,300,315] At least one study has also shown more widespread expression of FFA4 within the intestine, extending to the intestinal epithelial cells in addition to the enteroendocrine cells.[316] Interestingly, several factors seem to regulate FFA4 expression in the intestine, and these may be species and/or strain dependent. Most notably, FFA4 expression was upregulated in diet-induced obesity (DIO) rat models,[316,317] while it was downregulated in C57Bl6 mouse DIO models.[318] In studies measuring expression of FFA4 mRNA in human duodenum, a direct correlation was observed between body mass index and FFA4 transcript levels,[319] seeming to match well with the observations in DIO rats. Interestingly, FFA4 expression levels in cultured Caco-2 intestinal epithelial cells appear to be regulated by different species of gut microbiota, perhaps suggesting that FFA4 may have an indirect role in linking gut microbiota to health.[320]

The presence of FFA4 in adipose tissue is also now well established, with expression increasing during adipocyte differentiation in both mouse and human cells.[312,314,321] Alterations in FFA4 adipose expression have been reported in obese individuals; however, these studies have generated conflicting results, with one suggesting upregulation[322] and the other downregulation[323] of FFA4 in obesity. Obesity is often associated with systemic inflammatory state, and FFA4 appears to play an important role linking inflammation to obesity because FFA4 expression on human adipocytes appears to be significantly upregulated by exposure to macrophage secretions.[324] FFA4 is also expressed on multiple immune cell types, but to date the most widely studied of these have been monocytes and macrophages.[312,325]

Despite showing some of the highest levels of FFA4 expression, few studies have assessed the function of FFA4 in lung. To date, a single study has suggested FFA4 is expressed specifically in airway smooth muscle,[326] and significantly more work will be needed to establish both the full cell-type distribution and the biological function of FFA4 in lung. FFA4 is expressed in pancreatic islets, with limited reports finding expression and regulation of hormone release in each of the α,[327] β,[328] and δ[304] cell types. Importantly, a systems genetics approach also found that FFA4 expression in human islets is positively correlated with insulin secretion and content and may protect against lipotoxicity.[329]

Interestingly, FFA4 expression has also been demonstrated on taste buds,[330,331] where some studies have suggested it plays a role in fat taste preference.[331,332] However, several other studies have found that although FFA4 is expressed in taste buds and its expression levels are regulated by dietary LCFAs,[333,334] FFA4 itself is not the primary receptor for oral fat taste preference.[333,335,336] Instead, a fatty acid transporter, CD36, was found to mediate oral fat taste preference.

FFA4 has also been reported in a number of cancers and cancer cell lines. While studies in prostate cancer cell lines have suggested FFA4 to have protective anticancer effects,[337] in pancreatic cancer cell lines FFA4 enhanced motility, invasion, and tumorigenicity.[338] Most interestingly, FFA4 expression appeared to be significantly induced in human colorectal carcinoma cells and tissues, and indeed, FFA4 appears to function as a tumor-promoting receptor in these cells.[339] Taken together, these findings suggest FFA4 may represent a target for novel anticancer therapeutics, but the mode of action (agonism vs antagonism) is not yet entirely clear and may vary between specific cancer types.

4.2. FFA4 Splice Variation

FFA4 can exist as multiple splice variants, although this is apparently species dependent. Humans express both "long" and "short" forms that differ by a 16 amino acid insertion in the third intracellular loop of the long form.[302] Interestingly, the long isoform was shown to be unable to elevate intracellular $[Ca^{2+}]$ and, therefore, presumably is unable to interact effectively with $G_{q/11}$-family proteins (Figure 26).[303] There are no specific data at this point that relate to the ability of this isoform to interact with other G protein classes. Although not fully explored at this point, the long isoform may display rather limited tissue expression patterns compared to the short isoform.[331] Indeed, in these studies the long isoform was only detected in colon. At odds with the studies of Watson et al.,[303] Galindo et al. did observe a capacity of the long isoform to cause Ca^{2+} elevation in response to various fatty acids, and in the cases of the more high efficacy ligands, 18:2 and 18:3, differences between the splice variants were negligible.[331] By contrast, for MCFAs including 8:0 and particularly 10:0, activation via the long isoform was substantially less effective.[331] It must be noted, however, that while the studies by Galindo et al. cotransfected a very uncommonly used and non-native G protein chimera in order to generate strong signals,[331] Watson
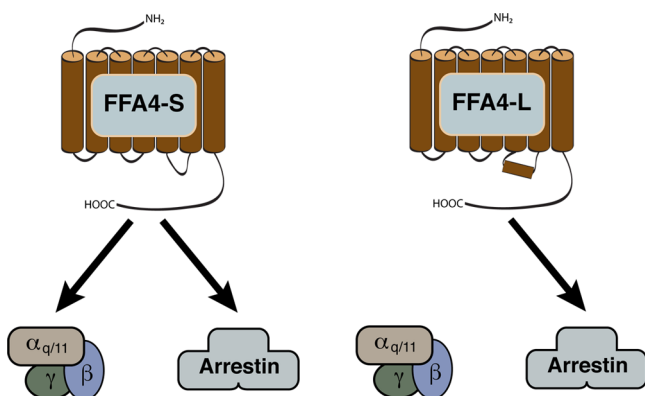
Figure 26. Comparison of FFA4 splice variant signaling. In human FFA4 exists as two distinct isoforms, distinguished by the presence or absence of 16 amino acids within IL3. While the more common short isoform couples effectively to both $G_{q/11}$ and arrestin pathways, the long isoform couples only to arrestin pathways.[291]

et al. did not.[303] In contrast to humans, rodents appear to have only a single variant that corresponds to the short isoform of human FFA4, and this is also the case for cynomolgus monkey.[302] As such, the functional and physiological importance of the long isoform remains uncertain.

4.3. FFA4 Genetic Polymorphisms

Like FFA1, FFA4 has received significant interest as a potential target for metabolic disease. In the case of FFA4 this has been driven largely by genetic evidence linking FFA4 dysfunction in both mouse and human to obesity and insulin resistance.[322] Most notably, a single-nucleotide polymorphism in FFA4 that produces the R254H^{IL3} variant in the short isoform of FFA4 (or the R270H^{IL3} variant in the long isoform) has been linked to obesity in a European population.[322] Early studies suggested that the R270H^{IL3} version of this polymorphism was nonfunctional, and loss of function in a $G_{q/11}$-dependent Ca^{2+} assay was demonstrated.[322] However, more recently an examination of the effect of this mutation on the more common short FFA4 isoform demonstrated that although the R254H variant did display reduced function in both $G_{q/11}$- and $G_{i/o}$-FFA4 signaling, clearly some function remained.[340] Further, this study found that FFA4-arrestin interactions were not altered at all in this variant,[340] which given the link between FFA4-arrestin signaling and inflammation[312,325] may indicate that the R270H/R254H variant will affect the metabolic but not the anti-inflammatory effects of FFA4. It should also be noted that one follow-up study using a European population found that although this polymorphism did appear to link with fasting glucose levels, no link was found to type II diabetes,[341] while another using specifically a Danish population found no link to either glucose levels or type II diabetes.[340] Further, this polymorphism is rare, and its frequency varies with population. For example, in a European population a minor allele frequency (MAF) of 3% was reported, while in a Japanese population only a single heterozygous carrier for the allele was identified in a study of 1500 people, equating to a MAF less than 0.001%. Taken together, although potentially very interesting, significantly more work is needed to understand the pharmacology and function of this and other FFA4 polymorphisms and their influence on metabolism and inflammation.

4.4. LCFAs at FFA4

Although it has become popular to describe FFA4 as the receptor for $n-3$-PUFAs[312,342] and the initial studies of Hirasawa et al. did indicate that certain $n-3$-PUFAs, e.g., aLA acid (18:3$n-3$), were among the most potent LCFAs,[300] more extensive studies provide only modest support for this idea. For example, analysis of the widest group of fatty acids yet reported found that nearly all MCFAs and LCFAs tested had potencies falling within the range of 1–20 μM, and indeed, even the SFAs, which typically have not been associated with FFA4 agonism, have comparable potency to the $n-3$ PUFAs at FFA4.[129] However, it must be noted that although the potency does appear to be broadly similar across the MCFAs and LCFAs, efficacy differences among the fatty acids are apparent. Most notably the SFAs have consistently been found to be partial agonists at FFA4 compared to the PUFAs.[129,327,331] Given these efficacy differences, it is therefore likely that SFAs and PUFAs will produce different biological responses through FFA4, and indeed, this is likely to have contributed to the continued view that FFA4 is primarily a receptor for PUFAs. A recent study has also suggested that FFA4 is activated by a novel class of branched fatty acid, the FAHFAs.[25] However, this work has yet to be replicated, and given the low abundance of FAHFAs in biological systems and their poor potency at FFA4, the importance of FAHFAs as FFA4 ligands remains uncertain.

As with FFA1, the carboxylate of fatty acids appears to be integral to recognition of the MCFAs and LCFAs by FFA4. This was demonstrated by initial studies testing the methyl ester of aLA, which was found to be inactive.[300] Likewise, alcohols also appear to be unable to activate FFA4, as both oleyl alcohol and linoleyl alcohol were unable to activate FFA4, despite their corresponding fatty acids, oleic acid (18:1$n-9$) and linoleic acid (18:2$n-6$), each having good activity.[331] FFA4 shares very little sequence identity with FFA1 and does not possess the same key positively charged residues shown to coordinate orthosteric ligand binding to FFA1. Instead, it appears that the carboxylate of LCFAs interacts with FFA4 through Arg99$^{2.64}$. Mutation of this residue has repeatedly been shown to result in a complete loss of LCFA function.[203,303,310]

4.4.1. LCFAs, FFA4, and Gut Hormone Secretion. Much of the initial focus examining the biological function of LCFAs through FFA4 was on a role in GLP-1 secretion from enteroendocrine cells. In particular, the initial study deorphanizing FFA4 as a receptor for LCFAs also reported that LCFAs stimulated secretion of GLP-1 from the STC-1 mouse enteroendocrine cell line, and this effect was reduced by FFA4 knockdown.[300] Several in vitro studies have reproduced similar effects in mouse and human enteroendocrine cells,[301,310,343] although in some studies FFA4 involvement has only been implied through pharmacological interventions and not through genetic knockdown. In contrast, studies that have attempted to determine whether FFA4 stimulates GLP-1 secretion in vivo have generated mixed results. While some studies have found that orally administered LCFAs stimulate GLP-1 secretion,[200,313] the only study employing genetic knockout indicated that the effect was mediated by FFA1 and not FFA4.[200] In addition to a possible role in stimulating GLP-1 secretion from enteroendocrine cells, one study has also suggested that FFA4 is responsible for LCFA-mediated protection from apoptosis in these cells.[344] Finally, while GLP-1 secretion is classically associated with enteroendocrine cells, pharmacological evidence also appears to suggest that LCFAs can stimulate GLP-1 secretion from mouse taste buds

through activation of FFA4.[345] In addition to GLP-1, FFA4 has also been implicated in LCFA-mediated secretion of CCK from STC-1 cells.[346] However, a subsequent in vivo study concluded that LCFA-mediated CCK secretion was most likely via FFA1 and not FFA4.[124] In contrast, LCFA-stimulated GIP secretion in vivo does appear to be through FFA4 because it was eliminated in FFA4$^{-/-}$ mice.[315]

The hunger regulating gastric hormone, ghrelin, is also regulated by LCFAs through FFA4. However, unlike GLP-1, CCK, and GIP, ghrelin secretion is negatively regulated by the LCFAs[347] and inhibition of ghrelin secretion in vitro by LCFAs has been linked to FFA4 both through pharmacological approaches and siRNA knockdown.[259,348] Although LCFAs have been found to inhibit ghrelin secretion in vivo and this has been suggested to be via FFA4,[347] the only study utilizing FFA4$^{-/-}$ mice to assess this found that, at least when delivered orally as triglycerides in olive oil, FFA4 was not involved in regulating in vivo ghrelin secretion.[259] Interestingly, the signal transduction pathway leading to FFA4-mediated inhibition of ghrelin in vitro appears to be via $G_{i/o}$,[259] while the pathways leading to the stimulation of GLP-1 and other hormone secretion are likely through $G_{q/11}$. At present it is unclear what factors differ between cell types allowing FFA4 to couple to $G_{q/11}$ in enteroendocrine L, K, and I cells yet to $G_{i/o}$ in the gastric ghrelin cells.

4.4.2. LCFAs, FFA4, and Adipocyte Function. FFA4 is expressed in adipocytes and appears to increase in expression during adipocyte differentiation. This observation led Gotoh et al. to consider whether FFA4 may play a role in adipogenesis, finding that adipocyte markers and lipid accumulation in 3T3-L1 cells were significantly reduced by siRNA knockdown of FFA4.[321] Similarly, adipogenesis has also been shown to be inhibited in cells derived from FFA4$^{-/-}$ mice.[322] To establish the function of FFA4 in mature adipocytes, the primary focus has been on how LCFAs acutely enhance glucose uptake via FFA4 in cultured 3T3-L1 adipocytes.[203,312] This effect of the LCFAs resulted from GLUT4 translocation to the adipocyte membrane and appears to be mediated by FFA4-$G_{q/11}$ signaling.[312] Longer term siRNA knockdown studies in 3T3-L1 cells have found that FFA4 may also regulate the expression levels of key glucose metabolism genes, including GLUT4.[349]

In addition to effects on glucose metabolism, the $n-3$ fatty acids in particular also produce anti-inflammatory effects in adipocytes, for example, inhibiting lipopolysaccharide (LPS)-stimulated release of the pro-inflammatory adipokines: monocyte chemoattractant protein-1 (MCP)-1 and IL-6.[350] While similar effects were observed with a synthetic FFA4 agonist,[350] significantly more work is needed to directly confirm the involvement of FFA4 and determine the signaling pathways mediating these effects. Finally, the $n-3$ PUFA eicosapentaenoic acid (EPA) also appears to regulate expression of vascular endothelial growth factor-A in 3T3-L1 adipocytes through activation of both FFA4 and PPARγ,[351] which may be important to the vascularization of adipose tissue.

4.4.3. LCFAs, FFA4, and Inflammation. The anti-inflammatory properties of the $n-3$ PUFAs are now very well established,[352] and a significant amount of effort has been aimed at determining if these effects are mediated by FFA4.[353] The first key finding in this area came with the observation that LPS-stimulated secretion of the cytokines TNF-α and IL-6 in RAW264.7 macrophages was inhibited by DHA and that this was dependent on FFA4.[312] To explore the mechanism underlying this effect, Oh et al. looked at the transforming

growth factor-β-activated kinase 1 (TAK1) signaling pathway leading to NFκB activation. To activate this pathway TAK1 needs to interact with TAK1 binding protein (TAB1), and it was found that FFA4 activation inhibited this by causing arrestin 3 to sequester TAB1, thus preventing its interaction with TAK1. A subsequent study looking further downstream of FFA4-DHA signaling in macrophages found that inhibition of NFκB leads to a downregulation in cyclooxygenase 2 (COX2) expression and a reduction in the levels of prostaglandin E$_2$ (PGE$_2$).[354] This was also found to be arrestin 3 dependent. Hence, the general consensus is that anti-inflammatory effects of FFA4 are arrestin but not G protein mediated. Contradicting this central hypothesis, a study by Liu et al. found that anti-inflammatory effects of DHA were mediated by both $G_{q/11}$- and arrestin-3-dependent FFA4 pathways.[355] Confusingly, this study found the anti-inflammatory FFA4-$G_{q/11}$ pathway involved activation of cytosolic phospholipase A2 (cPLA2), which in turn activated COX2, leading to an increase in PGE$_2$.[355] This is in direct contrast to an earlier study suggesting DHA reduces COX2 expression and PGE$_2$ levels.[354] Although at present it is difficult to fully reconcile these two findings, it must be noted that PGE$_2$ is known to serve both pro- and anti-inflammatory roles[356] and that the experimental conditions of these two studies were markedly different. In particular, the study indicating DHA increased PGE$_2$ levels measured relatively short-term effects (6 h),[355] while the study demonstrating decreased PGE$_2$ assessed longer treatments (24 h).[354]

DHA has also been shown to inhibit the inflammasome, a protein complex involved in the maturation of the pro-inflammatory cytokine IL-1β, in both human THP1 and mouse bone marrow-derived macrophages.[357,358] The effect of DHA was partially reduced in macrophages derived from arrestin 3$^{-/-}$ mice, suggesting that although arrestin 3 is involved there are other pathways contributing to the effect as well.[358] Interestingly, in these studies although the $n-3$ PUFA, DHA, produced inhibition, the $n-6$ and $n-9$ LCFAs tested did not.[358] Considering that all of these LCFAs are able to activate FFA4 with similar potency,[129] it is difficult to understand immediately why DHA is able to produce an effect while the others are not. Similarly, in a study assessing TNF-α release from mouse RAW264.7 macrophages it was found that aLA was not as effective at inhibiting LPS-stimulated TNF-α release as DHA,[203] and here both aLA and DHA are $n-3$ PUFAs. Taken together, such observations that suggest apparently unique effects of specific $n-3$ fatty acids in producing anti-inflammatory effects via FFA4 do not fully match the current understanding of the basic pharmacology of these ligands at FFA4. It will be important for future studies to assess in more detail whether individual, specific $n-3$ PUFAs activate unique signaling pathways via FFA4, serving in effect as endogenous "biased" agonists of FFA4, or if other mechanisms account for their unique effects.

Given the established link between inflammation and insulin resistance,[359] it is not surprising that several studies have also examined whether the anti-inflammatory effects mediated by FFA4 may link to altered insulin sensitivity. A key finding in this area is the observation in two independent studies that FFA4$^{-/-}$ mice fed a high-fat diet developed insulin resistance.[312,322] Importantly, in one of these studies the ability of diet supplementation with $n-3$ PUFAs to improve insulin resistance was absent in the FFA4$^{-/-}$ mice, suggesting FFA4 activation by these compounds improved systemic insulin

sensitivity.[312] However, conflicting studies have found no effect of FFA4 genetic knockout on insulin resistance[327,360] and that any effects of the n−3 PUFAs on insulin sensitivity are not mediated by FFA4.[360] Although more work is needed to address these discrepancies, it must be noted that the FFA4$^{-/-}$ mice from these studies were from different genetic backgrounds, and as noted earlier in relation to FFA2 and FFA3, this may have contributed to the different observations.[360]

4.5. Synthetic Ligands for FFA4

Developing potent and selective ligands for FFA4 has proved challenging. In particular, despite sharing very limited sequence similarity with FFA1, it has been difficult to identify ligands that are highly selective for FFA4 over FFA1. Considering that the majority of current biological evidence has suggested that agonism of FFA4 would be beneficial for the treatment of metabolic and/or inflammatory disease,[1−4,7] the majority of work to date has focused on identifying synthetic FFA4 agonists. Indeed, several chemical classes of FFA4 synthetic agonist have now been reported with at least some level of selectivity for FFA4 over FFA1. By contrast, only a single, poorly characterized compound has been described to date as an FFA4 antagonist.

4.5.1. Orthosteric Agonists. Because LCFAs activate both receptors, characterization of synthetic agonists at FFA1 generally also assess potential activity at FFA4 in parallel. Thus, although GW9508 (**1**; Figure 9) is markedly selective for FFA1, as noted earlier, this ligand is certainly a moderate potency agonist of FFA4 as well.[130,203] Thus, in a number of studies on the functional roles of FFA4, GW9508 has been used as agonist if expression of FFA1 could not be detected in the relevant cells or tissue and/or if knockdown of FFA4 mRNA levels provided support that GW9508 was acting as an agonist of FFA4 in that setting.[303,312,328,337,348,355,361−364]

Efforts to identify ligands selective for FFA4 were initiated by modification of known PPARγ active chemicals, with NCG21 (**55**) (Figure 27), being described as having modest selectivity for FFA4 over FFA1 and a lack of activity at PPARγ.[365] A follow-up study focused on NCG21 and developed a receptor homology model that was able to predict the correlation between calculated binding affinity of a ligand series to FFA4 and the ability of the ligands to activate ERK1/2 MAP kinases in FFA4 expressing cells.[366] A screen of a number of naturally derived molecules highlighted grifolic acid (**56**) as a modest potency, partial agonist of FFA4 but with limited activity at FFA1.[301]

The first major advance in identifying a highly potent and selective FFA4 agonist reported in the primary literature was the identification and development of TUG-891 (**57**), derived from an initial series of dihydrocinnamic acid-based ligands that have activity at FFA1.[118] In an assay based on recruitment of arrestin 3 to agonist-occupied human FFA4 TUG-891 was a potent full agonist that displayed greater than 1000-fold selectivity over FFA1. Although somewhat less selective in Ca^{2+}-based assays, TUG-891 still displayed >50-fold selectivity over FFA1 and was slightly more potent at mouse FFA4 than at the human ortholog.[118] Interestingly, despite the high potency of TUG-891 at mouse FFA4, it also displays increased potency at mouse FFA1,[203] and, therefore, in mouse this ligand acts essentially as a dual FFA4/FFA1 agonist, at least in end points that reflect Ca^{2+}-mediated signaling.

Although the patent literature discloses a somewhat broader range of FFA4 active ligands,[193,367] the details of function,
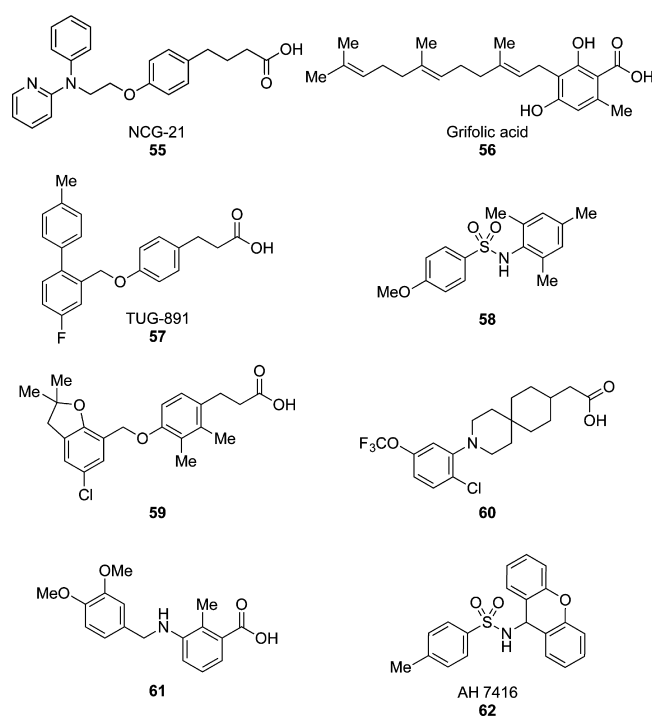
Figure 27. FFA4 ligands.

selectivity, and potency of many of the chemicals described have not been reported to date in peer-reviewed studies. However, a distinct class of diarylsulfonamide-based FFA4 ligands, identified initially in a high-throughput screen, has been reported and described by GlaxoSmithKline.[368] These lack the carboxylate moiety long considered a hallmark of agonists at free fatty acid receptors. Moreover, compounds such as **58** showed good selectivity against the other fatty acid receptors and activity across key species orthologs of FFA4 but were found unsuitable for in vivo studies because of poor solubility.[368]

A further markedly selective FFA4 ligand is **59**,[369] which is reported to have high potency and to be more than 1000-fold selective for this receptor over FFA1.[259] This compound inhibited release of ghrelin with micromolar potency, an effect lacking in cells derived from FFA4 knockout mice, and inhibited release of somatostatin from primary mouse gastric mucosal cells.[370] **60** (Figure 27) has been reported as an orally available, small-molecule FFA4 agonist also with high selectivity for FFA4 over FFA1.[325] This compound displayed high potency at human and mouse orthologs of FFA4 in a G$_{q/11}$-dependent assay system, while somewhat lower potency was observed in an arrestin 3 interaction assay.[325] In addition to being relatively potent and selective for FFA4, compared to DHA, **60** also appeared to have significantly greater efficacy when assessed in a serum response element promoter-based assay.[325] More interestingly, in mice fed a high-fat diet, **60** improved glucose handling in oral GTT in wild-type but not FFA4$^{-/-}$ mice. Treatment also decreased hyperinsulinemia, increased insulin sensitivity, and decreased hepatic steatosis. Moreover, as FFA4 in macrophages is known to provide anti-inflammatory signals (see section 4.4.3), **60** also inhibited macrophage chemotaxis, altered M1/M2 polarization, and modified expression of a range of inflammatory markers.[325] Such characteristics, if replicated in man, will provide strong

Figure 28. Similar binding poses for TUG-891 and **60** but not GW9508 in a FFA4 homology model. Binding poses of TUG-891 and **60** within the FFA4 homology model described by Hudson et al.[298] are sh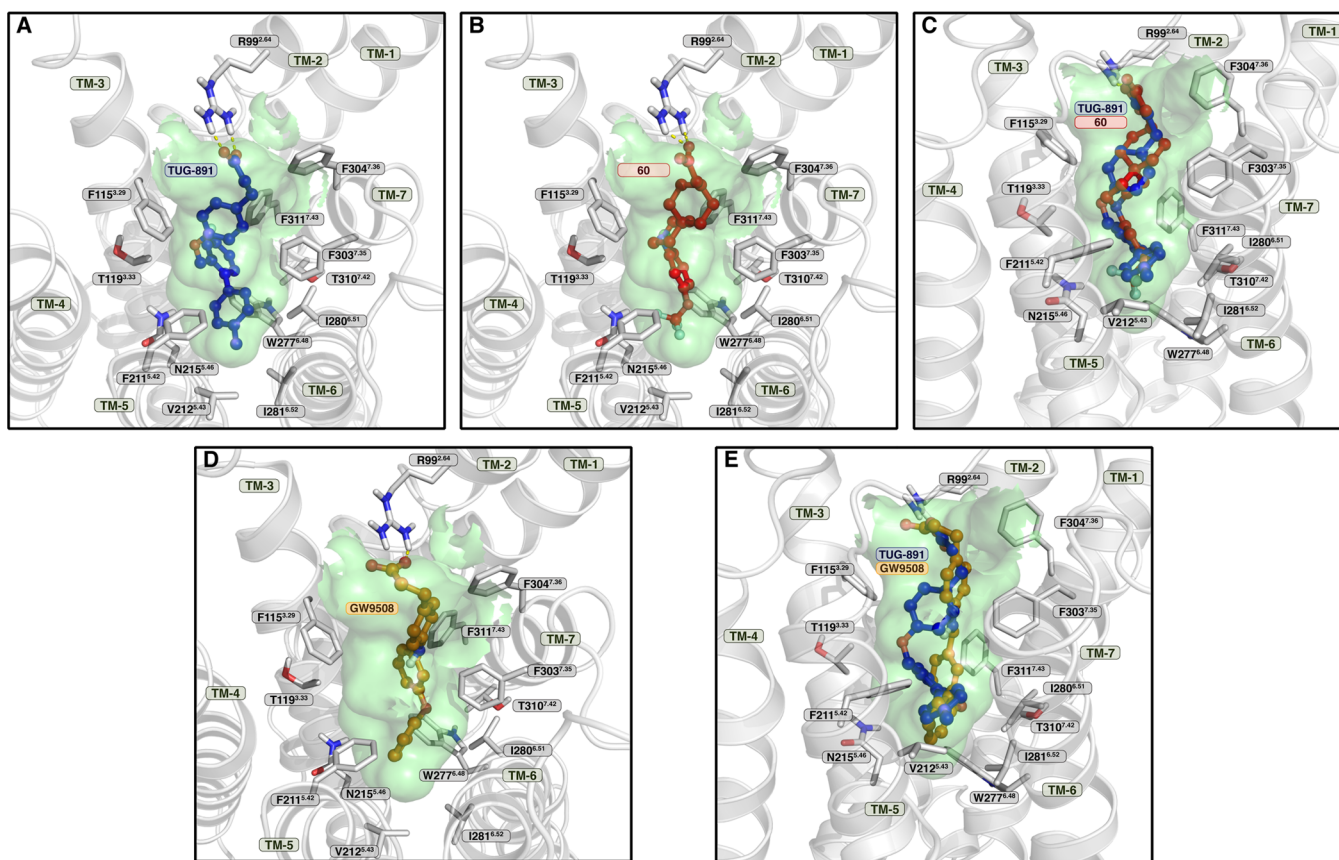own both independently (A and B) and superimposed (C). The binding pose of GW9508 is also shown independently (D) and superimposed with TUG-891 (E). Key residues lining the binding pocket that affected TUG-891 potency when mutated to alanine are shown and labeled in gray, while the surface area of the pocket is shown in green.

support for the concept that agonism of FFA4 would be of considerable benefit in treatment of type II diabetes.[1,193,371−373]

4.5.2. Mode of Ligand Interaction with FFA4. The molecular basis for agonist interaction with FFA4 has been explored in some detail through both molecular modeling and mutation studies.[310] As no crystal structure of FFA4 has been published to date, a homology model was constructed using the crystal structure of a nanobody-stabilized active state of the β_2-adrenoceptor (PDB code 3P0G)[374] as template with the sequence of the human FFA4 short isoform. The model was used in combination with mutagenesis data and ligand SAR studies to probe the mode of ligand binding. The studies were performed using aLA, the widely reported FFA1/FFA4 agonist **1**, as well as the potent and selective FFA4 agonist **57** and its analogs. Interestingly, unlike FFA1, this model suggests FFA4 ligands bind within the core 7TM structure of the receptor. Indeed, comparing the binding pose of two of the key synthetic FFA4 agonists, **57** and **60**, suggests that despite structural differences these compounds adopt very similar binding poses (Figure 28A−C).

As FFA4 is distantly related to FFA1−FFA3, as noted earlier, it does not have the positively charged arginine residues near the top of the TM domains at position 5.39 and 7.35 present in the other members of the FFA receptor family. However, a positively charged Arg99[2.64] residue located at the top of TMII has been implicated in several studies as a critical residue involved in interaction between FFA4 and the carboxylate moiety of many ligands.[303,310,366] On the basis of this key

residue, several models of the putative FFA4 orthosteric binding pocket have been constructed. The binding pocket was indicated to be located between TMII, TMIII, and TMV−VII. Docking of ligands into the refined binding pocket resulted in strong correlation between observed potency in a receptor-arrestin-3 interaction assay and calculated relative binding energies, suggesting the model to be robust, as least for the chemical series studied.[310] Alanine mutations at several residues predicted by the model to be in close proximity to the binding pose of **57** revealed several key residues lining the orthosteric binding pocket. Among these W104A, F115A[3.29], F211A[5.42], W277A[6.48], and F304A[7.36] completely abolished response to all tested ligands in the receptor-arrestin-3 interaction assay.[310] In support of previous studies described earlier the model effectively predicted hydrogen-bond interactions between the carboxylate moiety of the ligands and the positively charged residue Arg99[2.64].[310] The binding pose of **57** and other ligands from the same chemical series highlighted hydrophobic interactions between the phenylpropionate moiety of the ligands and aromatic residues, including Phe115[3.29], Phe303[7.35], and Phe304[7.36], of the receptor. The *o*-biphenyl moiety was surrounded by a narrow binding pocket lined by residues Phe88[2.53], Thr119[3.33], Gly122[3.36], Phe211[5.42], Val212[5.43], Asn215[5.46], Trp277[6.48], Ile280[6.51], Ile281[6.52], and Thr310[7.42]. However, docking of GW9508 into the refined model suggested a slightly different mode of binding within the narrow hydrophobic binding pocket (Figure 28D and 28E). GW9508 (**1**) exhibited $\pi−\pi$ stacking interaction with

Phe311[7.43], suggesting the importance of Phe311[7.43] for the binding of **1**. However, Phe311[7.43] failed to show prominent interactions with both the PUFA aLA and other synthetic ligands used in the study. Results from mutagenesis of predicted key residues in the receptor-arrestin-3 interaction assay were essentially reproduced in a $G_{q/11}$-dependent G-protein-mediated Ca^{2+} mobilization assay, which suggest that binding of ligands within the pocket predicted from the model would probably not display bias in arrestin vs G protein-mediated signaling. It is interesting, therefore, that Li et al. described an FFA4 agonist series, including **61** (Figure 27), suggested to be significantly biased to favor signaling via arrestin-mediated pathways.[375] Although the bias of these compounds at FFA4 has yet to be verified, it will if proven be interesting to consider how **61** docks in the model described above.[310]

4.5.3. FFA4 Antagonists. Much like FFA1, the focus on therapeutic targeting of FFA4 has been almost entirely on agonism. As such there has been little effort to identify FFA4 antagonists. To date, only a single compound, AH 7614 (**62**), has been described as an FFA4 antagonist.[368] In the reported studies this compound blocked the function of both **58** and of LA in Ca^{2+} elevation assays. It also was used to confirm FFA4 involvement in effects of **58** on both GSIS in mouse MIN6 insulinoma cells and GLP-1 release from human intestinal NCI-H716 cells[368] and shown to block agonist-mediated phosphorylation of mouse FFA4.[309] However, the pharmacology of the effect was clearly consistent with a noncompetitive mode of action, and thus, **62** most likely binds at an allosteric site on FFA4, which is yet to be defined.

5. OTHER POTENTIAL FATTY ACID-RESPONSIVE RECEPTORS

In addition to the recognized members of the FFA family of receptors there are at least three additional receptors that have been reported to be activated by fatty acids. These are the orphan receptor GPR84, reported to be activated by MCFAs, the olfactory receptor Olfr78/OR51E2, which has been found to respond to SCFAs, and the HCA_2 receptor, reported to respond to butyrate.

5.1. GPR84

Although GPR84 officially remains an orphan receptor, a number of studies have highlighted the ability of MCFAs to activate this GPCR. The significant expression profile of this receptor in bone marrow, lung, and peripheral blood leukocytes, particularly in neutrophils and eosinophils,[376] first initiated potential interest in GPR84. Deorphanization studies highlighted MCFAs with chain length between C9 and C14 as agonists,[377] with the highest potency noted for C10 and C11, while compounds longer than C14 and shorter than C9 were inactive. Susuki et al. also indicated that 2- and 3-hydroxylated forms of MCFAs are also activators of GPR84.[378] In general, GPR84 is considered a pro-inflammatory receptor,[377,378] while in GPR84 knockout mice, responses to an inflammatory insult are impaired.[379] As such, there is a potential for blockade of GPR84 to be a useful therapeutic strategy. In contrast, again based on a GPR84 knockout mouse model, Audoy-Rémus et al. suggest that blockade of GPR84 might enhance cognitive decline in diseases such as Alzheimer-type dementia,[380] so clearly much remains to be clarified and the development and use of high-affinity and selective chemical tools will be vital to do so. It is also important to note that several commonly used

inbred mouse strains carry a putative loss of function mutation in GPR84,[381] indicating selection of the appropriate mouse strain for in vivo studies on this receptor may be absolutely critical.

5.1.1. Synthetic Ligands for GPR84. Even in advance of the demonstration of MCFAs as agonists at GPR84, Takeda et al. identified both **63** and **64** (Figure 29) as low-potency
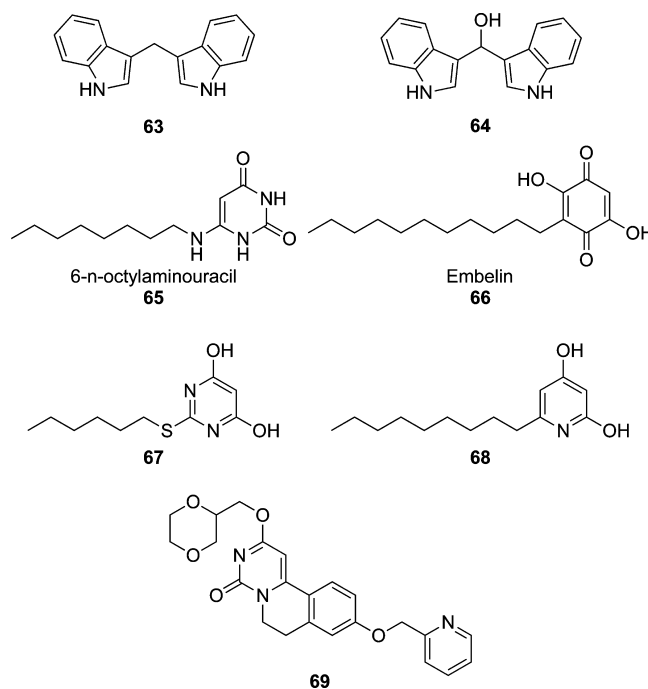
Figure 29. GPR84 ligands.

activators of this receptor (which at that time was designated *GPCR4*) using a screen in which binding of [^{35}S]GTPγS to a receptor-G_{i1} fusion protein was measured.[382] However, the poor potency (EC$_{50}$ 10−100 μM) of these ligands would appear to significantly limit their practical use in characterizing functional roles of GPR84. Despite this, **63** was shown to increase secretion of the pro-inflammatory cytokine IL-12B subunit from LPS-stimulated, macrophage-like RAW264.7 cells, suggesting a pro-inflammatory role for GPR84.[377] The identification of an additional, more potent, surrogate agonist of GPR84, **65** (6-n-octylaminouracil) has further confirmed a pro-inflammatory function for this receptor.[378] In these studies, **65** was shown to induce chemotaxis of polymorphonuclear leukocytes as well as macrophages while also enhancing LPS-stimulated IL-8 and TNF-α production.[378] In addition to these reported surrogate agonists, embelin (**66**) has also been described in the patent literature as an agonist of GPR84.[383] However, little has been described in terms of its potency at GPR84, and its likely utility as a tool compound is limited by known off-target effects, including antagonism of X-linked inhibitor of apoptosis protein (XIAP).[384] Most recently, two additional GPR84 agonists have been reported that are structurally related to embelin, **67**[385] and **68**,[386] with **68** in particular reported to have subnanomolar potency at the receptor.

Hara et al. noted in passing that derivatives of dihydropyr-imidinoisoquinolinones are GPR84 antagonists[3] and **69** is reported as a potent and selective antagonist of GPR84, inhibiting GPR84 activation in a functional GTPγS binding

assay as well as GPR84-induced neutrophil and macrophage migration.[387] Moreover, in the dextran sulfate sodium mouse inflammatory bowel disease model, **69** dose-dependently prevented disease progression.[387] Galapogos progressed this chemical into a Phase 2 proof-of-concept study in ulcerative colitis in January 2015; however, the company recently reported (but has not yet published) no clinical improvement in these studies. As Nicol et al. have shown that GPR84 knockout mice do not develop mechanical or thermal hypersensitivity subsequent to partial sciatic nerve ligation,[379] it may be interesting to examine the effect of GPR84 antagonists in models of nerve cell injury.

5.1.2. Mode of Ligand Interaction with GPR84.

At the current time insight into the basis of ligand binding to GPR84 is at an early stage. Nikaido et al. performed a limited mutagenesis and modeling study using capric acid and **63** as agonists.[388] Given the very distinct chemical structures of the two ligands, unsurprisingly, differences in the potential modes of binding were uncovered. Perhaps surprisingly, given the contribution of key arginine residues in the binding of fatty acids to all the other FFA receptors, no amino acids with fixed positive charged were identified or implicated in binding of capric acid. Two arginine residues that were mutated in these studies, R94K[3.26] and R319A[6.35], did not affect the potency of this fatty acid, while a L100D[3.32], but not a L100N[3.32], mutation largely eliminated function of capric acid but not of diindolylmethane.[388] Finally, a N357D[7.40] mutant also all but eliminated response to capric acid but not to **63**. While the above results indicate that these two ligands almost certainly interact at distinct sites on GPR84,[388] detailed examination of if and how these ligands may allosterically modulate the function of each other is currently lacking.

5.2. Olfr78/OR51E2

While historically it has only been the nonolfactory GPCRs that have received attention in drug development, there has been a growing appreciation that some designated olfactory receptors are also expressed outside the olfactory system and regulate various biological processes.[26,389−392] One such olfactory receptor expressed in kidney, mouse Olfr78 (OR51E2 in human), is reported to be activated by micromolar concentrations of the SCFAs acetate and propionate to regulate blood pressure.[274,393] Expression of this receptor has also been described in PYY-secreting enteroendocrine cells,[394] although its physiological function in these cells remains unknown. More recently, very specific expression of Olfr78 has been described in glomus cells of the carotid body, an oxygen-sensing organ in the carotid artery bifurcation.[395] However, in this study it was demonstrated that Olfr78 is activated by lactate in addition to the SCFAs, and although the potency for lactate and the SCFAs were approximately equal, this study proposed lactate as the more likely endogenous ligand, based on its higher concentrations in blood.[395] This remains an unanswered question, and it may be the case that Olfr78 responds to lactate in cells that interface directly with the blood while responding to the SCFAs in other tissues, such as the gut, where the SCFA concentrations are likely to be higher.

5.3. HCA$_2$

The HCA$_2$ receptor is a member of the hydroxycarboxylic acid family and typically is associated with β-hydroxybutyrate (**70**; Figure 30) as the endogenous agonist. However, it was earlier indicated that fatty acids between C4 and C8 have high micromolar to low millimolar potency at both human and
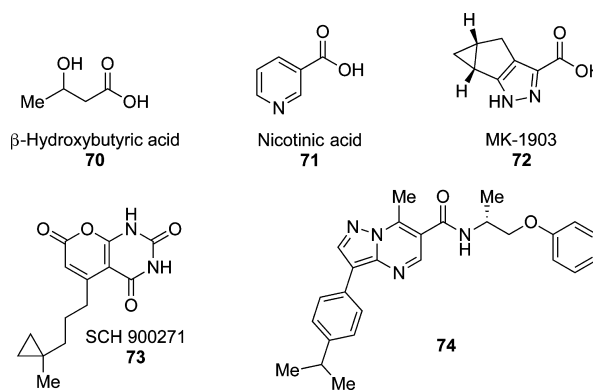

Figure 30. HCA$_2$ ligands.

mouse orthologs of this receptor.[296] HCA$_2$ is expressed highly in adipocytes, where it regulates lipolysis,[95] as well as in various immune cells and in the colon, where its activation appears to result in anti-inflammatory effects.[396] Given the relatively low potency of butyrate (C4) at HCA$_2$ and indeed a higher potency for its more traditional endogenous agonist, β-hydroxybutyrate, the importance of HCA$_2$ as a SCFA receptor remains unclear. Perhaps it is most likely that if HCA$_2$ is acting as a receptor for butyrate it would do so in the colon, where the concentration of this SCFAs is highest. Indeed, several studies have examined the biological effects of butyrate through HCA$_2$ in the colon, for example, demonstrating that the butyrate acts as a tumor suppressor in the colon via activation of HCA$_2$[397] and implicating the receptor as a metabolite sensor of dietary fiber, through its fermentation to butyrate, to regulate the inflammasome.[398] In addition to β-hydroxybutyrate and butyrate, nicotinic acid (**71**) as well as several more potent synthetic agonists, for example, **72**[399] and **73**,[400] of HCA$_2$ have been described. At least one series of allosteric agonist has been described for HCA$_2$, exemplified by **74**.[401] Although no studies have explored the basis of butyrate interaction with HCA$_2$, Tunaru et al. did explore the basis for nicotinic acid interaction with this receptor.[402] Of particular note, Arg111[3.36] appears to anchor the carboxylate of HCA$_2$ agonists in much the same way that arginine residues at positions 5.39/7.35 and 2.64 do for FFA1−3 and FFA4, respectively. To our knowledge, there are not currently any antagonists of the HCA$_2$ receptor described. Going forward, it will be important to determine whether or not HCA$_2$ represents a functional receptor for butyrate in vivo.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

In recent times understanding of the pharmacology of the GPCR family has expanded immensely. This has included an appreciation that pharmacological regulation of this important family of receptors extends far beyond simple competitive agonism or antagonism. Such concepts have been critical in uncovering the complex pharmacology of the FFA family of receptors (see Table 1) and importantly is beginning to define how these receptors may be targeted therapeutically for the treatment of metabolic and/or inflammatory disease.

Although understanding of the FFA family has expanded exponentially since their deorphanization in the early 2000s, several critical questions remain to be answered. Most notably, there are still many unresolved issues about how these receptors function in a physiological setting. For example, how do FFA1 and FFA4 produce biological responses to uncommon LCFAs, the levels of which seem to be

Table 1. Summary of GPCRs Activated by Free Fatty Acids

	fatty acids	signaling[a]	key residues[b]	key tissue expression	common biological functions	synthetic agonists	synthetic antagonists
fatty acid receptor family							
FFA1	MCFAs LCFAs	G_q, G_i, G_s, arrestin	$Arg183^{5.39}$, $Asn244^{6.55}$, $Arg258^{7.35}$	pancreas islet β-cells	enhance GSIS	orthosteric and allosteric	reported, mode of action not clearly defined
				enteroendocrine cells	stimulate gut hormone secretion		
FFA2	SCFAs	G_q, **G_i**, arrestin	$Arg180^{5.39}$, $His242^{6.55}$, $Arg255^{7.35}$	immune cells	neutrophil chemotaxis	orthosteric and allosteric	orthosteric antagonists described only for human ortholog
				enteroendocrine cells	gut hormone secretion		
				adipocytes	inhibition of lipolysis		
				pancreatic β-cells	regulation of insulin secretion		
FFA3	SCFAs	G_i	$Arg185^{5.39}$, $His245^{6.55}$, $Arg258^{7.35}$	enteroendocrine cells	gut hormone secretion	allosteric only	allosteric NAMs reported
				pancreatic β-cells	inhibition of insulin secretion		
				sympathetic ganglia			
FFA4	MCFAs LCFAs	G_q, G_i, **arrestin**	$Arg99^{2.64}$	enteroendocrine cells	gut hormone secretion	orthosteric only	single noncompetitive antagonist reported
				adipocytes	glucose uptake		
				immune cells, macrophages	inhibiting cytokine secretion		
				pancreas islets	regulating hormone secretion		
other possible fatty acid receptors							
GPR84	MCFAs	G_i	$Leu100^{3.32}$, $Asn357^{7.40}$	immune cells	pro-inflammatory	reported with mode of action not clearly defined	reported with unknown mode of interaction
Olfr78	SCFAs	G_{olf}, G_s	ND^c	kidney	regulate blood pressure	NR^d	NR^d
				enteroendocrine cells			
HCA$_2$	butyrate	G_i, arrestin	$Arg111^{3.36}$	adipocytes	inhibition of lipolysis	orthosteric and allosteric	NR^d
				colon	anti-inflammatory, tumor suppressor		

[a]Most commonly linked signaling pathways are in bold. [b]Residues reported as key to agonist function are indicated. [c]Has not been determined. [d]No ligands have been reported.

incompatible with the concentrations required to activate the receptors observed in vitro? Moreover, these uncommon LCFAs are present within a complex mixture of LCFA of which they are quantitatively minor components. For FFA2 and FFA3, it remains challenging to clearly attribute biological effects to one receptor over the other. Moreover, it still remains to be determined if GPR84, Olfr78, HCA$_2$, or any other as yet unidentified receptors also respond to FFAs in physiological settings.

In order to progress understanding of these receptors there is a clear need for better pharmacological tool compounds. In particular, antagonist ligands that show function across species and are suitable for in vivo use will be critical. For many of the receptors there still remains a strong need for novel potent and selective agonists as well, most notably for FFA3 where potent and selective orthosteric agonists remain to be described and characterized. A key goal going forward may also be to develop novel ligands with expanded chemical diversity, given that at present that vast majority of orthosteric ligands targeting the FFA receptors retain the carboxylate functionality. Such expanded chemical diversity is likely to identify ligands with novel and perhaps bias signaling properties, which may well be critical in defining how best to target these receptors to treat disease. Taken together, the complex pharmacology of the FFA

receptors continues to make this family an exciting possibility for the development of novel therapeutics to treat metabolic and inflammatory disease.

On a positive note, it is likely that clinical trials of further ligands that target FFA1 will be initiated and there is growing confidence that either selective FFA4 agonists or, potentially, molecules with combined FFA1/FFA4 activity will also be developed. Further research is needed to provide a convincing case for therapeutic intervention that might target FFA2 and/or FFA3 and indeed whether agonism or antagonist might be most effective in distinct therapeutic indications. New developments in chemical ligands targeting these receptors will be integral in providing the answers.

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Notes

Biographies

Graeme Milligan's research interests focus on the function, structure, and regulation of G protein-coupled receptors (GPCRs) and their interacting proteins. He obtained his Ph.D. degree in 1982 from the University of Nottingham; following a 3 year stay as a Fogarty International Visiting Fellow at the National Institute of Mental Health Bethesda, MD, he moved to the University of Glasgow in 1986, where he is currently Professor of Molecular Pharmacology and Gardiner Professor of Biochemistry. He was elected to the Fellowship of the Royal Society of Edinburgh in 1998. He has published more than 500 peer-reviewed articles, and his research has been cited more than 22 000 times.

Bharat Shimpukade obtained his M.Sc. degree in Organic Chemistry from University of Pune, India, in 2004. He worked as a medicinal chemist from 2005 to 2010, first at Aurigene Discovery Technologies, India, and then at Albany Molecular Research Inc., Singapore. He received his Ph.D. degree in Medicinal Chemistry (2014) from the University of Southern Denmark, under the supervision of Prof. Trond Ulven. His Ph.D. research was focused on developing free fatty acid receptor 4 (FFA4) agonists for the treatment of metabolic disorders. Currently, he is working with Prof. Ulven as a postdoctoral research fellow. His current research work is focused on developing novel agonists for the free fatty acid receptors.

Trond Ulven obtained his Doctoral Engineering degree in Organic Chemistry in 1999 from the Norwegian University of Science and Technology. He then moved to the Scripps Research Institute as a postdoctoral researcher on an NFR fellowship before relocating to Denmark in 2001 to the startup company 7TM Pharma A/S. In 2005, he joined the University of Southern Denmark, where he now is Professor of Pharmaceutical Chemistry with special responsibilities in entrepreneurship. His research interests include drug discovery and medicinal chemistry with special focus on 7-transmembrane receptors, inflammation and metabolic diseases, and development of synthetic methods aimed at drug discovery.

Brian Hudson obtained his Ph.D. degree in 2010 from Dalhousie University in Halifax, Canada. He then began a postdoctoral fellowship with Prof. Graeme Milligan at the University of Glasgow, focused on the pharmacology of the short chain fatty acid receptors FFA2 and FFA4. In 2011 he was awarded a fellowship from the Canadian Institutes of Health Research to continue his work on the FFA family, switching focus to the long chain fatty acid receptors FFA1 and FFA4. He was recently awarded funding from the University of Glasgow to work as a research fellow and build an independent research program focused on developing novel biosensor technologies to study the FFA family of receptors.

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