Pharmacological Tool Compounds for the Free Fatty Acid Receptor 4 (FFA4/GPR120)

Steffen V.F. Hansen and Trond Ulven

Contents

Abstract

The free fatty acid receptor 4 (FFA4), also known as GPR120, is a G protein-coupled receptor that is activated by long-chain fatty acids and that has been associated with regulation of appetite, release of insulin controlling hormones, insulin sensitization, anti-inflammatory and potentially anti-obesity activity, and is progressively appearing as an attractive potential target for the treatment of metabolic dysfunctions such as obesity, type 2 diabetes and inflammatory disorders. Ongoing investigations of the pharmacological functions of FFA4 and validation of its potential as a therapeutic target depend critically on the appropriateness and quality of the available pharmacological probes or tool compounds. After a brief summary of the pharmacological functions of FFA4 and some general considerations on desirable properties for these pharmacological tool compounds, the individual compounds that have been or are currently

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being used as tools for probing the function of FFA4 in various in vitro and in vivo settings will be discussed and evaluated.

Keywords
Chemical probes • FFA4 • GPR120 • Tool compound

1 Introduction

The G protein-coupled receptor (GPCR) GPR120 was deorphanized in 2005 when it was found to be activated by long-chain fatty acids (LCFAs) (Hirasawa et al. 2005). Despite a sequence only distantly related to the other free fatty acid receptors FFA1-3, GPR120 was later renamed free fatty acid receptor 4 (FFA4) (Davenport et al. 2013). FFA4 is expressed in the gastrointestinal tract, lungs, adipose tissue, macrophages, pancreas, bone-related cells, taste buds and in the brain and has since its deorphanization become subject of intensive interest (Milligan et al. 2016; Moniri 2016; Offermanns 2014; Oh da and Olefsky 2016; Ulven and Christiansen 2015). Several studies have convincingly suggested FFA4 to be a promising potential target for treatment of obesity, type 2 diabetes (T2D) and related inflammatory diseases. For example, FFA4 is reported to regulate the secretion of a range of hormones, such as glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP), cholecystokinin (CCK), ghrelin, somatostatin, and glucagon, all implicated in regulation of glucose metabolism and appetite (Ulven and Christiansen 2015). Inflammation is playing an important role in the pathogenesis of T2D and obesity (Donath and Shoelson 2011; Talukdar et al. 2011). It has been shown in several studies that activation of FFA4 leads to anti-inflammatory and insulin sensitizing effects, and it is believed that part of the established anti-inflammatory effects of omega-3 polyunsaturated fatty acids (PUFAs) derive from interaction with FFA4 (Oh da and Olefsky 2016). Many of these studies depend on the use of synthetic ligands as tools to clarify the biological role of FFA4. This chapter seeks to give an overview of the different tool compounds that have been (and continue to be) used in studies of FFA4 pharmacology. However, it is instructive to begin the chapter with a discussion on what makes a good tool compound and how a tool compound is distinct from a drug.

2 What Is a Good Tool Compound?

A tool compound or chemical probe is a small molecule that can assist researchers in making specific inquiries about a biological target. To generate trustworthy answers, there are certain requirements that the chemical probes should fulfil (Table 1) (Arrowsmith et al. 2015; Bunnage et al. 2013; Frye 2010; Kodadek 2010; Workman and Collins 2010). As the purpose of a chemical probe is to study the biological role or function of a specific target, it is required to specifically

Table 1 Generally desirable properties of tool compounds

Potency	Sufficient to ensure full target engagement at concentrations that do not cause off-target effects. Typically EC_{50} or $IC_{50} < 100$ nM
Selectivity	Sufficient to ensure full target engagement at concentrations that do not cause off-target effects. Typically >100-fold selective over off-targets
Chemical stability	Stable under the assay conditions for the extent of the study. Devoid of reactive and/or promiscuous functional groups
Solubility	Sufficient for intended use (e.g. avoid DMSO concentrations that may interfere with the assay)
In vivo pharmacokinetics	Information on bioavailability and formulation necessary for selection of appropriate dose and method of administration. Distribution and clearance properties that ensure target exposure for the appropriate period of time
Availability	Commercially or synthetically available

engage the target in question without concurrent interactions with other targets that may cause effects that can interfere with the readout and confuse the interpretation. To achieve this, the probe must be able to reach its target (e.g. it should be sufficiently soluble and chemically stable for in vitro studies), it should have sufficient in vitro potency and selectivity to specifically engage its target without causing off-target effects, and its mode of action (e.g. agonistic vs. antagonistic, orthosteric vs. allosteric, signalling bias) must be well defined. It is prudent to avoid groups generally associated with reactivity and promiscuity, including pan-assay interfering compounds (PAINS) (Baell and Holloway 2010; Pouliot and Jeanmart 2016). For in vivo studies, the compound must have a distribution and clearance profile that ensures sufficient target exposure for a sufficient period of time, implying a certain metabolic stability. The compound should not give rise to biologically active metabolites. It is usually desirable to have good oral bioavailability to avoid administration routes that can be stressful to the animal, even if intravenous (iv) or intraperitoneal (ip) administration can be viable alternatives in case of insufficient oral bioavailability.

It is important to keep in mind that a drug and a chemical probe are two very different things with different purposes. The ultimate goal for a drug is not to answer questions about biology but to elicit an effect of therapeutic value. A drug might well have several modes of action – drugs can even be designed to engage multiple mechanisms against a disease (Anighoro et al. 2014). The usefulness of a drug does not depend on a well-characterized mode of action. In contrast, the role of a tool compound is to probe the function of one specific biological target and should therefore ideally only have one well-defined mode of action. Another set of rules applies for drugs; a drug needs to have good pharmacokinetic properties and to be safe for human consumption at the dose required for the biological activity. There has previously been a mentality in drug design of "one drug, one target", which is changing to "one drug, multiple targets" (Anighoro et al. 2014; Peters 2013; Reddy and Zhang 2013). The concept of polypharmacology is used to describe such ligands with activity on more than one biological target (Anighoro et al. 2014;

Peters 2013; Reddy and Zhang 2013). Many drugs currently on the market are of polypharmacological nature, for example the new antidepressant vortioxetine (Bang-Andersen et al. 2011). In contrast, multiple activities will in most cases be inappropriate for a tool compound, with the exception of well-characterized dual activities where the study aims to investigate concomitant modulation of both targets.

An ideal tool compound will reach and specifically bind to and either activate or inhibit a specific target without causing any effect that is not target related. This task is more challenging to fulfil for in vivo studies because of the more complex biological system with a larger variety of off-targets, natural barriers, efflux pumps and metabolic enzymes designed to eliminate xenobiotics. It is rare that all these properties can be found in a tool compound. Due to the sheer number of potential biological targets, it is impossible to guarantee that a probe does not have any unforeseen off-target effects. Use of the tool in combination with knockout or gene silencing techniques is one way to avoid the risk of confusing off-target effects and build confidence and robustness into the study. Like chemical probes, the variety of techniques available to disrupt normal gene expression can have off-target effects in addition to the desired on-target effects (Boettcher and McManus 2015), however, these will typically be different from off-target effects of chemical probes and the use of both methods in parallel will usually result in more robust studies and conclusions that can be drawn with greater confidence (Workman and Collins 2010).

Another approach to add confidence to the link between target engagement and the observed effects is to use a second probe belonging to a structurally different chemical class that therefore would be unlikely to share the same activity profile (referred to as a structurally orthogonal probe) (Bunnage et al. 2013). The complementary strategy is to include a structurally close analogue of the chemical probe that is likely to have a similar activity profile but is confirmed to be devoid of activity on the target of interest. Candidates for such inactive analogues can frequently be found in the original publications describing the discovery of the probe. These approaches can preferably be used in combination.

One important point that has often been overlooked is the variability of activity between species orthologues of the protein target. Since early target validation frequently involves rodent models and many immortalized cell lines used in research are derived from rodents, a tool compound's activity in rodents is typically of equal and frequently of higher interest than its activity in humans. High potency of a compound on the human receptor is, however, no guarantee of activity on the murine or other species orthologues. Examples of this are known from ligands of free fatty acid receptor 2 (FFA2/GPR43), where the Euroscreen compound CATPB and Galapagos' GLPG0974, a previous clinical candidate for inflammatory bowel disease, both are potent on the human orthologue but devoid of activity in several other species including mouse (Pizzonero et al. 2014; Sergeev et al. 2015). GLPG0974 is therefore unusual in progressing to clinical trials without having been through studies in rodent disease models. A potent and selective tool compound in humans can also have preserved potency on the target of interest but

Table 2 Checklist for design of experiments with chemical probes

Target activity
• The potency and selectivity of the probe should be adequate for the intended study • The applied concentration should be appropriate relative to the potency of the probe on the target • Evidence that the probe engages the intended target
Specific effect
• Implementation of structurally unrelated active compounds and/or structurally related inactive compounds used to support target specific effects • Use of knockout or gene silencing techniques
Chemical quality
• Known chemical structure and confirmed identity of the probe • Confirmed purity and stability
In vivo pharmacokinetics
• Sufficient target exposure – Appropriate formulation and route of administration (e.g. oral administration requires bioavailability) – Confirmed appropriate plasma concentration and half-life • Absence of interfering metabolites

increased activity on off-targets in other species of the study, rendering the compound unselective. The FFA4 agonist TUG-891 is an example of this, as will be detailed below. It is therefore essential to characterize the tool compound on the receptor for the relevant species. The issue of potency variability between species orthologues of the FFA receptors has been discussed in detail elsewhere (Hudson et al. 2013a). Inspired by Arrowsmith et al. (2015), Table 2 provides a list of points to consider in the design of experiments with chemical probes.

3 Controlling FFA1-Mediated Effects

FFA1 is another GPCR activated by LCFAs that is also established as a target for treatment of T2D and has been connected to regulation of insulin and secretion of incretin hormones (Defossa and Wagner 2014; Milligan et al. 2015). Some degree of co-activity on FFA1 is a property shared by all FFA4-activating LCFAs (Ulven and Christiansen 2015), and is also an issue for many of the synthetic tool compounds (see below). As a strategy to control confounding effects related to co-activation of FFA1 in studies with FFA4 agonists, it is common and relevant to include control experiments using a specific FFA1 antagonist. This has been particularly relevant in conjunction with the use of probes that are equipotent on FFA4 and FFA1 or even have up to two orders of magnitude higher potency on FFA1, such as LCFAs or GW9508, respectively (see below). Several FFA1 antagonists are known (Fig. 1). The first published antagonist was GW1100 with $pIC_{50} = 5.5–6.0$ against GW9508 and PUFAs at the human FFA1. The compound is also active on the mouse orthologue, as only 1 μM GW1100 completely blocked the

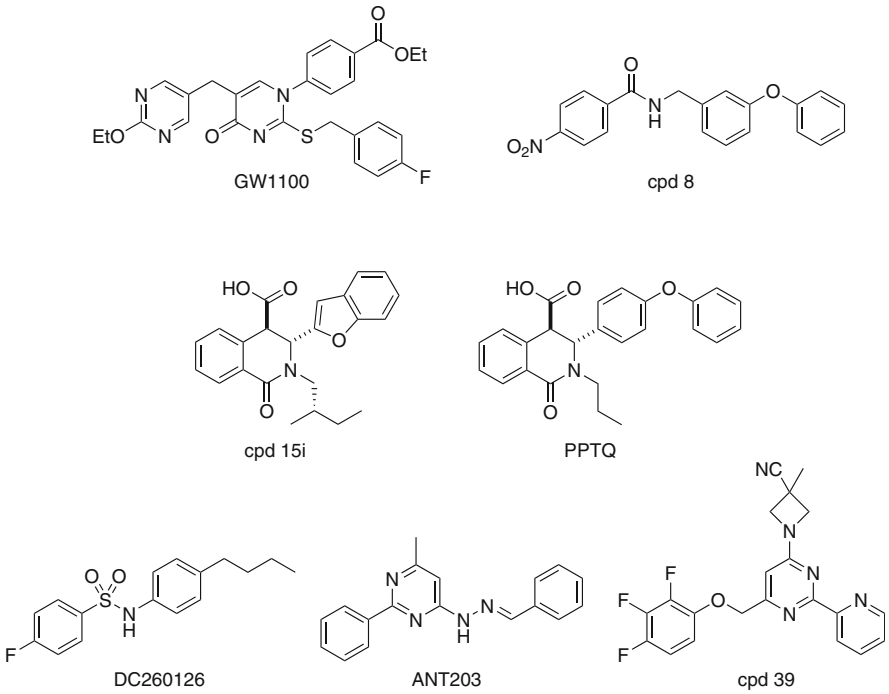

Fig. 1 FFA1 antagonists

enhancement of glucose-stimulated insulin secretion from the mouse-derived MIN6 β-cell line produced by up to 20 μM GW9508 (Briscoe et al. 2006). Importantly, GW1100 is confirmed not to affect the activity of agonists on FFA4 (Briscoe et al. 2006), however, the compound is otherwise incompletely characterized and may exert off-target activity on its own. GW1100 is not confirmed to be active in vivo and the metabolically labile ester functionality of the compound may represent a liability in this respect.

There are other FFA1 antagonists structurally orthogonal to GW1100 that are worthy of consideration. Another early antagonist is cpd 8, reported with $IC_{50} = 2.8$ μM against 100 nM GW9805 (Tikhonova et al. 2008). A series of 1,2,3,4-tetrahydroisoquinolin-1-ones (THIQs) were disclosed as FFA1 antagonists by Pfizer (Humphries et al. 2009). Optimization of pharmacokinetic properties led to identification of compound *15i*, which show satisfactory pharmacokinetic properties in rat. A representative from the series, PPTQ, has been used to specifically block FFA1 activity on mouse-derived β-cell lines and exhibited $pIC_{50} = 5.1–5.3$ in the inhibition of FFA1 agonists (Christiansen et al. 2011, 2012, 2013). These antagonists have, however, not been profiled on other receptors, including FFA4. The sulfonamide DC260126 was reported as a low potency FFA1 antagonist and has been used in vivo (Sun et al. 2013). However, as this compound recently was found to also behave as a β-arrestin-biased FFA4 agonist (Azevedo

et al. 2016), its use as an FFA1 antagonist within FFA4 studies cannot be recommended. The hydrazone ANT203 has been described as an FFA1 antagonist that at 2 μM concentration protected against palmitate-induced lipotoxicity (Kristinsson et al. 2013). However, as might have been expected from a hydrazone (Baell and Holloway 2010), the compound resulted in β-cell apoptosis above 5 μM, lacks bioavailability and is unsuitable for in vivo studies (Kristinsson et al. 2013). Waring and co-workers recently reported their discovery of compound 39, a potent FFA1 antagonist with $pIC_{50} = 6.8$ and 7.2 on the human and mouse orthologue, respectively, and good pharmacokinetic properties that makes it suitable for in vivo studies (Waring et al. 2015). Compound 39 is relatively well characterized as a chemical probe as it has been screened against 144 protein targets in the Cerep panel, unfortunately not including FFA4. Only seven targets showed measurable activity and only pig Na+/K+ ATPase had sub 1 μM activity. Compound 39 is likely to become the preferred FFA1 antagonist if lack of interference with FFA4 can be confirmed.

4 Chemical Probes for Studies of FFA4

FFA4 has seen a surge of interest in recent years, and the growing number of FFA4 ligands found in the patent literature has recently been the subject of reviews (Formicola et al. 2015; Halder et al. 2013). The following sections will discuss the compounds that have been used as tools to investigate FFA4 in vitro and in vivo and evaluate their properties as chemical probes. The current FFA4 probes (Fig. 2) can overall be classified as either carboxylic acid probes or sulfonamide probes.

4.1 Carboxylic Acid Probes

4.1.1 Long-Chain Fatty Acids

LCFAs are recognized to be the endogenous ligands for FFA4 (Davenport et al. 2013). The initial publications highlighted unsaturated fatty acids as functional endogenous FFA4 agonists and also recognized that saturated fatty acids of 14 carbon atoms or longer activate the receptor but without translation into GLP-1 secretion (Hirasawa et al. 2005). In particular omega-3 fatty acids have been associated with FFA4 and their anti-inflammatory effects have been linked to the receptor (Oh et al. 2010). A subsequent study on a broader selection of fatty acids found both saturated fatty acids with carbon chain length at least ten and unsaturated fatty acids to activate the receptor (Christiansen et al. 2015). Several studies have employed unsaturated LCFAs, typically docosahexaenoic acid (DHA) and α-linolenic acid (aLA) (Fig. 2), as tool compounds for study of FFA4 pharmacology. Initially, this was out of necessity, as no high-quality synthetic agonists were available. This is no longer the case and the use of LCFAs as exclusive pharmacological tool compounds cannot be recommended. Despite being the endogenous ligands for FFA4, LCFAs are not optimal as tool compounds owing to their low

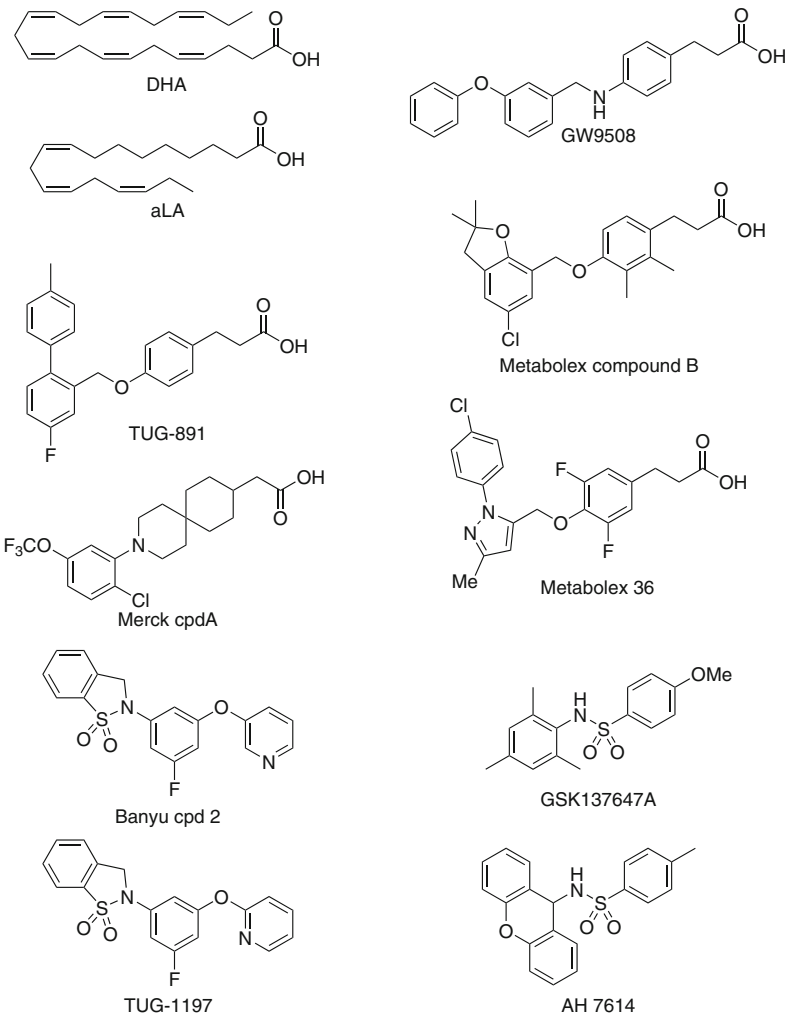

Fig. 2 FFA4 probes

potency and multiple other biological effects (Calder 2012). Due to their importance as fuel sources, the body is equipped with several proteins just for shuttling and metabolising LCFAs. Moreover, LCFAs are known to modulate several biological targets directly. For example, at the concentration needed to activate FFA4 [$EC_{50} = 4$ μM (Ulven and Christiansen 2015)], DHA also has significant activity on other targets e.g. FFA1 [$EC_{50} = 1–8$ μM (Ulven and Christiansen 2015)], retinoid X receptor α [$EC_{50} = 5–10$ μM, (Lengqvist et al. 2004)] and the peroxisome proliferator-activated receptors [$EC_{50} = 145$ nM on PPARα (Hostetler et al. 2006)]. In addition, PUFAs act as precursors for important messenger molecules with biological activity, such as the prostaglandins, leukotrienes,

lipoxins, protectins, marseins, and resolvins (Serhan 2014; Ulven and Kostenis 2006). The physicochemical properties of LCFAs, including poor solubility and the tendency to micelle formation in the relevant concentration range, also make them unsuitable as tool compounds. Nevertheless, studies with LCFAs for the elucidation of the ability of, for example, dietary-derived fatty acids to cause effects through these receptors are of high interest and relevance.

4.1.2 GW9508

The first synthetic ligand used in studies of FFA4 was GW9508 (Fig. 2), disclosed as a potent and selective FFA1 ligand a decade ago and also described in the same publication as a low potency agonist for FFA4 with approximately the same potency as the endogenous LCFAs (Briscoe et al. 2006). Despite only moderate potency on FFA4 and a 100-fold higher potency on FFA1, the compound has been frequently used as a tool compound for studies of FFA4, initially due to a paucity of better tools. GW9508 is well characterized as a chemical probe for FFA1 as it has been shown in either functional or binding assays to be at least 100-fold selective against 220 other GPCRs, 60 kinases, 63 proteases, 7 integrins and 20 nuclear receptors including PPARα, δ and γ (Briscoe et al. 2006). Unfortunately, since GW9508 is also approximately 100-fold selective for FFA1 over FFA4, little is known about the selectivity for FFA4 over off-targets. Notably, GW9508 was reported to have $pEC_{50} = 4.9$ on PPARγ (Briscoe et al. 2006), implying only four-fold selectivity for FFA4. Nevertheless, due to lack of better alternatives, GW9508 has frequently been used as a tool compound in studies of FFA4 and can with appropriate controls still generate meaningful data.

GW9508 was, for example, used to study the anti-osteoclastogenic role of FFA4 in RAW264.7 cells, where the expression levels of FFA1 were found to be 100-fold lower than of FFA4 (Cornish et al. 2008). The compound has also been used as a "functionally specific" FFA4 agonist to study FFA4 in macrophages and adipocytes since these do not express FFA1 (Oh et al. 2010), the compound was found to elicit anti-inflammatory effects that were ablated upon FFA4 knockdown.

GW9508 can be used to rule out that an effect is related to either FFA1 or FFA4: using GW9508 and GW1100 as tools, Garrel et al. showed that neither receptor is involved in fatty acid induced secretion of the gonadotropin hormone LH although both are expressed in LβT2 gonadotrope cells (Garrel et al. 2011). Murine RAW264.7 cells were used to study if FFA4 is involved in DHA-mediated cyclo-oxygenase-2 (COX-2) induction (Li et al. 2013a). Here, GW9508 and PPAR specific ligands were used in concert to demonstrate that DHA attenuates COX-2 expression in a PPAR-independent manner but, at least in part, through FFA4. At a concentration of 100 μM GW9508 was found to reverse homocysteine-induced inflammation in adipocytes (Li et al. 2013b). Since human colorectal carcinoma cells have been shown to not express FFA1, GW9508 was used as a "functionally specific" FFA4 agonist on these cells (Wu et al. 2013). Backed up by knockdown of FFA4, this study showed activation of FFA4 to be promoting angiogenesis in vitro and in vivo. Yan et al. used GW9508 and knockdown of FFA1, FFA4 or both in

their study of the NLRP3 inflammasome in human THP-1 derived macrophages that express both receptors (Yan et al. 2013).

In a study on ghrelin producing SG-1 cells, GW9508 and knockdown approaches were used to show that activation of FFA4 and not of FFA1 leads to reduced secretion of this hunger-promoting hormone (Gong et al. 2014). GW9508 and DHA were used to further study the mechanism of FFA4 signalling through phospholipase A_2 in murine RAW264.7 cells (Liu et al. 2014). FFA4 was found to be expressed on Kupffer cells, hepatic stellate macrophages, and activation of FFA4 on these cells with GW9508 promoted an anti-inflammatory state which appeared to be beneficial against hepatic ischemia reperfusion injury and possibly other liver injuries (Raptis et al. 2014). The neuronal cell line rHypoE-7, derived from rat hypothalamus, expresses FFA4 at abundant levels but does not express FFA1. GW9508 has therefore been used as a "functionally specific" FFA4 tool in this cell model. Both GW9508 and DHA were found to produce anti-inflammatory effects mediated through FFA4, results that were supported by siRNA-mediated FFA4 knockdown (Wellhauser and Belsham 2014).

PANC-1 is a pancreatic cancer cell line that expresses both FFA1 and FFA4. GW9508 and a combination of GW1100 and FFA1 and FFA4 knockdown were used to study the role of the receptors in these cells (Fukushima et al. 2015). The authors found that FFA4 stimulates and FFA1 inhibits motility, invasion and tumorigenicity of pancreatic cancer cells. Using the same tools, the group has also studied the effects of FFA4 and FFA1 on ethionine induced liver epithelial cell motility (WB-F344 cells) and the role of the receptors in several lung cancer cell lines (Ishii et al. 2015a, b; Kita et al. 2016). Kim et al. reported that FFA4 negatively regulates osteoclast differentiation, survival, and function, by using GW9508 as an FFA4 probe despite expression of FFA1 at a very low level, and provided support for the conclusion through the use of shRNA-mediated FFA4 knockdown (Kim et al. 2015).

Tsukahara and co-workers found both FFA4 and FFA1 to be overexpressed in enteroendocrine L cells of the inflamed ileum of Crohn's disease patients. The combination of GW9508 and GW1100 helped them to elucidate that FFA1 had stimulatory, and FFA4 inhibitory, effects on the production of the anti-inflammatory glucagon-like peptide-2 (GLP-2) from L-cells (Tsukahara et al. 2015). Using GW9508 and GW1100 as tools, Konno et al. investigated the expression and functions of FFA4 on human eosinophils, where 100 μM GW9508 induced the release of IL-4 and inhibited apoptosis, effects that were not affected by pre-treatment with 10 μM GW1100, thus leading the authors to conclude that the effects were mediated by FFA4 and not FFA1 (Konno et al. 2015).

As testified by the studies mentioned above, GW9508 is probably the most extensively used tool compound in studies of FFA4. It is commercially available and thus easily accessible. However, its modest potency on FFA4, similar potency on PPARγ and 100-fold higher potency on FFA1 render GW9508 a suboptimal tool compound and unsuitable for such studies when used alone. The high concentration necessary to ensure full activation of FFA4 implies that off-target activity beyond FFA1 cannot be excluded. It is therefore recommended that any use of this

compound as a probe for FFA4 also includes receptor silencing and/or an FFA4-specific antagonist such as AH 7416 (see below).

4.1.3 TUG-891

Screening of a series of FFA1 agonists and optimization of a hit that exhibited selectivity for FFA4 led to the identification of TUG-891 (Fig. 2) as the first potent and selective FFA4 agonist (Shimpukade et al. 2012). Depending on the type of assay, TUG-891 is 1.7–3.2 orders of magnitude selective for hFFA4 over hFFA1 and has no detectable activity on the two short-chain fatty acid receptors FFA2 and FFA3. Unfortunately, whereas the compound is even more potent on the murine orthologue of FFA4, its selectivity over FFA1 in this species is only 3-fold and 50-fold selective in a calcium mobilization and β-arrestin-2 recruitment assay, respectively (Hudson et al. 2013b). TUG-891 has also been used for investigation of the porcine variant of FFA4, where on the wild-type receptor it has pEC_{50} of 5.7–5.9 in the nuclear factor of activated T-cell response element (NFAT) and serum response element (SER) based reporter systems (Song et al. 2015). Despite the less pronounced selectivity between FFA4 and FFA1 at the mouse orthologues, the compound still represents a useful tool compound for both in vitro and in vivo studies of the biology of FFA4.

Following its discovery, TUG-891 was confirmed to promote Ca^{2+} mobilization, β-arrestin recruitment and ERK1/2 phosphorylation via FFA4, but with a clear bias for signalling through Ca^{2+} and β-arrestin (Hudson et al. 2013b). As also observed for LCFAs and GW9508 (Hirasawa et al. 2005; Watson et al. 2012), activation of FFA4 by TUG-891 was followed by rapid receptor internalization and desensitization, effects that were equally rapidly reversed after washout. TUG-891 was also tested together with the selective FFA1 agonist TUG-905 on the enteroendocrine cell lines STC-1 and GLUTag that express both receptors, and it was found that GLP-1 release could be ascribed predominantly to FFA4 activation (Hudson et al. 2013b). In the same study TUG-891 was reported to be as efficacious as aLA in causing inhibition of lipopolysaccharide-induced TNF-α release from RAW264.7 macrophages (Hudson et al. 2013b). TUG-891 was later used in a mass spectrometry-based proteomics study of FFA4 in an effort to map the sites of phosphorylation and residues interacting with arrestin-3, it was found that activation of the receptor with this agonist leads to phosphorylation of Thr^{347}, Thr^{349}, Ser^{350} Ser^{357} and Ser^{360} (Butcher et al. 2014). Mutagenesis and modelling studies have confirmed that TUG-891 is an orthosteric ligand that binds to essentially the same pocket as aLA (Hudson et al. 2014).

A hallmark of osteoporosis is an imbalance in the osteoblast/adipocyte ratio. TUG-891 has recently been used as a tool to investigate the role of FFA4 in relation to differentiation of bone marrow mesenchymal stem cells (Gao et al. 2015). It was found that low concentrations (0.1–1 μM) of the FFA4 agonist led to increased adipogenesis whereas high concentrations (30–100 μM) led to increased osteogenesis. Furthermore, local injection of high doses of TUG-891 to the proximal femur of ovariectomized mice rescued the animals from oestrogen-deficient bone loss. In light of the activity of TUG-891 on mFFA1 and the expression of also this receptor

in bone-related cells (Wauquier et al. 2013), inclusion of either a specific antagonist or gene silencing would have been interesting to confirm and clarify the roles of the receptors.

TUG-891 and GW9508 were used as selective FFA4 and FFA1 agonists, respectively, in a study of the function of the two receptors in human and guinea pig airway smooth muscle, where it was concluded that, of these two, FFA1 is the sole receptor responsible for ligand-induced airway smooth muscle contraction (Mizuta et al. 2015). The paper claims that TUG-891 is a highly selective FFA4 agonist without providing support for this. The claim is true for the human but not for the murine orthologue, whilst data on the guinea pig orthologue is lacking. The compounds are therefore more appropriately used as selective agonists on human than murine cells and tissue. The same two agonists have been used as tool compounds in a study of human prostate cancer cell lines, where it was found that TUG-891 acted as a potent inhibitor of proliferation induced by lysophosphatidic acid or serum (Liu et al. 2015).

Obstructive sleep apnoea (OSA) is a common health problem that is associated with the presence of insulin resistance and altered lipid homeostasis. Chronic sleep fragmentation can be used to mimic the disrupted sleep patterns of OSA and it has been shown to induce deregulation of appetite and promote the emergence of obesity and insulin resistance as well as adipose tissue inflammation in mice being fed normal diets (Hakim et al. 2015a, b). A recent study employing TUG-891 found that the compound attenuated these effects when dosed to mice subjected to chronic sleep fragmentation, suggesting that FFA4 agonists may in the future become valuable to combat some of the side effects of OSA, although it was correctly noted that further examination of causal pathways is justified (Gozal et al. 2016).

As part of their effort to clarify the mechanism for FFA4-induced adipogenesis of 3T3-L1 adipocytes Song et al. employed TUG-891 as well as the PUFAs DHA, aLA and linoleic acid (Song et al. 2016). Addition of 1, 10 or 100 μM TUG-891 led to dose-dependent increase of triglyceride accumulation and PPARγ expression in differentiated 3T3-L1, effects that disappeared upon FFA4 knockdown. Using a reporter assay, the authors were able to conclude that TUG-891 has no direct effect on PPARγ.

Because TUG-891 is commercially available and a relatively potent and selective FFA4 agonist it has become a frequently employed tool compound in both in vitro and in vivo studies. The activity and potency of TUG-891 has been well characterized on both arrestin and G protein-dependent signalling pathways, which is noteworthy since both pathways have been implicated in important functions of the receptor. The compound should, however, be used with care in mice and murine-derived cells, since only a three-fold selectivity has been observed over FFA1 in a calcium mobilization assay (Hudson et al. 2013b). Furthermore, a broader characterization of the selectivity profile of the compound, including on PPARs, is still lacking.

4.1.4 Metabolex Compounds

The company Metabolex (now Cymabay Therapeutics) has developed and patented FFA4 agonists (Ma et al. 2010; Shi et al. 2010). Two of these agonists, referred to as Metabolex 36 and compound B (example 209 in the patent), respectively, have been used as tool compounds in peer reviewed studies (Fig. 2) (Egerod et al. 2015; Engelstoft et al. 2013; Stone et al. 2014).

Metabolex 36 (as well as two proprietary FFA4 agonists from AstraZeneca, AZ-423 and AZ-670 with pEC_{50} 5.8–6.4 on mFFA4, that have undisclosed structures and thereby violate an obvious requirement for tool compounds) was used by Stone et al. to study the function of FFA4 in pancreatic delta cells (Stone et al. 2014). The compound was found to have pEC_{50} 6.0 (DMR) and 5.9 (FLIPR) on mFFA4 and the pEC_{50} on mFFA1 was found to be <4.0 (FLIPR) (Stone et al. 2014). Metabolex 36 lowered both basal and glucose induced somatostatin secretion from murine pancreatic islets with maximum inhibition reached at 30 μM. The agonist was found to be without effect on somatostatin levels when tested on islets isolated from FFA4 knockout mice. The authors did not observe any change in insulin secretion under either basal or elevated glucose conditions after dosing of Metabolex 36. Interestingly DHA was found in the same study to be without influence on somatostatin secretion, whereas this endogenous agonist did influence the insulin levels in a dose-dependent manner in islets from wild-type mice but not from FFA4-knockout mice.

Compound B was used by Engelstoft et al. in their study of the role of FFA4 in ghrelin secreting cells (Engelstoft et al. 2013). The compound was found to have $EC_{50} = 15$ nM on FFA4 and >1,000-fold selectivity over FFA1 in an inositol trisphosphate assay in transfected COS7 cells. Compound B dose-dependently inhibited ghrelin secretion in isolated primary gastric mucosal cells, an effect that was absent in cells isolated from FFA4 knockout mice. Oral dosing of compound B to fasting wild-type mice also significantly and dose-dependently decreased ghrelin levels (Engelstoft et al. 2013). The same group subsequently used compound B in their investigation of primary gastric mucosal cells where they found significant and dose-dependent inhibition of somatostatin release from cells isolated from wild-type but not from FFA4 knockout mice (Egerod et al. 2015).

Whereas Metabolex 36 appears to be only moderately potent, compound B may be the most potent compound used as an FFA4 probe. However, the compound was tested in a different assay than the others. To reliably compare potency, the compounds should be tested head-to-head in the same assay. Furthermore, only data from a G protein-dependent assay is currently available. Potency in an arrestin-dependent assay would also be relevant since the anti-inflammatory effects of FFA4 are believed to be mediated by this signalling pathway (Talukdar et al. 2011). Furthermore, the data is only from one unspecified receptor orthologue. The selectivity of compound B over FFA1 appears to be excellent but no further counter-screening appears to have been performed. Data disclosed in the patent, where oral dose of 30 mg/kg of compound B led to 43.6% reduction of area under the curve (AUC) in an intraperitoneal glucose tolerance test (Shi et al. 2010), indicates that compound B also is suitable for in vivo studies. Neither Metabolex 36 nor compound B is commercially available and both compounds require multistep synthesis.

4.1.5 Merck cpdA

This selective FFA4 agonist (Fig. 2) entered the public domain in early 2014 in a patent from Merck, in which it was reported to have a $pEC_{50} = 6.1$ on hFFA4 in FLIPR Ca^{2+} assays (Chelliah et al. 2014). Shortly thereafter, it was published in the peer-reviewed literature and reported with $pEC_{50} = 7.6$ on hFFA4 and negligible activity on hFFA1 up to 1 μM in FLIPR assays (Oh da et al. 2014). The compound was also tested in a β-arrestin-2 recruitment assays on human and murine FFA4 with $pEC_{50} \approx 6.5$ in both assays. In this report, cpdA and DHA were used to investigate FFA4 mediated anti-inflammatory properties in vitro. Both compounds exerted potent anti-inflammatory effects on macrophages from wild-type mice but not from FFA4-knockout mice. CpdA was then tested in both wild-type and FFA4-knockout high-fat diet (HFD) fed mice at a dose of 30 mg/kg, resulting in markedly improved glucose and insulin tolerance, decreased insulin secretion in the wild-type mice, but not in the FFA4-knockout mice. Furthermore, cpdA was shown to improve systemic and hepatic insulin sensitivity and beneficial effects were also seen on hepatic lipid metabolism (decrease in hepatic steatosis, liver triglyceride and diacylglycerol levels and fewer saturated free fatty acids). No effects on GLP-1 levels were found upon treatment of HFD-fed mice with cpdA (Oh da et al. 2014).

CpdA is an FFA4 probe with high selectivity over FFA1 that is proven to be useful in both in vitro and in vivo assays. As for the other tool compounds above, data from more extensive profiling is still lacking and its use therefore requires compensating control studies such as with FFA4-knockout mice as used by Oh et al. The compound is commercially available.

4.2 Sulfonamide Probes

4.2.1 GSK137647A

A medium throughput screening campaign at GlaxoSmithKline yielded a diarylsulfonamide that after optimization led to the discovery of GSK137647A (Fig. 2), an FFA4 agonist with comparable potency across species [$pEC_{50} = 6.3$ (human), 6.2 (mouse) and 6.1 (rat) in FLIPR assays] (Sparks et al. 2014). The compound was shown to be quite selective for FFA4, having pEC_{50} of less than 4.5 on both on human and rodent orthologues of FFA1, FFA2 and FFA3. The agonist was also screened and found to have minimal activity on 58 other targets, including PPAR α, δ and γ. The compound thus represents a good and selective probe for in vitro studies and is the currently best profiled FFA4 probe. Unfortunately, the compound suffers from low solubility in simulated intestinal fluid (FASSIF) (2.9 μg/mL), which limits its usefulness for in vivo studies (Sparks et al. 2014). The agonist was tested on MIN6 cells and found to produce a dose-dependent increase in glucose-stimulated insulin secretion. GSK137647A was also shown to cause a modest increase in GLP-1 release from a human intestinal cell line (NCI-H716) (Sparks et al. 2014). Before the discovery of GSK137647A was published, the compound was used as a tool compound to investigate FFA4 as a lipid sensor in mouse taste buds (Martin et al. 2012). At a concentration of 50 μM,

the agonist led to a small but significant increase in GLP-1 release from freshly isolated mouse circumvallate papillae. To date, this compound has only been used in these two studies, but as it is commercially available and easily synthesized, this may change. The compound is suitable as a structurally orthogonal probe in combination with any of the carboxylic acid probes above.

4.2.2 Cyclic Sulfonamides, Banyu cpd 2 and TUG-1197

A patent from Banyu Pharmaceutical disclosed a series of cyclic sulfonamide FFA4 agonists where cpd 2 (Fig. 2) appeared as the most potent ($pEC_{50} = 6.7$ in a FLIPR assay) of two compounds with activity data (Arakawa et al. 2010). The compound was found to be unbiased in β-arrestin recruitment and a Ca^{2+} mobilization assays with $pEC_{50} = 6.4$ and 6.5, respectively (Azevedo et al. 2016). Interestingly, and in contrast to all other known FFA4 agonists (including GSK137647A above, which has a weakly acidic function), the scaffold in this series contains no acidic group and cannot be negatively charged under physiological conditions. In light of its distinct structure, it is also interesting that cpd 2 also binds to the orthosteric site of FFA4 (Azevedo et al. 2016).

Structure-activity explorations starting with cpd 2 led Azevedo et al. to identify the closely related TUG-1197 (Fig. 2), a compound with $pEC_{50} = 6.9$ and 6.6 in the human FFA4 in a β-arrestin recruitment and a Ca^{2+} mobilization assay, respectively, as well as $pEC_{50} = 6.3$ and 6.8 in the same assays on the murine FFA4. Both TUG-1197 and cpd 2 were found active in an acute oral tolerance test in normal mice after oral dosing at 10 mg/kg. TUG-1197 was investigated further in diet-induced obese (DIO) mice, where TUG-1197 again significantly lowered the glucose level in wild-type but not in FFA4-knockout mice. The compound was investigated further in a chronic study in DIO mice where it caused significant insulin sensitization and a moderate but significant bodyweight loss, effects that were not observed in FFA4-knockout mice (Azevedo et al. 2016).

Cpd 2 and especially TUG-1197 are reasonably potent compared to the other FFA4 agonists and none of these compounds show activity on FFA1 at up to 100 μM concentration. TUG-1197 is also confirmed to lack activity on FFA2 and FFA3 (Azevedo et al. 2016). A more comprehensive profiling is still lacking. The compounds are non-acidic and structurally distinctly different from the carboxylic acid containing FFA4 agonists (see above), and would therefore be expected to have different activity profiles (preliminarily confirmed by lack of activity on FFA1). They would therefore be suitable as orthogonal FFA4 probes in conjunction with carboxylic acid probes (Bunnage et al. 2013). The absence of a charge causes low aqueous solubility but not to a degree that it interfered with the in vivo studies (Azevedo et al. 2016). Neither cpd 2 nor TUG-1197 is currently commercially available, but they are both synthetically easily accessible by a three-step sequence from saccharin (Azevedo et al. 2016).

4.3 Antagonists

4.3.1 AH 7614

During their work towards GSK137647A, Sparks et al. also identified the sulfonamide AH 7614 (compound 39) as a potent FFA4 antagonist with pIC_{50} of 7.1, 8.1 and 8.1 on the human, mouse and rat orthologue, respectively (Fig. 2) (Sparks et al. 2014). The compound was also confirmed to lack agonist activity on hFFA4 and either agonist or antagonist activity on hFFA1. The antagonist counteracted effects of GSK137647A on insulin and GLP-1 secretion in MIN6 and NCI-H716 cells, respectively. The exact mode of antagonism for AH 7614 is yet to be clarified. However, the compound lacks properties appropriate for utilization in an in vivo setting (Sparks et al. 2014), presumably due to low solubility. As AH 7614 is the only FFA4 antagonist published to date, it is nevertheless an important tool compound with confirmed high potency across human and rodent FFA4, and it is commercially available. The antagonist was recently used in a study on the phosphorylation patterns of mFFA4, where the compound was reported to effectively block FFA4 activation of TUG-891 (Prihandoko et al. 2016).

5 Outlook

FFA4 has in recent years appeared as a hot target for new potential drugs aimed at the treatment of metabolic and inflammatory disorders such as T2D, obesity and potentially non-alcoholic steatohepatitis. Both selective FFA4 agonists and polypharmacological activities, such as dual FFA1/FFA4 agonists, are of interest. There is, however, still many unanswered questions regarding the physiology and pharmacology of FFA4 and its interplay with other receptors. Tool compounds with appropriate properties are therefore required for further investigations. As outlined above, several reasonably potent FFA4 agonists are now available, thus, there is no longer any need to use low potency and non-selective agonists like the LCFAs or GW9508 in future studies where specific FFA4 activation is desired. We are now also in a position where structurally orthogonal FFA4 agonists that may be presumed to have non-overlapping activity profiles are available (even if this remains to be supported by broader profiling). Thus, inclusion of a carboxylic acid based agonist such as TUG-891, a Metabolex compound or Merck's cpdA, may be used together with sulfonamide based agonists such as GSK137647A or a Banyu-derived compound to provide strong pharmacological evidence for specific engagement of FFA4. Furthermore, the first antagonist for FFA4 was recently disclosed and has already been employed as an in vitro tool in the study of FFA4.

Despite this, there is still a need for better profiling of all current FFA4 probes, as well as a need for further improved probes. The properties of the current FFA4 probes are summarized in Table 3. Since FFA4 is linked to effects mediated by both G protein and β-arrestin pathways, it is critical that tools are characterized on both of these. A more extensive profiling is currently only available for GSK137647A. Properties such as solubility, chemical stability and the more detailed

Table 3 Properties of currently used FFA4 tool compounds

	FFA4 potency	Off-target activity (*if known*)	Solubility	In vivo PK	Commercial availability (06/2016)
Agonists	*pEC$_{50}$*	*pEC$_{50}$*			
LCFAs	<6[1]	<6[1]	Generally poor[1]	Rapidly metabolized[1]	
GW9508	h: 5.5[a,2] m: 6.5[b,4]	hFFA1: 7.3[a,2] PPARγ: 4.9[2]	ND	ND Used in several mouse studies	✔
TUG-891	h: 7.4–7.0[a,3], 7.2–6.8[b,3,4], 5.8[c,4], 7.3[d,4] m: 7.8[a,3,4], 7.0[b,4]	hFFA1: 4.2[b,3] mFFA1: 5.9[b,4] FFA2, FFA3: <4[3]	ND	ND Used orally in mice (20 mg/kg)[5]	✔
Metabolex compound B	m: 7.8 (IP)[6]	mFFA1: <5 (IP)[6]	ND	ND Used in mice (30 mg/kg)[6]	✘
Metabolex 36	m: 6.0[e,7], 5.9[d,7]	mFFA1: <4[d,7]	ND	ND	✘
Merck cpdA	h: 7.6[d,8], 6.1[d,9], 6.5[b,8] m: 6.5[b,8]	hFFA1: <5[d,8]	ND	ND Used in mice[8]	✔
GSK137647A	h: 6.3[d,10] m: 6.2[d,10] r: 6.1[d,10]	h/m/rFFA1/2/3: <4.5[d,10]	<10 μm (FASSIF)[10]	ND Deemed unsuitable for in vivo studies[10]	✔
Banyu cpd 2	h: 6.5[a], 6.4[b]	hFFA1: <4[a]	11 μm (PBS)[11]	ND Used in mice[11]	✘
TUG-1197	h: 6.6[a], 6.9[b] m: 6.3[b]	hFFA1: <4[a]	14 μm (FASSIF), 1.3 μm (PBS)[11]	ND Used in mice[11]	✘

(continued)

Table 3 (continued)

	FFA4 potency	Off-target activity (*if known*)	Solubility	In vivo PK	Commercial availability (06/2016)
Antagonists	pIC_{50}				
AH 7614	h: 7.1[d,10] m: 8.1[d,10] r: 8.1[d,10]	hFFA1: $pEC_{50} < 4.3$[d,10] $pIC_{50} < 4.6$[d,10]	ND	ND Deemed unsuitable for in vivo studies[10]	✔

Source: [1]Ulven and Christiansen (2015); [2]Briscoe et al. (2006); [3]Shimpukade et al. (2012); [4]Hudson et al. (2013b); [5]Gozal et al. (2016); [6]Engelstoft et al. (2013); [7]Stone et al. (2014); [8]Oh da et al. (2014); [9]Chelliah et al. (2014); [10]Sparks et al. (2014); [11]Azevedo et al. (2016)
h human, *m* mouse, *r* rat, *ND* no data available
[a]Calcium mobilization assay
[b]β-Arrestin recruitment assay
[c]pERK
[d]Internalization
[e]Dynamic mass redistribution assay and FLIPR Ca^{2+} assay

pharmacokinetic properties, including oral bioavailability, metabolic stability and propensity to enter the CNS, are still mostly unknown for all known probes, even if several have been successfully used in vivo. It is clear that although TUG-891, Metabolex compound B and Merck cpdA are of reasonable potency, agonists with even higher potency, and thereby increased chance of appropriate target exposure and reduced risk of off-target effects and pharmacokinetic issues, are still desirable.

Likewise, the FFA4 antagonist AH 7614 has appreciable potency at human and especially at rodent receptors, and represents a significant advance since the compound enables studies with pharmaceutical ablation of FFA4 and an alternative method for controlling specific activity of FFA4 agonists. However, further characterization is required to establish specificity, chemical stability, and pharmacological mode of action. It seems clear that the compound has certain suboptimal properties, including poor solubility, that interfere with its use in vivo (Sparks et al. 2014). Additional FFA4 antagonists with complementary properties and improved solubility are therefore still desirable.

None of the current FFA4 probes are sufficiently characterized to make them optimal for in vivo studies, even though several of the compounds have been used in such studies. For in vivo studies in general, information on bioavailability, preferred formulations, expected plasma concentrations following specific doses, clearance, major metabolites and their activity profiles, is useful but rarely available information. The widespread expression and multiple functions of FFA4 make this a particularly challenging case. It is difficult to ensure complete and concomitant target engagement in all organs relevant for regulation of the metabolic state, including intestines (where exposure from the vascular or luminal side will not necessarily result in the same effect), pancreas, adipose tissue and the CNS, and imbalanced exposure may affect the response. The role of FFA4 in the brain is not as well studied as in other tissues, but studies in which intracerebroventricular (icv) injection of LCFAs has been performed indicate that the receptor is mediating anti-inflammatory effects (Cintra et al. 2012). Probes with well-characterized CNS penetration are therefore desirable for in vivo studies, where the preferred compound would either be completely excluded from the CNS or have good penetration that could engage FFA4 in the CNS and enable studies of their effects without the need for icv injection.

In conclusion, although none of the FFA4 probes described hitherto are completely characterized or have ideal properties, tool compounds are available that are of sufficient quality for exploration of FFA4 provided that appropriate precautions are taken in the experimental design. Strategies to minimize the risk of confounding readouts include concomitant use of structurally orthogonal probes, for example both a carboxylic acid and a sulfonamide, FFA4 antagonists, of which only one currently is available, and gene knockout or knockdown techniques. Meanwhile, the high current activity in the field is likely to result in new probes with further improved properties that will contribute to the further elucidation of the function of FFA4 and its potential as a drug target.

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