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REVIEW

Characterizing pharmacological ligands to study the long-chain fatty acid receptors GPR40/FFA1 and GPR120/FFA4

G Milligan[1], E Alvarez-Curto[1], K R Watterson[1], T Ulven[2] and B D Hudson[1]

[1]*Molecular Pharmacology Group, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, Scotland, UK, and* [2]*Department of Physics, Chemistry and Pharmacy, University of Southern Denmark, Odense, Denmark*

Correspondence
Graeme Milligan, Molecular Pharmacology Group, Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, UK. E-mail: graeme.milligan@glasgow.ac.uk

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The free fatty acid receptors (FFA) 1 (previously designated GPR40) and FFA4 (previously GPR120) are two GPCRs activated by saturated and unsaturated longer-chain free fatty acids. With expression patterns and functions anticipated to directly or indirectly promote insulin secretion, provide homeostatic control of blood glucose and improve tissue insulin sensitivity, both receptors are being studied as potential therapeutic targets for the control of type 2 diabetes. Furthermore, genetic and systems biology studies in both humans and mouse models link FFA4 receptors to diabetes and obesity. Although activated by the same group of free fatty acids, FFA1 and FFA4 receptors are not closely related and, while the basis of recognition of fatty acids by FFA1 receptors is similar to that of the short-chain fatty acid receptors FFA2 and FFA3, the amino acid residues involved in endogenous ligand recognition by FFA4 receptors are more akin to those of the sphingosine 1 phosphate receptor $S1P_1$. Screening and subsequent medicinal chemistry programmes have developed a number of FFA1 receptor selective agonists that are effective in promoting insulin secretion in a glucose concentration-dependent manner, and in lowering blood glucose levels. However, the recent termination of Phase III clinical trials employing TAK-875/fasiglifam has caused a setback and raises important questions over the exact nature and mechanistic causes of the problems. Progress in the identification and development of highly FFA4 receptor-selective pharmacological tools has been less rapid and several issues remain to be clarified to fully validate this receptor as a therapeutic target. Despite this, the ongoing development of a range of novel ligands offers great opportunities to further unravel the contributions of these receptors.

LINKED ARTICLES

This article is part of a themed section on 5th BPS Focused Meeting on Cell Signalling. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-13

Abbreviations

aLA, α-linolenic acid; AMG-837, (*S*)-3(-4-((4′-(trifluoromethyl)-[1,1′-biphenyl]-3-yl)methoxy)phenyl)hex-4-ynoic acid; AMG-1638, (*S*)-3-cyclopropyl-3-(3-((2-(5,5-dimethylcyclopent-1-en-1-yl)-2′-fluoro-5′-methoxy-[1,1′-biphenyl]-4-yl) methoxy)phenyl)propanoic acid; ANT203, (4-(2-benzylidenehydrazinyl)-6-methyl-2-phenylpyrimidine; AS2034178, 2-[(4-{[4′-(2-hydroxyethoxy)-2′-methyl[1,1′-biphenyl]-3-yl]methoxy}phenyl)methyl]-3,5-dioxo-1,2,4-oxadiazolidine; DC260126, *N*-(4-butylphenyl)-4-fluorobenzenesulfonamide; DHA, docosahexaenoic acid; FFA1–4, free fatty acid receptors 1–4; GLP-1, glucagon-like peptide-1; GW1100, 1-(4-ethoxycarbonylphenyl)-2-(4-fluorobenzylthio)-5-(2-ethoxy-5-pyrimidinylmethyl)-4-pyrimidinone; GW9508, 4-[[(3-phenoxyphenyl)methyl]amino]benzenepropanoic acid; NCG21, 4-(4-{2-[phenyl(pyridin-2-yl)amino]ethoxy}phenyl)butanoic acid; TAK-875 (fasiglifam), [(3*S*)-6-({2′,6′-dimethyl-4′-[3-(methylsulfonyl)propoxy]biphenyl-3-yl}methoxy)-2,3-dihydro-1-benzofuran-3-yl]acetic acid; TMD, transmembrane

domain; TUG-424, 4-[2-(2-methylphenyl)ethynyl]benzenepropanoic acid; TUG-469, 3-(4-(((2′-methyl-[1,1′-biphenyl]-3-yl)methyl)amino)phenyl)propanoic acid ; TUG-770, 3-(4-((2-(cyanomethyl)phenyl)ethynyl)-2-fluorophenyl)propanoic acid; TUG-891, 4-[(4-fluoro-4′-methyl[1,1′-biphenyl]-2-yl)methoxy]-benzenepropanoic acid

Tables of Links

TARGETS
GPCRs[a]
GPR40, FFA1 receptor
GPR120, FFA4 receptor
Nuclear hormone receptors[b]
PPAR-γ
Enzymes[c]
TAK1 kinase

LIGANDS	
AMG-837	α-Linolenic acid
DHA, docosahexaenoic acid	NCG21
Eicosapentaenoic acid	Pioglitazone
GLP-1	TAK-875/fasiglifam
GW1100	TUG-424
GW9508	TUG-770
Insulin	TUG-891
Linoleic acid	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

GPCRs are the molecular targets of a wide range of medicines employed clinically to treat both acute and chronic diseases. It has been noted, however, that the number of GPCRs that act as the primary targets for approved medicines remains modest. This is despite the large number of GPCRs encoded within the human genome that are known or anticipated to respond to endogenously generated regulators of homeostatic function. The advent of 'reverse pharmacology', designed to either pair 'orphan' GPCRs with endogenous modulators or to identify surrogate, low MW chemical ligands useful to interrogate the function of these receptors (Yoshida *et al.*, 2012; Civelli *et al.*, 2013), promised to expand significantly the proportion of GPCRs that could be considered as validated therapeutic targets. At least in part, this effort has begun to deliver. This has included work on receptors that are activated by free fatty acids, including the two GPCRs that provide the focus of the current review. Four GPCRs, free fatty acid receptors 1–4 (FFA1–4) are currently defined as receptors for free fatty acids (Stoddart *et al.*, 2008) while a further receptor, GPR84, although clearly activated by medium-chain fatty acids, officially remains an 'orphan'. FFA2 and FFA3 receptors are activated by short-chain fatty acids that are produced in high concentrations by bacterial fermentation of dietary fibre (Milligan *et al.*, 2009), whereas FFA1 and FFA4 receptors, although displaying only limited relatedness to each other, are both activated by medium- and long-chain saturated and unsaturated fatty acids derived from dietary triglycerides (Hudson *et al.*, 2011).

FFA1 receptor agonists: from identification to clinical studies

The 'orphan' receptor GPR40 [subsequently renamed systematically as the FFA1 receptor (Stoddart *et al.*, 2008)] was initially shown to be expressed selectively by beta cells of rat islets (Briscoe *et al.*, 2003). In parallel with these studies, ligand fishing experiments using FFA1 receptors demonstrated this receptor to be activated by a broad range of both medium- and longer-chain saturated and unsaturated fatty acids (Briscoe *et al.*, 2003). Interestingly, within this group of ligands, only modest variation in potency was observed (Briscoe *et al.*, 2003), and therefore, in an *in vivo* context, it might be anticipated that FFA1 receptor-mediated effects of fatty acids at the level of the pancreas would largely reflect their relative circulating concentrations. There is a substantial literature on the health benefits of various fatty acids, including ω-3 fatty acids derived from fish oils and other sources (Calder, 2013). However, the relatively high overall concentration of circulating fatty acids might, therefore, be anticipated to limit the effectiveness of fatty acids provided as dietary supplements, unless key effects are produced largely within the gut, for example, or at targets other than the GPCRs that are activated by the broader group of fatty acids (Dranse *et al.*, 2013). FFA1 receptors are also expressed by a range of gut enteroendocrine cells (Edfalk *et al.*, 2008; Liou *et al.*, 2011) that generate, store and release hormones such as glucagon-like peptide-1 (GLP-1) and cholecystokinin. Initial de-orphanization studies also demonstrated the high-level expression of FFA1 receptors in a broad range of regions of the human brain (Briscoe *et al.*, 2003). Expression of this receptor in rodent brain and its potential function in the CNS has subsequently been a matter of debate. Recently, however, a number of studies have used combinations of *in situ* hybridization and receptor-selective pairs of agonist and antagonist to provide substantial support for regional expression and function (Zamarbide *et al.*, 2014), although the exact role of FFA1 receptors here remains uncertain (Yamashima, 2012). Equally, FFA1 receptors are expressed in osteoclastic cells (Cornish *et al.*, 2008) and regulation of apoptosis of such cells (Mieczkowska *et al.*, 2012) and inhibition of osteoclast differ-

entiation (Wauquier *et al.*, 2013) by fatty acids and synthetic FFA1 receptor agonists has hinted at other applications of FFA1 receptor ligands, although these ideas have yet been explored in any detail.

By contrast with these findings, expression of FFA1 receptors in the pancreas was rapidly linked to the capacity of free fatty acids to acutely amplify glucose-stimulated insulin secretion (Itoh *et al.*, 2003; Salehi *et al.*, 2005). This resulted in a desire to assess the action of synthetic FFA1 receptor ligands in both animal models of glucose dysregulation, and potentially, for the treatment of diabetes in humans. This subject, and the development of arguments for the use of either FFA1 receptor agonists or antagonists, based in part on potentially contradictory results from analysis of FFA1 receptor 'knockout' mouse lines, have been reviewed both excellently and extensively (see Stoddart *et al.*, 2008, Mancini and Poitout, 2013), and therefore, will not be reiterated here. However, of some note, although viewed generally as selective activators of PPAR-γ, the capacity of the thiazolidinedione 'glitazone' drugs to activate FFA1 receptors (Kotarsky *et al.*, 2003; Smith *et al.*, 2009) has recently been suggested to be linked directly to the beneficial effect of pioglitazone on lipotoxicity (Wu *et al.*, 2010). Moreover, certain 5-aryloxy-2,4-thiazolidinedione compounds have recently been described as potent FFA1 receptor agonists (Zhou *et al.*, 2010).

Based on the overwhelming view that agonists of FFA1 receptors would certainly be beneficial acutely and not detrimental in the longer term (Mancini and Poitout, 2013), a broad patent portfolio of FFA1 receptor agonists has been developed (see Defossa and Wagner, 2014) (Figure 1), and a number of ligands have entered clinical development. Of

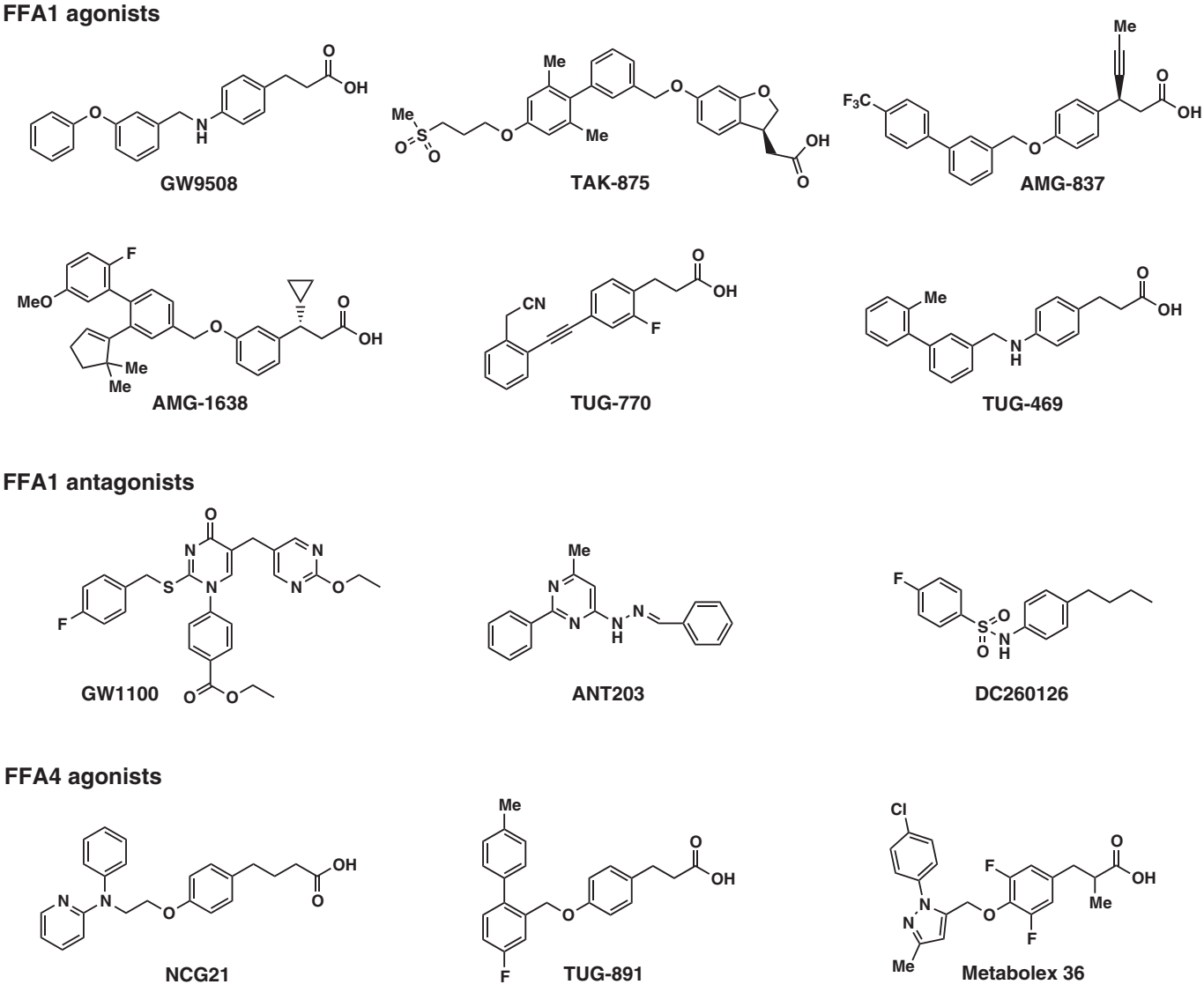

Figure 1

Representative FFA1 and FFA4 receptor ligands. The structures of representative FFA1 and FFA4 receptor-selective ligands discussed in the main text are displayed.

these, the one that has attracted the greatest attention is TAK-875/fasiglifam, which entered Phase III studies after meeting key end points in glycaemic control in Phase II studies (Kaku *et al.*, 2013). Although early reports indicated promising efficacy in the Phase III trials, these studies were unexpectedly terminated in December 2013 with feedback indicating that the clinical 'risk to benefit' potential for treatment with this compound was not acceptable. The only other FFA1 receptor agonist currently reported to have advanced into Phase II studies is JTT-851, a compound of unpublished structure from Japan Tobacco.

FFA1 receptors: mode(s) of ligand binding

Initial studies to explore the mode of binding of ligands to FFA1 receptors (Sum *et al.*, 2007; Tikhonova *et al.*, 2007) centred on both the C18 polyunsaturated (18:2 n-6) linoleic acid and the first reported potent and selective synthetic agonist GW9508 (Briscoe *et al.*, 2006). Mutation of either of a pair of Arg residues located near the extracellular surface, one in each of transmembrane domains (TMDs) V and VII (positions 5.39 and 7.35 in the Ballesteros and Weinstein relative residue numbering system) caused large reductions in potency of both GW9508 (Sum *et al.*, 2007; Tikhonova *et al.*, 2007) and linoleic acid (Sum *et al.*, 2007). This was also the case following mutation of Asn6.55 (Sum *et al.*, 2007; Tikhonova *et al.*, 2007), with each of these three residues anticipated to contribute to binding of the carboxylate of both the endogenous fatty acid and the synthetic ligand. Based on modelling studies, a series of further residues was selected for mutagenesis and, as more of these changes reduced the potency of GW9508 than linoleic acid, this was explained on the basis that the higher potency of GW9508 would require more points of interaction with the receptor (Sum *et al.*, 2007). The usefulness of the homology model was then tested by the capacity to identify further ligands in an *in silico* virtual screen (Tikhonova *et al.*, 2008). As well as synthetic fatty acid-like molecules, this search also identified certain 4-thiazolidinone derivatives. Direct mutagenesis studies have also been consistent with such 'glitazone' ligands binding in a manner akin to the fatty acids, and requiring the TMD V and TMD VII arginine residues for function (Smith *et al.*, 2009).

As the vast majority of reported FFA1 receptor agonists, including both the clinically trialled ligands TAK-875/fasiglafam and AMG-837 for which chemical structures are known, contain a carboxylate moiety, or at least an obvious acidic bioisosteric group, there was general acceptance that their mode of binding would be similar to both each other and that of the fatty acids. It was, therefore, a considerable surprise when detailed studies using [^{3}H]AMG-837 and another synthetic agonist, [^{3}H]AMG-1638 provided strong evidence that these ligands bound allosterically with respect to one another, and with respect to the endogenous fatty acid docosahexaenoic acid (DHA) (Lin *et al.*, 2012). Although still to be fully rationalized these data were consistent with the set of ligands tested interacting with three distinct binding sites and producing both positive and negative allosteric effects upon the binding and function of each other (Lin *et al.*, 2012). Moreover, although effects of AMG-837 were all but abolished after mutation of either of the TMDV or TMDVII arginine residues described above, this was not the case for AMG-1638, where effects on potency of these alterations were small (Lin *et al.*, 2012). Although not reported to date, the requirement or otherwise of the carboxylate moiety of AMG-1638 for affinity or potency would be interesting to explore. These studies emphasize the need for careful pharmacological analysis to be employed with a broad range of apparently related chemical series and also indicate, although it is now often challenging to obtain appropriate ligands, the level of insight that can be obtained via the use of [^{3}H]radioligand binding studies. Subsequently, TAK-875 has also been described as an 'ago-allosteric', rather than an orthosteric, regulator of FFA1 receptors (Yabuki *et al.*, 2013). Intriguingly, the recently reported crystal structure of a thermally stabilized form of the human FFA1 receptor complexed to TAK-875 provides some novel insights into this issue, but does not answer all the questions outlined earlier (Srivastava *et al.*, 2014). As anticipated the carboxylate does indeed interact with the key TMDV and TMDVII arginine residues as well as with a pair of tyrosine residues. However, unexpectedly, the rest of the ligand lies largely along the line of the membrane axis and protrudes between TMDIII and TMDV. Coupled with the capping of entry to the traditional, canonical binding site that is found in many other GPCRs by a 'lock' provided by the organization of the second extracellular loop, this arrangement of the ligand is consistent with entry to the receptor from the lipid phase of the membrane rather than directly from the aqueous phase (Srivastava *et al.*, 2014). This may have implications for the general lipophilicity of FFA1 receptor agonist ligands and, indeed, the mesylpropoxy moiety of TAK-875 lies completely outside the seven TMD helix bundle and does not contribute to ligand binding. This is in good agreement with previous studies on analogues with and without the mesylpropoxy tail, which showed that the group contributes by significantly reducing lipophilicity and providing improved pharmacokinetic and toxicological properties (Christiansen *et al.*, 2012; Negoro *et al.*, 2012). Beyond such detailed molecular studies, in an *in vivo* setting, the insulinotropic effect of TAK-875 in diabetic rats was suppressed by an inhibitor of lipolysis, which acted to reduce plasma free fatty acid levels (Yabuki *et al.*, 2013). It is, therefore, reasonable to assume at least certain FFA1 receptor agonists potentiate insulin release in conjunction with free fatty acids rather than by simply replacing them. A second key feature uncovered in the studies described earlier was that both TAK-875 and AMG-837 functioned as partial agonists (Lin *et al.*, 2012; Yabuki *et al.*, 2013) compared with endogenous fatty acids and AMG-1638, again stressing the need for strong basic pharmacology and not only reliance on highly receptor over-expressing systems that might display marked receptor reserve. Interestingly, a subsequent report both confirmed the partial agonist nature of AMG-837 and indicated that AMG-1638 displayed higher efficacy at FFA1 receptors and was able to both promote secretion of GLP-1 from intestinal enteroendocrine cells and enhance glucose-dependent secretion of insulin from pancreatic islets (Luo *et al.*, 2012) (Figure 2). The compound also improved glucose control in the high fat-fed streptozotocin-treated mouse model of type 2 diabetes (Luo *et al.*, 2012).

Identification and functional assessment of further FFA1 receptor ligands

Agonists. Other FFA1 receptor agonists identified by commercial organizations have not been as fully described in the

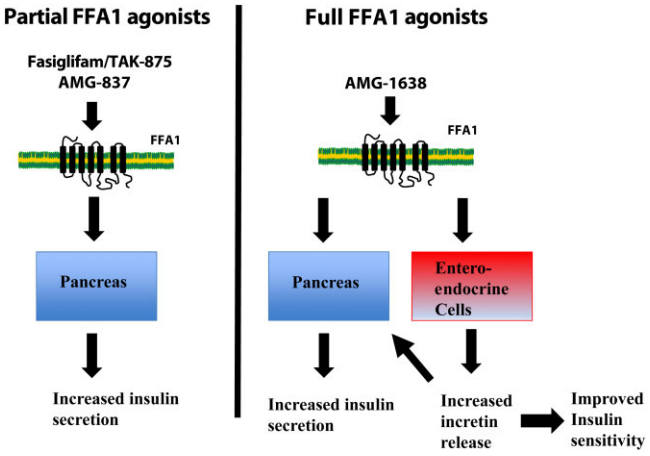

Figure 2

Physiological effects of partial and full synthetic FFA1 receptor agonists. A series of studies have indicated the non-equivalence of various FFA1 receptor agonists including their detailed mode of binding and extent of efficacy (see text for details). Although both TAK-875 and AMG-837 entered clinical trials and are able to enhance secretion of insulin and thus lower blood glucose levels, both have subsequently been shown to act as partial agonists. In contrast, AMG-1638 is reportedly a full agonist, and as well as increasing insulin secretion, is also able to promote secretion of incretins from gut enteroendocrine cells and improve insulin sensitivity. Whether such dual actions will be a general feature of FFA1 receptor full agonists and the implications for further clinical development of FFA1 receptor agonists remains to be established.

academic literature as GW9508, which, because it was both the first described ligand and can be purchased, has become a standard ligand in the field. Reports on other ligands, including AS2034178 (Tanaka *et al.*, 2013) have provided further support of the ability of such ligands to improve glucose homeostasis and maintain or enhance islet beta cell function. Academic groups have also reported on novel FFA1 receptor ligands. Christiansen and co-workers have developed a series of selective and potent FFA1 receptor agonists. A number of these have been based on a 4-phenethynyldihydrocinnamic acid structure, the prototype of which was 4-[2-(2-methylphenyl)ethynyl]benzenepropanoic acid (TUG-424) (Christiansen *et al.*, 2008). Improvements in this scaffold have produced compounds with lower lipophilicity, good *in vitro* metabolic stability and permeability, complete oral bioavailability, and appreciable efficacy on glucose tolerance in mice (Christiansen *et al.*, 2013a). Optimization generated TUG-770 as a ligand that normalized glucose tolerance in diet-induced obese mice, an effect that was fully sustained after a month of chronic dosing (Christiansen *et al.*, 2013b). The same groups have also described the effectiveness of a different ligand, TUG-469 (Christiansen *et al.*, 2010), on glucose tolerance in pre-diabetic New Zealand obese mice (Urban *et al.*, 2013).

Antagonists. Given the focus on the agonists of FFA1 receptors for potential therapeutic use in diabetes, there has been far less effort devoted to the identification and characterization of antagonist ligands. However, in terms of target validation, these can, at a minimum, be highly useful pharmacological tools. Examples include the early identification of GW1100 (Briscoe *et al.*, 2006), which continues to be used as a tool compound (Stoddart *et al.*, 2007; Hudson *et al.*, 2013a; Nakamoto *et al.*, 2013), as well as ANT203 (Kristinsson *et al.*, 2013), DC260126 (Hu *et al.*, 2009; Sun *et al.*, 2013) and a series of 1,2,3,4-tetrahydroisoquinolin-1-ones (Humphries *et al.*, 2009). Although treatment with these would not be likely to reduce hyperglycaemia, it has been suggested that DC260126 may protect against pancreatic beta cell dysfunction and might increase insulin sensitivity possibly via alleviation of hyperinsulinaemia, at least in genetically diabetic *db/db* mice (Sun *et al.*, 2013). Interestingly, although compounds from the Pfizer 1,2,3,4-tetrahydroisoquinolin-1-one series were tested and optimized for stability and *in vivo* clearance in rat (Humphries *et al.*, 2009), no clear assessment of their potency at FFA1 receptors from relevant animal model species was provided. However, as Hudson *et al.* (2013b) have reported an approximately 100-fold lower affinity of a compound from this series to inhibit the agonist function of TUG-424 at mouse compared with human FFA1 receptors, whereas the affinity of GW1100 was equivalent at these species orthologues, it is vital to explore potential differences in the affinity/potency of ligands across model species. Such species orthologue comparisons of affinity have not been reported for the other currently FFA1 receptor antagonists.

Nevertheless, a representative compound from the Pfizer series (*trans*-1-oxo-3-(4-phenoxyphenyl)-2-propyl-1,2,3,4-tetrahydroisoquinoline-4-carboxylic acid, PPTQ) has proven useful for demonstrating FFA receptor-mediated activity in the mouse-derived beta cell line INS-1E of a synthetic selective agonist (Christiansen *et al.*, 2011) and of conjugated linoleic acids (Schmidt *et al.*, 2011).

FFA4 receptors

The GPR120 [subsequently renamed systematically as the FFA4 receptor (Davenport *et al.*, 2013)] was initially defined as a receptor for unsaturated long-chain free fatty acids (Figure 3). Highly expressed in the gut (Hirasawa *et al.*, 2005; Paulsen *et al.*, 2014), initial studies also implicated FFA4, rather than FFA1, receptors as promoting secretion of GLP-1 from enteroendocrine cells (Hirasawa *et al.*, 2005). In significant part, interest in this receptor as a potential therapeutic target has been driven by the reported capacity to promote secretion of incretins, including GLP-1. Moreover, that a non-synonomous polymorphism in human FFA4 receptors is linked to obesity (Ichimura *et al.*, 2012), that an FFA4 receptor knockout line of mice developed obesity when fed a high fat diet (Ichimura *et al.*, 2012), and that a further FFA4 receptor knockout line are hyperglycaemic and glucose intolerant (Suckow *et al.*, 2014) have added impetus for further studies. Equally, a systems genetics approach identified markedly lower levels of FFA4 receptor mRNA in islets from diabetic or hyperglycaemic individuals, and knock-down of FFA4 receptor mRNA levels in islets limited the capacity of eicosapentaenoic acid, an ω-3 fatty acid activator of FFA4 receptors, to prevent palmitate-induced cell apoptosis (Taneera *et al.*, 2012). Although certainly expressed in pancreatic islets of both humans (Taneera *et al.*, 2012) and mice (Stone *et al.*, 2014; Suckow *et al.*, 2014), the exact cell types expressing this

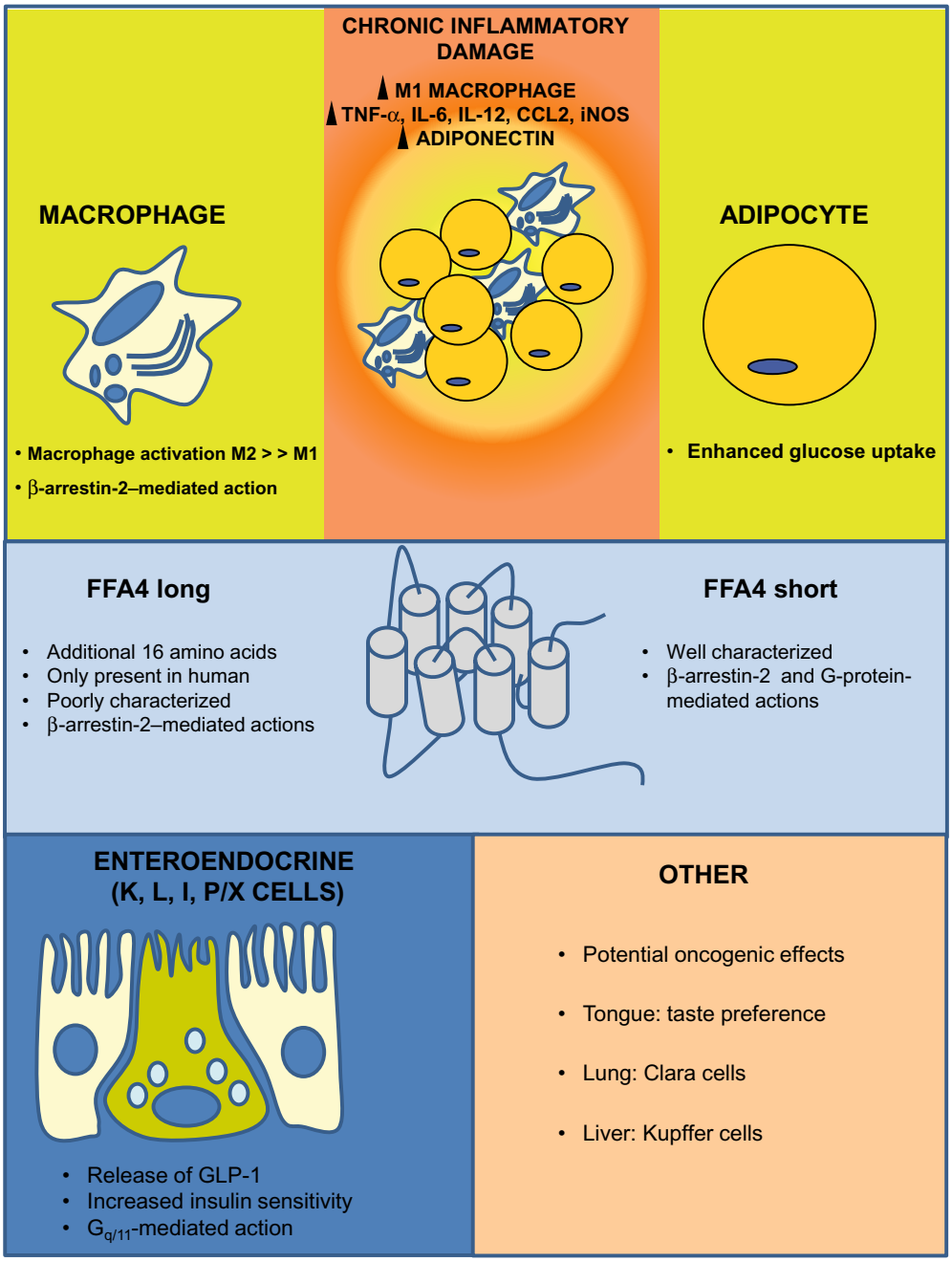

Figure 3

Expression and roles of FFA4 receptors. In humans, two isoforms, long and short, of FFA4 receptors have been reported. Key sites of expression include macrophages, where FFA4 receptor-mediated effects are anti-inflammatory and reported to be mediated via β-arrestin 2 scaffolding and in adipocytes and various enteroendocrine cells, where key signals reflect activation of G_q/G_{11} (and possibly G_i) family G-proteins. Although not discussed within the review FFA4 receptors are also known to be expressed in a range of other tissues, including the tongue where it plays an important role in the perception of fats, and in the lungs, where the function of the receptor is poorly explored.

receptor remain uncertain with evidence to favour both delta (Stone *et al.*, 2014) and alpha (Suckow *et al.*, 2014) cells. The allelic frequency of the human polymorphic variant initially linked to obesity is relatively low (Hudson *et al.*, 2013b), and indeed, appears to be very low in some populations, with detection only once in a recent Japanese study of 1585 subjects (Waguri *et al.*, 2013). Moreover, at least in rat models, Paulsen *et al.*, (2014) failed to observe increased circulating GLP-1 levels using the combined FFA1–4 receptor active fatty acid α-linolenic acid (18:3 n-3; aLA), and therefore, further work to define the importance of FFA4 receptors to specifically promote systemic GLP-1 levels requires further investigation. An interesting twist in man is the reported presence of both 'long' and 'short' isoforms of the receptor (Watson *et al.*,

2012), a feature not found in rodents or other species widely used for pharmacological studies, including cynomolgus monkey (Moore *et al.*, 2009). The current view appears to be that the long isoform, which contains an insert of 16 amino acids within the third intracellular loop, has rather limited tissue expression (Galindo *et al.*, 2012), and therefore, may be of limited consequence for therapeutic consideration. However, expression has been reported in the colon (Galindo *et al.*, 2012), and this variant does appear to display distinct differences in signal properties compared with the short isoform (see later).

A topic that has attracted almost as much attention as the possible effects of activation of FFA4 receptors on incretin release is the role of and mode of action of these receptors in macrophages. Activation here is anti-inflammatory and improves systemic insulin sensitivity in wild-type mice with these effects lacking in FFA4 receptor knockout animals (Oh *et al.*, 2010). Reviews stemming from these studies have focussed particularly, and potentially excessively, on the capacity of FFA4 receptors to be 'selective' for ω-3 fatty acids (e.g. Saltiel, 2010, Talukdar *et al.*, 2011, Im, 2012). This is not fully justified by data showing that FFA4 receptors are activated by a broad range of fatty acids. However, there is great interest in the ways in which ω-3 fatty acids produce health benefits (Calder, 2013). Furthermore, FFA4 receptors are also expressed by white adipocytes and studies have shown that in differentiated 3T3-L1 adipocytes both fatty acids (Oh *et al.*, 2010; Hudson *et al.*, 2013a) and the synthetic agonists GW9508 (Oh *et al.*, 2010) and TUG-891 (Hudson *et al.*, 2013a) can enhance uptake of deoxyglucose. However, it should be noted that at least in the studies of Hudson *et al.* (2013a), these effects were modest in extent compared with the effect of insulin. Despite this, Oh *et al.* (2010) have noted a capacity of DHA to promote uptake of deoxyglucose in primary adipose tissue from wild-type, but not FFA4 receptor knockout mice, and that the extent of this effect was undiminished by the presence of insulin, suggesting the potential for additive or even synergistic effects.

Identification and characterization of FFA4 receptor ligands

Because of the marked overlap in activation of FFA4 and FFA1 receptors by fatty acid ligands there has been a need to identify and develop selective FFA4 receptor agonists (Figure 1) to elucidate roles of this receptor and to employ these in concert with, or instead of, various knock-down and knockout strategies. Conceptually, this initially appeared likely to be highly tractable because, despite sharing the same group of fatty acids as agonists, the two receptors are not closely related [see *FFA4 receptors: mode(s) of ligand binding*]. Initial studies on the FFA1 receptor agonist GW9508 highlighted that, although markedly selective for FFA1 receptors, this compound does activate FFA4 receptors at higher concentrations (Briscoe *et al.*, 2006). As such, in the absence of other synthetic ligands, GW9508 has been used as an FFA4 receptor agonist in cell systems in which PCR-based studies have failed to identify co-expression of FFA1 receptors (Oh *et al.*, 2010). Initial efforts to find ligands with FFA4 receptor selectivity centred on the modification of PPAR-γ agonists (Suzuki *et al.*, 2008). However, these were of both modest potency and selectivity, and although the compound NCG21 was reported to be more potent (Sun *et al.*, 2010), it also displays modest potency and very limited selectivity (Shimpukade *et al.*, 2012; Hudson *et al.*, 2013a). TUG-891 was thus the first ligand reported with nanomolar potency at human FFA4 receptors and greater than 100-fold selectivity over FFA1 receptors (Shimpukade *et al.*, 2012). Optimization of FFA4 receptor ligands that resulted in the development of TUG-891 was directed largely via the use of an FFA4-β-arrestin-2 interaction assay (Shimpukade *et al.*, 2012) and might have resulted in ligand 'bias' towards this end point. Such assays are used in many GPCR ligand screening programmes (Chen *et al.*, 2012), but importantly, strong correlations between potency in this assay and ligand-induced elevation of $[Ca^{2+}]$, which reflects activation of the G_q/G_{11} family of heterotrimeric G-proteins, have been observed (Hudson *et al.*, 2013a). Moreover, in direct studies employing mutants of human FFA4 receptors, no obvious differences in ligand regulation of β-arrestin-2 recruitment and Ca^{2+} mobilization were noted (Hudson *et al.*, 2014). A number of other FFA4 agonist series have been reported in the patent literature (Halder *et al.*, 2013), but to date, very limited information (Engelstoft *et al.*, 2013; Stone *et al.*, 2014) on any of these has appeared in the primary scientific literature, and therefore, information on activity, potency and selectivity remain largely unverified. A compound denoted 'Metabolex 36' (3-(3,5-difluoro-4-((3-methyl-1-phenyl-1H-pyrazol-5-yl)methoxy)phenyl)-2-methylpropanoic acid) (Stone *et al.*, 2014) has been reported to show >100-fold selectivity for FFA4, over murine FFA1 receptors, while 3-(4-((5-chloro-2,2-dimethyl-2,3-dihydrobenzofuran-7-yl)methoxy)-2,3-dimethylphenyl)propanoic acid (compound B) is also reported to be markedly selective for FFA4 over murine FFA1 receptors (Engelstoft *et al.*, 2013).

The availability of TUG-891 allowed Hudson *et al.* (2013a) to explore the role of FFA4 receptors in a number of model cell systems. When human FFA4 receptors were expressed in a Flp-In T-REx 293 cell background, TUG-891 potently promoted elevation of $[Ca^{2+}]_i$ and, with distinctly lower potency, phosphorylation of ERK and MAPK in a predominantly G_q/G_{11}, but not β-arrestin-2-dependent fashion. Internalization was associated with receptor desensitization, but function and cell surface location of the receptor was rapidly recovered with washout of TUG-891 (Hudson *et al.*, 2013a). Others have also noted rapid internalization of the receptor in response to fatty acids (Watson *et al.*, 2012), and indeed, ligand-induced internalization was a key assay employed in de-orphanization of this receptor (Hirasawa *et al.*, 2005). However, unlike with TUG-891, internalization of the receptor in response to oleic acid was not rapidly reversed (Watson *et al.*, 2012). The basis for this difference has not been explored directly, but variation in the lipophilicity of the ligands and/or the washout protocols employed may provide at least part of the answer. This may then hint at possible differences in response and desensitization of the receptor *in vivo* when exposed chronically to ligands of different classes, but this is another topic that has yet to be addressed directly.

Potentially linked to receptor internalization, and as also shown for fatty acid agonists of FFA4 receptors (Hudson *et al.*, 2013a; Burns *et al.*, 2014; Sánchez-Reyes *et al.*, 2014), TUG-891 also promotes phosphorylation of the receptor (Hudson *et al.*, 2013a). Burns and colleagues extended initial studies

using a stepwise mutational approach to identify three residues in the C-terminal tail of the receptor that became phosphorylated. Butcher *et al*. (2014) have extended such studies to use combinations of mass spectrometry and mutagenesis to identify both the three same residues as Burns *et al*. (2014), and also two further amino acids in this region that were phosphorylated and had to be eliminated before TUG-891-mediated phosphorylation of the receptor was fully ablated.

Combinations of studies with TUG-891 and the highly selective FFA1 receptor agonist TUG-905 allowed Hudson *et al*. (2013a) to demonstrate that although FFA4 and FFA1 receptors are co-expressed by both STC-1 and GLUTag enteroendocrine cells, the release of GLP-1 in response to the fatty acid aLA predominantly reflects activation of FFA4 receptors. Moreover, although, as noted earlier, modest in efficacy compared with insulin, TUG-891 was able to promote deoxyglucose uptake in differentiated 3T3-L1 adipocytes (Hudson *et al*., 2013a). Furthermore, in terms of anti-inflammatory potential, TUG-891 was as efficacious as aLA in causing inhibition of LPS-induced TNF-α release from RAW264.7 macrophages (Hudson *et al*., 2013a). Interestingly, although the effectiveness of aLA and TUG-891 in this assay was equivalent, this was substantially lower than the efficacy of DHA in parallel experiments. DHA had been used in such assays previously (Oh *et al*., 2010), and although its enhanced effect has yet to be fully explored, Hudson *et al*. (2013a) speculated on both possible off-target effects of DHA and also the potential of high concentrations of this ligand to damage the cells under study and hence produce artefactual effects.

FFA4 receptors: mode(s) of ligand binding. As noted earlier, FFA4 receptors are not closely related to FFA1 receptors, despite being activated by the same broad range of fatty acids. This is highlighted by the lack of conservation of the pair of arginine residues (positions 5.39 and 7.35) and the asparagine (position 6.55) that are key components of the orthosteric binding pocket of the GPR40/FFA1 receptor. Potential charge partner residues in FFA4 receptors for the carboxylate of the fatty acids were Arg^{99} (residue position 2.64) and Arg^{178} (residue 4.65). Direct mutational studies have shown that Arg^{178} is not part of the orthosteric binding pocket (Watson *et al*., 2012; Hudson *et al*., 2013a), but that mutation of Arg^{99} eliminates function of ligands including fatty acids (Watson *et al*., 2012; Hudson *et al*., 2013a), GW9508 (Watson *et al*., 2012; Hudson *et al*., 2013a) and TUG-891 (Hudson *et al*., 2013a). Modelling studies have also indicated the potential of the carboxylate of both fatty acids and synthetic ligands to interact with Arg 2.64 (Sun *et al*., 2010; Shimpukade *et al*., 2012; Hudson *et al*., 2014). Use and further development of such homology models allowed Hudson and colleagues to select some 20 further residues predicted to be in contact with or in close proximity to bound TUG-891. Systematic mutational studies showed that conversion to alanine of the majority of these reduced or abolished the potency of TUG-891, and as discussed earlier for FFA1 receptors, subsets of these residues also affected potency of both GW9508 and aLA (Hudson *et al*., 2014). By examining a series of ligands closely related to TUG-891, but containing alterations within the biphenyl moiety that enhances potency and selectivity for FFA4 receptors, Hudson *et al*. (2014) went on to develop and employ a further model that included insights from the initial mutational studies and a series of alterations of the size and physical properties of amino acid 281 (residue 6.52), which is isoleucine in the wild-type receptor sequence. This model was used to explain the importance of the 4′-methyl group of TUG-891 for high-potency effects. Furthermore, this model also identified the contributions of Phe^{211} (residue position 5.42) and Val^{212} (5.43) in providing a hydrophobic pocket to accommodate the 4′-toluyl moiety of the ligand. In the absence of atomic level structures of FFA4 receptors, these studies set the stage for efforts in virtual screening and structure-based drug design to identify novel ligand classes for this receptor.

At this time, only agonist ligands of FFA4 receptors have been described. However, a recent report on the potential of FFA4 receptor agonists to promote angiogenesis in colorectal carcinoma cells (Wu *et al*., 2013) both hints at a potential concern in targeting this receptor, but more positively, also at a therapeutic rationale for the identification and characterization of antagonists of FFA4 receptors. This report suggests that FFA4 receptor activation promotes angiogenesis by stimulating release of VEGF, IL-8 and COX-2-derived PGE_2. These results are somewhat surprising in light of the findings that ω-3 fatty acids have been found to significantly reduce the risk of colorectal cancer (Kantor *et al*., 2014), and that FFA4 receptor activation is generally associated with anti-inflammatory effects (Oh *et al*., 2010) and down-regulation of COX-2 (Li *et al*., 2013), and therefore this issue requires further study. Assuming that FFA4 receptor antagonists display the equivalently high selectivity as already described for FFA1 receptor antagonists, they would also be of great use in further unravelling the roles and contribution of FFA4 receptors to GPCR-mediated effects of longer-chain fatty acids.

FFA4 receptors: mode(s) of signal transduction. Activation of FFA4 receptors results in rapid elevation of intracellular $[Ca^{2+}]$, and as this is not affected by cellular pretreatment with *Pertussis* toxin (Watson *et al*., 2012), was anticipated to reflect activation of G_q/G_{11} G-proteins. This has been confirmed, at least in Flp-In T-REx 293 cells transfected to express FFA4 receptors, by the capacity of the G_q/G_{11} inhibitor YM-254890 to block TUG-891-mediated increases in $[Ca^{2+}]_i$ (Hudson *et al*., 2013a). Moreover, although a role for β-arrestin-mediated scaffolding is often associated with phosphorylation of the ERK–MAPK (Luttrell and Miller, 2013), at least in the Flp-In T-REx 293 cell system employed, TUG-891-mediated effects on the ERK–MAPK were not affected by β-arrestin 2 knock-down (Hudson *et al*., 2013a). Although such G_q/G_{11}-mediated signalling is likely to be responsible for a broad range of the functional effects of FFA4 receptors, including potential effects on incretin-secretion and glucose update, at least for inhibition of release of inflammatory mediators from macrophages, a direct and central role for β-arrestin 2-mediated scaffolding has been elucidated. Selective knock-down of β-arrestin 2, but not β-arrestin 1 in RAW 264.7 cells blocked effects of GW9508 (used as an FFA4 receptor ligand in the absence of FFA1 receptor expression) to inhibit LPS-mediated inflammatory signals (Oh *et al*., 2010). This reflected that effects were channelled to the key kinase TAK1 via the β-arrestin 2-binding partner TAB1 (Oh *et al*., 2010). The results mentioned earlier suggest that anti-inflammatory

effects of FFA4 receptor ligands would be lacking in macrophages derived from β-arrestin 2 knockout mice, but that G–protein-mediated signals should be preserved. Given this rather obvious prediction and the relatively widespread availability and use of β-arrestin 2 knockout mice, it is surprising that such studies have not been reported to date.

Interestingly, a role for G_i-mediated signalling induced by FFA4 receptor activation has recently been uncovered. Both FFA4 receptor-mediated regulation of release of somatostatin from murine islets (Stone *et al.*, 2014) and of ghrelin from primary gastric mucosal cells (Engelstoft *et al.*, 2013) have been shown to be prevented by treatment with *Pertussis* toxin.

As noted earlier, agonist-induced internalization of FFA4 receptors is both rapid and extensive in model cell systems. However, truncation of the entire receptor C-terminal tail or mutation of a combination of hydroxyl amino acids and those with a negative charge, is sufficient to eliminate interactions with β-arrestin 2 and to prevent agonist-induced internalization of the receptor (Butcher *et al.*, 2014). As such, a further key assessment of the importance of β-arrestin 2-mediated signalling for FFA4 receptor-induced anti-inflammatory effects may be produced via knock-in of such a β-arrestin 2 interaction-deficient form of the receptor and subsequent studies on macrophages isolated from these animals.

Genetic variants of FFA4 receptors. As with other GPCRs activated by fatty acids, a number of open-reading frame, non-synonomous single-nucleotide polymorphisms have been reported for FFA4 receptors (Hudson *et al.*, 2013b). The most common of these is the Arg67Cys variant, where the minor Cys allele is reported to occur with some 15% frequency. No links of this variant to disease or substantial alteration in function have been reported (e.g. Ichimura *et al.*, 2012). However, although the minor allele frequency reported in the '1000 genomes' database is below 1% (Hudson *et al.*, 2013b), substantially more attention has been paid to the Arg254His (or Arg270His in the long isoform of the receptor) variant (Ichimura *et al.*, 2012). This reflects a combination of genetic linkage of the minor allele that, within a French population of adults and children displaying extreme obesity was in the region of 3% (although the population size was only 312), with a tendency to obesity in Europeans, and that the His containing variant when transfected alone appears to be both less effective in producing Ca^{2+} elevation in response to aLA, and when co-expressed with the major allele suppressed its signalling capacity (Ichimura *et al.*, 2012). Once again, independent confirmation of these results is awaited with interest.

In addition, there is also expression in humans of a 'long' isoform of the FFA4 receptor that has a 16-amino acid segment inserted into the third intracellular loop. The importance of the long isoform and even, indeed, its expression profile remains unclear, although it does not seem to be expressed widely. However, at least when expressed in a heterologous cell line, marked differences in signal transduction have been noted. Most interestingly, the long isoform is reported to be unable to cause agonist-induced elevation of $[Ca^{2+}]_i$, but to be internalized and interact with β-arrestin 2 as effectively as the short isoform (Watson *et al.*, 2012). Moreover, the apparent lack of G-protein engagement of the long isoform is supported by 'label-free' dynamic mass redistribution experiments (Watson *et al.*, 2012), which have been shown previously to integrate and report information from activation of all G-protein subclasses (Schröder *et al.*, 2011).

Summary and future directions

FFA1 and FFA4 receptors continue to attract great attention as potential therapeutic targets for both metabolic and inflammatory diseases. In the near future, attention is likely to focus on the progress of current clinical trials of FFA1 receptor agonists, and whether increased understanding of both variable efficacy and the modes of agonist action of different FFA1 receptor ligands results in further and distinct ligands entering clinical trials. For FFA4 receptors, further analysis of the roles of this receptor and to what extent these overlap with functions of FFA1 receptors, as well as the development and use of a broader range of more potent, more selective and more drug-like agonist ligands will be required before a full appreciation of the potential utility of this receptor as a therapeutic target is obtained.

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All authors wrote or contributed to the writing of this review.

Conflict of interest

None.

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