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Development of Free Fatty Acid Receptor 4 (FFA4/GPR120) Agonists in Health Science

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Abstract

Till the 21st century, fatty acids were considered as merely building blocks for triglycerides, phospholipids, or cholesteryl esters. However, the discovery of G protein-coupled receptors (GPCRs) for free fatty acids at the beginning of the 21st century challenged that idea and paved way for a new field of research, merged into the field of receptor pharmacology for intercellular lipid mediators. Among the GPCRs for free fatty acids, free fatty acid receptor 4 (FFA4, also known as GPR120) recognizes long-chain polyunsaturated fatty acids such as DHA and EPA. It is significant in drug discovery because it regulates obesity-induced metaflammation and GLP-1 secretion. Our study reviews information on newly developed FFA4 agonists and their application in pathophysiologic studies and drug discovery. It also offers a potency comparison of the FFA4 agonists in an AP-TGF-α shedding assay.

Key Words: GPR120, FFA4, G protein-coupled receptor, Agonist, Drug development

INTRODUCTION

Fatty acids have been long considered as merely building blocks for triglycerides, phospholipids, or cholesteryl esters. However, the discovery of G protein-coupled receptors (GPCRs) for the free fatty acids at the beginning of the 21st century opened up a new research field in pharmacology, merged into the field of receptor pharmacology for intercellular lipid mediators (Im, 2013). Free fatty acid receptor 1 (FFA1, also known as GPR40), highly expressed in pancreatic β cells, regulates insulin secretion in response to medium and long-chain fatty acids in blood (Li *et al*., 2020). Free fatty acid receptors 2 and 3 (FFA2 and FFA3, also known as GPR43 and GPR41, respectively) recognize short-chain fatty acids produced by gut microbiota and regulate immune responses (Sun *et al*., 2017; Tan *et al*., 2017). Free fatty acid receptor 4 (FFA4, also known as GPR120) was initially cloned as an orphan GPCR (Fredriksson *et al*., 2003; Davenport *et al*., 2013). In 2005, FFA4 was found to regulate gut incretin glucagon-like peptide-1 (GLP-1) secretion in enteroendocrine L cells (Hirasawa *et al*., 2005). Considering that GLP-1 is an incretin hormone, the therapeutic potential of FFA4 agonists is drawing great attention in the treatment of diabetes by enhancing glucose-dependent secretion of insulin from pancreatic β cells. In 2010, FFA4 was found to suppress the inflammatory response in macrophages and regulate insulin-sensitizing and antidiabetic effects of omega-3 polyunsaturated fatty acids (Oh *et al*., 2010). Later, exon sequencing of FFA4 in obese European subjects revealed a deleterious non-synonymous mutation (p.R270H), inhibiting FFA4 signaling activity (Ichimura *et al*., 2012). FFA4 regulation of GLP-1 in the gut and obesity-induced metaflammation in the adipose tissue indicates promising applications of FFA4 agonists in metabolic disorders (Oh *et al*., 2010). In this article, we review the development of FFA4 agonists and compared their potencies in an AP-TGF-α shedding assay system (Inoue *et al*., 2012).

DEVELOPMENT OF SYNTHETIC FFA4 AGONISTS

GW9508

GW9508, (3-[4-({[3-(phenyloxy)phenyl]methyl}amino)phenyl] propanoic acid), was the first synthetic dual agonist of FFA1 and FFA4 (Fig. 1) (Briscoe *et al*., 2006). GW9508 stimulated intracellular Ca^{2+} mobilization in HEK293 cells expressing FFA1 and FFA4 (pEC_{50} values of 7.32 and 5.46, respectively). Its potency towards FFA4 was 100-fold lower compared to FFA1 (Briscoe *et al*., 2006). GW9508 was used to study the role of FFA1 recep-

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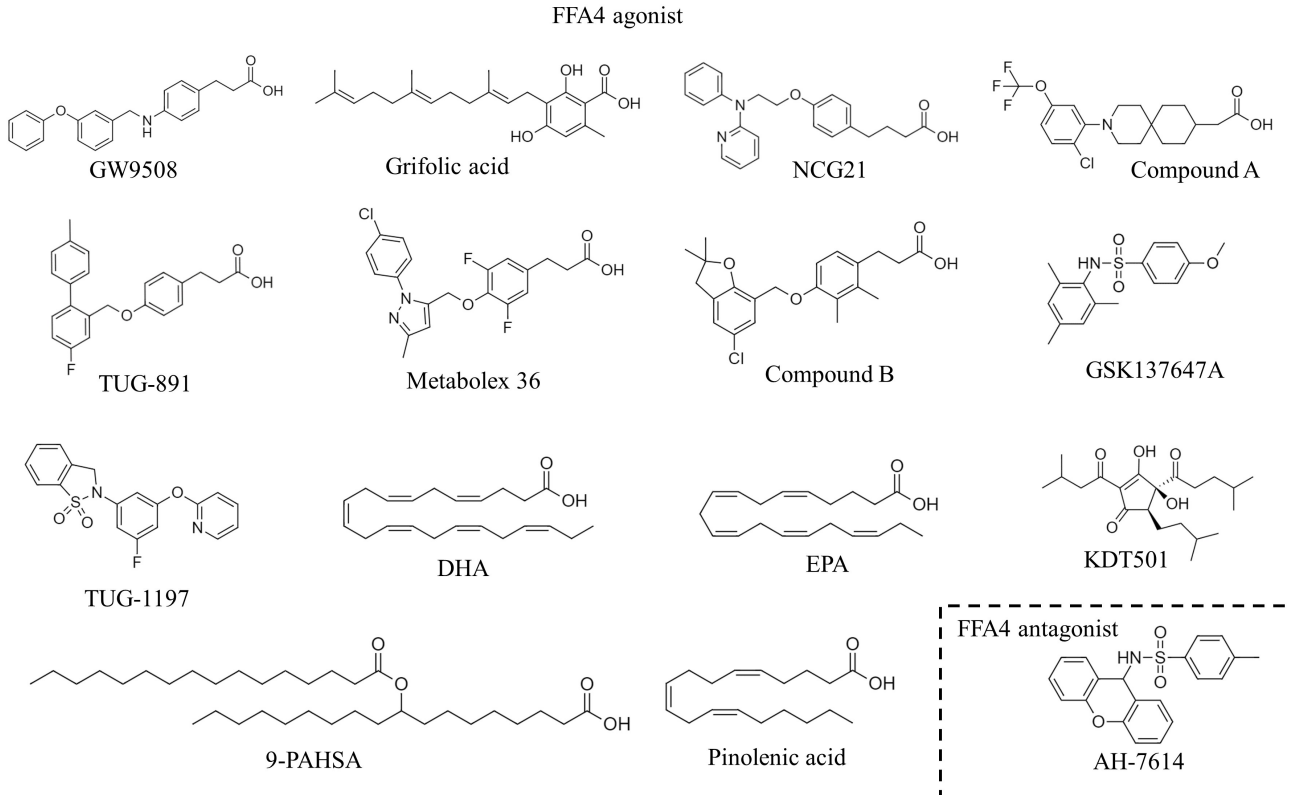

Fig. 1. Structures of FFA4 agonists and antagonist. DHA, EPA, pinolenic acid, 9-PAHSA, GW9508, grifolic acid, NCG21, compound A, TUG891, metabolex 36, and compound B have a carboxylic acid moiety, while GSK137647A, TUG-1197, KDT501 and AH-7614 have a sulfone amide moiety or an enolic acid moiety.

tors in processes such as the free fatty acid enhancement of glucose-stimulated insulin release and type 2 diabetes (Graciano *et al*., 2013). In glucose tolerance studies, the administration of GW9508 significantly reduced plasma glucose and augmented insulin release in mice (Moran *et al*., 2014).

GW9508 application in only FFA4-expressing cells: Despite the dual agonism of GW9508, it was used to specifically study FFA4 functions on several occasions. One occasion was when FFA1 expression was not detected or was negligible such as in ghrelin-producing cells, rat hypothalamic cells, murine bone marrow cultures, and human eosinophils (Cornish *et al*., 2008; Gong *et al*., 2014; Wellhauser and Belsham, 2014; Konno *et al*., 2015). Ghrelin is produced in the gut, and is often called the hunger hormone, because ghrelin signals the brain to become hungry and seek out food (Nakazato *et al*., 2001). Endogenous ghrelin levels are increased by fasting and decreased immediately after feeding. The activation of FFA4 by long-chain polyunsaturated fatty acids was studied as a mechanism of plasma ghrelin decrement after feeding (Gong *et al*., 2014). In ghrelin-producing SG-1 cells, mRNA of FFA4 is expressed, but not FFA1 (Gong *et al*., 2014). Treatment with GW9508 or α-linolenic acid (αLA) significantly inhibited ghrelin secretion *in vitro*, and this inhibition was absent in FFA4 knockdown cells (Gong *et al*., 2014). In addition, administration of GW9508 prevented fasting-induced plasma ghrelin elevation *in vivo*. Therefore, the decrease in plasma ghrelin levels after feeding is partially controlled by FFA4 activation in response to long-chain polyunsaturated fatty acids in the food (Gong *et al*., 2014).

In rHypoE-7 cells, an immortalized hypothalamic neuronal cell line, FFA4 expression was high but FFA1 was not detectable (Wellhauser and Belsham, 2014). Therefore, GW9508 was used to validate DHA FFA4 activation to prevent neuronal inflammation (Wellhauser and Belsham, 2014). GW9508 was used as a dual FFA1/FFA4 agonist on osteoclastogenesis in murine bone marrow cultures, and it mimicked the anti-osteoclastogenesis actions of stearic acids (Cornish *et al*., 2008). Because FFA4 expression was 100-fold higher than FFA1 expression, FFA4 was proposed as the receptor for the anti-osteoclastogenic effects of fatty acids (Cornish *et al*., 2008). This was later confirmed using FFA4 knockdown cells and KO mice (Ahn *et al*., 2016; Kim *et al*., 2016). Due to the negligible expression of FFA1 in eosinophils, GW9508 was used to study FFA4 functions in human eosinophils, where FFA4 activation significantly induced IL-4 secretion from eosinophils (Konno *et al*., 2015). GW9508 was also used to study the role of FFA4 signaling in promoting angiogenic switching and motility of human colorectal carcinoma cells, because there was no detectable expression of FFA1 (Wu *et al*., 2013). FFA4 expression is induced in colorectal carcinoma tissues and cell lines in association with tumor progression (Wu *et al*., 2013).

GW9508 application in combination with siRNA technique: GW9508 was also used to demonstrate the role of FFA4 in the regulation of inflammatory response in cyclophosphamide-

induced interstitial cystitis. Interstitial cystitis is a clinical syndrome characterized by urinary frequency, nocturia, and pelvic pain with unknown etiology (Patnaik *et al*., 2017). GW9508 treatment inhibited cyclophosphamide-induced inflammatory responses in RT4 urothelial cells through suppression of NF-κB translocation from the cytosol to the nucleus (Chen *et al*., 2018a). This anti-inflammatory effect was abolished by FFA4 antagonist AH7614 or siRNA transfection of FFA4 (Chen *et al*., 2018a). Furthermore, the significantly increased inflammatory cell infiltration as well as the impaired urothelial integrity of the bladder after cyclophosphamide treatment were reversed *in vivo* by GW9508 treatment in combination with extracorporeal shock wave (Chen *et al*., 2018a). Treatment with GW9508 *in vivo* protected the liver against ischemia-reperfusion injury, and pretreatment with FFA4-siRNA suggested FFA4 in Kupffer cells as a mediator of the effects in the liver (Raptis *et al*., 2014).

GW9508 application in combination with GW1100 (FFA1 antagonist): Although FFA1 expression was detected, GW9508 was utilized in combination with GW1100, an FFA1 antagonist in colon cancer cells, osteosarcoma, pituitary cultures, and enteroendocrine L cells (Garrel *et al*., 2011; Wu *et al*., 2013; Takahashi *et al*., 2017, 2018). In colon cancer DLD1 cells, treatment with GW9508 in combination with FFA4 knockdown cells and GW1100 treatment revealed that both FFA1 and FFA4 are involved in the regulation of cellular function during tumor progression by inhibiting cell motility (Takahashi *et al*., 2018).

Therefore, GW9508 has been utilized to find out the functions of FFA4 in multiple cells and organs, although it is a dual agonist of FFA1 and FFA4. In several cell lines, FFA1 expression was not detectable or was negligible, and in other cases, FFA4 knockdown cells or KO mice were used to support the function of FFA4. Sometimes, the FFA1 antagonist GW0011 was useful to exclude the possibility of FFA1 involvement.

NCG21

NCG21, [4-{4-[2-(Phenyl-pyridin-2-yl-amino)-ethoxy]-phenyl}-butyric acid], was developed as a selective agonist of FFA4 by screening derivatives of peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists (Fig. 1) (Suzuki *et al*., 2008). It has a 10-fold higher selectivity to FFA4 over FFA1 (Suzuki *et al*., 2008). NCG21 activates extracellular signal-regulated kinase (ERK) in FFA4 expressing HEK293 cells, and increases intracellular Ca^{2+} concentrations and GLP-1 secretion in murine enteroendocrine STC-1 cells, which endogenously express FFA4 (Suzuki *et al*., 2008). Administration of NCG21 into the mouse colon resulted in an increase in plasma GLP-1 levels, implying *in vivo* efficacy on enteroendocrine L cells (Sun *et al*., 2010).

Grifolic acid

Grifolic acid, [2,4-Dihydroxy-6-methyl-3-[(2E,6E)-3,7,11-trimethyl-2,6,10-dodecatrien-1-yl]-benzoic acid], was found to be a partial agonist of FFA4 by screening lipids of acidic structure (Fig. 1) (Hara *et al*., 2009). It activated ERK and increased intracellular Ca^{2+} concentrations in FFA4-expressing cells, but not in FFA1 cells (Hara *et al*., 2009). However, its activation of FFA4 was not the same as that of αLA, and it inhibited the αLA-induced activation of ERK and Ca^{2+} response in FFA4-expressing cells (Hara *et al*., 2009). Grifolic acid was used to test whether FFA4 activation affects ghrelin secretion *in vitro* in MGN3-1 ghrelinoma cells, but no effect was observed (Janssen *et al*., 2012). However, administration of grifolic acid increased plasma ghrelin levels *in vivo* (Janssen *et al*., 2012).

TUG-891

TUG-891, 3-(4-((4-fluoro-4'-methyl-[1,1'-biphenyl]-2-yl)methoxy)phenyl)propanoic acid, has been reported as a potent selective agonist for FFA4 (Fig. 1) (Shimpukade *et al*., 2012). In various assay systems, such as Ca^{2+} mobilization, β-arrestin recruitment, and ERK phosphorylation, TUG-891 showed properties similar to αLA in human FFA4-expressing cells (Hudson *et al*., 2013). FFA4 activation by TUG-891 induces rapid phosphorylation of FFA4 and subsequent internalization (Hudson *et al*., 2013). TUG-891 showed potent agonism on both mouse and human FFA4. However, its selectivity for mouse FFA4 over mouse FFA1 was not high, resulting in limitation of its use in rodent species *in vivo* (Hudson *et al*., 2013; Suckow and Briscoe, 2017). Although TUG-891 is an orally available, potent, and selective agonist for human FFA4, its use in experimental therapeutics should be carefully interpreted, because its action could result from FFA1 in rodents (Hudson *et al*., 2013; Suckow and Briscoe, 2017).

Nevertheless, oral administration of TUG-891 through water intake not only ameliorated inflammation in visceral white adipose tissue and insulin resistance, but also suppressed increased food intake and weight gain induced by sleep fragmentation in mice (Gozal *et al*., 2016). FFA4 is highly expressed in brown adipose tissue and its expression increases further under cold conditions (Christian, 2020). TUG-891 administration induced stimulation of mitochondrial breathing in brown adipose tissue, resulting in enhancement of fat oxidation and reduction of fat mass in mice (Schilperoort *et al*., 2018). TUG-891 stimulation of brown adipocytes induces an intracellular Ca^{2+} increase, resulting in elevated consumption of O_2 and mitochondrial fission and depolarization (Christian, 2020). Therefore, FFA4 activation by TUG-891 increased fat oxidation and reduced fat mass in mice (Christian, 2020).

TUG-891 was used to elucidate FFA4 function in the tongue in relation to taste preference for fats. In cultured mouse and human taste bud cells, TUG-891 treatment induced a rapid increase in intracellular Ca^{2+}, induced ERK1/2 phosphorylation, and enhanced release of GLP-1 (Murtaza *et al*., 2020). Direct lingual application of TUG-891 altered circulating concentrations of cholecystokinin and adipokines, associated with decreased circulating LDL in conscious mice (Murtaza *et al*., 2020). Binding of TUG-891 to lingual FFA4 seems to activate the tongue-brain-gut axis and modulate taste preference for fats (Murtaza *et al*., 2020). TUG-891 was also used in a cisplatin-induced acute kidney injury model. Treatment with TUG-891 ameliorated cisplatin-induced acute kidney injury by repressing ER stress and apoptosis in tubular epithelial cells through FFA4 activation (Huang *et al*., 2020). In mouse alveolar macrophages, TUG-891 was used to investigate FFA4 function in phagocytosis. Treatment with TUG-891 inhibited the motility and phagocytosis of alveolar macrophages via a Gq protein-PLC-Ca^{2+} release pathway (Su *et al*., 2020). FFA4-mediated phagocytosis was counteracted by LPS treatment (Zhao *et al*., 2019). LPS treatment inhibited FFA4 expression in the mouse macrophage cell line, Ana-1, and in mouse alveolar macrophages both *in vitro* and *in vivo* (Zhao *et al*., 2019).

Based on the two findings that FFA4 activation induces M2 polarization in macrophages and M2 phenotype macrophages protect the kidney against renal interstitial fibrosis, an interesting *in vivo* experiment was conducted. Peritoneal

macrophages from rats were incubated with TUG-891 *in vitro* for 24 h, and then transplanted autologously into the kidney with ureteral obstruction by intrarenal injection for 7 days on the same day following unilateral ureteral obstruction operation (Wang *et al*., 2019). TUG891-programmed macrophages upregulated the expression of M2 markers CD206 and arginase-1, while the expression of M1 markers IL-6 and TNF-α was downregulated (Wang *et al*., 2019). Increased renal interstitial fibrosis following the operation was markedly alleviated by TUG891-programmed macrophages, but not by untreated macrophages (Wang *et al*., 2019). To interpret the relevant function of FFA4 in the differentiation of 3T3-L1 adipocytes, TUG-891 was used because natural ligands DHA and αLA showed different effects in the cells (Song *et al*., 2016). FFA4 expression was increased along with the adipogenic differentiation of 3T3-L1 adipocytes, and the adipogenic ability of TUG-891 was significantly inhibited in FFA4 knockdown cells (Song *et al*., 2016). Although TUG-891 has a highly selective activity on human FFA4 over FFA1, its selectivity for mouse FFA4 over mouse FFA1 was not convincing. However, in combination with siRNA technology and KO mice, TUG-891 has been used as a tool for FFA4.

GSK137647A

GSK137647A, [4-methoxy-N-(2,4,6-trimethylphenyl) benzenesulfonamide], was developed by GlaxoSmithKline as the first non-carboxylic FFA4 agonist (pEC_{50}=6.3) (Fig. 1) (Sparks *et al*., 2014). Based on this report, it has 50-fold higher selectivity for FFA4 over FFA1 and preserved activity across species (Sparks *et al*., 2014).

GSK137647A was used to investigate FFA4 function on the osteogenic and adipogenic differentiation of bone mesenchymal stem cells of db/db mice (Wang *et al*., 2020). GSK-137647A showed a significant increase in mineralization of differentiated osteoblasts compared to the control group and elevated alkaline phosphatase activity in a time-dependent manner, while it suppressed the adipogenic differentiation of bone mesenchymal stem cells (Wang *et al*., 2020).

In vitro and *ex vivo* treatments of pancreatic β MIN6 cells and islets isolated from wild-type mice, GSK137647A restored pancreatic duodenal homeobox-1 expression levels and β-cell function by inhibiting palmitic acid-induced elevation of proinflammatory chemokines and activation of NF-κB, JNK1/2, and p38MAPK signaling pathways (Wang *et al*., 2019). The protective effects were not observed in FFA4 knockdown MIN6 cells and islets isolated from FFA4 KO mice (Wang *et al*., 2019). GSK137647A supplementation ameliorated glucose tolerance and insulin sensitivity as well as improved pancreatic duodenal homeobox-1 expression and islet inflammation in mice with diet-induced obesity, but not in FFA4 KO mice (Wang *et al*., 2019).

GSK137647 attenuated both basal and LPS-induced production of IL-6 and CCL2 in 3T3-L1 adipocytes (Hasan *et al*., 2017). The expression of MMP-9, MMP-3, and tissue inhibitor of metalloproteinase-1 were attenuated in RAW 264.7 macrophages grown in conditioned medium collected from GSK137647-treated adipocytes (Hasan *et al*., 2017). FFA4 activation in adipocytes could reduce macrophage migration via a paracrine mechanism (Hasan *et al*., 2017).

Compound A

Compound A was developed by Merck as an orally available, selective FFA4 agonist (EC_{50}=~0.35 μM) (Fig. 1) (Oh *et al*., 2014). Its activation on FFA1 is negligible (Oh *et al*., 2014). Compound A showed anti-inflammatory effects in macrophages *in vitro* (Oh *et al*., 2014). Administration of compound improved glucose tolerance, decreased hyperinsulinemia, increased insulin sensitivity, and decreased hepatic steatosis in obese mice fed a high-fat diet (Oh *et al*., 2014). Recently, compound A was used to verify that beneficial effects of DHA on atopic dermatitis is mediated through FFA4 activation and $Foxp3^+$ Treg increase in combination with FFA4 KO mice (Son *et al*., 2020).

Metabolex compounds

Metabolex 36 and metabolex compound B, developed by the company Metabolex (now Cymabay Therapeutics) have been used as FFA4 agonists in several studies (Fig. 1). Metabolex 36 was used to study FFA4 function in pancreatic δ cells along with AstraZeneca compounds (AZ-423 and AZ-670) (Stone *et al*., 2014). Glucose-induced somatostatin secretion from murine islets was inhibited by FFA4 agonists, but not in FFA4 KO mice (Stone *et al*., 2014). Metabolex 36 has a pEC_{50} value of 5.9 on FFA4 and less than 4.0 on FFA1 (Stone *et al*., 2014). Compound B was used to investigate the role of FFA4 in ghrelin secretion and somatostatin release in combination with FFA4 KO mice (Engelstoft *et al*., 2013; Egerod *et al*., 2015). Compound B had EC_{50}=15 nM on FFA4 and 1000-fold selectivity over FFA1 in inositol triphosphate accumulation assays in FFA4-transfected COS7 cells (Engelstoft *et al*., 2013). Compound B inhibited basal ghrelin secretion from primary gastric mucosal cells but not in cells isolated from FFA4 KO mice (Engelstoft *et al*., 2013). Oral administration of compound B decreased plasma ghrelin under fasting conditions in mice (Engelstoft *et al*., 2013). Compound B also inhibited somatostatin release from primary gastric epithelial cells, but was not in cells isolated from FFA4 KO mice (Egerod *et al*., 2015).

TUG-1197

TUG-1197 (compound 34 in the paper) was developed from a series of cyclic sulfonamide FFA4 agonists disclosed by Banyu Pharmaceutical (Fig. 1) (Azevedo *et al*., 2016; Hansen and Ulven, 2017). TUG-1197 has pEC_{50}=6.6 and 6.8 in Ca^{2+} mobilization assay on human FFA4 and murine FFA4, respectively (Azevedo *et al*., 2016; Hansen and Ulven, 2017). In mice with diet-induced obesity, TUG-1197 treatment improved glucose tolerance and increased insulin sensitivity in an FFA4-dependent manner (Azevedo *et al*., 2016).

KDT501

KDT501 is an orally administered isohumulone structure derived from hop extracts and designed to help control impaired glucose and insulin regulation in patients with insulin resistance. The agonist activity of KDT501 on FFA4 was estimated as EC_{50}=30.3 μM and a partial agonism on PPARγ [US patents 2012/0108671A1]. Nonclinical studies have shown that administration of KDT501 improved insulin sensitivity and glucose regulation as well as reduced proinflammatory signals (Konda *et al*., 2014).

NATURAL AGONISTS OF FFA4

Natural long-chain fatty acids are recognized as endogenous ligands of FFA4 (Davenport *et al*., 2013). Initially, saturated fatty acids with a chain length of C14 to C18 and unsaturated fatty acids with chain length of C16 to C22 were found to activate FFA4 (Hirasawa *et al*., 2005), which was later confirmed (Christiansen *et al*., 2015). Omega-3 polyunsaturated fatty acids, including αLA, DHA, EPA, and pinolenic acid, showed higher potency than other fatty acids, and natural polyunsaturated fatty acids are all dual agonists for FFA4 and FFA1 (Christiansen *et al*., 2015; Ulven and Christiansen, 2015). Natural fatty acids such as DHA and EPA have also been utilized to determine the functions of FFA4 in various cells and animal models. However, caution should be exercised while using DHA and EPA as tools for FFA4 activation because they could exert actions via other molecular targets such as FFA1, PPARα, RXRα, or their conversion to pro-resolving mediators such as resolvin E series, protectin D1, and maresin 1 (Im, 2012; Ulven and Christiansen, 2015; Serhan *et al*., 2018). Nevertheless, DHA, EPA, and αLA have been used as natural ligands for FFA4.

EPA treatment alleviated cerebral ischemia injury by preventing acute cerebral infarction-induced inflammation via the inhibition of NLRP3 inflammasome activation, which was not observed in FFA1 and FFA4 double knockout mice (Mo *et al*., 2020). Treatment with EPA or DHA significantly increased the protein level of FFA4 in RAW264.7 cells and increased the proliferation index in RAW264.7 cells (Han *et al*., 2017). Using DHA supplementation and FFA4 KO mice, FFA4 was identified as an inhibitor of dendritic cell maturation (Zhao *et al*., 2020). DHA induced a tolerogenic dendritic cell phenotype and enhanced regulatory T cell population *in vivo* through FFA4 (Zhao *et al*., 2020).

Administration of DHA showed promising results in experimental chronic colitis and body weight loss improvement. IL-10 KO mice display the most similar characteristics to those of human Crohn's disease (Zhao *et al*., 2017). In the DHA-treated mice, enhanced expression and improved distribution integrity of protein FFA4 were observed, which was probably associated with the regulation of the TAK1/IKK-α/IκB-α/p65 pathway, which might be an important target for Crohn's colitis (Zhao *et al*., 2017). FFA4 levels were downregulated in osteoarthritis patients compared with control subjects, whereas FFA4 activation with DHA exhibited anti-inflammatory effects in primary human chondrocytes *in vitro* (Chen *et al*., 2018b). FFA4 agonism with DHA enhanced wound repair and healing of a skin defect model in mice, as shown by the downregulation of $CD68^+$ cell numbers (Chen *et al*., 2018b).

In 2014, lipidomic analysis of mice overexpressing the Glut4 glucose transporter in adipocytes revealed structures of branched fatty acid esters of hydroxy fatty acids (e.g., palmitic-acid-9-hydroxy-stearic-acid, 9-PAHSA) as endogenous ligands for FFA4 (Yore *et al*., 2014). In adipocytes, 9-PAHSA enhanced insulin-stimulated glucose uptake through FFA4 (Yore *et al*., 2014).

FFA4 ANTAGONIST

AH-7614

AH-7614 (compound 39 in the paper) was first described as an antagonist of FFA4, that is, AH-7614 inhibited FFA4 activation by GSK137647A and linoleic acid (Fig. 1) (Sparks *et al*., 2014). AH-7614 is a negative allosteric modulator of FFA4 (Sparks *et al*., 2014). GSK137647A inhibited differentiation towards adipocyte phenotype in a mouse mesenchymal stem cell line and consistently inhibited endogenous signaling of FFA4 (Watterson *et al*., 2017). Effects of arachidonic acid on FFA4 are also inhibited by AH-7614 (Villegas-Comonfort *et*

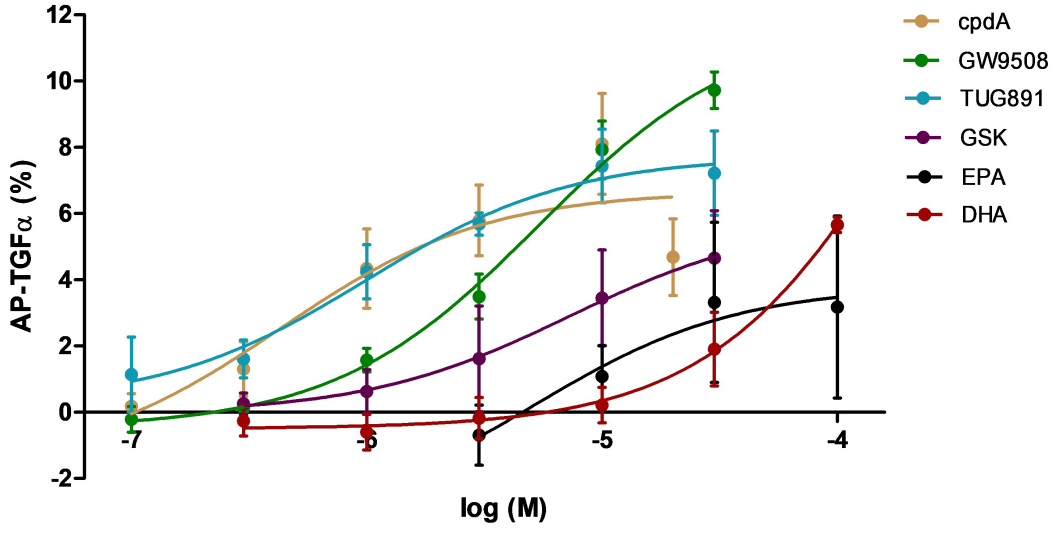

Fig. 2. Concentration-response curves of FFA4 agonists in an AP-TGF-α shedding assay. HEK-293 cells were transfected with plasmids (an AP fusion protein of TGF-α and human FFA4) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The following day, ligands were added at different concentrations in transfected HEK-293 cells, and the plate was incubated for 1 h. Para-nitrophenyl phosphate (substrate of AP)-containing solution was added to the conditioned medium plate and to the cell plate. The absorbance of the plate contents was measured for 405 nm. The ratio of the two absorbance values was used as a measure of GPCR activation (Inoue *et al*., 2012).

al., 2017). AH-7614 was used to prove FFA4 involvement in cyclophosphamide-induced interstitial cystitis by inhibiting the inflammatory response in bladder cells (Chen *et al*., 2018a). Protective effect of DHA on non-alcoholic fatty liver disease was studied in an *in vitro* model of liver X receptor-mediated hepatocellular steatosis. DHA-induced inhibition of lipid accumulation was blunted by treatment of AH-7614 and not observed in primary hepatocytes from FFA4 deficient mice (Kang *et al*., 2018).

COMPARISON OF FFA4 AGONIST ACTIVITIES

Commercially available agonists for FFA4 are limited. We compared the available potency of FFA4 agonists in an AP-TGF-α shedding assay system (Inoue *et al*., 2012). As shown in Fig. 2, compound A and TUG-891 showed the highest potency, followed by GW9508. GSK137647A was not as potent as compound A and TUG-891, and its efficacy was not as high as that of GW9508 (Fig. 2). Natural fatty acids DHA and EPA showed some activity, but their potency was lower than that

Table 1. Synthetic FFA4 agonists and their applications in FFA4-mediated pathophysiologic functions

Synthetic agonist	FFA4-mediated functions	Reference
GW9508	Enhancement of glucose-stimulated insulin release	Graciano *et al*., 2013
	Reduction of plasma glucose	Moran *et al*., 2014
	Inhibition of inflammatory responses	Raptis *et al*., 2014; Chen *et al*., 2018a
	Decrease of ghreline secretion	Gong *et al*., 2014
	Prevention of neuronal inflammation	Wellhauser and Belsham, 2014
	Anti-osteogenesis	Ahn *et al*., 2016; Kim *et al*., 2016
	Induction of IL-4 secretion from eosinophils	Konno *et al*., 2015
	Promotion of angiogenesis and motility in colorectal carcinoma	Wu *et al*., 2013
NCG21, grifolic acid	Increase of GLP-1 secretion	Suzuki *et al*., 2008; Sun *et al*., 2010; Janssen *et al*., 2012
TUG-891	Amelioration of metaflammation and insulin resistance	Gozal *et al*., 2016
	Increase of fat oxidation and reduction of fat mass	Schilperoort *et al*., 2018; Christian, 2020
	Increase of GLP-1 secretion	Murtaza *et al*., 2020
	Decrease of circulating LDL	Murtaza *et al*., 2020
	Amelioration of acute kidney injury	Huang *et al*., 2020
	Inhibition of motility and phagocytosis in alveolar macrophages	Zhao *et al*., 2019
	Induction of M2 polarization	Wang *et al*., 2019
	Induction of adipogenic differentiation	Song *et al*., 2016
GSK137647A	Increase in mineralization of differentiated osteoblasts	Wang *et al*., 2020
	Suppression of adipogenic differentiation of mesenchymal stem cells	Wang *et al*., 2020
	Protection of pancreatic β cell dysfunction	Wang *et al*., 2019
	Inhibition of islet inflammation	Wang *et al*., 2019
	Attenuation of IL-6 and CCL2 production in adipocytes	Hasan *et al*., 2017
Compound A	Anti-inflammation in macrophages	Oh *et al*., 2014
	Improvement of glucose tolerance,	Oh *et al*., 2014
	Decrease of hyperinsulinemia	Oh *et al*., 2014
	Increase of insulin sensitivity	Oh *et al*., 2014
	Decrease of hepatic steatosis	Oh *et al*., 2014
	Amelioration of atopic dermatitis	Son *et al*., 2020
Metabolex 36, Compound B	Suppression of somatostatin secretion from pancreatic δ cells	Stone *et al*., 2014
	Inhibition of ghrelin secretion	Engelstoft *et al*., 2013; Egerod *et al*., 2015
	Inhibition of somatostatin secretion from gastric epithelial cells	Egerod *et al*., 2015
TUG-1197	Improvement of glucose tolerance	Azevedo *et al*., 2016
	Increase of insulin sensitivity	Azevedo *et al*., 2016
KDT501	Improvement of insulin sensitivity	Konda *et al*., 2014
	Improvement of glucose regulation	Konda *et al*., 2014
	Reduction of proinflammatory signals	Konda *et al*., 2014
	Reduction of plasma triglycerides	Kern *et al*., 2017
	Increase of plasma adiponectin	Kern *et al*., 2017

of other synthetic FFA4 agonists (Fig. 2). 9-PAHSA did not show any activity in the AP-TGF-α shedding assay system (not shown in the Figure).

CLINICAL TRIALS TARGETING FFA4

There are completed and ongoing clinical trials with natural and synthetic FFA4 agonists. The ongoing human trial is titled as "Effects of pine nut and olive oil as FFA1/FFA4 and GPR119 agonists on glucose tolerance in healthy overweight or obese subjects" NCT03774095 (https://www.clinicaltrials.gov). Pine nut oil contains about 20% pinolenic acid, which has been shown to be an equipotent natural dual agonist of FFA1 and FFA4, and also improves glucose tolerance in mice (Christiansen *et al*., 2015). Olive oil contains mono-unsaturated oleic acid (61-80%), which can be transformed during digestion to 2-oleoylglycerol, an agonist of GPR119 (Hansen *et al*., 2011). Activation of three receptors (FFA1/FFA4 and GPR119) with pinolenic acid and 2-oleoylglycerol from pine nut oil and olive oil, respectively, may enhance or synergize GLP-1 secretion from enteroendocrine cells and enhance glucose-stimulated insulin secretion from pancreatic β cells. Outcomes of the clinical trial would be useful for building therapeutic strategies for diabetes and obese patients.

The other completed clinical trial related to FFA4 was "Effects of KDT501 on metabolic features in insulin-resistant subjects", that is, NCT02444910 (https://www.clinicaltrials.gov). KDT501 was well tolerated in nine insulin-resistant prediabetic humans, and treatment with KDT501 reduced plasma triglycerides and TNF-α, while plasma adiponectin increased significantly (Kern *et al*., 2017). The systemic effects might come from KDT501 ability to potentiate β-adrenergic signaling and enhance mitochondrial function in adipocytes of subcutaneous white adipose tissue (Finlin *et al*., 2017).

CLOSING REMARKS

Since the identification of FFA4 as a receptor for polyunsaturated fatty acids in 2005, many efforts have been made to synthesize selective and potent agonists of FFA4. Among natural agonists, DHA and EPA are the most potent, while compound A and TUG-891 are the most potent synthetic agonists. Despite having dual agonism for FFA1 and FFA4, GW9508 has been primarily used as a tool for FFA4 research. The limitation of FFA1/4 dualism has been overcome by combining GW9508 with GW1100 (FFA1 antagonist) or combination with FFA4 knockdown cells or mice. In certain cell types, FFA1 expression was null or not detectable, resulting in observation of only FFA4-activated functions with GW9508. Therefore, GW9508 has been efficiently applied to elucidate FFA4 functions. However, for the therapeutic applications, FFA4 agonists with better pharmacokinetic and pharmacodynamic properties should be developed, especially for human trials.

In summary, there are accumulating data to support the beneficial functions of FFA4 in metabolic and inflammatory diseases (Table 1). Pharmacological tools for FFA4 research are available to apply, but not many. Clinical trials with natural agonists for FFA1/FFA4/GPR119 or synthetic derivatives for FFA4 and PPARγ are progressing for obesity and diabetes. We would like to close this article with the hope to find more pathophysiological significance of FFA4 in human bodies and to see FFA4 agonists (natural or synthetic) in use for the treatment of human diseases in the future.

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