# Pharmacological potential of novel agonists for FFAR4 on islet and enteroendocrine cell function and glucose homeostasis

A. G. McCloskey, M. G. Miskelly, P. R. Flatt, A. M. McKillop

School of Biomedical Sciences, Ulster University, Cromore Road, Coleraine, BT52 1SA, Northern Ireland.

**Short Title:** Selective FFAR4 agonists and beta cell function

**Corresponding author**

Aine M. McKillop, School of Biomedical Sciences, Ulster University, Cromore Road, Coleraine, BT52 1SA, Northern Ireland. Tel: +44 (0)28 70123066. Fax: +44(0)2870124965. Email: am.mckillop@ulster.ac.uk

Word count:

Number of references: 34

Number of figures: 5

Number of tables: 0

## Abstract

**Background:** To investigate the metabolic effects of FFAR4-selective agonists on islet and enteroendocrine cell hormone release and the combined therapeutic effectiveness with DPP-IV inhibitors.

**Methods:** Insulinotropic activity and specificity of FFAR4 agonists were determined in clonal pancreatic BRIN-BD11 cells. Expression of FFAR4 was assessed by qPCR and western blotting following agonist treatment in BRIN-BD11 cells and by immunohistochemistry in mouse islets. Acute *in-vivo* effects of agonists was investigated after intraperitoneal (i.p.) or oral administration in lean and HFF-obese diabetic mice.

**Results:** GSK137647 ($10^{-11}$-$10^{-4}$ M) and Compound-A ($10^{-10}$-$10^{-4}$ M) stimulated insulin secretion at 5.6mM ($p<0.05$-$p<0.001$) and 16.7mM ($p<0.05$-$p<0.001$) glucose in BRIN-BD11 cells, with no cytotoxicity effects as assessed by MTT. FFAR4 antagonist (AH-7614) abolished the insulintropic effect of GSK137647 ($p<0.05$-$p<0.001$), whilst FFAR1 antagonist (GW1100) had no effect. Incubation of BRIN-BD11 cells with GSK137647 and Compound-A increased FFAR4 ($p<0.01$) gene expression at 16.7 mM glucose, with a corresponding increase in FFAR4 ($p<0.01$) protein concentrations. FFAR4 upregulation was attenuated under normoglycaemic conditions. Immunohistochemistry demonstrated co-localisation of FFAR4 and insulin in mouse islets. Orally administered GSK137647 or Compound-A (0.1 μmol/kgBW) monotherapy and combinational therapy with Sitagliptin improved glucose tolerance ($p<0.001$), increased plasma insulin ($p<0.001$), GLP-1 ($p<0.05$), GIP ($p<0.05$), decreased DPP-IV activity ($p<0.01$-$p<0.001$) and induced satiety ($p<0.001$) in HFF mice.

**Conclusions:** Specific FFAR4 agonism improves glucose tolerance through insulin and incretin secretion, with enhanced DPP-IV inhibition in combination with Sitagliptin.

**General significance:** These findings have for the first time demonstrated that selective FFAR4 activation regulates both islet and enteroendocrine cell function with agonist combinational therapy, presenting a promising strategy for the treatment of type-2-diabetes.

**Keywords:** FFAR4, specificity, insulin, incretin, DPP-IV inhibition, combinational therapy

## 1 Introduction:

FFAR4 (GPR120) is a rhodopsin-like G-protein coupled receptor that is activated by unsaturated fatty acids (C16-22) and long chain saturated fatty acids (C14-18) [1-2]. The human FFAR4 gene is encoded on chromosome 10.q23.3 [3]. With respect to tissue distribution, FFAR4 is extensively expressed in peripheral tissues, intestines, lungs, spleen and pro-inflammatory macrophages [4]. Furthermore, recent studies have demonstrated that FFAR4 is abundantly expressed in the pancreatic islet, with further analysis demonstrating its expression in clonal pancreatic β-cell lines, including MIN6, RINm5f and INS-1E [5-7].

Previously considered as orphan receptors, recent studies have shown FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41), FFAR4 (GPR120) and GPR84 to be activated by free fatty acid (FFA) molecules [3, 4, 8]. FFAR3 and FFAR2 exhibit specificity towards short chain fatty acids, GPR84 is activated by medium chain fatty acids, whereas FFAR1 and FFAR4 are activated by long chain fatty acids [3, 4]. Furthermore, FFAR4 and FFAR1 share 10% sequence homology and can be activated by similar endogenous ligands (Omega-3-fatty acids), which warrants the utilisation of receptor specific agonists to evaluate the therapeutic potential of FFAR4 [2, 8].

FFAR4 has been hypothesised to act as a lipid sensor in the body, and has been proven to have involvement in the regulation of inflammation, adipogenesis, and glucose homeostasis [1, 7, 9]. Interestingly, it has been reported that a mutation in the FFAR4 gene (R270H) is linked with the development of obesity. The p.R270H variant impairs the signalling response of FFAR4 upon FFA binding, with subsequent defects observed to intracellular calcium mobilisation and GLP-1 secretion in intestinal cells [10, 11]. Further studies have demonstrated that FFAR4 knockdown with siRNA impaired the anti-apoptotic effects of omega-3 fatty acids in serum-starved STC-1 cells. Thus, indicating the potential proliferative and anti-apoptotic effects of FFAR4 in pancreatic beta cells [12].

Numerous studies have identified the involvement of FFAR4 in the gastrointestinal (GI) tract, including the mediation of glucagon-like peptide-1 (GLP-1), gastric inhibitory polypeptide (GIP) and cholecystokinin (CCK) secretion from intestinal L-cells, K-cells and I-cells, with high FFAR4 expression observed in the intestinal STC-1 and GLUTag cell lines [1, 12, 13]. FFAR4 activation has been shown to mediate GLP-1 secretion when tested with its endogenous agonist α-linolenic acid [1, 14, 15]. However, other studies suggest that FFAR4 has no role in GLP-1 release [16]. Previous findings have shown FFAR4 to mediate insulin-sensitising and anti-inflammatory properties in peripheral tissues [9].

The expression and biological function of FFAR4 in the intestinal tract has been heavily documented, however the role of FFAR4 in pancreatic beta cell function was not investigated until recently [7]. A number of FFAR4 agonists were demonstrated to have regulatory role in glucose dependent insulin secretion in mouse islets, including endogenous docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), alpha-linolenic acid (ALA) and synthetic GW-9508 [7, 8]. In addition, these agonists demonstrated insulinotropic and glucose lowering properties in *in-vivo* [7]. However, the selectivity of endogenous FFAR4 agonists (ALA, DHA, EPA)

remains uncertain as activation of FFAR1 may contribute to the effects observed, whilst synthetic GW9808 has been shown to exhibit 100-fold greater potency towards FFAR1 over FFAR4 [17, 18].

Upon activation, FFAR4 primarily couples to Gαq, which stimulates an array of secondary messenger signalling pathways through phospholipase C (PLC), including intracellular calcium and mitogen-activated protein kinases [4, 7]. The mechanism of FFAR4 mediated insulin secretion from the pancreatic beta cell is not conclusive; however, studies have shown a range of FFAR4 agonists to induce intercellular calcium release, indicating the potential involvement of inositol trisphosphate on intracellular calcium stores through PLCβ signalling [7]. FFAR4 activation with ALA and DHA leads to the rapid and transient phosphorylation of the receptor of HEK293 cells [19]. Although FFAR4 has been shown to act predominately through PKC signalling, DHA has also shown to activate G-protein coupled receptor kinase (GPK6) upon FFAR4 phosphorylation, with Thr(347), Ser (350), and Ser(357) shown to be major phosphorylation sites in the C-terminal tail of FFAR4 [19].

Recently, Oh *et al.,* have reported an orally available, selective, high affinity, small FFAR4 agonist (Compound A) that exhibits a range of anti-diabetic effects [20]. Oral administration of Compound A improved glucose tolerance, insulin sensitivity and exerted anti-inflammatory effects on macrophages in high fat fed obese mice [20]. Sparks *et al.,* recently identified a potent FFAR4 agonist GSK137647 [21] and preliminary *in-vitro* analysis has demonstrated that GSK137647 augmented insulin secretion in MIN6 cells, with a modest increase in GLP-1 secretion from the NCl-H716 intestinal cell line [21, 22]. *In-vivo* findings showed that GSK137647 induced GLP-1 release by mouse circumvallate papillae [22]. The highly selective properties of this agonist suggested that it was suitable to evaluate FFAR4 activation in pancreatic beta cells.

Due to the regulatory role of FFAR4 activation on insulin and incretin secretion [1, 7, 14], a promising approach using selective FFAR4 agonists combined with a dipeptidyl peptidase-4 (DPP-IV) inhibitor may offer therapeutic potential [23, 24]. The present study has assessed the effect of potent DPP-IV inhibitor (Sitagliptin) in combination with Compound A and GSK137647 on glucose tolerance and insulin secretion in high fat fed (HFF)-induced diabetic mice. This research aims to investigate the acute metabolic effects and of FFAR4 agonist monotherapy and combinational therapy on islet and enteroendocrine cell function, using pancreatic cells and diabetic mice.

## 2 Materials and methods:

### 2.1 Materials:

FFAR4 agonists Compound A and GSK137647 were purchased from Cayman Chemicals (Michigan, USA) and Tocris (Bristol, UK) respectively. Sitagliptin phosphate monohydrate was obtained from Apexbio Technology LLC (Texas, USA). Thiazolyl blue tetrazolium bromide (MTT) was received from Sigma (Poole, UK). Rabbit anti-GPR120 polyclonal IgG antibody (H-155) was purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA) and guinea pig anti-insulin from Abcam (Cambridge, UK).

### 2.2 Insulin secretion:

Generation and characterization of the insulin-secreting BRIN-BD11 cells were outlined previously [25]. BRIN-BD11 cells were cultured with RPMI-1640 media (11.1 mM glucose) containing antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 10% foetal calf serum at 37$^{o}$C in 95% air and 5% carbon dioxide. For acute insulin secretion studies, cells were detached using trypsin/EDTA and incubated overnight in 24-well plates with 150,000 cells per

well. Cells were then pre-incubated for 40 min at 1.1 mmol/l glucose in Krebs buffer (comprising 4.7 mmol/l KCL, 115 mmol/l NaCl, 1.28 mmol/$CaCl_2$, 10 mmol/l $NaHCO_3$, 5 g/l bovine serum albumin, 1.2 mmol/l $KH_2PO_4$, 1.2 mmol/l $MgSO_4$.7H2O, pH 7.4). Test incubations were then performed at 37ºC for 20 min. Supernatants were removed, then frozen at −20ºC until determination of insulin by radioimmunoassay [26]. All FFAR4 ligands (1 mg/ml) were dissolved in 50% DMSO prior to preparation in Krebs buffer. Compound A and GSK137647 at $10^{-12}$-$10^{-4}$ mol/l were tested at 5.6 and 16.7 mmol/l glucose. To determine the selectivity of the agonists towards FFAR4, the FFAR1 antagonist GW1100 ($10^{-5}$ mol/l), and FFAR4 antagonist AH-7614 ($10^{-5}$ mol/l) were utilised to antagonise the respective receptors. Receptor antagonists were co-incubated with Compound A and GSK137647 on BRIN-BD11 cells, with insulin secretory responses determined.

### 2.3 Cytotoxicity assessment by tetrazolium (MTT):

BRIN-BD11 cells were cultured overnight in 96-well plates. Incubations were performed as described for insulin secretory analysis. Test solutions were decanted, then 1mg/ml of working MTT solution was added and incubated for 2 h at 37ºC. MTT solution was removed and replaced with 200 µl of DMSO. The plate was placed on an orbital shaker for 5 min to mix the formazan into the solvent. The optical density of each well was recorded at 560 nm with the background absorbance at 670 nm removed. The resultant optical density was proportional to the viable cell quantity.

### 2.4 Acute effects of FFAR4 agonists *in-vivo*:

All animal experiments were carried out in accordance with the UK Animal (Scientific Procedures) Act 1986. Male lean and HFF Swiss TO mice (Harlan UK, 30-34 weeks old, 55–67 g) were individually housed in an air-conditioned room at 22 ± 2ºC with a 12-h light: 12-h dark cycle. Drinking water was supplied ad libitum. Animals were maintained on a high fat

diet (45% fat, 20% protein, 35% carbohydrate; percent of total energy 26.15 kJ/g; Dietex International Ltd., Witham, UK) from 8 weeks of age for a total of 150 days to evoke dietary-induced obesity-diabetes (DIO). Another group of mice was maintained on standard rodent diet (10% fat, 30% protein, 60% carbohydrate; percent of total energy 12.99 kJ/g, Trouw Nutrition, Cheshire, UK) and used as a model of normal controls. Similar high-fat diets, containing a large percentage of energy from fat, are used routinely in obesity-diabetes research [27-29]. Mice (n=6, fasted 18 h) received an oral or IP administration of glucose alone (18 mmol/kg body weight) or in combination with FFAR4 agonists (0.1 μmol/kg body weight). All FFAR4 ligands (1 mg/ml) were dissolved in 50% DMSO prior to preparation in saline. Blood samples were obtained by a cut from the tip of the tail vein of conscious mice at the times indicated in Figures, and centrifuged at 16,060 × *g* for 3 min at 4ºC. Plasma glucose was measured using an automated glucose oxidase procedure with a Beckman glucose analyser (Beckman-Coulter, High Wycome, UK) and insulin determined by radioimmunoassay [26]. Intestinal hormone secretion was assessed using ELISA; total GLP-1 (Millipore) and total GIP (Millipore). DPP-IV activity was evaluated by Gly-Pro-AMC cleavage [30]. In a second series of experiments, 18 h fasted normal mice were used to assess the effects of agonist treatment on food intake. Mice received oral administration of saline alone (0.9% (w/v) NaCl) or in combination with agonist (0.1 μmol/kg body weight) and food intake measured at 30 min intervals.

**2.5 Gene expression analysis by qPCR:**

mRNA was extracted from clonal pancreatic BRIN-BD11 cells following exposure to agonist treatment, using an RNeasy Mini kit adhering to manufacturer's protocol (Qiagen, UK). Isolated mRNA (3 μg) was converted to cDNA using SuperScript II Reserve Transcriptase. Amplification parameters were set at 95ºC for denaturation, 58ºC for primer annealing and 72ºC for elongation for a total of 40 cycles, followed with melting curve analysis, with

187 temperature range set at 60ºC to 90ºC. Values were analysed using the Livak method and
188 normalised to GAPDH expression.

189 **2.6 FFAR4 protein concentrations using western blotting:**

190 BRIN-BD11 cells were seeded at a density of 1,000,000 cells per well in 6-well plates and
191 allowed to attach overnight. After 4 h exposure to $10^{-4}$M GSK137647 and Compound A, total
192 protein was extracted at 4ºC for 10 min using RIPA buffer containing 150 mM NaCl, 1.0%
193 Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 7.6 and protease
194 inhibitor cocktail (Sigma, UK). Total protein concentration was determined using Bradford
195 reagent (Sigma, UK). Equal amounts of protein were prepared in aliquots with Laemmli buffer
196 (1 µg/µl), then boiled at 95ºC for 10 min. Samples (25 µg per well) were loaded onto pre-cast
197 gels (NUPAGE 4–12% Bis–Tris gels, Invitrogen, UK) and subjected to SDS-PAGE (70 V, 90
198 min). After transfer to nitrocellulose membrane for 16 h at 90 mA, membranes were blocked
199 with 5% skimmed milk and probed with rabbit anti-FFAR4 (1:150) (Santa Cruz, US)/mouse
200 anti-β-actin (1:2500) (Cell signalling, US). Membranes were probed with ECL horseradish
201 peroxidase donkey anti-rabbit IgG/ECL horseradish peroxidase sheep anti-mouse IgG
202 (1:10000) (GE Healthcare, UK) and detected using Luminata Forte HRP substrate (Millipore,
203 UK), with images captured using the G:BOX Chemi XX9 imager (Syngene, UK). Data were
204 normalised to β-actin and presented relative to untreated control.

205 **2.7 Tissue distribution of FFAR4 by immunohistochemistry:**

206 Pancreatic tissue from lean and HFF NIH Swiss mice was excised and cut at 8µm using a
207 microtome. Sections were placed on slides and dried for 2 h on a hot plate at 37ºC. After
208 incubation, wax was removed and tissue re-hydrated in ethanol (100%), ethanol (95%), ethanol
209 (80%) and distilled water for 5 min each. Slides were incubated in 50 mM sodium citrate for
210 20 min at 90ºC for antigen retrieval. BSA (2.5%) was added to each slide (200 µl) for 45 min.

Primary antibodies (200 µl) at optimal dilutions (FFAR4 1:100, Insulin 1:300) were added and incubated at 4ºC overnight. Slides were washed, followed by secondary antibody (1:400) incubation at 37ºC for 45 min. The slides were washed, then DAPI (0.1 µg/ml) added and incubated at 37ºC for 15 min. Slides were washed, then mounted.

### 2.8 Statistics

All data was analysed with Prism (v.5.0, GraphPad Software Inc. CA, USA) and expressed as mean ± S.E.M. All *in-vivo* glucose tolerance test data (glucose, insulin, GLP-1, GIP and DPP-IV activity) were analysed using two-way analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test. Area under the curve (AUC) was calculated using trapezoidal rule with baseline correction. All other data including AUC were analysed using Student's *t*-test (non-parametric, with two-tailed P values and 95% confidence interval). $p<0.05$ was considered to be statistically significant.

## 3 Results:

### 3.1 Determination of FFAR4 agonist selectivity on insulin secretion.

The insulinotropic response and specificity of the novel synthetic FFAR4 agonists (Compound A, GSK137647) at $10^{-12}$-$10^{-4}$ mol/l were assessed using clonal pancreatic BRIN-BD11 cells. At 5.6 mM glucose, Compound A at $10^{-10}$-$10^{-4}$ mol/l augmented insulin secretion by 1.2- to 1.9-fold ($p<0.05$-$p<0.001$), with a half maximal effective concentration ($EC_{50}$) of 2.9 x $10^{-7}$ mol/l, while GSK137647 was more potent ($EC_{50}$ of 2.2 x $10^{-7}$ mol/l) with a 1.5- to 2.1-fold increase at $10^{-8}$-$10^{-4}$ mol/l ($p<0.05$-$p<0.001$) (Figure 1 A, B). At 16.7 mM glucose, both agonists exhibited enhanced insulinotropic activity. Compound A at $10^{-7}$-$10^{-4}$ mol/l augmented insulin secretion from 1.8- to 2.4 fold ($p<0.05$-$p<0.001$) and GSK137647 at $10^{-11}$-$10^{-4}$ mol/l

enhanced insulin secretion by 1.4- to 2.8-fold ($p<0.05$-$p<0.001$), with $EC_{50}$ values of $3.0 \times 10^{-8}$ mol/l (Compound A) and $1.2 \times 10^{-10}$ mol/l (GSK137647) (Figure 1 C, D). Neither agonist affected cell viability when assessed by MTT (Figure 1).

To investigate the selectivity of the agonists, both Compound A and GSK137647 were co-incubated with selective FFAR1 and FFAR4 antagonists. The insulin secretory response of Compound A and GSK137647 was not influenced by incubation with the FFAR1 antagonist GW1100 ($10^{-5}$ mol/l), with similar insulinotropic responses being observed in the presence and in the absence of GW1100 (Figure 1). In contrast, the FFAR4 antagonist AH-7614 ($10^{-5}$ mol/l) significantly impaired the insulinotropic responses of Compound A and GSK137647. At 5.6 mM glucose in the presence of the FFAR4 antagonist, Compound A ($10^{-6}$-$10^{-4}$ mol/l) augmented insulin secretion by 1.25- to 1.3-fold ($p<0.05$), corresponding to a 65% decrease in insulin output compared with control. Furthermore, the insulinotropic effect of GSK137647 was abolished in the presence of the FFAR4 antagonist (Figure 1 A, B). At 16.7 mM glucose in the presence of the FFAR4 antagonist, Compound A at $10^{-5}$-$10^{-4}$ mol/l increased insulin secretion by 1.7- to 1.8-fold ($p<0.05$), corresponding to a 65% reduction compared to agonist alone. GSK137647 at $10^{-7}$-$10^{-4}$ mol/l only augmented insulin secretion by 1.7- to 2.1 fold in the presence of the antagonist ($p<0.01$) (Figure 1 C, D), reflecting a 40% decrease in insulinotropic action.

### 3.2 Expression of FFAR4 in high fat fed pancreatic tissue and BRIN-BD11 cells:

Immunohistochemistry revealed high expression and areas of co-localisation of FFAR4 and insulin in pancreatic islets from lean and HFF mice (Figure 2 A-H). The pancreatic BRIN-BD11 cell line was used to confirm FFAR4 gene expression in pancreatic beta cells. At 5.6 mM glucose, FFAR4 agonists Compound A ($p<0.05$) and GSK137647 ($p<0.05$) downregulated FFAR4 receptor mRNA expression in BRIN-BD11 cells (Figure 2 I). When

exposed to 16.7 mM glucose, FFAR4 agonists (Compound A ($p<0.01$) and GSK137647 ($p<0.01$) increased FFAR4 mRNA expression (Figure 2 J). Western blotting was conducted to determine complimentary FFAR4 protein concentrations after agonist treatment. GSK137647 upregulated FFAR4 protein by 1.9 fold ($p<0.05$) in hyperglycaemic conditions, whilst Compound A had no significant effect (Figure 2 L). FFAR4 protein concentrations were not altered by either agonist under normoglycaemic (5.6 mM glucose) conditions (Figure 2 K).

### 3.3 Acute effects of FFAR4 agonists on glucose tolerance and insulin secretion *in-vivo*

An oral glucose tolerance test (OGTT) was performed to assess the anti-diabetic activity of Compound A and GSK137647 in fasted HFF mice. Compound A and GSK137647 were assessed alone or in combination with the DPP-IV inhibitor (Sitagliptin). FFAR4 antagonist AH-7614 was utilised to determine agonist specificity *in-vivo*. Oral administration of Compound A and GSK13647 improved glucose tolerance ($p<0.05$-$0.001$) (Figure 3 A, C), with AUC data showing decreases with Compound A and GSK137647 by 26% ($p<0.05$) and 18% ($p<0.05$) respectively (Figure 3 E, G). In combination with Sitagliptin, these compounds exhibited a further improvement on glucose excursion by 5-11%. The FFAR4 antagonist impaired the glucose lowering properties of Compound A and GSK137647 by 77% ($p<0.05$) and 89% ($p<0.05$) respectively (Figure 3 A, C).

These effects on blood glucose control were accompanied by relative changes in insulin secretion. Agonising FFAR4 with Compound A ($p<0.05$) and GSK137647 ($p<0.001$) increased plasma insulin by 20% and 54% when assessed with AUC data (Figure 3 F, H). Compound A in combination with Sitagliptin demonstrated an additive 12% insulinotropic effect compared to Compound A alone, whilst GSK137647 combinational therapy resulted in a 9% reduction compared to agonist alone (Figure 3 B, D). The FFAR4 antagonist AH-7614 inhibited the insulinotropic response of Compound A by 30% and GSK137647 by 76% ($p<0.001$).

GSK137647 increased plasma GLP-1 ($p<0.05$) and GIP ($p<0.05$), whilst Compound A increased plasma GLP-1 ($p<0.05$) but had no effect GIP secretion (Figure 4 A, B, D, E). GSK137647 ($p<0.01$) and Compound A ($p<0.01$) reduced DPP-IV activity, with activity further diminished when administered in combination with Sitagliptin ($p<0.001$) (Figure 4 C, F).

In addition to oral treatment, Compound A and GSK137647 were administered also by intraperitoneal injection to lean mice (Figure 5 A). Compound A significantly improved glucose excursion ($p<0.01$), with GSK137647 ($p<0.05$) eliciting a similar response.

### 3.4 Acute effects of FFAR4 agonists on appetite suppression in lean Swiss TO mice

Compound A induced satiety after 30 min with further effects observed over the 3 h time period ($p<0.05$-$p<0.001$) (Figure 5 B). Sitagliptin impaired the satiation effect of Compound A at 60 min. GSK137647 inhibited food intake after 30 min, with lasting effects throughout the experimental timeframe ($p<0.01$-$p<0.001$) (Figure 5 C). Combination of GSK137647 with Sitagliptin impaired the appetite suppressive effects of the agonist from 60 min to 180 min ($p<0.05$-$p<0.001$).

## 4 Discussion:

Recent interest in long chain fatty acid receptors has intensified due to identification of their involvement in the maintenance of glucose homeostasis through GPCR signalling. FFAR1 (GPR40) [31], GPR55 [32, 33] and GPR119 [33, 34] have been previously reported to regulate islet function and hormone secretion. In particular, orally administered FFAR1 agonist TAK-875 (Fasiglifam) entered stage III clinical trials with promising anti-diabetic effects of equal

potency to the sulphonylurea glimepiride, whilst omitting the risk of hypoglycaemia [35, 36]. However, signs of liver toxicity were observed towards the latter stages of the trial [37].

FFAR4 (GPR120) is a rhodopsin (class A) type receptor which has recently been shown to have anti-diabetic properties through the regulation of insulin and incretin secretion [1, 7, 8]. Recent studies have identified the expression and involvement of FFAR4 in the pancreatic islet and intestinal tract, with expression also identified in adipose tissue, lungs and pro-inflammatory macrophages. FFAR4 shares 10% homology with FFAR1 and has high affinity for long chain fatty acids. However, many FFAR4 agonists can act as dual agonists with FFAR1, such as synthetic GW9508, therefore the identification of selective FFAR4 agonists is required to fully evaluate the role of the receptor in the maintenance of glucose homeostasis [7, 8].

In the present study, the therapeutic potential of two novel synthetic agonists (Compound A, GSK137647), which have been shown to exhibit selective properties towards FFAR4, were assessed [20-21]. Insulin secretion studies demonstrated that both agonists enhanced glucose stimulated insulin secretion from pancreatic BRIN-BD11 cells. Compound A and GSK13647 displayed similar potencies at basal glucose levels ($EC_{50}$ of ~$10^{-7}$ mol/l). Stimulatory glucose concentrations, GSK137647 exhibited an $EC_{50}$ of $10^{-10}$ mol/l, compared with an $EC_{50}$ of $10^{-8}$ mol/l for Compound A. Neither agonist imparted adverse effects on cell viability as assessed using MTT.

To demonstrate the selectivity of the novel agonists towards FFAR4, potent antagonists for FFAR4 and FFAR1 were employed. In the presence of FFAR1 antagonist (GW1100), the insulin secretory responses to both Compound A and GSK137647 was relatively unaffected. In contrast, the FFAR4 antagonist (AH-7614) impaired the insulinotropic properties of both agents, suggesting that the two agonists stimulate glucose dependent insulin secretion through

FFAR4 and not FFAR1 in the pancreatic beta cell. Numerous endogenous FFAR4 ligands have been shown to stimulate other fatty acids receptors, including FFAR1 [3, 8].

Immunohistochemistry demonstrated the highly abundant expression and co-localisation of FFAR4 and insulin in both the pancreas of lean and diabetic mice. Consistent with this, gene expression analysis demonstrated an upregulation of FFAR4 in BRIN-BD11 cells exposed to hyperglycaemia, suggesting that FFAR4 may have a regulatory role in islets exposed to diabetic stress factors. Interestingly, agonist treatment under normoglycaemic conditions significantly attenuated FFAR4 gene expression, with no effect demonstrated on FFAR4 protein concentrations. The glucose responsive properties of the receptor expression promotes FFAR4 as a novel therapeutic target.

In HFF mice, both Compound A and GSK137647 demonstrated acute glucose lowering and insulinotropic properties. Previously, FFAR4 activation was shown to augment glucagon release from pancreatic alpha cells [38]. Although glucagon opposes the biological actions of insulin, improved glucose tolerance is exhibited upon FFAR4 agonist treatment. This indicates superior secretory actions of FFAR4 agonism on insulin releasing beta cells. Interestingly, FFAR4 activation has also been shown to inhibit somatostatin release from pancreatic delta cells, which may have indirectly enhanced the insulinotropic actions of FFAR4 agonists observed in this study [39]. Although numerous glucoregulatory hormones are released upon FFAR4 activation, the anti-inflammatory effects and potentiation of glucose uptake in peripheral tissues may have also attributed to the glucose lowering effects exhibited by FFAR4 [9].

Conflicting results have been reported of the effect of FFAR4 activation of GLP-1 secretion [1, 16]. In the present study, GSK137647 was shown to induce GLP-1 and GIP secretion, whilst Compound A only stimulated GLP-1 secretion. To prolong the bioactivity of endogenously

352 released incretins, the DPP-IV inhibitor Sitagliptin was examined in combination with the
353 FFAR4 agonists. Under these conditions, Compound A and GSK137647 exhibited enhanced
354 glucose lowering capabilities, by stimulating incretin (GLP-1, GIP) and insulin secretion
355 through FFAR4 activation, supplemented with prolonged incretin action through DPP-IV
356 inhibition. DPP-IV inhibition however countered the inhibitory effects of both FFAR4 agonists
357 on feeding activity. Further studies are required to fully understand the mechanism but
358 inhibition of DPP-IV-mediated degradation of PYY(1-36) to the active form PYY(3-36) seems
359 likely [40].

360 To establish that the glucose lowering properties of the agonists was not based solely on
361 incretin secretion, each agonist was administered i.p. in combination with glucose. Both
362 GSK137647 and Compound A improved glucose excursion confirming that FFAR4 activation
363 also directly stimulates beta cell function. The selectivity of the novel agonists was tested using
364 the FFAR4 antagonist AH-7614, which significantly impaired the insulinotropic and glucose
365 lowering capabilities of GSK137647 and Compound A.

366 In conclusion, selective FFAR4 agonists enhance glucose stimulated insulin secretion in a
367 concentration-dependent manner, whilst exhibiting no affinity for FFAR1. Expression analysis
368 demonstrated glucose responsive properties of FFAR4 expression under hyperglycaemic
369 stress, a novel finding which may aid the development of future anti-diabetic therapeutics.
370 Activation of FFAR4 was associated with acute stimulatory effects on GLP-1 and GIP
371 secretion. GSK137647 was the most potent agonist in terms of insulin and incretin secretion,
372 suggesting that this agonist should be considered for further investigation. In addition, it has
373 been shown for the first time that FFAR4 agonist combinational therapy with sitagliptin further
374 improves glucose tolerance and may provide a novel approach for the treatment of type 2
375 diabetes.

**Acknowledgement**

These studies were supported by Diabetes UK. AGMC conducted the investigation, methodology, validation, formal analysis and writing – original draft. MGM conducted the investigation. PRF involved in the writing- review and editing and supervision. AMMK involved in the conceptualization, formal analysis, supervision writing – review and editing.

**References**

1. Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M *et al.* Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med* 2005 **11** 90-94.
2. Ulven, T. and Christiansen, E. Dietary Fatty Acids and Their Potential for Controlling Metabolic Diseases Through Activation of FFA4/GPR120. *Annu Rev Nutr* 2015 **35(1)** 239-263.
3. Ichimura A, Hirasawa A, Hara T, Tsujimoto G. Free fatty acid receptors act as nutrient sensors to regulate energy homeostasis. *Prostaglandins Other Lipid Mediat* 2009 **89** 82-88.
4. Milligan G, Shimpukade B, Ulven T. and Hudson, B. Complex Pharmacology of Free Fatty Acid Receptors. *Chem. Rev.*2017 **117(1)** 67-110.
5. Dhayal S, Welters HJ, Morgan NG. Structural requirements for the cytoprotective actions of mono-unsaturated fatty acids in the pacreatic beta-cell line, BRIN-BD11. *Br J Pharmacol* 2008 **153** 1718-1727.
6. Gehrmann, W., Elsner, M. and Lenzen, S. Role of metabolically generated reactive oxygen species for lipotoxicity in pancreatic β-cells. *Diabetes Obes Metab* 2010 **12** 149-158.

7. Moran BM, Abdel-Wahab YH, Flatt PR, McKillop AM. Evaluation of the insulin-releasing and glucose-lowering effects of GPR120 activation in pancreatic beta-cells. *Diabetes Obes Metab* 2014 **16** 1128-1139.
8. Moran BM, Flatt PR, McKillop AM. G protein-coupled receptors: signalling and regulation by lipid agonists for improved glucose homoeostasis. Acta Diabetol 2016 **53** 177-188.
9. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W *et al*. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell* 2010 **142** 687-698.
10. Ichimura A, Hirasawa A, Poulain-Godefroy O, Bonnefond A, Hara T, Yengo L *et al*. Dysfunction of lipid sensor GPR120 leads to obesity in both mouse and human. *Nature* 2012 **483** 350-354.
11. Bonnefond A, Lamri A, Leloire A, Vaillant E, Roussel R, Levy-Marchal C *et al*. Contribution of the low-frequency, loss-of-function p.R270H mutation in FFAR4 (GPR120) to increased fasting plasma glucose levels. *J Med Genet* 2015 **52** 595-598.
12. Iakoubov R, Izzo A, Yeung A, Whiteside CI, Brubaker PL. Protein kinase Czeta is required for oleic acid-induced secretion of glucagon-like peptide-1 by intestinal endocrine L cells. *Endocrinology* 2007 **148** 1089-1098.
13. Sankoda A, Harada N, Iwasaki K, Yamane S, Murata Y, Shibue K *et al*. Long-Chain Free Fatty Acid Receptor GPR120 Mediates Oil-Induced GIP Secretion Through CCK in Male Mice. *Endocrinology* 2017 **158** 1172-1180.
14. Tanaka T, Yano T, Adachi T, Koshimizu TA, Hirasawa A, Tsujimoto G. Cloning and characterization of the rat free fatty acid receptor GPR120: in vivo effect of the natural ligand on GLP-1 secretion and proliferation of pancreatic beta cells. *Naunyn Schmiedebergs Arch Pharmacol* 2008 **377** 515-522.

15. Bhaswant, M., Poudyal, H. and Brown, L. Mechanisms of enhanced insulin secretion and sensitivity with n-3 unsaturated fatty acids. *J. Nutr. Biochem* 2015 **26(6)** 571-584.
16. Paulsen SJ, Larsen LK, Hansen G, Chelur S, Larsen PJ, Vrang N. Expression of the fatty acid receptor GPR120 in the gut of diet-induced-obese rats and its role in GLP-1 secretion. *PLoS One* 2014 **9**: e88227.
17. Briscoe C, Tadayyon M, Andrews J, Benson W, Chambers J, Eilert M *et al*. The Orphan G Protein-coupled Receptor GPR40 Is Activated by Medium and Long Chain Fatty Acids. *J. Biol. Chem* 2003 **278(13)** 11303-11311.
18. Christiansen E, Watterson K, Stocker C, Sokol E, Jenkins L, Simon K *et al*. Activity of dietary fatty acids on FFA1 and FFA4 and characterisation of pinolenic acid as a dual FFA1/FFA4 agonist with potential effect against metabolic diseases. *Br J Nutr* 2015 **113(11)** 1677-1688.
19. Burns RN, Singh M, Senatorov IS, Moniri NH. Mechanisms of homologous and heterologous phosphorylation of FFA receptor 4 (GPR120): GRK6 and PKC mediate phosphorylation of Thr(3)(4)(7), Ser(3)(5)(0), and Ser(3)(5)(7) in the C-terminal tail. *Biochem Pharmacol* 2014 **87** 650-659.
20. Oh DY, Walenta E, Akiyama TE, Lagakos WS, Lackey D, Pessentheiner AR *et al*. A Gpr120-selective agonist improves insulin resistance and chronic inflammation in obese mice. *Nat Med* 2014 **20** 942-947.
21. Sparks SM, Chen G, Collins JL, Danger D, Dock ST, Jayawickreme C *et al*. Identification of diarylsulfonamides as agonists of the free fatty acid receptor 4 (FFA4/GPR120). *Bioorg Med Chem Lett* 2014 **24** 3100-3103.
22. Martin C, Passilly-Degrace P, Chevrot M, Ancel D, Sparks SM, Drucker DJ *et al*. Lipid-mediated release of GLP-1 by mouse taste buds from circumvallate papillae: putative involvement of GPR120 and impact on taste sensitivity. *J Lipid Res* 2012 **53** 2256-2265.

447 23. Drucker D, Easley C, Kirkpatrick P. Sitagliptin. *Nat Rev Drug Discov* 2007 **6** 109-110.
448 24. Tanaka H, Yoshida S, Minoura H, Negoro K, Shimaya A, Shimokawa T *et al*. Novel
449 GPR40 agonist AS2575959 exhibits glucose metabolism improvement and synergistic
450 effect with sitagliptin on insulin and incretin secretion. *Life Sci* 2014 **94** 115-121.
451 25. McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YH, O'Harte FP, Yoon TW,
452 Swanston-Flatt SK & Flatt PR. Characterization of a novel glucose-responsive insulin-
453 secreting cell line, BRIN-BD11, produced by electrofusion. *Diabetes* 1996 **45** 1132-1140.
454 26. Flatt PR, Bailey CJ. Abnormal plasma glucose and insulin responses in heterozygous lean
455 (ob/+) mice. *Diabetologia* 1981 **20** 573-577.
456 27. West DB, Waguespack J, McCollister S. Dietary obesity in the mouse: interaction of strain
457 with diet composition. *Am. J. Physio*l 1995 **268** 658–665.
458 28. Black BL, Croom J, Eisen EJ, Petro AE, Edwards CL, Surwit RS. Differential effects of
459 fat and sucrose on body composition in A/J and C57BL/6 mice. *Metabolism*. 1998 **47** 1354–
460 1359.
461 29. Buettner R, Schölmerich J, Bollheimer LC. High-fat diets: modeling the metabolic
462 disorders of human obesity in rodents. *Obesity (Silver Spring)* 2007 **15** 798–808.
463 30. Davis JA, Singh S, Sethi S, Roy S, Mittra S, Rayasam G *et al*. Nature of action of
464 Sitagliptin, the dipeptidyl peptidase-IV inhibitor in diabetic animals. *Indian J Pharmacol*
465 2010 **42** 229-233.
466 31. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S *et al*. Free fatty acids
467 regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* 2003 **422** 173-
468 176.
469 32. McKillop AM, Moran BM, Abdel-Wahab YH, Flatt PR. Evaluation of the insulin releasing
470 and antihyperglycaemic activities of GPR55 lipid agonists using clonal beta-cells, isolated
471 pancreatic islets and mice. *Br J Pharmacol* 2013 **170** 978-990.

33. McKillop AM, Moran BM, Abdel-Wahab YH, Gormley NM, Flatt PR. Metabolic effects of orally administered small-molecule agonists of GPR55 and GPR119 in multiple low-dose streptozotocin-induced diabetic and incretin-receptor-knockout mice. *Diabetologia* 2016 **59** 2674-2685.
34. Moran BM, Abdel-Wahab YH, Flatt PR, McKillop AM. Activation of GPR119 by fatty acid agonists augments insulin release from clonal beta-cells and isolated pancreatic islets and improves glucose tolerance in mice. *Biol Chem* 2014 **395** 453-464.
35. Marcinak J, Cao C, Lee D, Ye Z. Fasiglifam for glycaemic control in people with type 2 diabetes: A phase III, placebo-controlled study. *Diabetes Obes Metab* 2017 **19** 1714-1721.
36. Peng XV, Marcinak JF, Raanan MG, Cao C. Combining the G-protein-coupled receptor 40 agonist fasiglifam with sitagliptin improves glycaemic control in patients with type 2 diabetes with or without metformin: A randomized, 12-week trial. *Diabetes Obes Metab* 2017 **19** 1127-1134.
37. Otieno MA, Snoeys J, Lam W, Ghosh A, Player MR, Pocai A *et al*. Fasiglifam (TAK-875): Mechanistic Investigation and Retrospective Identification of Hazards for Drug Induced Liver Injury. *Toxicol Sci* 2018 **163** 374-384.
38. Suckow A, Polidori D, Yan W, Chon S, Ma J, Leonard J *et al*. Alteration of the Glucagon Axis in GPR120 (FFAR4) Knockout Mice. *J. Biol. Chem* 2014 **289(22)** 15751-15763.
39. Stone V, Dhayal S, Brocklehurst K, Lenaghan C, Sörhede Winzell M, Hammar M, *et al*. GPR120 (FFAR4) is preferentially expressed in pancreatic delta cells and regulates somatostatin secretion from murine islets of Langerhans. 2014 *Diabetologia*, **57(6)** 1182-1191.
40. Batterham RL, Bloom SR. The gut hormone peptide YY regulates appetite. *Ann N Y Acad Sci* 2003 **994** 162-168.

## Figure Legends

**Figure 1.** Acute insulinotropic effects of FFAR4 agonists (A, C) Compound A and (B, D) GSK137647 alone and in combination with FFAR4 antagonist AH-7614 ($10^{-5}$ mol/l) or FFAR1 antagonist GW1100 ($10^{-5}$ mol/l) at 5.6 mM and 16.7 mM glucose in clonal pancreatic BRIN-BD11 cells. Alanine (10 mmol/l) was used as positive control. MTT cell viability analysis demonstrating cytotoxicity of (A, C) Compound A and (B, D) GSK137647 on BRIN-BD11 cells. Hydrogen peroxide (1 mmol/l) was used as a positive control. Values are mean ± SEM (n=8) for insulin secretion and (n=4) for cell viability. *p<0.05, **p<0.01, ***p<0.001, compared to saline control. †p<0.05, ††p<0.01, †††p<0.001, compared to agonist alone.

**Figure 2.** Localisation of (A, B) 4', 6 diamidino-2-phenylindole (DAPI) nuclear stain, (C, D) FFAR4, (E, F) insulin and (G) double immunofluorescence of FFAR4 and insulin in high fat fed (A, C, E, G) and lean (B, D, F, H) pancreatic islets at X40 magnification. Examples of double immunofluorescence indicated by white arrows. qPCR and western blot analysis demonstrating the effect of FFAR4 agonist treatment on FFAR4 mRNA and protein concentrations at (I, K) 5.6 mM and (J, L) 16.7 mM in clonal pancreatic BRIN-BD11 cells after 4 h treatment. Expression was normalised to GAPDH for qPCR (n=3) and β-actin for western blotting (n=2; two independent experiments with two technical replicates). Values are presented as mean ± SEM.*p<0.05, **p<0.01, compared to glucose control.

**Figure 3.** Acute effects of FFAR4 agonists Compound A and GSK137647 on plasma glucose (A, C), insulin (B, D) and respective AUC (E-H). Glucose (18 mmol/kg bw) was administered orally alone or in combination with FFAR4 agonist Compound A or GSK137647 (0.1 µmol/kg bw) and either the FFAR4 antagonist AH-7614 (0.1 µmol/kg bw) or Sitagliptin (50 mg/kg bw) to HFF mice (n = 6). Values are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001,

compared to HFF glucose control. †p<0.05, ††p<0.01, †††p<0.001, compared to agonist alone.

**Figure 4.** Acute effects of FFAR4 agonists Compound A and GSK137647 (0.1 μmol/kg bw) on circulating (A, D) total GLP-1, (B, E) total GIP and (C, F) DPP-IV activity. Glucose (18 mmol/kg bw) was administered orally alone or in combination with FFAR4 agonist to HFF mice (n = 6). Values are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, compared to HFF glucose control.

**Figure 5.** Acute effects of i.p. administration of FFAR4 agonists Compound A and GSK137647 (0.1 μmol/kg bw) on (A) glucose tolerance and (B, C) cumulative food intake. Glucose (18 mmol/kg bw) was administered i.p. in combination with FFAR4 agonist to lean Swiss TO mice for glucose tolerance (n = 6). For satiety analysis, FFAR4 agonists were orally administered alone or in combination with DPP-IV inhibitor (Sitagliptin) to 18 h fasted lean Swiss TO mice. *p<0.05, **p<0.01, ***p<0.001, compared to saline control. †p<0.05, ††p<0.01, †††p<0.001, compared to agonist alone.

## Supplementary data

**Figure 1.** Effects of Compound A and GSK137647, with half maximum effective concentration ($EC_{50}$) values, on insulin release from clonal pancreatic BRIN-BD11 cells at (A) 5.6 mM and (B) 16.7 mM glucose concentrations. Results are the mean ± SEM (n=8).

**Figure 2.** Effect 150-day high fat fed diet on glucose tolerance and insulin secretion in 18 h fasted Swiss TO mice. Animals were subjected to an oral glucose tolerance test (18 mmol/kg bw) with (A, B) glucose tolerance, (C, D) insulin secretory response, (E) fasting plasma glucose

and (F) bodyweight determined. Results are the mean ± SEM (n=6). *p<0.05, **p<0.01, ***p<0.001 compared to lean control.

**Figure 3.** Effect 150-day high fat fed diet on glucose tolerance and insulin secretion in 18 h fasted Swiss TO mice. Animals were subjected to an insulin sensitivity test (40U/kg bodyweight; dissolved in 0.9% saline, i.p. injection). (A) Plasma glucose and (B) respective AOC are shown. Results are mean ± SEM (n=8). **p<0.01, compared to lean control.

**Figure 1**

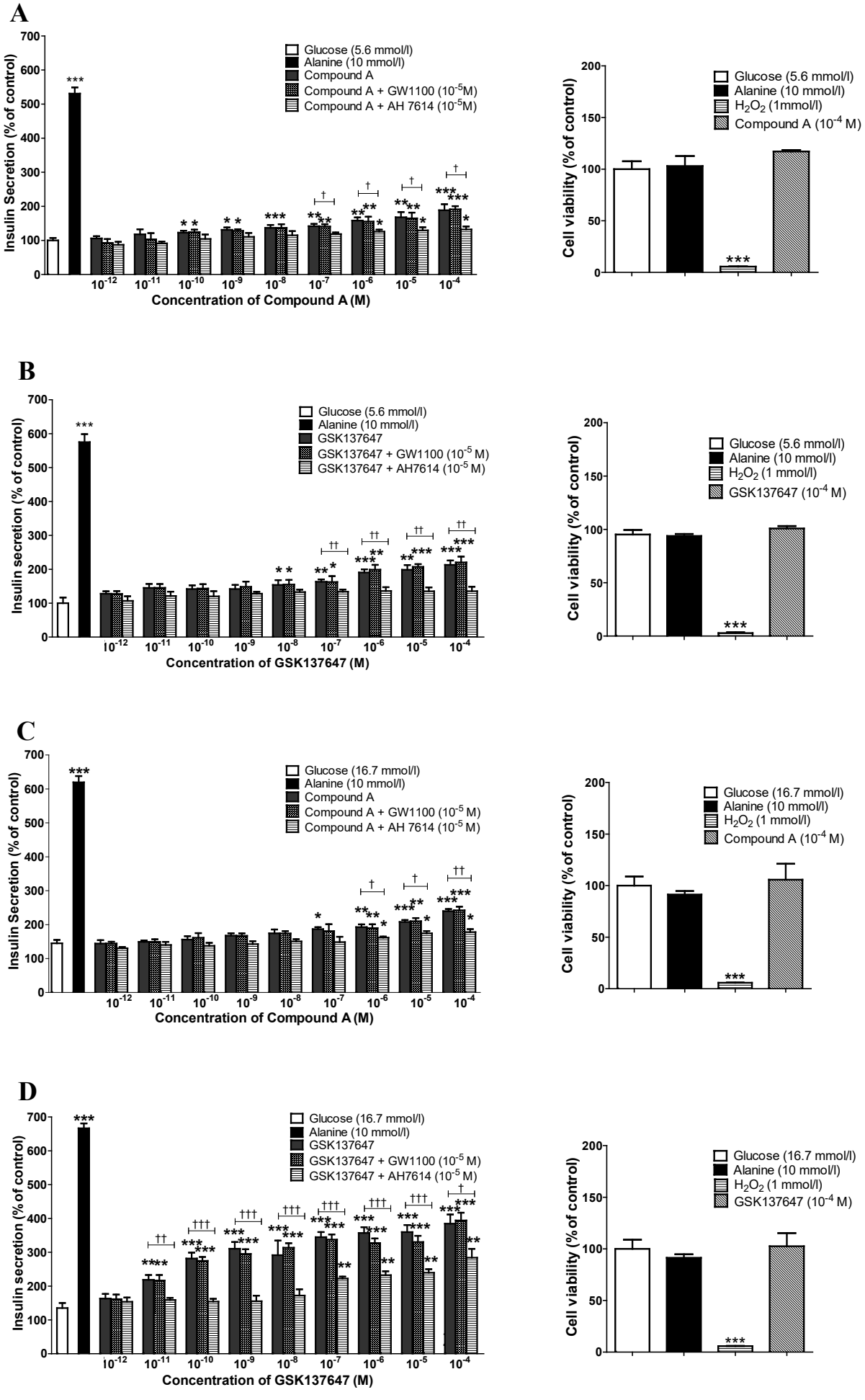
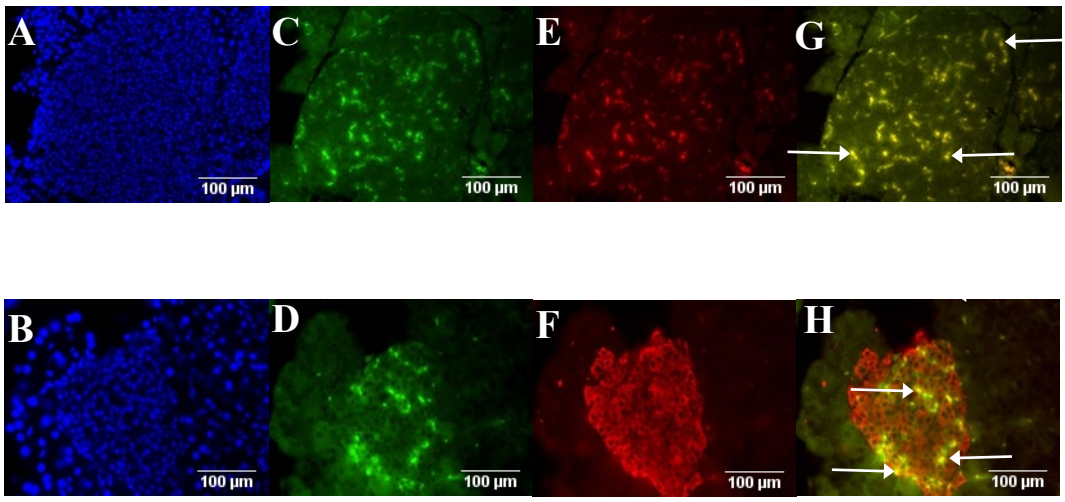
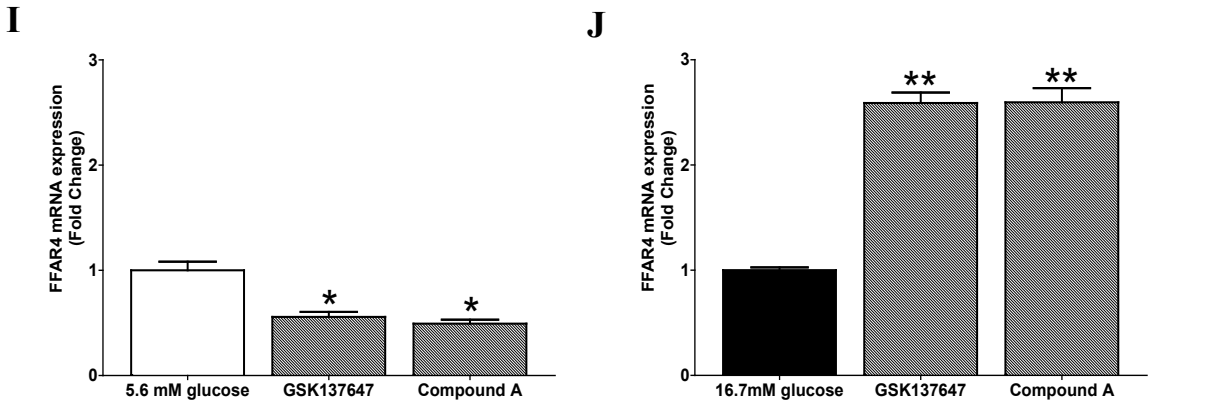

A
Glucose (5.6 mmol/l)
Alanine (10 mmol/l)
Compound A
Compound A + GW1100 (10-5M)
Compound A + AH 7614 (10-5M)
Insulin Secretion (% of control)
Concentration of Compound A (M)
Glucose (5.6 mmol/l)
Alanine (10 mmol/l)
H2O2 (1mmol/l)
Compound A (10-4 M)
Cell viability (% of control)
B
Glucose (5.6 mmol/l)
Alanine (10 mmol/l)
GSK137647
GSK137647 + GW1100 (10-5 M)
GSK137647 + AH7614 (10-5 M)
Insulin secretion (% of control)
Concentration of GSK137647 (M)
Glucose (5.6 mmol/l)
Alanine (10 mmol/l)
H2O2 (1 mmol/l)
GSK137647 (10-4 M)
Cell viability (% of control)
C
Glucose (16.7 mmol/l)
Alanine (10 mmol/l)
Compound A
Compound A + GW1100 (10-5 M)
Compound A + AH 7614 (10-5 M)
Insulin Secretion (% of control)
Concentration of Compound A (M)
Glucose (16.7 mmol/l)
Alanine (10 mmol/l)
H2O2 (1 mmol/l)
Compound A (10-4 M)
Cell viability (% of control)
D
Glucose (16.7 mmol/l)
Alanine (10 mmol/l)
GSK137647
GSK137647 + GW1100 (10-5 M)
GSK137647 + AH7614 (10-5 M)
Insulin secretion (% of control)
Concentration of GSK137647 (M)
Glucose (16.7 mmol/l)
Alanine (10 mmol/l)
H2O2 (1 mmol/l)
GSK137647 (10-4 M)
Cell viability (% of control)

**Figure 2.**


A
C
E
G
B
D
F
H
100 μm


I
FFAR4 mRNA expression (Fold Change)
5.6 mM glucose
GSK137647
Compound A
J
FFAR4 mRNA expression (Fold Change)
16.7mM glucose
GSK137647
Compound A

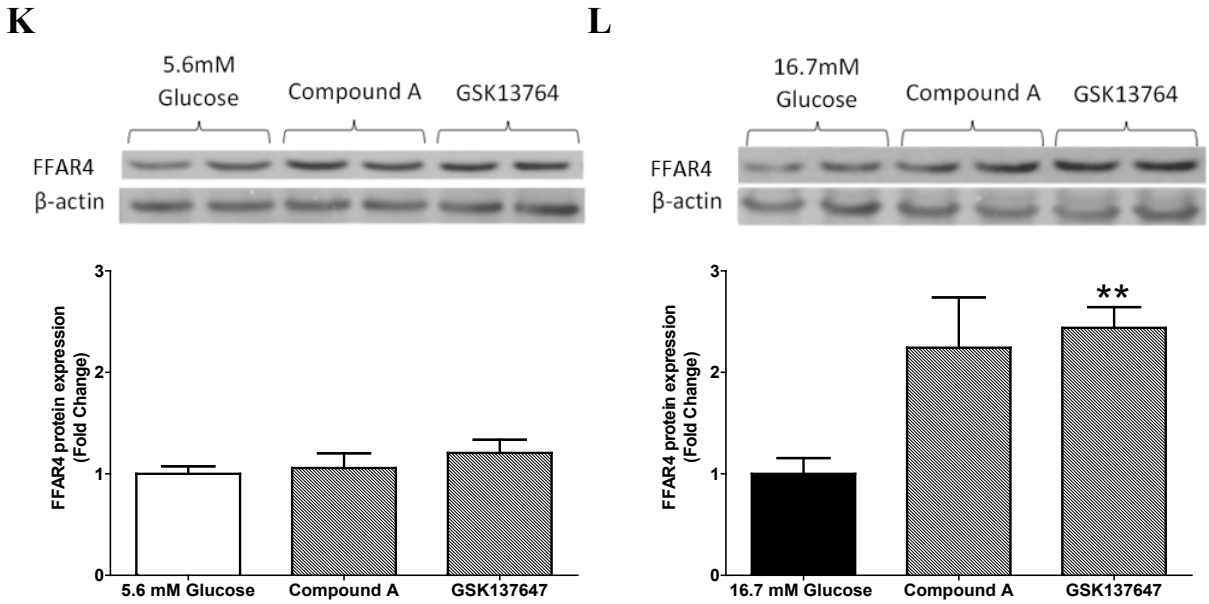

K
5.6mM Glucose
Compound A
GSK13764
FFAR4
β-actin
FFAR4 protein expression (Fold Change)
5.6 mM Glucose
Compound A
GSK137647
L
16.7mM Glucose
Compound A
GSK13764
FFAR4
β-actin
FFAR4 protein expression (Fold Change)
16.7 mM Glucose
Compound A
GSK137647

**Figure 3.**

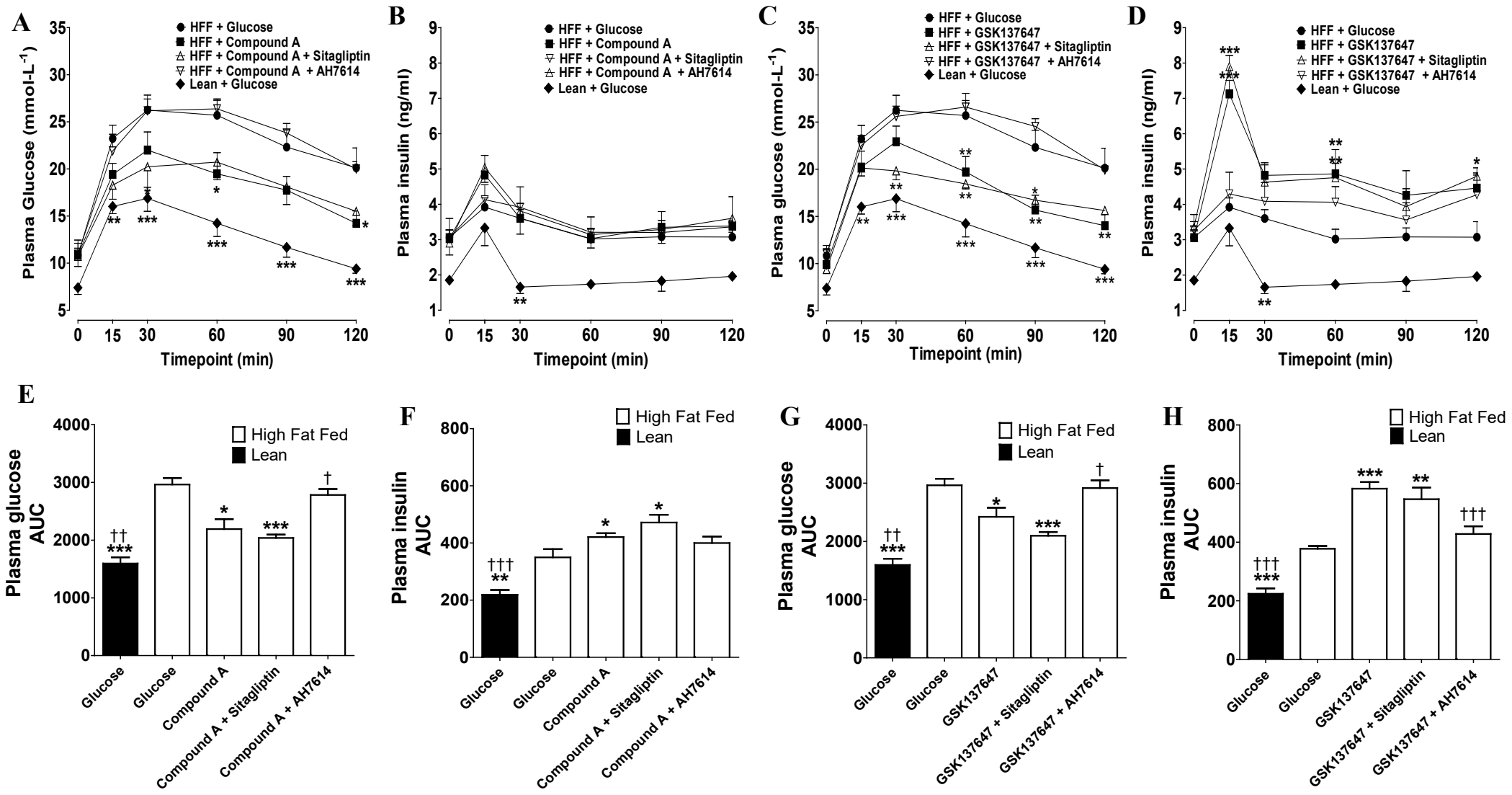

A
Plasma Glucose (mmol-L-1)
Timepoint (min)
HFF + Glucose
HFF + Compound A
HFF + Compound A + Sitagliptin
HFF + Compound A + AH7614
Lean + Glucose
B
Plasma insulin (ng/ml)
Timepoint (min)
HFF + Glucose
HFF + Compound A
HFF + Compound A + Sitagliptin
HFF + Compound A + AH7614
Lean + Glucose
C
Plasma glucose (mmol-L-1)
Timepoint (min)
HFF + Glucose
HFF + GSK137647
HFF + GSK137647 + Sitagliptin
HFF + GSK137647 + AH7614
Lean + Glucose
D
Plasma insulin (ng/ml)
Timepoint (min)
HFF + Glucose
HFF + GSK137647
HFF + GSK137647 + Sitagliptin
HFF + GSK137647 + AH7614
Lean + Glucose
E
Plasma glucose AUC
High Fat Fed
Lean
Glucose
Glucose
Compound A
Compound A + Sitagliptin
Compound A + AH7614
F
Plasma insulin AUC
High Fat Fed
Lean
Glucose
Glucose
Compound A
Compound A + Sitagliptin
Compound A + AH7614
G
Plasma glucose AUC
High Fat Fed
Lean
Glucose
Glucose
GSK137647
GSK137647 + Sitagliptin
GSK137647 + AH7614
H
Plasma insulin AUC
High Fat Fed
Lean
Glucose
Glucose
GSK137647
GSK137647 + Sitagliptin
GSK137647 + AH7614

**Figure 4.**

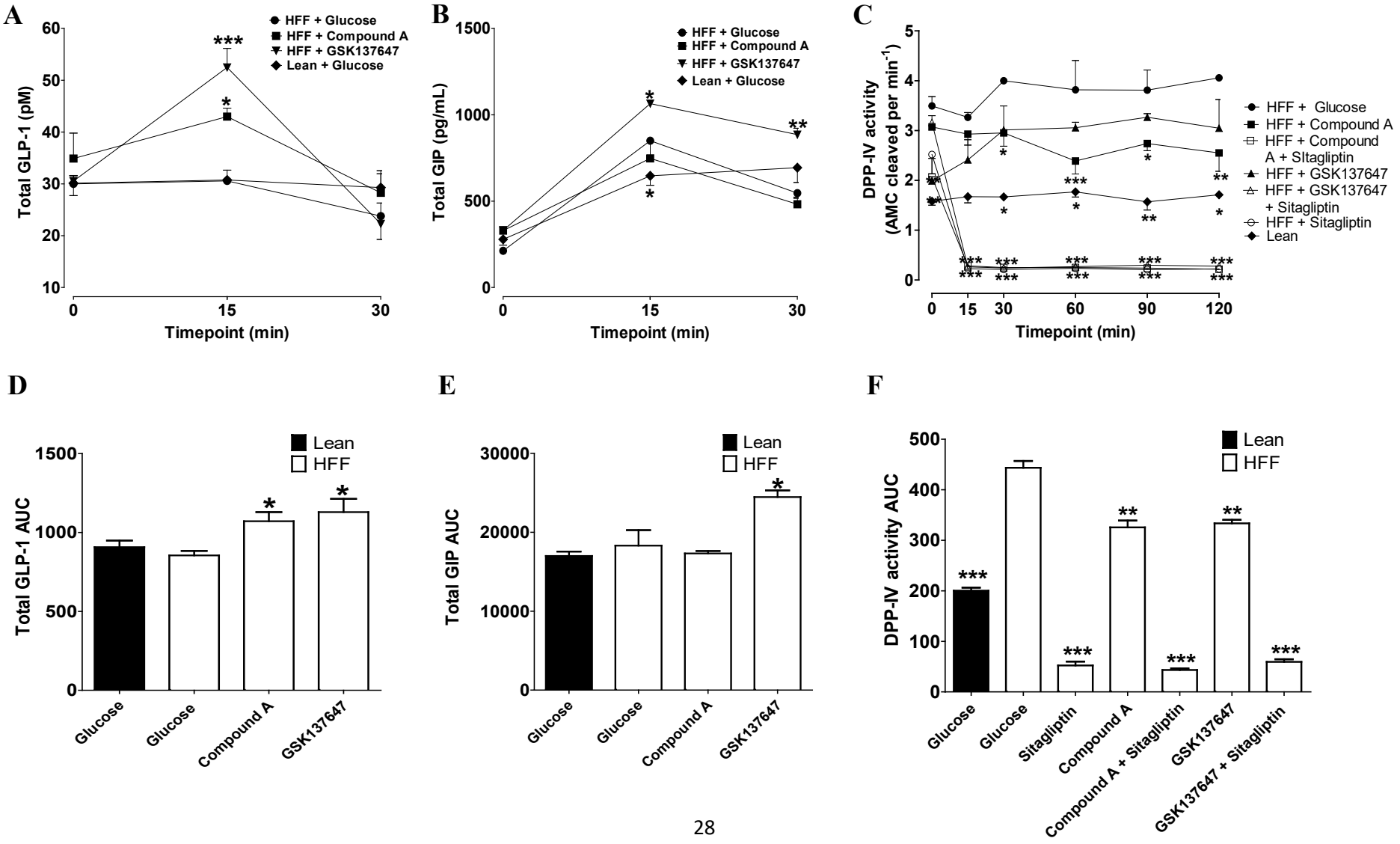
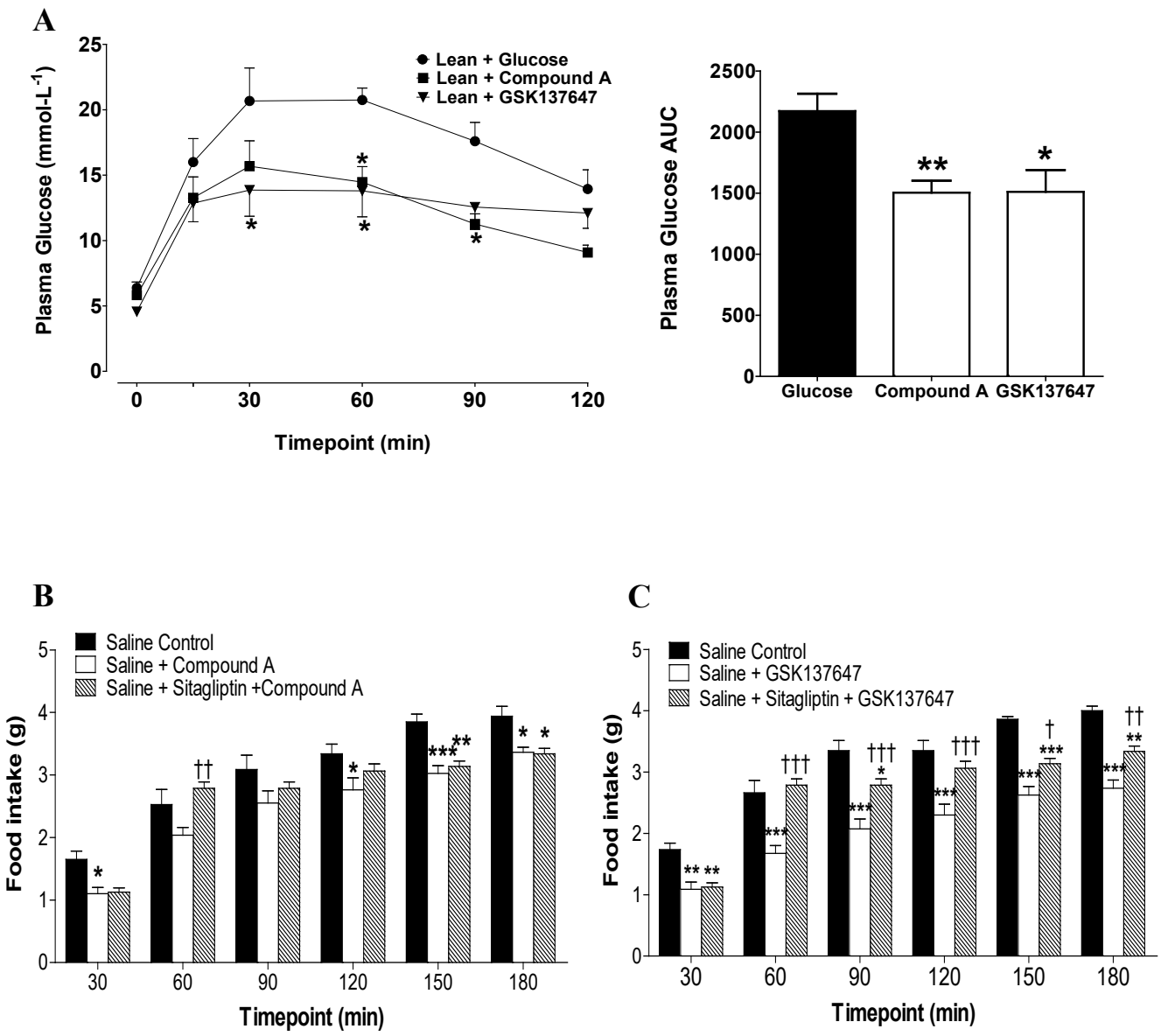

A
HFF + Glucose
HFF + Compound A
HFF + GSK137647
Lean + Glucose
Total GLP-1 (pM)
Timepoint (min)
B
Total GIP (pg/mL)
Timepoint (min)
C
DPP-IV activity (AMC cleaved per min-1)
HFF + Glucose
HFF + Compound A
HFF + Compound A + SItagliptin
HFF + GSK137647
HFF + GSK137647 + Sitagliptin
HFF + Sitagliptin
Lean
Timepoint (min)
D
Lean
HFF
Total GLP-1 AUC
Glucose
Glucose
Compound A
GSK137647
E
Total GIP AUC
F
DPP-IV activity AUC
Glucose
Glucose
Sitagliptin
Compound A
Compound A + Sitagliptin
GSK137647
GSK137647 + Sitagliptin

**Figure 5**


A
Plasma Glucose (mmol-L-1)
Lean + Glucose
Lean + Compound A
Lean + GSK137647
Timepoint (min)
Plasma Glucose AUC
Glucose
Compound A
GSK137647
B
Saline Control
Saline + Compound A
Saline + Sitagliptin +Compound A
Food intake (g)
Timepoint (min)
C
Saline Control
Saline + GSK137647
Saline + Sitagliptin + GSK137647
Food intake (g)
Timepoint (min)

**Supplementary material**

**Figure 1**

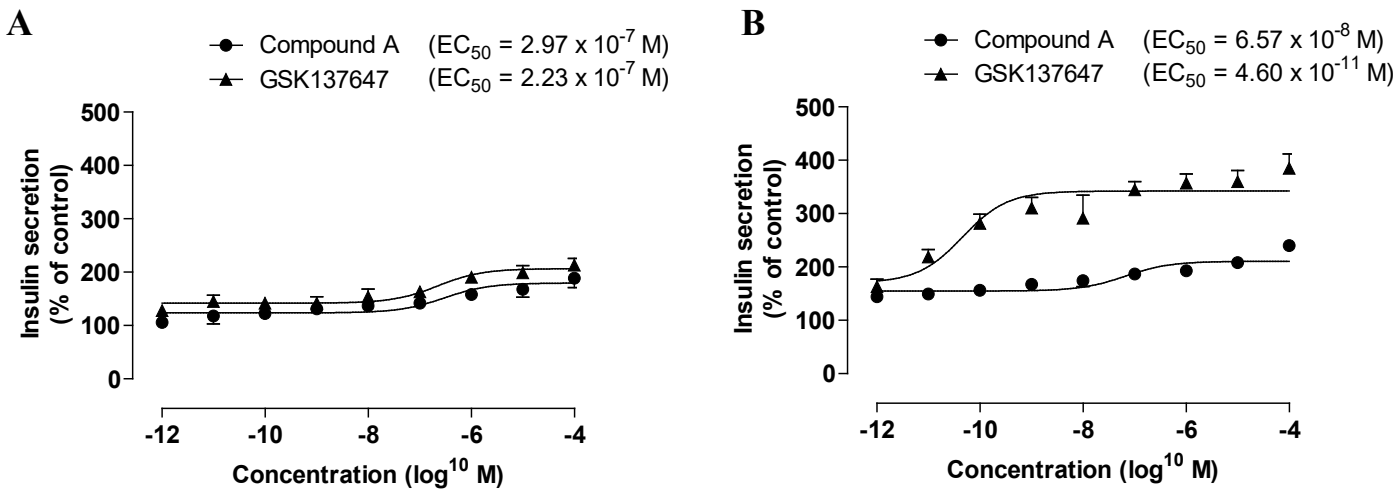


**Figure 2**

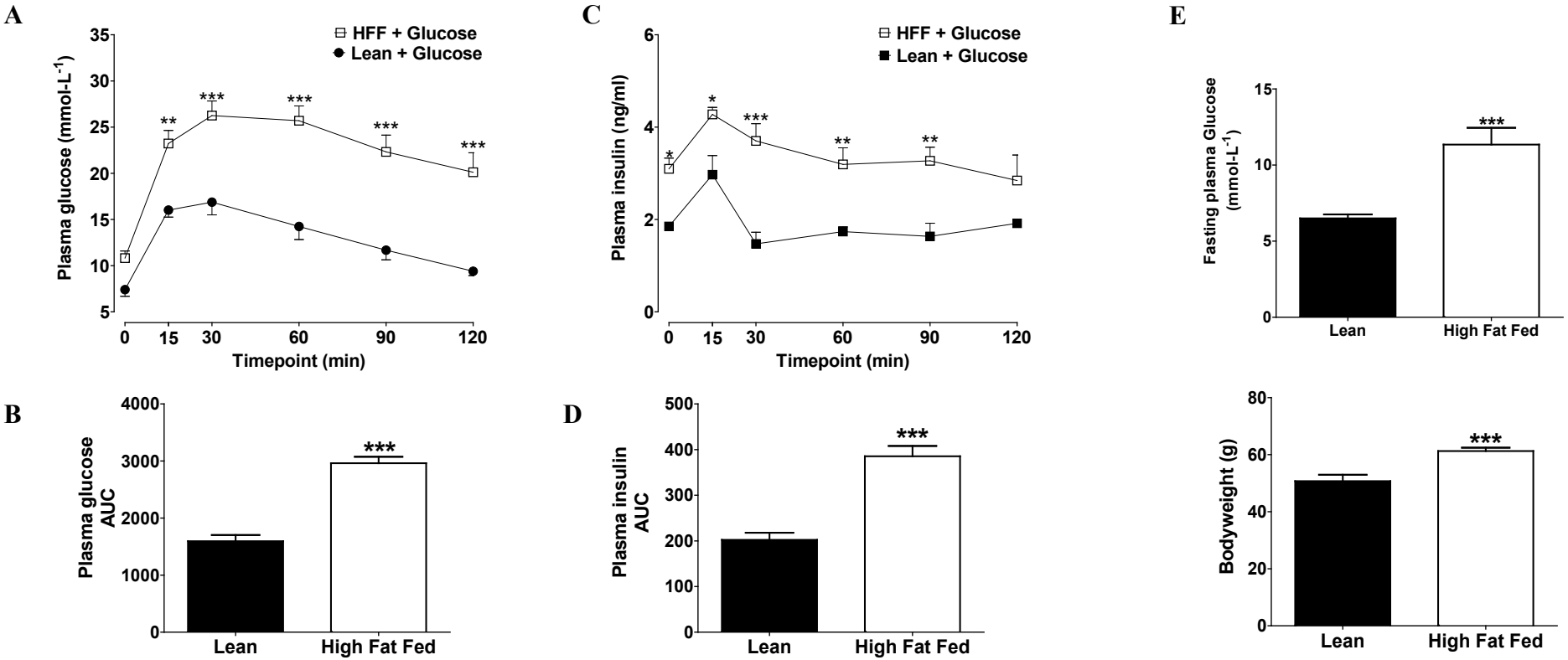
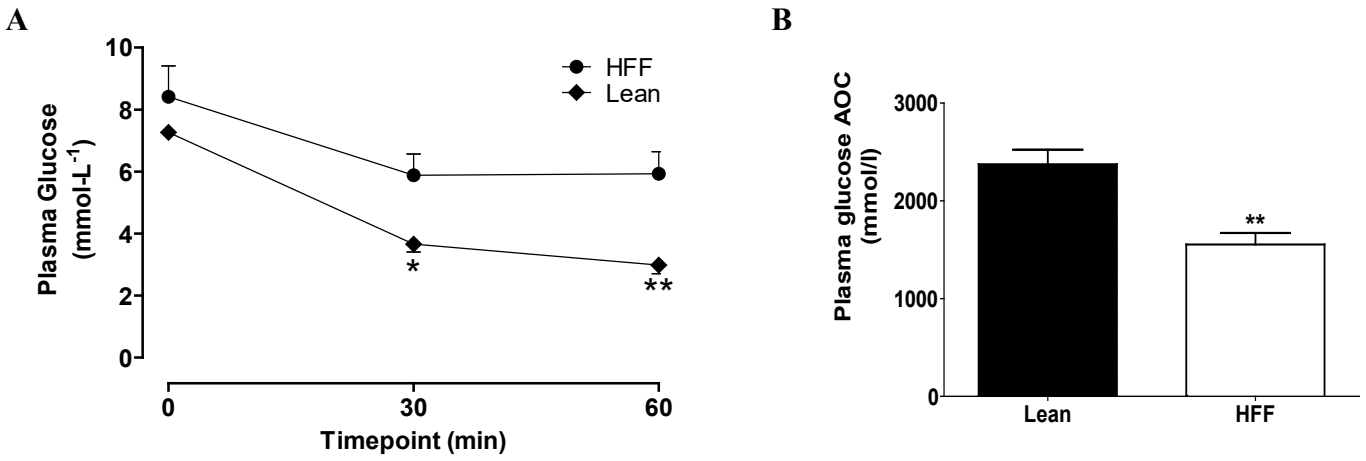

A
HFF + Glucose
Lean + Glucose
Plasma glucose (mmol-L-1)
Timepoint (min)
C
HFF + Glucose
Lean + Glucose
Plasma insulin (ng/ml)
Timepoint (min)
E
Fasting plasma Glucose (mmol-L-1)
Lean
High Fat Fed
B
Plasma glucose AUC
Lean
High Fat Fed
D
Plasma insulin AUC
Lean
High Fat Fed
Bodyweight (g)
Lean
High Fat Fed

**Figure 3**


A
Plasma Glucose (mmol-L-1)
10
8
6
4
2
0
HFF
Lean
*
**
0
30
60
Timepoint (min)
B
Plasma glucose AOC (mmol/l)
3000
2000
1000
0
**
Lean
HFF