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ORIGINAL ARTICLE

# In vitro and mouse in vivo characterization of the potent free fatty acid 1 receptor agonist TUG-469

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**Abstract** Activation of the G protein-coupled free fatty acid receptor 1 (FFA1; formerly known as GPR40) leads to an enhancement of glucose-stimulated insulin secretion from pancreatic β-cells. TUG-469 has previously been reported as a potent FFA1 agonist. This study was performed to confirm the higher in vitro potency of TUG-469 compared to the reference FFA1 agonist GW9508 and to prove in vivo activity in a pre-diabetic mouse model. The *in vitro* pharmacology of TUG-469 was studied using $Ca^{2+}$-, cAMP-, and impedance-based assays at recombinant FFA1 and free fatty acid receptor 4, formerly known as GPR120 (FFA4) expressing 1321N1 cells and the rat insulinoma cell line INS-1. Furthermore, we investigated the systemic effect of TUG-469 on glucose tolerance in pre-diabetic New Zealand obese (NZO) mice performing a glucose tolerance test after intraperitoneal administration of 5 mg/kg TUG-469. In comparison to GW9508, TUG-469 showed a 1.7- to 3.0-times higher potency in vitro at 1321N1 cells recombinantly expressing FFA1. Both compounds increased insulin secretion from rat insulinoma INS-1 cells. TUG-469 is > 200-fold selective for FFA1 over FFA4. Finally, a single dose of 5 mg/kg TUG-469 significantly improved glucose tolerance in pre-diabetic NZO mice. TUG-469 turned out as a promising candidate for further drug development of FFA1 agonists for treatment of type 2 diabetes mellitus.

**Keywords** Diabetes · FFA1 · Free fatty acids · GPR40 · TUG-469

**Abbreviations**

| | |
|---|---|
| FFA | Free fatty acids |
| FFA1 | Free fatty acid receptor 1, formerly known as GPR40 |
| FFA4 | Free fatty acid receptor 4, formerly known as GPR120 |
| GSIS | Glucose-stimulated insulin secretion |
| NZO | New Zealand obese |
| T2DM | Diabetes mellitus type 2 |

## Introduction

The G protein-coupled free fatty acid receptor 1 (FFA1) is highly expressed in pancreatic β-cells , and its activation by medium- and long-chain free fatty acids or synthetic FFA1 agonists like GW9508 results in the enhancement of glucose-stimulated insulin secretion (Briscoe et al. 2006; Itoh et al. 2003). The $G_q$ coupling affects the phospholipase C signaling pathway leading to a mobilization of intracellular calcium and insulin secretion from pancreatic β-cells (Feng et al. 2006; Kebede et al. 2009). FFA1 is furthermore expressed on gut enteroendocrine cells. Stimulation of FFA1 in enteroendocrine cells results in glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) release (Edfalk et al. 2008). Luo et al. (2012) have recently described a potent class of full agonists at FFA1 stimulating insulin secretion both directly by FFA1 activation on pancreatic β-cells and indirectly via GLP-1 and GIP release from enteroendocrine cells (Luo et al. 2012). Partial

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agonists such as AMG-837 were however not able to stimulate GLP-1 and GIP release (Luo et al. 2012).

In vivo studies have shown that FFA1 agonists display a positive impact on glucose tolerance in rats and mice, especially in the case of adipose animal models (Doshi et al. 2009; Lin et al. 2011; Sasaki et al. 2011; Tsujihata et al. 2011; Zhou et al. 2008). Overexpression of FFA1 in transgenic mice improves glucose tolerance (Nagasumi et al. 2009). Therefore, many groups claim that FFA1 agonists might be useful for the treatment of type 2 diabetes mellitus (T2DM) (Holiday et al. 2011; Garrido et al. 2006; Lin et al. 2011; Zhou et al. 2010). TAK-875 is the first FFA1 agonist to undergo clinical development for the treatment of T2DM and has shown a high efficacy in patients (Burant et al. 2012). We have in recent years aimed at developing FFA1 agonists for treatment of T2DM (Christiansen et al. 2008; 2010; 2011; 2012; 2013a; 2013b). TUG-469 was among the most active FFA1 agonists within this series of papers (Christiansen et al. 2010). In this study, we present pharmacological data of TUG-469 at recombinant FFA1 and another related free fatty acid receptor 4 (FFA4; formerly known as GPR120) in comparison to the standard FFA1 agonists GW9508 and TAK-875 (Hudson et al. 2013). Furthermore, we report the first in vivo study on the effect of TUG-469 on glucose tolerance in pre-diabetic New Zealand obese (NZO) mice.

## Methods

### Cell culture and cell lines

The 1321N1 cell line, stably expressing the human FFA1 receptor (named in this manuscript "1321N1-hFFA1") was obtained from Euroscreen (Gosselies, Belgium) and cultivated in Dulbecco's modified Eagle's medium 1640 + GlutaMAX™-I supplemented with 10 % (*v*/*v*) fetal calf serum (FCS) (both Gibco, Karlsruhe, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin (both Sigma Aldrich, Taufkirchen, Germany), and 400 μg/ml G-418 (Calbiochem, San Diego, CA, USA). The rat pancreatic β-cell line INS-1 was cultivated in RPMI 1640 + GlutaMAX™-I supplemented with 10 % (*v*/*v*) FCS (both Gibco, Karlsruhe, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin (both Sigma Aldrich, Taufkirchen, Germany), 1 mM sodium pyruvate (Applichem, Darmstadt, Germany), and 50 μM 2-mercaptoethanol (Roth, Karlsruhe, Germany). Both cell lines were cultivated with 5 % $CO_2$ at 37 °C in a humidified atmosphere and subcultured once a week.

### Generation of FlpIn-cell lines

pFRT/lacZeo plasmid (Invitrogen, Carlsbad, CA, USA) was stably transfected in 1321N1 wild type cell line according to the manufacturer's protocol to generate a 1321N1-FlpIn host cell line. Receptor-expressing cell lines were generated by transfection with pcDNA5/FRT-hFFA1- or pcDNA5/FRT-hFFA4-constructs, respectively (hFFA1 was kindly provided by Prof. Evi Kostenis, University of Bonn, Germany; hFFA4 was obtained from Biocat, Heidelberg, Germany), both cotransfected with pOG44 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Positively transfected cell lines were selected by addition of 200 μg/ml Zeocin for the host cell line or 250 μg/ml hygromycin B for both receptor-expressing cell lines. Cell lines were named "1321N1-FlpIn-FFA1" or "1321N1-FlpIn-FFA4", respectively.

### Preparation of test compound solutions for in vitro studies

The compounds TUG-469, GW9508, racemic TUG-761 and racemic TAK-875 were synthesized as described previously (Christiansen et al. 2010; 2011; 2012; Negoro et al. 2010). All compounds were dissolved in dimethyl sulfoxide at a concentration of 10 mmol/l (stock solution). For calcium mobilization assay, stock solution was diluted with Krebs-HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffer (please see "Calcium mobilization assay") to the final concentration. For cAMP reporter gene assay, measurement of cellular impedance and insulin secretion, stock solution was dissolved to the final concentration in cell culture medium (please see "Cell culture and cell lines") containing 10 % (*v*/*v*) FCS.

### Calcium mobilization assay

Calcium measurements were performed using a NOVOstar® microplate reader with a built-in pipetor (BMG LabTech, Offenburg, Germany) as previously described (Kassack et al. 2002). Briefly, 1321N1 cells were seeded in 96-well tissue culture plates at a density of 30,000–40,000 cells per well. After 24 h, cells were washed twice in Krebs-HEPES buffer (KHB; 118.6 mM NaCl, 4.7 mM KCl, 1.2 mM $KH_2PO_4$, 4.2 mM $NaHCO_3$, 11.7 mM D-glucose, 10 mM HEPES (free acid), 1.3 mM $CaCl_2$, and 1.2 mM $MgSO_4$, pH 7.4) and loaded with Oregon Green 488 BAPTA-1 AM (Molecular Probes, Eugene, OR, USA) to a final concentration of 1.5 μM and 0.03 % Pluronic F127 (Invitrogen, Karlsruhe, Germany) for 1 h (37 °C, 5 % $CO_2$). After addition of KHB, microplates were subsequently transferred to Novostar® and kept at 37 °C under exclusion of light for 15 min until the measurement was started. For testing of agonists, 20 μl of a tenfold concentrated test compound solution was injected sequentially into wells, and fluorescence was measured at 520 nm (bandwidth 25 nm) for 50 intervals of 0.4 s each. The excitation wavelength was 485 nm (bandwidth 25 nm). In studies involving the antagonist TUG-761 (*trans*-1-oxo-3-(4-phenoxyphenyl)-2-propyl-1,2,3,4-tetrahydroisoquinoline-4-carboxylic acid), TUG-761 solution was incubated 20 min

prior to the injection of agonist. No effect was observed with dimethyl sulfoxide (VWR, Darmstadt, Germany) up to 3 % (corresponding to a compound concentration of 31.6 μM).

### cAMP reporter gene assay

Intracellular cAMP levels were estimated using a cAMP reporter gene assay as previously described (Hamacher et al. 2006). 1321N1 cells were transfected with 3 μg/ml pCRE-luc plasmid (Stratagene, La Jolla, CA, USA) using 8 μl/ml PolyFect (Qiagen, Hilden, Germany) as transfection reagent for 24 h (37 °C, 5 % $CO_2$) in a T75-cell culture flask with normal culture medium at a confluence of around 50 %. Cells were then harvested and seeded on white 96-well μClear microplates (Greiner, Frickenhausen, Germany) for 24 h (37 °C, 5 % $CO_2$). Culture medium was then exchanged by fresh medium without FBS, and the test compounds were injected. Cells were incubated for 3 h at 37 °C, 5 % $CO_2$, washed once with Krebs-HEPES buffer and lysed by 100 μl lysis reagent (8 mM Tricin, 2 mM EDTA, 1 mM dithiothreitol, 5 % (*v*/*w*) Triton X-100, pH 7.8). Luminescence was measured upon injection of luciferase assay reagent (30 mM Tricin, 10 mM $MgSO_4 \times 7\ H_2O$, 0.5 mM EDTA, 10 mM dithiothreitol, 0.5 mM ATP, 0.5 mM coenzyme A, 0.5 mM D-luciferin, pH 7.8) using a LUMIstar® microplate reader (BMG LabTech, Offenburg, Germany).

### Measurement of cellular impedance

Cellular impedance was measured using the xCELLigence® RTCA DP Analyzer (Roche, Penzberg, Germany) with 16-well per plate format. Cells were seeded in 16-well E-plates at a density of 20,000 (for 1321N1 cells) or 70,000 (for INS-1 cells) cells per well in 195 μl culture medium containing 10 % FCS, and cell index was measured under normal cell culture conditions (37 °C, 5 % $CO_2$ in a humidified atmosphere). Then, after 16 h (for INS-1 cells) or 24 h (for 1321N1 cells), 5 μl solution of test compound was injected, and cell index was measured at an interval of 20 s for at least 2 h followed by an interval of 15 min for an additional 3–5 h after test compound injection. Antagonist solution was incubated 1 h prior to the injection of test compounds.

### Insulin secretion and insulin radioimmunoassay

INS-1E cells were seeded in 96-well tissue culture plates at a density of 120,000–150,000 cells per well and kept for 48 h under cell culture conditions (11 mM glucose). Cells were then washed twice with Krebs-Ringer bicarbonate HEPES buffer (KRBH; 135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM $KH_2PO_4$, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES (free acid), pH 7.4) containing 0.5 % (*w*/*v*) bovine serum albumin (free of fatty acids) and 0.5 mM glucose. After 30 min of incubation with KRBH containing 0.5 mM glucose, cells were loaded with glucose-free KRBH and stimulated with test compound and 16.7 mM glucose simultaneously for 30 min. Stimulation was stopped by bedding wells on ice. Supernatants were centrifuged at 10.000×*g* for 5 min and stored at −80 °C until insulin concentration was estimated using a radioimmunoassay assay (Millipore, Billerica, MA, USA), performed according to manufacturer's instructions using a gamma counter (Berthold Technologies, Bad Wildbad, Germany).

### Preparation of TUG-469 stock solution for in vivo mouse studies

TUG-469 was dissolved in dimethyl sulfoxide at a concentration of 66.7 mg/ml (stock solution). Stock solution was diluted in sterile 0.9 % NaCl solution containing 0.5 % (*w*/*v*) Tween®20 (Roth, Karlsruhe, Germany) as a solubilizer to a concentration of 0.5 mg TUG-469/ml freshly 1 day prior to the experiment.

### Glucose tolerance tests

Sixteen male NZO mice at the age of 180–220 days were fasted for 6 h prior to the experiment. The average body weight per mouse was 60.6 g±6.8 g (mean ± SD). Ten microliter per gram of body weight of vehicle or test compound solution containing 0.5 mg TUG-469/ml were injected intraperitoneally 1 h prior to intraperitoneal administration of 2.5 g glucose/kg body weight using a 40 % sterile glucose solution (B. Braun, Melsungen, Germany). Blood samples were collected from the tail vein. Plasma glucose levels were measured 60 min prior to ip glucose administration and 0, 30, 60, 90, 120, and 180 min after glucose administration using the blood glucose monitoring system Precision Xceed Xtra Plus (Abbott, Witney, Oxon, UK). All procedures in the animal studies complied with the German national guidelines for animal care (article 8 passage 1) and were approved by the ethics committee for animal experiments at the German Diabetes Center.

### Data analysis

In functional assays (calcium mobilization, cAMP reporter gene), effects of single concentrations of FFA1 agonists were expressed as percentage of the maximum response of TUG-20, a reported FFA1 agonist (Christiansen et al. 2010) if not otherwise stated. $pEC_{50}$ values were derived from pooled concentration–effect curves fitted to the nonlinear four-parameter logistic equation (Prism® 4.03; GraphPad Software, San Diego, CA, USA). All experiments were performed in triplicate assays and at least twice repeated if not otherwise stated. In cellular impedance assays, cell index (CI) values were basal-normalized according to manufacturer's protocols. In order to eliminate the effect of the solvent DMSO at high concentrations of test compounds, cell index

curves after DMSO control injections were monitored and used for correction. For calculation of $pEC_{50}$ values, an interval of 2 h after injection of test compound was selected to determine the maximum increase in cell index. Insulin concentrations were calculated according to manufacturer's protocols and normalized to the stimulation with "control" (control: Krebs-Ringer bicarbonate HEPES buffer containing the same amount of DMSO as compound solution). Data from glucose tolerance test (GTT) were expressed as change in blood glucose concentration from baseline at glucose application. For evaluation of significant differences between means, a two-tailed and unpaired *t* test was used (Prism® 4.03; GraphPad Software, San Diego, CA, USA). Values for area under the curve (AUC) are the mean ± standard deviation of AUCs from single curves, calculated for the interval between 0 and 120 min using Prism® 4.03.

## Results

TUG-469 (Fig. 1) is a highly potent FFA1 agonist in vitro (Christiansen et al. 2010). 3D-QSAR analysis (adaption of fields for molecular comparison—AFMoC) based on a homology model of FFA1 suggests a similar binding mode of TUG-469 to that of GW9508 (Gohlke and Klebe 2002; Tikhonova et al. 2007) (data not shown). However, the predicted potency of TUG-469 was higher than that of GW9508: $pEC_{50}$-predicted-TUG-469, 7.64; $pEC_{50}$-predicted-GW9508, 7.18. Indeed, the structural modifications between TUG-469 and GW9508 (Fig. 1) result—in all applied assays—in a small but significantly improved in vitro potency of TUG-469 (Figs. 2, 3, 4, Table 1). Figure 2a shows a 1.7-fold higher potency of TUG-469 compared to GW9508 in the $Ca^{2+}$ mobilization assay, and Fig. 2b shows a 2.7-fold higher potency in the cAMP assay. Next, changes in impedance measurements were used to monitor the effects of TUG-469 and GW9508 in FFA1 expressing cell lines. Figure 2c shows a concentration-dependent increase in cellular impedance after injection of TUG-469 to 1321N1 cells recombinantly expressing FFA1. The maximum increase of CI was selected for constructing a concentration–effect curve displayed in Fig. 2d for TUG-469 and GW9508. Again, TUG-469 showed a 3.0-fold higher potency compared to GW9508 in the cellular impedance assay (Fig. 2d). Although the slope factor of TUG-469 curve in Fig. 2d is significantly lower than that for GW9508, other functional data give slope factors of the same range (Fig. 2a, b). However, the potency of both compounds was four or seven times lower (TUG-469 or GW9508) compared to the data from the calcium mobilization assay, but in the same range as data from the cAMP assay. The leftward shift of concentration–response curves measured in calcium mobilization versus cAMP and cell impedance assay is probably due to absence of FCS in calcium studies preventing unspecific substance binding, thus leading to higher potency. All $pEC_{50}$ values determined at 1321N1 cells for TUG-469 and GW9508 are listed in Table 1. Even though differences in potency between TUG-469 and GW9508 are rather small (between 1.7 and threefold), TUG-469 is significantly more potent than GW9508 in all assays ($p<0.01$, *t* test). Although results represented in Fig. 2a, d point toward a slightly higher efficacy of TUG-469 versus GW9508, this finding was not confirmed by results shown in Figs. 2b and 3b.

In order to prove that the observed effects result from FFA1 stimulation, the selective FFA1 antagonist TUG-761 (Christiansen et al. 2011) was used in calcium mobilization experiments. Figure 2e shows the concentration-dependent inhibition by TUG-761 of TUG-469- and GW9508-induced calcium mobilization. $K_i$ values of TUG-761 were similar using the agonist TUG-469 ($K_i$, 97.7 nM) or GW9508 ($K_i$, 123 nM) (Fig. 2e).

Since TUG-469 (clog*P*=4.94) and GW9508 (clog*P*=4.95) with a calculated log*P* value (log of distribution coefficient as ratio of compound concentrations in a mixture of octanol/water at equilibrium) of around 5 are lipophilic FFA1 ligands (Christiansen et al. 2012), they are prone to high serum albumin binding. Increasing bovine serum albumin (fatty acid-free) concentrations reduce the functional potency of TUG-469 as measured in the calcium mobilization assay (Fig. 2f). The potency of TUG-469 (calcium mobilization assay) is reduced by 15-fold from 17 nM in the absence of albumin to 250 nM in the presence of 2.5 % albumin (Fig. 2f). These data were used to adjust the TUG-469 doses used in the glucose tolerance test in mice when switching from serum albumin-free cellular experiments to in vivo systems (mouse model).

Next, the effect of TUG-469 on the rat insulinoma cell line INS-1 was studied. INS-1 did not respond upon FFA1 stimulation in calcium mobilization and cAMP assays. Applying an impedance-based assay, it was possible to quantify cellular effects of FFA1 stimulation. Cellular impedance indicates a cellular event due to changes in the attachment of cells to the surface and is thus suited to analyze GPCR-mediated cellular effects, i.e., GPCR activation. Figure 3a shows the concentration-dependent increase in cellular impedance after TUG-469 administration to INS-1 cells. The biphasicity observed in this time course of cellular impedance (Fig. 3a) may reflect kinetics of insulin release remains speculative since a possible correlation between cellular impedance and insulin secretion was not studied. Corresponding concentration–effect curves of GW9508 and TUG-469 are given in Fig. 3b. TUG-469 is 3.7-fold more potent than GW9508, a similar result as obtained at recombinantly expressed FFA1 (Fig. 2d). The rightward shift of concentration–effect curves of TUG-469 and GW9508 at INS-1 (Fig. 3b) versus 1321N1 cells (Fig. 2d) may be explained by lower FFA1 expression levels in the native INS-1 cell line. Efficacies of TUG-469 and GW9508 are comparable at INS-1

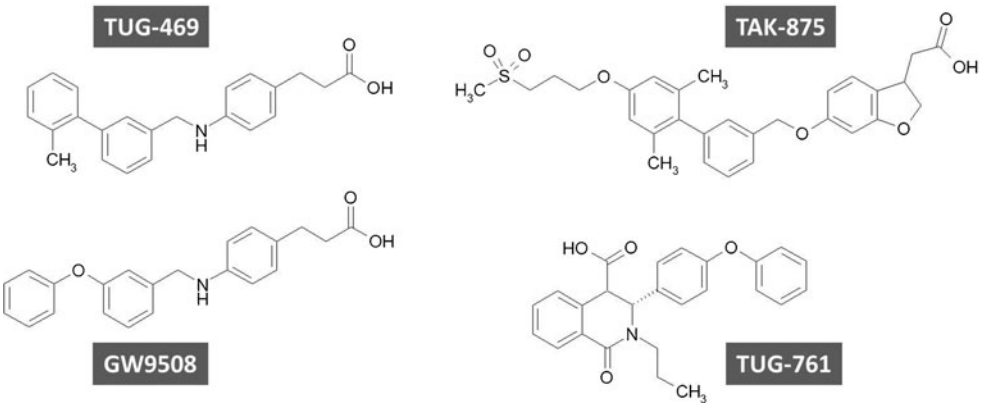



**Fig. 1** Structural formulas of TUG-469, standard FFA1 agonists GW9508 and TAK-875 and FFA1 antagonist TUG-761. In this study, TAK-875 and TUG-761 were used as a racemic mixture

cells (Fig. 3b). The impedance-based signal of TUG-469 seen in Fig. 3a, b was inhibited by the FFA1 antagonist TUG-761 (Fig. 3c). Furthermore, stimulation of INS-1 cells by 5 μM TUG-469 in the presence of high glucose concentration (16.7 mM) led to a significantly increased insulin secretion (Fig. 3d). Stimulation with 5 μM GW9508 gave similar results. Adenylate cyclase activation by forskolin served as positive control and led to a massive insulin secretion.

TUG-469 is among the most potent FFA1 agonists (Christiansen et al. 2010; 2012). Here, we present data on the selectivity of TUG-469 for FFA1 over FFA4 compared to GW9508 and TAK-875 (Fig. 4). Figure 4a, b illustrate the activity of TUG-469, GW9508, and racemic TAK-875 at 1321N1-FlpIn host cell lines either recombinantly expressing FFA1 or FFA4. All three compounds showed activity at FFA1 (Fig. 4a) and FFA4 (Fig. 4b) but no signal (calcium) in an empty vector control cell line (data not shown). However, TUG-469 was not only the most potent FFA1 ligand but also showed the highest selectivity for FFA1 compared to FFA4 under BSA free conditions as can be seen from a selectivity index of 223 for TUG-469 compared to selectivity indices of 7 (GW9508) or 58 (TAK-875) (Fig. 4c). In addition, the lower maximal effect of TUG-469 at FFA4 compared with GW9508 and TAK-875 indicates that TUG-469 may be a partial agonist at FFA4.

These data encouraged us to initiate—in addition to the in vitro characterization—an in vivo study on the effects of TUG-469 on glucose tolerance in pre-diabetic NZO mice. An intraperitoneal glucose tolerance test in NZO mice gave a difference between TUG-469 and vehicle-treated mice: the increase in blood glucose concentrations after intraperitoneal administration of 2.5 g/kg glucose was significantly affected by application of 5 mg/kg TUG-469 60 and 90 min after intraperitoneal glucose administration (Fig. 5a). Figure 5b illustrates means and single data from each mouse of changes in blood glucose concentration 60 and 90 min after glucose administration. Both time points show a significant reduction in blood glucose in TUG-469-treated mice. When analyzing the difference in blood glucose concentration from 0 to 120 min after glucose application by calculating the AUC, again a significant reduction in glucose concentration was observed for TUG-469-treated mice (Fig. 5c). However, there was no significant difference in basal blood glucose levels of both groups of mice. Prior to substance application, the mean basal blood glucose level was 216±68 mg/dl for TUG-469-treated mice and 244±58 for vehicle-treated mice, respectively.

## Discussion

TUG-469 was recently described as the most potent FFA1 agonist among a series derived from the initial hit TUG-20 and GW9508 (Christiansen et al. 2010). With an $EC_{50}$ of 17 nM (Figs. 2a and 4a) (Christiansen et al. 2010), TUG-469 belongs to the most potent FFA1 agonists so far described and displays selectivity over FFA2, FFA3, and FFA4 (Fig. 4) (Christiansen et al. 2010; 2012). The compound was also recently found to protect against apoptosis of pancreatic β-cells (Wagner et al. 2013) Here, we report on a further in vitro characterisation of TUG-469 in comparison to the lead compound GW9508 and TAK-875, and more importantly, present first evidence for an in vivo effect of TUG-469 on glucose tolerance in mice.

TUG-469 was chosen for the in vivo study on glucose tolerance due to its high selectivity for FFA1 over FFA4 (Fig. 4), thus reducing the likelihood of effects through other free fatty acid receptors than FFA1. Further, TUG-469 belongs to the most potent FFA1 agonists known so far and is superior to GW9508 as seen from Fig. 2. This result is in accordance with molecular modeling data obtained from our AFMoC model (Gohlke and Klebe 2002; Tikhonova et al. 2007) constructed on the basis of published agonist data (data on the AFMoC model will soon be presented elsewhere) (Christiansen et al. 2008; 2010; 2011). The higher potency of TUG-469 over GW9508 was predicted (TUG-469: $pEC_{50}$-predicted, 7.64; GW9508: $pEC_{50}$-predicted, 7.18) and is in accordance with this and the previous study (Fig. 2) (Christiansen et al. 2010). Besides data from calcium mobilization and dynamic mass redistribution assays (Christiansen

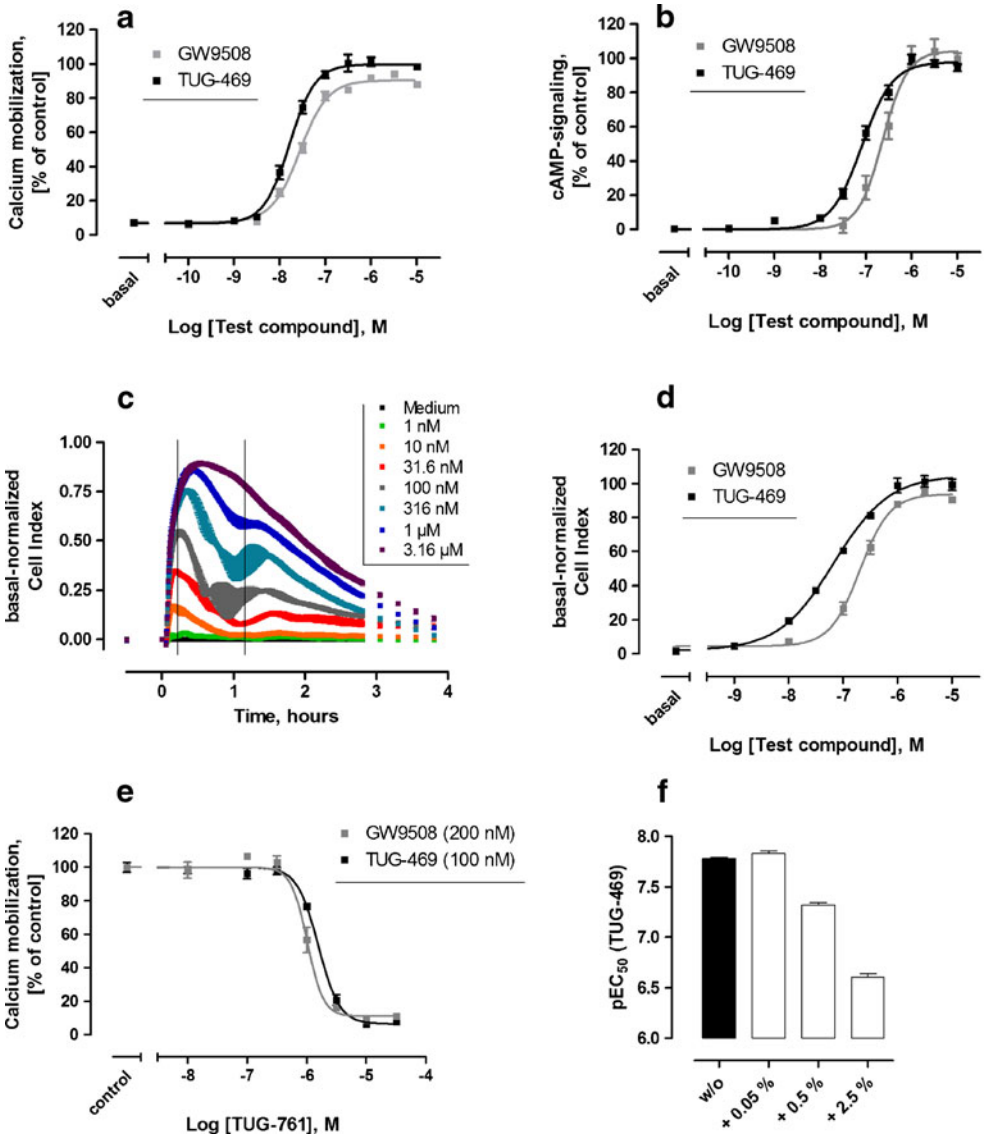



**Fig. 2** Concentration–effect curves of TUG-469 (*black curves*) and GW9508 (*gray curves*) at 1321N1 hFFA1 expressing cell line measured with $Ca^{2+}$ mobilization assay (**a**), cAMP signaling assay (**b**), and cell impedance assay (**d**). Top plateau of TUG-469-induced signals were set as 100 % control. All data points are mean of at least three independent experiments assayed in triplicates. **a** $pEC_{50}$ ± standard error (SE): GW9508 (7.56±0.02), TUG-469 (7.78±0.03); $E_{max}$ ± SE: GW9508 (90.5±1.0), TUG-469 (99.9±1.5); $n_H$ ± SE: GW9508 (1.42±0.10), TUG-469 (1.55±0.14). **b** $pEC_{50}$ ± SE: GW9508 (6.63±0.05), TUG-469 (7.08±0.03); $E_{max}$ ± SE: GW9508 (104.3±4.5), TUG-469 (97.4±1.7); $n_H$ ± SE: GW9508 (1.61±0.27), TUG-469 (1.30±0.11). **c** TUG-469-induced concentration-dependent cell responses in cell impedance assay. **d** Data points are mean of two independent experiments assayed in duplicates. $pEC_{50}$ ± SE: GW9508 (6.69±0.03), TUG-469 (7.17±0.04); $E_{max}$ ± SE: GW9508 (93.8±1.5), TUG-469 (105.0±2.3); $n_H$ ± SE: GW9508 (1.57±0.14), TUG-469 (0.85±0.07). **e** Inhibition of TUG-469 (*black curve*)- or GW9508 (*gray curve*)-induced signal by the competitive hFFA1-antagonist TUG-761 at 1321N1 hFFA1 expressing cell line measured with $Ca^{2+}$ mobilization assay. Control means 100 nM TUG-469- or 200 nM GW9508-induced hFFA1-signal. $pIC_{50}$ ± SE, 5.80±0.02, $K_i$=97.7 nM; $I_{max}$ ± SE: 6.44±1.70; $n_H$ ± SE:−2.45±0.21 (for TUG-469-induced signal) and $pIC_{50}$ ± SE: 5.99±0.02, $K_i$=123 nM; $I_{max}$ ± SE: 11.03±2.40; $n_H$ ± SE:−3.21±1.31 (for GW9508-induced signal). All data points are mean of at least two independent experiments assayed in triplicates. $K_i$ values were calculated according to the Cheng–Prusoff equation [$K_i$=$IC_{50}$/(1+(agonist concentration/$EC_{50}$))]. **f** Effect of bovine serum albumin (fatty acid-free) on $pEC_{50}$ of TUG-469 at 1321N1 hFFA1 cells performed with $Ca^{2+}$ mobilization assay. All data points are mean of two independent experiments assayed in triplicates

et al. 2010), this paper presents in vitro pharmacological data based on cAMP and impedance measurements (Figs. 2 and 3). Upon FFA1 stimulation, an increase in cAMP levels was observed (Fig. 2b). These data pointing to a $G_s$ coupling are in accordance with studies from Feng et al. (2006; Kebede et al. 2009) who also described cAMP increase upon FFA1 stimulation even though $G_s$ coupling is unlikely to present the main signaling pathway. $EC_{50}$ values derived from cAMP assays were higher than $EC_{50}$ from calcium studies (Table 1) which is also in accordance with a preferential coupling of FFA1 to the $G_q$ pathway. Furthermore, changes in the cellular attachment as measured in impedance-based assays (Figs. 2c

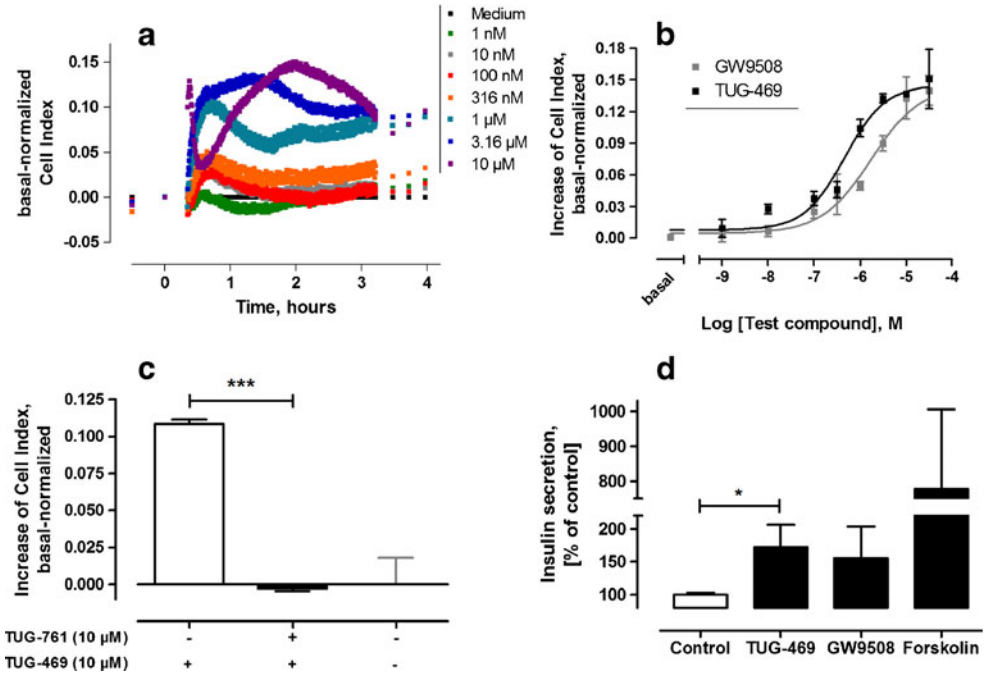
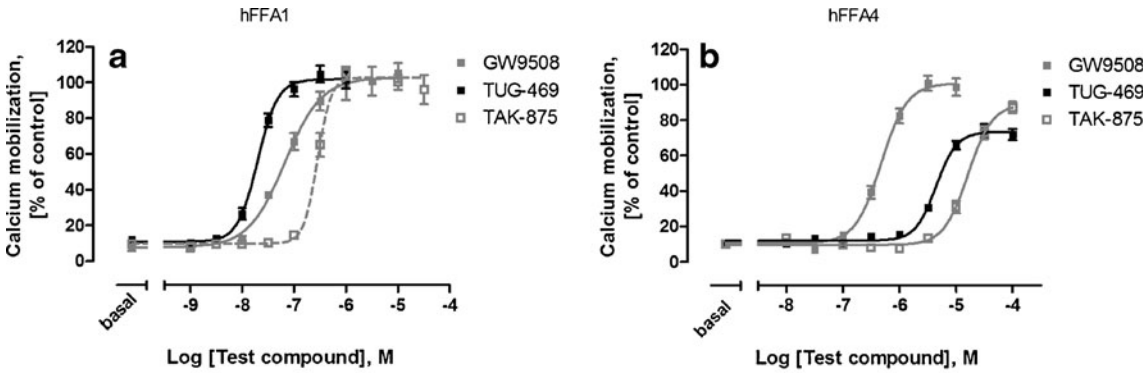



**Fig. 3** **a** TUG-469-induced concentration-dependent cell responses in a cell impedance assay at rat INS-1 cells. For evaluation of the effects basal-normalized cell index was calculated. **b** Concentration–effect curves of TUG-469 (*black curves*) and GW9508 (*gray curves*) at rat INS-1 cells using cellular impedance assay. $pEC_{50}$ ± SE: GW9508 (5.76±0.26), TUG-469 (6.34±0.11); $E_{max}$ ± SE: GW9508 (0.15±0.03), TUG-469 (0.15±0.01); $n_H$ ± SE: GW9508 (0.80±0.24), TUG-469 (0.98±0.20). Data points are mean of four independent experiments assayed in duplicates. **c** Inhibition of TUG-469-induced cell response in cell impedance assay by 10 µM TUG-761 (Fig. 2e). **d** 16.7 mM Glucose induced short-term insulin secretion from pancreatic INS-1E cells for 30 min. Shown are means±SE of at least three independent experiments assayed in duplicates. TUG-469 (5 µM), 173±33 % of control; GW9508 (5 µM), 156±48 % of control. Control means injection of assay buffer including 0.05 % DMSO. Unpaired *t* tests, *$p \leq 0.05$, ***$p \leq 0.0001$




| c | TUG-469 | GW9508 | TAK-875 (rac.) |
|---|---|---|---|
| $EC_{50}$ (hFFA1) | 1.99 x $10^{-8}$ M | 6.63 x $10^{-8}$ M | 2.80 x $10^{-7}$ M |
| $EC_{50}$ (hFFA4) | 4.44 x $10^{-6}$ M | 4.76 x $10^{-7}$ M | 1.63 x $10^{-5}$ M |
| SI | **223** | **7** | **58** |

**Fig. 4** Selectivity of FFA1 agonists at hFFA1 and hFFA4. Activities of TUG-469, GW9508, and TAK-875 were measured in the absence of bovine serum albumin at 1321N1 FlpIn host cells, recombinantly expressing the hFFA1 (**a**) or hFFA4 (**b**). Pharmacological data in **a** $pEC_{50}$ ± SE: GW9508 (7.18±0.06), TUG-469 (7.70±0.04), TAK-875 (6.55±0.02); $E_{max}$ ± SE: GW9508 (102.7±3.0), TUG-469 (101.8±2.2), TAK-875 (102.8±2.5); $n_H$ ± SE: GW9508 (1.24±0.21), TUG-469 (2.25±0.30); TAK-875 (3.32±0.98). Pharmacological data in **b** $pEC_{50}$ ± SE: GW9508 (6.32±0.04), TUG-469 (5.35±0.03), TAK-875 (4.79±0.04); $E_{max}$ ± SE: GW9508 (100.8±2.7), TUG-469 (73.5±1.7), TAK-875 (89.2±3.1); $n_H$ ± SE: GW9508 (1.90±0.26), TUG-469 (2.37±0.35), TAK-875 (2.02±0.25). All data points are mean of at least two independent experiments assayed in triplicates. **c** $EC_{50}$ values and selectivity index ($EC_{50}$ for hFFA4 / $EC_{50}$ for hFFA1)

**Table 1** In vitro potencies of TUG-469 and GW9508 as $pEC_{50}$ ± SE. Each value was calculated from at least three independent experiments

| | $Ca^{2+}$ mobilization | cAMP reporter gene | Cell impedance |
|---|---|---|---|
| TUG-469 | 7.78±0.03 | 7.06±0.03 | 7.17±0.04 |
| GW9508 | 7.56±0.02 | 6.63±0.05 | 6.69±0.03 |

and 3a) may reflect both $G_s$ and $G_q$ coupling of FFA1. TUG-469 displays higher potency than GW9508 in all assay systems at recombinantly and natively expressed FFA1 in the rat insulinoma cell line INS-1. The increase in potency is significant (*t* test in all assays, $p<0.01$). The specificity of the TUG-469 interaction with FFA1 was shown by inhibition of TUG-469 signaling with the competitive FFA1 antagonist TUG-761, both in cells recombinantly (1321N1) or natively (INS-1) expressing FFA1 (Figs. 2e and 3c). Taken together, this study confirms the high in vitro potency of TUG-469, which is in the same range as AMG-837 or TAK-875, both FFA1 agonists progressed to clinical evaluation (Houze et al. 2012; Lin et al. 2011; Sasaki et al. 2011; Zhou et al. 2010).

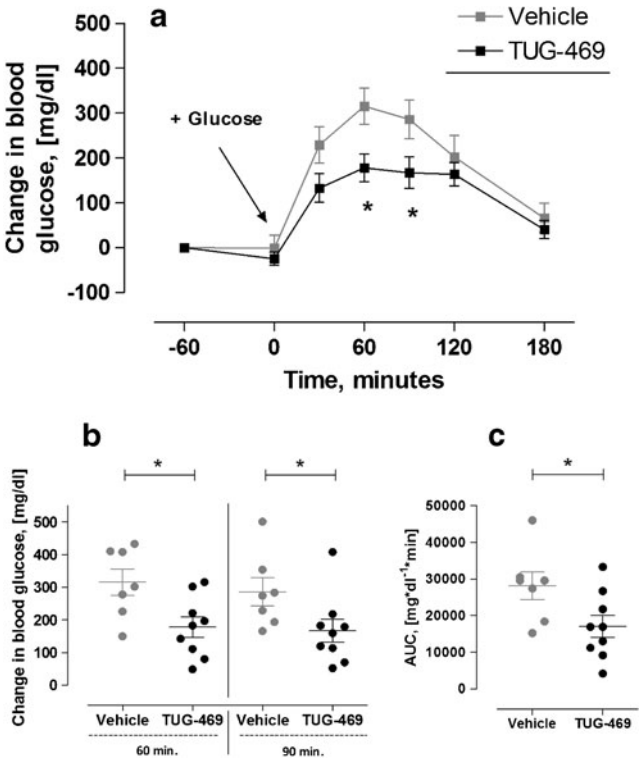



**Fig. 5** Glucose tolerance test (GTT) in male NZO mice. Nine NZO mice (TUG-469 administration), respectively seven NZO mice (vehicle administration) at the age of 180–220 days were used. The body weight per mouse was 60.6±1.8 g (mean±SE). Administration of vehicle or 5 mg/kg TUG-469 was performed intraperitoneally 60 min prior to glucose administration. **a** Change in blood glucose level means difference between blood glucose concentration at the distinct time points and the time point 60 min prior to glucose administration (−60 min). Shown are mean and SE. **b** Change of blood glucose levels after 60 and 90 min of glucose administration. Shown are mean ± SE and single values. **c** Difference in blood glucose levels measured as AUC from time point 0 to 120 min of glucose administration. Shown are mean ± SE and single values. *Asterisk* indicates a significant difference (unpaired *t* test; $p<0.05$)

TUG-469 shows high selectivity for FFA1 over FFA4 and only partial agonist activity at FFA4, a further free fatty acid receptor with preference for similar ligands, favoring TUG-469 for in vivo studies aimed at evaluation of FFA1-induced signaling. Thus, TUG-469 was selected for an in vivo study in mice to explore its effects on glucose tolerance. Through analysis of the effect of serum albumin on the $EC_{50}$ of TUG-469 (Fig. 2f), it was confirmed that the rather lipophilic compound TUG-469 is capable of stimulating FFA1 even at high serum albumin concentrations, thus assuming that high serum albumin concentrations in vivo will not constrain biological activity. Indeed, the potency of TUG-469 at INS-1, natively expressing FFA1, was in the submicromolar range ($EC_{50}$=462 nM) using cellular impedance measurement (Fig. 3b), and a significantly higher insulin secretion was found after INS-1 stimulation by 5 μM TUG-469 compared to control. TUG-469-treated pre-diabetic NZO mice showed a significant improvement of glucose tolerance at a dose of 5 mg/kg after ip glucose administration (Fig. 5). Furthermore, TUG-469 administration showed no influence on blood glucose levels in the fasted state (1 h prior to glucose administration) (Fig. 5a). This result suggests that TUG-469—similar to TAK-875—could enhance insulin secretion without increasing the risk of hypoglycemia in diabetic patients (Tsujihata et al. 2011).

Luo et al. (2012) have recently described full agonists at FFA1 stimulating insulin secretion by a dual mechanism, acting on both, FFA1 expressed in enteroendocrine cells (resulting in incretin release) and directly on FFA1 expressed in pancreatic β-cells. This dual action seems limited to full agonists at FFA1. AMG-837 and TAK-875 served as controls and turned out—in their study—as partial FFA1 agonists compared to fatty acids (linoleic acid, arachidonic acid). In our study, however, TUG-469, GW9508, and TAK-875 behaved as full agonists compared to free fatty acids in all assays (data not shown for free fatty acids). Thus, it remains unclear whether in our in vivo study TUG-469 acts as partial agonist (direct pancreatic insulin release) or full agonist (additional insulin secretion via enteroinsular axis: GLP-1/GIP release). Insulin secretion in mice by TUG-469-stimulation of FFA4 in enteroendocrine cells (via GLP-1/GIP release) is rather unlikely due to the partial agonism and low potency of TUG-469 at FFA4. Consequently, improvement of glucose tolerance in mice by TUG-469 (Fig. 5) presumably results from FFA1-mediated direct stimulation of insulin release rather than from interaction with FFA4 on enteroendocrine cells.

In conclusion, this study shows that the highly potent and selective FFA1 agonist TUG-469 improves glucose tolerance in pre-diabetic NZO mice. TUG-469 might thus

represent a promising lead compound for further preclinical development of novel FFA1-based therapeutics against T2DM.

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**Conflict of interest** The authors declare that they have no conflict of interest.

The animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All persons gave their informed consent prior to their inclusion in the study.

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