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RESEARCH PAPER

Effects of pyrazole partial agonists on HCA_2-mediated flushing and VLDL-triglyceride levels in mice

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BACKGROUND AND PURPOSE

Niacin can effectively treat dyslipidaemic disorders. However, its clinical use is limited due to the cutaneous flushing mediated by the nicotinic acid receptor HCA_2. In the current study, we evaluated two partial agonists for HCA_2, LUF6281 and LUF6283, with respect to their anti-dyslipidaemic potential and cutaneous flushing effect.

EXPERIMENTAL APPROACH

In vitro potency and efficacy studies with niacin and the two HCA_2 partial agonists were performed using HEK293T cells stably expressing human HCA_2. Normolipidaemic C57BL/6 mice received either niacin or the HCA_2 partial agonists (400 $mg \cdot kg^{-1} \cdot day^{-1}$) once a day for 4 weeks for evaluation of their effects *in vivo*.

KEY RESULTS

Radioligand competitive binding assay showed K_i values for LUF6281 and LUF6283 of 3 and 0.55 μM. [^{35}S]-GTPγS binding revealed the rank order of their potency as niacin > LUF6283 > LUF6281. All three compounds reduced plasma VLDL-triglyceride concentrations similarly, while LUF6281 and LUF6283, in contrast to niacin, did not also exhibit the unwanted flushing side effect in C57BL/6 mice. Niacin reduced the expression of lipolytic genes HSL and ATGL in adipose tissue by 50%, whereas LUF6281 and LUF6283 unexpectedly did not. In contrast, the decrease in VLDL-triglyceride concentration induced by LUF6281 and LUF6283 was associated with a parallel >40% reduced expression of APOB within the liver.

CONCLUSIONS AND IMPLICATIONS

The current study identifies LUF6281 and LUF6283, two HCA_2 partial agonists of the pyrazole class, as promising drug candidates to achieve the beneficial lipid lowering effect of niacin without producing the unwanted flushing side effect.

Abbreviations

APOB, apolipoprotein B; ATGL, adipose triglyceride lipase; HCA_2, hydroxy-carboxylic acid receptor 2; HDL, high-density lipoprotein; HSL, hormone-sensitive lipase; GTC, guanidinium thiocyanate; LDL, low-density lipoprotein; MTP, microsomal triacylglycerol transfer protein; TG, triglycerides; VLDL, very-low-density lipoprotein

Introduction

Niacin, also known as nicotinic acid, is the most effective agent currently available to treat dyslipidaemic disorders (Benhalima and Muls, 2010). It lowers plasma levels of pro-atherogenic lipids, including chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and triglycerides (TG) in normolipidaemic as well as hypercholesterolaemic subjects (Carlson, 2004). Several clinical trials have shown that nicotinic acid reduces cardiovascular disease and

myocardial infarction incidence, providing a solid rationale for the use of niacin in the treatment of atherosclerosis (Lee *et al.*, 2009; Taylor *et al.*, 2009). The GPCR GPR109A, also known as PUMA-G in mouse and HM74A in humans, has been identified as a high-affinity receptor for niacin (Lorenzen *et al.*, 2001; Wise *et al.*, 2003). We now know that the endogenous ligand for GPR109A is 3-hydroxybutyrate, and this receptor has recently been renamed as hydroxycarboxylic acid receptor 2 (HCA_2) (Offermanns *et al.*, 2011).

Despite its established cardiovascular benefits, the clinical use of niacin has been limited due to the cutaneous flushing, a well-recognized adverse skin effect from niacin therapy. Flushing has been cited as the major reason for the discontinuation of this therapy (Davidson, 2008). The nicotinic acid receptor HCA_2 expressed in the skin is a critical mediator of niacin-induced flushing (Benyó *et al.*, 2005). Niacin stimulates HCA_2 in epidermal Langerhans cells and keratinocytes, causing the cells to produce vasodilators PGD_2 and PGE_2, which leads to cutaneous vasodilatation (Morrow *et al.*, 1992; Cheng *et al.*, 2006; Dunbar and Gelfand, 2010; Hanson *et al.*, 2010).

For the past decade, the pharmacology of HCA_2 has been studied and full or partial agonists for HCA_2 have been developed in an attempt to achieve the beneficial effects of niacin while avoiding the unwanted flushing side effect (Wanders and Judd, 2011). Based on the structure–activity relationship of niacin-related molecules, several potent agonists for HCA_2 have been identified, including acipimox, acifran, 3-pyridine-acetic acid, 5-methylnicotinic acid, pyridazine-4-carboxylic acid and pyrazine-2-carboxylic acid (Kamanna and Kashyap, 2007; Soudijn *et al.*, 2007). However, so far HCA_2 partial agonists have failed to mimic the beneficial effects of niacin on LDL-cholesterol, triglycerides or HDL-cholesterol despite the absence of flushing events in clinical studies (Lai *et al.*, 2008). Further elucidation of the medicinal chemistry of HCA_2 is needed to pharmacologically dissociate the anti-lipolytic and vasodilator effects of niacin induced by its action on HCA_2.

In the current study, we assessed the properties of two HCA_2 partial agonists, LUF6281 and LUF6283, of the pyrazole class, which were developed in our laboratory (van Herk *et al.*, 2003). We first characterized these two compounds *in vitro*, using a radioligand binding assay, [^{35}S]-GTPγS assay and ERK phosphorylation assay. The ERK phosphorylation assay was included because it has been suggested that ERK1/2 phosphorylation downstream from HCA_2 correlates positively with skin flushing (Richman *et al.*, 2007). Subsequently, we determined the cutaneous flushing effect and the lipid-lowering potential of these two partial agonists in normolipidaemic C57BL/6 mice, which represent a good mouse model to study the VLDL-triglyceride lowering effect of niacin (Hernandez *et al.*, 2007).

Methods

Materials

[^{3}H]-nicotinic acid (60 Ci mmol^{-1}) was obtained from BioTrend (Koeln, Germany). [^{35}S]-GTPγS (1250 Ci mmol^{-1}) was obtained from PerkinElmer (Waltham, MA).

Cell culture and membrane preparation

HEK 293T cells stably expressing human HCA2 were cultured in DMEM supplemented with 10% newborn bovine serum, 0.4 mg·mL^{-1} G418, 50 IU·mL^{-1} penicillin and 50 µg·mL^{-1} streptomycin. The cells were harvested by scraping in cold PBS, centrifuged at 1000× *g* for 10 min and re-suspended in cold 50 mM Tris–HCl buffer, pH 7.4. Then a DIAX 900 electrical homogenizer (Heidolph, Schwabach, Germany) was used for 15 s to obtain cell lysis. The suspension was centrifuged at 225 000× *g* for 20 min at 4°C, and the supernatant was discarded. The pellet was re-suspended in Tris–HCl, and the homogenization and centrifugation steps were repeated. The membranes were re-suspended in cold assay buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4), and the protein content was determined using BCA assay (Thermo Scientific, Waltham, MA). During membrane preparation, the suspension was kept on ice at all times. Membrane aliquots were stored at –80°C until the day of use.

[^{3}H]-nicotinic acid displacement assay

Membranes of our stable HEK293T-HCA2 cell line (50 µg protein per tube) were incubated for 1 h at 25°C with 20 nM [^{3}H]-nicotinic acid and with increasing concentrations of the test compounds in assay buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4). The total assay volume was 100 µL. To assess the total binding, a control without test compound was included. The non-specific binding was determined in the presence of 10 µM unlabelled nicotinic acid. Final DMSO concentration in all samples was ≤0.25%. The incubation was terminated by filtering over GF/B filters using a 24-sample harvester (Brandel, Gaithersburg, MD). The filters were washed three times with 2 mL cold buffer (50 mM Tris–HCl, pH 7.4). Filters were transferred to counting vials and counted in a PerkinElmer LSA Tri-Carb 2900TR counter after 2 h of extraction in 3.5 mL Emulsifier Safe liquid scintillation cocktail (PerkinElmer).

[^{35}S]-GTPγS binding assay

This assay was performed in 96-well format in 50 mM Tris–HCl, 1 mM EDTA, 5 mM $MgCl_2$, 150 mM NaCl, pH 7.4 at 25°C with 1 mM DTT, 0.5% BSA and 50 µg·mL^{-1} saponin freshly added. HEK-HCA_2 membranes (5 µg protein per well in 25 µL) were pre-incubated with 25 µL of 40 µM GDP and 25 µL of increasing concentrations of the test compounds, for 30 min at room temperature. Then, 25 µL [^{35}S]-GTPγS was added (final concentration 0.3 nM), and the mixture was incubated for 90 min at 25°C with constant shaking. The incubation was terminated by filtration over GF/B filter plates on a FilterMate harvester (PerkinElmer). The filters were dried and 25 µL Microscint 20 (PerkinElmer) was added to each filter. After ≥3 h of extraction, the bound radioactivity was determined in a Wallac Microbeta Trilux 1450 counter (PerkinElmer).

ERK1/2 phosphorylation assay

The assay was performed using the AlphaScreen SureFire Phospho-ERK1/2 kit (PerkinElmer), following the kit protocol. Briefly, a 96-well cell culture plate was coated with poly-D-lysine, and HEK cells stably expressing human HCA_2 were seeded at 50 000 cells per well in 200 µL DMEM supple-

mented with 10% newborn bovine serum, 0.4 mg·mL^{-1} G418, 50 IU·mL^{-1} penicillin and 50 μg·mL^{-1} streptomycin. After overnight incubation, the cells were deprived of serum for 4 h in the same medium (but lacking the serum), and then the medium was replaced by 90 μL pre-warmed PBS and incubated for an additional 30 min. Increasing concentrations of the test compounds were diluted in pre-warmed PBS, and 10 μL was added per well for stimulation. After 5 min, the stimulation solution was removed from the plates, the wells were washed once in ice-cold PBS and 100 μL lysis buffer was added per well. After 15 min of incubation and shaking at room temperature, the lysates were mixed by pipetting, and 4 μL was transferred to a 384-well OptiPlate (PerkinElmer). The reaction mix was prepared according to the kit protocol (60 μL reaction buffer and 10 μL activation buffer with 1 μL of the donor and acceptor beads each), and 7 μL mix was added to each proxyplate well. After 2 h, the plate was read on an EnVision multilabel plate reader (PerkinElmer).

Animals

Twelve-week-old female C57BL/6 mice were fed a regular cholesterol-free chow diet containing 4.3% (w w^{-1}) fat (RM3, Special Diet Services, Witham, UK). Mice were kept, 4 mice per cage, in a climate controlled room of 20–22°C with a 12h/12h light-dark cycle. They had *ad libitum* access to food and water. The total number of mice used was 32. Mice received niacin, or HCA_2 partial agonists LUF6281 and LUF6283 [400 mg·kg^{-1}·day^{-1} in 50% (v v^{-1}) DMSO in PBS] once a day for 4 weeks via oral gavage. After being killed, mice were bled via orbital exsanguination and perfused *in situ* through the left cardiac ventricle with ice-cold PBS (pH 7.4) for 20 min. Liver and fat were dissected and snap-frozen in liquid nitrogen. Animal care and procedures were performed in accordance with the national guidelines for animal experimentation. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Measurement of skin flushing

Cutaneous flushing in C57BL/6 mice was assessed by monitoring the change in the skin temperature at the mouse paw location. Temperature measurements were recorded using a non-contact infrared thermometer (Pro Exotics PE-1 Infrared Temp Gun, Littleton, CO). The probe was held at a distance of 1 to 2 mm from the metacarpal pad of the mouse paw, and temperature readings were taken from a circular area approximately 3 mm in diameter. Animals were habituated to handling and to the infrared probe before use. Skin temperature was initially recorded from the abdominal area, tail, ear and paw; after which it was determined that mouse paw skin temperature yielded the most reliable and consistent results. During the experiment, the animals were dosed with niacin or partial agonists LUF6281 and LUF6283 (400 mg·kg^{-1}·day^{-1}) via oral gavage (10:00–11:00h), and the paw temperature was measured every 10 min for a period of 60 min in total. Three readings from the centre area of mouse paw were recorded routinely for each time point. Baseline paw temperature was recorded just before animals were dosed. All the administration was performed in conscious mice to avoid the interference of the anaesthetics on skin temperature.

Plasma lipid analysis

The distribution of cholesterol over different lipoproteins in plasma was determined by fast protein liquid chromatography (FPLC) through a Superose 6 column (3.2 × 30 mm; Smart-System, Pharmacia, Uppsala, Sweden). Cholesterol content of the lipoprotein fractions was measured using the enzymatic colorimetric assay (Roche Diagnostics, Mannheim, Germany).

Quantitative real-time PCR

Total RNA was isolated using acid guanidinium thiocyanate (GTC)–phenol–chloroform extraction. Briefly, 500 μL of GTC solution (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine) was added to each sample, followed by acid phenol : chloroform extraction. RNA in the aqueous phase was precipitated with isopropanol. The quantity and purity of isolated RNA were examined using an ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE). One microgram of RNA from each sample was converted into cDNA by reverse transcription with RevertAid™ M-MuLV Reverse Transcriptase (Promega, Madison, WI). Negative controls without addition of reverse transcriptase were prepared for each sample. Quantitative real-time PCR was carried out using ABI Prism 7700 Sequence Detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. 36B4, β-actin and GAPDH were used as internal housekeeping genes. The gene-specific primer sequences used are listed in Table 1. Amplification curves were analysed using 7500 Fast System SDS software V1.4 (Applied Biosystems).

Statistical analysis

Analysis of the *in vitro* studies was performed using Prism 5.0 software (GraphPad Software Inc., La Jolla, CA). Nonlinear regression was used to determine IC_{50} values from competition binding curves. The Cheng–Prusoff equation was then applied to calculate K_i values. [^{35}S]-GTPγS and pERK curves were analysed by nonlinear regression to obtain EC_{50} values. For the *in vivo* studies, data were analysed by *t*-test or one-way ANOVA with Student–Newman–Keuls post test (Instat GraphPad software, San Diego, CA) where appropriate. Statistical significance was defined as $P < 0.05$. Data are expressed as means ± SEM.

Results

The chemical structures of niacin and the HCA_2 partial agonists LUF6281 and LUF6283 are shown in Figure 1. The affinity of the compounds for HCA_2 was determined by a competitive binding assay using radiolabelled nicotinic acid and a HEK293T cell line stably expressing the human HCA_2 nicotinic acid receptor (Figure 2). The K_i values determined for LUF6281 and LUF6283 were 3.1 and 0.55 μM, respectively (Table 2).

As functional readout, the potencies and intrinsic efficacies of niacin, LUF6281, and LUF6283 were measured by their ability to stimulate [^{35}S]-GTPγS binding. The results show that

Table 1

Primers used for quantitative real-time PCR analysis

Gene	Forward primer	Reverse primer
36B4	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
β-actin	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
GAPDH	TCCATGACAACTTTGGCATTG	TCACGCCACAGCTTTCCA
ATGL	TGCCCTCAGGACAGCTCC	TTGAACTGGATGCTGGTGTTG
HSL	CTGACAATAAAGGACTTGAGCAACTC	AGGCCGCAGAAAAAAGTTGAC
APOB	ATGTCATAATTGCCATAGATAGTGCCA	TCGCGTATGTCTCAAGTTGAGAG
MTP	AGCTTTGTCACCGCTGTGC	TCCTGCTATGGTTTGTTGGAAGT

Table 2

In vitro biochemical characterization of the HCA_2 agonists niacin, LUF6281 and LUF6283

	K_i (μM)	EC_{50}-[35S]-GTPγS (μM)	EC_{50}-pERK1/2 (μM)	EC_{50} ratio GTPγS/pERK1/2
Niacin	0.04 ± 0.02	0.41 ± 0.11	0.02 ± 0.004	21
LUF6281	3.1 ± 0.5	8.60 ± 1.00	1.37 ± 0.31	6
LUF6283	0.55 ± 0.01	3.10 ± 0.13	0.32 ± 0.06	10

Values are means ± SEM ($n \geq 3$).

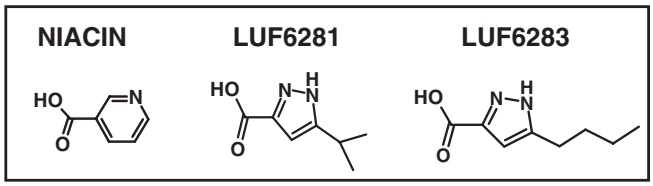

Figure 1

Chemical structures of niacin, LUF6281 and LUF6283.

LUF6281 and LUF6283 are partial agonists compared to niacin (100%), with intrinsic efficacies of 55 ± 4% and 76 ± 3%, respectively ($n = 3$). The rank order of their potency was niacin > LUF6283 > LUF6281, with EC_{50} values of 0.41, 3.1 and 8.6 μM, respectively (Figure 3; Table 2).

The second functional assay monitored ERK1/2 phosphorylation upon HCA_2 activation by the different compounds. All compounds appeared to be high efficacy full agonists (Figure 4; Table 2). The EC_{50} values obtained here were 20 nM for niacin, 1.4 μM for LUF6281 and 0.32 μM for LUF6283. Thus, all compounds seemed to be more potent in the pERK1/2 assay than in the [^{35}S]-GTPγS assay. Importantly, this difference was much more pronounced for niacin (21-fold) than for LUF6283 (10-fold) and LUF6281 (6-fold) (Table 2).

To examine the vasodilator effects of these compounds *in vivo*, we used normolipidaemic C57BL/6 mice to assess the cutaneous flushing. Normal paw skin temperature of C57BL/6 mice is approximately 26.4°C ($n = 30$). Flushing was

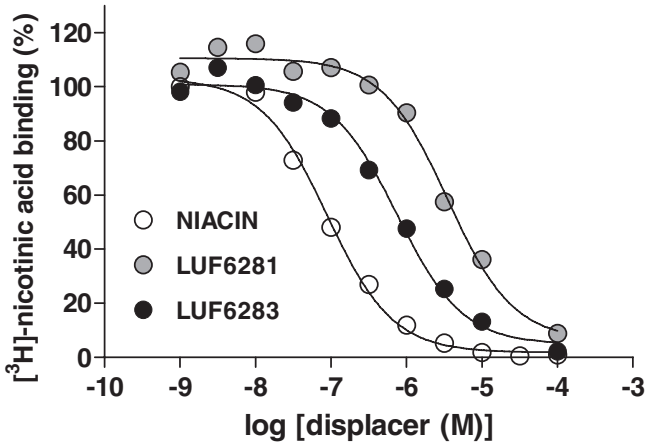

Figure 2

Competitive radioligand binding assay using 20 nM [^{3}H]-nicotinic acid revealing the relative affinities of niacin, LUF6281 and LUF6283. The assay was performed on HEK293T-HCA_2 membranes (50 μg per tube). A representative experiment is shown (of $n = 3$).

measured as absolute increase in mouse paw skin temperature. As anticipated, niacin treatment induced a strong increase in skin temperature (+3°C; $P < 0.001$). However, neither of the partial agonists displayed a significant temperature rise. At 20 min after compound administration, LUF6281 and LUF6283 induced a temperature increase of maximally 0.6°C ($n = 10$ per group), which was significantly lower than

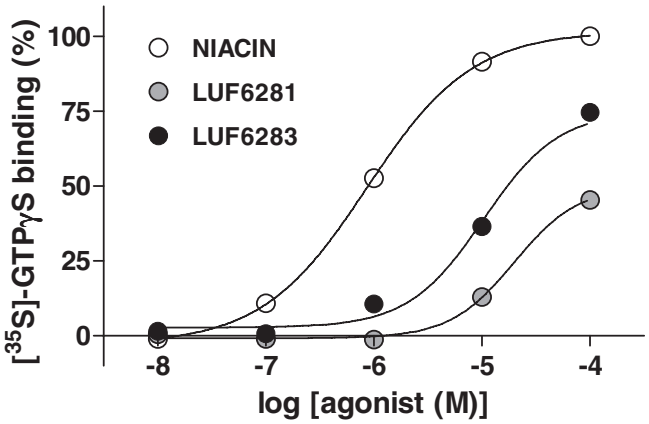

Figure 3

Concentration–response curves of niacin, LUF6281 and LUF6283 in a [^{35}S]-GTPγS binding assay, showing the relative potencies and intrinsic efficacies. Niacin is a full agonist, whereas LUF6281 and LUF6283 are partial agonists in this assay. The assay was performed on HEK293T-HCA_2 membranes (5 μg per tube). A representative experiment is shown (of n = 3).

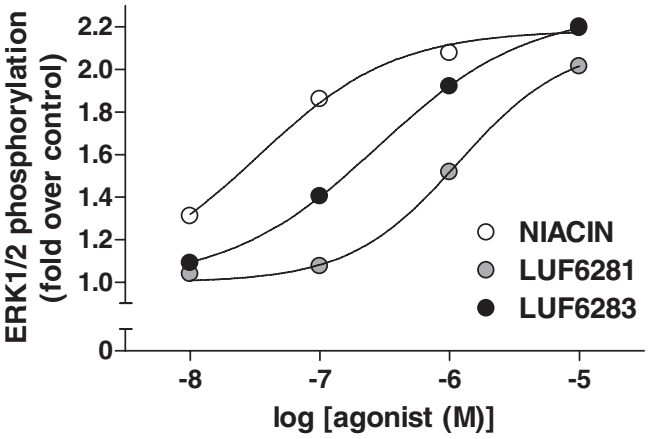

Figure 4

Concentration–response curves of niacin, LUF6281 and LUF6283 in an ERK1/2 phosphorylation assay, showing the relative potencies and intrinsic efficacies. All ligands are full agonists in this assay. The assay was performed on attached HEK293T-HCA_2 cells. A representative experiment is shown (of n = 3–5).

the temperature rise observed in the niacin group ($P < 0.001$; Figure 5) and not different from the temperature rise induced by mouse handling alone. Both of the HCA_2 partial agonists thus avoided the unwanted flushing side effect seen upon niacin exposure in mice.

To evaluate the lipid lowering potential of the LUF compounds, we tested their effect on plasma lipid levels. Although treatment with niacin or the LUF compounds for 4 weeks did not alter the plasma total cholesterol or triglyceride concentrations (data not shown), separation of plasma lipoproteins by FPLC in combination with analysis of the lipid content across the FPLC fractions showed that both niacin

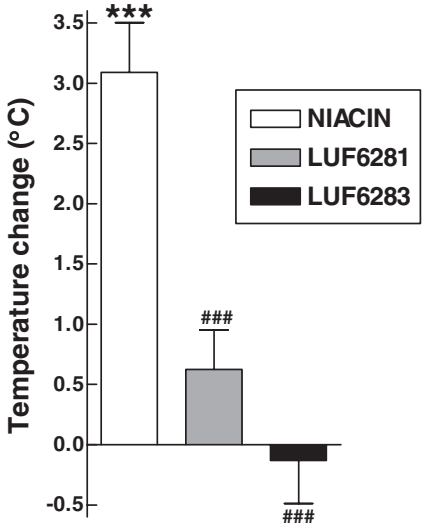

Figure 5

Mouse flushing after administration of niacin, LUF6281 and LUF6283. The cutaneous vasodilatation was determined by change in paw skin temperature in C57BL/6 mice. Mice received niacin, LUF6281 or LUF6283 (400 mg·kg^{-1}·day^{-1}) via oral gavage. Data are expressed as the change in skin temperature at 20 min after compound administration (n = 10 per group). ***$P < 0.001$ versus untreated mice; ###$P < 0.001$ versus niacin-treated mice.

and the two partial agonists greatly reduced plasma VLDL-triglyceride concentrations in C57BL/6 mice (Figure 6).

It is well established that niacin lowers plasma triglycerides through its anti-lipolytic action in adipocytes. In accord with this, we detected a marked decrease in the relative mRNA expression level of key lipolytic enzymes adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) in white adipose tissue of niacin-treated mice (Figure 7). Strikingly, no effect of the partial agonists on adipose tissue ATGL/HSL expression was noted. In contrast, in liver, LUF6281 and LUF6283 significantly reduced (–50%; $P < 0.05$) the expression of apolipoprotein B (APOB), the essential protein moiety of triglyceride-rich VLDL/LDL particles, while niacin did not (Figure 8). None of the treatments significantly changed the expression level of microsomal triacylglycerol transfer protein (MTP) in livers of C57BL/6 mice, suggesting that the loading of APOB with lipids was not affected. Combined, these findings suggest that the partial agonists lower plasma VLDL-triglycerides levels by interfering with hepatic VLDL production rather than inhibiting adipocyte lipolysis.

Discussion and conclusions

Although niacin can effectively lower lipid levels through inhibition of adipocyte lipolysis, its clinical use has been restricted by its off target cutaneous flushing effect that is mediated by HCA_2 located in skin Langerhans cells and keratinocytes (Zhang *et al.*, 2005; Hanson *et al.*, 2010). To overcome this problem, a variety of niacin derivates have been developed in the past that act as partial or biased ago-

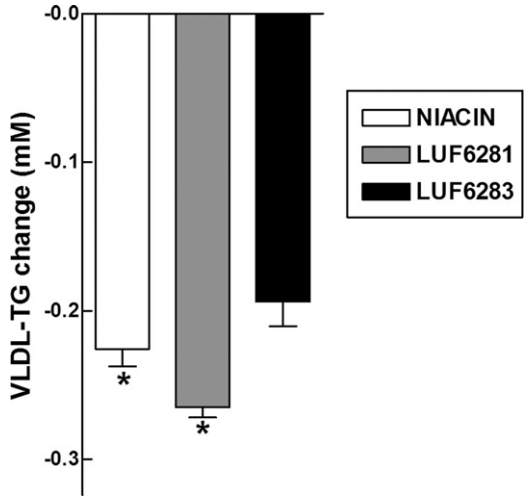

Figure 6

Effect of niacin, LUF6281 and LUF6283 on the plasma VLDL-triglyceride concentration in C57BL/6 mice. Mice were fed a regular chow diet and received either niacin or HCA_2 partial agonists LUF6281 and LUF6283 (400 $mg \cdot kg^{-1} \cdot day^{-1}$) once a day for 4 weeks. Plasma lipoproteins were separated by FPLC, and the triglyceride level was measured in each fraction. Fractions 2 to 7 represented VLDL. Values are means ± SEM (n = 3/4 per group). *P < 0.05 versus untreated mice.

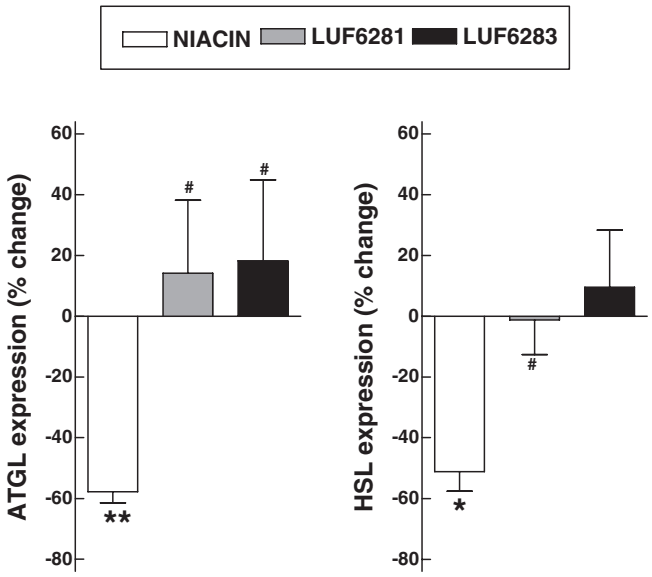

Figure 7

Effect of niacin, LUF6281 and LUF6283 on relative gene expression levels of ATGL and HSL in white adipose tissue of C57BL/6 mice. Data are presented as % change relative to the untreated group. Values are means ± SEM (n = 5/6 per group). *P < 0.05 **P < 0.01 versus untreated mice; #P < 0.05 versus niacin-treated mice.

nists for HCA_2 (van Herk *et al.*, 2003; Richman *et al.*, 2007; Semple *et al.*, 2008). To date, the most promising lead compound has been the pyrazole MK-0354, which was shown to stimulate the G-protein pathway associated with anti-

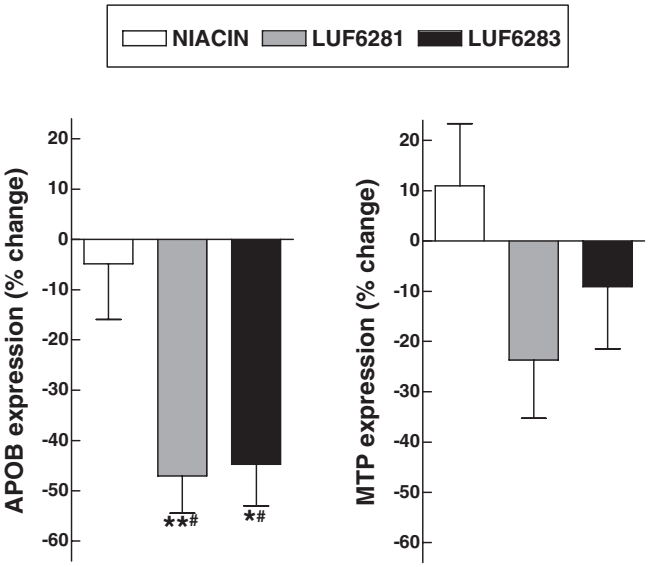

Figure 8

Effect of niacin, LUF6281 and LUF6283 on hepatic relative gene expression levels of APOB and MTP in C57BL/6 mice. Data are presented as % change relative to the untreated group. Values are means ± SEM (n = 5/6 per group). *P < 0.05 **P < 0.01 versus untreated mice; #P < 0.05 versus niacin-treated mice.

lipolysis in adipocytes without inducing parallel ERK1/2 phosphorylation, an *in vitro* measure for flushing (Semple *et al.*, 2008). Also in pre-clinical mouse studies MK-0354 showed promising results as no vasodilatation (flushing) was observed while the anti-lipolytic activity was retained. Unfortunately, in clinical trials, this compound eventually failed since it did not provide the beneficial effect of niacin on plasma lipid levels (Lai *et al.*, 2008). Although the clinical failure of MK-0354 argues against a high potential for this class of compounds in general, our current studies indicate that there is still hope for pyrazoles. More specifically, here we present data on the potential of two novel partial HCA_2 agonists of the pyrazole class to lower plasma VLDL-triglyceride levels without causing the off target flushing response.

Our *in vitro* studies suggest that niacin, LUF6281 and LUF6283 may all have a certain bias, since these compounds all show a higher potency for ERK1/2 phosphorylation than for G-protein activation. Furthermore, LUF6281 and LUF6283 were both partial agonists in the [^{35}S]-GTPγS assay but high efficacy full agonists in the ERK1/2 phosphorylation assay. The fold difference in potency was dependent on the compound; niacin was 21-fold more potent for ERK phosphorylation, while LUF6283 and LUF6281 were, respectively, 10-fold and only 6-fold more potent. The high potency of niacin for activation of the MAPK pathway may explain why this compound causes flushing so effectively. The previous findings of Walters *et al.* (2009) have suggested an important role for an additional pathway (i.e. β-arrestin-dependent signalling) in ERK phosphorylation and the flushing response associated with niacin treatment. Since we do not possess data on the effect of our compounds on β-arrestin membrane recruitment, we cannot draw a firm conclusion a to whether

our compounds are biased partial agonists. However, since all three compounds in the current study were more potent in the ERK phosphorylation assay (lower EC_{50} values), we do not anticipate that biased agonism is the reason for their negligible flushing effect. Although, unlike MK-0354, our pyrazole compounds were still active in the ERK1/2 assay, we hypothesize that their relatively low ratio of GTPγS over ERK1/2 might still attenuate the flushing response. Indeed, our *in vivo* findings in C57BL/6 mice confirm that the pyrazoles do not provoke a similar flushing response to niacin.

Both pyrazoles, however, induced a similar level of VLDL-triglyceride lowering as that observed with niacin treatment, suggesting that they do also exhibit the anti-lipolytic activity of niacin. Strikingly, niacin treatment decreased the relative expression level of the lipolytic genes ATGL and HSL in adipose tissue, while the two LUF compounds did not. From these combined findings, it seems that the novel partial agonists – in contrast to niacin – actually do not execute their VLDL-triglyceride lowering action by modulating adipocyte lipolysis. Our further gene expression analysis suggests that, instead, the compounds lower plasma triglyceride levels by inhibiting VLDL production, since they decrease the hepatic relative expression level of APOB. The assembly and secretion pathway of VLDL in the liver involves the transfer of lipid by MTP to APOB during translation and then the fusion of APOB-containing precursor particles with triglyceride droplets to generate mature VLDL particles (Davis, 1999; Shelness and Sellers, 2001). APOB is thus essential for proper assembly and secretion of APOB-containing lipoproteins (Davidson and Shelness, 2000), and several novel lipid-lowering therapies are therefore based on decreasing APOB transcription and mRNA stability by antisense oligonucleotides (Crooke *et al.*, 2005; Straarup *et al.*, 2010). As our new HCA_2 partial agonists target another metabolic pathway (i.e. hepatic VLDL production), as compared with both niacin and MK-0354 (inhibition of adipocyte lipolysis), we anticipate that they may still also effectively achieve lipid lowering in the human situation. However, their efficacy in the clinical setting remains to be validated.

In conclusion, the current study identifies the two HCA_2 partial agonists LUF6281 and LUF6283 as promising drug candidates to achieve the beneficial lipid lowering effect of niacin without producing the unwanted flushing side effect.

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Conflict of interest

The authors have nothing to disclose.

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