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ARTICLE

A novel chemical uncoupler ameliorates obesity and related phenotypes in mice with diet-induced obesity by modulating energy expenditure and food intake

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Abstract
Aims/hypothesis Decreasing mitochondrial coupling efficiency has been shown to be an effective therapy for obesity and related metabolic symptoms. Here we identified a novel mitochondrial uncoupler that promoted uncoupled respiration in a cell type-specific manner and investigated its effects on modulation of energy metabolism in vivo and in vitro.
Methods We screened a collection of mitochondrial membrane potential depolarising compounds for a novel chemical uncoupler on isolated skeletal muscle mitochondria using a channel oxygen system. The effect on respiration of metabolic cells (L6 myotubes, 3T3-L1 adipocytes and rat primary hepatocytes) was examined and metabolic pathways sensitive to cellular ATP content were also evaluated. The chronic metabolic effects were investigated in high-fat diet-induced obese mice and standard diet-fed (SD) lean mice.
Results The novel uncoupler, CZ5, promoted uncoupled respiration in a cell type-specific manner. It stimulated fuel oxidation in L6 myotubes and reduced lipid accumulation in 3T3-L1 adipocytes but did not affect gluconeogenesis or the triacylglycerol content in hepatocytes. The administration of CZ5 to SD mice increased energy expenditure (EE) but did not affect body weight or adiposity. Chronic studies in mice on high-fat diet showed that CZ5 reduced body weight and improved glucose and lipid metabolism via both increased EE and suppressed energy intake. The reduced adiposity was associated with the restoration of expression of key metabolic genes in visceral adipose tissue.
Conclusions/interpretation This work demonstrates that a cell type-specific mitochondrial chemical uncoupler may have therapeutic potential for treating high-fat diet-induced metabolic diseases.

Keywords Cell type-specific · Chemical uncoupler · Metabolic diseases · Obesity

Abbreviations

Δψm	Mitochondrial membrane potential
ACC	Acetyl-CoA carboxylase
AMPK	AMP-activated protein kinase
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
DNP	2,4-Dinitrophenol
EE	Energy expenditure
EI	Energy intake
FAO	Fatty acid oxidation
FDA	Food and Drug Administration
HFD	High-fat diet-fed
IKKβ	Inhibitor of κB kinase β
MC	Methylcellulose
mtDNA	Mitochondrial DNA
NF-κB	Nuclear factor-κB

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OXPHOS	Oxidative phosphorylation
REE	Resting EE
RQ	Respiratory quotient
SD	Standard diet-fed
SKM	Skeletal muscle
UCP	Uncoupling protein
WAT	White adipose tissue

Introduction

The increasing obesity epidemic, primarily driven by overnutrition and a sedentary lifestyle, has become a serious worldwide public health concern. Obesity is one of the most important pathogenic factors causing type 2 diabetes mellitus and other metabolic disorders [1]. Thermodynamically, therapeutic approaches to correct this energy imbalance should ameliorate obesity. However, lifestyle modification via diet and exercise or pharmacological interventions to reduce energy intake (EI) are inadequate and there has been limited approval for anti-obesity drugs in the past decade [2–4].

Mitochondria are the powerhouse of the cell, coupling the majority of energy derived from fuel substrates to ATP turnover and uncoupling less in the form of heat. In humans and rodents with obesity and related type 2 diabetes, fuel overload in the mitochondrial respiratory system and impaired mitochondrial oxidative capacity have been observed in energy-using organs and energy storage organs, such as skeletal muscle (SKM) [5–7] and white adipose tissue (WAT) [8, 9]. Increasing cellular energy expenditure (EE) by decreasing mitochondrial coupling efficiency has been proposed to be an appealing therapeutic alternative [10, 11]. Experiments using transgenic mice overexpressing uncoupling proteins (UCPs) in metabolic tissues showed that locally uncoupling oxidative phosphorylation (OXPHOS) could combat obesity and improve glucose homeostasis to varying extents [12–14]. Yet it remains to be verified how these UCPs are regulated by physiological ligands [15]. Pharmacological evidence for the effectiveness of uncoupling includes the potent anti-obesity effect observed with 2,4-dinitrophenol (DNP), a chemical uncoupler with a non-specific effect widely used in the 1930s [16]. However, severe side effects, such as uncontrolled hyperthermia at toxic doses, prevented the further development of other anti-obesity uncoupling agents largely due to their narrow therapeutic window [17].

In the present study, we screened compounds that depolarise mitochondrial membrane potential and identified a novel chemical uncoupler, CZ5, which potently uncouples respiration selectively in myocytes and adipocytes. The chronic administration of CZ5 induced beneficial metabolic effects in high-fat diet-fed (HFD) mice, not only by increasing EE but also by restricting EI.

Methods

Cell culture L6 myoblasts and HepG2 cells were cultured in DMEM containing 10% FBS. For differentiation of L6 myoblasts, the concentration of FBS was decreased to 2%. The culture and differentiation of 3T3-L1 cells was conducted as described previously [18]. Rat hepatocytes were isolated using Selgen's two-step perfusion method [19] and maintained in DMEM.

Mitochondrial membrane potential assay This assay was based on a previous report [20]. Briefly, L6 myotubes were treated with compounds for 40 min before being stained with 0.1% JC-1 for another 20 min. The ratio of red to green fluorescence reflects the mitochondrial membrane potential ($\Delta\psi m$).

Measurement of respiration in isolated mitochondria and intact cells Mitochondria were isolated from rat liver and cells as previously described [21]. Respiration measurements were conducted using a Clark-type oxygen electrode (Strathkelvin Instruments, Motherwell, UK) as described [22]. The procedure of respiration measurement is described in electronic supplementary material (ESM) Methods.

2-Deoxy-[^{3}H]D-glucose uptake 2-Deoxyglucose uptake was measured as described previously [23].

Measurement of glucose and fatty acid oxidation The effects of treatments on these oxidative metabolisms in differentiated L6 myotubes were traced with respective isotope-labelled substrate. Full details are provided in ESM Methods.

Glucose production assay After overnight attachment, glucose production was measured in primary hepatocytes following a 3 h pre-treatment. Cells were washed three times with phosphate-buffered saline and incubated in the gluconeogenic medium (glucose and phenol red-free DMEM containing 20 mmol/l sodium lactate and 2 mmol/l sodium pyruvate) for 3 h. The glucose concentration of the medium was measured with a colorimetric glucose assay kit (Fudan-Zhangjiang, Shanghai, China).

Oil red O staining and determination of triacylglycerol content Cell staining with oil red O and triacylglycerols were determined in cell lysates using a colorimetric assay as described previously [24].

Sulforhodamine B cytotoxicity assay L6 myotubes, rat hepatocytes and 3T3-L1 fibroblasts were treated with CZ5 followed by SRB assay as previously described [25].

Animal experiments All animal experiments were approved by the Animal Care and Use Committee of the Shanghai

Institute of Materia Medica (Shanghai, China). Six-week-old male C57BL/6J mice (Shanghai SLAC Laboratory Animal Co., Shanghai, China) were housed in a temperature-controlled room (22±2°C) with a light/dark cycle of 12 h. For chronic treatment, mice were fed high-fat diets (60% calories from fat; Research Diets, New Brunswick, NJ, USA) or standard diets ad libitum. At 14 weeks of age, mice were randomly assigned to treatment groups. For the study of chronic effect on standard diet-fed (SD) mice, either vehicle (0.5% methylcellulose, MC) or CZ5 (30 mg kg^{-1} day^{-1}) was orally administered for 25 days. The metabolic effects were investigated (see ESM Methods). Hepatic and muscular triacylglycerol content was measured following a Folch extraction [26].

Measurement of adenine nucleotide, glutathione and glutathione disulfide levels As described in ESM Methods, adenine nucleotide concentrations in cell or tissue extracts were determined by HPLC and reduced and oxidised glutathione levels in tissue extracts were measured using an enzymatic recycling method.

Quantitative RT-PCR for RNA, mitochondrial DNA (mtDNA) and genomic DNA copy number These assays were performed as described in ESM Methods. The primer sequences used are described in ESM Table 1.

Immunoblotting Total proteins were prepared in RIPA buffer (50 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl, 1% NP-40, 1 mmol/l Na_3VO_4, 1 mmol/l phenylmethanesulfonyl fluoride, 1 mmol/l dithiothreitol, 1 mmol/l EDTA and 1 mmol/l EGTA) containing complete protease inhibitors (Roche, Basel, Switzerland). Protein (20 μg per sample) was electrophoresed through SDS-PAGE after boiling for 5 min in SDS loading buffer. The antibodies for AMPK, phospho-AMPK (Thr172), total ACC, phospho-ACC (Ser79), AS160, phospho-AS160 (Thr642), IKKβ and phospho-IKKα(Ser176/180)/IKKβ (Ser177/181) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Acute toxicity study Overnight-fasted C57BL/6J male mice weighing 20–25 g each were gavaged orally with vehicle or CZ5 and thereafter observed continuously for the first 4 h and then at 6 h intervals for the following 48 h and once daily for the following 7 days, to observe any death or changes in general behaviour and other physiological activities.

Statistical analysis Results represented means ± SEM. All the in vitro experiments were conducted at least three times. Differences between two groups were examined using the unpaired two-tailed Student's *t* test. The EE data in mice were assessed by analysis of covariance (ANCOVA) with body mass as a covariate. $p<0.05$ was regarded as significant.

Results

Identification of a novel cell type-specific chemical uncoupler, CZ5 A canonical chemical uncoupler is characterised as an artificial proton carrier that forces protons to re-enter the mitochondrial matrix across the inner membrane, bypassing ATP synthase. Initially, we used our established high-throughput screening assay for Δψm [20] and obtained a collection of Δψm depolarising compounds. Within this collection, we observed that a novel compound, named CZ5 (Fig. 1a), potently depolarised Δψm of L6 myotubes (Fig. 1b) and that it significantly increased uncoupled respiration at a concentration of 1 μmol/l and achieved a fully uncoupled rate at 10 μmol/l in isolated mitochondria from L6 myotubes (Fig. 1c). Given the lack of cellular context in respiration measurements of isolated mitochondria [27], we investigated whether CZ5 correspondingly stimulated uncoupled respiration in intact major metabolic cells. Compared with the non-specific effects of the conventional uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), CZ5 unexpectedly exhibited cell type-specific uncoupling characteristics at a certain concentration range. In L6 myotubes and 3T3-L1 adipocytes CZ5 achieved CCCP-maximised respiration at a concentration of 10 μmol/l (Fig. 1d, e), whereas it did not elicit a significant increase compared with the same concentration of CCCP that caused maximal uncoupling in rat primary hepatocytes (Fig. 1f). Consistently, CZ5 increased the ADP/ATP ratio robustly in myotubes and modestly in adipocytes but not in hepatocytes, suggesting that CZ5 lowered the efficiency of transformation from ADP to ATP through ATP synthase in a cell type-specific manner (Fig. 1g–i).

Selective metabolic effects induced by CZ5 in myotubes and adipocytes Secondary to increased proton leak, the accelerated electron flux along the respiratory chain can lead to increased consumption of reducing equivalents derived from glucose and fatty acids. In L6 myotubes, CZ5 dose-dependently increased both glucose and palmitate oxidation (Fig. 2a, b). Furthermore, we observed that CZ5 increased glucose uptake dose-dependently in L6 myotubes (Fig. 2c).

In response to low ATP/ADP ratios, cells generally tend to decrease anabolism to maintain energy homeostasis. To fulfil their important role in storing energy, adipocytes have the unique capabilities of adipogenesis and lipogenesis, two pathways dependent on cellular energy supply. We treated white adipocytes with CZ5 during differentiation (Fig. 2d, e) and after differentiation (Fig. 2f, g). CZ5 caused a dramatic reduction in lipid droplet accumulation compared with DMSO-treated adipocytes displaying normal differentiation (Fig. 2d). In fully differentiated adipocytes, CZ5-treatment also reduced intracellular triacylglycerols (Fig. 2g).

CZ5 had a minimal effect on the energy-sensitive metabolic pathway of gluconeogenesis and lipid accumulation in

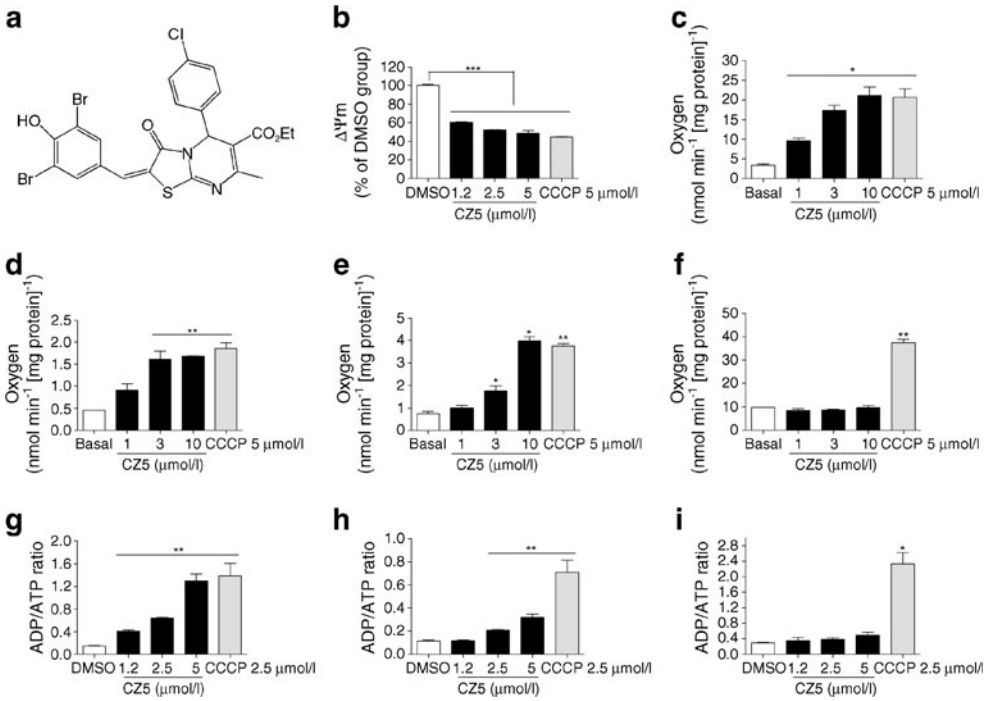

Fig. 1 Identification of a cell type-specific chemical uncoupler CZ5. (**a**) Chemical structure of compound CZ5. (**b**) L6 myotubes were first treated with CZ5 for 40 min and then stained with JC-1 for another 20 min. A fall in Δψm is provoked dose-dependently by CZ5. CCCP was used as a positive control for Δψm depolarisation. (**c**) Concentration-dependent stimulation of uncoupled O_2 consumption of isolated mitochondria from L6 myotubes induced by CCCP or CZ5. Basal uncoupled (addition of oligomycin) and chemical-uncoupled O_2 consumption rates were recorded sequentially. The fold change induced by CCCP at the indicated dose represents the greatest stimulated value in its dynamic range. (**d**–**f**) Concentration-dependent stimulation of uncoupled O_2 consumption in intact L6 myotubes (**d**), 3T3-L1 adipocytes (**e**) and rat hepatocytes (**f**) induced by CZ5. Cellular O_2 consumption was measured in the presence of 4 μg/ml oligomycin. The fold change induced by CCCP at the indicated dose represents the greatest stimulated value in its dynamic range. (**g**–**i**) Dose-dependent effects of CZ5 on ADP/ATP ratio after incubation for 4 h in L6 myotubes (**g**), 3T3-L1 adipocytes (**h**) and rat hepatocytes (**i**). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with DMSO/basal group

hepatocytes (Fig. 2h, i), even though there was some degree of uncoupled respiration in HepG2 hepatoma cells at the same working concentrations used in other cell types (far lower than CCCP) (Fig. 2j). These findings are consistent with the lack of a significant or weak uncoupling effect and an unchanged ADP/ATP ratio in hepatocytes.

The barely detectable cytotoxicity excluded the possibility that cell death interfered with the evaluation of metabolic effects (ESM Fig. 1).

CZ5 promoted EE but not weight loss in SD mice Before the in vivo efficacy study, we confirmed that CZ5 had a favourable pharmacokinetic profile in rats (ESM Fig. 2), and the oral bioavailability was calculated to be 58%. The short-term oral administration of CZ5 to SD mice led to an obvious increase in EE during both the light phase and the dark phase (Fig. 3a, b). To control for the influence of body mass on EE, we adjusted EE for differences in body mass by ANCOVA and found that there were also a significant difference between the groups (Fig. 3c). Reduced respiratory quotient (RQ) caused by CZ5 treatment is indicative of a relative shift from carbohydrate to lipid substrates for oxidation in vivo (Fig. 3d). However, CZ5 did not sufficiently affect body temperature (Fig. 3e). Spontaneous locomotor activity did not differ between the CZ- and vehicle-treated groups, suggesting that EE can be increased by CZ5 in vivo under this condition (Fig. 3f). These results suggest that CZ5 acts to increase EE in vivo similar to its actions in vitro in myocytes and adipocytes. Despite the increase in EE induced by CZ5, chronic treatment of SD mice with CZ5 did not result in any significant change in body weight or fat mass or in glycaemia and insulinaemia (ESM Fig. 3a–d). In contrast, hyperphagia occurred (ESM Fig. 3e). There was tendency toward plasma leptin level reduction after CZ5 treatment, accompanied by downregulated leptin gene expression in epididymal fat (ESM Fig. 3f, g). We also found that the phosphorylation of hypothalamic AMP-activated protein kinase (AMPK) was increased by CZ5 treatment (ESM Fig. 3h). These results indicate that peripheral enhanced EE possibly informs the hypothalamus that the body is in a starvation state, resulting in an upregulation of the phosphorylation of hypothalamic AMPK and promoted feeding behaviour.

Chronic treatment with CZ5 in HFD mice for 5 weeks produced an anti-obesity effect To test the chronic effects of CZ5 on

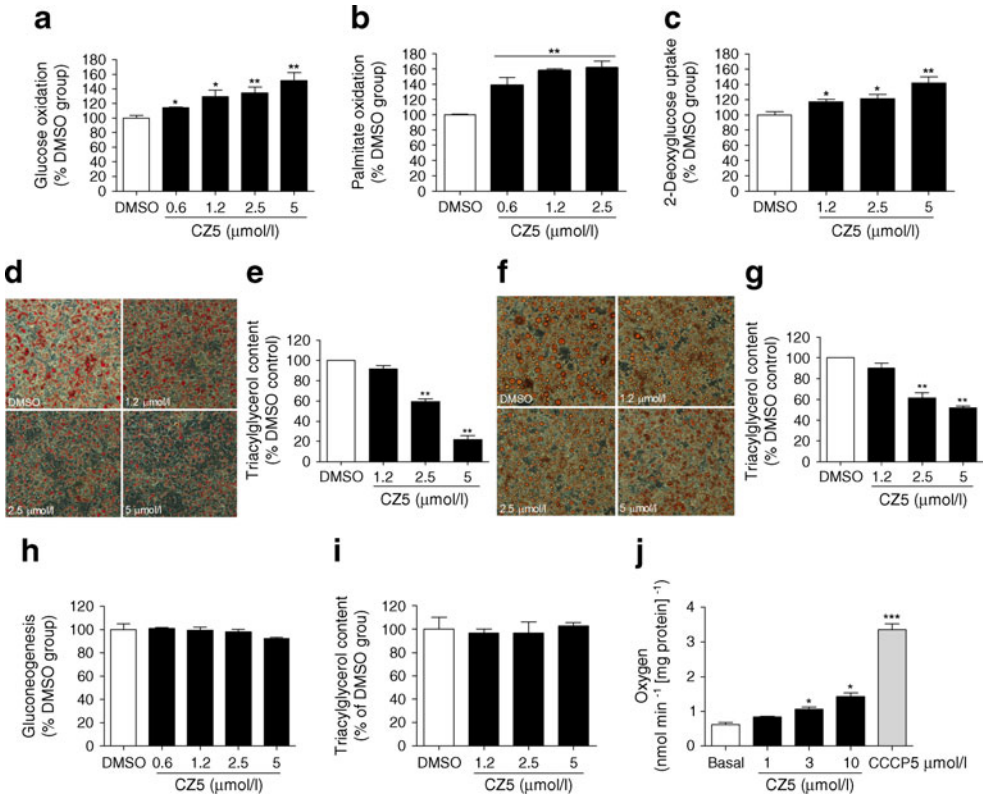

Fig. 2 Selective effects of CZ5 on respective energy-sensitive pathways in metabolically relevant cells. (**a**) Dose-dependent effect of CZ5 on glucose uptake. L6 myotubes were treated for 4 h with different doses of CZ5. (**b**, **c**) Dose-dependent effect of CZ5 on glucose oxidation (**c**) and palmitic acid oxidation (**d**) in L6 myotubes treated for 4 h. (**d**–**g**) Effect of CZ5 on lipid accumulation in 3T3-L1 adipocytes. 3T3-L1 cells were treated with or without CZ5 during adipocyte differentiation and then stained with oil red O (**d**) or lysed for determination of total lipids (**e**) on day 8 after differentiation. Fully differentiated adipocytes were treated with or without CZ5 for 6 days and then stained (**f**) or lysed for determination of total lipids (**g**). Cells were visualised and photographed at 200× magnification. (**h**) Effect of CZ5 on glucose production in rat primary hepatocytes after 6 h treatment. (**i**) Effect on lipid content. HepG2 cells were starved in serum-free medium overnight and treated with different doses of CZ5 for 24 h before assay. (**j**) Concentration-dependent stimulation of uncoupled O_2 consumption in HepG2 cells induced by CZ5. Uncoupled respiration was measured in the presence of 4 μg/ml oligomycin. The fold change induced by CCCP at the indicated dose represents the greatest stimulated value in its dynamic range. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with DMSO/basal group

obesity and related metabolic disorders, we administered CZ5 to mice that had been fed on high-fat diets for 8 weeks. At the end of the 5 weeks of treatment, the obese mice treated with CZ5 weighed markedly less (20%) than the obese mice treated with vehicle (Fig. 4a). Interestingly, CZ5 decreased the cumulative food intake by 11% throughout the treatment course (Fig. 4b). Given that a comparable plasma concentration and distribution of CZ5 were observed in the SD and HFD mice (ESM Fig. 4), it is unlikely that the disparate effect of CZ5 on EI in lean and obese mice is due to the levels of exposure to the compound. To determine the role of food intake suppression in the efficacy of CZ5, we employed a pair-fed obese control group (HFD-PF). HFD-PF mice displayed less body weight gain than the HFD-Veh mice during CZ5 treatment, but this difference was not significant. Thus, it can be proposed that the increased EE may be mainly responsible for the body-weight loss in CZ5-treated mice rather than decreased food intake. At the end of the 5 week treatment, the CZ5-treated obese mice also showed reduced white and brown fat pad mass (Fig. 4c). The weight of the liver and the pancreas of CZ5-treated group was unaltered by CZ5. Thus, the body-weight loss following CZ5 treatment is likely the result of reduced fat mass. In contrast, no significant reduction in adipose tissue weight was observed in the pair-fed obese group compared with the HFD-Veh group.

CZ5 affects glucose and lipid homeostasis Consistent with enhanced energy metabolism, 5 weeks of treatment with CZ5 led to a significant reduction in fasting blood glucose and plasma insulin levels (Fig. 5a, b). Moreover, both the glucose tolerance and response to insulin were improved in mice receiving CZ5, as demonstrated by glucose (Fig. 5c, d) and insulin tolerance tests (Fig. 5e, f). The food restriction in the HFD-PF group induced a significant degree of improvement in both hyperinsulinaemia and insulin sensitivity, but these effects were smaller than those induced by treatment

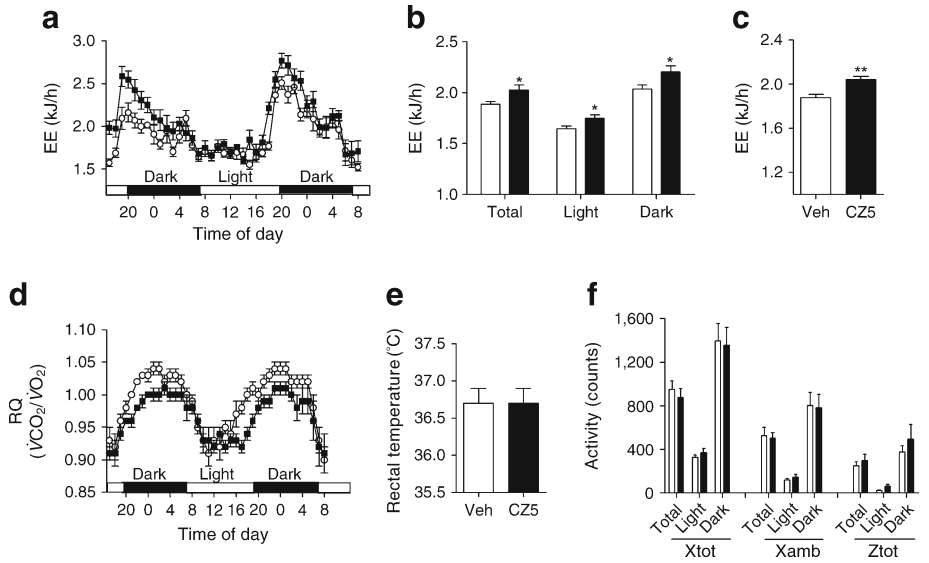

Fig. 3 Modulation of energy metabolism by CZ5 in vivo. For indirect calorimetry measurement, SD lean mice treated with vehicle (Veh) or CZ5 (30 mg kg^{-1} day^{-1}) for 5 days were placed in metabolic chambers. (**a**) Changes in the hourly EE per mouse over the indicated periods. White circles, vehicle; black squares, CZ5. (**b**) The average hourly EE during the total 40 h, light phase and dark phase. (**c**) The average hourly EE adjusted for body mass by ANCOVA. (**d**) Change in RQ throughout the indicated periods of monitoring. (**e**) Core (rectal) temperatures were measured 6 h after administration of the morning dose on day 7. (**f**) Physical activity was recorded by assessing *x*- and *z*-axis activity during the total, light and dark periods. Xtot, Xamb and Ztot each denote the total activity counts along the *x*-axis, ambulatory activity along the *x*-axis, and the total activity counts along the *z*-axis. White bars, vehicle; black bars, CZ5. (n=8 per group). *p<0.05 and **p<0.01 compared with vehicle group

with CZ5. Thus, these data suggest that CZ5 improved insulin sensitivity of HFD mice through a combined effect on EE and EI. To investigate the mechanism for increased whole-body glucose disposal after CZ5 treatment, we examined the phosphorylation of AS160 at Thr642, which has been demonstrated to play a key role in SKM glucose uptake stimulated by insulin and contraction [28]. CZ5 treatment significantly increased phosphorylation of this site in gastrocnemius muscle (Fig. 5g).

After the analysis of various plasma indicators of metabolism, we found that CZ5 treatment reversed the high-fat diet upregulated LDL-cholesterol/HDL-cholesterol ratios and the levels of cholesterol and adipokine leptin (Table 1). The pair-fed mice exhibited minimal improvement in the above-mentioned circulating variables. Despite the increased whole-body EE, the unchanged plasma lactate level suggests that CZ5 did not elicit overall energy starvation. The high-fat diet induced SKM and hepatic lipid accumulation with a fivefold increase in the triacylglycerol content. Treatment with CZ5 significantly improved this excessive lipid accumulation in muscle, but not liver (Table 1).

Chronic effects of CZ5 in metabolic tissues of HFD mice The beneficial effects of CZ5 on metabolic variables in HFD mice correlated with its regulatory potential on energy state and redox status. We found that CZ5 diminished energy efficiency, which was demonstrated by increased AMP/ATP and ADP/ATP ratios, most markedly in WAT

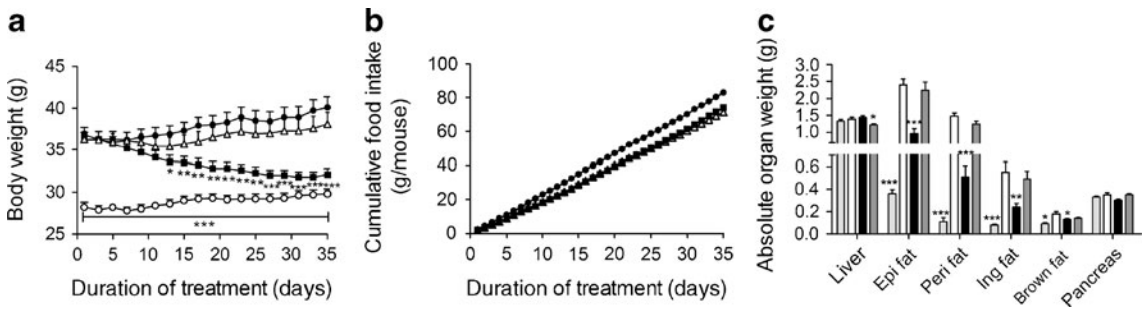

Fig. 4 Anti-obesity effect after 5 weeks of chronic treatment with CZ5 in HFD mice. (**a**) Changes in body weight. (**b**) Cumulative food intake. Black circles, HFD-vehicle (Veh); black squares, HFD-CZ5; white triangles, HFD-PF; white circles, SD-Veh. (**c**) Absolute weights of liver and epididymal (epi), perirenal (peri) and inguinal (ing) fat pads, interscapular brown fat and pancreas. Light grey bars, SD-Veh; white bars, HFD-Veh; black bars, HFD-CZ5; dark grey bars, HFD-PF. n=8 per group; *p<0.05, **p<0.01 and ***p<0.001 compared with HFD-Veh group

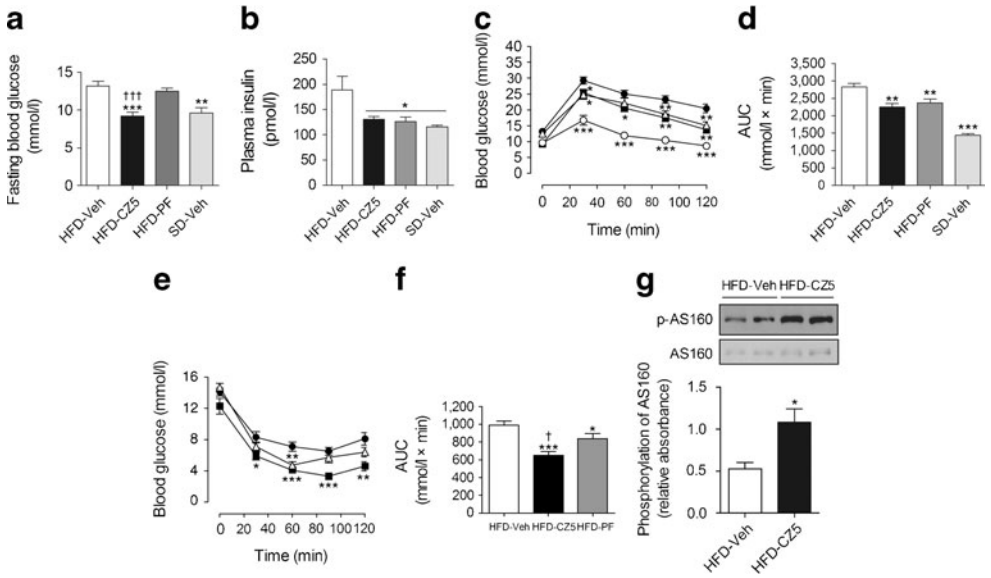

Fig. 5 Glucose homeostasis and insulin sensitivity was affected by CZ5 in HFD mice. Fasting blood glucose (**a**) and plasma insulin (**b**) after 5 weeks of treatment. Tail-vein blood was used for glucose assay after 6 h fasting. (**c**) Intraperitoneal glucose tolerance test was conducted on week 4 of treatment. Blood glucose values were assessed before i.p. injection of glucose (time 0), and at 30, 60, 90 and 120 min following the injection. The area under the glucose curve was calculated (**d**). (**e**) Insulin tolerance test was performed on HFD mice on week 4 of treatment. Blood glucose values were assessed before the test (time 0) and at 30, 60, 90 and 120 min afterwards. The area under the glucose curve was calculated (**f**). Black circles, HFD-Veh; black squares, HFD-CZ5, white triangles, HFD-PF; white circles, SD-Veh. (n=8 per group). (**g**) Immunoblot of AS160 phosphorylation in the gastrocnemius muscle of HFD mice at the end of treatment. A representative blot is shown. The blot signal strength was quantified and presented after normalisation to respective AS160 protein level (n=6 per group). *p<0.05, **p<0.01 and ***p<0.001 compared with HFD-Veh group; $^{\dagger}p$<0.05 and $^{\dagger\dagger\dagger}p$<0.001 compared with HFD-PF group

while having milder effects in SKM. Consistent with the result at the cellular level, chronic treatment with CZ5 did not obviously change hepatic energy state even though it had much higher distribution in the liver (Fig. 6a, b). In turn, the extent of AMPK phosphorylation in the three tissues was consistent with that of local alterations in energy state (Fig. 6c).

Mitochondrial uncoupling has been shown to reduce the production of reactive oxygen species [11], so to determine whether CZ5 affects oxidative stress levels we examined cellular glutathione status. CZ5 enhanced the GSH:GSSG ratio significantly in WAT and had a tendency (p=0.07) to do so in SKM, which resulted from more than halved GSSG (Fig. 6d, e). These findings indicated that CZ5 reduced oxidative stress.

To understand the molecular basis of the markedly decreased white fat mass, we found that the expression levels of master transcription regulators of adipogenesis (*Pparg* and *Srebf1*) and mitochondrial biogenesis (*Pgc-1α* [also known as *Ppargc1a*]) and their respective downstream targets genes

Table 1 Metabolic variables of SD mice (n=6) and vehicle-treated, CZ5-treated or pair-fed HFD mice (n=8 mice per group)

Variable	SD-Veh	HFD-Veh	HFD-CZ5	HFD-PF
Plasma cholesterol (mmol/l)	2.30±0.08***	4.79±0.09	4.20±0.25*	4.58±0.21
Plasma LDL-cholesterol/HDL-cholesterol	0.98±0.07**	1.64±0.15	1.24±0.08*	1.38±0.13
Plasma triacylglycerols (mmol/l)	0.59±0.03*	0.80±0.07	0.76±0.05	0.73±0.05
Plasma NEFA (mmol/l)	0.67±0.04	0.72±0.05	0.68±0.05	0.60±0.02*
Plasma lactic acid (mmol/l)	5.75±0.45	5.58±0.45	4.71±0.22	5.18±0.26
Plasma leptin (ng/ml)	2.25±0.24***	41.03±7.00	8.58±2.15***,†††	33.67±5.47
Muscle triacylglycerols (μmol/g)	11.88±2.51***	69.80±9.59	23.61±4.46***,††	53.63±6.60
Liver triacylglycerols (μmol/g)	10.45±0.52***	45.90±6.95	47.58±6.17	46.25±4.17

Data are means ± SEM. Mice were induced into obesity after 8 weeks on a high-fat diet and treated with vehicle or CZ5 orally at a dose of 10 mg kg^{-1} day^{-1} or were pair-fed. In parallel, a series of mice were maintained on a standard diet and orally administered with vehicle as controls. Blood samples were collected, after 4 h fasting at the end of 5 weeks of treatment, for measurement of metabolic variables. *p<0.05, **p<0.01 and ***p<0.001 vs HFD-Veh group. $^{\dagger\dagger}p$<0.01 and $^{\dagger\dagger\dagger}p$<0.001 vs HFD-PF group

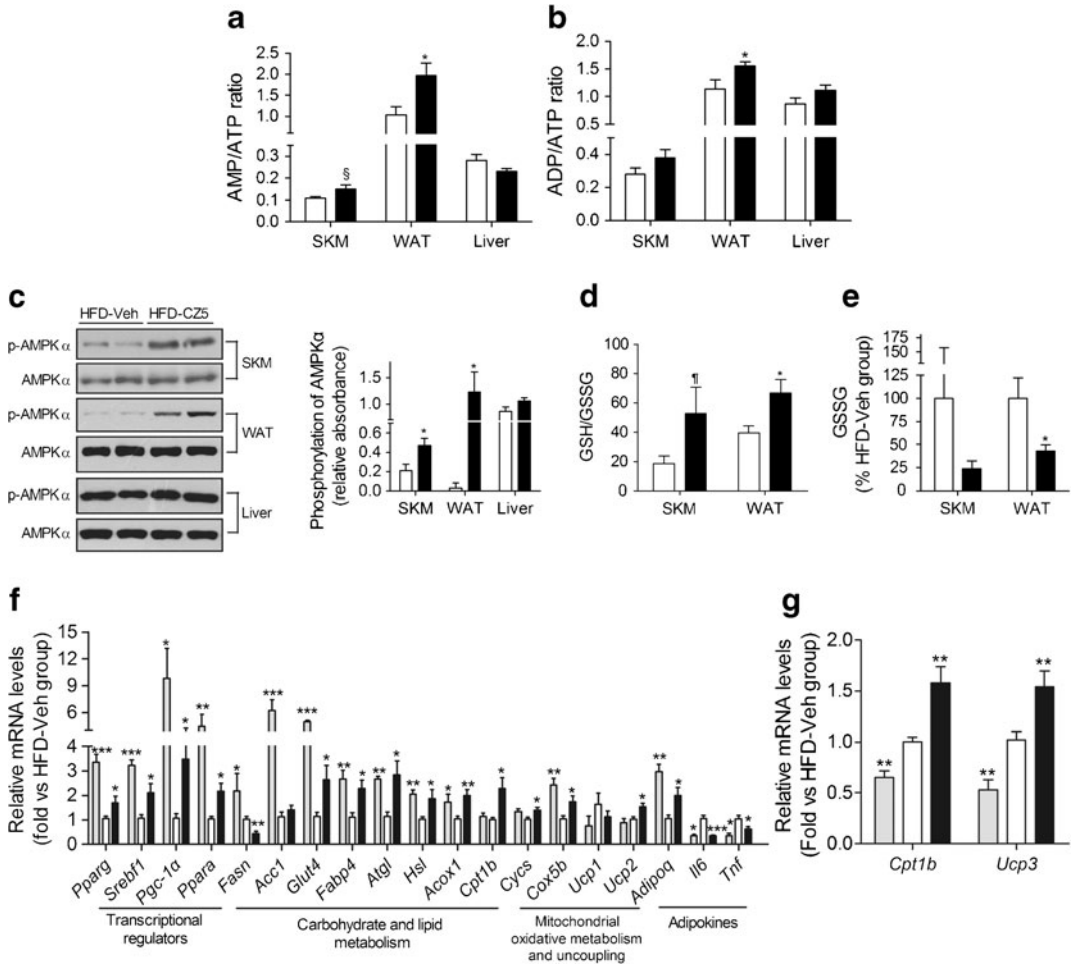

Fig. 6 Regulation of energy state, redox status and metabolic gene expression by CZ5 in vivo. (**a**, **b**) HFD mice were treated orally with vehicle or CZ5 for 2 weeks followed by collection of gastrocnemius muscle (SKM), epididymal fat (WAT) and liver for determination of AMP, ADP and ATP content and calculation of AMP/ATP (**a**) and ADP/ATP ratios (**b**) (n=6 per group). (**c**) Immunoblot of AMPK phosphorylation in the indicated tissues collected from HFD mice treated for 5 weeks. A representative blot is shown. The blot signal strength was quantified and expressed after normalisation against respective AMPKα protein level. (n=6 per group). GSH/GSSG ratio (**d**) and GSSG content (**e**) were measured in the indicated tissues of HFD mice treated for 2 weeks (n=5 per group for SKM measurement; n=8 per group for WAT measurement). (**f**, **g**) Transcriptional changes in epididymal WAT (**f**) and gastrocnemius muscle (**g**) of HFD mice treated with vehicle or CZ5 and SD mice treated with vehicle as control for 5 weeks. Data are means ± SEM (n=8 per HFD group, n=6 per SD group). Results were expressed as relative fold change compared with HFD-Veh group. Light grey bars, SD-Veh; white bars, HFD-Veh; black bars, HFD-CZ5. *p<0.05, **p<0.01 and ***p<0.001 vs HFD-Veh group. $^{§}p$=0.06 and $^{¶}p$=0.07 compared with HFD-Veh group

involved in lipid metabolism (e.g. *Fasn*, *Glut4* [also known as *Slc2a4*], *Fabp4*, *Atgl* [also known as *Pnpla2*]) and mitochondrial OXPHOS (e.g. *Cycs* and *Cox5b*) were downregulated in mice fed high-fat diets compared with those fed standard diets (Fig. 6f), consistent with previous reports [8, 29–31]. Treatment with CZ5 restored the impaired expression of these genes, with the exception of *Fasn* mRNA, which was further decreased. However, CZ5 treatment did not significantly affect mtDNA copy number (ESM Fig. 5). *Ppara* plays a key role in the transcriptional control of genes involved in cellular fatty acid oxidation (FAO). Although the expression of *Ppara* was decreased in the epididymal fat of HFD mice, not all of the detected target genes (*Acox1* and *Cpt1b*) were downregulated in comparison with SD mice. CZ5 not only upregulated *Ppara* mRNA but also induced its target genes. Obesity-induced chronic inflammation in adipose tissue has been suggested to be critical for the development of insulin resistance [32, 33]. HFD significantly increased the mRNA expression of *Tnf* and *Il6*, two key cytokines for obesity-induced insulin resistance in WAT, whereas the induction was largely reduced in CZ5-treated mice. *Ucp3* is known to mediate FAO specifically in SKM. In contrast to the effects on FAO gene expression in WAT, high-fat diets increased the expression levels of *Cpt1b* and *Ucp3* in SKM. CZ5 further promoted the expression of these two genes (Fig. 6g). For the majority of genes detected, their expression in the HFD-PF group was not substantially different from that in the obese controls (data not shown).

Discussion

Uncoupling of oxidation from phosphorylation in mitochondria is an effective way to treat obesity mainly via augmenting EE [34]. However, it is undesirable for a chemical uncoupler to cause widespread disturbance in ATP production in multiple organs [17]. The capacity to reduce coupling efficiency selectively in muscle or fat may allow a chemical uncoupler to be used in obesity treatment [11]. SKM, as a large organ and a major site of facultative thermogenesis, contributes significantly to resting EE (REE) [35]. WAT is a major site of energy storage and does not directly contribute a large amount to REE, but has the capacity to indirectly influence whole-body metabolic efficiency via a range of secreted regulatory factors (adipokines) [8, 36, 37]. It has been shown in tissue-specific gain-of-function studies in mice that overexpression of UCPs in SKM increased REE and reduced body weight, accompanied by hyperphagia [14, 38], whereas UCP overexpression in WAT did not significantly raise REE and reduced body weight only in mice on high-fat diets [39].

CZ5 displayed selective potent uncoupling effects on myocytes and adipocytes compared with the uniform effects of the classic chemical uncoupler CCCP, which also uncoupled respiration in hepatocytes. Consistent with these findings a previous study reported suppression of hepatic gluconeogenesis with CCCP [40], while CZ5 did not affect glucose output in hepatocytes. The present study demonstrated that upregulation of EE may be responsible for CZ5-induced weight loss, by lowering fat mass and improving lipid metabolism in HFD mice. Consistent with the cell type-specific uncoupling effects of CZ5 in vitro, we found that in vivo markers of energy status, as well as triacylglycerol content, was altered in WAT and SKM. However, CZ5 had no remarkable effects on body weight and adiposity in SD mice, as the enhanced EI was able to compensate for the increased EE. These therapeutic effects induced by CZ5 appear to mimic those produced by overexpression of UCPs in SKM and WAT [12–14], which are in contrast to those effects reported for obesity treatment with non-specific uncouplers in humans or rodents [16, 17].

WAT is known to play an important role in the maintenance of whole-body glucose homeostasis through the sequestering of excess triacylglycerols and fatty acids. The increased expression of key adipogenic transcriptional factors and their downstream target genes involved in adipogenesis and lipogenesis indicated that CZ5 treatment restored the ‘de-differentiation’ induced by high-fat diets. The simultaneous increase in mitochondrial biogenesis regulator *Pgc-1α* may co-activate these adipogenic transcriptional factors [41]. The induction of *Pgc-1α* expression may be a compensatory consequence of increased intracellular calcium concentrations levels, secondary to a compromised energy status induced by CZ5 [42]. Combined with the upregulated expression of lipid oxidation genes, it can be speculated that CZ5 may enhance oxidative metabolism in WAT, leading to loss of white fat mass. We investigated in vivo whether changes in the expression of genes involved in metabolism were at least partially mediated by cell-autonomous effects in adipocytes. Consistent with the action of positive uncouplers described in a previous report [43], CZ5 enhanced *Pgc-1α* expression and reduced *Leptin* expression in adipocytes via incubation during differentiation (ESM Fig. 6).

Another interesting result is related to appetite regulation. In the chronic study in HFD mice, CZ5-treated obese mice exhibited suppression of food intake from the onset of therapy, but did not show any behavioural abnormalities related to compound-induced toxicity. There was also no obvious alteration in plasma markers of liver and kidney toxicity (ESM Table 2). The initial pharmacokinetic study showed that the plasma-to-brain concentration ratio was nearly 100 at the time of peak plasma concentration after a single oral dose (ESM Fig. 4), suggesting a limited blood–brain barrier permeability to CZ5. High-fat diet-induced obese rodents are known to harbour blunted central leptin sensitivity in comparison with SD rodents [44, 45]. Overexpression of UCP1 in epididymal fat after the development of obesity led to decreased food intake and reversed leptin resistance [46]. In agreement, we observed that plasma leptin level and leptin mRNA expression in epididymal fat tissues were markedly decreased, though white fat mass was not significantly altered in obese mice treated by CZ5 for 2 weeks (ESM Fig. 7a–c). A leptin tolerance test showed that the acute anorexigenic effect of leptin was more potent in CZ5-treated obese mice, while no difference was observed between the control and pair-fed group (ESM Fig. 7d). These results suggest that increased leptin sensitivity might be one of the causes of the anorexic effect induced by CZ5, rather than the anorexia being a secondary effect of food restriction. The basal phosphorylation of the AMPK direct substrate acetyl-CoA carboxylase (ACC), downstream of leptin’s action in the hypothalamus [47, 48], was significantly decreased in CZ5-treated mice, despite phosphorylation levels of AMPK remaining unaltered (ESM Fig. 7e). This suggests the inhibition of hypothalamic AMPK activity may, in part, mediate the CZ5-induced reduction in EI. Furthermore, previous studies reported that overnutrition can induce leptin-insensitive hyperphagia through activation of inhibitor of κB kinase β (IKKβ)/nuclear factor-κB (NF-κB) [49]. We found that hypothalamic phosphorylation of IKKβ was reduced after CZ5 treatment (ESM Fig. 7f). CZ5 treatment also weakened the expression of the *Socs3* gene, another common inhibitor for leptin signalling (ESM Fig. 7g) [50]. Meanwhile, chronic treatment of SD mice led to hyperphagia, which mimics the effects of overexpression of UCPs in

SKM [14]. This suggests that mitochondrial uncoupling in muscle or adipose tissue can regulate appetite in different ways, which might relate to leptin sensitivity and body composition.

The in vitro cell type-specific effects might allow CZ5 to have a milder uncoupling effect than that observed with the non-specific uncoupler DNP at the whole-body level. Examination for potential toxic cardiac effects after 5 weeks of administration of CZ5 to SD mice at a dose of 30 mg kg^{-1} day^{-1} showed that there were no obvious alterations of echocardiographic variables (ESM Table 3). In a small-scale acute toxicity study, all of the mice survived without any signs of compound-induced toxicity after a single oral dose 50-fold greater than that administered in the chronic study (ESM Table 4). Although the potential systemic toxicity of CZ5 requires a more complete evaluation, we initially conclude that CZ5 is a relatively safe chemical uncoupler, at least in rodent models.

Regarding the mechanism of the cell type-specific uncoupling effects, we found that discrepancies among uncoupling capacities in the three types of isolated mitochondria may partially contribute to the unusual characteristic of this compound. In isolated mitochondria from L6 myotubes and 3T3-L1 adipocytes, CZ5 stimulated the lowest and the maximal uncoupling respiration at lower concentrations than in isolated liver mitochondria (ESM Fig. 8). It is possible that CZ5 uncouples OXPHOS via its chemical properties, namely lipophilicity and weak acidity. Additionally CZ5 may also interact with certain proteins that mediate proton leak and are expressed specifically in myocytes and adipocytes [15]. However, neither GDP nor carboxyatractyloside was able to attenuate the uncoupling effects of CZ5 (data not shown), suggesting no involvement of UCPs or the adenine nucleotide translocase in these effects. Although we favour the aforementioned explanation, the possibility of cell type-specific membrane permeability to CZ5 contributing to its various metabolic effects in the three cell types cannot be excluded.

Overall, we discovered a novel cell type-specific chemical uncoupler that has potential as an anti-obesity therapy by increasing EE and suppressing EI. Our findings provide a proof of concept for increasing mitochondrial uncoupling selectively in SKM and WAT as a novel approach for the treatment of obesity and type 2 diabetes.

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