Contrasts between the effects of zinc- α_2 -glycoprotein, a putative $\beta_{3/2}$ -adrenoceptor agonist and the $\beta_{3/2}$ -adrenoceptor agonist BRL35135 in C57Bl/6 (*ob/ob*) mice

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Abstract

Previous studies by Tisdale *et al.* have reported that zinc- α_2 -glycoprotein (ZAG (AZGP1)) reduces body fat content and improves glucose homeostasis and the plasma lipid profile in Aston (*ob/ob*) mice. It has been suggested that this might be mediated via agonism of β_3 - and possibly β_2 -adrenoceptors. We compared the effects of dosing recombinant human ZAG (100 μ g, i.v.) and BRL35135 (0.5 mg/kg, i.p.), which is in rodents a 20-fold selective β_3 - relative to β_2 -adrenoceptor agonist, given once daily for 10 days to male C57Bl/6 *Lep^{ob}/Lep^{ob}* mice. ZAG, but not BRL35135, reduced food intake. BRL35135, but not ZAG, increased energy expenditure acutely and after sub-chronic administration. Only BRL35135 increased plasma concentrations of glycerol and non-esterified fatty acid. Sub-chronic treatment with both ZAG and BRL35135 reduced fasting blood glucose and improved glucose tolerance, but the plasma insulin concentration 30 min after administration of glucose was lowered only by BRL35135. Both ZAG and BRL35135 reduced β_1 -adrenoceptor mRNA levels in white adipose tissue, but only BRL35135 reduced β_2 -adrenoceptor mRNA. Both ZAG and BRL35135 reduced β_1 -adrenoceptor mRNA levels in brown adipose tissue, but neither influenced β_2 -adrenoceptor mRNA, and only BRL35135 increased β_3 -adrenoceptor and uncoupling protein-1 (*UCP1*) mRNA levels in brown adipose tissue. Thus, ZAG and BRL35135 had similar effects on glycaemic control and shared some effects on β -adrenoceptor gene expression in adipose tissue, but ZAG did not display the thermogenic effects of the β -adrenoceptor agonist, nor did it increase β_3 -adrenoceptor or *UCP1* gene expression in brown adipose tissue. ZAG does not behave as a typical $\beta_{3/2}$ -adrenoceptor agonist.

Key Words

- ▶ zinc- α_2 -glycoprotein
- ▶ β_3 -adrenoceptor agonist
- ▶ *ob/ob* mice
- ▶ obesity
- ▶ glucose tolerance
- ▶ thermogenesis
- ▶ BRL35135

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Introduction

Zinc- α_2 -glycoprotein (ZAG (AZGP1)), which is identical to the previously named lipid-mobilising factor (LMF) (Hirai *et al.* 1998), is a 40 kDa single-chain polypeptide similar to

the class I major histocompatibility complex heavy chain. It is secreted by both brown and white adipocytes (Bing *et al.* 2010), as well as other cells, and in humans, ZAG

gene expression in white adipose is negatively correlated with BMI, fat mass, plasma insulin, C-reactive peptide and leptin and positively correlated with adiponectin expression (Marrades *et al.* 2008, Ceperuelo-Mallafre *et al.* 2009, Mracek *et al.* 2010a). Similarly, circulating levels of ZAG are low in obese subjects (Selva *et al.* 2009).

Urine-derived LMF and plasma-derived or recombinant ZAG affect energy balance, body composition and metabolic homeostasis in mice and rats in ways that are in many respects similar to those of β_3 -adrenoceptor agonists (Arch *et al.* 1991). For example, ZAG/LMF (termed here ZAG) reduces body weight and body fat content without reducing food intake (Hirai *et al.* 1998, Russell *et al.* 2004). ZAG also decreases plasma concentrations of glucose, triglycerides and non-esterified fatty acids (NEFAs), partly independent of any anti-obesity effect, and, like β_3 -adrenoceptor agonists, it increases fat oxidation (Russell & Tisdale 2002, 2010a, Arch 2008). There are similarities between the effects of ZAG and 'fat mobilising substance', which was purified previously from urine (Beaton *et al.* 1964, Kekwick & Pawan 1967), but it is not clear whether they are the same substance.

There has, for many years, been an interest in β_3 -adrenoceptor agonists for the treatment of obesity and type 2 diabetes because they retain the metabolic effects of non-selective sympathomimetic agents and avoid cardiovascular effects due to stimulation of β_1 - or β_2 -adrenoceptors. In rodents, both β_3 -adrenoceptor agonists and ZAG stimulate lipolysis, increase the expression of uncoupling protein-1 (UCP1) in brown adipose tissue and increase core temperature, suggestive of increased energy expenditure (Granneman *et al.* 2003, Inokuma *et al.* 2006, Russell & Tisdale 2010a, 2011, 2012a). β_3 -Adrenoceptor agonists have not been developed for the treatment of metabolic disease in humans, partly because the β_3 -adrenoceptor is less dominant in human adipose tissue, especially white adipose tissue, than in rodent adipose tissue, and there is less brown adipose tissue in humans than in rodents (Arch 2011a). ZAG, however, may have more potential for the treatment of metabolic diseases because, unlike β_3 -adrenoceptor agonists, ZAG is reportedly equally effective (relative to isoprenaline) as a lipolytic agent in human adipocytes (Hirai *et al.* 1998, Russell & Tisdale 2011).

Russell *et al.* (2002, 2004) have presented evidence that ZAG elicits its metabolic effects by acting as a β_3 -adrenoceptor agonist. Some of this evidence involves the use of a high concentration of a marginally selective β_3 -adrenoceptor antagonist (Manara *et al.* 1996, Arch 2011b); however, it is unclear why neither a β_3 -adrenoceptor agonist nor isoprenaline corrected decreased lipolysis in

adipocytes from ZAG knockout mice (Rolli *et al.* 2007). One criticism – that ZAG is equally effective as a lipolytic agent in human and rodent white adipocytes despite the β_3 -adrenoceptor playing at best a minor role in human white adipocytes – has recently been countered by a report that ZAG is also a β_2 -adrenoceptor agonist but not a β_1 -adrenoceptor agonist (Russell & Tisdale 2012a). Treatment of 3T3-L1 adipocytes with β_3 -adrenoceptor agonists increases ZAG mRNA expression (Bing *et al.* 2004), opening the possibility that ZAG might mediate the metabolic effects of β_3 -adrenoceptor agonists.

Although many of the metabolic effects of ZAG are similar to those described for β_3 -adrenoceptor agonists, it has not been reported whether ZAG elicits an acute increase in energy expenditure, which is an invariable effect of β -adrenoceptor agonists. Therefore, in this study, we have investigated whether a dose and duration of treatment with ZAG that is reported to elicit other metabolic effects also increases energy expenditure in C57Bl/6 *Lep^{ob}/Lep^{ob}* mice. We have used mice that had the defined C57Bl/6 background rather than the more undefined Aston background. As a positive control, we have used the standard β_3 -adrenoceptor agonist prodrug BRL35135. BRL35135 is rapidly de-esterified to the β_3 -adrenoceptor agonist BRL37344 when it is administered to rodents and only BRL37344 is found in the systemic circulation (Cawthorne *et al.* 1992). Like ZAG, BRL37344 has some effect at the β_2 -adrenoceptor but is about 20-fold less potent as an agonist of this receptor compared with the β_3 -adrenoceptor (Arch & Kaumann 1993).

We have compared the effects of ZAG and BRL35135 on energy balance, glucose tolerance and plasma metabolites, as well as energy expenditure. We have also compared the effects of ZAG and BRL35135 on the gene expression in adipose tissue of β -adrenoceptors and UCP1.

Materials and methods

Materials

Reagents were obtained from Sigma–Aldrich, unless otherwise stated. Endotoxin-free recombinant human ZAG was synthesised by EzBiolab (Carmel, CA, USA) using established methodology (Mracek *et al.* 2010b). Briefly, full-length human ZAG cDNA in the pcDNA 3.2 vector (Invitrogen) was transfected into HEK-293 cells. ZAG-conditioned medium was concentrated into sterile PBS using a centrifugal filter (Merck Millipore, Billerica, MA, USA). The purity of the concentrated protein was assessed by total protein staining by SDS–PAGE, and ZAG

protein concentration was then quantified using a human ZAG ELISA kit (BioVendor, Karasek, Brno, Czech Republic) according to the manufacturer's instructions (Gao *et al.* 2010). On receipt, the endotoxin concentration was <0.0001 ng/ml (HEK-Blue endotoxin detection kit; InvivoGen, Toulouse, France). ZAG was shipped and stored at 4 °C throughout the experiment.

Animals

Animal procedures were conducted in accordance with the University of Buckingham project licence under the UK Animals (Scientific Procedures) Act (1986) and as approved by the University's Ethics Review Board. Male obese C57/Bl6 (*Lep^{ob}/Lep^{ob}*) mice (Charles River, Margate, Kent, UK) aged 6–7 weeks on arrival were fed a standard laboratory chow diet that contained 10% fat, 70% carbohydrate and 20% protein by energy (Beekay Feed, B&K Universal Ltd., Hull, UK). They were housed in pairs at 21–23 °C with lights on from 0700 to 1900 h and dosed by i.v. administration into the tail vein with vehicle (saline) or ZAG (100 μ g/mouse per day) or by i.p. administration of BRL35135 ((R*,R*-($\pm$)-methyl-4-(2-(2-hydroxy-2-(3-chlorophenyl) ethyl-amino)-propyl)-phenoxyacetate hydrobromide); 0.5 mg/kg body weight; Tocris Bioscience, Bristol, UK) at 0900 h daily for 10 days, which is the maximal dosing regimen for i.v. dosing into the tail vein as approved by our Home Office Licence. The dose and route of administration of ZAG were based on the previous reports that showed effects of this compound in *Lep^{ob}/Lep^{ob}* mice (Russell & Tisdale 2010a,b, 2011, 2012a), while that of BRL35135 has been used by a number of workers, including ourselves (Cawthorne *et al.* 1992, Virtanen *et al.* 1997, Bing *et al.* 1998). Food and water intakes for each cage of two mice were measured daily at 0900 h and body weight of each mouse was measured every 3 days. On day 9, they were fasted from 0800 h, dosed with ZAG or BRL35135 at 0900 h and then orally with glucose (2 g/kg body weight) at 1300 h. Glucose, insulin, NEFA and glycerol were measured as described previously (Wargent *et al.* 2005). On the final day of dosing (day 10), plasma was taken at 30-min intervals following dosing in the fed state for the measurement of concentrations of glycerol and NEFAs. The mice were then killed, and tissues and plasma were harvested and stored at -80 °C before analysis.

Adipocytes from 20 lean and 15 diet-induced obese male C57/Bl6 mice (Charles River) were used to demonstrate that the human recombinant ZAG could stimulate lipolysis. The concentrations of ZAG were based on

previous reports, which include the effect of ZAG on lipolysis in adipocytes from wild-type and *Lep^{ob}/Lep^{ob}* mice (Russell & Tisdale 2011). The concentrations of isoprenaline (1 μ M and BRL37344, (R*,R*-($\pm$)-4-(2-(2-(3-chlorophenyl)-2-hydroxyethyl)amino)propyl)phenoxyacetic acid, sodium salt; 1 μ M) were chosen to give maximal stimulation of lipolysis (Arch *et al.* 1984). Mice aged 6–8 weeks on arrival were maintained on either standard laboratory chow or a high-fat diet that contained 60% fat, 20% carbohydrate and 20% protein by energy (Research Diets, Inc., New Brunswick, NJ, USA) for 16 weeks.

Body composition

The body composition of each mouse was measured at the termination of the experiment using dual-energy X-ray absorptiometry (Lunar PIXImus 2 mouse densitometer and version 1.46 Software, GE Medical, Bedford, UK).

Energy expenditure

Energy expenditure was measured for 24 h after dosing on days 1 and 8 by open-circuit indirect calorimetry with both mice in their home cage, as described previously (Stocker *et al.* 2007).

Adipocyte isolation and incubation

The bioactivity of ZAG was assayed by measuring the amount of glycerol released from epididymal adipocytes over 1 h in the presence or absence of ZAG, using BRL37344 (Tocris Bioscience) and isoprenaline as positive controls (Zaibi *et al.* 2010).

RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated as described previously (Zaibi *et al.* 2010) and analysed using a NanoDrop ND1000 (Thermo Fisher Scientific, Loughborough, UK). RNA from white and brown adipose tissues was used for gene expression analysis of β -adrenoceptors and UCP1 with GAPDH and β -actin as internal controls. Real-time PCR (StepOne, Applied Biosystems) was carried out using Assay on Demand pre-designed primer and probe sets for β_1 -, β_2 -adrenoceptors and UCP1 (Applied Biosystems) and a custom-designed primer and probe set for the β_3 -adrenoceptor (forward: 5'-CTCCAACATGCCCTATGCG-3', reverse: 5'-ACGAAGAGCATCACAAGGAGG-3', probe: 5'-FAM-TGCTCTCCTCCTCCGTCTCCTTCTACC TTC-3'-TAMRA) as described previously (Mracek *et al.* 2010a).

Data were analysed using the comparative ΔC_t method, comparing both ZAG- and BRL35135-treated animals with vehicle-dosed controls. All procedures were carried out in accordance with the manufacturer's recommendation.

Statistical analysis

Data were analysed using GraphPad Prism Software, version 5.0 (GraphPad Prism, La Jolla, CA, USA). Lipolysis data were analysed by two-way ANOVA followed by Bonferroni comparisons against the control conditions for lean or obese mice. Food intake, body weight and glucose tolerance, and plasma glycerol and NEFA concentrations were analysed using repeated measures two-way ANOVA followed by Bonferroni's multiple comparison test. Blood glucose at each time point, area under the glucose curve, total energy expenditure and body composition data were analysed using one-way ANOVA followed by Dunnett's multiple comparison test. The Kruskal-Wallis test was used if variances were unequal according to Bartlett's test. Tissue weights were compared using the Student's *t*-test. Real-time PCR of gene expression data were analysed using one-way ANOVA followed by Dunnett's test compared with the control group. Results are presented as mean $\pm$ S.E.M. Statistical significance for effects in the figures is given as **P* < 0.05; ***P* < 0.01 and ****P* < 0.001.

Results

In vitro bioactivity of ZAG

ZAG increased lipolysis in adipocytes from both lean and diet-induced obese mice (Fig. 1). The effect was statistically significant at concentrations of 10 and 25 μ g/ml in adipocytes from lean mice and at 5, 10 and 25 μ g/ml in adipocytes from obese mice. The effect of 25 μ g/ml ZAG was 25 and 29% of that of isoprenaline in lean and obese mice respectively. The effect of 10 μ g/ml ZAG appeared slightly less than that of 25 μ g/ml ZAG in lean mice (19% of isoprenaline), but in obese mice, these concentrations had almost identical effects, indicating that 10 μ g/ml elicited a maximal lipolytic effect.

Food intake, body weight and body composition

Food consumption was significantly reduced by treatment with ZAG, but not by BRL35135 throughout the 10-day dosing period (Fig. 2). However, neither ZAG nor BRL 35135 reduced body weight, body fat content, percentage

Figure 1

Effect of ZAG on lipolysis from isolated adipocytes from lean and diet-induced obese mice. Glycerol release into the medium was measured after a 1-h incubation with ZAG, BRL37344 or isoprenaline. *n* = 4 experimental replicates. C, control; B, BRL37344 (1 μ M); I, isoprenaline (1 μ M); Z, hrZAG at 5, 10 and 25 μ g/ml. **P* < 0.05; ***P* < 0.01 and ****P* < 0.001 for differences between lean and obese mice compared with unstimulated lean and obese control adipocytes.

body fat or epididymal fat pad weight significantly. Neither treatment affected body lean content. There was a trend towards a reduction in gastrocnemius weight in both the ZAG- and the BRL35135-treated groups, but this did not reach statistical significance (*P* = 0.1 and *P* = 0.09 respectively; Table 1).

Energy expenditure and *in vivo* lipolysis

The first dose of BRL35135 increased energy expenditure during the light period, but ZAG had no effect. The eighth dose of BRL35135 elicited a greater thermogenic effect during the light period than the first dose (Fig. 3). ZAG was again ineffective, although after the eighth dose, energy expenditure of the ZAG-treated mice was similar to that of the BRL35135-treated mice during the dark period (Fig. 3C). Neither compound had an obvious effect on the activity of the animals throughout the treatment period. Plasma glycerol and NEFA levels were elevated after the final dose of BRL35135 but not after ZAG (Fig. 4).

Glucose tolerance

Blood glucose was lower in the BRL35135-treated mice than in the control mice before BRL35135 was administered on day 9, 30 min before the administration of glucose. Both ZAG and BRL35135 reduced fasting blood glucose 30 min after they were administered (just before

Figure 2

Cumulative food intake following administration of ZAG or BRL35135. Food intake was measured daily for 10 days during administration of ZAG (filled square), BRL35135 (filled triangle) or vehicle (open circle) to *ob/ob* mice. $n=4-5$ cages per group containing two mice per cage. Food intake per pair of mice has been divided by two to give the mean value per mouse for each cage. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$ for differences between ZAG- and BRL35135-treated mice compared with vehicle-treated mice.

administration of glucose). Both compounds improved glucose tolerance (Fig. 5A). The areas under the curve were significantly reduced even after correction for the lower baseline glucose levels just before administration of glucose (control: 12.8 ± 1.5 ; ZAG: 7.8 ± 1.1 , $P<0.05$; BRL35135: 5.4 ± 2.5 mM, 120 min, $P<0.01$). The plasma insulin concentrations measured 30 min after glucose administration was lowered in mice dosed with BRL35135 but not ZAG (Fig. 5B). The fasting glucose $\times$ fasting insulin concentration, which is an indicator of insulin resistance, was reduced by both ZAG (63% decrease, $P<0.05$) and BRL35135 (75% decrease, $P<0.01$).

Gene expression of β -adrenoceptors and UCP1

Both BRL35135 and ZAG reduced the β_1 -adrenoceptor mRNA level in white adipose tissue (Fig. 6A) and BRL35135 reduced the β_2 -adrenoceptor mRNA level (Fig. 6B). Neither agent influenced β_1 -adrenoceptor gene expression in brown adipose tissue (Fig. 7A), but both BRL35135 and

ZAG significantly reduced the β_2 -adrenoceptor mRNA level (Fig. 7B). BRL35135, but not ZAG, increased the UCP1 mRNA level (Fig. 7C). Neither BRL35135 nor ZAG changed the expression of the β_3 -adrenoceptor gene in white adipose tissue (Fig. 8A). However, BRL35135 but not ZAG increased the β_3 -adrenoceptor mRNA level in brown adipose tissue (Fig. 8B).

Discussion

This study compares the effects of ZAG, which according to Tisdale *et al.* is a putative β_3 -adrenoceptor agonist that has also some β_2 -adrenoceptor agonist activity, with the standard β_3 -adrenoceptor agonist BRL35135, which also has some activity at β_2 -adrenoceptors. The BRL35135 metabolite BRL37344 is 20-fold more potent as an agonist of the β_3 - than the β_2 -adrenoceptor. Others and we regard this difference in potency as sufficient to describe BRL37344 as a 'selective' β_3 -adrenoceptor agonist, a term that should not be confused with 'specific' (Mencher & Wang 2005). It has been argued that BRL37344 is of limited value as a pharmacological tool because it is a muscarinic and α_1 -adrenoceptor antagonist (Vrydag & Michel 2007). Its potency at these receptors is, however, 1000- to 10 000-fold lower than its potency ($pEC_{50}=8.7$) as a stimulant of β_3 -adrenoceptor-mediated lipolysis in rat white and brown adipocytes (Arch *et al.* 1984, Hollenga *et al.* 1990, Kubota *et al.* 2002, Brahmadevara *et al.* 2004, Briones *et al.* 2005, Leblais *et al.* 2005). Moreover, antagonists of muscarinic receptors and α_1 -adrenoceptors do not share the metabolic effects of BRL35135 demonstrated here (in particular its acute thermogenic and lipolytic activity), whereas other β_3 -adrenoceptor agonists do.

Numerous β_3 -adrenoceptor agonists – some more selective than BRL35135 – have been shown to elicit similar metabolic effects to those of BRL35135, but rather than contrasting our findings on ZAG with those previously published for various animal models and

Table 1 Weight gain and body composition of *Lep^{ob}/Lep^{ob}* mice treated with saline, BRL35135 or ZAG for 10 days

	Control	BRL35135	ZAG
Initial body weight (g)	35.7 ± 1.3	34.8 ± 0.7	35.8 ± 1.2
Weight gain (g)	5.86 ± 0.46	5.96 ± 0.79	5.87 ± 0.57
Body fat content (g)	22.2 ± 0.9	19.8 ± 1.0	20.8 ± 0.8
Body lean content (g)	18.3 ± 0.3	18.2 ± 0.2	18.0 ± 0.5
Body fat (%)	54.6 ± 0.8	51.9 ± 1.1	53.5 ± 0.8
Fat pad weight (g)	1.57 ± 0.08	1.45 ± 0.05	1.44 ± 0.09
Gastrocnemius muscle weight (mg)	37 ± 3	30 ± 2	31 ± 2

Figure 3

Twenty-four-hour energy expenditure following administration of ZAG or BRL35135. Energy expenditure per animal (A) was measured for 24 h after the first dose and (B) area under the curve for the first 6-h post-treatment. Energy expenditure (EE) per animal (C) was measured for 24 h after the eighth dose and (D) area under the curve for the first 6-h post-treatment after daily administration of ZAG (filled square), BRL35135 (filled triangle) or vehicle (open circle) to *ob/ob* mice. $n=4-5$ cages per group containing two animals per cage. Energy expenditure per pair of mice has been divided by two to give the mean value per mouse for each cage.

*** $P<0.001$ for differences between ZAG- and BRL35135-treated mice compared with vehicle-treated mice.

periods of compound administration, we included BRL35135 as a positive control in this study. Both the dose of ZAG (100 μg , i.v.) and period of its administration (10 days) were double those previously reported to elicit various metabolic effects in *Lep^{ob}/Lep^{ob}* mice (Russell & Tisdale 2010a,b, 2012a), except that one study employed the same dose that we employed over 15 days (Russell & Tisdale 2011). The i.v. route of administration was the same as in most previous studies, our work being conducted before a recent report that oral ZAG has similar effects to i.v. ZAG (Russell & Tisdale 2012b). The metabolic effects of ZAG in these previous studies include an anti-obesity effect, which is the least sensitive of the metabolic effects of β_3 -adrenoceptor agonists (Hashimoto *et al.* 1996, Arch 2002). Therefore, if ZAG elicits its metabolic effects by stimulating the β_3 - and possibly also the β_2 -adrenoceptor, one might expect to see other effects typical of such compounds. The dose of BRL35135 was the same as that used in previous studies (Cawthorne *et al.* 1992, Virtanen *et al.* 1997, Bing *et al.* 1998). It may have resulted in the stimulation of the β_2 - as well as the β_3 -adrenoceptor. However, the metabolic effects of this dose of BRL35135 are similar to those of the much more selective (reasonably described as 'specific') but less potent β_3 -adrenoceptor agonist CL316243 (Largis *et al.* 1994, Gavrilova *et al.* 2000, Granneman *et al.* 2003).

ZAG and β_3 -adrenoceptor agonists share a number of metabolic effects when they are dosed repeatedly over a number of days to obese rodents. These include increased fat oxidation, reduced body fat content and decreased plasma concentrations of glucose, triglycerides and NEFA (Arch *et al.* 1991, Hirai *et al.* 1998, Russell & Tisdale 2002, 2010a, 2011, Russell *et al.* 2004, Arch 2008). Neither ZAG nor BRL35135 reduced body weight gain or fat content in the current study, but this was not an unexpected result for BRL35135: reductions in body weight and fat content in response to β_3 -adrenoceptor agonists typically require more than 10 days' dosing to achieve statistical significance when group sizes are in the range used here, and the 2.4 g difference between the mean body fat contents of the control and BRL35135-treated mice after 10 days' dosing (Table 1) is consistent with the 4–5 g difference in body fat content generally found after 28 days (Arch *et al.* 1991). It was surprising, however, that ZAG did not increase gastrocnemius muscle weight – in fact, there was a trend to a reduction – and that it reduced energy intake. Both findings are inconsistent with previous reports, albeit for *Lep^{ob}/Lep^{ob}* mice on a different background strain (Russell & Tisdale 2010a, 2011). In one study, ZAG elicited a remarkable 2.8-fold increase in gastrocnemius

Figure 4

In vivo lipolysis assay following administration of ZAG or BRL35135. Plasma glycerol levels (A) and plasma NEFA levels (B) were measured for 2 h following the final daily dose of ZAG (filled square), BRL35135 (filled triangle) or vehicle (open circle) to *ob/ob* mice. $n=8$ or 10 per group. *** $P<0.001$ for differences between ZAG- and BRL-treated mice compared with vehicle-treated mice.

muscle weight in *Lep^{ob}/Lep^{ob}* mice after only 5 days' treatment (Russell & Tisdale 2010a), which is a far greater effect even than that of the β_2 -adrenoceptor agonist clenbuterol (Yang & McElligott 1989, Arch *et al.* 1995).

Both ZAG and BRL35135 improved glucose tolerance. It is well established that the insulin sensitising and anti-diabetic effects of β_3 -adrenoceptor agonists become apparent before their anti-obesity effects, possibly due to stimulation of fatty acid oxidation (Arch 2002). However,

ZAG, in contrast to BRL35135, did not decrease the plasma insulin concentration 30 min after administration of glucose, in contrast to a previous study (Russell & Tisdale 2010a). Thus, we have only partly reproduced previous findings on the metabolic effects of ZAG in *Lep^{ob}/Lep^{ob}* mice and we have found differences between the effects of ZAG and BRL35135.

Russell *et al.* (2002, 2004) have presented evidence that ZAG is a β_3 -adrenoceptor agonist. Recently, these workers have suggested that ZAG is also a β_2 -adrenoceptor agonist (Russell & Tisdale 2012a). One important property

Figure 5

Oral glucose tolerance following administration of ZAG or BRL35135. Glucose tolerance (A) was measured 9 days after daily administration of ZAG (filled square), BRL35135 (filled triangle) or vehicle (open circle) to obese *ob/ob* mice. Plasma insulin levels before and in response to the glucose load (B) in control mice (open columns) and 9 days after daily administration of ZAG (hatched columns) or BRL35135 (filled columns) to *ob/ob* mice. $n=8$ or 10 animals per group. * $P<0.05$; ** $P<0.01$ and *** $P<0.001$ for differences between ZAG- and BRL-treated mice compared with vehicle-treated mice.

Figure 6

β -Adrenoceptor gene expression in white adipose tissue from mice following administration of ZAG or BRL35135. Gene expression was measured using real-time PCR of (A) β_1 -adrenoceptor and (B) β_2 -adrenoceptor in white adipose tissue following the final daily dose of ZAG or BRL35135 to *ob/ob* mice. $n=8$ or 10 animals per group. *** $P<0.001$ for differences between ZAG- and BRL35135-treated mice compared with vehicle-treated mice.

of β_3 -adrenoceptor agonists has not been demonstrated, however. This is the ability to rapidly raise energy expenditure. ZAG raises body temperature, which may be a consequence of increased energy expenditure, but this takes up to 3 days to develop (Russell & Tisdale 2011, 2012a). If ZAG behaves like a typical β_3 -adrenoceptor agonist, one would expect it to raise energy expenditure rapidly after injection into rodents. Others have reported that it stimulates lipolysis over 2 h when it is incubated with adipocytes (Hirai *et al.* 1998, Russell *et al.* 2002, 2004) and it stimulated lipolysis over 1 h in the current study, so it seems to activate its receptor within an hour. Moreover, binding to β_3 -adrenoceptors has been demonstrated over 1 h and elevation of the concentration of cAMP in β_3 -adrenoceptor-transfected cells over 30 min (Russell *et al.* 2002, Russell & Tisdale 2011, 2012a). In the current study, the first dose of ZAG did not stimulate energy expenditure in marked contrast to the thermogenic effect

of BRL35135. It is unlikely that ZAG had an intrinsic thermogenic effect that was masked by reduced locomotor activity because energy expenditure was measured during the light period, when baseline locomotor activity is already low. We used a dose of 0.5 mg/kg body weight for BRL35135, but as little as 0.002 or 0.010 mg/kg body weight has been shown to elicit an acute increase in energy expenditure in rats (Carlisle & Stock 1993) or mice

Figure 7

β -Adrenoceptor and UCP1 gene expression in brown adipose tissue from mice following administration of ZAG or BRL35135. Gene expression was measured using real-time PCR of (A) β_1 -adrenoceptor, (B) β_2 -adrenoceptor and (C) UCP1 in brown adipose tissue following the final daily dose of ZAG or BRL35135 to *ob/ob* mice. $n=8$ or 10 animals per group. ** $P<0.01$; *** $P<0.001$ for differences between ZAG- and BRL-treated mice compared with vehicle-treated mice.

Figure 8

β_3 -Adrenoceptor gene expression in white and brown adipose tissue from mice following administration of ZAG or BRL35135. Gene expression was measured using real-time PCR of (A) β_3 -adrenoceptor in white adipose tissue and (B) in brown adipose tissue following the final daily dose of ZAG or BRL35135 to *ob/ob* mice. $n=8-10$ animals per group. *** $P<0.001$ for differences between ZAG- and BRL-treated mice compared with vehicle-treated mice.

(Cawthorne *et al.* 1992) respectively. Other β_3 -adrenoceptor agonists, including the highly selective β_3 -adrenoceptor agonist CL316243, also elicit a marked and rapid increase in energy expenditure (Gavrilova *et al.* 2000, Granneman *et al.* 2003). These effects are associated with activation of brown adipocyte thermogenesis but not with increased locomotor activity (Inokuma *et al.* 2006, Stemmelin *et al.* 2008). The β_2 -adrenoceptor agonists salbutamol and clenbuterol elicit rapid and marked increases in oxygen consumption in rats, but they lack potency (Holloway *et al.* 1991, Oriowo *et al.* 1994). Thus, ZAG did not share one of the main *in vivo* effects of β_3 -adrenoceptor agonists. There was an indication that sub-chronic administration of ZAG might have a small thermogenic effect after the eighth dose. Such an effect, together with decreased food intake, might contribute to the improved glucose tolerance that was observed in response to ZAG.

ZAG and BRL35135 had similar, though not identical, effects on the expression of the β_1 - and β_2 -adrenoceptor

genes, reducing expression of the former in white adipose tissue and the latter in brown adipose tissue. BRL35135 also reduced the expression of the β_2 -adrenoceptor gene in white adipose tissue. cAMP-mediated mechanisms by which β_1 - and β_2 -adrenoceptors are down-regulated in response to β -adrenoceptor agonists are well established (Lafontan & Berlan 1993). BRL35135, but not ZAG, increased the expression of the β_3 -adrenoceptor and *UCP1* genes in brown adipose tissue. By contrast, others have reported that administration of ZAG to *Lep^{ob}/Lep^{ob}* mice of the Aston strain for 7 days had no effect on the expression of β_1 - or β_2 -adrenoceptors in either white or brown adipose tissue and that ZAG increased the expression of the β_3 -adrenoceptor and *UCP1* genes in brown adipose tissue (Russell & Tisdale 2012a). Thus, in this study, ZAG shared only some of the effects of BRL35135 on the expression of β -adrenoceptor and *UCP1* genes.

ZAG is unstable, in particular being inactivated by freezing and thawing (Russell & Tisdale 2012a). The ZAG used for the current study was therefore shipped and stored at 4 °C. Its bioactivity was demonstrated by showing that it stimulated lipolysis by epididymal adipocytes. ZAG stimulated lipolysis by 42 and 62% above the basal level and its maximum effects were 25 and 29% of those of isoprenaline in adipocytes from lean and diet-induced obese mice respectively. Similar maximum effects of recombinant human ZAG relative to those of isoprenaline have been described for stimulation of lipolysis by visceral adipocytes from lean and *Lep^{ob}/Lep^{ob}* mice (Russell & Tisdale 2011). In another study, the lipolytic effect of various ZAG batches was described as always less than that of isoprenaline, the ZAG effect illustrated being 58% of the isoprenaline effect (Russell *et al.* 2004). Moreover, the maximum effect of murine LMF was only about 40% of that of isoprenaline for stimulation of lipolysis by murine adipocytes (Hirai *et al.* 1998). Other experiments have been described in which the effects of ZAG and isoprenaline were similar, but in these experiments, isoprenaline stimulated lipolysis by about tenfold above the basal level (Russell & Tisdale 2011), contrasting with its roughly twofold effect in the current work. The intrinsic activity of ZAG relative to isoprenaline may decrease as receptor numbers or the efficiency of signalling mechanisms between cAMP and lipolysis decrease, as is predicted by the classical receptor theory for low-efficacy agonists (Kenakin 1984). The effects on lipolysis *in vitro* of the ZAG used in the current study are therefore consistent with the effects of the ZAG used by others.

Some of the evidence that ZAG is a β_3 -adrenoceptor agonist is based on the evidence that its *in vitro* effects are antagonised by the β_3 -adrenoceptor antagonist SR59230 (Russell *et al.* 2002, 2004, Sanders & Tisdale 2004a,b, Russell & Tisdale 2005). However, SR59230 was used at a concentration of 10 μ M in many of these experiments, which is not a concentration at which it is a selective β_3 -adrenoceptor antagonist: as little as 0.1 μ M antagonises the rodent β_1 -adrenoceptor (Manara *et al.* 1996). We do not claim to have excluded the possibility that ZAG is a β_3 - or $\beta_{3/2}$ -adrenoceptor agonist, but only that it does not behave like other β_3 - or $\beta_{3/2}$ -adrenoceptor agonists at the dose that we used. Nevertheless, as ZAG affected glycaemic control and β -adrenoceptor expression, it is pertinent to ask whether it might have other mechanisms of action. Both injection of mice with LMF over 48 h and treatment of 3T3-L1 adipocytes with LMF for 24 h increased the amount of G α s protein in adipocyte membranes (Islam-Ali *et al.* 2001). While G α s might not be its primary target, we speculate that ZAG/LMF elicits its effects by increasing the amount of G α s and thereby the cellular concentration of cAMP, independently of β -adrenoceptor stimulation.

An involvement of sympathetic activation in the metabolic effects of ZAG in *Lep^{ob}/Lep^{ob}* mice is suggested by reports that propranolol blocks the effects of both i.v. and oral ZAG (Russell & Tisdale 2012a,b). This could be because propranolol blocks the binding of ZAG to the β_3 -adrenoceptor, but it is difficult to explain the effects of oral ZAG because oral ZAG is not absorbed. The authors proposed that oral ZAG binds to an oesophageal β_3 -adrenoceptor and that this raises the endogenous ZAG plasma concentration by an undefined mechanism (Russell & Tisdale 2012b). It seems equally likely that oral ZAG raises sympathetic activity by an undefined mechanism. If stimulation of oesophageal β_3 -adrenoceptors can elicit, via endogenous ZAG, the same effects in rodents as well-absorbed β_3 -adrenoceptor agonists, this raises the question of why the oral bioavailability of β_3 -adrenoceptor agonists has been a major obstacle to their development, as discussed in a number of publications (Arch 2011a). On the other hand, sympathetic activity is normally very low in *Lep^{ob}/Lep^{ob}* mice, so that its capacity for elevation is high (Wilson *et al.* 1984).

We acknowledge two limitations in the design of our experiment. First, we gave BRL35135 and ZAG by different routes and controlled only for the ZAG (i.v.) route. We felt that the use of an i.p. dosed group for the BRL35135-treated mice could not be ethically justified because BRL35135 and other β_3 -adrenoceptor agonists have been studied many times before. We expected to reproduce

these findings and the most important effects to be far greater than might reasonably be explained by administration of the vehicle. More importantly, we used only one dose of both ZAG and BRL35135 and did not conduct dose–response curves. Much lower doses of BRL35135 retain thermogenic activity (Cawthorne *et al.* 1992, Carlisle & Stock 1993), but we cannot exclude the possibility that higher doses of ZAG might also elicit such an effect.

In conclusion, ZAG and the $\beta_{3/2}$ -adrenoceptor agonist prodrug BRL35135 elicited some effects in common in *Lep^{ob}/Lep^{ob}* mice: both compounds improved glucose tolerance and decreased the expression of β_1 -adrenoceptor in white adipose tissue and the expression of β_2 -adrenoceptor in brown adipose tissue. However, only BRL35135 stimulated thermogenesis acutely, raised plasma glycerol and NEFA concentrations, lowered the plasma insulin concentration, increased the expression of *UCP1* in brown adipose tissue and reduced the expression of the β_2 -adrenoceptor in white adipose tissue. Thus, ZAG does not behave as a typical $\beta_{3/2}$ -adrenoceptor agonist.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Mr E T W and Dr C J S planned the study and carried out the in-life phase and physiological measurements. Dr J F O assisted with the optimisation, running and analysis of the real-time gene expression studies. Dr M S Z performed the *in vitro* lipolysis study. Drs C B and D G assisted with the quantification of the recombinant ZAG protein and provided the real-time PCR assays for the β_3 adrenoceptor. Prof. J R S A, M A C and P T provided valuable advice in the design of these studies and in the preparation of this manuscript. Prof. J R S A and Dr C J S prepared the current manuscript.

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