

RESEARCH PAPER

Chronic glucokinase activator treatment at clinically translatable exposures gives durable glucose lowering in two animal models of type 2 diabetes

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BACKGROUND AND PURPOSE

Pharmacological activation of glucokinase (GK) lowers blood glucose in animal models and humans, confirming proof of concept for this mechanism. However, recent clinical evidence from chronic studies suggests that the glucose-lowering effects mediated by glucokinase activators (GKAs) are not maintained in patients with type 2 diabetes (T2D). Existing preclinical data with GKAs do not explain this loss of sustained glucose-lowering efficacy in patients. Here, we have assessed the effects of chronic (up to 11 months) treatment with two different GKAs in two models of T2D.

EXPERIMENTAL APPROACH

Two validated animal models of T2D, insulin-resistant obese Zucker rats and hyperglycaemic $gk^{wt/del}$ mice, were treated with two different GKAs for 1 or 11 months respectively at exposures that translate to clinical exposures in humans. Blood glucose, cholesterol, triglycerides and insulin were measured. GKA pharmacokinetics were also determined.

KEY RESULTS

Treatment with either GKA provided sustained lowering of blood glucose for up to 1 month in the Zucker rat and up to 11 months in hyperglycaemic $gk^{wt/del}$ mice, with maintained compound exposures. This efficacy was achieved without increases in plasma or hepatic triglycerides, accumulation of hepatic glycogen or impairment of glucose-stimulated insulin secretion.

CONCLUSIONS AND IMPLICATIONS

Chronic treatment with two GKAs in two animal models of diabetes provided sustained lowering of blood glucose, in marked contrast to clinical findings. Therefore, either these animal models of T2D are not good predictors of responses in human T2D or we need a better understanding of the consequences of GK activation in humans.

Abbreviations

GK, glucokinase; GKA, glucokinase activator; GSIS, glucose-stimulated insulin secretion; HF, high fat; OGTT, oral glucose tolerance test; PD, pharmacodynamics; PK, pharmacokinetics; T2D, type 2 diabetes; ZDF rats, Zucker diabetic fatty rats

Introduction

Glucokinase activators (GKAs) bind to an allosteric binding site of glucokinase (GK) and stabilize a conformation of the enzyme that promotes the binding of glucose and its phosphorylation to glucose 6-phosphate (Coghlan and Leighton, 2008; Matschinsky, 2009). The glucose-lowering efficacy of GKAs is mediated through GK activation in pancreas and liver. In the pancreas, GK acts as the 'glucose sensor' controlling glucose-stimulated insulin secretion (GSIS). Hepatic GK, together with the GK regulatory protein, is a key regulator of glucose uptake by the liver (Futamura *et al.*, 2006; Agius, 2008; Bourbonais *et al.*, 2012) and hence of net hepatic glucose balance.

Recent clinical data demonstrate that GKAs can acutely lower glucose levels in non-diabetic and type 2 diabetic subjects (Bonadonna *et al.*, 2010; Ericsson *et al.*, 2012; Morrow *et al.*, 2012; Norjavaara *et al.*, 2012; Sjöstrand *et al.*, 2013). Repeat dose studies have shown glucose control can be maintained for 4 weeks (Morrow *et al.*, 2012) and up to 14 weeks (Meininger *et al.*, 2011). However, the glucose-lowering efficacy of GKAs over the longer term has been called into question (Meininger *et al.*, 2011; Kiyosue *et al.*, 2013; Wilding *et al.*, 2013).

A number of acute (1 day; Grimsby *et al.*, 2003; Efanov *et al.*, 2005; Coope *et al.*, 2006) and repeat dose (9–35 days; Fyfe *et al.*, 2007; Ohyama *et al.*, 2010; Eiki *et al.*, 2011; Sörhede Winzell *et al.*, 2011; Futamura *et al.*, 2012) pre-clinical studies have demonstrated effective glucose lowering by GKAs in both non-diabetic and diabetic animal models. Two chronic studies report sustained glucose lowering with two different GKAs at 20 and 40 weeks (Nakamura *et al.*, 2009; 2011). However there are no data describing exposure in these studies, making it difficult to relate these results to clinical observations. In addition to the issue of a sustained glucose response, questions have recently been raised about the effect of chronic GKA administration on hepatic and plasma triglyceride levels (De Ceuninck *et al.*, 2013; Rees and Gloyn, 2013). It is also unclear what might be the long-term consequences of GKA activation on beta cell function, (Bebemitz *et al.*, 2009) given that sulphonylureas gradually lose efficacy in humans (Kahn *et al.*, 2006).

In order to better understand whether or not GKAs can deliver robust and sustained glucose lowering under diabetic conditions, we have investigated the chronic efficacy of two GKAs (AZD1656 and GKA71) in two animal models of type 2 diabetes (T2D) at clinically relevant exposures. Sustained glucose lowering was observed over a 4 week study in male obese Zucker *fa/fa* rats that are insulin-resistant, hyperinsulinaemic and hyperlipidaemic (but not markedly hyperglycaemic), and also over 11 months in high-fat (HF)-fed male *gk^{wt/del}* mice (Gorman *et al.*, 2008; Baker *et al.*, 2014) that are insulin-resistant and hyperglycaemic but not hyperlipidaemic. Reductions in blood glucose were achieved without increases in plasma or hepatic triglycerides or hepatic glycogen content and without adverse effects on GSIS after 48 weeks of dosing in *gk^{wt/del}* mice.

Methods

Animals

All animal care and experimental procedures were approved by the UK Home Office and carried out in accordance with the Animals (Scientific Procedures) Act 1986. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 75 animals were used in the experiments described here. Male obese Zucker *fa/fa* rats and male *gk^{wt/del}* mice (Gorman *et al.*, 2008) were supplied from the AstraZeneca Biological Animal Breeding Unit. Zucker *fa/fa* rats were received at 9–10 weeks of age, housed in groups of 3–4 at 19–21°C on a reverse 12 h : 12 h light : dark cycle (lights off 08:00 h) and maintained on RM3 diet (Special Diets Services, Witham, Essex, UK) for 3 weeks before the start of the study. Body weights at the start of the study were 440–535 g. Male *gk^{wt/del}* mice or wild-type littermates were received at 8 weeks of age, housed in groups of three at 21–23°C on a reverse 12 h : 12 h light : dark cycle (lights off 09:00 h), and maintained on standard chow RM1 diet (Special Diets Services) for 3 weeks acclimatization. Animals had *ad libitum* access to food and tap water. Blood samples for in-study glucose (~5 µL) and pharmacokinetic (PK) analyses (20 µL) were obtained at the same time from the tail vein, as previously described (Baker *et al.*, 2014).

Experimental designs and measurements

Details of individual study designs are provided in the Supporting Information. Doses of GKAs were selected to provide translatable drug exposures based on known PK properties in the rodent species and known tolerated and therapeutically relevant exposures and PK in humans (Ericsson *et al.*, 2012; Morrow *et al.*, 2012). Correction was made for species differences in plasma protein binding and potency against species-specific GK isoforms to ensure comparable free plasma exposure.

Study 1: chronic efficacy of AZD1656 in male obese Zucker fa/fa rats. Rats received daily oral doses of vehicle [1% Pluronic F127 (Sigma-Aldrich, Gillingham, Dorset, UK) in water; $n = 9$] or corresponding solutions of AZD1656 at either 3 mg·kg⁻¹ ($n = 9$) or 10 mg·kg⁻¹ ($n = 7$) over 29 days. Free-feeding blood glucose and PK profiles were measured over 12 h post-dose on days 1 and 28, and an oral glucose tolerance test (OGTT) was performed on day 14. Terminal blood samples were taken on day 29 for clinical chemistry analysis.

Study 2: long-term efficacy of GKA71 in HF-fed male gk^{wt/del} mice. Thirteen days prior to first administration of compound, 30 male *gk^{wt/del}* mice and 10 male wild-type mice were transferred from RM1 chow diet to a 60% HF diet (RD12492i, Research Diets, New Brunswick, NJ, USA). Another group of 10 male wild-type mice remained on RM1 chow. Body weights at the start of the study were in the range 26–33 g; average 29.7 g (*gk^{wt/del}*, HF diet), 30.2 g (wild-type, HF diet) and 30.6 g (wild-type, chow diet). *gk^{wt/del}* mice on HF diet were randomized into three groups ($n = 10$ per group) based on body weight, and baseline 24 h free-feeding glucose profiles

were measured at day 3. Three days later (day 0), two of these groups were switched to HF diet supplemented with GKA71 (formulated in RD12492i by Research Diets) to provide a daily dose of 2.5 or 5 mg·kg⁻¹, based on historical records of food intake in these mice. The third group was maintained on unsupplemented HF diet. Food intake was checked at the start and midway through the study. Compound exposures were confirmed by PK measurements throughout the course of the study.

Beginning on day 3 and continuing weekly up to week 49, blood glucose measurements were taken on all mice in the study, 1 h before start of dark phase. On day 5, and thereafter monthly up to month 11 (week 41), 24 h free-feeding blood glucose profiles were measured on *gk^{wt/del}* mice only, and on all mice at month 12 (week 45). PK profiles were measured in *gk^{wt/del}* mice for months 1–11. The study was terminated at the start of week 50, and blood samples were taken for blood chemistry analysis. Tissue samples (eyes, sciatic nerves, kidneys, liver, heart, skeletal muscle and pancreas) were collected from each animal and fixed in 10% neutral buffered formalin prior to processing into paraffin wax. Sections (4 µm thick) were stained with haematoxylin and eosin and evaluated by light microscopy.

Three *gk^{wt/del}* mice (all from 2.5 mg·kg⁻¹·day⁻¹ group) were withdrawn from this study on weeks 18, 19 and 39 due to injury sustained from fighting. The incidence of fighting is in line with our historical records for this animal model. Two wild-type mice from the HF diet control group were also withdrawn in weeks 32 and 43 for welfare reasons unrelated to treatment. In both cases, data from these mice were excluded from statistical analysis.

Table 1

GKA potencies in mouse, rat and human

Compound	AZD1656								GKA71		
	Human				Rat				Human	Mouse	
Dose (mg·kg ⁻¹ ·day ⁻¹) ^a	0.6	–	2.3	–	3	–	10	–	–	2.5	5
	1656	Met ^b	1656	Met	1656	Met	1656	Met			
GKA EC ₅₀ (µM) ^c	0.09	0.04	0.09	0.04	0.1	0.05	0.1	0.05	0.1	0.4	0.4
f _u ^d	0.056	0.136	0.056	0.136	0.041	0.041	0.041	0.041	0.013	0.0021	0.0021
C _{max} (µM)	1.1	0.12	5	0.47	3.5	0.87	6.9	2.6	–	33.7	53.1
C _{ss,av} (µM) ^e	0.3	0.1	1.7	0.2	0.3	0.2	1.6	0.7	–	26.2	47.1
C _{max,free} (µM)	0.062	0.016	0.28	0.064	0.14	0.036	0.28	0.11	–	0.071	0.11
C _{ss,av,free} (µM)	0.019	0.008	0.096	0.031	0.013	0.006	0.066	0.027	–	0.055	0.099
C _{max,free} /EC ₅₀	0.68	0.41	3.1	1.6	1.4	0.71	2.8	2.1	–	0.18	0.28
C _{ss,av,free} /EC ₅₀	0.21	0.21	1.1	0.76	0.13	0.13	0.66	0.54	–	0.14	0.25
Total C _{max,free} /EC ₅₀ ^f	1.1		4.7		2.1		5.0		–	0.18	0.28

^aHuman dose based on 70 kg individual (Morrow *et al.*, 2012), oral twice daily. Rat dose oral once daily. Mouse dosed with compound in diet.

^bMet = active metabolite AZD5658; (Morrow *et al.*, 2012).

^cEquipotent against hepatic and pancreatic forms of GK.

^df_u = fraction unbound.

^eC_{ss,av} = mean drug concentration over 24 h at steady state.

^fCombined AZD1656 + active metabolite activity.

Blood glucose measurements and OGTT

Free-feeding blood glucose profiles from blood microsamples were measured using a hand-held Accu-chek® glucose monitor (Roche Diagnostics, Burgess Hill, West Sussex, UK). For OGTTs, food was withdrawn 4 h before the test. OGTTs were performed by administration of 2 g·kg⁻¹ bolus of glucose (5 mL·kg⁻¹) 2 h after compound dosing (Baker *et al.*, 2014). Accu-chek blood glucose readings were taken at –120 min (baseline) and 5–90 min (after glucose bolus). Free-feeding blood glucose profile AUCs were calculated as total AUC, OGTT glucose and insulin. AUC values were corrected for differences in baseline at *t* = 0 min.

PK methods

To measure compound exposure, 20 µL blood samples were collected and mixed with 80 µL PBS pH 7.0. Protein precipitation with acetonitrile was used to extract compounds from plasma matrix. GKAs (including active metabolite) were quantified by HPLC-MS/MS. HPLC methods used a Phenomenex (Macclesfield, Cheshire UK) Synergi 4 µm max-RP column (50 × 2.0 mm), with a gradient of 5% methanol/95% 10 mM aqueous ammonium acetate (or formic acid), to 95% methanol/5% 10 mM aqueous ammonium acetate (or formic acid) at a flow rate of 0.7 mL·min⁻¹ over 4 min. Detection used a Waters (Elstree, Hertfordshire, UK) tandem quadrupole mass spectrometer.

Previously, we have shown that a GKA will lower blood glucose by an amount proportional to free drug concentration relative to the GK EC₅₀ (Coope *et al.*, 2006). Values for compounds GKA71 and AZD1656 (and its active metabolite AZD5658) are detailed in Table 1.

Blood and plasma chemistry

Insulin concentrations were measured using ELISA [Insulin (Mouse) Ultrasensitive ELISA, ALPCO Diagnostics, Salem, NH, USA]. Assays for total plasma cholesterol, lactate and triglycerides were performed as previously described (Gorman *et al.*, 2008). HbA1c was measured on a Variant II analyser (Bio-Rad, Hercules, CA, USA).

Tissue analysis

Hepatic triglyceride and glycogen concentrations were determined as described previously (Poucher *et al.*, 2007; Sörhede Winzell *et al.*, 2011).

Data analysis

Data collected on free-feeding glucose AUC measurements in male $gk^{wt/del}$ mice demonstrate that a 10% difference at $P < 0.05$ with 79% power can be detected with a group size of $n = 6$. Animals were randomized to initial experimental groups based on body weight. All results are presented as mean values \pm SEM for the indicated number of animals. Statistical analysis was performed using ANOVA adjusting for treatment groups, followed by pairwise *t*-tests using pooled estimates of variance. One-sided tests were performed for reductions in blood glucose and OGTT AUC values, and for changes in metabolic parameters. A two-sided test was applied to changes in body weight. Differences were considered significant at $P < 0.05$. Studies were powered assuming no multiple comparison procedures would be used, and statistical significance was judged based on raw *P*-values. Multiple comparison adjustments such as Bonferroni did not materially change the conclusions.

Materials

AZD1656 and GKA71 have been described previously (Waring *et al.*, 2011b; 2012) and were provided by AstraZeneca (Macclesfield, UK). EC₅₀ values against recombinant forms of GK are shown in Table 1.

Results

Study 1: chronic efficacy of AZD1656 in male obese Zucker fa/fa rats

Effect of AZD1656 on body weight. Male obese Zucker rats dosed orally with AZD1656 at either 3 or 10 mg·kg⁻¹·day⁻¹ showed a slight (non-significant) increase in body weight compared with animals receiving vehicle (Figure 1) over the 28 days of the study.

Effect on free-feeding glucose profiles. AZD1656 dose-dependently decreased glucose AUC_(0–12 h) in free-feeding blood glucose profiles after 1 day (Figure 2A,B) and 28 days (Figure 2C,D) dosing, compared with vehicle. Rats dosed with AZD1656 at 10 mg·kg⁻¹·day⁻¹ for 28 days also showed a small but significant reduction in pre-dose glucose levels compared with vehicle (5.6 ± 0.3 vs. 6.3 ± 0.2 mM, $P < 0.05$). AZD1656 forms an active metabolite AZD5658 *in vivo* that has equivalent glucose-lowering efficacy (Morrow *et al.*, 2012; Waring *et al.*, 2012). At both days 1 and 28, total free combined levels

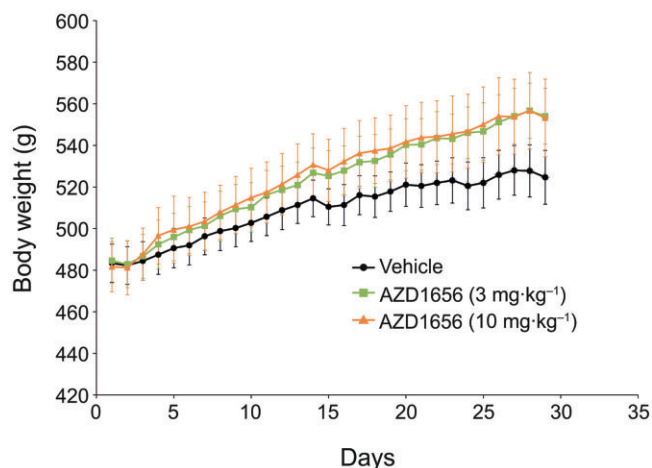


Figure 1

Effect of AZD1656 at 3 mg·kg⁻¹·day⁻¹ ($n = 9$) and 10 mg·kg⁻¹·day⁻¹ ($n = 7$) or vehicle ($n = 9$) on body weight gain over 29 days in male obese (*fa/fa*) Zucker rats. Data are mean \pm SEM.

of AZD1656 and active metabolite exceeded the efficacy threshold for 5–6 h at 3 mg·kg⁻¹ and > 12 h at 10 mg·kg⁻¹ doses.

Effect of AZD1656 on OGTT. AZD1656 gave a dose-dependent reduction in baseline-corrected glucose excursion (Figure 3A, B) in an OGTT performed at day 14. AZD1656 did not further stimulate insulin secretion at 20 min post-glucose bolus compared with vehicle (Figure 3C).

Effect of AZD1656 on lipids. Analysis of terminal blood samples (day 29) showed that AZD1656 had no effect on plasma cholesterol, lactate or triglyceride concentrations (Table 2).

Study 2: chronic efficacy of GKA71 in HF-fed male $gk^{wt/del}$ mice

Effect of GKA71 on body weight gain. The body weights of $gk^{wt/del}$ mice on HF diet increased in a biphasic manner consistent with established diet-induced obesity (Sörhede Winzell and Ahrén, 2004), with a slower rate of increase after about 17 weeks. GKA71 at 2.5 mg·kg⁻¹·day⁻¹ did not have a significant effect on body weight gain over the study period (Figure 4). At a dose of 5 mg·kg⁻¹·day⁻¹ GKA71, there was a downward trend in mean body weight for the treatment group after week 41 attributable to weight decrease in three animals, such that significance ($P < 0.05$) for the group as a whole was reached at week 49.

Effect of GKA71 on weekly blood glucose levels. Baseline blood glucose values in $gk^{wt/del}$ mice on HF diet were 16.0 ± 0.2 mM, compared with 9.9 ± 0.5 mM for wild-type mice on HF diet and 8.2 ± 0.2 mM on chow diet. Over the 49 weeks of the study, mean blood glucose values of $gk^{wt/del}$ mice receiving HF diet alone remained within a range of 13.5–18.3 mM

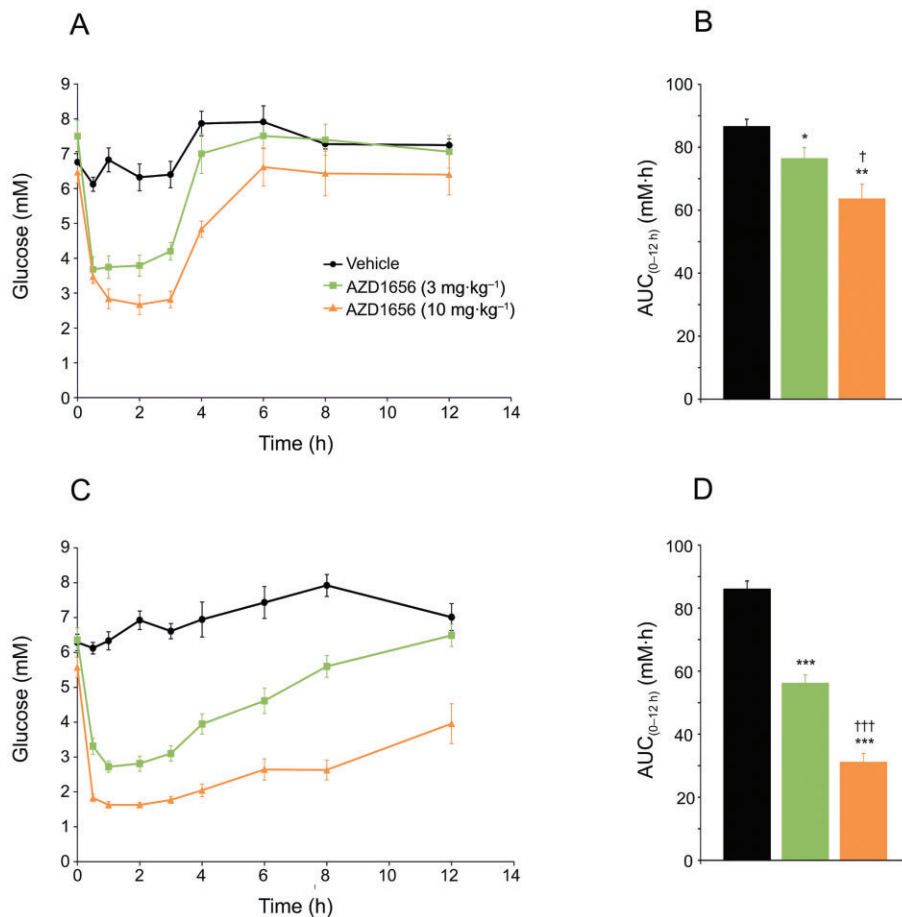


Figure 2

Blood glucose lowering in male obese (*fa/fa*) Zucker rats after daily dosing of AZD1656 at 3 mg·kg⁻¹·day⁻¹ (*n* = 9) and 10 mg·kg⁻¹·day⁻¹ (*n* = 7) or vehicle (*n* = 9). (A) 12 h free-feeding blood glucose profiles at day 1. Sample sizes: 3 mg·kg⁻¹·day⁻¹, *n* = 9; 10 mg·kg⁻¹·day⁻¹, *n* = 6; vehicle, *n* = 9. (B) Glucose AUC_(0-12h) values (mM·h) at day 1. (C) 12 h free-feeding blood glucose profiles at day 28. Sample sizes: 3 mg·kg⁻¹·day⁻¹, *n* = 9; 10 mg·kg⁻¹·day⁻¹, *n* = 7; vehicle, *n* = 9. (D) Glucose AUC_(0-12h) values (mM·h) at day 28. Data are mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 versus vehicle; †*P* < 0.05, †††*P* < 0.001 versus AZD1656 at 3 mg·kg⁻¹·day⁻¹.

Table 2

Study 1: terminal metabolic parameters in plasma samples from obese (*fa/fa*) and lean (*fa/+*) male Zucker rats

Genotype	Treatment	Total cholesterol (mM) (<i>n</i>)	Lactate (mM) (<i>n</i>)	Triglycerides (mM) (<i>n</i>)
<i>fa/fa</i>	Vehicle	18.76 ± 5.37 (9)	4.69 ± 0.19 (8)	4.92 ± 1.98 (9)
<i>fa/fa</i>	AZD1656 (3 mg·kg ⁻¹ ·day ⁻¹)	13.91 ± 3.16 (8)	5.09 ± 0.50 (8)	5.93 ± 2.07 (8)
<i>fa/fa</i>	AZD1656 (10 mg·kg ⁻¹ ·day ⁻¹)	15.64 ± 4.46 (7)	4.91 ± 0.60 (7)	4.75 ± 1.72 (7)
<i>fa/+</i>	Vehicle	2.41 ± 0.11 ^a (10)	4.58 ± 0.43 (10)	1.00 ± 0.10 ^b (10)

Values are mean ± SEM.

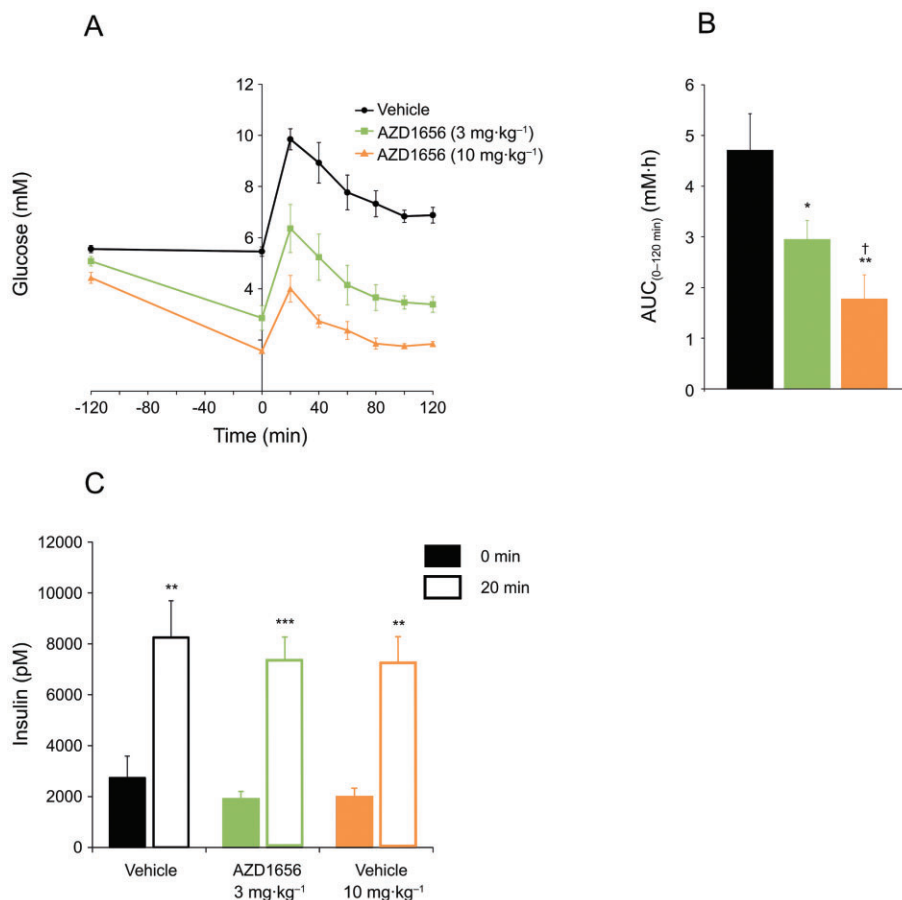
^a*P* < 0.01 versus *fa/fa* vehicle.

^b*P* < 0.05 versus *fa/fa* vehicle.

(Figure 5). The range for wild-type mice on HF diet was 7.1–9.9 mM, and on standard chow diet 7.0–10.5 mM.

Glucose lowering stabilized within 2 weeks in animals receiving GKA71 in the diet (Figure 5). Over the remaining 47 weeks of the study, mean glucose values for *gk^{wt/del}* mice

receiving GKA71 in the diet at either dose were significantly lower (*P* < 0.001) than animals on HF diet alone (Table 3). GKA71 reduced mean blood glucose by 2.4 ± 0.3 mM at 2.5 mg·kg⁻¹·day⁻¹ and 3.2 ± 0.3 at 5 mg·kg⁻¹·day⁻¹, compared with control *gk^{wt/del}* mice on HF diet. There was a difference

**Figure 3**

Blood glucose and insulin levels following OGTT in male obese (*fa/fa*) Zucker rats after 14 days dosing of AZD1656 at 3 mg·kg⁻¹·day⁻¹ (*n* = 9) and 10 mg·kg⁻¹·day⁻¹ (*n* = 7) or vehicle (*n* = 9). (A) Glucose levels during OGTT. Sample sizes: 3 mg·kg⁻¹·day⁻¹, *n* = 9; 10 mg·kg⁻¹·day⁻¹, *n* = 7; vehicle, *n* = 9. (B) Glucose AUC_(0-12 h) values (mM·h). Data are mean ± SEM. **P* < 0.05, ***P* < 0.01 versus vehicle, †*P* < 0.01, versus AZD1656 at 3 mg·kg⁻¹·day⁻¹. (C) Insulin levels at *t* = 0 and *t* = 20 min during OGTT. Sample sizes: 3 mg·kg⁻¹·day⁻¹, *n* = 8; 10 mg·kg⁻¹·day⁻¹, *n* = 8; vehicle: *n* = 9 (*t* = 0 min); 3 mg·kg⁻¹·day⁻¹, *n* = 8; 10 mg·kg⁻¹·day⁻¹, *n* = 6; vehicle, *n* = 9 (*t* = 20 min). Data are mean ± SEM. ***P* < 0.01, ****P* < 0.001 versus *t* = 0 min.

Table 3

Study 2: mean blood glucose levels in male *gk^{wt/del}* and wild-type mice over weeks 2–49

Genotype	Diet	Mean blood glucose (mM) (<i>n</i>)	Mean total GKA71 over 24 h interval (μM)	Free drug/EC ₅₀
<i>gk^{wt/del}</i>	HF	15.1 ± 0.2 (10)	–	–
<i>gk^{wt/del}</i>	HF + GKA71 (2.5 mg·kg ⁻¹ ·day ⁻¹)	12.7 ± 0.1 ^{a,b} (7)	21.64 ± 1.20	0.21 ± 0.01
<i>gk^{wt/del}</i>	HF + GKA71 (5 mg·kg ⁻¹ ·day ⁻¹)	11.9 ± 0.7 ^a (10)	39.70 ± 2.35	0.11 ± 0.006
<i>gk^{wt/wt}</i>	HF	8.4 ± 0.1 (8)	–	–
<i>gk^{wt/wt}</i>	Chow	8.8 ± 0.1 (10)	–	–

Values are mean ± SEM.

^a*P* < 0.001 versus *gk^{wt/del}* on HF diet.

^b*P* < 0.01 versus *gk^{wt/del}* on HF diet + GKA71 (5 mg·kg⁻¹·day⁻¹).

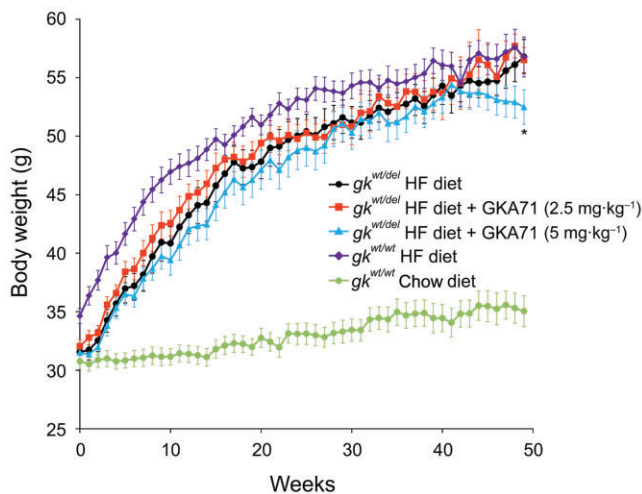


Figure 4

Body weight gain over 49 weeks in male $gk^{wt/del}$ mice on HF diet ± GKA71 and male wild-type mice on HF or chow diets: $gk^{wt/del}$ mice on HF diet alone ($n = 10$), $gk^{wt/del}$ mice on HF + GKA71 at $2.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ($n = 7\text{--}10$), $gk^{wt/del}$ mice on HF + GKA71 at $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ($n = 10$), wild-type mice on HF alone ($n = 8\text{--}10$), wild-type mice on chow diet alone ($n = 10$). Data are mean ± SEM. * $P < 0.05$ versus $gk^{wt/del}$ mice on HF diet alone.

($0.8 \pm 0.3 \text{ mM}$, $P < 0.01$) in mean blood glucose levels between the two dose groups over the study. Blood glucose levels were lower compared with the HF diet control group for all weeks except weeks 5, 28, 29, 34, 36, 44 and 46–49 for the $2.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ GKA71 group, and weeks 29, 34, 36 and 47 for the $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ GKA71 group.

Mean daily systemic exposure of GKA71 obtained for weeks 1–45 is shown in Table 3. Exposure was proportional to dose. Drug concentration in animals receiving $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ was above the efficacy threshold at all time points (Figure 5B), while for the group dosed at $2.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, 24 h drug concentration was significantly lower over the entire experiment, and only reached the efficacy threshold at weeks 17, 21 and 25 (Figure 5A).

Effect of GKA71 on monthly 24 h free-feeding blood glucose profiles. Once a month, 24 h free-feeding blood glucose profiles (–1 to 23 h) were measured. $gk^{wt/del}$ mice randomized to groups receiving HF diet alone, or HF + 2.5 or $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ GKA71 showed no significant difference in pre-study baseline glucose profiles (Figure 6A). Free-feeding glucose profiles at months 1, 3, 6, 9 and 12 are shown in Figure 6B–F. For animals receiving $2.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ GKA71, significant glucose lowering was achieved at all time points of the monthly glucose profile except for months 1 and 12 (see Figure 6B,F). For mice dosed at $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, significant glucose lowering was achieved at all time points at all months. Glucose $\text{AUC}_{(-1 \text{ to } 23 \text{ h})}$ values for all monthly glucose profiles are shown in Figure 6G. The mean glucose $\text{AUC}_{(-1 \text{ to } 23 \text{ h})}$ value averaged over the 12 month treatment period for mice receiving HF diet + $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ GKA71 ($260.2 \pm 3.9 \text{ mM}\cdot\text{h}$) was lower than that of mice on HF diet + $2.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (283.2 ± 2.9 , $P < 0.001$), and both values were lower compared with mice on HF diet alone

(328.5 ± 2.6 , $P < 0.001$). It should be noted that the glucose $\text{AUC}_{(-1 \text{ to } 23 \text{ h})}$ value for mice receiving HF diet alone at month 12 was significantly lower than the mean $\text{AUC}_{(-1 \text{ to } 23 \text{ h})}$ value for this group over the whole study ($P < 0.01$).

Effect of GKA71 on glucose tolerance. At week 49, an OGTT was given to all mice in the study. Four hours fasted blood glucose levels were lower for mice dosed at $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ GKA 71 ($14.4 \pm 0.5 \text{ mM}$) versus controls (16.9 ± 1.0 , $P < 0.05$), but not at $2.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ ($15.7 \pm 0.6 \text{ mM}$). Glucose clearance was slower in both $gk^{wt/del}$ and wild-type mice on HF diet compared with chow diet, consistent with diet-induced insulin resistance in this model (Gorman *et al.*, 2008), and similar with untreated and treated $gk^{wt/del}$ mice (Figure 7A). Glucose $\text{AUC}_{(0\text{--}90 \text{ min})}$ values for $gk^{wt/del}$ mice on HF diet + GKA71 were not significantly lower than mice on HF diet alone. As expected, the $\text{AUC}_{(0\text{--}90 \text{ min})}$ value for wild-type littermates on HF diet was significantly lower than HF-fed $gk^{wt/del}$ mice ($P < 0.001$) and also lower than wild-type mice on chow diet (Figure 7B).

Insulin levels during the OGTT are shown in Figure 7C. The insulin excursions in animals on HF + 2.5 or $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ GKA71 were not significantly different than mice on HF diet alone (Figure 7D).

Effect of GKA71 on lipids and glycated haemoglobin (HbA1c). GKA71 at 2.5 and $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ did not affect terminal concentrations of plasma cholesterol, plasma triglyceride, hepatic triglyceride or hepatic glycogen content compared with control $gk^{wt/del}$ mice on HF diet alone (Table 4). At the end of the study, GKA71 at both doses had significantly reduced blood % HbA1c levels compared with controls (Table 4).

Histopathology. A slight reduction in hepatocyte vacuolation from ‘marked’ in the $gk^{wt/del}$ mice given HF diet to ‘moderate’ in HF-fed $gk^{wt/del}$ animals on $5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ was seen histologically. No significant differences were observed in other tissues examined. HF feeding of $gk^{wt/wt}$ or $gk^{wt/del}$ mice resulted in substantially elevated hepatocellular cytoplasmic lipid vacuolation in comparison with the former strain-fed chow diet, which is reflected in elevated hepatic triglycerides (Table 4).

Discussion

Recent dose-ranging clinical studies with GKAs in differing type 2 diabetic cohorts (Meininger *et al.*, 2011; Kiyosue *et al.*, 2013; Wilding *et al.*, 2013) have raised questions about whether this class of agents can elicit chronic glucose control. We have explored the ability of GKAs (AZD1656 and GKA71) to deliver sustained glucose control in two different animal models of insulin resistance and T2D. The male obese Zucker (*fa/fa*) rat was chosen to explore the effects of a GKA on long-term glucose control and lipid metabolism in a hyperlipidaemic environment (Kasiske *et al.*, 1992; King, 2012). However, Zucker rats are not hyperglycaemic. Therefore, HF-fed male $gk^{wt/del}$ mouse was selected to investigate the effects of chronic GKA administration in the context of stable hyperglycaemia (Baker *et al.*, 2014).

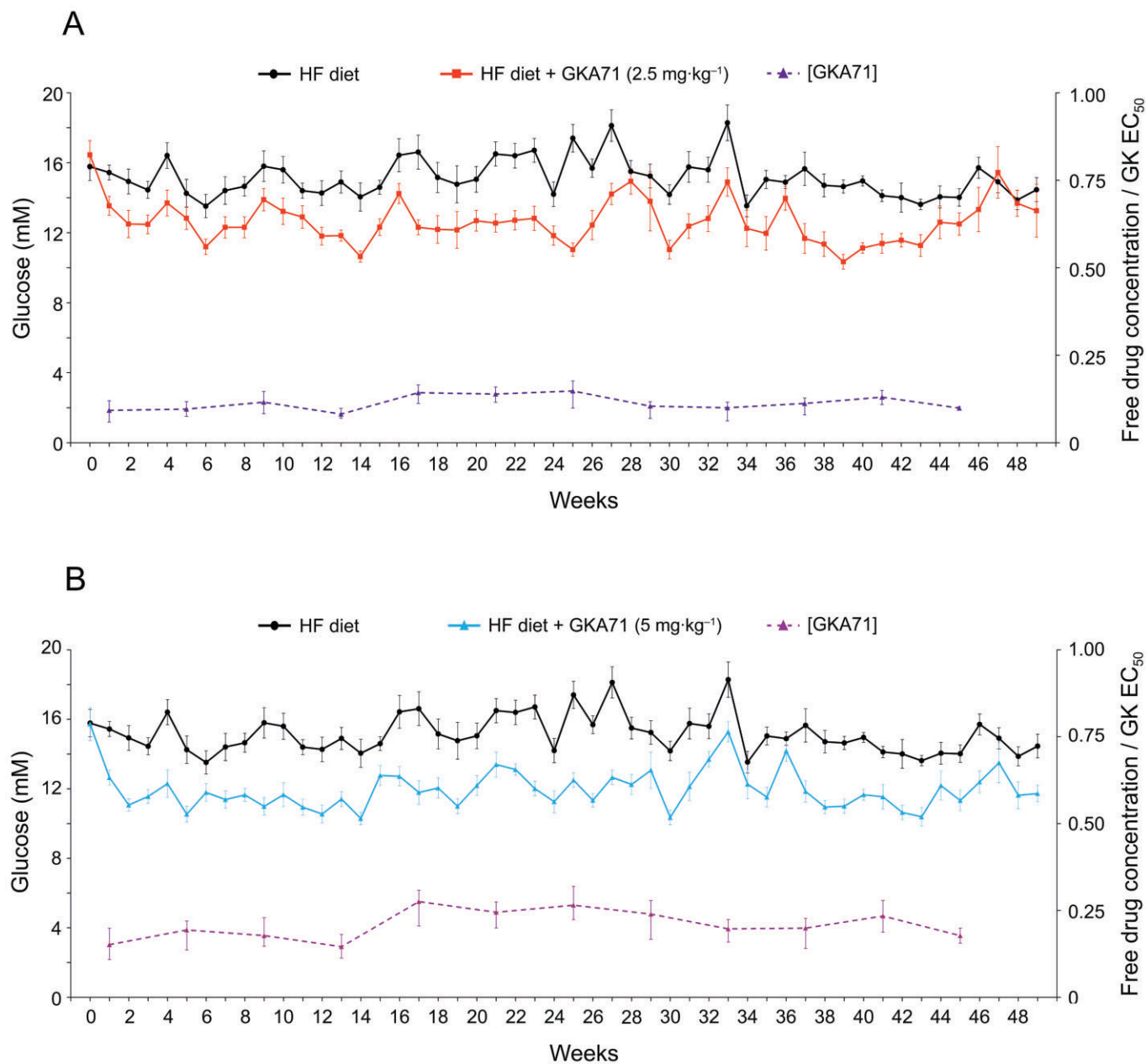


Figure 5

Weekly blood glucose values (1 h before start of dark phase) and compound exposures over 49 weeks in male *gk^{wt/del}* mice on HF diet ± GKA71. (A) Blood glucose values for mice on HF diet alone ($n = 10$) or HF diet + GKA71 at 2.5 mg·kg⁻¹·day⁻¹ ($n = 7-10$), and compound exposure, expressed as a multiple of the mouse EC₅₀. (B) Blood glucose values for mice on HF diet alone or HF diet + GKA71 at 5 mg·kg⁻¹·day⁻¹ ($n = 10$), and compound exposure. Blood glucose values are mean ± SEM. Compound exposure ratios are mean values over 24 h. Error bars indicate C_{max} and C_{min}. Week 0 values show mean baseline glucose in control and treatment groups at day -3 relative to diet switch. Three animals from the 2.5 mg·kg⁻¹·day⁻¹ GKA71 group were removed during the study as noted under Methods.

AZD1656 and GKA71 have very similar physico-chemical properties and are close analogues (Waring *et al.*, 2011b; 2012), AZD1656 being slightly more potent. Acute studies in rodents have demonstrated that these compounds have similar PK/pharmacodynamic (PD) relationships (unpublished results). AZD1656 has been characterized in both acute and repeat-dose studies in humans (Ericsson *et al.*, 2012;

Morrow *et al.*, 2012; Norjavaara *et al.*, 2012; Kiyosue *et al.*, 2013; Wilding *et al.*, 2013).

In the obese Zucker *fa/fa* rat, AZD1656 gave a sustained reduction in free-feeding glucose profiles over 28 days, but only delivered a modest reduction in pre-dose glucose levels at 10 mg·kg⁻¹·day⁻¹, where compound exposure is sufficient to drive glucose-lowering efficacy for >12 h. At this dose, the

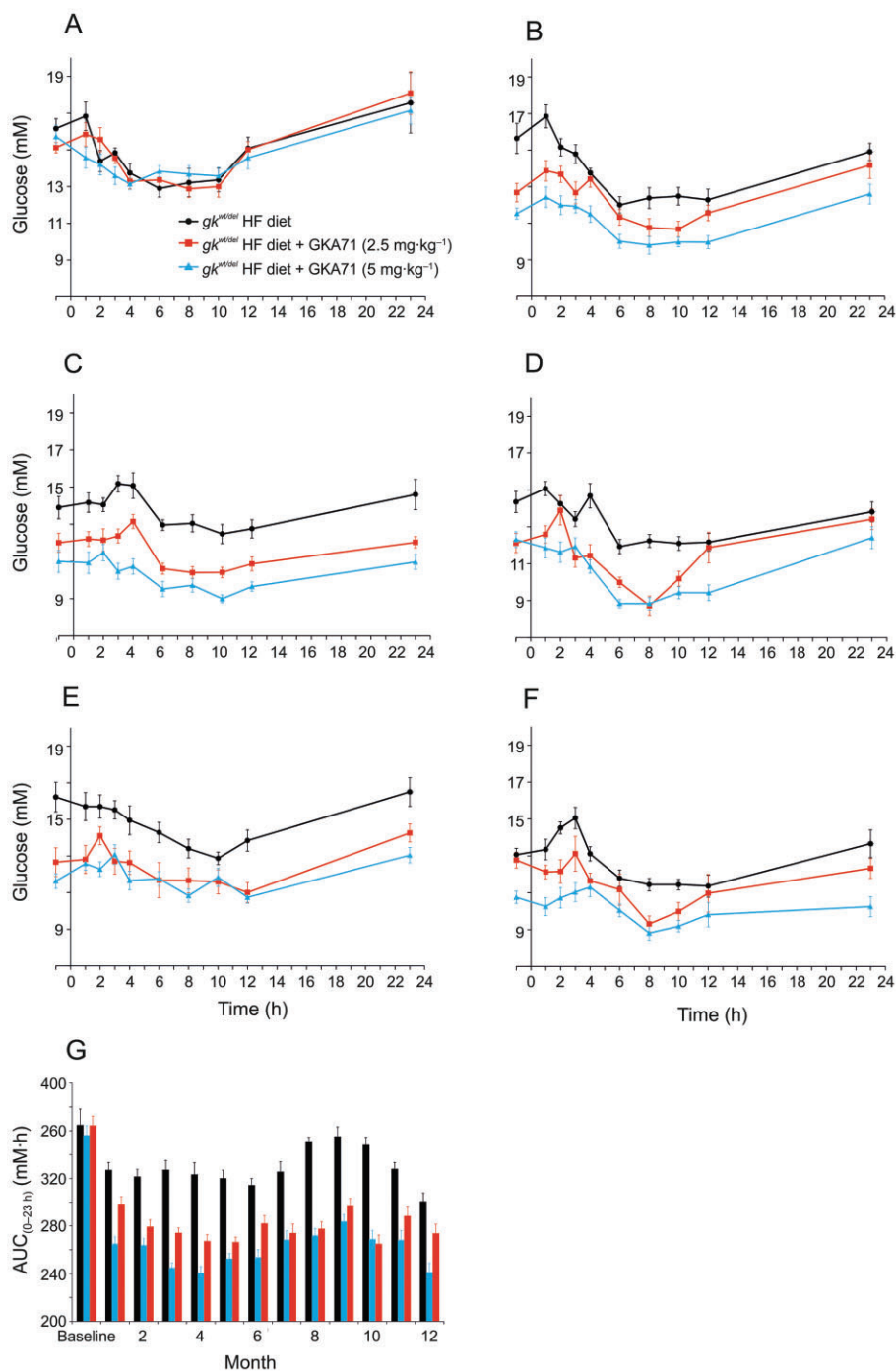
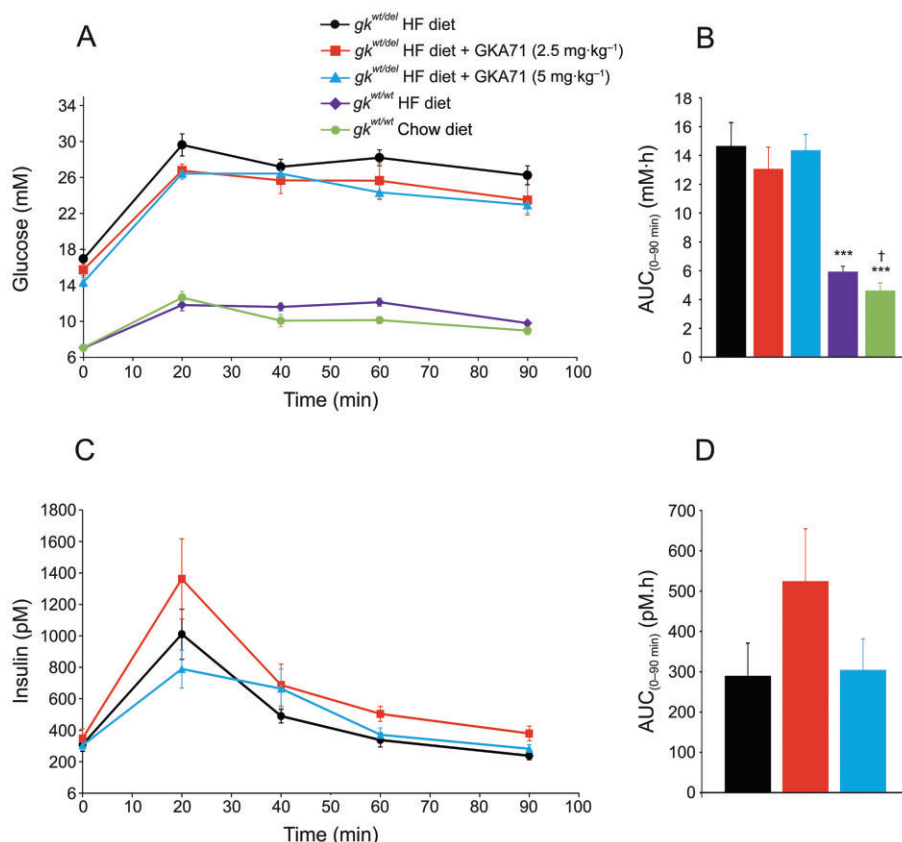


Figure 6

Twenty-four hour free-feeding blood glucose profiles for male $gk^{wt/del}$ mice on HF diet \pm GKA71. (A) Baseline, (B) 1 month, (C) 3 months, (D) 6 months, (E) 9 months, (F) 12 months. Mice on HF diet alone ($n = 10$), HF diet + GKA71 at 2.5 mg·kg⁻¹·day⁻¹ ($n = 7-10$), HF diet + GKA71 at 5 mg·kg⁻¹·day⁻¹ ($n = 10$). Data are mean \pm SEM. (G) Glucose AUC_(-1 to 23 h) values (mM·h) for all monthly free-feeding glucose profiles over 12 months. Data are mean \pm SEM.

free drug level of AZD1656 is comparable with the highest dose used in the multiple ascending dose study reported by Morrow *et al.*, (2012), and is at the higher end of the dose range used in dose-finding studies (Kiyosue *et al.*, 2013; Wilding *et al.*, 2013). In normal rats and mice, AZD1656 and

other GKAs give sustained reductions in blood glucose (Ohyama *et al.*, 2010; unpublished results), but obese Zucker rats are extremely insulin-resistant (Kasiske *et al.*, 1992; King, 2012) and GKAs have a smaller effect on pre-dose blood glucose.

**Figure 7**

Blood glucose and insulin levels following OGTT in male $gk^{wt/del}$ mice on HF diet \pm GKA71 and wild-type littermates on HF or chow diet after 49 weeks. (A) Glucose levels during OGTT. $gk^{wt/del}$ mice on HF diet alone ($n = 10$), HF diet + GKA71 at 2.5 mg·kg⁻¹·day⁻¹ ($n = 7$), HF diet + GKA71 at 5 mg·kg⁻¹·day⁻¹ ($n = 10$). Wild-type littermates on HF diet alone ($n = 8$), chow diet alone ($n = 10$). (B) Glucose AUC_(0-90 min) values (mM·h). (C) Insulin levels during OGTT. (D) Insulin AUC_(0-90 min) values (pM·h). Data are mean \pm SEM. *** $P < 0.001$ versus $gk^{wt/del}$ mice on HF diet alone, † $P < 0.05$, versus wild-type on HF diet.

Table 4

Study 2: terminal metabolic parameters from male $gk^{wt/del}$ mice and $gk^{wt/wt}$ littermates

Genotype	Diet	Plasma total cholesterol (mM) (n)	Plasma triglycerides (mM) (n)	Hepatic triglycerides (μ mol·g ⁻¹) (n)	Hepatic glycogen (μ mol·g ⁻¹) (n)	HbA1c (%) (n)
$gk^{wt/del}$	HF	6.40 \pm 0.63 (7)	1.02 \pm 0.06 (7)	0.19 \pm 0.04 (8)	26.34 \pm 7.46 (9)	7.97 \pm 0.16 (9)
$gk^{wt/del}$	HF + GKA71 (2.5 mg·kg ⁻¹ ·day ⁻¹)	5.96 \pm 0.40 (6)	1.03 \pm 0.08 (6)	0.29 \pm 0.06 (6)	23.84 \pm 3.35 (7)	7.37 \pm 0.22 ^a (7)
$gk^{wt/del}$	HF + GKA71 (5 mg·kg ⁻¹ ·day ⁻¹)	5.29 \pm 0.72 (8)	1.00 \pm 0.14 (8)	0.25 \pm 0.04 (10)	25.95 \pm 6.00 (10)	7.09 \pm 0.29 ^a (7)
$gk^{wt/wt}$	HF	8.41 \pm 0.41 ^a (6)	1.02 \pm 0.12 (6)	0.34 \pm 0.06 ^a (8)	19.59 \pm 5.27 (7)	5.73 \pm 0.09 ^b (7)
$gk^{wt/wt}$	Chow	2.88 \pm 0.10 ^b (9)	1.26 \pm 0.07 (9)	0.06 \pm 0.01 ^b (9)	7.70 \pm 3.62 ^a (8)	6.22 \pm 0.07 ^b (8)

Values are mean \pm SEM.

^a $P < 0.05$ versus $gk^{wt/del}$ on HF diet.

^b $P < 0.001$ versus $gk^{wt/del}$ on HF diet.

To explore the long-term efficacy of a GKA at human translatable exposures in a rodent model with stable hyperglycaemia, we selected the HF-fed male $gk^{wt/del}$ mouse model (Baker *et al.*, 2014). GKA71 has previously been shown to

reduce basal glucose over 4 weeks of dietary dosing in the DIO mouse (Sörhede Winzell *et al.*, 2011). However, the mice in this study were not markedly hyperglycaemic (8.8 \pm 0.4 mM) and the glucose phenotype in the DIO model

changes following prolonged HF feeding (Sörhede Winzell and Ahrén, 2004), making it unsuitable for an extended study. $gk^{wt/del}$ mice in study 2 remained consistently hyperglycaemic with an average blood glucose concentration in the HF diet control group over the study period of 15.1 ± 0.2 mM. GKA71 at both 2.5 and 5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ delivered a sustained and significant reduction in plasma glucose for at least 45 weeks, together with a significant reduction in AUC values for monthly free-feeding blood glucose profiles up to week 45 and a significant reduction ($P < 0.05$) in glycated haemoglobin of ~ 0.6 to 0.9% respectively. Free exposure of GKA71, as a multiple of the EC_{50} , at 5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, was consistently at levels associated with glucose lowering based on the defined relationship between exposure and *in vitro* GK enzyme potency (Coope *et al.*, 2006), and slightly lower than the multiple obtained with AZD1656 in the clinic (Table 1). Exposure at 2.5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ was significantly lower ($P < 0.05$; approximately 50% in magnitude), thus accounting for the correspondingly lower glucose-lowering efficacy observed at this dose.

Between weeks 45 and study termination, there is a suggestion in Figure 5A that efficacy may be diminishing in the mice receiving 2.5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ GKA71, although this may also be interpreted as random fluctuation in glucose levels, as similar transient reductions in efficacy are seen over the course of the study. Unfortunately we did not measure drug exposure or food intake after week 45, but there was no decline in body weight at this dose during the last 4 weeks of the study, suggestive of no decrease in food intake and therefore drug dosage. However, we cannot be sure that there was no change in drug exposure between weeks 45 and 49. Efficacy is still present in the group receiving 5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, although in this group there is a downward trend in mean body weight for the final few weeks of the study. The free-feeding glucose profile at week 45 gave $AUC_{(-1 \text{ to } 23 \text{ h})}$ values for both doses of GKA71 in line with corresponding values from previous months (Figure 7). However, the $AUC_{(-1 \text{ to } 23 \text{ h})}$ value at week 45 for mice on HF diet alone was significantly lower than the study mean for this group. Some strains of mice (including C57Bl/6) maintained for a long period on HF diet ($>> 4$ weeks) are known to adapt and show improvements in glucose homeostasis (Sörhede Winzell and Ahrén, 2004; Park *et al.*, 2005; Ko *et al.*, 2011; Sakurai *et al.*, 2012). It is possible that we are seeing a similar effect beginning to appear in this study, albeit after a much longer period of stability.

Study 2 clearly demonstrated that GKA71 delivered sustained exposure-dependent efficacy in the male $gk^{wt/del}$ mouse model for at least 45 weeks. This finding is consistent with other chronic studies with GKAs reported in rodent models. Nakamura *et al.* (2009, 2011) showed that GKAs gave sustained glucose lowering up to 40 weeks in hyperglycaemic (~ 11 mM) beta cell-specific GK knockout mice on HF diet. A different GKA gave sustained glucose lowering over 5 weeks at a dose of 10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ but not at 3 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ in the progressively diabetic male Zucker diabetic fatty (ZDF) model (Futamura *et al.*, 2012).

After 4 weeks treatment with AZD1656 in obese Zucker rats, we found neither increase in plasma total cholesterol and plasma triglycerides, nor any increase in plasma lactate levels. After 50 weeks dosing with GK71 in HF-fed $gk^{wt/del}$ mice, we also found neither increase in plasma total cholesterol and

triglycerides, nor did we find any evidence for increased hepatic steatosis compared with that due to HF diet alone. We also found no evidence for elevation of hepatic glycogen above that associated with the HF diet alone. These findings are consistent with previous studies (Nakamura *et al.*, 2009; 2011; Eiki *et al.*, 2011; Sörhede Winzell *et al.*, 2011; Futamura *et al.*, 2012). Recently, De Ceuninck *et al.* (2013) have reported that a number of GKAs related to the benzoyl amino nicotinic acid GKA50 (McKerrecher *et al.*, 2006) increase hepatic and plasma triglycerides. We have also observed elevated plasma triglycerides after dosing GKA50 (3 and 10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) in obese Zucker rats (8 days) and ZDF rats (35 days; unpublished results). However, GKA50 and some other compounds from the benzoyl amino nicotinic acid series were found to have secondary pharmacology and a glucose-lowering efficacy that did not follow the established PK/PD relationship (Coope *et al.*, 2006). GKA50, for example, is both a retinoic acid receptor antagonist (Waring *et al.*, 2011a) and agonist. These findings led to the discovery and development of the neutral benzamide GKAs (Waring *et al.*, 2011b; 2012), including AZD1656 and GKA71. Both GKAs are free of this secondary pharmacology with no effect on plasma lipids or hepatic triglyceride levels in efficacy studies such as those reported here, or at higher doses in safety studies performed in rats and dogs (unpublished results). In the clinic, AZD1656 induced a small transient rise in triglycerides in a diverse European and Latin American population (Wilding *et al.*, 2013), but had no effect on triglycerides as monotherapy in a Japanese cohort (Kiyosue *et al.*, 2013). The issue of triglyceride accumulation with GKAs therefore appears inconclusive, and is likely to benefit from further investigation.

AZD1656 gave a significant improvement in the glucose excursion profile after an OGTT at 2 weeks in the obese Zucker rat, as assessed by total glucose AUC and AUC after adjusting for differences in baseline glucose at $t = 0$ min. In contrast, although GKA71 lowered the total glucose AUC in an OGTT in HF-fed $gk^{wt/del}$ mice at 49 weeks there was neither significant reduction in the AUC after correcting for differences in baseline glucose, nor was there any change in the glucose elimination constant (K_e ; Sörhede Winzell and Ahrén, 2004). In study 1, AZD1656 was administered orally to fasted rats 120 min before the glucose bolus, and the free drug concentration over the period of the OGTT was significantly above the efficacy threshold. In study 2, drug was administered in the diet, which was withdrawn 4 h before the OGTT, so free drug levels during the glucose challenge are predicted to be below the efficacy threshold for both doses. This would in part explain the lack of marked efficacy in the OGTT. However, the lack of any effect of GKA71 on glucose tolerance over the 49 weeks treatment period, despite the sustained reduction in pre-feeding blood glucose, indicated that this GKA did not improve overall insulin sensitivity. Nakamura *et al.* (2009) have reported that a different GKA did improve glucose tolerance after 20 weeks treatment in the beta cell haploinsufficient $gk^{wt/del}$ mouse, although exposure was not reported. Neither AZD1656 nor GKA71 gave any improvement in insulin secretion in response to the oral glucose challenge, suggesting that these GKAs may be controlling blood glucose concentration primarily by a reduction in net hepatic glucose output. Histopathological examination

of pancreatata from mice in study 2 showed no detrimental findings associated with treatment with GKA71. Hence, we conclude that long-term treatment with GKAs has no significant adverse functional effects on the aging pancreas. In support of this conclusion, it has recently been reported that a GKA can still increase beta cell proliferation rates in 1- to 2-year-old mice (Stolovich-Rain *et al.*, 2012).

There is a growing view that GKA-mediated glucose-lowering efficacy diminishes over time in diabetic patients. However, almost all preclinical animal model data, including current studies, support sustained efficacy for GKA-mediated glucose-lowering efficacy. Compelling evidence on human target validation from GK-activating mutations in humans also indicates that GK activation delivers sustained glucose lowering (Osbaek *et al.*, 2009). Therefore, these contradictory data question either the value of animal models of diabetes as predictors of long-term responses in patients or the extent of our understanding of this mechanism in humans. GKAs mediate short-term perturbations in fluxes in and out of the hepatic triglyceride and glycogen pools (Nissim *et al.*, 2012), and it is certainly possible that these will differ between rodents and humans. For example, triglyceride synthesis in man relies less on *de novo* lipogenesis than in rodents (Murphy, 2006) and also varies significantly between lean and obese human subjects (Donnelly *et al.*, 2005). Further studies on hepatic metabolic flux *in vivo* may be the key to a better understanding of how GKAs achieve glycaemic control in rodents and humans, and their potential for safe and sustained glucose lowering in patients with T2D.

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Conflicts of interest

Authors are either past or present employees of AstraZeneca and hold stock in the company.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.12504>

Figure S1 Study design: Study 1.

Figure S2 Study design: Study 2.