

RESEARCH PAPER

Characterization of the heterozygous glucokinase knockout mouse as a translational disease model for glucose control in type 2 diabetes

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BACKGROUND AND PURPOSE

The global heterozygous glucokinase (GK) knockout ($gk^{wt/del}$) male mouse, fed on a high-fat (60% by energy) diet, has provided a robust and reproducible model of hyperglycaemia. This model could be highly relevant to some facets of human type 2 diabetes (T2D). We aimed to investigate the ability of standard therapeutic agents to lower blood glucose at translational doses, and to explore the glucose-lowering potential of novel glucokinase activators (GKAs) in this model.

EXPERIMENTAL APPROACH

We measured the ability of insulin, metformin, glipizide, exendin-4 and sitagliptin, after acute or repeat dose administration, to lower free-feeding glucose levels in $gk^{wt/del}$ mice. Further, we measured the ability of novel GKAs, GKA23, GKA71 and AZD6370 to control glucose either alone or in combination with some standard agents.

KEY RESULTS

A single dose of insulin (1 unit·kg⁻¹), metformin (150, 300 mg·kg⁻¹), glipizide (0.1, 0.3 mg·kg⁻¹), exendin-4 (2, 20 µg·kg⁻¹) and GKAs reduced free-feeding glucose levels. Sitagliptin (10 mg·kg⁻¹), metformin (300 mg·kg⁻¹) and AZD6370 (30, 400 mg·kg⁻¹) reduced glucose excursions on repeat dosing. At a supra-therapeutic dose (400 mg·kg⁻¹), AZD6370 also lowered basal levels of glucose without inducing hypoglycaemia.

CONCLUSION AND IMPLICATIONS

Standard glucose-lowering therapeutic agents demonstrated significant acute glucose lowering in male $gk^{wt/del}$ mice at doses corresponding to therapeutic free drug levels in man, suggesting the potential of these mice as a translatable model of human T2D. Novel GKAs also lowered glucose in this mouse model.

Abbreviations

DPP-IV, dipeptidylpeptidase-IV; GK, glucokinase; GKA, glucokinase activator; GLP-1, glucagon-like peptide-1; HF, high fat; HGO, hepatic glucose output; OGTT, oral glucose tolerance test; PK, pharmacokinetic; T2D, type 2 diabetes; ZDF, Zucker diabetic fatty

Introduction

Many animal models of type 2 diabetes (T2D) such as Zucker diabetic fatty (ZDF) rats, and *ob/ob* or *db/db* mice have defects in circulating leptin or leptin signalling. This leptin dependency weakens the translational value of such models as humans with T2D are likely to be obese and leptin-resistant (Miyazaki *et al.*, 2002; DeFronzo, 2010). In the male ZDF rat, there is a rapid rate of progression to diabetes such that pharmacological treatments frequently have to be initiated within a limited age window, and differences in timing can affect experimental outcomes (Smith *et al.*, 2000). In the *ob/ob* or *db/db* mouse model, there is an inherent variability in body weight and blood glucose levels that can require large group sizes for statistically valid comparisons of treatment effects (Young *et al.*, 1999; Ljung *et al.*, 2002; Tozzo *et al.*, 2007).

An alternative rodent model of obesity with insulin and leptin resistance is the high-fat (HF)-fed diet-induced obese C57Bl/6 mouse model. However, this model is not markedly hyperglycaemic and the phenotype is subject to a number of variables such as background sub-strain, sex, dietary composition and duration of HF diet (Rossmeisl *et al.*, 2003; Sörhede Winzell and Ahrén, 2004; Park *et al.*, 2005; Nicholson *et al.*, 2010). Overall, this can limit the ability to compare study outcomes, even within the same laboratory.

While the C57Bl/6 mouse on a HF diet is mildly hyperglycaemic (Sörhede Winzell and Ahrén, 2004), we have previously shown that the male $gk^{wt/del}$ mouse containing a global disruption of one allele for the gene encoding glucokinase (GK), on a C57Bl/6 background, displays a wide but stable hyperglycaemia similar to human levels, when maintained on a HF diet (Gorman *et al.*, 2008). The homozygous $gk^{del/del}$ mouse is neonatally or perinatally lethal (Bali *et al.*, 1995; Grupe *et al.*, 1995). Other commonly used animal models of T2D (*ob/ob* and *db/db* mice, male ZDF rats) have a wide but unstable hyperglycaemic range. In man, heterozygous inactivating mutations in the *gk* gene lead to mature onset of diabetes in the young, where patients present with mild fasting hyperglycaemia, while homozygous inactivating mutations produce the much more severe phenotype of permanent neonatal diabetes mellitus (Osbaek *et al.*, 2009).

We have examined the potential for male $gk^{wt/del}$ global knockout mice on HF diet to act as a translatable model for some facets of human T2D. GK is believed to act as a glucose sensor in both the pancreas and liver (Coghlan and Leighton, 2008; Matschinsky, 2009) and patients with T2D display defects in GK expression in pancreatic islets (Del Guerra *et al.*, 2005). On a HF diet, the $gk^{wt/del}$ mouse has reduced islet GK activity and defects in glucose-stimulated insulin secretion (Terauchi *et al.*, 2007; Gorman *et al.*, 2008). Patients with T2D also exhibit high rates of hepatic glucose output (HGO) and reportedly have significantly lower GK activity in liver (Caro *et al.*, 1995; Basu *et al.*, 2000; 2001). Basu *et al.* (2000; 2001) showed that despite equal or higher glucose and insulin concentrations, splanchnic glucose uptake and flux through UDP-glucose during enteral glucose feeding were lower in type 2 diabetic than in non-diabetic subjects, and that this defect in hepatic glucose uptake appears to reside at the level of GK. Similarly, hepatic GK activity in male $gk^{wt/del}$ mice was decreased by 35–50% (Rossetti *et al.*, 1997) and this contrib-

uted directly to elevated HGO during hyperglycaemia in addition to the defects in insulin secretion. Importantly, the increased rate of HGO in these mice was not dependent on leptin defects for its phenotype. Such evidence suggests that the reduction in GK expression and activity in T2D is better represented by the HF-fed $gk^{wt/del}$ global knockout model, which displays a phenotype similar to that of human T2D. $gk^{wt/del}$ mouse lines containing tissue-specific disruptions of either the beta cell or liver *gk* gene have been used to study the contributions of pancreatic and hepatic GK to overall glucose control (Terauchi *et al.*, 1995; 2007; Postic *et al.*, 1999), and mice haploinsufficient for beta cell *gk* have been used to explore the effects of antidiabetic compounds on glucose control (Nakamura *et al.*, 2009; Shirakawa *et al.*, 2011), but this model does not allow an assessment of any effects arising from defective hepatic GK function on overall glucose homeostasis.

To consider the HF-fed male $gk^{wt/del}$ global knockout mouse as a model translatable to humans with T2D, it is important to establish that agents known to modulate glycaemic control in the clinic by a variety of mechanisms demonstrate similar effects in this model at comparable levels of free drug exposure. In this investigation, we have explored the ability of two therapeutic insulins, insulin detemir and insulin glargine (Evans *et al.*, 2011), and glipizide, a sulphonylurea that directly stimulates pancreatic insulin secretion (Rendell, 2004), to lower blood glucose levels in the HF-fed male $gk^{wt/del}$ mouse. We have also investigated the efficacy of metformin, an agent that controls glucose levels by reducing HGO (Hundal *et al.*, 2000; Natali and Ferrannini, 2006). Turning to more recently introduced therapies, we also studied the effects of exendin-4, a glucagon-like peptide-1 (GLP-1) analogue that potentiates pancreatic insulin secretion (Flatt *et al.*, 2009; Tahrani *et al.*, 2011) and sitagliptin, a dipeptidylpeptidase-IV (DPP-IV) inhibitor that increases endogenous GLP-1 levels (Baetta and Corsini, 2011; Subbarayan and Kipnes, 2011). Finally, we examined the efficacy of GK activators (GKAs), GKA23, GKA71 and AZD6370, representing a new class of potential therapeutic agents that increase GK activity in liver and pancreatic beta cells, and lower blood glucose in type 2 diabetics (Meininger *et al.*, 2011; Kiyosue *et al.*, 2013; Wilding *et al.*, 2013). Overall, our results show that the HF-fed male $gk^{wt/del}$ mouse responds as expected to a range of current antidiabetic agents with a low level of variability.

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 148 animals were used in the experiments described here. Male $gk^{wt/del}$ mice were derived on a C57Bl/6 background (Gorman *et al.*, 2008) and supplied from the AstraZeneca Biological Animal Breeding Unit. Animals were received at 8 weeks of

age and housed in groups of three at 21–23°C. The mice were acclimatized to a reverse 12 h:12 h light : dark cycle (lights off 09:00) for a minimum of 3 weeks prior to the start of the study. One week prior to experimentation, mice were transferred from standard chow diet (RM1 diet from Special Diets Services, Witham, Essex, UK) to a 60% HF diet (RD12492i, Research Diets, New Brunswick, NJ, USA), which robustly induces insulin resistance and hyperglycaemia (Gorman *et al.*, 2008). Unless specified otherwise, the mice were maintained on this diet for the duration of the study. Body weights at the start of studies were typically in the range 30–40 g and were monitored routinely during studies. Animals had *ad libitum* access to food and tap water. Unless specified otherwise, blood samples for glucose (~5 µL) and pharmacokinetic (PK) analyses (20 µL) were obtained at the same time from the tail vein using a microsampling technique with EDTA-coated capillary tubes (Sarstedt Ltd., Leicester, UK) to minimize stress to the mice.

Experimental designs and measurements

Details of individual study designs are provided in the Supporting Information.

Study 1: effect of insulin. Two groups of mice ($n = 6$ per group) received a single subcutaneous dose of insulin glargine or insulin detemir, (Andrews Pharmacy, Macclesfield, UK) at 1 unit·kg⁻¹. Insulins were formulated as solutions in a vehicle of 0.3% BSA in sterile saline and given in volumes of 10 µL·g⁻¹ body weight using pre-siliconized syringes. A third group of mice ($n = 6$) received saline only. Animals were treated 30 min before the start of the dark phase.

Study 2: effect of metformin. Mice were given (p.o.) an aqueous solution of metformin (Sigma-Aldrich, Gillingham, Dorset, UK) at 150 or 300 mg·kg⁻¹, or water once daily for 14 days ($n = 6$ per group). Animals were treated 1 h before the start of the dark phase. Eight hours free-feeding glucose profiles were obtained on days 1 and 14.

Study 3: effect of glipizide and GKA71. Glipizide (Sigma-Aldrich) was formulated into the 60% HF diet (RD12492i) by Research Diets at concentrations that provided daily doses of 0.1 and 0.3 mg·kg⁻¹ based on a daily food intake of 10% body weight. GKA71 was formulated in the same diet by Research Diets to provide a daily dose of 5 mg·kg⁻¹. Exposures were confirmed by PK analysis. Animals received either HF diet alone ($n = 20$) or HF diet supplemented with glipizide ($n = 30$ per dose group) for 10 days. At this point, animals on HF diet + glipizide were re-randomized on glucose levels into three different groups ($n = 10$ per group) and received a HF diet alone or HF diet supplemented with either glipizide (dose equivalent 0.1 or 0.3 mg·kg⁻¹·day⁻¹) or GKA71 (dose equivalent 5 mg·kg⁻¹·day⁻¹) for a further 5 days. Free-feeding glucose profiles were measured over 24 h on days 1, 9 and 15.

Study 4: effect of exendin-4. Mice were randomized to three groups for measurement of free-feeding blood glucose profiles, oral glucose tolerance test (OGTT) or exposure. Animals in the free-feeding glucose profile and OGTT groups received a single i.p. dose of exendin-4 (Bachem H-8730, Bachem AG,

Bubendorf, Switzerland) in aqueous solution at 0.2, 2 and 20 µg·kg⁻¹ ($n = 6$ per dose group) or vehicle (water; $n = 7$), 1 h before the start of the dark phase. Free-feeding glucose profiles were measured over a 7 h period after dosing. A satellite group of animals ($n = 2$ per time point per dose group) were dosed as above for measurement of exendin-4 concentrations at 0.5, 3 and 7 h.

Study 5: effect of sitagliptin and GKA23. Mice were pre-dosed orally with either sitagliptin (Sigma-Aldrich; 10 mg·kg⁻¹·days⁻¹, $n = 18$), or vehicle [1% Pluronic F127 (Sigma-Aldrich) in water] ($n = 6$) for 6 days, 1 h before start of the dark phase. On day 7, the mice pre-dosed with sitagliptin were re-randomized based on glucose and body weight to three groups ($n = 6$ per group). One group received GKA23 (formulated in 1% Pluronic F127 in water) at 3 mg·kg⁻¹·day⁻¹, a second group continued on sitagliptin, and the third group were dosed with a combination of sitagliptin and GKA23. Free-feeding blood glucose profiles were measured over a period of 8 h post-dose on day 7 and an OGTT was performed on day 13.

Study 6: effect of AZD6370. GKA AZD6370 (formulated in 1% Pluronic F127 in water) was given p.o., daily at 30 and 400 mg·kg⁻¹ ($n = 4$ or 5 per dose group respectively). A third group of mice ($n = 5$) received vehicle only. Treatments were given 1 h before the start of the dark phase. Free-feeding glucose profiles were obtained over a 12 h period post-dose on days 1 and 7.

Blood glucose measurements and oral glucose tolerance test

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 (see Supporting Information). Accu-chek® blood glucose readings were taken at -120 min (baseline) and 5–90 min after glucose bolus). AUC values from free-feeding glucose profiles were calculated as total AUC. AUC values from OGTTs were corrected for differences in baseline glucose 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. Glipizide, sitagliptin and AZ GKAs 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. Quantification of metformin used laser diode thermal desorption coupled with a Thermo Scientific (Hemel Hempstead, Hertfordshire, UK) tandem quadrupole mass spectrometer as described previously (Swales *et al.*, 2010).

For determination of exendin-4 levels, cardiac blood samples were taken from mice ($n = 2$ per time point per dose) under CO_2/O_2 narcosis and the mice then killed. Blood samples were dispensed into lithium-heparin-coated tubes (Sarstedt Ltd.) containing aprotinin (Sigma-Aldrich) and DPP-IV inhibitor [Millipore (UK) Ltd., Watford, Hertfordshire, UK], immediately placed on ice until chilled and centrifuged at $9300 \times g$ for 10 min. The clear plasma fraction was aliquoted into siliconized tubes. Samples were frozen at -20°C prior to analysis. Exendin-4 concentrations were determined using an Exendin-4 Ultra-sensitive RIA kit from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA).

PK parameters were determined using non-compartmental analysis in WinNonlin (Pharsight Corporation, Mountain View, CA, USA). Human exposures were simulated using 2-compartmental analysis in WinNonLin based on published clinical data. Mouse compound-in-diet dose predictions were simulated using murine daily feeding patterns combined with in-house assessment of total daily food consumption (data not shown) and a custom in-house simulation algorithm executed in Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA, USA). Translational doses were defined by calculating unbound plasma concentrations based on human clinical data at a therapeutically relevant dose and plasma protein binding values from published data or as determined within AstraZeneca using an equilibrium dialysis method (see Table 1). Rodent doses were defined to obtain similar unbound plasma concentrations *in vivo* in terms of $C_{\text{max free}}$ and $\text{AUC}_{(0-24 \text{ h free})}$.

Data analysis

Data collected on free-feeding glucose AUC 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 $n = 6$. In study 6, we used $n = 4$ or $n = 5$ in the treatment groups because historical data on the magnitude of glucose lowering with

GKAs showed this was sufficient. 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 significance was evaluated using a one-sided Student's unequal variance *t*-test. Differences were considered significant at $P < 0.05$.

Materials

GKA23 is the free alcohol derivative of AZD1656 (Waring *et al.*, 2012). The structures of GKA71 and AZD6370 have been previously reported (Waring *et al.*, 2011; Norjavaara *et al.*, 2012). EC_{50} values against human recombinant GK are 34 nM (GKA23), 110 nM (GKA71) and 45 nM (AZD6370). All GKAs (including GKA23) were synthesized by AstraZeneca (Macclesfield, UK).

Results

Study 1: effect of insulins detemir and glargine

Insulin detemir and insulin glargine caused comparable and significant glucose lowering compared with vehicle when administered as a single dose ($1 \text{ unit} \cdot \text{kg}^{-1}$) to male $gk^{wt/del}$ mice (Figure 1A, B). Insulin detemir had a slightly slower onset of action and a delayed nadir in the glucose response (2 h), compared with insulin glargine (1 h).

Study 2: effect of metformin

In male $gk^{wt/del}$ mice, a single oral dose of metformin at 150 or $300 \text{ mg} \cdot \text{kg}^{-1}$ gave a dose-dependent reduction in free-feeding blood glucose over a 6 h period following administration (Figure 2A, 2B). The reduction in glucose $\text{AUC}_{(0-6 \text{ h})}$ was significant at $300 \text{ mg} \cdot \text{kg}^{-1}$. Metformin exposure in male $gk^{wt/del}$ mice gave free drug levels slightly higher than those

Table 1

Comparison of experimentally derived, unbound (free drug) plasma exposure, defined as $C_{\text{max free}}$ and $\text{AUC}_{(0-24 \text{ h free})}$ in male $gk^{wt/del}$ mice with clinical free plasma concentrations

Compound	Glipizide		Metformin		Sitagliptin		Exendin-4		GKA ADZ6370	
Species	Human	Mouse	Human	Mouse	Human	Mouse	Human	Mouse	Human	Mouse
Dose per day (mg)	40	0.3 kg^{-1}	2250	300 kg^{-1}	100	10 kg^{-1}	0.020	0.002 kg^{-1}	180	25 kg^{-1}
Doses per day	Divided 2 ^a	1	Divided 3 ^a	1	1 ¹	1	Divided 2	1	1	1
f_u	0.013 ^b	0.0299 ^b	0.9 ^c	0.95 ^b	0.77 ^b	0.71 ^b	ND	ND	0.15 ^b	0.019 ^b
$C_{\text{max free}}$ ($\text{ng} \cdot \text{mL}^{-1}$)	22.18 ^g	18.81 ^b	1380 ^g	6652 ^b	228 ^d	859 ^b	0.35 ^e	0.17 ^b	207 ^f	112 ^b
$\text{AUC}_{(0-24 \text{ h free})}$ ($\text{ng} \cdot \text{h} \cdot \text{mL}^{-1}$)	179.1 ^g	248 ^b	14706 ^g	27743 ^b	2224 ^d	4595 ^b	ND	ND	428 ^f	475 ^b

^aHuman dose selected at upper end of therapeutic range.

^bDetermined in AstraZeneca by equilibrium dialysis.

^cVallner, 1977.

^dBergman *et al.*, 2007.

^eKolterman *et al.*, 2005.

^fNorjavaara *et al.* (2012), Sjöstrand *et al.* (2013).

^gCalculated levels assuming dose linearity.

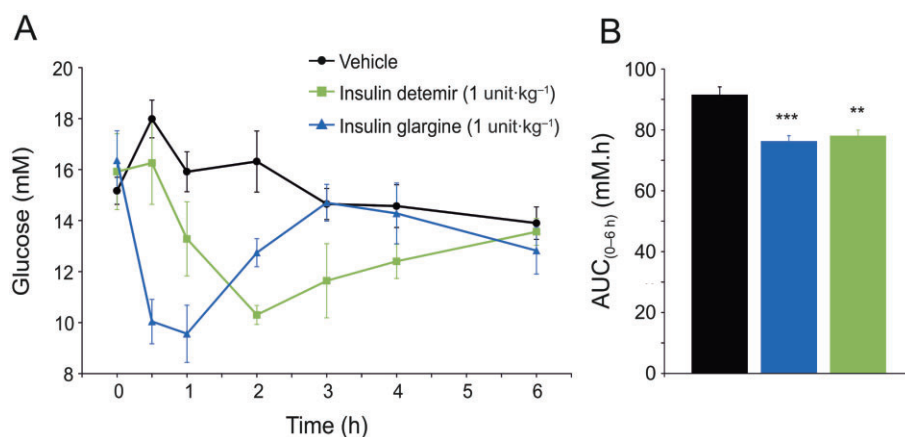


Figure 1

Blood glucose lowering in HF-fed male $gk^{wt/del}$ mice after a single subcutaneous dose of vehicle, insulin detemir at 1 unit·kg⁻¹ or insulin glargine at 1 unit·kg⁻¹. (A) 6 h free-feeding blood glucose profile. (B) Glucose AUC_(0-6 h) values (mM·h). Results are the mean ± SEM ($n = 6$ per treatment group). ** $P < 0.01$, *** $P < 0.001$ versus vehicle.

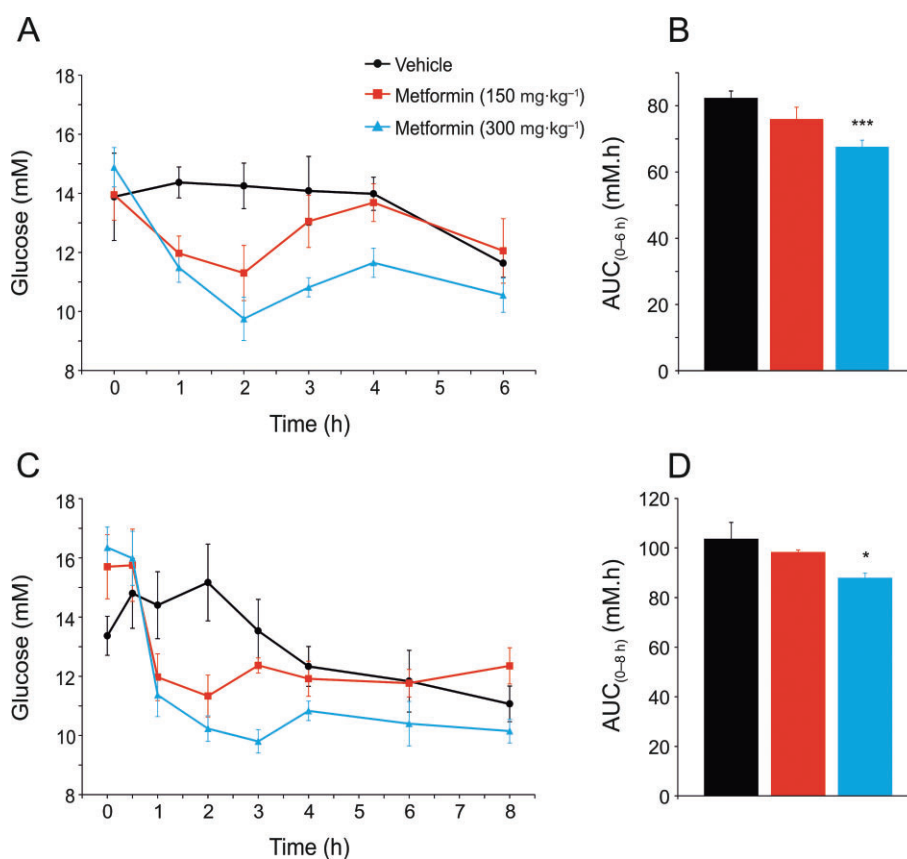


Figure 2

Repeat administration of vehicle, metformin at 150 mg·kg⁻¹·day⁻¹, or metformin at 300 mg·kg⁻¹·day⁻¹ to HF-fed male $gk^{wt/del}$ mice. (A) 6 h free-feeding blood glucose profile on day 1. (B) Glucose AUC_(0-6 h) values (mM·h) on day 1. (C) 8 h free-feeding blood glucose profile on day 14. (D) Glucose AUC_(0-8 h) values (mM·h) on day 14. Results are the mean ± SEM ($n = 6$ per treatment group). * $P < 0.05$, *** $P < 0.001$ versus vehicle.

observed in man at therapeutic doses (Table 1). After 14 days dosing, both doses of metformin continued to deliver a dose-dependent reduction in free-feeding blood glucose profiles (Figure 2C), but only metformin at 300 mg·kg⁻¹·day⁻¹ significantly reduced the glucose AUC_(0-8 h) (Figure 2D). Neither dose of metformin lowered pre-dose blood glucose levels at day 14 compared with day 1 (Figure 2B). There was no effect of metformin on body weight compared with vehicle at either dose over the study period.

Study 3: effect of glipizide and GKA

To select an appropriate translational dose of glipizide in the male *gk^{wt/del}* mouse, the predicted PK profile for compound delivered in the diet was modelled to match as closely as possible the clinical profile observed in man (Figure 3). Based on these simulations, a dietary formula was selected to give an overall dose of 0.1 or 0.3 mg·kg⁻¹·day⁻¹. Pilot PK studies were conducted to ensure systemic compound exposures would reach expected levels with these diets. Subsequent PK measurements in Study 3 confirmed that the predicted exposure profiles were achieved (Figure 3), and maintained on repeat dosing.

At the start of the study, 23 h free-feeding glucose profiles were obtained at day 8 (see Supporting Information) to ensure all animals had similar baseline glucose profiles (Figure 4A). Glipizide delivered in the diet at 0.1 or

0.3 mg·kg⁻¹·day⁻¹ gave an acute and significant lowering of blood glucose after day 1 of dosing (Figure 4B, 4D). However, by day 9, glucose control in both glipizide treatment groups had worsened compared with vehicle (Figure 4C, 4D).

To see if the loss of efficacy observed with glipizide treatment could be reversed by a GKA, *gk^{wt/del}* mice that had been dosed with 0.3 mg·kg⁻¹·day⁻¹ glipizide were re-randomized on blood glucose to three new groups on day 10. One group was switched to diet without glipizide, one group continued with glipizide and the third group received GKA71 formulated in the diet to deliver a daily dose of 0.3 mg·kg⁻¹. After a further 5 days on these diets, a final free-feeding glucose profile was measured over 23 h (Figure 4D). Mice that were taken off glipizide treatment displayed a glucose profile that was essentially the same as mice that had been maintained on unsupplemented HF diet for the entire study. Mice that had continued with glipizide showed a further elevation in pre-dose glucose levels (Figure 4E, 4F). Mice that were switched from glipizide to GKA71 showed a significant improvement in free-feeding glucose profile and a significant reduction in AUC_(0-23 h) value (Figure 4F). None of the treatment groups showed any significant changes in body weight compared with animals on HF diet alone.

A similar protocol was applied to mice that had received glipizide at 0.1 mg·kg⁻¹·day⁻¹ for 10 days. The results (see Supporting Information) were similar to those shown in Figure 4E and 4F.

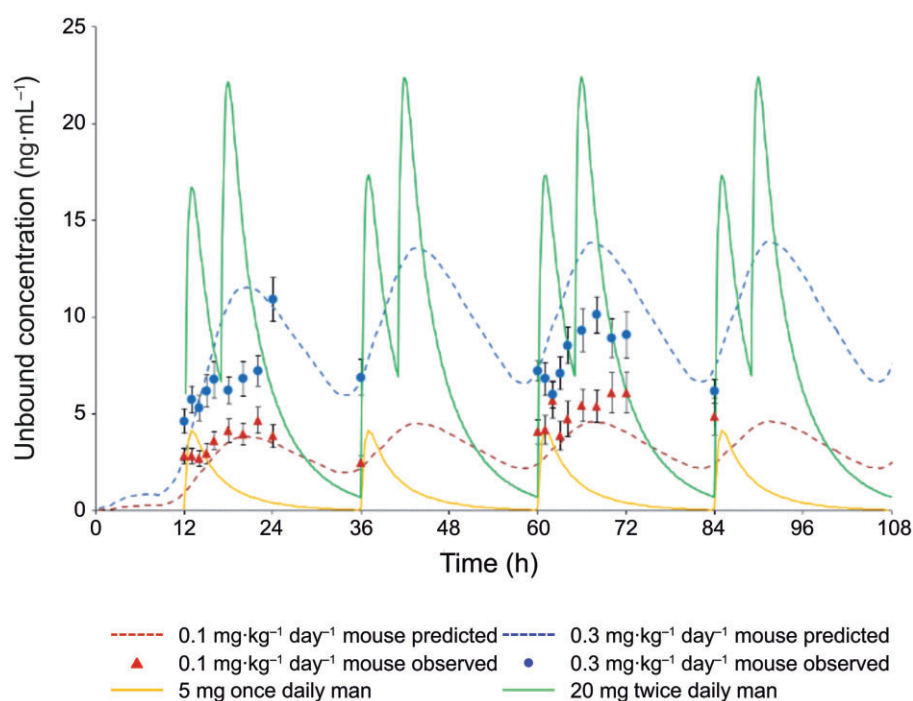


Figure 3

Predicted and measured systemic concentrations of glipizide in mouse, based on drug in diet simulations accounting for diurnal murine free-feeding pattern. Simulated human PK profiles of unbound drug shown for a representative low (5 mg once daily) and high (20 mg twice daily) human therapeutic dose of glipizide based on published 5 mg once daily dose data (Melander and Wahlin-Boll, 1983). Protein binding for glipizide in human (1.3% unbound) and mouse (2.9% unbound) plasma measured by equilibrium dialysis. Predicted murine PK profiles shown for doses of 0.1 and 0.3 mg·kg⁻¹·day⁻¹ glipizide in diet. Observed glipizide unbound drug concentrations (mean ± SEM) in mouse plasma following dosing at 0.1 mg·kg⁻¹·day⁻¹ (*n* = 12) and 0.3 mg·kg⁻¹·day⁻¹ (*n* = 11) in diet were measured on days 1 and 3 following switch to diet with drug.

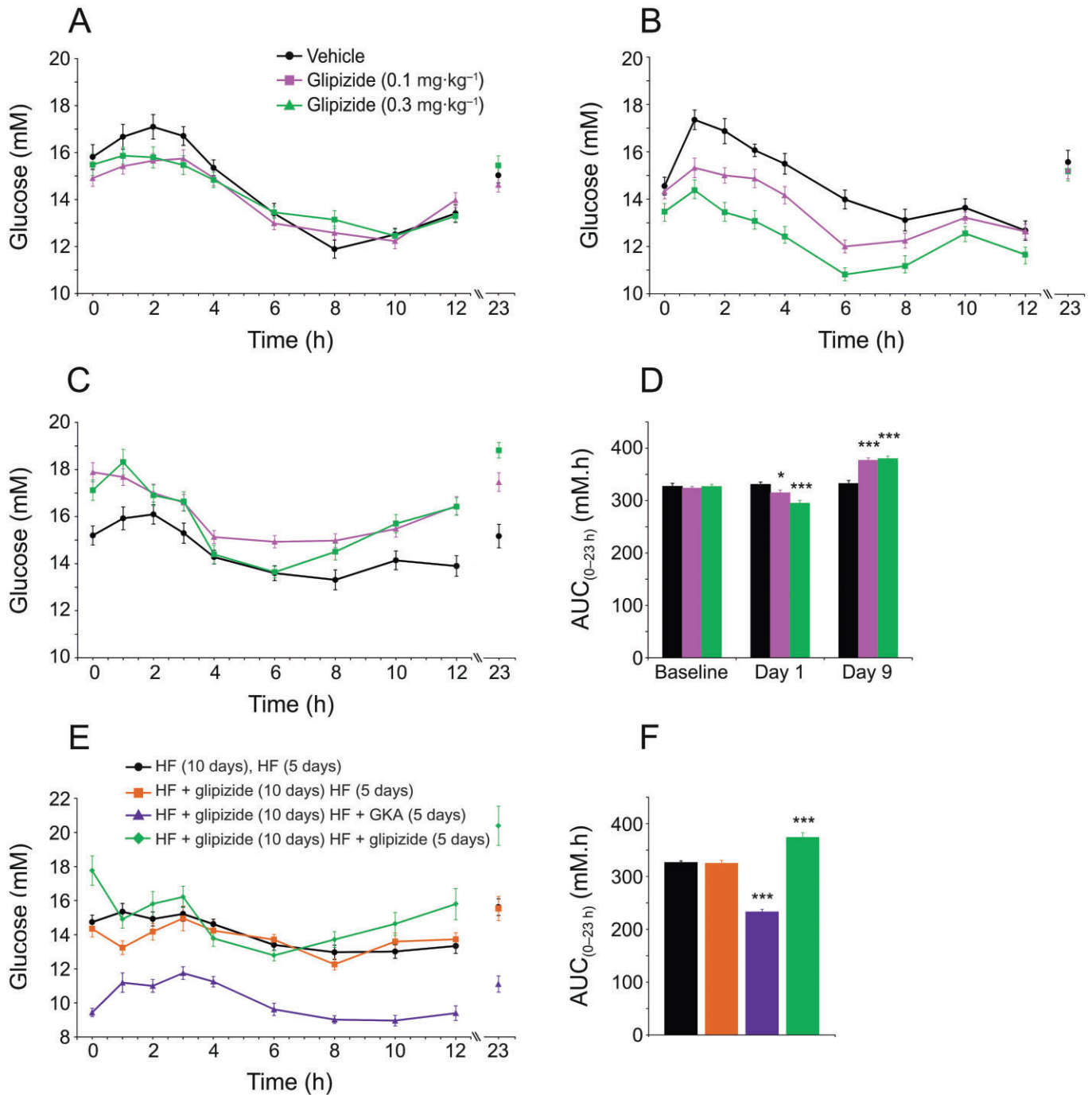


Figure 4

Dose-dependent glucose lowering with glipizide in HF-fed male $gk^{wt/del}$ mice. 23 h free-feeding blood glucose profiles in mice receiving standard 60% HF diet ($n = 20$) or HF diet + glipizide at a dose equivalent of 0.1 mg·kg⁻¹·day⁻¹ ($n = 30$) or 0.3 mg·kg⁻¹·day⁻¹ ($n = 30$) at (A) baseline (day 8 before start of glipizide treatment), (B) 1 day of glipizide treatment, (C) 9 days of glipizide treatment. (D) Glucose AUC_(0-23h) values (mM·h) for free-feeding blood glucose profiles at days 8, 1, 9 of dosing. * $P < 0.05$, *** $P < 0.001$ versus HF diet group on same day. At day 10, animals that had been dosed with glipizide at 0.3 mg·kg⁻¹·day⁻¹ were split into three groups ($n = 10$ per group) and received either standard HF diet, HF diet + GKA71 at a dose equivalent of 5 mg·kg⁻¹·day⁻¹ or HF diet + glipizide at a dose equivalent of 0.3 mg·kg⁻¹·day⁻¹ for 5 days. Mice that had received HF diet only continued on this diet for a further 5 days. (E) 23 h free-feeding blood glucose profiles 5 days after diet switch. (F) Glucose AUC_(0-23h) values (mM·h) Results are the mean $\pm$ SEM for each group. *** $P < 0.001$ versus HF diet only.

Study 4: effect of exendin-4

Acute i.p. administration of exendin-4 at 0.2, 2 or 20 $\mu\text{g}\cdot\text{kg}^{-1}$ showed significant dose-dependent blood glucose lowering in male $gk^{wt/del}$ mice (Figure 5A, 5B).

Exendin-4 also produced a dose-dependent improvement in acute glucose tolerance by (Figure 5C). The reduction in OGTT $\text{AUC}_{(0-90\text{ min})}$ values corrected for differences in baseline at $t = 0$ min (Figure 5D) was however only significant at the 20 $\mu\text{g}\cdot\text{kg}^{-1}$ dose. Systemic levels of exendin-4 achieved at a dose of 2 $\mu\text{g}\cdot\text{kg}^{-1}$ were similar to exposure levels reported in man (Table 1).

Study 5: effect of sitagliptin and GKA, alone or in combination

A single acute oral dose of sitagliptin at 10 $\text{mg}\cdot\text{kg}^{-1}$ in male $gk^{wt/del}$ mice gave a slight reduction in a 6 h free-feeding glucose profile (A. Atkinson, unpubl. data). At this dose, free drug levels of sitagliptin are slightly higher than that achieved in man at the common therapeutic dose of 100 mg (i.e. ~ 1.0 – $1.25\text{ mg}\cdot\text{kg}^{-1}$; Table 1). After 6 days pre-dosing with sitagliptin at 10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, a further dose of sitagliptin at 10 $\text{mg}\cdot\text{kg}^{-1}$ on day 7 showed a significant reduction in the free-feeding blood glucose profile and $\text{AUC}_{(0-8\text{ h})}$ value (Figure 6A, 6B). A

single dose of GKA23 at 3 $\text{mg}\cdot\text{kg}^{-1}$ to mice pre-dosed with sitagliptin also produced significant glucose lowering over 8 h. A single dose of the combination of sitagliptin (10 $\text{mg}\cdot\text{kg}^{-1}$) and GKA23 (3 $\text{mg}\cdot\text{kg}^{-1}$) to mice pre-dosed with sitagliptin showed a similar level of blood glucose lowering.

After a further 5 days of dosing, sitagliptin, but not GKA23, showed significant blood glucose lowering in male $gk^{wt/del}$ mice after an acute oral glucose challenge (Figure 6C, 6D). The OGTT $\text{AUC}_{(0-60\text{ min})}$ values ($\text{mM}\cdot\text{h}$) for sitagliptin (17.4 ± 0.9), and GKA23 (14.2 ± 1.2) were significantly lower versus vehicle (22.3 ± 0.6 ; $P < 0.01$ and $P < 0.001$ respectively). The combination of sitagliptin with GKA produced a reduction in baseline-corrected $\text{AUC}_{(0-60\text{ min})}$ similar to that obtained with sitagliptin alone. Body weights in the treatment groups did not change significantly relative to the vehicle group over the course of the study.

Study 6: effect of GKA AZD6370

GKA AZD6370 (30 or 400 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or vehicle were given p.o. to male $gk^{wt/del}$ mice for 7 days. Unbound exposure at a dose of 30 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ in these mice is similar to that required for therapeutically relevant glucose lowering in clinical trials (Table 1). A single oral dose of AZD6370 at

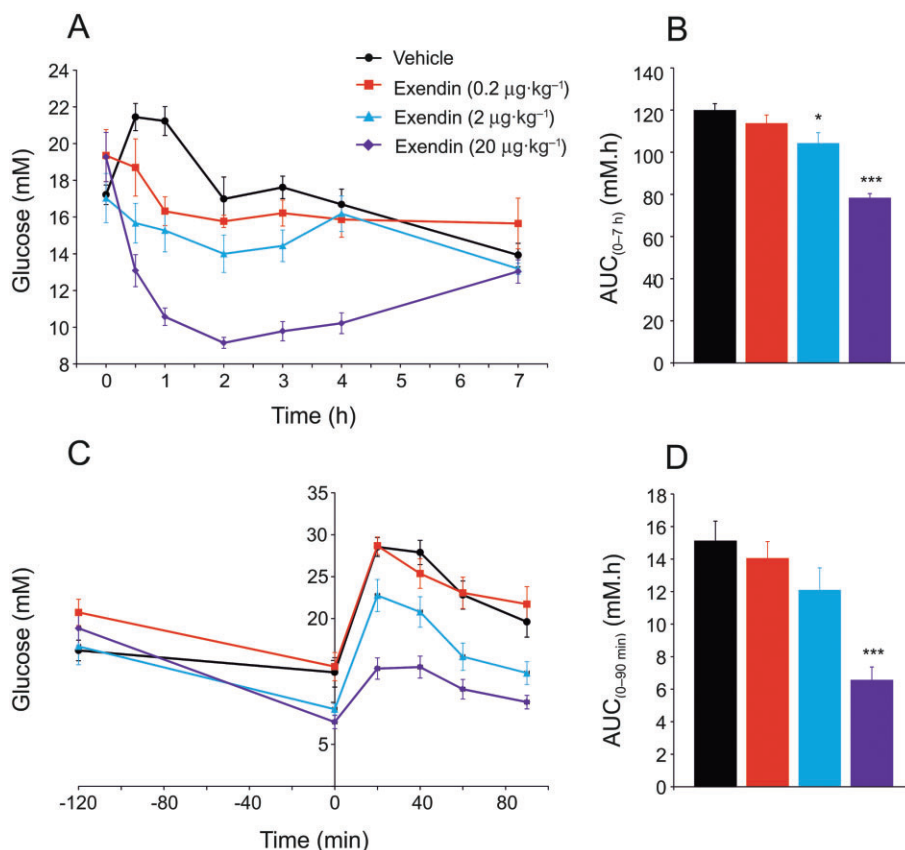


Figure 5

Glucose-lowering effect of exendin-4 in male $gk^{wt/del}$ mice. (A) 7 h free-feeding blood glucose profiles after a single i.p. dose of exendin-4 at 0.2, 2 or 20 $\mu\text{g}\cdot\text{kg}^{-1}$ or vehicle. (B) Glucose $\text{AUC}_{(0-7\text{ h})}$ values ($\text{mM}\cdot\text{h}$). (C) OGTT 2 h after receiving an i.p. dose of exendin-4 at 0.2, 2, 20 $\mu\text{g}\cdot\text{kg}^{-1}$ or vehicle. (D) Glucose $\text{AUC}_{(0-90\text{ min})}$ values ($\text{mM}\cdot\text{h}$) corrected for differences in baseline at $t = 0$ min. Results are the mean $\pm$ SEM for at least six mice per treatment group. * $P < 0.05$, *** $P < 0.001$ versus vehicle.

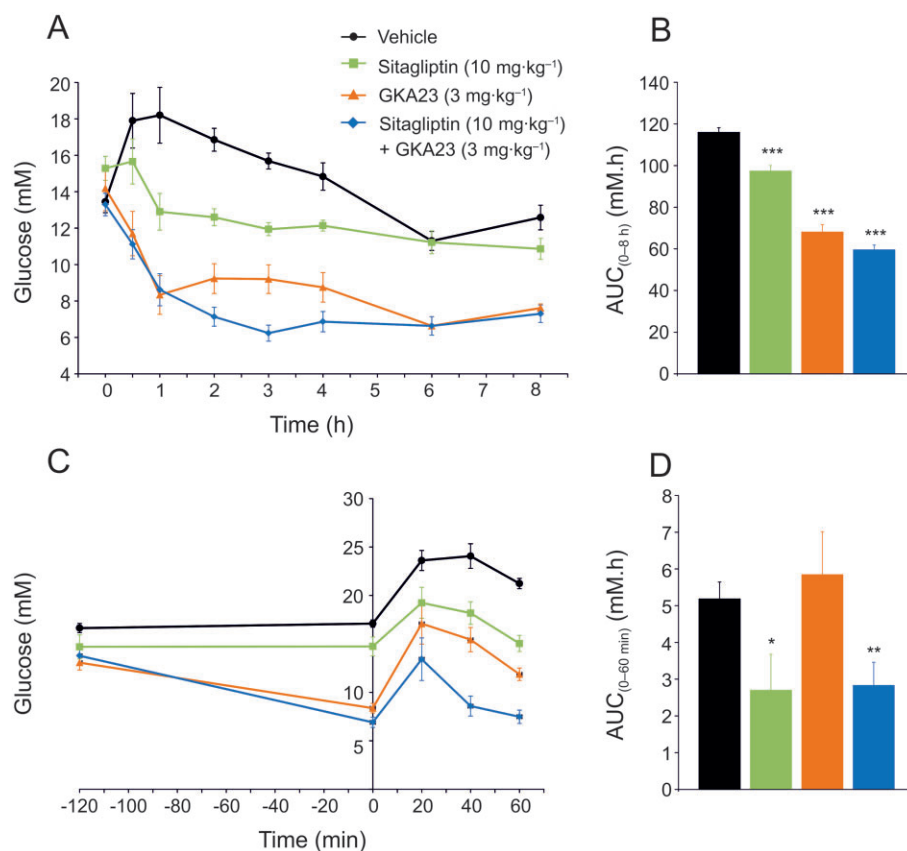


Figure 6

Glucose-lowering effect of sitagliptin in male $gk^{wt/del}$ mice. Mice received either vehicle ($n = 6$) or sitagliptin at $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ($n = 18$) for 6 days. At day 7, the group receiving sitagliptin was re-randomized into three subgroups ($n = 6$ per group), which then received either sitagliptin at $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, GKA23 at $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ or a combination of sitagliptin at $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ and GKA23 at $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. (A) 8 h free-feeding blood glucose profile on day 7. (B) Glucose AUC_(0-8 h) values (mM·h). (C) OGTT on day 13, 2 h after receiving the final dose of sitagliptin, GKA23 or sitagliptin + GKA23. (D) Glucose AUC_(0-60 min) values (mM·h) corrected for differences in baseline at $t = 0$ min. Results are the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus vehicle.

$30 \text{ mg} \cdot \text{kg}^{-1}$ or $400 \text{ mg} \cdot \text{kg}^{-1}$ significantly lowered free-feeding blood glucose (data not shown). After 7 days treatment, AZD6370 at $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ had no significant effects on pre-dose glucose levels, but did significantly reduce blood glucose in the 12 h period post-dose (Figure 7A, 7B). After 7 days dosing at $400 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, AZD6370 significantly lowered fasting blood glucose from $12.5 \pm 0.4 \text{ mM}$ to $9.1 \pm 1.0 \text{ mM}$ ($P < 0.05$) and achieved a sustained and significantly lower free-feeding blood glucose profile (Figure 7A, 7B). There were no significant changes in body weight over the study period for animals receiving either dose of AZD6370 compared with vehicle.

Discussion and conclusions

The rationale for doses of drugs chosen for preclinical animal experiments is often driven by the desire to achieve efficacy (e.g. blood glucose lowering), rather than to establish the boundaries of efficacy in the context of free drug exposures in humans. We have now set out to characterize the effects of current therapeutic agents representing the range of com-

monly prescribed antidiabetic drugs in the male $gk^{wt/del}$ mouse. In our experimental design, we have taken particular care to use systemic free drug exposures in the male $gk^{wt/del}$ mouse that are therapeutically relevant to those achieved in humans with T2D (see Table 1), such that the level of effect in relation to exposure achieved in man can be assessed effectively. In these studies, we have profiled the ability of a number of currently used glucose-lowering agents to modulate glucose control in the male $gk^{wt/del}$ mouse at translatable free-drug exposures. Our first key finding was that insulin detemir or insulin glargine acutely lowered blood glucose levels in male $gk^{wt/del}$ mice (Figure 1). These are long-acting versions of insulin designed to provide 24 h glucose lowering in man (Evans *et al.*, 2011). Insulin glargine has a much shorter pharmacodynamic profile in diabetic rodents (Schneider *et al.*, 2004), consistent with our observations that both insulins produce a maximal glucose-lowering effect 1–2 h after injection, with a return to basal values within 6 h. The doses of insulin used in this study ($1 \text{ unit} \cdot \text{kg}^{-1}$) are slightly higher than the typical dose range for man (~ 0.2 – $0.8 \text{ units} \cdot \text{kg}^{-1}$; Swinnen *et al.*, 2009) to adjust for the differences in pharmacodynamic profile in rodents.

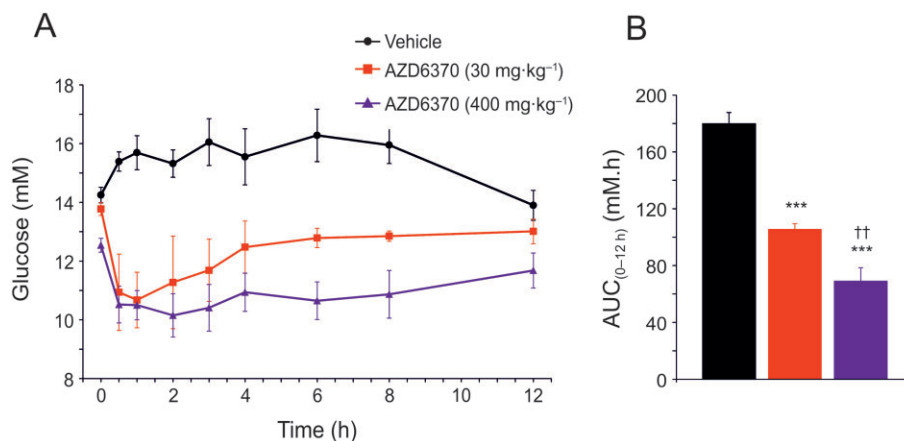


Figure 7

Glucose-lowering efficacy of AZD6370 in male $gk^{wt/del}$ mice. Mice were dosed orally with AZD6370 at 30 mg·kg⁻¹·day⁻¹ or 400 mg·kg⁻¹·day⁻¹, or vehicle for 7 days (A) 12 h free-feeding blood glucose profile at day 7. (B) Glucose AUC_(0-12 h) values (mM·h). Results are the mean ± SEM ($n = 4-5$ per group). *** $P < 0.001$ versus vehicle, †† $P < 0.01$ versus AZD6370 at 30 mg·kg⁻¹·day⁻¹.

Having demonstrated the ability of insulin to lower blood glucose levels in this model, we next explored the effects of metformin, a drug that is well established as a first-line therapy for the treatment of T2D. The mechanism of action of metformin is not fully understood, but one of its effects may be to reduce HGO by antagonizing the effect of glucagon (Miller *et al.*, 2013). We explored the efficacy of metformin at two doses (150 and 300 mg·kg⁻¹·day⁻¹), which achieve somewhat higher free drug exposures in the male $gk^{wt/del}$ mouse than typically found in man. Both doses of metformin acutely lowered blood glucose after dosing, but after 14 days only the 300 mg·kg⁻¹ dose of metformin was able to maintain significant post-dose glucose lowering in this model. Neither dose was able to achieve a pre-dose lowering of blood glucose. Other studies have shown that repeated high doses of metformin are usually required to achieve basal glucose lowering in hyperglycaemic rodent models such as *db/db* mice (Konstantopoulos *et al.*, 2012), or in models of progressive pancreatic failure such as diabetic ZDF rats (Sreenan *et al.*, 1996; Atkinson *et al.*, 2008).

Sulphonylureas such as glipizide are well known to directly stimulate insulin secretion from the pancreas through inhibition of the K_{ATP} ion channel (Rendell, 2004). Glipizide is typically administered b.i.d. in man, so we chose to provide compound in the diet to minimize stress on mice receiving subacute oral gavage twice daily. To study the effects of glipizide in the $gk^{wt/del}$ mouse model, we modelled the predicted PK profile for dietary intake and selected dietary drug concentrations that were predicted to deliver free drug levels at therapeutically relevant exposures (Figure 3). Subsequent PK analysis confirmed that we achieved the desired exposures. Glipizide at doses equivalent to 0.1 or 0.3 mg·kg⁻¹·day⁻¹ acutely lowered post-dose blood glucose levels (compare Figure 4A, 4B), but efficacy was lost by 5 days of treatment (Figure 4C). We confirmed that loss of efficacy was not due to increased elimination of glipizide on repeat dosing. It is well known that continued administration of sulphonylureas in man eventually leads to a desensitization, which manifests itself as loss of efficacy after the first

6–9 months of treatment (Kahn *et al.*, 2006). Desensitization to sulphonylureas in normoglycaemic and hyperglycaemic rodents has been reported previously (Mu *et al.*, 2006; Ohyama *et al.*, 2010), although at doses that may not reflect therapeutically relevant free drug levels (Mu *et al.*, 2006). The reason for sulphonylurea desensitization in man is not fully understood, nor is it clear that the mechanisms for this process are the same in man as in rodents, but the behaviour of glipizide in hyperglycaemic male $gk^{wt/del}$ mice at translational doses is consistent with reported effects of sulphonylureas in other rodent models (Ohyama *et al.*, 2010).

Importantly, GKA71 was able to restore glucose control after glipizide desensitization (Figure 4D). Activation of pancreatic GK stimulates insulin secretion by increasing intracellular [ATP]:[ADP] ratios through increased glucose oxidation, but this mechanism would be compromised by loss of K_{ATP} channel activity. Ohyama and co-workers also reported that a GKA could reverse the effect of glimepiride desensitization in Sprague Dawley rats (Ohyama *et al.*, 2010), and speculated that this reversal could be due to increased hepatic glucose uptake. Our data indicate that a GKA will effectively control blood glucose even at the very high blood glucose levels we observed after glipizide desensitization, and are consistent with a significant hepatic effect.

We next evaluated the effects of exendin-4, a mimetic of the incretin hormone GLP-1. GLP-1 is produced in the lower gut in response to dietary glucose and potentiates glucose-stimulated insulin secretion from the pancreas (Brubaker, 2010). Exendin-4 is a GLP-1 mimetic that is resistant to inactivation by the peptidase DPP-IV and is efficacious at controlling glucose levels in man (Flatt *et al.*, 2009; Tahrani *et al.*, 2011). In HF-fed male $gk^{wt/del}$ mice, a single dose of exendin-4 produced a dose-dependent decrease in blood glucose levels (Figure 5A), and an improvement in glucose tolerance (Figure 5B). The dose of 20 µg·kg⁻¹ for exendin-4 compares with similar i.p. doses in other mouse models (Young *et al.*, 1999; Doyle *et al.*, 2005; Lamont and Drucker, 2008).

A single dose of sitagliptin (10 mg·kg⁻¹), which raises active blood GLP-1 levels by inhibiting DPP-IV (Baetta and

Corsini, 2011; Subbarayan and Kipnes, 2011; Muscelli *et al.*, 2012), had a limited effect on free-feeding glucose levels in male $gk^{wt/del}$ mice. Acute effects of DPP-IV inhibitors in rodents are more readily demonstrated by OGTT (Waget *et al.*, 2011), where a bolus of oral glucose is more effective at inducing GLP-1 secretion than a meal. A dose of $10 \text{ mg} \cdot \text{kg}^{-1}$ sitagliptin achieved free drug levels in mice comparable to the therapeutic dose in man ($\sim 1 \text{ mg} \cdot \text{kg}^{-1}$, see Table 1). After 7 days oral dosing with sitagliptin, there was a modest reduction in the free-feeding glucose profile (Figure 6A, 6B). A single dose of GKA23 also effectively lowered free-feeding blood glucose in sitagliptin-pretreated animals, either alone or in combination with sitagliptin. Seven days dosing of GKA23 (alone or in combination with sitagliptin) lowered total glucose AUC in an OGTT, but did not significantly reduce the glucose AUC after correction for differences in baseline at $t = 0$, even though systemic drug levels of GKA23 2–3 h post-dose are sufficient to lower free-feeding glucose (Figure 6A). The lack of a marked effect of GKAs on baseline-corrected glucose excursions in OGTTs has been observed in other studies (Baker *et al.*, 2014), suggesting that GKA-mediated inhibition of HGO is likely to be the main mechanism for lowering free-feeding glucose, with limited effects on net glucose handling following an oral glucose challenge.

Finally, we have investigated the ability of a GKA, AZD6370, to control blood glucose in the $gk^{wt/del}$ model with either acute or subacute dosing. AZD6370 acutely lowered blood glucose in male $gk^{wt/del}$ mice in a dose-dependent manner. AZD6370 has also been shown to acutely increase whole body glucose disposal in man. After 7 days dosing at $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$, AZD6370 significantly reduced blood glucose in male $gk^{wt/del}$ mice over the period 0.8–8 h post-dose, reaching a nadir of 5–6 mM glucose 0.5–1 h post-dose from a pre-dose baseline of 11–12 mM. A dose of $30 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ of AZD6370 in mice corresponds to a therapeutically relevant acute dose in man (Table 1; Norjavaara *et al.*, 2012; Sjöstrand *et al.*, 2013). At a much higher dose ($400 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$), glucose levels were lowered for a longer period but importantly, no hypoglycaemia was observed. This is consistent with the proposed mechanism of action of GKAs, which shift the dose response for glucose activation of GK to lower glucose concentrations, but still permit allosteric regulation of the enzyme in response to systemic glucose concentrations (Coghlan and Leighton, 2008; Matschinsky, 2009). Under controlled clamp conditions in healthy male volunteers, AZD6370 induces hypoglycaemia (Norjavaara *et al.*, 2012), and produces the expected central counter-regulatory response. Hypoglycaemia has also been observed in safety studies in normal mice and rats dosed with supra-therapeutic levels of GKAs (B. Leighton, unpubl. data). However, the HF-fed male $gk^{wt/del}$ mouse model has much higher basal glucose, and high doses of GKAs such as AZD6370 do not result in hypoglycaemia. We have now extended our translational design paradigm and used this model to explore the chronic efficacy (1 year) of GK activators using this model (Baker *et al.*, 2014). The stable hyperglycaemic nature of the HF-fed male $gk^{wt/del}$ mouse model suggests it may have utility in safety studies with glucose-lowering agents, and permits its use for crossing with other transgenic lines to explore the contribution of additional genes to the overall diabetic phenotype.

In summary, we have shown that the HF-fed male $gk^{wt/del}$ mouse model has a robust and reproducible phenotype representing key features of human T2D and have demonstrated that a range of therapeutic agents known to control glucose levels in patients with T2D by a variety of mechanisms also achieve glucose-lowering efficacy in this model at clinically translatable efficacious exposures with acute or subacute dosing. This model therefore provides a valuable advance in the portfolio of diabetic animal models available for preclinical studies of potential therapeutic agents.

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Conflict 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.12498>

Figure S1 Study design schematics and OGTT protocol. Design schematics for Studies 1–6 and OGTT protocol.

Figure S2 Study 3: Effect of glipizide at 0.1 mg kg⁻¹ day⁻¹ and GKA71. Free-feeding glucose profiles in male $gk^{wt/del}$ mice after 10 d dosing with glipizide at 0.1 mg kg⁻¹ day⁻¹ followed by 5 d dosing with GKA71 at 5 mg kg⁻¹ day⁻¹ or glipizide at 0.1 mg kg⁻¹ day⁻¹.

Figure S3 Study 6: Effect of sub-acute dosing of GKA AZD6370 on pre-dose blood glucose levels. Change in pre-dose glucose levels in male $gk^{wt/del}$ mice over 7 d on daily dosing of AZD6370 at 30 and 400 mg kg⁻¹ day⁻¹.