Combining a basal insulin and a GLP-1RA: a new paradigm in insulin intensification

A virtual symposium featuring Professor Roopa Mehta, Professor Ildiko Lingvay & Professor Kamlesh Khunti

Tuesday 20 October
16:00 CEST / 14:00 GMT

Wednesday 21 October
19:00 CEST / 17:00 GMT

The information presented at this virtual symposium is scientific information for European Healthcare professionals only, excluding Denmark
Chronic glucokinase activator treatment activates liver Carbohydrate response element binding protein and improves hepatocyte ATP homeostasis during substrate challenge

Brian E. Ford PhD1 | Shruti S. Chachra PhD1 | Ahmed Alshawi PhD1 | Alfie Brennan PhD2 | Suzannah Harnor PhD2 | Celine Cano PhD2 | David J. Baker PhD3 | David M. Smith PhD4 | Rebecca J. Fairclough D.Phil.4 | Loranne Agius D.Phil.1

1Biosciences Institute, Newcastle University, Medical School, Newcastle upon Tyne, UK
2Newcastle Drug Discovery, Newcastle Centre for Cancer, School of Natural and Environmental Sciences, Newcastle University, Newcastle Upon Tyne, UK
3Bioscience, Research and Early Development, Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Cambridge, UK
4Emerging Innovations Unit, Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Cambridge, UK

Correspondence
Loranne Agius, D.Phil., Biosciences Institute, Newcastle University, Medical School, Newcastle upon Tyne, UK.
Email: loranne.agius@ncl.ac.uk

Funding information
Medical Research Council UK, Grant/Award Number: MR/P002854/1

Peer Review
The peer review history for this article is available at https://publons.com/publon/10.1111/dom.14111.

Abstract
Aim: To test the hypothesis that glucokinase activators (GKAs) induce hepatic adaptations that alter intra-hepatocyte metabolite homeostasis.
Methods: C57BL/6 mice on a standard rodent diet were treated with a GKA (AZD1656) acutely or chronically. Hepatocytes were isolated from the mice after 4 or 8 weeks of treatment for analysis of cellular metabolites and gene expression in response to substrate challenge.
Results: Acute exposure of mice to AZD1656 or a liver-selective GKA (PF-04991532), before a glucose tolerance test, or challenge of mouse hepatocytes with GKAs ex vivo induced various Carbohydrate response element binding protein (ChREBP) target genes, including Carbohydrate response element binding protein beta isoform (ChREBP-β), Gckr and G6pc. Both glucokinase activation and ChREBP target gene induction by PF-04991532 were dependent on the chirality of the molecule, confirming a mechanism linked to glucokinase activation. Hepatocytes from mice treated with AZD1656 for 4 or 8 weeks had lower basal glucose 6-phosphate levels and improved ATP homeostasis during high substrate challenge. They also had raised basal ChREBP-β mRNA and AMPK-α mRNA (Prkaa1, Prkaa2) and progressively attenuated substrate induction of some ChREBP target genes and Prkaa1 and Prkaa2.
Conclusions: Chronic GKA treatment of C57BL/6 mice for 8 weeks activates liver ChREBP and improves the resilience of hepatocytes to compromised ATP homeostasis during high-substrate challenge. These changes are associated with raised mRNA levels of ChREBP-β and both catalytic subunits of AMP-activated protein kinase.

KEYWORDS
drug mechanism, energy regulation, liver, pharmacodynamics, type 2 diabetes
1 | INTRODUCTION

The recognition of the unique role of glucokinase in control of blood glucose homeostasis through its dual function in liver and pancreatic islets led to the development of glucokinase activators (GKAs) as candidate drugs for type 2 diabetes.\(^1\,^2\) GKAs were identified that were very effective at lowering blood glucose in animal models and during short-term treatment in human diabetes.\(^3\,^5\) However, in phase 2 clinical trials, efficacy declined during chronic therapy.\(^6\,^8\)

Three hypotheses have been proposed for this decline in efficacy.\(^3\,^5\) The most widely held hypothesis is that liver glucokinase activation leads to increased hepatic production of triglycerides, aggravating fatty liver disease.\(^9\,^11\) A second hypothesis is that liver glucokinase activation raises hepatic glucose 6-phosphate (G6P) and downstream metabolites with consequent activation of metabolite-responsive mechanisms, including the transcription factor Carbohydrate response element binding protein (ChREBP)\(^5\) and induction of its target genes such as the Carbohydrate response element binding protein beta-isoform (ChREBP-\(\beta\)).\(^13\,^14\) A third hypothesis is that chronic overstimulation of pancreatic \(\beta\)-cell glucokinase causes \(\beta\)-cell failure by glucotoxicity.\(^4\) The latter, together with the risk of hypoglycaemia by over-stimulating insulin secretion, could be mitigated by development of liver-selective GKAs.\(^2\)

The arguments supporting the first hypothesis are: first, that blood triglycerides were moderately raised in some phase 2 studies with GKAs\(^6\,^7\); second, elevation in liver triglycerides occurred in some preclinical models\(^9\,^11\); and third, that a common variant in the GCKR gene associates with raised blood lipids and fatty liver disease, possibly through raised glucokinase activity.\(^12\) The arguments against are: first, that loss of GKA efficacy in humans also occurred in the absence of raised blood triglycerides\(^6\); second, that several preclinical GKA models did not show raised blood or liver triglycerides\(^15\,^21\); and third, that metabolites of some GKAs that caused hepatic steatosis were later found to have target-independent hepatotoxicity.\(^22\)

A key caveat to the second hypothesis is that hepatic changes result from induction of inherent adaptive mechanisms involving repression of liver Gck and/or induction of ChREBP target genes to safeguard intrahepatic metabolite homeostasis\(^23\,^24\) rather than cytotoxicity by excess lipid production. The aim of this study was to test the second hypothesis in C57BL/6 mice treated for 8 weeks with AZD1656, a GKA with a good safety record.\(^25\) We chose 8-week exposure because the decline in glycaemic efficacy with AZD1656 became evident within this interval\(^8\) and we used a standard rodent diet because raised hepatic triglyceride levels by GKA therapy is more likely to manifest at low hepatic fat.\(^9\,^10\) We show that hepatocytes from mice treated chronically with AZD1656 have raised basal mRNA levels of ChREBP-\(\beta\) and AMP-activated protein kinase catalytic subunit (AMPK-\(\alpha\)) and improved ATP homeostasis during substrate challenge, together with attenuated induction of ChREBP target genes and the catalytic unit of AMPK (Prkaa1,2). The improved ATP homeostasis supports a beneficial role for ChREBP activation in the hepatic adaptive response to the GKA.

2 | MATERIALS AND METHODS

2.1 | Glucokinase activators

AZD1656 has been described\(^26\) and was provided by AstraZeneca (Cambridge, United Kingdom). Synthesis of PF-04991532\(^27\) (+) and (--) enantiomers for ex vivo studies is described in the Supplementary methods in Appendix S1 and PF-04991532 for in vivo studies was from Tocris Bioscience (C10363).

2.2 | Animals

Animal procedures conformed to Home Office Regulations and were approved by Newcastle University Animal Welfare Ethics Review Board. Male C57BL/6JOlaHSD mice were from Envigo, United Kingdom.

2.3 | Experimental design

For the acute dose study (Figure 1), food was withdrawn 4 hours before glucose gavage. Blood was sampled after 2-hour fasting, mice were gavaged with AZD1656, blood was sampled after 2 hours, followed by glucose gavage (2 g/kg body weight) and blood sampling at the times indicated. For PF-04991532, mice were gavaged with the drug 60 minutes before glucose gavage. Mice were cullad after the last blood sample and the livers snap-frozen in liquid N\(_2\) for RNA extraction and mRNA analysis (quantitative RT-PCR). For the chronic studies (Figure 3) mice received a powdered diet (SDS. 801723. CRM) without or with GKA added to the diet at the doses indicated for the diet consumed (4 g/d). Free-feeding blood samples were collected from the tail vein for glucose, insulin and triglyceride analysis. For drug tolerance tests mice were fasted for 2 hours, a blood sample was collected followed by gavage with AZD1656 and blood was sampled after 2 hours. For the 4- and 8-week studies mice were either cullad (n = 6) for whole liver analysis or used for hepatocyte isolation (n = 3–5).

2.4 | Blood and liver analysis

Blood glucose was determined with a Roche Glucometer; plasma insulin by ELISA (Mercodia #10–1247-01); plasma triglycerides and liver triglycerides, extracted by the method of Bligh and Dyer,\(^28\) were determined with a WAKO kit (290–63 701). Glucokinase activity (Figure 3D,L) was determined on liver 100 000–g supernatant by kinetic assay at 100 and 0.5 mM glucose as described\(^29\) and GKA efficacy (Figure 2A,B) using the same assay at 0.5 mM glucose.\(^29\)

2.5 | Hepatocyte studies

Hepatocytes were isolated by collagenase perfusion of the liver, seeded in 24-well plates and cultured overnight in serum-free.
minimum essential medium (MEM). Parallel incubations were then performed in MEM with the substrates and metabolic inhibitors indicated either for 1 hour for metabolite analysis or 4 hours for mRNA analysis by quantitative RT-PCR of the genes indicated (primers listed in Table S1). Metabolites are expressed as nmol/mg cell protein and mRNA levels as percentage of respective 5 mM glucose control and also normalized to vehicle-treated controls.

3 | RESULTS

3.1 | Mouse liver gene expression after single exposure to AZD1656 or PF-04991532 in vivo

The effects of a GKA on the liver in vivo could be direct on the hepatocyte or indirect through altered blood insulin or glucagon caused by the GKA targeting the pancreas. We compared the effects of AZD1656 with those of PF-04991532, a liver-selective GKA, on liver gene expression after a single exposure to the GKA followed by an oral glucose tolerance test. AZD1656 (2–9 mg/kg), administered 2 hours before the oral glucose tolerance test, lowered blood glucose and glucose excursion (Figure 1A.C.D) and raised insulin (Figure 1E.G.H), whereas PF-04991532 (100 mg/kg) modestly decreased blood glucose (Figure 1B.C) with no effect on insulin (Figure 1F.G). Liver mRNA levels for various ChREBP target genes including ChREBP-β, G6pc, Pklr, Acly, Acac and Gpd2 were increased by PF-04991532 and AZD1656 (Figure 1I).

3.2 | Induction of ChREBP target genes by (+)PF-04991532 but not (−)PF-04991532 in hepatocytes

To test whether liver gene regulation by the GKAs in vivo is consequent to glucokinase activation, we compared the two enantiomers of PF-04991532 on glucokinase activity and gene regulation in mouse
hepatocytes ex vivo. In the glucokinase assay (−)-PF-04991532 was inactive whereas (+)-PF-04991532 (10 μM) was a potent GKA (Figure 2A) and caused greater activation than AZD1656 (Figure 2B). This concurs with the dual effect of PF-04991532 on $S_{0.5}$ and maximum activity (1.6-fold), whereas AZD1656 only affects $S_{0.5}$.

**FIGURE 2** Comparison of AZD1656 and PF-04991532 (+)/(−) enantiomers with substrate challenge on gene expression in mouse hepatocytes. A–D, Comparison of (+) and (−) enantiomers of PF-04991532 and AZD1656 on glucokinase activity assayed at 0.5 mM glucose (A) and gene expression in hepatocytes in minimum essential medium (MEM) with 5 mM glucose (C,D). E, Pathway showing xylitol metabolism to fructose 6-P (F6P) and triose phosphates and sites of action of: AZD1656 and PF-04991532 [glucokinase activators (GKAs)]; S4048 [inhibitor of glucose 6-phosphate (G6P) hydrolysis]; amino-oxyacetate (AOA; inhibitor of oxidation of cytoplasmic NADH). F–N, Hepatocytes from C57BL/6 mice were incubated for either 1 hour for metabolite analysis (F,G) or 4 hours for mRNA analysis (H–O) in MEM containing 5 mM glucose (5G) and the additions indicated: 10 μM AZD1656 (AZD); 10 μM PF(+)-04991532 (PF); xylitol (2 mM, XY); AOA (0.1 mM) or with 25 mM glucose without (25G) or with 0.4 μM S4048 (25GS). Cell G6P and glycerol 3-P (G3P) are expressed as nmol/mg protein and cell ATP as % 5 mM glucose control. mRNA levels are normalized to control incubations with 5 mM glucose alone. Values are means ± SEM for n = 4 (AOA, xylitol) or n = 8 (all other conditions) hepatocyte preparations. *P <0.05 relative to 5 mM glucose alone. https://doi.org/10.25405/data.ncl.12550541
including ChREBP-β, whereas the (-) enantiomer had no effect (Figure 2C), confirming that gene induction is consequent to glucokinase activation. AZD1656 caused similar gene induction but mostly with lower efficacy (Figure 2D), consistent with the lower glucokinase activation (Figure 2B).

3.3 | Comparison of GKAs with substrate challenge on cell metabolites and gene regulation in hepatocytes

The induction of ChREBP target genes is linked to raised metabolites, such as G6P and other phosphate esters.23,31,32 We next compared the effects of the GKAs on cell metabolites [ATP, G6P, glycerol 3-P (G3P)] and ChREBP target gene induction with substrate challenges known to raise G6P (eg, high glucose) or more distal metabolites. For the latter we used xylitol (2 mM) which is metabolized independently of glucokinase and, similarly to fructose, lowers ATP because of excessive accumulation of phosphate esters, mainly as G3P.36,37 We used an inhibitor of G6P hydrolysis (S4048, a chlorogenic acid derivative)31,32 to further raise G6P with 25 mM glucose and an inhibitor of NADH shuttling to mitochondria [amino-oxyacetate (AOA)] to raise G3P with xylitol30 (Figure 2E). PF-04991532 at 5 mM glucose raised G6P more than AZD1656 or 25 mM glucose but less than 25 mM glucose with S4048 (Figure 2F). However, xylitol, either alone or with AOA, had modest effects on G6P but markedly raised G3P (Figure 2F), as expected.30,36,37 Cell ATP was not affected by the GKAs but was

---

**FIGURE 3** Chronic effects of AZD1656 on blood glucose, liver triglycerides and glucokinase activity. A–D, C57BL/6 mice were treated for 1 week without or with AZD1656 (at 0.3, 1, 3 and 9 mg/kg body weight): A, blood glucose; B, plasma insulin at the times indicated; C, liver mRNA levels; D, liver glucokinase activity. Means ± SEM for n = 6, *P <0.05 vs vehicle. E–L, C57BL/6 mice were treated for 4 or 8 weeks without (0) or with AZD1656 (1 or 3 mg/kg body weight): E, blood glucose; F, plasma insulin; G, acute (120 min) blood glucose-lowering by AZD1656 (drug tolerance test); H, blood triglycerides (TAGs); I, liver triglycerides (TAGs); J, liver mRNA after 4 weeks; K, liver mRNA after 8 weeks; L, liver glucokinase activity. Means ± SEM for n = 10–12, E–H) or n = 6 (I–L). https://doi.org/10.25405/data.ncl.12550574
lowered by xylitol and by high glucose with S4048 (Figure 2G). ATP depletion by xylitol is analogous to fructose challenge and lowers mitochondrial ATP production by trapping phosphate in G3P.37 ChREBP-α mRNA was modestly raised at the highest G6P (Figure 2H), whereas ChREBP-β, a downstream target of ChREBP-α,38 was induced two- to five-fold at raised G6P or G3P and was decreased with AOA alone (Figure 2I). Gck, which is not a ChREBP target gene,31 was repressed by raised G6P but not G3P (Figure 2J). Gckr, Pklr, G6pc, Txnip and FGF21, which are ChREBP target genes, were induced in association with raised G6P or G3P (Figure 2K–O), with G6pc and FGF21 more strongly induced by G6P and G3P, respectively. Cumulatively, induction of ChREBP target genes is linked to raised hexose-P or triose-P, and GKAs raise predominantly hexose-P rather than triose-P, without lowering ATP.

3.4 Effects of chronic exposure to AZD1656 on liver gene expression

We next performed a 1-week chronic study with four doses of AZD1656 (0.3, 1, 3 and 9 mg/kg) to select two doses for the 8-week study. Blood glucose-lowering by AZD1656 was dose-dependent, blood insulin was not significantly increased, ChREBP target gene mRNA levels were increased dose-dependently at or above 1 mg/kg and total liver glucokinase activity at or above 3 mg/kg (Figure 3A–D). Because the liver Gck gene is induced by raised insulin or by lowered glucagon,24 we infer that the raised total glucokinase at 3 to 9 mg/kg suggests liver exposure to a raised insulin/glucagon ratio. Accordingly, we selected 1 and 3 mg/kg AZD1656 for further study.

During 8-week treatment with AZD1656 body weight gain was unchanged (Figure S1), blood glucose-lowering was maintained and insulin was modestly but not significantly raised (Figure 3E,F). The response to an intra-gastric load of AZD1656 (drug tolerance test [DTT], 120 minutes) showed maintained blood glucose lowering at 4 and 8 weeks (Figure 3G) and stimulation of insulin secretion at 4 weeks but not 8 weeks (Figure S1). Liver triglycerides and liver triglyceride were unchanged (Figure 3H, I). Liver mRNA levels of ChREBP target genes that were induced by single G6P elevation during substrate challenge (Figure 4I). Elevation in G3P by substrate challenge was attenuated in the 1 mg/kg AZD1656 group (Figure 4J). Basal ATP was similar across groups (Figure 4K) but fractional ATP-lowering by xylitol + AOA or by 25 mM glucose + S4048 was attenuated in the AZD1656 treatments (Figure 4L). Basal mRNA levels at 5 mM glucose (Figure 4M) were modestly but significantly raised in the 3-mg/kg AZD1656 group for three genes including Gckr, whereas G6pc was decreased in the 1-mg/kg AZD1656 hepatocytes similar to 4-week treatment. The percentage induction of ChREBP-β, Gckr, Fasn and Acly with substrate challenge was attenuated in hepatocytes from AZD1656 treatments (Figure 4N–P, Figure S3), whereas other ChREBP target genes (Pklr, Gpd2, Txnip, G6pc, FGF21) and Gck repression were not different across groups (Figure S3). Cumulatively, chronic treatment with AZD1656 had three effects that manifest in hepatocytes ex vivo: (1) lower basal G6P at 5 mM glucose; (2) better preservation of ATP during substrate challenge with xylitol or 25 mM glucose+S4048; and (3) attenuated induction of ChREBP-β and other ChREBP target genes during substrate challenge.

To test for candidate mechanisms for the improved ATP homeostasis during substrate challenge, we determined mRNA levels of the catalytic subunits of AMPK (Prkaa1 and Prkaa2) because Prkaa2 was identified as a putative ChREBP target by chromatin immunoprecipitation sequencing.39 We found raised basal Prkaa1,2 mRNA in hepatocytes from AZD1656 (3 mg/kg)-treated mice relative to vehicle (Figure 5A,B) and 1.5-fold induction by ex vivo substrate challenge raising G6P or G3P in hepatocytes from vehicle-treated mice but not from AZD1656 (3 mg/kg)-treated mice (Figure 5C,D). This attenuated

3.5 Improved ATP homeostasis and blunted ChREBP-β induction in hepatocytes from AZD1656-treated mice

To test for changes in metabolite homeostasis, hepatocytes were isolated from the three groups of mice after the 4- or 8-week treatments and cultured overnight at 5 mM glucose, followed by substrate challenge for cell metabolite and gene expression analysis. For the 4-week treatment (Figure 4A–H) we used the same substrates as in Figure 2F,G. Hepatocytes from AZD1656-treated mice had lower basal G6P and G3P at 5 mM glucose but similar G6P elevation with substrate challenge (Figure 4A,B). Basal ATP at 5 mM glucose was similar across groups (Figure 4C). However, the fractional lowering of ATP by xylitol was greater in the untreated mice than in the AZD1656 treatments (67%, 82% and 90%, 0, 1 and 3 mg/kg, respectively). Likewise, with 25 mM glucose + S4048, fractional ATP-lowering was attenuated by AZD1656 treatments (82%, 87% and 97%; Figure 4D), indicating resilience to ATP-lowering. Basal mRNA levels were mostly similar across treatment groups except for G6pc and Gck (Figure 4E), which are known to be regulated oppositely by the direct GKA effect on liver versus indirect effect through pancreatic targeting.28 To assess the gene response to the substrate challenge ex vivo, mRNA levels were expressed as percentage of respective 5 mM glucose controls (Figure 4F–H, Figure S3A–D). The induction of ChREBP-β and Pklr by substrate challenge was attenuated in hepatocytes from 3 mg/kg AZD1656-treated mice (Figure 4F,G).
**DISCUSSION**

In the present study we explored the hepatic adaptations in C57BL/6 mice during 4 to 8 weeks of treatment with AZD1656, a GKA with an established safety record\(^{25}\) that has been studied preclinically\(^{18,19}\) and clinically, and showed a decline in glycaemic efficacy at approximately 4 to 8 weeks.\(^{8}\) Previous work with other GKAs in rat hepatocytes *in vitro* had shown induction of *G6pc* and *Pklr*, which are ChREBP target genes and repression of *Gck*, which is not a ChREBP target.\(^{13,14,31}\) Here we tested the hypothesis that chronic exposure to AZD1656 causes hepatic adaptations linked to ChREBP activation and liver *Gck* repression. Various sets of evidence support adaptive changes consequent to ChREBP activation. However, we found very modest changes in liver total glucokinase activity. During 8-week exposure to AZD1656, blood glucose-lowering efficacy was maintained and there were no changes in blood or liver triglycerides.
We compared AZD1656 with PF-04991532, a liver-selective GKA, to identify AZD1656 doses with optimal liver targeting and minimal targeting of pancreatic islet glucokinase, to minimize confounding effects from increased insulin exposure, which has converse effects on liver Gck and G6pc, to those expected from direct GKA effects on liver.13 We show that AZD1656 and PF-04991532 induce several ChREBP target genes, including ChREBP-β, after acute exposure in vivo and in mouse hepatocytes ex vivo (Figures 1 and 2). These effects are unique to the active enantiomer of PF-04991532 that functions as a GKA, and associate with the raised G6P. At doses of AZD1656 and PF-04991532 causing comparable ChREBP activation, AZD1656 was far more effective at lowering blood glucose. This greater glycaemic efficacy of AZD1656 is best explained by stimulatory effects of the GKA on insulin secretion and possible inhibitory effects on glucagon secretion,33,34 resulting in liver exposure to a raised insulin to glucagon ratio. Although we confirmed that Gck mRNA levels change inversely with raised G6P in mouse hepatocytes (Figure 2) similar to rat hepatocytes,13,31 we found very modest lowering of total glucokinase (18%) after 8 weeks with 1 mg/kg AZD1656 and no effect at 3 mg/kg AZD1656. Increased hepatic exposure to a raised insulin to glucagon ratio would promote induction of the Gck gene and counterbalance the repression through raised G6P and could explain the increase in total glucokinase activity in the 1-week study by higher doses of AZD1656 and possibly also the modest lowering of glucokinase by 1 mg but not 3 mg AZD1656 after 8 weeks. We infer that ChREBP activation has a more prominent role than Gck repression in the chronic hepatic adaptations to AZD1656.

ChREBP (encoded by Mlxipl) is a major transcriptional regulator in liver that is activated by high dietary carbohydrate and is frequently described as a “glucose sensor” because it is activated by high glucose.38 However, the stimulus for its activation is the raised phosphate esters and not glucose itself, as shown by inhibitors of hexokinases and G6P hydrolysis which modulate cell G6P and ChREBP target induction.23,31,32 ChREBP is expressed as two isoforms (α and β) by alternative splicing of the first exon.38 ChREBP-α protein accumulates in the cytoplasm at low glucose, and is regulated by an inhibitory domain which enables activation by metabolites causing translocation to the nucleus where it activates ChREBP target genes.38 ChREBP-β lacks the inhibitory domain and is constitutively active in the nucleus and moreover the ChREBP-β promoter is itself a target of ChREBP-α.38 Accordingly, conditions associated with raised phosphate esters, such as high-fructose diets, cause strong induction of ChREBP-β,38 making ChREBP-β mRNA a convenient read-out of ChREBP activation. Although ChREBP is often functionally described as a “lipogenic” transcription factor, its wide array of target genes,39 which include G6pc and Gckr,14,32 implicate a more complex role in metabolite homeostasis.23 This is supported by germ-line and liver-
selective ChREBP deletion models which have markedly raised phosphate esters and compromised ATP homeostasis, particularly when challenged with dietary fructose. The hypothesis that ChREBP activation is a component of the chronic effects of AZD1656, predicts changes in metabolite homeostasis in hepatocytes. To test this hypothesis we challenged hepatocytes isolated from mice treated chronically with AZD1656 with substrates that raise G6P or the more distal metabolite G3P. We used xylitol as a surrogate for fructose, because, like fructose, it compromises ATP homeostasis, but does so by raising G3P, which can be monitored accurately.

Three key findings emerged from the hepatocyte studies on mice treated chronically with AZD1656. First, there was lowering of basal G6P at 5 mM glucose but not maximal G6P levels in substrate-challenged conditions. Second, ATP depletion during substrate challenge was attenuated despite sustained elevation in G3P and G6P, Third, induction of ChREBP target genes (including ChREBP-β) by the substrate challenge ex vivo was attenuated despite lack of attenuation of the raised phosphate esters. The improved ATP homeostasis in the absence of attenuation of raised G6P or G3P was surprising. However, ATP homeostasis involves complex recovery mechanisms and moreover, the target genes of ChREBP include some of the seven subunits of AMPK which has an established role in ATP homeostasis, as shown by the greater ATP depletion at raised G6P in hepatocytes from AMPK-KO mice. The attenuated fractional induction of ChREBP-β in hepatocytes from AZD1656-treated mice is only in part explained by higher basal ChREBP-β mRNA. To our knowledge, this study is the first to demonstrate induction of the AMPK-α subunits (Prkaa1 and Prkaa2) at the mRNA level in hepatocytes with raised G6P or G3P. AMPK is an energy sensor but crucially also a negative regulator of lipogenesis. For both Prkaa1 and Prkaa2 the substrate induction was abolished in hepatocytes from the high-dose AZD1656 treatment, in association with raised basal Prkaa1 and Prkaa2 mRNA. The mechanism(s) by which metabolites of glucose or fructose activate ChREBP is far from understood and is highly unlikely to involve a single metabolite. A tentative conjectural hypothesis to explain the attenuated induction of candidate ChREBP target genes in substrate-challenged conditions despite sustained elevation in G6P or G3P, is that ChREBP activation may be a composite function of both raised phosphate esters (beyond the homeostatic range) and of compromised ATP homeostasis (adenine nucleotide phosphorylation potential), to varying degrees depending on the gene. The attenuated induction of some genes in association with improved ATP homeostasis may reflect their stronger regulatory links with ATP homeostasis as compared with raised phosphate esters. An analogous mechanism for composite control by a glucose metabolite and a signal from the electron transport chain, was recently reported for MondoA (encoded by Mxip), the parologue of ChREBP.

CONFLICTS OF INTEREST
B.E.F., S.S.C., A.A., A.B., S.H., C.C. and L.A. declare no conflict of interest. D.J.M., D.M.A. and R.J.F. are employees and shareholders of AstraZeneca.

AUTHOR CONTRIBUTIONS
B.E.F., S.S.C., A.A., A.B., S.H. and C.C. designed and performed experiments and analysed the data; L.A. directed the study, wrote the paper and is the guarantor of the work. D.J.B., D.M.S. and R.J.F. contributed to experimental design by providing guidance on AZD1656.

ORCID
Brian E. Ford https://orcid.org/0000-0003-0543-4978
Suzannah Harnor https://orcid.org/0000-0003-1646-593X
Celine Cano https://orcid.org/0000-0002-2032-2272
Loranne Agius https://orcid.org/0000-0002-6419-6503

REFERENCES
1. Sarabu R, Berthel SJ, Kester RF, Tilley JW. Novel glucokinase activators: a patent review (2008 - 2010). Expert Opin Ther Pat. 2011;21:13-33.
2. Filipski KJ, Pfefferkorn JA. A patent review of glucokinase activators and disruptors of the glucokinase–glucokinase regulatory protein interaction: 2011–2014. Expert Opin Ther Pat. 2014;24:875-891.
3. Matschinsky FM. GKAs for diabetes therapy: why no clinically useful drug after two decades of trying? Trends Pharmacol Sci. 2013;34:90-99.
4. Nakamura A, Terauchi Y. Present status of clinical deployment of glucokinase activators. J Diabetes Investig. 2015;6:124-132.
5. Agius L. Lessons from glucokinase activators: the problem of declining efficacy. Expert Opin Ther Pat. 2014;24:1155-1159.
6. Meininger GE, Scott R, Alba M, et al. Effects of MK-0941, a novel glucokinase activator, on glycemic control in insulin-treated patients with type 2 diabetes. Diabetes Care. 2011;34:2560-2566.
7. Wilding JP, Leinsson-Zachrisson M, Wessman C, Johnsson E. Dose-ranging study with the glucokinase activator AZD1656 in patients with type 2 diabetes mellitus on metformin. Diabetes Obes Metab. 2013;15:750-759.
8. Kiyosue A, Hayashi N, Komori H, Leinsson-Zachrisson M, Johnsson E. Dose-ranging study with the glucokinase activator AZD1656 as monotherapy in Japanese patients with type 2 diabetes mellitus. Diabetes Obes Metab. 2013;15:923-930.
9. De Ceuinck F, Kargar C, Charton Y, et al. S 50131 and S 51434, two novel small molecule glucokinase activators, lack chronic efficacy despite potent acute antihyperglycaemic activity in diabetic mice. Br J Pharmacol. 2013;169:999-1010.
10. De Ceuinck F, Kargar C, Ilic C, et al. Small molecule glucokinase activators disturb lipid homeostasis and induce fatty liver in rodents: a warning for therapeutic applications in humans. Br J Pharmacol. 2013;168:339-353.
11. Dhanesha N, Joharapurkar A, Shah G, et al. Treatment with exendin-4 improves the antidiabetic efficacy and reverses hepatic steatosis in glucokinase activator treated db/db mice. Eur J Pharmacol. 2013;714:188-192.
12. Brouwers MCGJ, Jacobs C, Bast A, Stehouwer CDA, Schaper NC. Modulation of Glucokinase regulatory protein: a double-edged sword? Trends Mol Med. 2015;21:583-594.
13. Al-Qanzi ZH, Fountana S, Mooinira T, et al. Opposite effects of a glucokinase activator and metformin on glucose-regulated gene expression in hepatocytes. Diabetes Obes Metab. 2017;19:1078-1087.
14. Ma L, Robinson LN, Towle HC. ChREBP*mlx is the principal mediator of glucose-induced gene expression in the liver. J Biol Chem. 2006;281:28721-28730.
15. Nakamura A, Terauchi Y, Ohyama S, et al. Impact of small-molecule glucokinase activator on glucose metabolism and beta-cell mass. Endocrinology. 2009;150:1147-1154.
16. Eliki J, Nagata Y, Futamura M, et al. Pharmacokinetic and pharmacodynamics properties of the glucokinase activator MK-0941 in rodent models of type 2 diabetes and healthy dogs. Mol Pharmacol. 2011;80:1156-1165.

17. Futamura M, Yao J, Li X, et al. Chronic treatment with a glucokinase activator delays the onset of hyperglycaemia and preserves beta cell mass in the Zucker diabetic fatty rat. Diabetologia. 2012;55:1071-1080.

18. Wintzell MS, Coghlan M, Leighton B, et al. Chronic glucokinase activation reduces glycaemia and improves glucose tolerance in high-fat diet fed mice. Eur J Pharmacol. 2011;663:80-86.

19. Baker DJ, Wilkinson GP, Atkinson AM, et al. Chronic glucokinase activator treatment at clinically translatable exposures gives durable glucose lowering in two animal models of type 2 diabetes. Br J Pharmacol. 2014;171:1642-1654.

20. Erion DM, Lapworth A, Amor PA, et al. The hepatoselective glucokinase activator PF-04991532 ameliorates hyperglycaemia without causing hepatic steatosis in diabetic rats. PLoS One. 2014;9:e97139.

21. Tsumura Y, Tsushima Y, Tamura A, et al. TMG-123, a novel glucokinase activator, exerts durable effects on hyperglycemia without increasing triglyceride in diabetic animal models. PLoS One. 2017;12:e0172252.

22. Sarabu R, Bizzarro FT, Corbett WL, et al. Discovery of piragliatin-6-(3-cyclopentyl-2-(4-[trifluoromethyl]-1H-imidazol-1-yl)propanamido)nicotinic acid as a hepatoselective glucokinase activator. J Med Chem. 2012;55:7021-7036.

23. Agius L. Dietary carbohydrate and control of hepatic gene expression: mechanistic links from ATP and phosphate ester homeostasis to the carbohydrate-response element-binding protein. Proc Nutr Soc. 2016;75:10-18.

24. Agius L. Hormonal and metabolite regulation of hepatic Glucokinase. Annu Rev Nutr. 2016;36:389-415.

25. Morrow LA, Leonsson-Zachrisson M, Ericsson H, et al. Safety, pharmacokinetics and pharmacodynamics of multiple-ascending doses of the novel glucokinase activator AZD1656 in patients with type 2 diabetes mellitus. Diabetes Obes Metab. 2012;14:1114-1122.

26. Waring MJ, Clarke DS, Fenwick MD, Godfrey L, Groombridge SD, Johnstone C. Property based optimization of glucokinase activators-discovery of the phase 1b clinical candidate AZD1656. Med Chem Comm. 2012;3:1077-1081.

27. Pfefferkorn JA, Guzman-Perez A, Litchfield J, et al. Discovery of (S)-6-(2-cyclopentyl-2-(4-[trifluoromethyl]-1H-imidazol-1-yl)propanamido)nicotinic acid as a hepatoselective glucokinase activator clinical candidate for treating type 2 diabetes mellitus. J Med Chem. 2012;55:1319-1333.

28. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959;37:911-917.

29. Davidson AL, Arion WJ. Factors underlying significant underestimations of glucokinase activity in crude liver extracts: physiological implications of higher cellular activity. Arch Biochem Biophys. 1987;253:156-167.

30. Alshawi A, Agius L. Low metformin causes a more oxidized mitochondrial NADH/NAD redox state in hepatocytes and inhibits gluconeogenesis by a redox-independent mechanism. J Biol Chem. 2019;294:2839-2853.

31. Arden C, Tudhope SJ, Petrie JL, et al. Elevated glucose represses liver glucokinase and induces its regulatory protein to safeguard hepatic phosphate homeostasis. Diabetes. 2011;60:3110-3120.

32. Arden C, Tudhope SJ, Petrie JL, et al. Fructose 2,6-bisphosphate is essential for glucose-regulated gene transcription of glucose-6-phosphatase and other ChREBP target genes in hepatocytes. Biochem J. 2012;443:111-123.

33. Basco D, Zhang Q, Salehi A, et al. α-cell glucokinase suppresses glucose-regulated glucagon secretion. Nat Commun. 2018;9:546.

34. Norjanaara E, Ericsson H, Sjöberg F, et al. Glucokinase activators AZD6370 and AZD1656 do not affect the central counterregulatory response to hypoglycemia in healthy males. J Clin Endocrinol Metab. 2012;97:3319-3325.

35. Brocklehurst KJ, Payne VA, Davies RA, et al. Stimulation of hepatocyte glucose metabolism by novel small molecule glucokinase activators. Diabetes. 2004;53:535-541.

36. Woods HF, Eggleton LV, Krebs HA. The cause of hepatic accumulation of fructose 1-phosphate on fructose loading. Biochem J. 1970;119:501-510.

37. Woods HF, Krebs HA. Xylitol metabolism in the isolated perfused rat liver. Biochem J. 1973;134:437-443.

38. Kim MS, Krawczyk SA, Doridot L, et al. ChREBP regulates fructose-induced glucose production independently of insulin signaling. J Clin Invest. 2016;126:4372-4386.

39. Poungvarin N, Chang B, Imamura M, et al. Genome-wide analysis of ChREBP binding sites on male mouse liver and white adipose chromatin. Endocrinology. 2015;156:1982-1992.

40. Burgess SC, Iizuka K, Jeoung NH, et al. Carbohydrate-response element-binding protein deletion alters substrate utilization producing an energy-deficient liver. J Biol Chem. 2008;283:1670-1678.

41. Shi JH, Lu JY, Chen HY, et al. Liver ChREBP protects against fructose-induced glycogenic hepatotoxicity by regulating L-type pyruvate kinase. Diabetes. 2020;69(4):591-602.

42. Zhang D, Tong X, VanDommelen K, et al. Lipogenic transcription factor ChREBP mediates fructose-induced metabolic adaptations to prevent hepatotoxicity. J Clin Invest. 2017;127:2855-2867.

43. Moonira T, Chachra SS, Ford BE, et al. Metformin lowers glucose 6-phosphate in hepatocytes by activation of glycolysis downstream of glucose phosphorylation. J Biol Chem. 2020;295(10):3330-3346.

44. Agius L, Ford BE, Chachra SS. The metformin mechanism on gluconeogenesis and AMPK activation: the metabolite perspective. Int J Mol Sci. 2020;21(9):3240.

45. Wilde BR, Ye Z, Lim TY, Ayer DE. Cellular acidosis triggers human MondoA transcriptional activity by driving mitochondrial ATP production. Elife. 2019;8:e40199

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Ford BE, Chachra SS, Alshawi A, et al. Chronic glucokinase activator treatment activates liver Carbohydrate response element binding protein and improves hepatocyte ATP homeostasis during substrate challenge. Diabetes Obes Metab. 2020;22:1985–1994. https://doi.org/10.1111/dom.14111