Small molecule glucokinase activators as novel anti-diabetic agents

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Abstract
The monomeric enzyme GK (glucokinase) has a low affinity for glucose and, quantitatively, is largely expressed in the liver and pancreatic β-cells, playing a key ‘glucose sensing’ role to regulate hepatic glucose balance and insulin secretion. Mutations of GK in man can be inactivating, to cause a form of diabetes mellitus, or activating, to lower blood glucose levels. Recently, models of GK protein structure have helped to elucidate the role of inactivating and activating mutations, with the latter revealing an allosteric binding site, possibly for an unknown physiological activator. However, this discovery was pre-dated by Drug Discovery projects that have identified small organic molecules that activate pancreatic and liver GK enzyme activity. These compounds stimulate insulin secretion in islets and glucose metabolism in hepatocytes. The profile of these GK activators, both in vitro and in vivo and the potential role that GK activators play in lowering blood glucose levels in Type II diabetes mellitus will be discussed.

T2D (Type II diabetes mellitus) and the role of glucose and insulin in HGB (hepatic glucose balance)
The regulation of blood glucose homoeostasis is complex. T2D is characterized by elevated blood glucose levels or hyperglycaemia, and results from failure of pancreatic β-cells to secrete sufficient insulin to overcome insulin resistance (mainly in liver, adipose and skeletal muscle). Anti-diabetic agents seek to reduce hyperglycaemia and, thus, diminish the elevated risk of micro- and macro-vascular disease in T2D patients. In non-diabetic humans, the liver plays a key role in glucose homoeostasis (see Figure 1), producing glucose during the post-absorptive period and taking up glucose for storage as glycogen during the post-prandial phase [1]. Blood borne glucose and insulin work in tandem to regulate HGB: increasing concentrations shut off HGP (HG production) and increase HGU (HG uptake); decreasing concentrations promote glucagon secretion from pancreatic α-cells, which drives HGP [1].

However, HGB is abnormal in T2D as hyperglycaemia fails to suppress HGP and to stimulate HGU [1,2]. This inability of glucose to regulate HGB in T2D is often referred to as decreased GE (‘glucose effectiveness’). Accumulating evidence suggests that decreased flux through hepatic GK (glucokinase) underlies the decrease in GE in people with T2D [2]. Impaired GE in T2D is compounded further by lower than normal rates of pancreatic insulin secretion; a defect that may also contribute to abnormal HGB. Pancreatic α-cells express GK, which when activated might decrease glucagon secretion, but this notion has yet to be tested.

Therefore, any agent that can stimulate pancreatic insulin secretion and restore GE should normalize HGB and have efficacy in decreasing hyperglycaemia in people with T2D. An overview of the regulation of GK activity, and the action of small organic molecule activators of GK on liver and pancreatic β-cell function is described. The hypothesis that activation of GK may be a valuable approach to diminish hyperglycaemia in people with diabetes is discussed further.

Introduction to GK
Conversion of glucose into glucose 6-phosphate is catalysed by GK (EC 2.7.1.1, also called hexokinase IV or D). Hexokinases differ, in terms of enzyme kinetics, from GK, which has a much lower affinity for glucose and sigmoidal kinetics. These properties underlie the role of GK as a ‘sensor’ of blood glucose concentrations [3]. So as blood glucose levels rise after a meal, GK activity is elevated to increase HGU and decrease HGP.

In the liver, but not in the pancreas, GK activity is regulated by a 68 kDa GKRP (GK regulatory protein), which inhibits GK competitively with respect to glucose [4]. The binding affinity of GKRP for GK is increased by fructose 6-phosphate and decreased by fructose 1-phosphate. GKRP also determines the subcellular location of GK and sequesters the enzyme in the nucleus of liver cells in the fasted state [5]. This mechanism enables rapid translocation of the enzyme to the cytoplasm in response to a rise in the extra-cellular concentration of glucose or fructose, a precursor of fructose 1-phosphate, resulting in a rapid increase in the rate of glucose phosphorylation and utilization, after a meal [5].

Intraportal infusion of fructose at a low dose during a hyperinsulinaemic hyperglycaemic clamp enhances HGU and hepatic glycogen storage in conscious dogs [1]. Low-dose...
fructose infusion decreased HGP in people with T2D and stimulates hepatic glycogen synthesis during a euglycaemic clamp in non-diabetic subjects [1]. These results are consistent with the notion that improving GK activity improves HGB by restoring hepatic GE.

**Target validation in mice and man**

A number of studies in rodents support a critical role for GK in glucose homeostasis. Mice die within days of birth of severe diabetes if there is global or pancreatic β-cell disruption of the GK gene, whereas the heterozygotes are only modestly hyperglycaemic [6]. Heterozygous disruption of hepatic GK in the mouse results in a loss of GE on HGP and a decrease in HGU [7]. In contrast, a β-cell selective decrease in GK activity causes decreased insulin secretion but no changes in fasting plasma glucose levels or glucose tolerance, with GE preserved [7]. Hepato-selective GK over-expression in non-diabetic mice improved glucose tolerance [8]. In high-fat diet-induced diabetic mice, adenoviral over-expression of GK improved glucose tolerance and decreased fasting blood glucose with concomitant decreased insulin secretion [9].

In humans, heterozygous loss-of-function mutations in the GK gene lead to a form of mild and stable chronic hyperglycaemia, called MODY2 (maturity onset diabetes of the young, Type II), which is associated with defective insulin secretion [3]. After a meal, liver glycogen content is lower in MODY2, compared with non-diabetic subjects, and this defect is associated with exaggerated post-prandial hyperglycaemia and impaired suppression of HGP. These observations are consistent with impaired GE in people with defective GK activity [10].

In contrast, rare activating mutations of GK in man cause hyperinsulinaemia with hypoglycaemia [3], but the effect of these mutations on HGP or GE has not been reported. Functional in vitro studies show that these mutations increase GK activity by increasing the affinity for glucose. Recent structural information has helped to elucidate the molecular mechanism by which these mutations activate GK. The crystal structure of GK has revealed a hydrophobic allosteric pocket, remote from the catalytic site (20 Å distant, 1 Å = 10^{-10} m), which is exposed to solvent when the kinase is bound to glucose in its ‘closed’ catalytically active state. The pocket is formed at the hinge region between the large and small domains when the kinase adopts the closed conformation upon glucose binding. The entropic penalty incurred through exposure of the hydrophobic pocket probably accounts for the greater stability of the ‘open’ form of the kinase. In this glucose unbound form, the hydrophobic pocket is buried within the opposed large and small domains. Interestingly, most of the activating mutations (T651I, Y214C, V455M, A456V) are predicted to stabilize the closed active conformation through reducing the hydrophobic surface area of the allosteric pocket exposed to solvent. The remaining activating mutations, which map outside of the pocket, are thought to stabilize the closed state by directly affecting domain–domain interactions.

**In vitro and in vivo GKA (GK activator) effects**

Several GKAs have been described during the past three years [11–13]. These compounds (GKA1, GKA2, RO-28-1675 and compound A) directly activate GK. These chemically distinct compounds, with potencies (EC_{50}) in the sub-micromolar range, act to increase the affinity of the kinase for glucose [11–13]. These compounds differ with respect to how they influence the other kinetic parameters of GK. For example, we reported that GKA1 and GKA2 did not affect in vitro enzymatic V_{max} [13], whereas others have reported GKA compounds (RO-28-1675 or compound A) that increase the V_{max} of GK [11,12].
Binding of a GKA causes subtle changes in structure that appear to stabilize the closed form of the enzyme and mimic, to some degree, the activating mutations. Co-crystallization of GKAs with GK shows that these compounds bind to the allosteric pocket [3,12]. GKAs do not activate other hexokinases as only GK possesses this binding pocket.

We have shown that GKA1 and GKA2 are potent activators (EC\textsubscript{50} of 1 and 2–3 \(\mu\)M, respectively) of glucose metabolism in hepatocytes, stimulating glucose phosphorylation, glycolysis, and glycogen synthesis to a similar extent but by a mechanism independent of GKRP [13]. Consistent with their effects on isolated GK, these compounds also increased the affinity of hepatocyte metabolism for glucose. GKA1 and GKA2 caused translocation of GK from the nucleus to the cytoplasm [13]. This effect was additive with the effect of sorbitol and is best explained by a ‘glucose-like’ effect of GKAs in translocating GK to the cytoplasm [13]. The compounds cause translocation of GK from the nucleus despite the fact that they do not dissociate GK from GKRP [13]. It has been generally assumed that the translocation induced by glucose is due to dissociation of GK from GKRP. However, we confirmed by size-exclusion chromatography that high glucose, like GKA1 and in contrast with fructose 1-phosphate, does not cause dissociation of GK from GKRP.

RO-28-1675 lowers the threshold for glucose-stimulated insulin release from isolated perfused rat or mouse islets [3] in a glucose dependent manner. There was no effect of RO-28-1675 on \(\alpha\)-ketoisocaproic acid stimulated insulin release (which causes glucose-independent insulin secretion). Consistent with this finding, RO-28-1675 stimulated an increase in cytosolic calcium in isolated cultured mouse pancreatic islets, but only when glucose was present in the medium.

Administration of a single acute oral dose of GKA1 to overnight fasted female mice caused a dose-dependent decrease in blood glucose levels (see Figure 2).

Similarly [3], acute oral administration of RO-28-1675 (15–50 mg/kg) decreased blood glucose levels and/or improved glucose tolerance in non-diabetic mice (C57BL/6J mice) and animal models of diabetes (ob/ob mice, KK-Ay mice, diet-induced obese C57BL/6J mice and Goto–Kakizaki rats). So, GKAs appear to be efficacious for blood glucose lowering in a range of rodent models of diabetes, and even in non-diabetic rodents. For example, RO-28-1675 decreased blood glucose levels to low values in non-fasted Wistar rats and C57BL/6J mice and caused a significant increase in plasma insulin levels in the mice (though similar observations were not described for other rodents). This might suggest that the glucose lowering effect is not solely due to pancreatic insulin secretion.

Strong evidence for direct effects of GKAs on hepatic glucose metabolism was obtained in 18 h fasted conscious Sprague-Dawley rats maintained on a pancreatic clamp (which keeps the contribution of insulin and glucose from the pancreas low and constant) [12]. Rats were clamped at constant levels of glucose (about 8 mM) and both insulin and RO-28-1675 increased the glucose infusion rate. Hyperglycaemia, via GE, decreased rates of HGP, but further treatment with a RO-28-1675 decreased rates of HGP still further and promoted HGB towards net glucose uptake [12]. Zucker diabetic rats, an animal model of diabetes, have very high rates of HGP. Acute administration of RO-281675 (30 mg/kg) to these diabetic rats return rates of HGP to near basal values [3].

**Strengths and limitation of small molecule GKAs as blood glucose lowering therapy in T2D**

Emerging scientific evidence (highlighted in previous sections) favours the notion that GKAs will affect the liver and pancreas, two tissues that heavily influence glucose homoeostasis. GKAs should enhance insulin secretion but also act on the liver to begin to normalize HGB in T2D patients. Although there may be concerns about the excessive accumulation of liver glycogen or increased conversion of glucose into fatty acids and triacylglycerols (as observed with massive over-expression of GK activity in the livers of rats [14]) none of these observations have been reported in people with activating mutations of GK or in rodents treated chronically with a GKA. For example, chronic treatment of diet-induced obese mice for 42 weeks with RO-28-1675 supplemented in the diet demonstrated no significant differences between body weights but the increases in blood glucose seen in the diet-induced obese group were not observed in the RO-28-1675-treated group [3].

In summary, small organic molecule activators of GK offer a potential therapeutic that could be a novel approach to decrease hyperglycaemia in people with diabetes.

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**Figure 2 | Fasted female mice were dosed orally with GKA1 in HPMC (0.5%)/Tween (0.1%)**

After 5 min, blood was sampled from a tail vein, and blood glucose levels were measured with a hand-held monitor. Results are compared with vehicle-treated animals using a Student’s t test.
References

3 Matschinsky, F.M. and Magnuson, M.A. (2004) Frontiers in Diabetes, Volume 16 (Chapter 1 - GK sensor; Chapter 4 - MODY2; Chapters 5–7-Activating mutations; Chapter 11 - Crystal structure; Chapter 28 Gks)
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