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ARTICLE TYPE

Optimizing Glucokinase Activator Binding Kinetics to Lower *in vivo* Hypoglycemia Risk

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Activation of glucokinase represents a promising strategy for the treatment of type 2 diabetes; however, drug candidates have failed in clinical trials due to narrow therapeutic index between glucose-lowering efficacy and hypoglycemia. Described herein is a novel strategy for the design of next generation glucokinase activators with increased therapeutic index, which involves the optimization of activator-enzyme binding kinetics (k_{on}, k_{off}). This approach is based on the idea that activator binding kinetics are relevant to pharmacodynamics since the affinity of activator binding to glucokinase is cooperative with glucose such that, the rate at which an activator dissociates may influence the enzyme's sensitivity to changes in physiological glucose concentrations. This study provides a compelling example of using fast-off binding kinetics for developing safe and effective activator drugs targeting glucokinase.

Introduction

Glucokinase functions as a key regulator of glucose homeostasis and small molecule allosteric activators of this enzyme have been shown to reduce blood glucose levels by enhancing glucose-stimulated insulin secretion in the pancreas while also increasing glucose uptake and reducing glucose production in the liver.[1,2] The development of glucokinase activators (GKAs) has been an active area of pharmaceutical research with several candidates advancing to early clinical studies. However, while these GKAs have shown promising early efficacy in T2DM patients, there has also been significant attrition driven by narrow therapeutic windows against hypoglycemia as well as concerns about durability and adverse effects on plasma and hepatic lipids.[3,4] For example, during a Phase 2 study of GKA MK-0941 (10 – 40 mg, TID) conducted in T2DM patients on basal insulin therapy, the incidence of hypoglycemia (glucose < 50 mg/dL) was 14-16% in the active treatment arms versus 8% for the placebo group. Furthermore, four patients on MK-0941 discontinued from the study due to hypoglycemia and one subject in the 20 mg group experienced an episode of severe hypoglycemia requiring medical assistance.[4a] Additionally, during this same study of MK-0941, tachyphylasis was observed after 14 weeks of treatment.[4a] A similar loss of efficacy was also recently reported in a Phase 2 study of GKA AZD-1656.[4b] The explanation for this tachyphylasis is currently unclear, and studies with additional activators in various patient populations are needed to further investigate the issue.

Given the potential therapeutic limitations and compliance issues (e.g. dose titration and/or dosing with meals) imposed by the risk of GKA-induced hypoglycemia, there is continued interest in drug design strategies to develop next generation agents with reduced risk. Biochemically, glucokinase catalyzes the conversion of glucose to glucose-6-phosphate, which is the first and rate limiting step in glycolysis and glycogen synthesis. It has a physiologically optimal affinity for glucose (K_m or $S_{0.5}$ = 8 mM) and has been shown to demonstrate positive cooperativity of glucose binding,[5] facilitated by multiple conformations of this monomeric protein in solution.[6,7] High resolution structures of glucokinase complexed with several activator chemotypes revealed an allosteric binding site, 20 Å remote from the catalytic site. Activators of glucokinase have been well characterized and are known to modulate enzyme activity by two parameters α and β, which are defined by a decrease in K_m and an increase in V_{max}, respectively.[8,9,10] Previously, we published an experimental assessment of the hypoglycemic risk associated with different α,β activation profiles and successfully identified a "partial activator" that demonstrated favorable efficacy and minimal hypoglycemia risk in preclinical studies.[9] As an extension of this work, we also began to consider how activator-enzyme binding kinetics might influence GKA residency time on the enzyme and influence

hypoglycemia safety.[11] For many enzyme systems, slow off rates and long dissociative half-lives (a direct measure of residence times) have been shown to improve target selectivity and reduce off-target safety risks.[12,13] However in the case of GKAs there is positive cooperativity between activator and glucose binding to the enzyme making the apparent potency of an activator dependent on glucose concentrations.[14] As a consequence of this profile, the rate at which an activator dissociates from the enzyme may play a critical role in the ability of glucokinase to rapidly respond to changing physiological glucose levels hence influencing hypoglycemic risk. Finally, while durability of GKA efficacy is not the focus of the current work, it should be acknowledged that understanding differences in activator binding kinetics could have additional, longer term, benefit for understanding durability differences between activators.

The influence of binding kinetics on hypoglycemic risk was investigated using a series of structurally diverse GKAs spanning a range of *in vitro* potencies (EC_{50} = 10 – 391 nM) for which, a detailed assessment of binding kinetic, enzyme activation and *in vivo* pharmacodynamic profiles were determined. Specifically, the binding affinities and kinetics of these activators were characterized against human recombinant glucokinase using surface plasmon resonance (SPR) as shown in Table 1. To enable this experiment, biotinylated glucokinase was expressed in *E. Coli*, purified as previously described[8,10] and captured onto a streptavidin sensor chip to levels ranging from 5000-6000 response units (RU). SPR binding experiments were carried out in duplicate for three activator concentrations with 3 fold dilution and in the presence of a saturating glucose concentration of 50 mM glucose (representative SPR sensorgrams shown in supplementary material Figure S1 for compounds **6** and **15**). The activator concentrations run in SPR were based on the EC_{50} with the top concentration chosen to be several fold over the EC_{50} to achieve saturation. Activator binding kinetics to glucokinase as a function of glucose concentrations were attempted but could not be rigorously determined in SPR due to conformational instability of glucokinase at low glucose concentrations.

To validate our SPR results obtained at 50 mM glucose, we compared binding affinity (K_D) data to activator potency (EC_{50}), which was defined as the ligand concentration required to show a half maximal reduction in K_m.[9] Determination of binding affinities (K_D) for compounds in Table 1 revealed a reasonable correlation with compound potency (supplementary material Figure S2). Our data also indicates that potency is predominantly driven by binding off rates (Figure 1), which can be readily converted to dissociative half-lives to assess its influence on hypoglycemia. SPR binding kinetics were also determined against rat recombinant glucokinase for compounds **6**, **7**, **15** and showed excellent correlation with those determined for human glucokinase (Table S1), thereby providing additional confidence in the translation of *in vitro* binding kinetics to *in vivo* pharmacodynamic studies in rats.

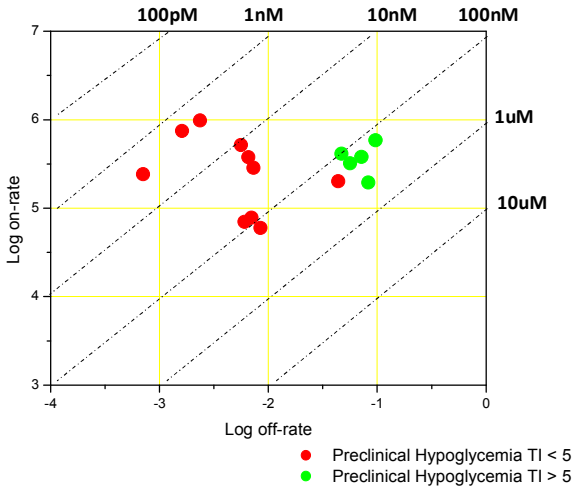


Figure 1: Kinetic plot showing relationship between off rates (k_{off}), on rates (k_{on}) and binding affinities (K_D) represented on the diagonal axis for compounds in Table 1, and determined in SPR. Off rates and on rates are plotted as logarithmic values. Only efficacious compounds are represented on plot.

To obtain a pharmacokinetic/pharmacodynamic (PK/PD) assessment of postprandial efficacy and hypoglycemia risk, these activators were evaluated in Wistar rats during an oral glucose tolerance test (OGTT) as described previously.[9] To evaluate efficacy, an oral glucose challenge (2 g/kg) was administered 60 min post dose and the effect of the activator at reducing the glucose AUC excursion during a hyperglycemic state was determined. $[GKA]_{efficacy}$ was defined as concentration of unbound (free) activator affording a 20% reduction in glucose AUC during an OGTT. $[GKA]_{hypoglycemia}$ was defined as the concentration of unbound (free) activator reducing fasting plasma glucose to <60 mg/dL. The hypoglycemic risk assessment was characterized by a therapeutic index (TI) < 5 as calculated by $[GKA]_{hypoglycemia}$ / $[GKA]_{efficacy}$ and listed as "no" or "yes" hypoglycemia in Table 1. A TI of 5 was selected to offer a reasonable margin of hypoglycemic safety relative to the C_{min} to C_{max} pharmacokinetic exposure range for a typical glucokinase activator. While the efficacy and hypoglycemia data from *in vivo* preclinical models may not directly translate to diabetic patients, we postulate that activators which are more efficacious in a hyperglycemic state and cause less hypoglycemia in a euglycemic state are likely to afford a wider therapeutic index in clinical studies.

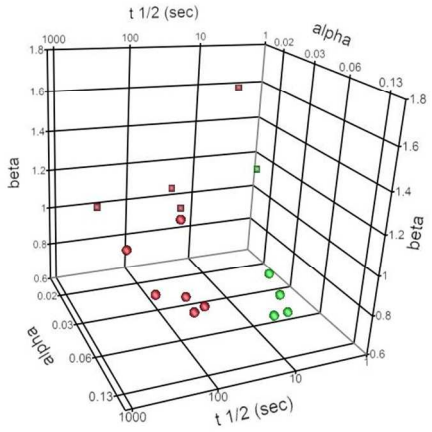


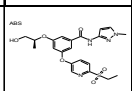
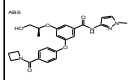
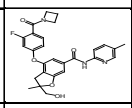
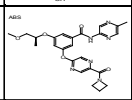
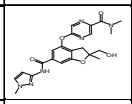
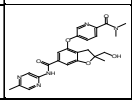
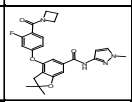
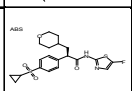
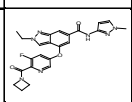
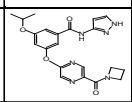
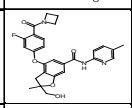
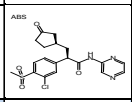
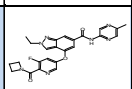
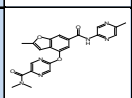
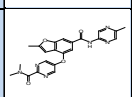
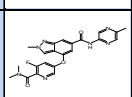
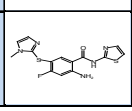
Figure 2: x,y,z plot showing correlation of alpha α (K_m) on the x-axis, beta β (V_{max}) on the y-axis and dissociative half-life ($t_{½}$ sec), plotted on a logarithmic scale on the z-axis for efficacious compounds in Table 1. Compounds shown in red presented with preclinical hypoglycemia with TIs < 5 while those in green had TIs > 5. Compounds with favorable α,β profiles are shown as circles and those with unfavorable profiles are shown as squares (See Reference 9 for definition and further discussion of favorable and unfavorable α,β).

Kinetic studies for these activators binding to glucokinase, resulted in on rates (k_{on}) which ranged from $0.70x10^5$ to $9.75x10^5$ (1/M*s) while off rates (k_{off}) ranged from $0.71x10^{-3}$ to $96.6\ x10^{-3}$ (1/s) providing a range of dissociative half-lives from 980 to 7.2 sec. If we consider the binding kinetics of these compounds, shown as a kinetic plot in Figure 1, off rates slower than approximately $4.4\ x10^{-2}$ sec^{-1} typically result in preclinical hypoglycemia with TIs < 5 despite similar binding affinities (K_D) in some cases. In our previous work, structural activity relationships for a series of 2-substituted benzofurans indicated *in vitro* efficacy and a reduced risk of hypoglycemia for partial activators with a moderate effect on K_m, indicated by α values in the range of 0.05-0.2 and a lesser effect on V_{max} with β values in the range of 0.8-1.2.[9] In the current study, compounds **1**, **7**, **8**, and **12** in Table 1 and shown as red squares in Figure 2, displayed unfavorable α,β profiles resulting in low therapeutic indexes (TI) against hypoglycemia as predicted based upon previous work.[9] Importantly, compounds **2-6** and **10** were identified with favorable α,β enzymatic profiles yet these compounds also exhibited unfavorable pharmacodynamic profiles reflected by preclinical hypoglycemia TI < 5 and shown as red, filled circles in Figure 2. This analysis clearly indicates the need for an additional preclinical predictive parameter, beyond α and β values, to mitigate hypoglycemia risk. For compounds **13-17** with reduced hypoglycemia risk (TI > 5), shown in green, the majority of them have favorable α,β profiles in addition to relatively short dissociation times. Based on this analysis, an upper limit to the off rate of approximately $5x10^{-2}$ sec^{-1} or faster in conjunction with favorable α,β profiles are proposed to minimize hypoglycemia risk.

The predictive value of including *in vitro* target residence time as key criteria in advancing GKAs is illustrated by the *in vivo* profiles of two activators, compounds **6** and **15** in Figure 3. Both compounds show dose dependent glucose lowering with oral dosing from 3-100 mg/kg. However, in the case of compound **6**, the free concentration of the activator normalized to its EC_{50}, which results in an efficacious reduction in plasma glucose, is very close to the concentration for which hypoglycemia is induced. Although the dissociation half-life of compound **6**, equal to 105.5 sec, is considered short compared with those of many known drugs,[12] it is still not short enough to allow for rapid dissociation of the compound from the receptor in response to highly varying glucose levels. This effect is particularly critical for glucokinase which displays cooperative binding of activators with increasing glucose levels, resulting in greater affinities, decreased off rates and longer residence times. As a result, the therapeutic windows are extremely narrow. In the case of compound **15**, the efficacious concentration, indicated by the green line, is significantly less than that predicted to result in hypoglycemia as indicated by the red line. Although the α,β enzymatic profiles are similar for both compounds, the residence time for compound **15** is an order of magnitude less than that for compound **6**. Based on a favorable preclinical efficacy and safety profile, compound **15** was advanced into clinical trials and is currently in Phase 2 studies for the treatment of T2DM.

This study suggests that optimizing the window between efficacy and hypoglycemia safety for GKAs will require careful attenuation of both enzyme activation profiles (K_m, V_{max} effects) as previously reported[9] as well as activator binding kinetics and more specifically, off rates and resultant residence times on the enzyme. Due to the positive cooperativity between activator potency (EC_{50}) and glucose concentrations as previously reported[9] and the correlation of EC_{50} to K_D/k_{off} in this work, we believe that at saturating glucose concentrations, activators will display their slowest inherent off rates. When GKAs start to lower *in vivo* glucose levels, they will have even faster target dissociation rates, thus reducing their glucose-lowering efficacy and limiting the risk of a hypoglycemic event. Consideration of off rate parameters and residence times may be particularly important for GKAs that have also been recently shown to activate glucokinase in a glucose independent manner.[22] The strategy described here provides a successful example of using fast-off binding kinetics to optimize therapeutic index for glucokinase activators, which may have broader implications for other enzyme activator or inhibitor approaches.

Table 1. Binding kinetics, potency and activation profiles of glucokinase activators s **1** – 17

Cmd	Ref.	Structure	Dissociation $t_{1/2}$ ± SE (s)[a]	k_{on} x 10^5 ± SE ($M^{-1}s^{-1}$)	k_{off} x 10^{-3} ± (s^{-1})	K_D ± *SE* (nM)	EC_{50} (nM)	α	β	$[GKA]_{hypoglycemia}$ / $[GKA]_{efficacy}$ <5
1	15		980 ± 22	2.41 ± 0.07	0.71 ± 0.02	2.93 ± 0.10	9.5	0.05	1.27	Yes
2	16		430 ± 4	7.46 ± 0.17	1.61 ± 0.02	2.16 ± 0.05	16.2	0.05	1.05	Yes
3[d, g]			294 ±5	9.75 ± 0.41	2.36 ± 0.04	2.42 ± 0.11	9.4	0.08	0.92	Yes
4	17		123.5 ± 0.8	5.17 ± 0.04	5.61 ± 0.04	10.8 ± 0.1	28	0.1	0.86	Yes
5[e, g]			114.6 ± 0.5	0.70 ± 0.01	6.05 ± 0.02	86.4 ± 1.1	121	0.06	1.2	Yes
6[d, g]			105.5 ± 0.7	3.76 ± 0.03	6.57 ± 0.04	17.5 ± 0.2	24	0.07	0.85	Yes
7	9		98.9 ± 0.7	0.78 ± 0.01	7.01 ± 0.05	89.9 ± 1.4	26	0.04	1.28	Yes
8	18		94.8 ± 0.3	2.85 ± 0.03	7.31 ± 0.02	25.6 ± 0.3	37	0.05	1.22	Yes
9	20		81.9 ± 0.8	0.60 ± 0.01	8.46 ± 0.08	141.5 ± 2.8	191	0.09	0.86	Lack of Efficacy[f]
10	19		69.8 ± 0.6	4.65 ± 0.11	9.93 ± 0.08	21.4 ± 0.5	52	0.08	0.82	Yes
11[d, g]			21.1 ± 0.2	3.40 ± 0.07	32.9 ± 0.3	96.8 ± 2.2	120	0.08	1	Lack of Efficacy[f]
12	21		15.8 ± 0.3	2.05 ± 0.05	43.9 ± 0.8	218.4 ± 6.6	364	0.04	1.73	Yes
13	20		14.7 ± 0.1	4.12 ± 0.07	47.0 ± 0.3	114.1 ± 2.1	114	0.12	0.82	No
14	9		12.3 ± 0.2	3.22 ± 0.09	56.3 ± 0.8	174.8 ± 5.3	210	0.09	0.96	No
15	9		9.7 ± 0.1	3.79 ± 0.07	72.7 ± 0.7	189.2 ± 3.7	174	0.1	0.87	No
16	20		8.4 ± 0.1	1.94 ± 0.04	82.9 ± 0.6	427.3 ± 8.8	391	0.11	0.8	No
17	10		7.2 ± 0.03	5.86 ± 0.03	96.6 ± 0.4	164.9 ± 1.2	80	0.04	1.33	No

[a]Dissociation $t1/2 = \ln 2/k_{off}$; kinetic parameters (k_{on}, k_{off}) were obtained by a global fit using three concentrations in duplicate; K_D determined by the standard equation, $K_D = K_{off}/K_{on}$; SE= Standard Error; [b]Potency (EC_{50}) and activation profile (α,β) determined as described and reported as the mean of n>2 independent determinations[9]; [c]Preclinical *in vivo* efficacy and hypoglycemia safety evaluated in Sprague-Dawley rat as described[4]. [d]Single stereoisomer, absolute stereochemistry not determined; [e]Racemic; [f]Activator did not achieve >20% AUC reduction during OGTT at highest dose (100 mg/kg) evaluated. [g]Synthetic protocols for compounds **3, 5, 6,and 11** are described in supporting information.

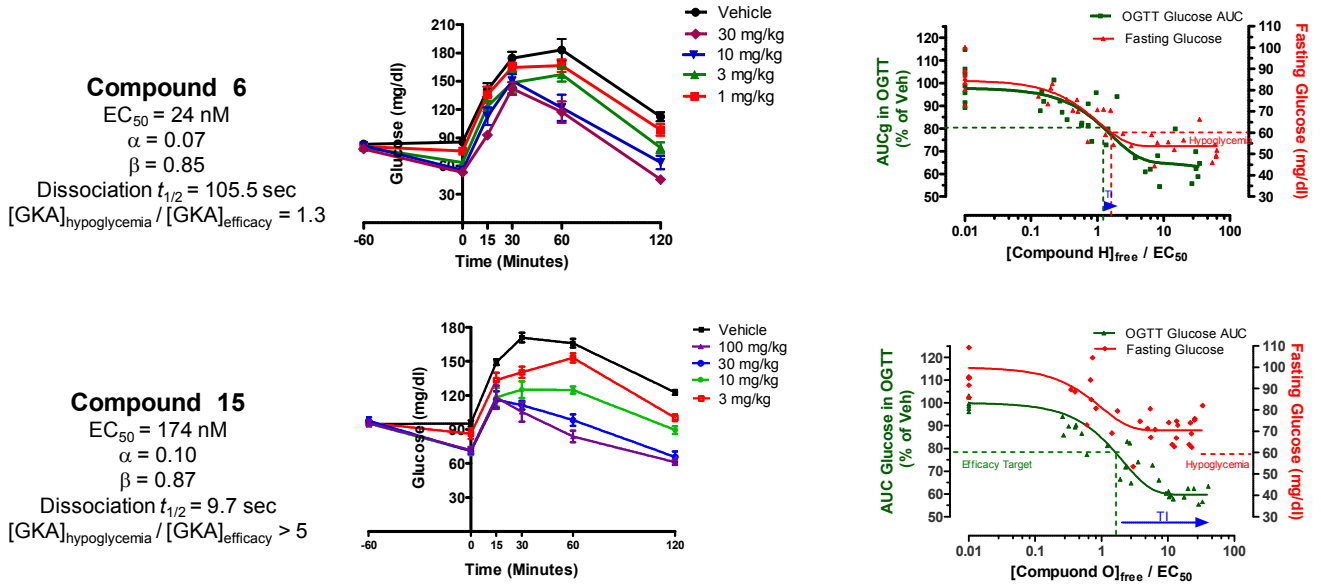


Figure 3: Potency (EC_{50}), α/β profiles, dissociative half-lives, therapeutic index (left), dose dependent effect on plasma glucose following a single dose at 3, 10, 30 and 100mg/kg during an oral glucose tolerance test (OGTT) (middle). Glucose excursion data expressed as Means ± Standard Error (n=7). PK/PD plots (right) describing plasma glucose AUC reduction following OGTT in green and fasting plasma glucose reduction in red vs. compound concentration normalized by the EC_{50} for compounds **6** and **15**. Data for compound **15** is reprinted with permission from the Royal Society of Chemistry.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [SPR profiles for compounds **6** and **15**, K_D and EC_{50} Correlation, Comparison of human and rat off rates for select activators, Synthetic protocols for compounds **3**, **5**, **6** and **11**].

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