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RESEARCH ARTICLE

An Aldehyde Responsive, Cleavable Linker for Glucose Responsive Insulins

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Abstract: A glucose responsive insulin (GRI) that responds to changes in blood glucose concentrations has remained an elusive goal. Here we describe the development of glucose cleavable linkers based on hydrazone and thiazolidine structures. We developed linkers with low levels of spontaneous hydrolysis but increased level of hydrolysis with rising concentrations of glucose, which demonstrated their glucose responsiveness *in vitro*. Lipidated hydrazones and thiazolidines were conjugated to the LysB29 side-chain of HI by pH-controlled acylations providing GRIs with glucose responsiveness confirmed *in vitro* for thiazolidines. Clamp studies showed increased glucose infusion at hyperglycemic conditions for one GRI indicative of a true glucose response. The glucose responsive cleavable linker in these GRIs allow changes in glucose levels to drive the release of active insulin from a circulating depot. We have demonstrated an unprecedented, chemically responsive linker concept for biopharmaceuticals.

Introduction

An estimated 463 M people currently live with diabetes of which approximately 10% have type 1 diabetes (T1D).[1,2] In T1D patients, little or no insulin is produced by the pancreas, and patients require daily insulin injections to maintain normoglycaemia. Several short- and long-acting insulins are marketed and management of T1D requires regular injections of basal (long-acting) as well as meal time (short-acting) insulin.[3,4] Some basal insulins, such as insulin glargine[5], precipitate after administration and form a subcutaneous depot from which they are gradually released. Others, like the lipidated insulin degludec, form polymers of hexamers that leads to slow release from the subcutaneous depot.[6] Upon release lipidated insulins are non-covalently bound to albumin as a circulating depot that provides further half-life extension.[6] While the basal insulins provide decent 12-24 h glucose control, none of them are responsive to circulating glucose fluctuations, which can lead to episodes of hypoglycemia if too much insulin is administered. Even with optimal insulin treatment, tight control of blood glucose is difficult. For decades the development of a glucose responsive insulin (GRI) has remained an elusive goal. One of the main obstacles in the development of GRIs is the very narrow range of glucose control in the order of mM and very narrow range of circulating insulin, in the order of pM, and hence a ratio between them in the order of millions.[7]

Current GRI approaches[8,9,10,11,12] can be divided into unimolecular GRIs and controlled release matrix GRIs. Unimolecular GRIs are insulin variants with a built-in functionality that in response to increasing glucose concentration, releases an active insulin molecule from an inactive form. Once the glucose concentration decreases, release of active insulin from the inactivated form decreases or stops. Hoeg-Jensen and coworkers reported an insulin modified with a polyol moiety and a phenylboronic acid conjugated to LysB29 that cross-linked insulin hexamers by formation of boronate groups from boronic acid and polyol moieties on different hexamers.[8] The high-molecular weight complex with boronate linkages disassembled by addition of the polyol sorbitol, thus releasing the insulin. However, glucose was less efficient in disassembling this complex. Chou et al reported an insulin variant with a phenylboronic acid at the end of an aliphatic chain attached to LysB29, the latter to facilitate binding to albumin.[9] This insulin conjugate provides glycemic control in a diabetic mouse model. A third approach used multivalent mannose conjugated to insulin at Lys B29 or at Gly A1 by acylation.[10] Controlled release matrices on the other hand are complex biomimetic GRI systems that contain both insulin and proteins directly interacting with glucose, by binding to it or catalyzing its oxidation. Proteins evaluated so far include lectins (e.g. concanavalin A) and glucose oxidase.[11,13,14]

Here we report a fundamentally new glucose responsive cleavable linker that exploits the fact that soluble glucose exists in an equilibrium between ring-closed forms (hemiacetal), primarily the α- and β-pyranose, and the open, linear aldehyde form (aldehydo-D-glucose). We demonstrate that glucose can accelerate the hydrolysis of a cleavable bond. The aldehyde group of carbohydrates readily reacts with α-nucleophiles such as hydrazides,

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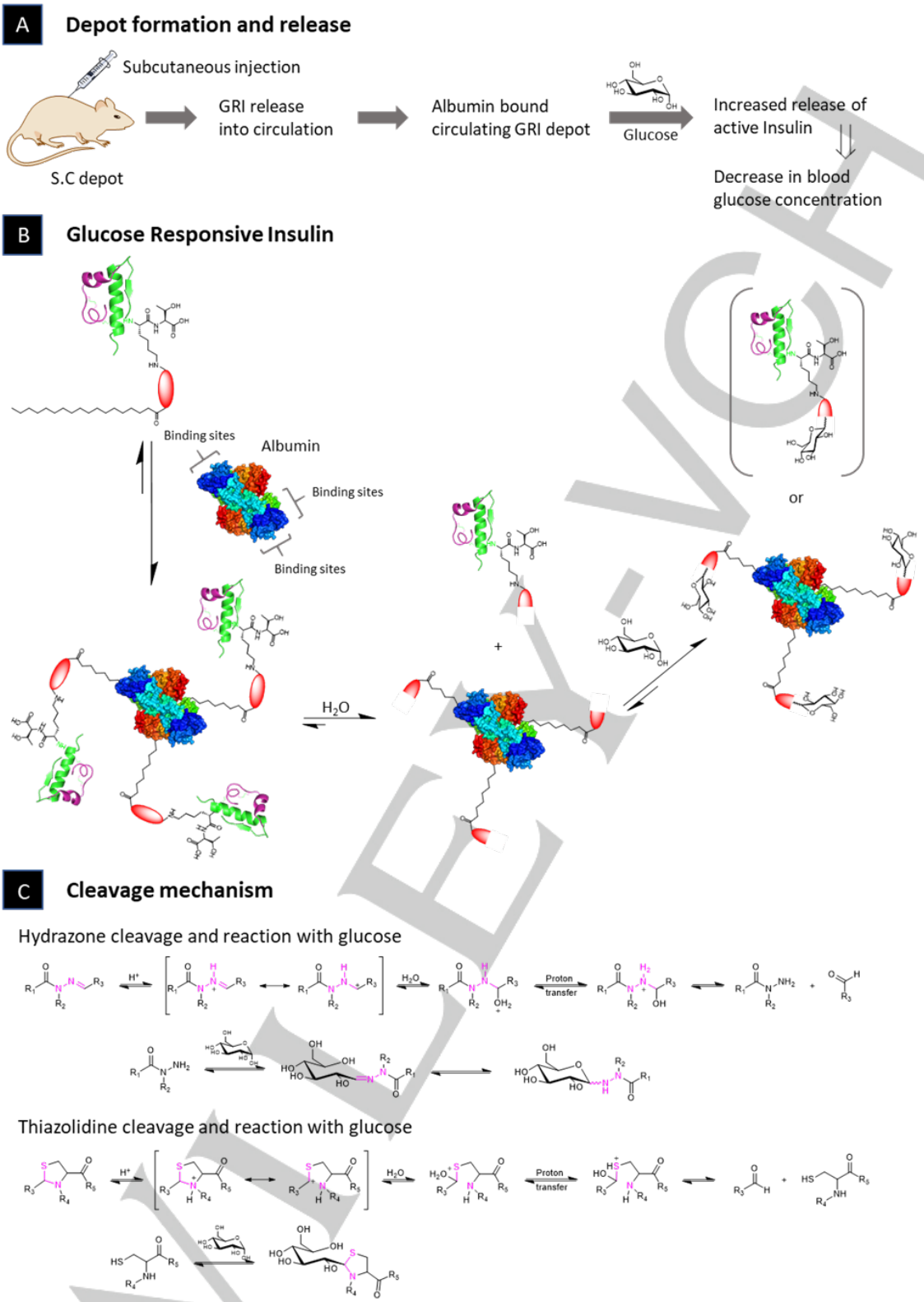


Figure 1: (A) Subcutaneous and circulating depots; (B) GRI concept based on a circulating depot, where the GRIs are non-covalently bound to one of the fatty-acid binding sites in albumin and released by hydrolysis of a cleavable linker; (C) Hydrazone: R_1 and R_3 are moieties that can be chosen to adjust the reactivity and glucose responsiveness of the hydrazone (Table 1). R_3 is derived from an aldehyde. In addition, R_1 and R_3 can provide anchor points for insulin and the fatty acid to form full GRIs (Fig. 2). Thiazolidine: R_3 and R_5 are moieties that can be chosen to adjust the glucose responsiveness of the thiazolidine (Table 2). In addition, R_3 and R_5 can provide anchor points for insulin and the fatty acid to form full GRIs (Fig. 2).

which are formed in the cleavage of this bond, to form carbohydrate hydrazones. In a series of experiments, we explored both hydrazones and thiazolidines as the cleavable groups and relied on lipidation as half-life extension method, as the lipidated insulin

forms both sub-cutaneous and albumin bound depots that can be adjusted according to the length of the lipid (Fig. 1a and 1b).
The hydrolyzable, cleavable hydrazone and thiazolidine groups will in a first equilibrium release a nucleophile, which in a second equilibrium can react with glucose (Fig. 1b). Glucose can react as the aldehyde driving the formation of a new hydrazone or a thiazolidine, respectively. The reactivity of the linker was adjusted using electron-withdrawing and -donating substituents, so the linker is hydrolyzed by glucose in a concentration dependent manner.

Human blood can contain aldehydes other than glucose, most notably glyceraldehyde and acetaldehyde. However, they generally occur in low or insignificant amounts, as low molecular weight aldehydes such as formaldehyde, acetaldehyde, acrolein, and methylglyoxal are generally toxic compounds and carcinogenic[15] (see the Section 1.8 in SI for a more extensive discussion).

Results

The initial step in the cleavage of the hydrazone[16] and thiazolidine groups is the protonation of a hydrazone or amine nitrogen, respectively (Fig. 1c). The pKa of this amine is influenced by nearby substituents and the reactivity can be adjusted through these substituents. Furthermore, the hydrolysis of the five-membered thiazolidine ring could potentially also release ring-strain, depending on its substitution pattern. Based on these considerations, we designed an ensemble of hydrazone and thiazolidine core structures to survey the structural requirements for possible glucose responsiveness (i.e. systematic variation of R_1, R_2, R_3 and R_4 substituents (Fig. 1c, Tables 1 and 2, Fig. 2). We chose hydrazides as precursors for the hydrazones due to their stability. The carbonyl precursors were in most cases aliphatic or aromatic aldehydes, with only a few being ketones. The reactivity was further adjusted by adding electron-withdrawing or electron-donating substituents to the aromatic side-chains. *N*-Alkylation of some hydrazones and a thiazolidine provided additional possibility to adjust the properties. Finally, the core structures were designed to allow further extension to lipidated core structures (here called linkers) that could be coupled to Lys B29 in insulin.

Simple hydrazone core structures were synthesized from aromatic and aliphatic hydrazide and aldehyde starting materials. The R_3 groups were varied, with 1-3 electron-donating substituents (-OMe, -NMe_2 and -OH) on the benzaldehyde precursors, while the R_1 and R_2 groups on the hydrazides were varied less (Table 1, **1**-**14**). Hydrazone core structures with electron-withdrawing substituents on the aromatic group were also synthesized but showed no reactivity towards glucose in a HPLC based assay (data not shown). Acyl hydrazones can be in *trans*- or cis-configurations, with the former generally being more stable.[17] In some cases we observed two isomers of the hydrazones, where the major form likely has the trans-configuration.

A smaller set of thiazolidine core structures were synthesized starting from aldehydes and 1,2-amino-thiols derived from cysteine. Both aliphatic and aromatic aldehydes were used, with electron-donating substituents such as -OMe on the aromatic side-chains (Table 2, **15**-**19**). The R- and S-form of the thiazolidines likely have similar reactivity. As controls, *N*-substituted oxazolidines and dioxolane were also synthesized (Table 2, **20** and **21**) but were either too labile (half-life less than 20h, dioxolanes) or too stable (no conversion, *N*-substituted oxazolidines), for further exploration.

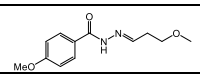
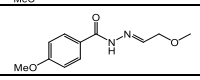
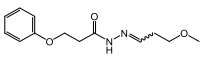
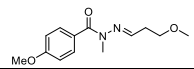
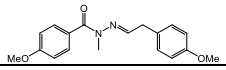
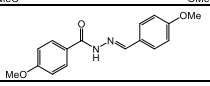
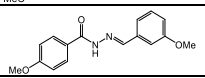
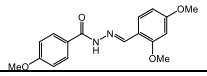
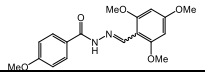
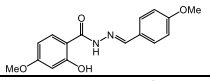
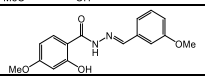
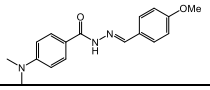
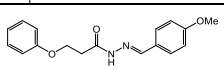
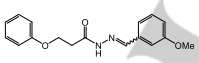
A key challenge for a glucose responsive linker is the large difference in the concentrations of glucose and insulin under physiological conditions, which are in the range of mM and pM, respectively.[7] This difference is difficult to mimic in an *in vitro* setting due to limitations in detecting pM concentrations of small molecules, such as the core structures. We chose to use an UV-HPLC based analysis and sub-mM concentrations of the core structures (For an overview of methods for testing glucose responsiveness see Fig 2S). The glucose reactivity of the core structures was evaluated with a glucose test, where they wer incutaed in varying concentrations (0.21 mM and 0.42 mM) with or without glucose (4.2 M) and analyzed by UV-HPLC. The glucose was added in two different molar ratios, a low ratio (10,000 equiv. Glc) and a high ratio (20,000 equiv. Glc). Spontaneous hydrolysis in the absence of glucose was also determined. For the hydrazone core structures, the disappearance of the starting material as well as the formation of hydrazide-glucose adduct were measured, and the half-life of the compounds calculated. To determine the differences for slow-reacting hydrazones, the percentage of formed hydrazide-glucose adduct was also measured at 72 h (Table 1).

For the glucose tests, the thiazolidine core structures were tested with two different glucose ratios, a low ratio (10,000 equiv.) and a high ratio (20,000 equiv.), as well as incubation without glucose. The thiazolidine-glucose adduct could not be detected, due to lack of UV activity or slow reaction rate, so the disappearance of the starting material was measured, and the half-life of the compounds was calculated (Table 2). In summary, we synthesized hydrazone and thiazolidine core structures of which a majority showed a glucose responsive cleavage (Table 1 and 2).

The vast majority of glucose is in the ring-closed hemiacetal form, with only a small fraction in the open-chain *aldehydo*-form. Reaching the equilibrium between these forms is relatively slow. We investigated an additional aldehyde, glyceraldehyde, which cannot ring-close (4% in aldehyde form, 96% as hydrate; see SI). The ratio between the aldehyde and the core structures and GRIs, could therefore be decreased. A glyceraldehyde test was established and applied for selected compounds. The core structures or GRIs were incubated with glyceraldehyde in two different ratios, 20 and 100 molar equivalence (Table 2S). Rewardingly, we observed a glyceraldehyde responsive cleavage.

Full GRIs were evaluated in *in vitro* experiments to enable a larger difference in concentration between insulin variants and glucose. In control experiments to bridge between HPLC-UV analyses and *in vitro* measurements of glucose responsiveness, we synthesized two intermediate insulin variants with a hydrazone core structure attached, but without a lipid. These insulin conjugates (SI **IC-1**, **IC-2**) were suitable for HPLC-UV studies with glucose equivalents similar as for the above studied core structures (Fig. 10S, 11S). We studied the cleavage of the hydrazone linkage in presence or absence of glucose or glyceraldehyde. Rewardingly, both **IC-1** and **IC-2** showed an increased cleavage in the presence of Glc and glyceraldehyde after 72 h (Table 3S). With increasing concentrations of glyceraldehyde (0, 20 and 200 equiv) and glucose (0 and 2000 equiv) an increased cleavage was observed after 72 h (Table 3S). This showed the hydrazone core structure retained the glucose responsiveness after coupling to insulin. **IC-1** and **IC-2** are regioisomers and their differences in activity also indicated that the glucose responsiveness could be adjusted by changing the substituents on the hydrazones.

Table 1: Glucose test of hydrazone core structure. Compound (0.21 or 0.42 mM) in 1x PBS buffer containing 10% DMSO, pH 7.4, 37°C; [a] Glc_{low} 10,000 equiv. glucose (4.2 M); [b] Glc_{high} 20,000 equiv. glucose (4.2 M); [c] Hydrazone-Glc was analyzed; [d] The hydrazide formed in the hydrolysis reaction was analyzed by UPLC.

Compound	Core structure	Glucose test				Hydrolysis (72h, %)[d]
		T½ (h)		Conversion % (72h)[c]		
		Glc_{low}[a]	Glc_{high}[b]	Glc_{low}[a]	Glc_{high}[b]	
1		22	14	79	87	7
2		13	4	94	99	17
3		17	12	88	95	18
4		45	18	42	54	23
5		21	1	68	78	-
6		>72	>72	33	36	7
7		>72	>72	3	28	4
8		>72	>72	40	47	8
9		4.4	2.6	77	89	22
10		>72	>72	15	23	-
11		>72	>72	3	10	2
12		>72	>72	4	4	1
13		>72	>72	5	2	1
14		>72	>72	1	1	1

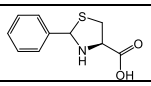
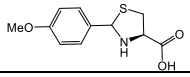
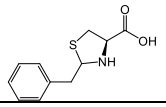
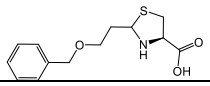
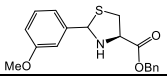
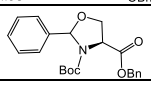
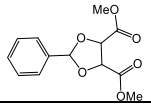
Subsequent studies were performed *in vitro* to allow higher equivalents of glucose, thus gradually approaching more physiological conditions.

We next set out to select core structures for lipidation (Fig. 1S). Core structures were chosen based on three parameters (*i*) low background hydrolysis, (*ii*) a shorter half-life at high than at low glucose concentration, and (*iii*) for some hydrazones, a higher rate of formation of new glucose hydrazones at high than at low glucose concentration. The corresponding linkers were synthesized, i.e. core structures with handles for introduction of a fatty acid and conjugation to HI. The core structures were appended with additional functional groups to enable attachment of the fatty acid and eventually insulin. The ease of chemical synthesis was also considered in the design. The core structures could have two opposite orientations in the linkers, as the aldehyde part could be either attached to the insulin side or to the fatty acid (Fig. 2a). Most of the linkers were synthesized with the hydrazide part of the hydrazone attached to insulin and the cysteine part of the thiazolidines attached to insulin. Aldehyde and hydrazide starting materials with a C18 fatty acid were prepared first, where after the hydrazone and thiazolidine linkages were synthesized (see SI). C18 was chosen as the preferred lipidation strategy as Insulin-C18, in contrast to Insulin- C14, has minimal blood glucose lowering effects and would thus serve as an ideal GRI depot upon normoglycemia, see Fig. 3S.

Acylation of insulin at LysB29 is generally well tolerated; insulin detemir and insulin degludec are both acylated at this position.[3] The Lys side-chain amine can be acylated with high selectivity in aqueous buffer at pH 10.5.[3a,17,18,19] Solubility problems and low yields for the acylation of LysB29, required the use of organic solvents, which on the other hand lowered the selectivity. Nevertheless, pure GRIs were obtained after chromatography (SI: Section 9).

Table 2: Glucose test of thiazolidine core structures. Compound (0.21 or 0.42 mM) in 1x PBS buffer containing 10% DMSO, pH 7.4, 37°C; [a]Glc_{low} 10,000 equiv. glucose (4.2 M); [b]Glc_{high} 20,000 equiv. glucose (4.2 M). Disappearance of starting material was analysed by UPLC

.

Compound	Core structure	Glucose test				Hydrolysis (24h, %)
		$T_{½}$ (h)		Conversion % (24h)		
		Glc_{low}[a]	Glc_{high}[b]	Glc_{low}[a]	Glc_{high}[b]	
15		11	13	77	71	42
16		5	7	98	94	70
17		8	8	66	75	10
18		12	9	56	69	9
19		60				30
20		>72	>72	0	0	-
21		1		100	100	100

For the *in vitro* studies of the GRI's we used CHO cells overexpressing the human insulin receptor b (hIRb) and a phospho-AKTassay to demonstrate an aldehyde-driven increase in insulin potency. GRIs were pre-incubated with either glyceraldehyde or glucose and added to the cells for the insulin receptor signaling assay. Basal hydrolysis was observed for all tested GRIs, causing slightly lower EC_{50} values post- than pre-incubation with glucose for 24 h, however at different rates (Fig. 5S-h). Pre-incubation with low concentrations of glucose relative to GRI had no effect on the potency of the GRI's (data not shown), however, increasing the equivalents of glucose to GRI, by either increasing the glucose concentrations (Fig. 3a, GRI-2) or decreasing the GRI concentration (Fig. 3b, GRI-2), or substituting glucose with glyceraldehyde (Fig. 3S c-d, GRI-2) led to increased potency of thiazolidine-based GRIs, confirming that our concept extended to the full GRI molecule. Notably, all tested thiazolidine-based GRIs with the aldehyde attached to insulin reacted with glyceraldehyde (SI-Fig. 5S a-h) and glucose (SI-Fig 6S a-d), whereas none of the tested thiazolidine GRIs with reversed orientation (SI-Fig. 7S a-d) or hydrazone-based GRIs reacted (Fig. 3e-f), with either with glucose nor glyceraldehyde. The ratio between the glucose-induced hydrolysis and the basal hydrolysis (termed "glucose potency") was used to rank the glucose responsiveness of GRIs with GRI-2 showing the highest score (Fig. 3g-h). Notably, substituting glucose with glyceraldehyde, resulted in the same overall ranking of thiazolidine-based GRIs (Fig. 3S g-h; Fig. 8S).

We next set out to test the most promising GRIs, GRI-2, (Fig. 3h) and a non-glucose responsive insulin, GRI-1, (Fig. 3e-f) in a T1D animal using 90% pancreatectomized rats displaying overt hyperglycemia.[20] HI was included as a positive control that had immediate blood glucose (BG) lowering effect (Fig. 4a-b). In contrast to HI-C18 (HI acylated with C18 lipid at Lys B29; HI-C18; Fig. 3S), which had no impact on blood glucose (BG) levels, GRI-2 and GRI-1 both caused a sustained reduction with significant effects after 1 and 2 h, respectively. Normoglycemic levels were ultimately reached after 8 h (Fig. 4a-b). Thus, the data shows that the two GRIs are potent in T1D model in contrast to acylated insulin (HI-C18), despite sharing same lipidation strategy (Fig. 4b), most likely explained by linker hydrolysis. This prompted us to conduct more sophisticated experiments to evaluate in vivo glucose responsiveness.

In order to assess the glucose responsiveness in a physiological setting, where blood glucose levels were controllable, we performed normo- (5 mM) and hyperglycemic (20 mM) clamp experiments in healthy rats (Fig. 9S, graphs showing similar BG for all groups throughout the study). For additional explanation of the clamp study see Fig 4S. Somatostatin was infused 30 min prior to GRI administration to suppress endogenous insulin secretion (see SI). When comparing GRI-1 and GRI-2 at equimolar concentrations (113 nmol/kg) we observed that a lower glucose infusion rate (GIR) was required in order to achieve normoglycemia (Fig. 4c-d), while an identical GIR was required to achieve hyperglycemia. This prompted us to double the GRI-2 dose aiming for a matched AUC GIR at normoglycemia. Interestingly, at the double dose of GRI-2 as compared to GRI-1 we observed that the GIR required to maintain normoglycemia remained unchanged (Fig.4c-d), while the GIR required to maintain hyperglycemia was significantly increased (15% in GIR AUC, Fig. 4d) indicative of a true glucose response.

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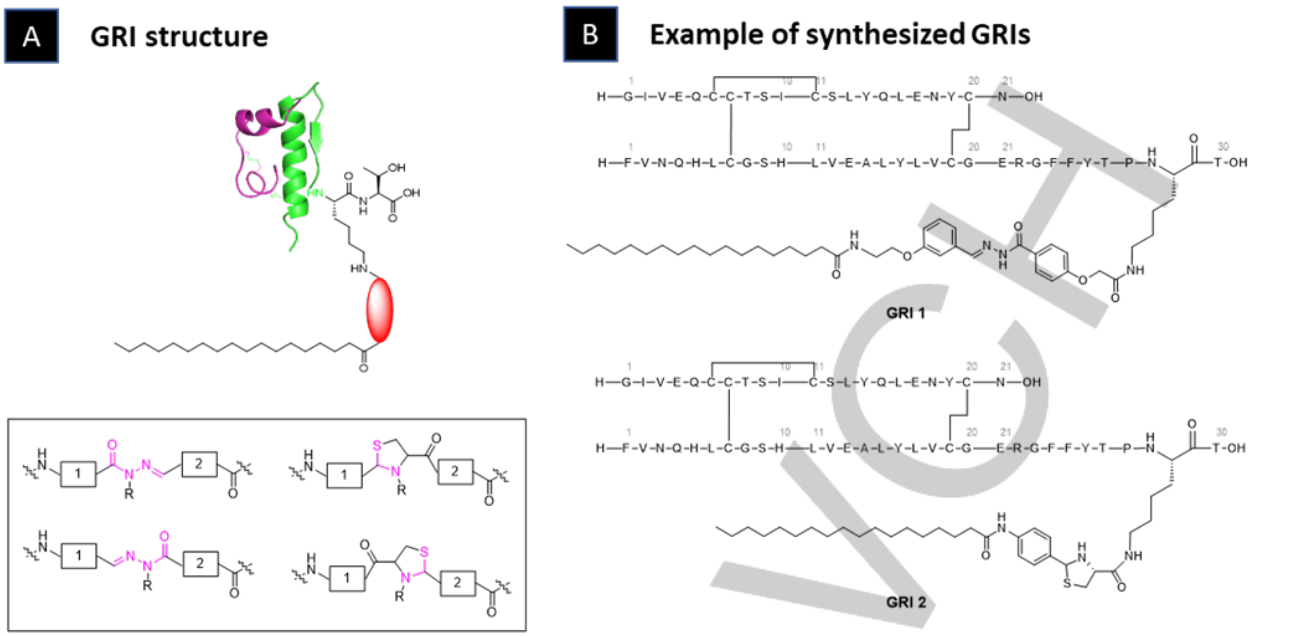

Figure 2: (**A**) General GRI structures; the hydrazone and thiazolidine moieties can have two different orientations between insulin and the lipid, with the aldehyde derivative being attached either to insulin or to the lipid; (**B**) Full structure of two GRIs.

Discussion

The first step in realizing this new GRI concept was the ability to adjust the reactivity of the cleavable linker. Hydrazones and thiazolidines were chosen as core structures for their well-documented chemistries and their different structures (the former has a central sp^2 carbon, the latter a sp^3 carbon). The desired reactivity was an initial hydrolysis that could be followed by reaction of the released nucleophile (hydrazide or aminothiol) with glucose. The glucose responsiveness would be a higher rate of cleavage of these hydrazone and thiazolidine core structures at higher glucose concentrations. Additionally, an increased rate of hydrolysis at higher glucose concentrations, without formation of a new glucose hydrazone would also support the GRI concept. We prepared core structures that reacted at fast, intermediate, or slow rates. Next, linkers were assembled that in addition to hydrazones or thiazolidines had a lipid moiety and a carboxylic acid enabling subsequent anchoring to insulin (see SI).

Glucose in solution exists in an equilibrium between ring-closed forms (hemiacetal), primarily the α- and β-pyranose, and the open, linear aldehyde form (aldehydo-D-glucose). The mutarotation between the α- and β-pyranose proceeds via the open aldehyde form (ring-chain tautomerism). The mutarotational half-life of glucose in pure water is 9.8 min at 37 °C, while in whole blood it is 2.3 min at 37 °C. This increased interconversion could be due to the presence of mutarotase, phosphate ions, and bicarbonate ions.[21] There is 0.0040% of the linear aldehyde present in this equilibrium together with 0.0059% of the corresponding hydrate.[22] In chemical reactions, glucose and other carbohydrates can be made to react quantitatively via the open-chain aldehyde form by a continuous sdisplacement of the equilibrium.[23]

The aldehyde moiety of carbohydrates readily reacts with α-nucleophiles such as hydrazides to form carbohydrate hydrazones. This reaction can be carried out in physiological conditions i.e. aqueous solutions at pH 7 and 37°C and has been used for bioconjugation reactions. The reaction between an aldehyde and a hydrazide is reversible, a feature which has been used in dynamic combinatorial chemistry for exchange reactions between hydrazones.[24] The exchange with another aldehyde means transfer of the hydrazide to this aldehyde, forming a new hydrazone. If the new aldehyde is present in excess, the formation of the new hydrazone can be driven to completion.[16] Aldehydes can also react with divalent nucleophiles such as 1,2-amino-thiols to form thiazolidines, a five-membered ring.[25]

Hydrazones were derived from hydrazides (i.e. hydrazine with a carbonyl substituent), while thiazolidines were conveniently derived from L-cysteine. Both were reacted with substituted benzaldehydes or aliphatic aldehydes. A challenge in designing the glucose response assays was the required concentration difference between the GRI/core structures and glucose, necessitating detection of the core structures and their cleavage products at low concentration. Blood glucose concentrations are 3.9 to 7.1 mmol/L in fasting, healthy persons,[7] with 0.0040% present as the linear aldehyde, while insulin concentrations are 45 to 280 pmol/L. An additional problem was that at very high glucose concentrations the water concentration would decrease markedly. This would be problem as the water is required for hydrolysis step.As an intermediate step we used glyceraldehyde as aldehyde since it cannot form a cyclic hemiacetal and thus has a significantly higher proportion of the *aldehydo*-form together with its hydrate (see SI). This allowed the use of fewer equivalents of the aldehyde in in the initial evaluation of hydrazone and thiazolidine. The first parameter was a relatively low basal hydrolysis in the

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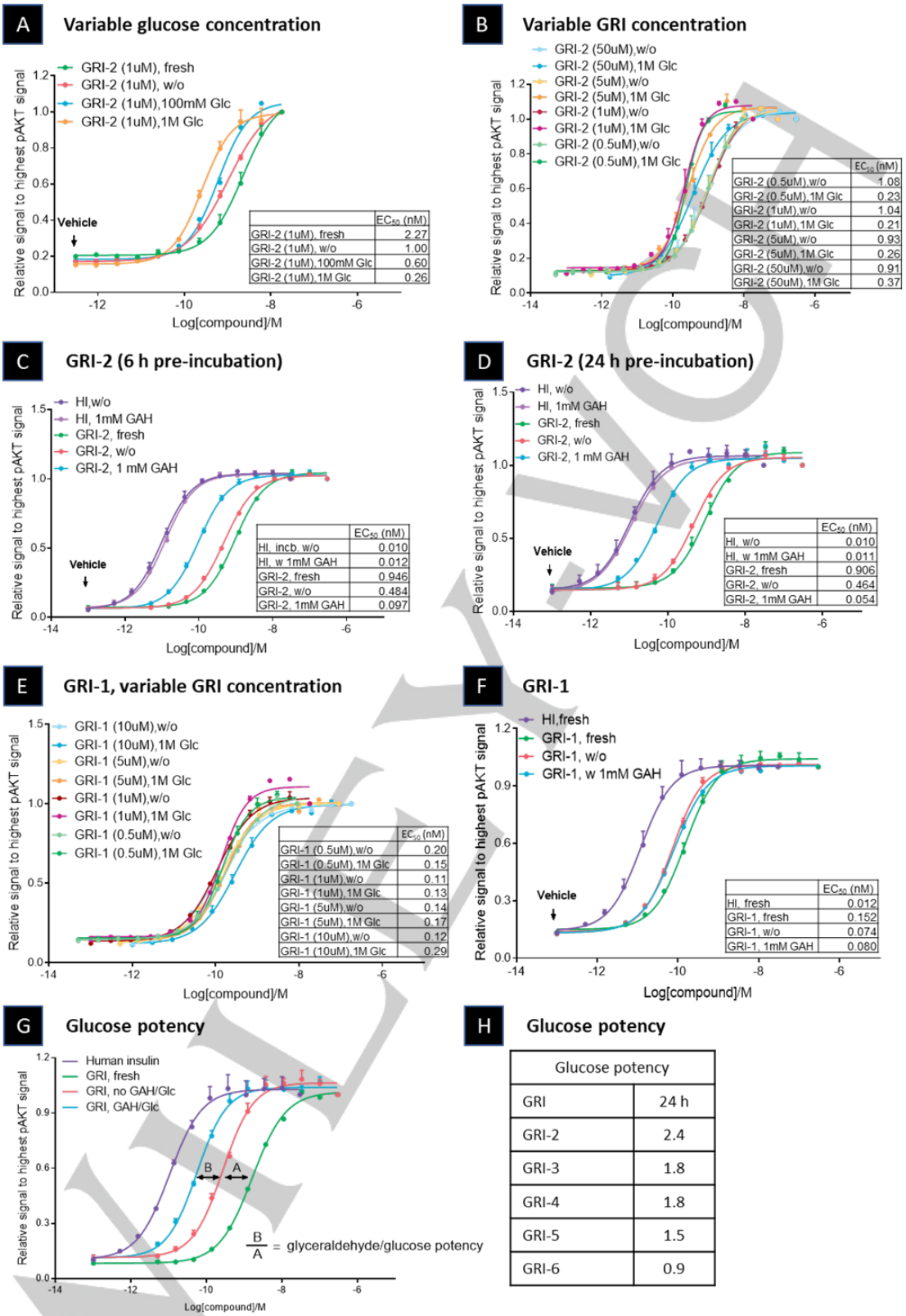

Figure 3: In vitro assay for evaluation of the aldehyde responsiveness of hydrazone and thiazolidine GRI's towards glyceraldehyde (GAH) or glucose using an insulin receptor (IR) assay. Human insulin (HI) was included as a positive control. (A) Constant GRI-2 concentration (1μM) incubated with varying glucose concentrations for 24 h. (B) Variable GRI-2 concentrations (0.5-10μM) incubated with or without 1M glucose for 24 h. (C) GRI-2 (50μM) incubated for 6 h with or without 1mM GAH. (D) GRI-2 (50μM) incubated for 24 h with or without 1mM GAH. (E) Variable GRI-1 concentrations incubated with or without 1M glucose for 24 h. (F) GRI-1 (50μM) incubated for 24 h with or without 1mM GAH. (G) Description of glyceraldehyde/glucose potency calculations. (H). GRIs ranked according to glucose potencies. Data are mean + SEM, n=2.

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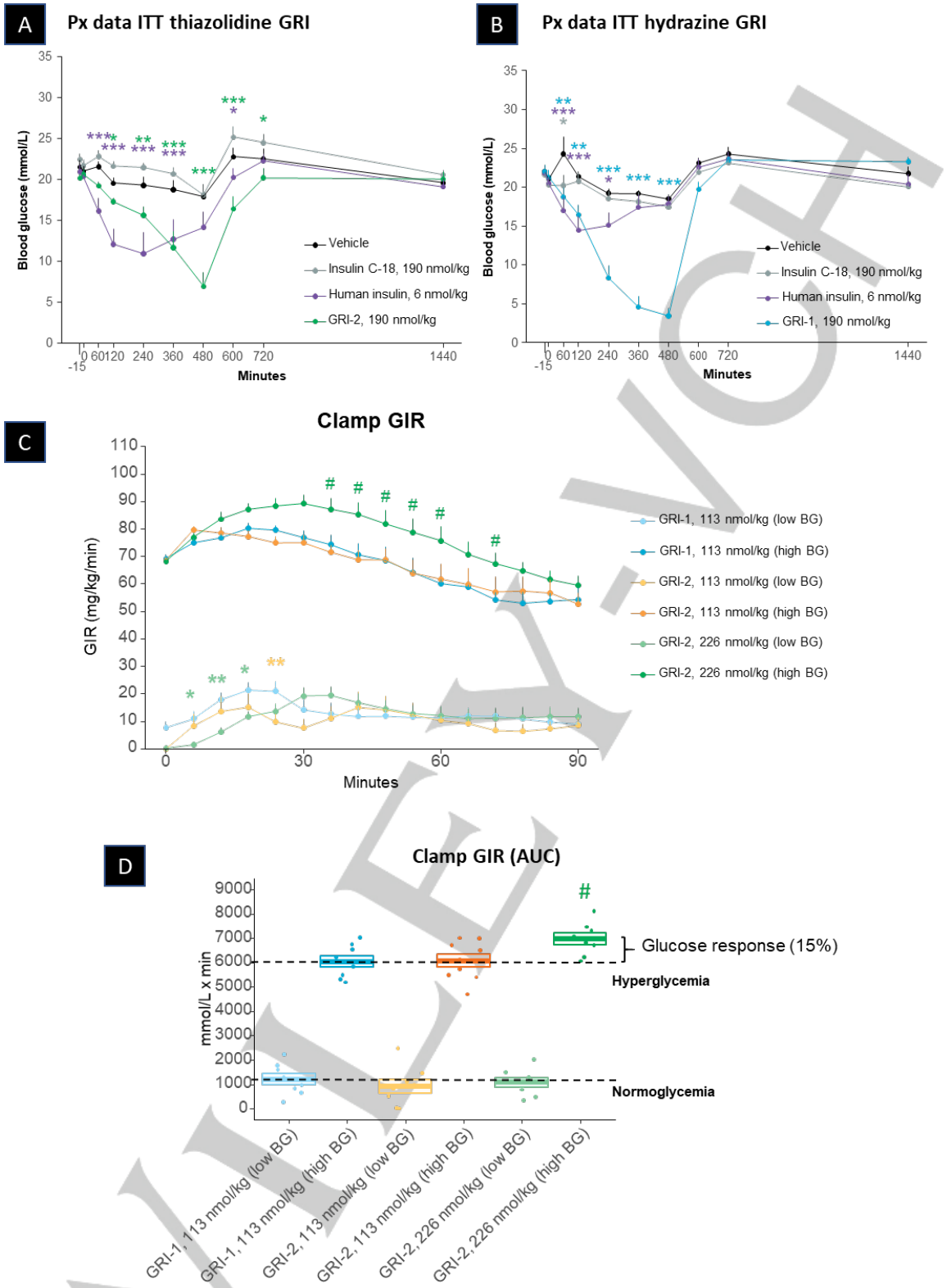

Figure 4: *In vivo* GRI testing. (**A**) Blood glucose during insulin tolerance test (ITT) in pancreatectomized rats. Rats were re-fed after 480 min. Values expressed as mean of n = 5-7 + SEM. Dunnett's test on two-factor linear model with interaction. *: P < 0.05, **: P < 0.01, ***: P < 0.001 compared to Insulin C-18; (**B**) Blood glucose during a second ITT. Rats were re-fed after 480 min. Values expressed as mean of n = 7-8 + SEM. Dunnett's test on two-factor linear model with interaction. *: P < 0.05, **: P < 0.01, ***: P < 0.001 compared to Insulin C-18; (**C**) Glucose infusion rate (GIR) from 0 min to 90 min. Values expressed as mean of n = 8-9 + SEM. Dunnett's test on two-factor linear model with interaction. *: P < 0.05, **: P < 0.01 compared to GRI-1 113 nmol/kg (low BG), #: P < 0.05, ##: P < 0.01 compared to GRI-1 113 nmol/kg (high BG); (**D**) Glucose infusion rate (GIR) AUC from 0-90 minutes. Values expressed as mean of n = 8-9 + SEM. Dunnett's test one-factor linear model. #: P < 0.05 compared to GRI-1 113 nmol/kg (high BG). Glucose responsiveness indicated.

absence of aldehyde. The second parameter was glucose responsiveness as determined by whether the core structures and linkers hydrolyzed at higher concentration of glucose and glyceraldehyde than at lower concentrations.

The glucose responsiveness of core structures was judged by three criteria (Tables 1 and 2): (i) the half-lives ($T_{1/2}$) of the core structures at low and high glucose ratios (a significant ratio or $T_{1/2}$ > 72 h); (ii) the conversion to the new glucose hydrazone at low vs. at high glucose concentration ratios; (iii) for some core structures an additional third criterion was based on the conversion to the new hydrazones in the presence of low or high ratios of glyceraldehyde.

In general, hydrazones derived from aliphatic aldehydes reacted very fast with glucose and showed a rate of basal hydrolysis that was too high to practically make them into full GRIs (Table 1, 1-5). However, N-methylation of these gave slightly more stable core structures (Table 1, **1** vs **4**). In contrast, hydrazones derived from benzaldehydes were more stable (Table 1, **6**-**14**). The reactivity of the hydrazone was increased by having a para- rather than a meta-methoxy substituent on the benzaldehyde side (Table 1, **7** vs **6** and **10** vs **11**). That electron-donation from the substituents near the hydrazone moiety increased reactivity was further shown by increasing the number of ortho/para-substituents (Table 1, **6**, **8**, **9**). An ortho hydroxy group on the hydrazide gave a less reactive hydrazone (Table 1, **6** vs **11**). Also, substituting a para-methoxy group on the phenyl with a dimethylamine (resulting in an aniline) reduced glucose reactivity (Table 1, **6** vs **12**), as did hydrazones derived from aliphatic hydrazides (Table 1, **13** and **14** vs **6** and **7**). Core structures **4**, **6**, **7**, **15**, and **16** were also tested with glyceraldehyde, where they reacted faster than with glucose (Table 2S).

Thiazolidines derived from benzaldehydes showed a promising reactivity (Table 2, **15**, **16**, **19**), while thiazolidines derived from aliphatic aldehydes showed slower reactivity and a lower rate of hydrolysis (Table 2, **17**-**18**). Electron-withdrawing substituents were also synthesized; a para-nitro substituted benzaldehyde thiazolidine was tested in the glucose test resulting in very slow reactivity (data not shown). The number of electron-donating substituents in para- and ortho positions were increased and gave very reactive thiazolidine core structures (data not shown or SI). Ortho-hydroxy groups gave side reactions for both hydrazones and thiazolidines. Acetals reacted too fast and were to hydrolytically labile (Table 2, **21**). Boc-protection of the oxazolidine amine gave an essentially unreactive structure (Table 2, **20**).

We selected hydrazone **7**, which had a low hydrolysis of 4% over 72 h and a significant difference in conversion in response to low and high glucose, 3% and 28%, respectively. Thiazolidine **15** had a hydrolysis of 20% over 24 h. Hydrazone **7** was derived from a benzoic acid hydrazide and an alkoxy-benzaldehyde, while thiazolidinie **15** was derived from cysteine and benzaldehyde.

Full GRI conjugates were obtained by acylation of HI at Lys B29 with linkers. The coupling reactions were performed in organic solvents as a precaution to prevent hydrolysis of the linker during preparation. Subsequent purification of the full GRI conjugates was achieved using C-4 reversed-phase columns under non-acidic conditions, using either medium-pressure high-performance flash chromatography or preparative HPLC. That the purified GRI was acylated at LysB29, rather GlyA1, was confirmed by reduction of the disulfides[26] or enzymatic cleavage with V8[27] followed by MS analysis.

In the design and evaluation of the full GRI the orientation of the core structure hydrazones and thiazolidines played a role (Fig. 2a). The core structure could be oriented in the linker so that either the aldehyde or the nucleophile (hydrazide, aminothiol, or glucose adduct) would remain attached to insulin LysB29 after cleavage. Cleavage of the linker thiazolidine forms an amino-thiol that could react with glucose to form a new thiazolidine. But a free thiol attached to insulin could potentially scramble the disulfide linkages in insulin. However, as active insulin has a short half-life after release (4-6 min) of HI[28], this is less of a concern *in vivo*.

The *in vitro* assay was based on measuring phosphorylation of AKT downstream of activation of the insulin receptor and preincubation of GRIs (5-50 μM) with different concentrations of glucose (0, 0.1, 1 M) or glyceraldehyde (0 or 1 mM) (Fig. 3), for 6 h or 24 h. Several key learnings were extracted from these controlled experiments. First, basal hydrolysis was seen for all GRIs, which we later recapitulated in our in vivo setting with pancreatectomized rats. Secondly, the ratio of GRI to glucose (equivalence) is essential for the concept; increasing the equivalences to levels that resembles physiological levels drives the hydrolysis, shown by both glucose and glyceraldehyde. Third, we could evaluate our GRIs on whether they responded to glucose/glyceraldehye or not and also their reactivity, which we termed “glucose potency”. We thus concluded that thiazolidine-, but not hydrazone-based GRIs indeed were glucose responsive insulins. The ratio between insulin concentration and glucose concentration was approaching physiological ratios. Thiazolidine-, but not hydrazone-based GRIs, responded to increased glucose concentrations in vitro. Since both classes retained potency in pancreatectomized rats, a clamp study comparing the most glucose-responsive thiazolidine, GRI-2, to the hydrazone GRI-1. When comparing GRI-2 and GRI-1 at equimolar concentrations (113 nmol/kg) during hyperglycemia, an identical GIR AUC was observed. At normoglycemia, a reduced GIR of GRI-2 compared to GRI-1 was observed (Fig. 4c). When doubling the dose of GRI-2 the GIR AUC at normoglycemia remained the same. Interestingly, the GIR AUC at hyperglycemic conditions at double dose was then increased by 15% compared to both GRI-1 and GRI-2 at low dose. This proved a true glucose response.

Initially, a GRI with a glucose responsive cleavable linker, as described here, will be administered by subcutaneous injections. Ultimately oral administration could be considered. It would form a subcutaneous depot, from where it would be gradually released and form a second, circulating, albumin-bound depot, GRI administration is well adjusted, we anticipate that fewer insulin injections will be required.

This GRI approach directly uses a reactive part of the glucose molecule, which makes it very specific. Thus, this GRI has a higher selectivity compared to the existing approaches that react with polyols in general.

Conclusion

We developed an unprecedented glucose responsive chemical linker that enabled a glucose responsive insulin (GRI). First, we developed hydrazone and thiazolidine core structures that could undergo an *aldehydo*-D-glucose promoted hydrolysis. This was achieved by tuning the stability and reactivity of the hydrazone and thiazolidine by adjusting the electron-donating and -withdrawing groups of nearby substituents. The resulting hydrazone and

thiazolidine core structures hydrolyzed faster when the glucose concentration increased. Since this was achieved for two different functional groups (i.e. cleavable groups), this points to the generality of the concept. The glucose sensitive core structures were incorporated in lipidated linkers coupled to the LysB29 amine in HI to form functional GRIs. The thiazolidine-based GRIs showed glucose responsiveness *in vitro*. *In vivo* studies of two GRIs in 90% pancreatomized rats showed a similar profile by lowering the blood glucose for a sustained period. Lastly, a physiological glucose response was demonstrated for a thiazolidine-based GRI. While clear differences and trends were identified in the core structures, the properties of the GRIs fell into three broad groups: aromatic and aliphatic, as well as N-methylated aliphatic for hydrazone. This new GRI concept is based on general organic-chemical principles and the core structures can likely be further adjusted for additional improvement of the GRI functionality. We envision that further fine-tuning of the GRI functionality could be achieved within the narrow physiological glucose range, based on the chemical principles disclosed here.

Experimental Section

Full experimental details for the synthesis of core structure/Insulin conjugates, characterization for all compound, in vitro assay for aldehyde responsiveness and in vivo studies for glucose lowering effects, clamp design/studies included in the Supporting Information. All animal experiments were performed according to the bioethical guidelines of Gubra (Hørsholm, Denmark) and in compliance with internationally accepted laboratory animal care and use principles under the personal licenses 2017-15-0201-01378 and 2017-15-0201-01383, which was approved by the Danish Animal Experimentation Council and issued by The Danish Animal Experiments Inspectorate.

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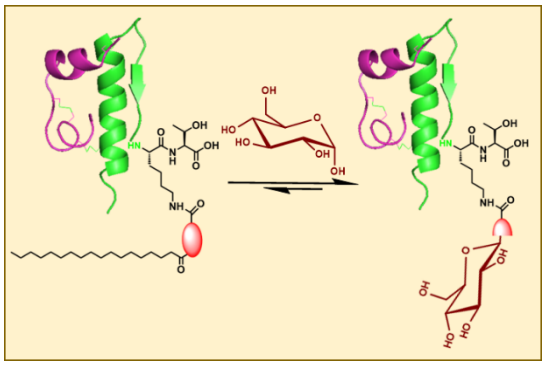
RESEARCH ARTICLE

An Aldehyde Responsive, Cleavable Linker for Glucose Responsive Insulins



463 M peo ple live with diabetes of which approximately 10% have type 1 diabetes. A glucose responsive insulin that responds to fluctuations in blood glucose concentrations has remained an elusive goal for decades. Here we describe the chemical development of hydrazones and thiazolidines that are cleavable in glucose concentration dependent manner. These were used to construct unprecedented glucose responsive insulins.

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