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RESEARCH

GZR101, a Novel Premixed Insulin for the Treatment of Diabetes Mellitus

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Abstract

Inadequate glycemic control in patients with current premixed insulin regimens is often due to the short half-life and pronounced peak-to-trough ratio of basal insulin. This study evaluated insulin GZR33 (GZR33), an ultra-long basal insulin, and insulin GZR101 (GZR101), which is a premix of GZR33 and postprandial insulin aspart. The binding affinity of GZR33 to the human insulin receptor (hIR) was analyzed using surface plasmon resonance. Potency was tested in vivo in streptozotocin-induced T1DM rats and db/db T2DM mice, using insulin degludec (degludec, IDeg) and insulin degludec/insulin aspart (IDegAsp) as controls. GZR33 was constructed by linking a C22 fatty diacid side chain to desB30 human insulin via a 6 × oligoethylene glycol (OEG) linker. GZR33 exhibited strong hIR-A and hIR-B binding (dissociation constants: 2.86E-6 M and 1.28E-6 M). It activated IRA and IRB with EC_{50} of 370.13 ± 94.86 nM and 477.48 ± 64.28 nM, respectively, in 0.5% human serum albumin (HSA), and induced adipogenesis with an EC_{50} of 2.45 ± 0.81 nM. GZR33 demonstrated a half-life twice as long as degludec (5.45 h vs 2.22 h in SD rats) and superior binding affinity to HSA (80-fold higher). In T1DM rats and T2DM mice, GZR33 outperformed IDeg in reducing HbA1c and improving glucose tolerance, demonstrating a potency at least three-fold higher than IDeg at the same molar concentration. GZR101 showed proportional glucose-lowering effects, combining the long-acting profile of GZR33 with the rapid action of insulin aspart. Therefore, GZR101 provides a promising once-daily premixed insulin candidate with potential to enhance glycemic control and patient adherence. Clinical trial number: not applicable.

Keywords Diabetes Mellitus · Premixed insulin · GZR101 · GZR33

Introduction

Diabetes mellitus is a metabolic disorder characterized by elevated blood glucose due to increased insulin insensitivity or insulin deficiency (Sharma et al. 2019). Approximately 537 million people worldwide live with diabetes, with the highest prevalence in China, estimated at 140 million (Diawara et al. 2023). Diabetes mellitus can be due to either a deficiency of insulin, known as Type 1 Diabetes Mellitus (T1DM), or an inability of insulin to control hyperglycemia, known as Type 2 Diabetes Mellitus (T2DM) (Banday et al. 2020). Management strategies include dietary and lifestyle adjustments, oral hypoglycemic agents, and insulin therapy (Asif 2014, Davies et al. 2018). Among these, (Wilcox 2005) insulin is the most effective approach to lowering blood glucose, making it the last resort in treating advanced diabetes (Freeland and Farber 2016) and indispensable for T1DM. The insulin analog of human insulin is structurally modified to mimic the effects of endogenous insulin, exhibiting absorption kinetics that more closely align with the rate of supply from the injection depot to meet dynamic physiological needs(Jonassen et al. 2012). Based on their pharmacokinetic (PK) properties, insulin analogs are roughly classified as rapid-acting and long-acting insulin (Sharma et al. 2019) used to address postprandial and fasting hyperglycemia, respectively.

Once-daily basal insulin regimens provide a simple and convenient tool for managing diabetes (Sarbacker and Urteaga 2016). However, its limitation in controlling

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postprandial glucose frequently results in suboptimal maintenance of target glycated hemoglobin (HbA1c) levels over time, necessitating higher insulin doses and transitioning to more complicated basal-bolus insulin regimens. Premixed insulin combines basal and rapid-acting components to regulate both fasting and postprandial blood glucose, reducing the number of injections, simplifying insulin administration regimens, providing convenience, and significantly improving adherence (Elizarova et al. 2014). Premixed insulin has the potential to improve glycemic control with less glucose level fluctuation, ultimately leading to an enhanced quality of life. Recent analyses have demonstrated that premixed insulin regimens are more effective at reducing HbA1c than basal insulin alone (Lasserson et al. 2009), with better patient adherence. However, the effectiveness of premixed regimens was limited by conventional basal insulins. Basal insulins typically have a relatively short half-life and higher peak-to-trough ratio, which increases the fluctuations in blood glucose management (Haahr and Heise 2014), including hypoglycemia and hyperglycemia when the insulin action wanes (Donnor and Sarkar 2000). Additionally, the variability in insulin action of current premixed insulin complicates glycemic control, as patients must time their meals and activities to match the insulin's action profile, which can be challenging to achieve (Sorli and Heile 2014).

Insulin GZR101 (GZR101) is an investigational premixed insulin which may address these challenges. GZR101 is comprised of a novel long-acting basal insulin analog, insulin GZR33 (GZR33) and a marketed insulin aspart (aspart, Asp) in a 50:50 ratio. This improved formulation is ideal because it includes a rapid-acting insulin and a basal insulin with an improved PK profile, an extended half-life, and fewer fluctuations, aiming to mimic the consistent and natural insulin release pattern of individuals without diabetes. Preclinical studies indicated that GZR33 exhibits a sustained and consistent glucose-lowering effect, characterized by an ultra-long duration of action in diabetic models compared to insulin degludec (degludec, IDeg). The preclinical PK and pharmacodynamic (PD) profile of GZR33 suggests that it offers better efficacy than degludec with diminished variability and enhanced dosing flexibility. This investigation evaluated and characterized the pharmacological attributes of GZR33 and GZR101, revealing their potential advantages in diabetes management.

Materials and methods

In vitro pharmacology and activity

Human serum albumin (HSA) binding assay

The binding affinity of GZR33 to HSA was determined with surface plasmon resonance (SPR) technology on Biacore™ 8 K. HSA was captured on the surface of a Series S Sensor Chip CM5 using an Amine Capture Kit according to the manufacturer's instructions (Cytiva), and a capture level of ~ 11,000 resonance units (RU) was used. Nine increasing GZR33 concentrations (0, 800, 1600, 3200, 6400, 12,800, 25,600, 51,200, 102,400 nM) were injected into the flow cells at a flow rate of 30 µL/min for 60 s, followed by a dissociation phase of 60 s. Binding kinetics parameters were calculated with the Biacore™ 8 K evaluation software using 1:1 binding model.

Insulin receptor binding assay

The binding affinity of GZR33 to human insulin receptor isoform A (hIR-A) and human insulin receptor isoform B (hIR-B) was determined with SPR technology on Biacore™ 8 K. Histidine-tagged hIR-A/hIR-B was captured on NTA sensor chip by chelation of Ni^{2+} through NTA on the surface and histidine residues in the hIR-A/hIR-B. Seven different concentrations of GZR33 (for hIR-A: 0, 800, 1600, 3200, 6400, 12,800, 25,600 nM; for hIR-B: 0, 100, 300, 900, 2700, 8100, 24,300 nM) were aliquoted into the flow cells at a flow rate of 30 µL/min for 60 s, followed by a dissociation phase of 60 s. Binding kinetics parameters were calculated with the Biacore™ 8 K evaluation software using 1:1 binding model.

Insulin receptor phosphorylation assay

Chinese Hamster Ovary (CHO) cells were obtained from Thermo Fisher Scientific (Cat No.: R75807) and overexpressed with IR-A to construct CHO-IR-A cells. CHO-IR-A cells were subcultured into a 96-well plate and maintained for 16–24 h. After starvation in a serum-free medium for 3–6 h, CHO-IR-A cells were stimulated with GZR33 for 30 min. IR-A phosphorylation was determined using IR Beta Phospho-Y1150/1151 Kit (PerkinElmer, Waltham, MA, USA).

CHO cells overexpressing hIR-B (CHO-IR-B) were obtained from American Type Culture Collection (ATCC) (Cat No.: CRL-3307) and were sub-cultured into a 96-well plate and maintained for 16–24 h. After starvation in a serum-free medium for 3–6 h, CHO-IR-B cells were stimulated with GZR33 for 30 min. IR-B phosphorylation was determined using IR Beta Phospho-Y1150/1151 Kit (PerkinElmer, Waltham, MA, USA).

Lipogenesis assay

3T3-L1 MBX cells were obtained from ATCC (Cat No.: CRL-3242) and differentiated into mature adipocytes. Mature 3T3-L1 MBX adipocytes were sub-cultured into a 96-well plate and maintained for 3–5 days. After starvation in a serum-free medium, adipocytes were stimulated with GZR33. Lipogenesis was determined by the Triglyceride-Glo Assay Kit (Promega, Madison, WI, USA).

Phosphorylated Akt assay

C2C12 cells were obtained from ATCC (Cat No.: CRL-1772) and differentiated into mature myotubes. The differentiated C2C12 cell line was subjected to a starvation process in serum-free medium for a period of three hours, followed by a 30-min treatment with human insulin (Cat.No.1342106, USP), degludec (Tresiba, Novo Nordisk), or GZR33. The expression of pAkt-s473/Akt was detected by Western Blot. The monoclonal β-actin antibody (sc-47778) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal Akt antibody (#4691) and polyclonal phospho-Akt (Ser473) antibody (#9271) were purchased from Cell Signaling Technology (Beverly, MA).

Pharmacokinetic (PK) study in Sprague Dawley (SD) rats and Beagle dogs

PK parameters of GZR33 were analyzed in both SD rats and Beagle dogs. SD rats of 205–296 g ($n = 8$ per group; four females and four males) and Beagle dogs of 5.26–11.26 kg ($n = 6$ per group; three females and three males) were treated with a single subcutaneous (s.c.) injection of GZR33 in multiple doses (SD rats 2, 6, 18 U/kg; Beagle dogs 0.3, 0.6, 1.2 U/kg) to assess the PK characteristics of GZR33 respectively. Meanwhile, 8 SD rats and 6 Beagle dogs received 0.6 U/kg GZR33 via intravenous (i.v.) administration.

Whole blood samples (~ 0.3 mL) were collected from the SD rats' retro-orbital sinus and Beagle dogs using intravenous lines in tubes containing dipotassium ethylenediaminetetraacetic acid (K_2-EDTA) anticoagulant, and centrifuged (2–8 °C, 2000 g, 10 min) to collect plasma samples. The anesthetic used for this process was isoflurane. No major adverse events were observed after blood sampling. For the SD rats, samples were analyzed at time points 0 (prior to administration), 2, and 15 min, and 1.5, 4, 8, 12, 30, and 36 h following s.c. injection of GZR33; and at 0, 2, and 15 min, and 1.5, 4, 8, 12, 30, and 36 h, following i.v. administration of GZR33. Beagle dogs blood samples were collected for analysis at time points 0 (prior to administration), 0.5, 1, 2, 4, 6, 8, 12, 24, 30, 36, and 48 h following s.c. administration of GZR33; and at 0, 2, 10, and 30 min, and 1, 2, 4, 6, 8, 12, 24, 30, 36, and 48 h following i.v. administration of GZR33.

The efficacy of GZR33 and GZR101

The antidiabetic effect of GZR33 and GZR101 in STZ-induced T1DM rats

SD rats, acquired from Changzhou Cavens Laboratory Animal Technology Co., Ltd, were used to establish a T1DM model by using streptozotocin (STZ), and rats with random blood glucose levels exceeding 16.7 mmol/L were selected into the study. The rats were subsequently stratified into four distinct groups ($n = 12$ each), encompassing a vehicle control group (administered GZR33 vehicle), a positive control group (treated with degludec at a dosage of 288 nmol/kg), and two GZR33 groups receiving varying dosages of GZR33 (72 nmol/kg and 144 nmol/kg) (Table S2). The regimen for the group receiving degludec entailed subcutaneous injections once daily, whereas the remaining groups were administered their respective treatments subcutaneously once every two days, over a continuous period of 28 days. Glycemic control was assessed through blood glucose measurements using a Roche blood glucose meter, and HbA1c levels were determined using an automated biochemical analyzer.

In the GZR101 study, STZ-induced T1DM rats were randomized into five groups, each comprising 12 animals. The groups included: 1) a vehicle control group, 2) the GZR101 group (36 nmol/kg, containing 18 nmol/kg GZR33 and 18 nmol/kg aspart), 3) a positive control insulin degludec/insulin aspart (IDegAsp) group (60 nmol/kg, composed by 42 nmol/kg degludec and 18 nmol/kg aspart), 4) GZR33 group administered at 18 nmol/kg, and 5) Aspart group administered at 18 nmol/kg (Table S2). Each group was subjected to s.c. injection of the respective substances once daily for 28 consecutive days. Glycemic control was monitored using a Roche blood glucose meter, and HbA1c levels were determined with a fully automated biochemical analyzer. All experimental procedures were conducted in compliance with relevant ethical guidelines and regulations.

The antidiabetic effect of GZR33 and GZR101 in db/db mice

Db/db mice, sourced from Changzhou Cavens Laboratory Animal Technology Co., Ltd, were selected based on a criterion of random blood glucose levels exceeding 20 mM. The mice were allocated into four distinct groups ($n = 10$ each) based on random blood glucose and HbA1c. These groups included a vehicle control group, two GZR33 groups (72 nmol/kg and 288 nmol/kg), and a degludec group (administered degludec at 288 nmol/kg). The groups undergoing GZR33 treatment received s.c. injections once every two days, while the group receiving degludec and placebo had subcutaneous injections once daily for 28 days. Glycemic monitoring was conducted using a Roche blood glucose meter, and HbA1c levels were quantified utilizing a fully automated biochemical analyzer. Following the completion of the experimental protocol, the mice were euthanized by ethical standards for animal research.

In the GZR101 study, mice were subsequently categorized into five groups, with stratification based on parameters including random blood glucose, HbA1c, and body weight. Each group comprised 10 mice, delineated as follows: 1) a vehicle control group receiving GZR33 vehicle, 2) a GZR101 group treated with GZR101 at dosages of 108 nmol/kg, 3) an IDegAsp group administered IDegAsp at 180 nmol/kg, 4) GZR33 group administered 54 nmol/

kg of GZR33, and 5) Aspart group administered 54 nmol/kg aspart (Table S2). The treatment protocol for all groups involved daily subcutaneous injections over 28 days, resulting in a total of 28 administrations. Glycemic monitoring was executed using a Roche blood glucose meter, while HbA1c levels were quantified with a fully automated biochemical analyzer. Following the completion of the experimental period, the mice were euthanized in accordance with ethical standards for humane treatment in animal research.

Adipogenic genes assay

Following the administration of multiple doses of insulin in db/db mice (Degludec 288 nmol/Kg, GZR33 18 & 72 & 288 nmol/Kg), samples were collected six/twenty-four hours after the final administration. The RNA was extracted from tissues and reverse transcribed for the purpose of conducting a quantitative polymerase chain reaction (QPCR) analysis of the expression of the fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC). Total RNA was extracted by RNAiso Plus (9108, Takara, Japan). To build the cDNA library, the PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A, Takara, Japan) was used. The QPCR was performed in triplicate with an CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad, USA) using SYBR Premix ExTaq II (RR820A, Takara, Japan). The mRNA level of the target genes was normalized to the Gapdh mRNA level in each sample. The standard $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression. We conducted three biological and three experimental replicates for all QPCR experiments.

Statistical analysis

Data represent the means ± standard error of mean (SEM). Statistical differences between two groups were evaluated by Student's *t*-test, and those among three groups or more were evaluated by one-way analysis of variance (ANOVA) using GraphPad Prism 8 software. The difference was statistically significant if the *p* value was less than 0.05. In instances where the ANOVA was statistically significant ($p < 0.05$) and the variance was homogeneous, the Tukey test was employed for the purpose of intergroup comparative analysis. In instances where the variance was found to be heterogeneous, the Dunnet's T3 test was employed for the purpose of conducting an intergroup comparative analysis.

Results

HSA binding affinity of GZR33

The novel basal insulin analog, GZR33, has been biochemically engineered through the conjugation of a C22 dicarboxylic acid via a 6 ×OEG linker to the ε-amino group of the lysine residue at position B29 of the desB30 human insulin molecule (illustrated in Fig. 1A). HSA is the most abundant plasma protein with a circulatory half-life of 19 days (Sleep 2015). After binding insulin analog to HSA, an essentially inactive depot forms, slowly releasing active insulin analog over time, resulting in the extension of the half-life of insulin analog (Kurtzhals et al. 1995). HSA binding affinity was determined through SPR technology, and representative sensorgrams were shown in Fig. 1B-C. The equilibrium dissociation constant (KD) of GZR33 for HSA was $6.45 \pm 0.03 \times 10^{-6}$ M, demonstrating an 80-fold higher affinity compared to degludec ($5.17 \pm 0.69 \times 10^{-4}$ M). This enhanced binding affinity likely contributes to the extended in vivo half-life observed.

Insulin receptor affinity of GZR33

Decreased insulin receptor binding affinity and rate of insulin receptor-mediated clearance is another perspective to extend the half-life of insulin analog (Kjeldsen et al. 2021). Insulin receptor binding affinity was determined through SPR technology, and representative sensorgrams were shown in Fig. 2A-D. The equilibrium KD of hIR-A binding for GZR33 was $2.86 \pm 0.029 \times 10^{-6}$ M, while for degludec it was $3.77 \pm 0.18 \times 10^{-7}$ M, indicating that the hIR-A binding affinity of GZR33 was 7.6-fold lower than that of degludec. Similar results were observed in hIR-B binding affinity (GZR33 $1.28 \pm 0.032 \times 10^{-6}$ M vs. degludec $4.14 \pm 0.072 \times 10^{-7}$ M).

Biological activity of GZR33

To characterize the biological properties of GZR33, IR-A and IR-B phosphorylation were determined in CHO-IR-A cells and CHO-IR-B cells. Lipogenesis was determined in 3T3 L1 MBX adipocytes. As shown in Fig. 3A-C, and Table 1, GZR33 stimulated IR-A phosphorylation (EC_{50}, 370.13 ± 94.86 nM), IR-B phosphorylation (EC_{50}, 477.48 ± 64.28 nM), and lipogenesis (EC_{50}, 2.45 ± 0.81 nM) in a similar dose-dependent manner as human insulin and degludec, indicating that GZR33 retained the biological properties of human insulin. In addition, treatment with GZR33 (1 nM, 10 nM, 100 nM) for 30 min increased the phosphorylation levels of Akt in a concentration-dependent manner (Fig. S1). Furthermore, in comparison to degludec, GZR33 has been observed to exhibit a reduced IR-A and IR-B phosphorylation yet displays a comparable p-Akt and an elevated lipogenesis. In the present study, the relative expression of adipogenic genes, namely FAS and ACC, was examined in db/db mice following the administration of multiple doses of degludec and GZR33. The results demonstrated that the GZR33 group exhibited a higher relative expression of these genes in comparison to the degludec group (Figs. S2-3).

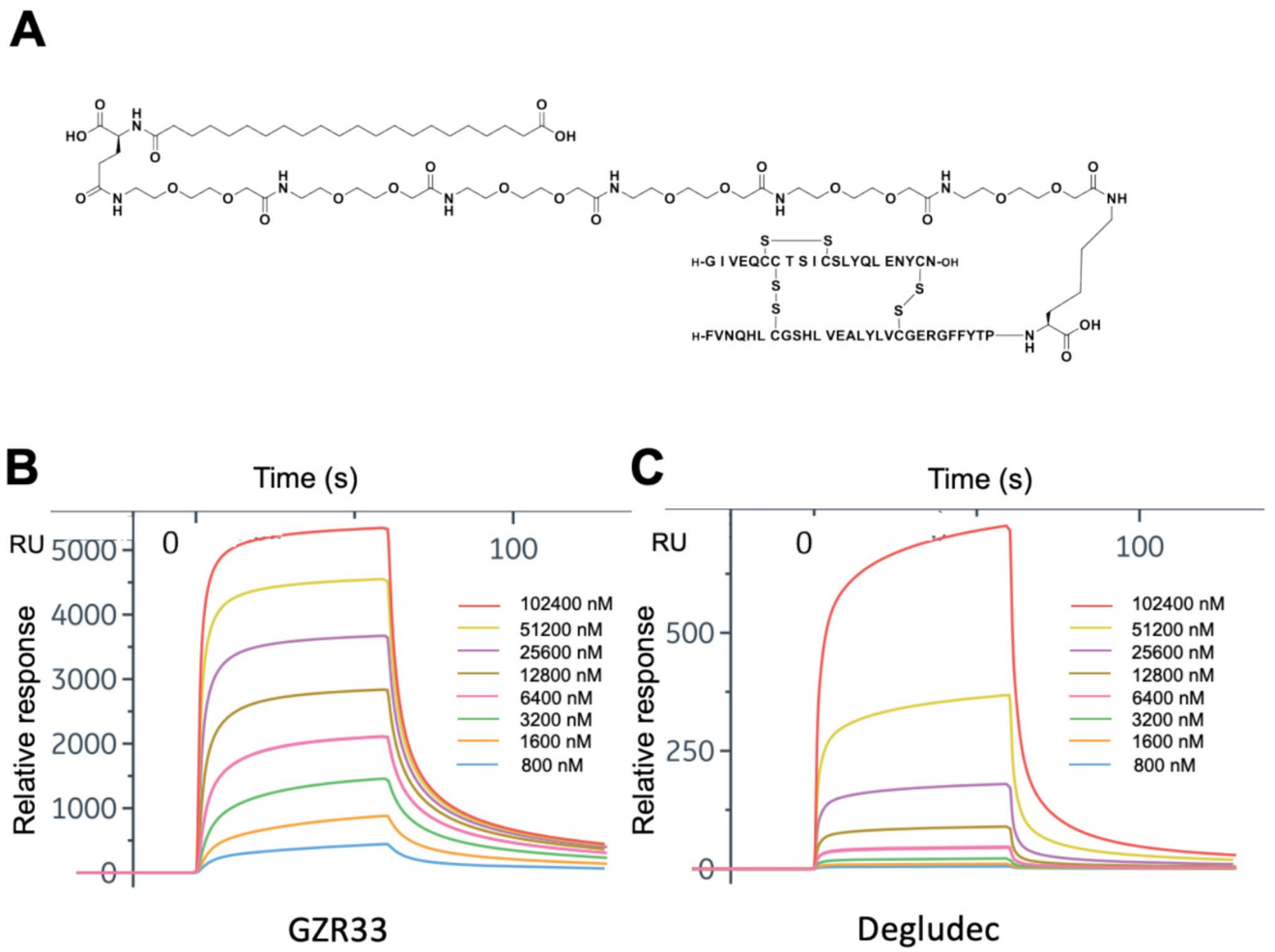

Fig. 1 HSA binding affinity of GZR33 and degludec. **A** Structure schematic of GZR33. **B** Representative sensorgrams of GZR33 binding to HSA. Nine increasing GZR33 concentrations (0, 800, 1600, 3200, 6400, 12,800, 25,600, 51,200, 102,400 nM) were conducted in the study. **C** Representative sensorgrams of degludec binding to HSA. Nine increasing Degludec concentrations (0, 800, 1600, 3200, 6400, 12,800, 25,600, 51,200, 102,400 nM) were conducted in the study

Antidiabetic efficacy of GZR33 in T1DM rats and T2DM mice

In STZ-induced T1DM rats, GZR33 dose-dependently improves the glycemic area under the curve (G-AUC) of the oral glucose tolerance test (OGTT) and HbA1c (Fig. 4A-B) at 72 and 144 nmol/kg. The glucose-lowering effect of GZR33 persisted for over 48 h, compared to 24 h for degludec, highlighting the prolonged duration of action for GZR33. In addition, the reduction of HbA1c in GZR33 at 144 nmol/kg (−6.3%) was superior to that in degludec at 288 nmol/kg (−3.3%). Therefore, GZR33 significantly outperformed daily administered degludec (288 nmol/kg), doubling its hypoglycemic potency. In total, the hypoglycemic duration of GZR33 notably surpassed that of degludec, with GZR33 efficacy than two times that of degludec in HbA1c reduction.

Likewise, dose-dependent efficacy of GZR33 was similar in T2DM db/db mice. GZR33 markedly improved the AUC of OGTT and reduced HbA1c at 12, and 288 nmol/kg, which was more pronounced than that of degludec (Fig. 4C-D). GZR33 at a dose of 72 nmol/kg (−1.4%) achieved a superior reduction in HbA1c levels compared to degludec at a dose of 288 nmol/kg (−1.0%). Additionally, the duration of the glucose-lowering effects of GZR33 exceeded those of degludec. The results of the two diabetic models indicate that GZR33 may offer a superior clinical solution for the management of diabetes mellitus.

Differential in vivo pharmacokinetic characteristics of GZR33

The PK characteristics of GZR33 were examined in dogs and rats. Absorption of GZR33 following s.c. administration took place immediately with maximal plasma concentration at 5–6 h after dosing in dogs. The terminal half-life of GZR33 was approximately 5.45 h in rats, significantly exceeding degludec

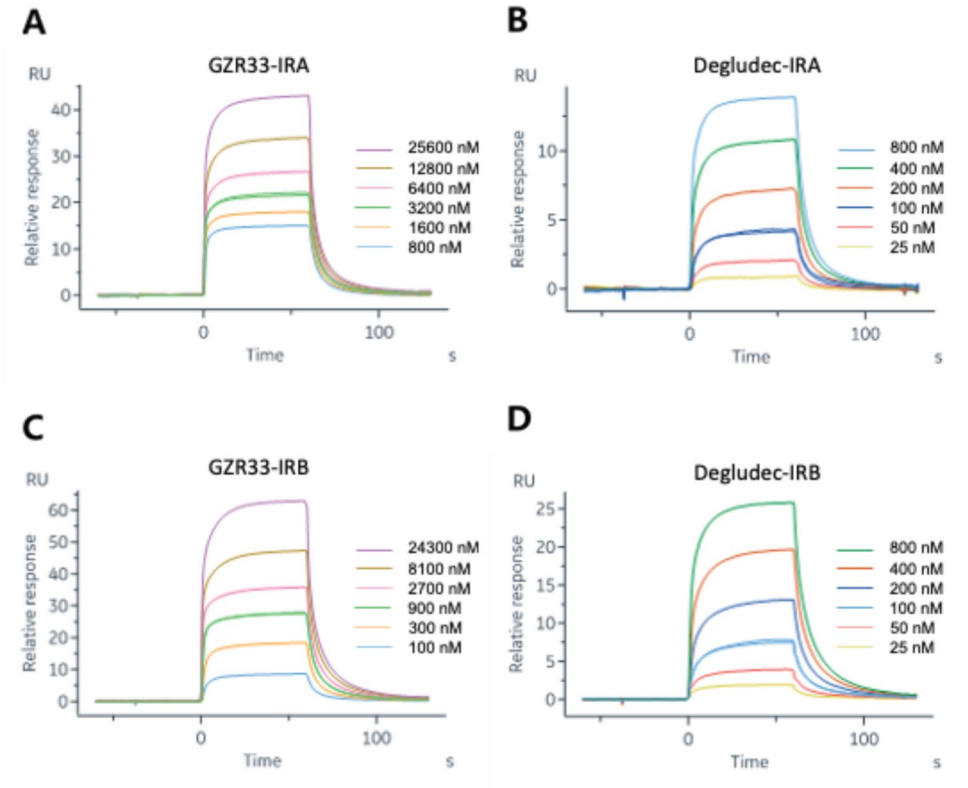

Fig. 2 Insulin receptor binding affinity of GZR33 and degludec. **A** Representative sensorgrams of GZR33 binding to hIR-A. Seven different concentrations of GZR33 (0, 800, 1600, 3200, 6400, 12,800, 25,600 nM) were conducted in the study. **B** Representative sensorgrams of degludec binding to hIR-A. Seven different concentrations of degludec (0, 25, 50, 100, 200, 400, 800 nM) were conducted in the study. **C** Representative sensorgrams of GZR33 binding to hIR-B. Seven different concentrations of GZR33 (0, 100, 300, 900, 2700, 8100, 24,300 nM) were conducted in the study. **D** Representative sensorgrams of degludec binding to hIR-B. Seven different concentrations of degludec (0, 25, 50, 100, 200, 400, 800 nM) were conducted in the study

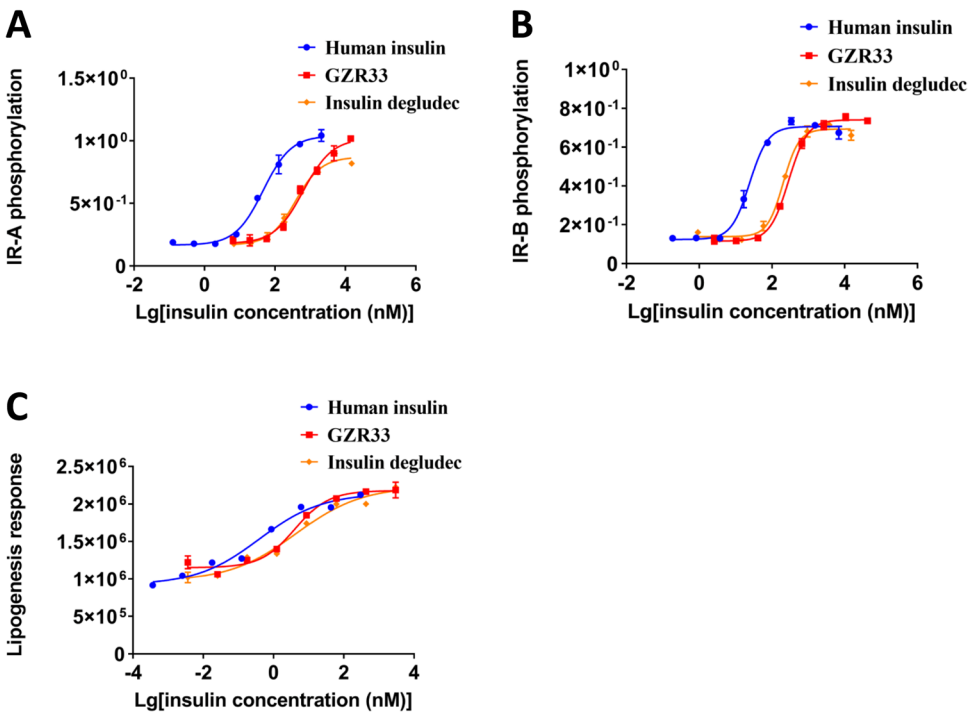

Fig. 3 Biological properties of GZR33 and degludec. **A** Representative dose–response curves for human insulin/GZR33/insulin degludec stimulated IR-A phosphorylation in CHO-IR-A cells. **B** Representative dose–response curves for human insulin/GZR33/insulin degludec stimulated IR-B phosphorylation in CHO-IR-B cells. **C** Representative dose–response curves for human insulin/GZR33/insulin degludec stimulated lipogenesis in 3T3-L1 MBX adipocytes. Data points are the average of two separate sets in one assay, and error bars represent SEM

(2.22 h). The terminal half-life of GZR33 was approximately 6 h in dogs, significantly exceeding degludec (3.2 h) (Fig. 5A-B). In corresponding with the longer in vivo pharmacodynamics effect, GZR33 demonstrated a prolonged half-life compared to degludec, both approximately 2 times longer. The longer T_{max} of GZR33 compared to degludec is shown in this study, with 2.3–6.2 h for GZR33 and 1.5 h for degludec in rats (Fig. 5, Table S1). The prolonged T_{max} and $T_{1/2}$ suggest slower absorption and clearance rates for GZR33, which contribute to its ultra-long-acting profile and reduced glycemic variability.

Table 1 Biological properties of human insulin, insulin GZR33, and insulin degludec (Mean ± SEM)

Insulin	lipogenesis (nM) EC_{50}	EC_{50} for IR-A phosphorylation (nM) EC_{50}	EC_{50} for IR-B phosphorylation (nM) EC_{50}
Human insulin	0.39 ± 0.05	24.53 ± 7.80*	26.78 ± 1.04*
Insulin GZR33	2.45 ± 0.81	370.13 ± 94.86*	477.48 ± 64.28*
Insulin degludec	10.00 ± 3.09	253.75 ± 51.57*	280.18 ± 27.48*

*In 0.5% human serum albumin

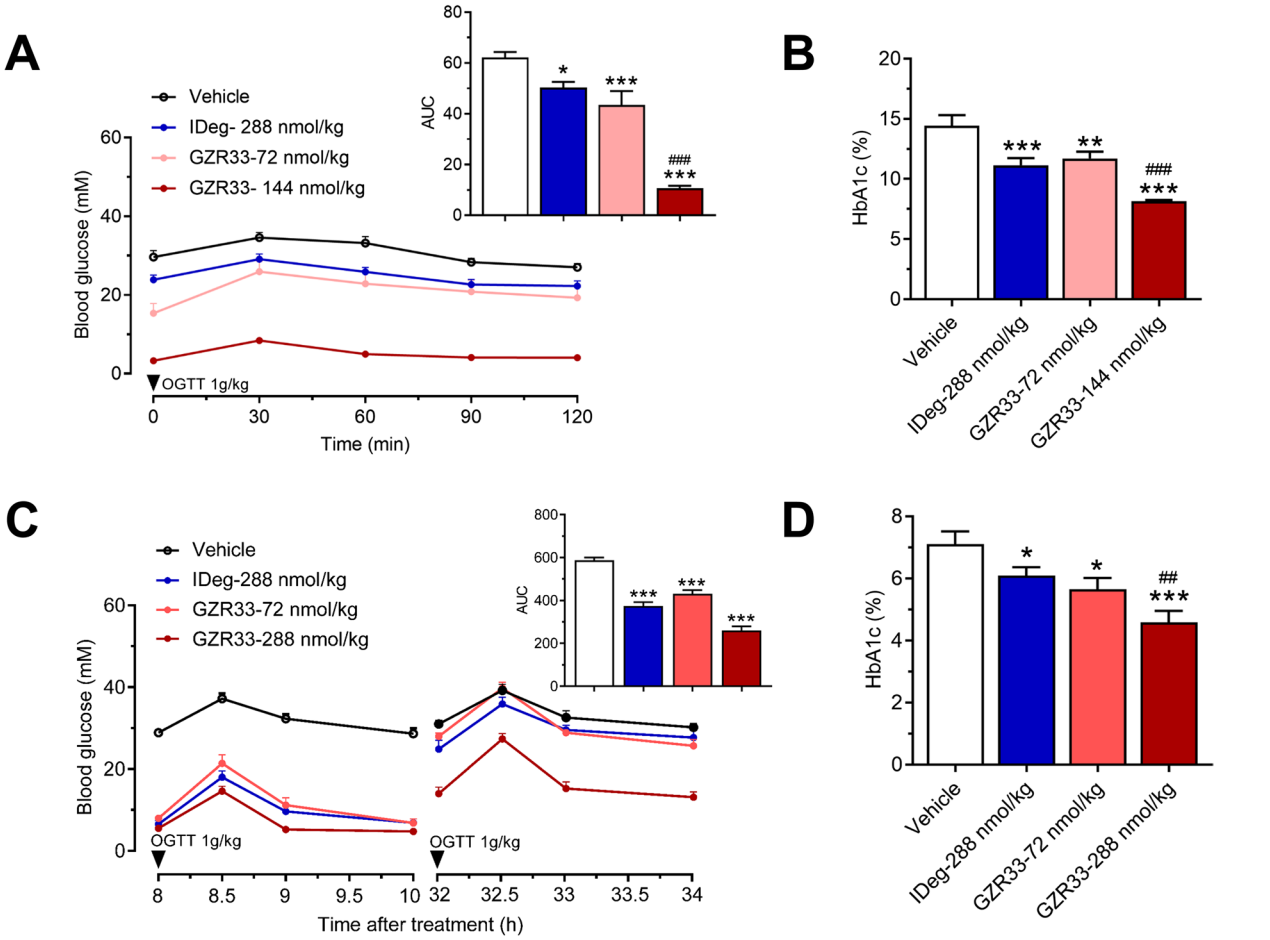

Fig. 4 The antidiabetic effect of GZR33 in T1DM rats and T2DM mice. **A** Effect of continuous administration of GZR33 on oral glucose tolerance test (OGTT) in male STZ-induced T1DM rats; **B** Effect of continuous administration of GZR33 for 29 days on HbA1c in male STZ-induced T1DM rats; **C** Effect of continuous administration of GZR33 on OGTT in male db/db mice; **D** Effect of continuous administration of GZR33 for 29 days on HbA1c in male db/db mice. The glucose response to the OGTT and area under the curve (AUC) on day 28 (**A**) or day 27–28 (**C**), OGTT was performed 24 h (**A**) or 8 h and 32 h (**C**) after drug administration; HbA1c on day 29 (**B/D**). Vehicle: GZR33 solvent control group. Data were expressed as Mean ± SEM. $n = 10$ in T2DM mice; $n = 12$ in T1DM rats, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Vehicle, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs IDeg

The PK curve of the GZR33 and aspart component when premixed together as GZR101 is comparable to that observed independently (Fig. 5C, D). These results demonstrate that GZR33 and aspart has no drug-drug interaction in the compound formulation GZR101. This establishes the compatibility of the two drugs while ensuring that each is capable of functioning independently.

Antidiabetic efficacy of GZR101 in T1DM rats and T2DM mice

Consisting of GZR33 and aspart, GZR101 has been demonstrated to exert a beneficial therapeutic effect on db/db mice and STZ-induced diabetic rats, with markedly enhanced oral glucose tolerance (Fig. 6A-C and B-D) and lowered HbA1c

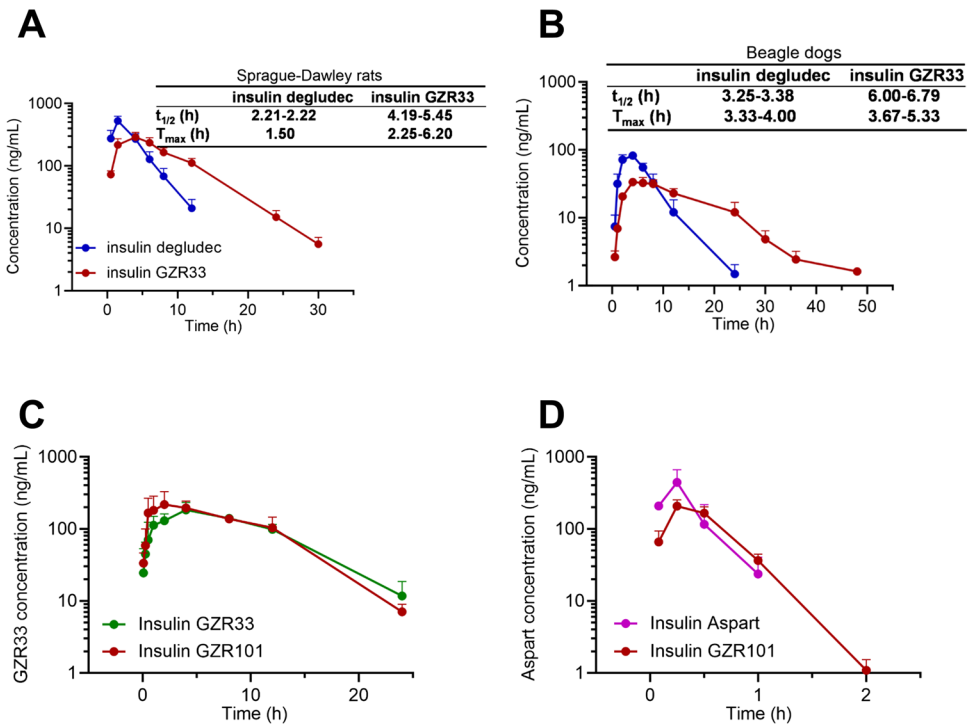

Fig. 5 The PK characteristics of GZR33 and GZR101 in vivo**. A** The PK characteristics of GZR33 in SD rats. **B** The PK characteristics of GZR33 in Beagle dogs. **C** Mean concentration for a single dose of 60 nmol/kg GZR101 compared to 30 nmol/kg GZR33 and 30 nmol/kg aspart (**D**) in male SD rats. Data were presented as Mean ± SEM

(about −2% in T2DM and −10% in T1DM compared to placebo) (Fig. 6E-F). The efficacy of GZR101 covers both postprandial and basal blood glucose levels. Relative to equivalent doses of aspart, GZR101 demonstrates superior efficacy than IDegAsp in improving glycemic levels, alleviating glucose intolerance, and prolonging glycemic control across both T1DM and T2DM models. Furthermore, the glucose-lowering effect of GZR101 is significantly more pronounced than that of GZR33 and aspart administered alone in the same molar concentration. Distinct from the monotherapeutic use of GZR33, GZR101 presents with an expedited hypoglycemic onset, thus effectively addressing the delayed action typical of long-acting basal insulins. Moreover, the duration of its therapeutic impact parallels that observed with GZR33, underscoring GZR101's potent and sustained pharmacological benefits in diabetic management. The results indicated that GZR101 may offer a more accurate representation of the physiological effect of glucose management, encompassing both postprandial and basal blood glucose levels, than that observed with the current treatments.

Discussion

Currently available basal insulin analogs, including insulin glargine, insulin detemir, and insulin degludec (Pollom et al. 2019), offer an improvement over neutral protamine Hagedorn (NPH) insulin in terms of duration of action and reduced peak effect (Peterson 2006). However, they still demonstrate suboptimal or nonideal PK and PD properties, leading to blood glucose fluctuations and complicating diabetes management by requiring frequent monitoring and dosage adjustments. The new long-acting basal insulin GZR33 provide a continuous and flat glucose-lowering effect over 48 h in T1DM rats, with a once every 2 days or once-daily dosing frequency for T1DM and T2DM models. The primary structure of GZR33 is designed to facilitate the formation of soluble multi-hexamer assemblies upon subcutaneous injection and robust reversible binding to HSA, thereby conferring an ultra-long peak-less PK profile. GZR101, a novel premixed insulin consisting of GZR33 and rapid insulin aspart, demonstrated good PK/PD profiles in these preclinical studies. Both GZR33 and GZR101 have shown superior glucose-lowing efficacy in T1DM and T2DM animal models.

Most premixed insulin products currently available are NPH premix insulin. NPH-premixed insulins are designed to reduce the number of daily injections. Still, there are several disadvantages associated with resuspension, variability, short action, multiple daily injections, and a high risk of hypoglycemia. Inadequate resuspension of NPH insulin is attributed to the day-to-day variability in the PK and PD profile of NPH insulin, which can result in hypoglycemia. The other long-acting insulin analogs are less suitable for incorporation into premixed insulin formulations due to their incompatibility with other insulins, such as insulin glargine and insulin aspart or lispro. The distinctive physicochemical properties of insulin glargine render it incompatible to mix

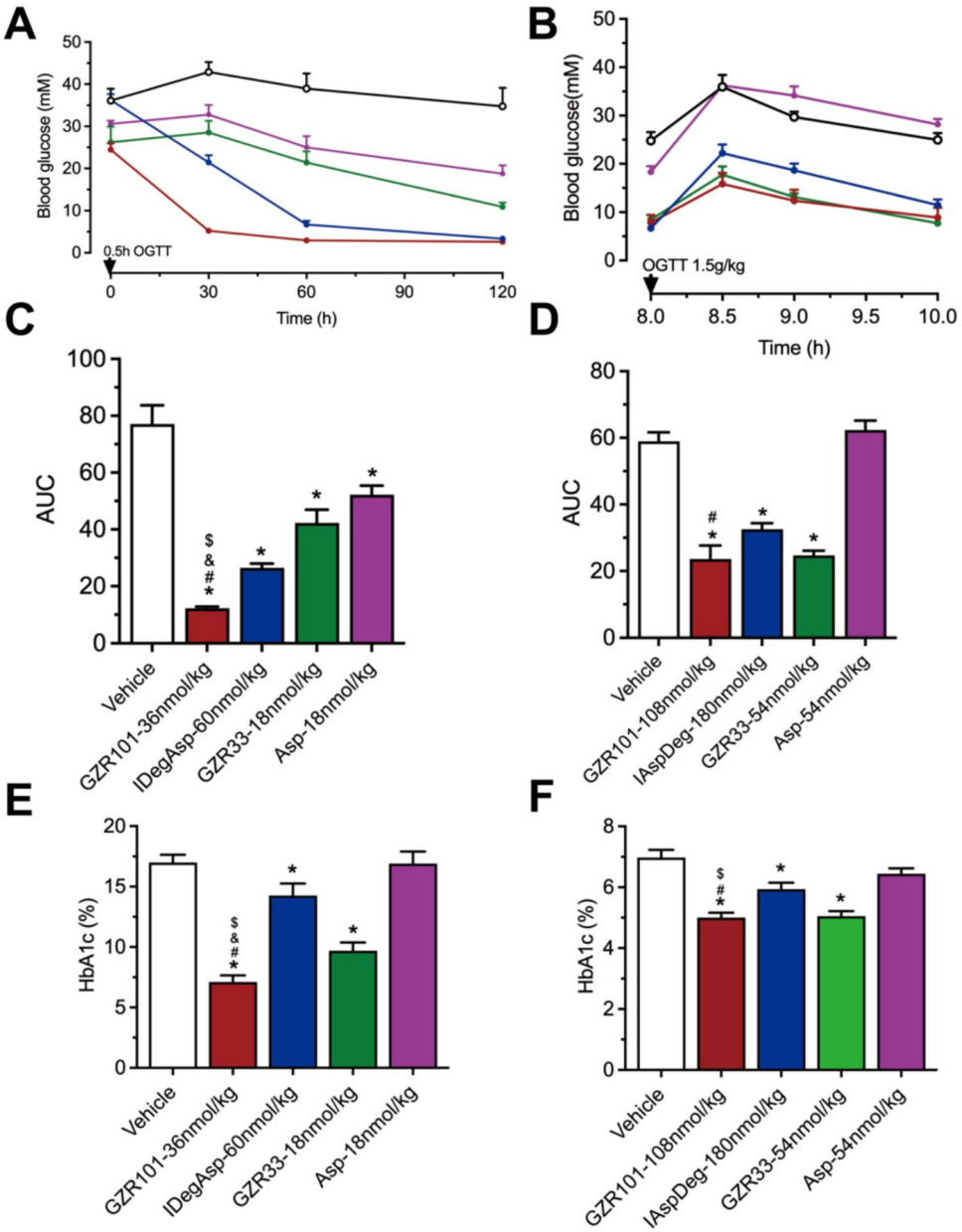

Fig. 6 The antidiabetic effects of GZR101 in T1DM rats and T2DM mice**. A** Blood glucose curve of GZR101 in OGTT on Day 28 after injection 0.5 h in STZ rats; **B** Blood glucose curve of GZR101 in OGTT on Day 28 after injection 8 h in db/db mice; **C** Effect of continuous administration of GZR101 for 28 days on AUC of OGTT after injection 0.5 h in STZ rats; **D** Effect of continuous administration of GZR101 on AUC of OGTT after injection 8 h in db/db mice; **E** and **F** Glucose-lowering efficacy of GZR101 given for 28 consecutive days. HbA1c on day 29 in STZ-induced T1DM rats (**E**) and db/db mice (**F**). Vehicle: GZR33 solvent control group. Data were expressed as Mean ± SEM. male and female, $n = 10$ in T2DM mice; $n = 12$ in T1DM rats. Asp = insulin aspart. $^{*}p < 0.05$ vs Vehicle, $^{\#}p < 0.05$ vs IDegAsp, $^{\&}p < 0.05$ vs GZR33, $^{\$}p < 0.05$ vs Asp

with rapid insulins (Meiffren et al. 2019). The distinctive pH of glargine may potentially interfere with the rapid insulins present in premixed insulin formulations. Consequently, such a formulation has not yet been successfully launched. The only product combination of rapid and basal insulin analogs that have been successfully commercialized worldwide is Ryzodeg (insulin degludec/insulin aspart). However, one of the issues associated with Ryzodeg is that the degludec has a relatively brief duration of action and a peak in its action profile. The half-life of degludec is approximately 24 h, with a peak occurring throughout this period.

The need for novel premix insulin formulations exists, particularly those comprising basal insulin with an extended half-life and reduced fluctuations. The most effective strategies for prolonging the half-life of basal insulin are reversible binding to albumin by a fatty acid side chain and reduction of insulin receptor binding by insulin substitutions. For degludec, the threonine residue at position B30 is deleted, and the ε-amino group of LysB29 is acylated with a 16-carbon fatty acid side-chain via a g-L-glutamic acid linker (Jonassen et al. 2012; Lane et al. 2018; Zhou et al. 2019), which results in a duration of action exceeding 24 h. This strategy has been developed based on the properties of insulin detemir, which has a 14 C acid with several recognized shortcomings, such as short action. In consideration of this strategy, GZR33 was designed with an increased fatty acid side chain from 16 to 22 C, which is postulated to enhance the affinity for HSA. Concurrently, the linker was

modified to 6× OEG, linking the C22 fatty diacid side chain to desB30 human insulin, which will increase the spatial distance between fatty acids and HSA, thereby facilitating the folding of insulin polypeptide and improving the stability of the molecule in circulation. This design significantly reduces receptor-mediated clearance, contributing to the extended glycemic control duration observed in preclinical models. Therefore, compared to degludec, GZR33 displays a more extended fatty acid side chain and OEG linker, which may result in augmented affinity for HSA while concurrently reducing binding affinity for the insulin receptor. Following HSA binding, an essential inactive depot is established, facilitating the gradual release of active GZR33 over an extended period. Consequently, binding to HSA can increase the plasma half-life of GZR33 and contribute to prolonging the action profile. In addition, the low IR binding affinity reduces the in vivo IR-mediated clearance rate, thereby further increasing the plasma half-life of GZR33. This may be the reason why GZR33 exhibits a prolonged half-life compared to degludec, with in vivo pharmacodynamic efficacy persisting for up to two days.

In the PK studies, the half-life of GZR33 was validated to be approximately two-fold longer than that of degludec in both rats and dogs. The prolonged T_{max} of GZR33, compared to degludec, indicates the gradual and sustained absorption of GZR33 from the site of administration into the circulation. Furthermore, the PK studies suggest that the absorption and clearance of GZR33 were slower than that of degludec. The steady-state PK profile of GZR33 shows minimal peaks, thus facilitating a more stable and predictable control of blood glucose levels. This attribute significantly reduces the risk of glycemic variability, potentially decreasing the incidence of hypoglycemic events and periods of hyperglycemia. Additionally, the PK profiles of GZR33 and aspart in GZR101 were observed to occur independently, without cross-interactions. The results indicate that GZR33 may exhibit a longer duration and lower fluctuations than degludec in T1DM rats and T2DM mice.

The prolonged efficacy of GZR33 was substantiated in rodent models of both T1DM and T2DM. In these diabetic models, the administration of GZR33 resulted in a dose-dependent reduction in the glucose area under the curve of the oral glucose tolerance test and HbA1c, with a glucose-lowering effect observed for a duration exceeding two days (longer than that of IDeg). In addition, GZR33 outperformed IDeg in the reduction of HbA1c and FBG, demonstrating a potency at least three times greater than that of IDeg. GZR33 has the potential to be used as a stand-alone basal insulin or as part of premixed insulin to provide glycemic control, with low variability and low incidence of hypoglycemia and other adverse effects both in Type 1 and Type 2 diabetes.

Because of the properties of GZR33, GZR101 has the potential for option treatment for once-daily premixed insulin. The formulation of GZR101 integrates the extended duration of action and steady PK characteristics of GZR33 with the rapid action of insulin aspart to regulate both fasting and postprandial glucose. GZR101 has been demonstrated to have a beneficial therapeutic impact on both T2DM and T1DM models, with a similar secretion pattern of physiological insulin to manage postprandial and basal glucose. Furthermore, its hypoglycemic effect and duration of efficacy are superior to those of IDegAsp and more effective than those of GZR33 and insulin aspart as single agents. Compared to the independent use of GZR33, the hypoglycemic effect of GZR101 is observed to manifest at an earlier point in time, effectively compensating for the deficiency in the slow-acting action profile of long-acting basal insulin. Furthermore, the effect of maintenance time was observed to be essentially similar to that of GZR33. By offering a nearly peak-less action profile and sustained glucose control throughout the day, GZR101 promises to enhance the predictability and convenience of insulin therapy, thereby reducing the risk of hypoglycemia and improving treatment adherence for patients with diabetes.

This study has several limitations that warrant acknowledgment. First, the prolonged mechanism of action associated with GZR33 requires comprehensive pharmacological evaluation to fully elucidate its therapeutic properties. Second, particular emphasis should be placed on molecular signaling pathways (such as, phosphorylation-IR). Finally, while preliminary findings appear promising, the therapeutic efficacy and safety profile of GZR101 will require rigorous assessment through well-designed clinical trials.

In conclusion, the preclinical results of GZR33 and GZR101 highlight their potential to address critical challenges in diabetes management, offering enhanced efficacy, reduced glycemic variability, and improved patient convenience.

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Data availability The data generated during the current study are available from the corresponding author on request.

Declarations

Competing interests The authors declare no competing interests.

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