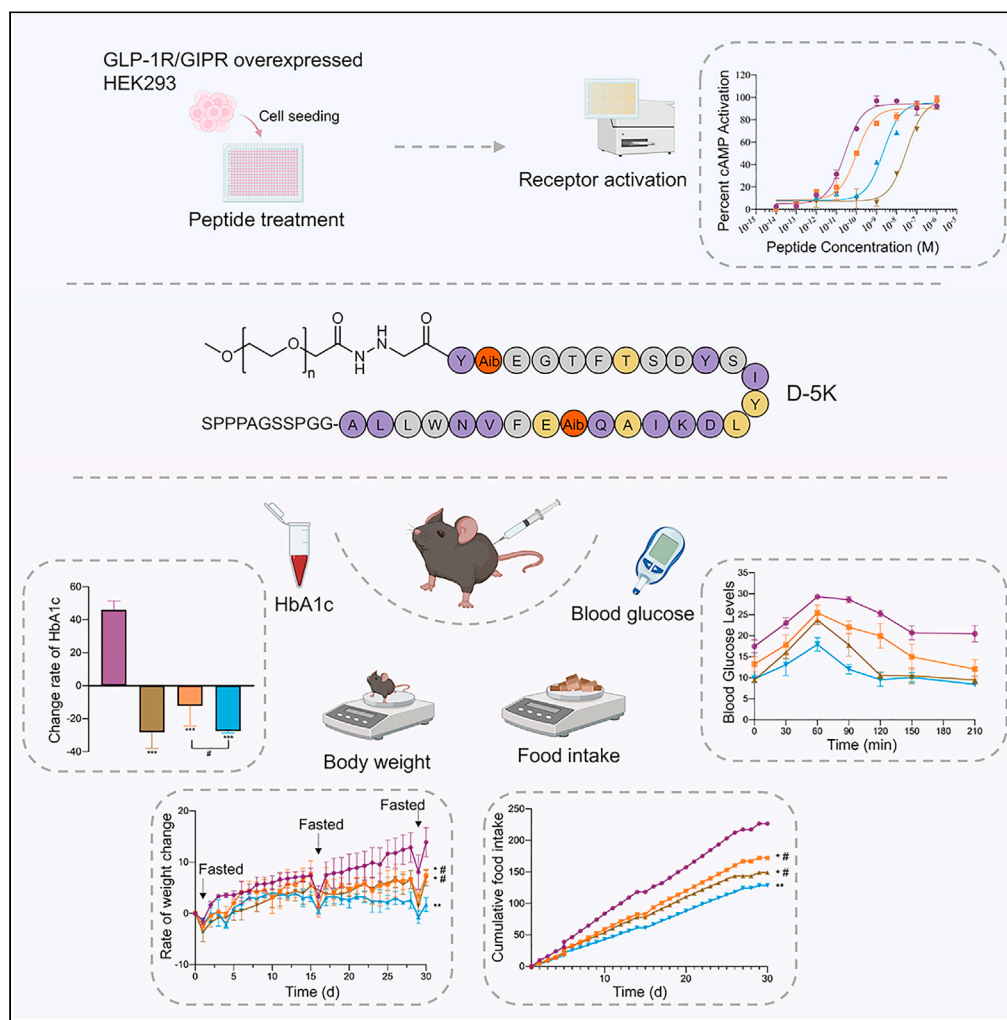**Article**

An imbalanced GLP-1R/GIPR co-agonist peptide with a site-specific N-terminal PEGylation to maximize metabolic benefits

Xuan Xia,
Qianmeng Lin,
Zhan Zhou,
Yongheng Chen

yonghenc@163.com

Highlights

Peptide **1** is a potent and imbalanced GLP-1R/GIPR co-agonist

Action time of peptide **1** was refined through a site-specific N-terminal PEGylation

D-5K is a long-acting GLP-1R/GIPR co-agonist

D-5K exhibits improved metabolic benefits, outperforming GLP-1R mono-agonists

Article

An imbalanced GLP-1R/GIPR co-agonist peptide with a site-specific N-terminal PEGylation to maximize metabolic benefits

Xuan Xia,[1,2,4] Qianmeng Lin,[1,2,4] Zhan Zhou,[3] and Yongheng Chen[1,2,5,*]

SUMMARY

Glycemic and body weight control gained from GLP-1R agonists remains an unmet need for diabetes and obesity treatment, leading to the development of GLP-1R/GIPR co-agonists. An imbalance in GLP-1R/GIPR agonism may extensively maximize the glucose- and weight-lowering effects. Hence, we prepared a potent and imbalanced GLP-1R/GIPR co-agonist, and refined its action time through a site-specific N-terminal PEGylation strategy. The pharmacological efficacy of these resulting long-acting co-agonists was interrogated both *in vitro* and *in vivo*. The results showed that peptide 1 possessed potent and imbalanced receptor-stimulating potency favoring GIP activity, but its hypoglycemic action was disrupted probably resulting from its short half-life. After PEGylation to improve the pharmacokinetics, the pharmacological effects were amplified compared to native peptide 1. Among the resulting derivatives, D-5K exhibited significant glycemic, HbA1c, body-weight, and food-intake control, outperforming GLP-1R mono-agonists. Based on its excellent pharmacological profiles, D-5K may hold the great therapeutic potential for diabetes and obesity treatment.

INTRODUCTION

Glucagon-like peptide-1 receptor agonists (GLP-1RAs) are widely used to treat type 2 diabetes mellitus (T2DM) and obesity due to their ability to improve glucose homeostasis and weight control.[1–4] However, the variable and incomplete activation of GLP-1R by different GLP-1RAs generally leads to subtherapeutic effects,[5] most often requiring combination with conventional oral agents.[6] Some patients even develop neutralizing antibodies against GLP-1RAs, which would result in the failure of glucose balance and weight control.[7] Unfortunately, higher dosages of GLP-1RAs are required to achieve greater efficacy and are accompanied by significant gastrointestinal adverse events, such as nausea and vomiting.[6,8] Therefore, there is a need to improve GLP-1RA therapy.

Glucose-dependent insulinotropic polypeptide (GIP) was the first incretin isolated and characterized in the 1970s as a potent insulin-releasing peptide in response to glucose intake.[9–11] Patients with diabetes seem to be insensitive to GIP therapy, leading to the peptide being mostly ignored.[12,13] However, when combined with GLP-1, GIP could potentiate not only glucose-lowering effects but also weight loss.[14–18] GIP may facilitate adipocyte differentiation and lipogenesis and additionally enhance the insulinotropic effects of GLP-1R activation on the pancreas.[15,17,18] GIP may also ameliorate the gastrointestinal effects of GLP-1R agonists, potentially allowing for higher therapeutic doses of GLP-1 analogs and decreasing adverse events.[15,17,19] These benefits of GIP make it a potential therapeutic option for treating T2DM and obesity. Thus, simultaneous activation of GLP-1R and GIPR has become a promising and effective treatment strategy for achieving improved therapeutic effects.

Dual GLP-1R/GIPR agonists have been introduced into the T2DM and obesity treatment.[15,20] Tirzepatide (LY3298176) is the first drug in this class approved by the FDA in May 2020 for T2DM treatment and later received the Fast Track designation for obesity treatment in October. It offered more than 20% weight loss in T2D treatment and even in obesity independent of diabetes.[21–27] Tirzepatide is an imbalanced GLP-1R/GIPR co-agonist that reduces GLP-1R activating activity and favors GIPR potency. As limited by the dose-related gastrointestinal adverse events, a low potency against GLP-1R may enhance the therapeutic index, while a high potency against GIPR could reduce GLP-1R-mediated gastrointestinal effects and exert synergism with GLP-1 activity to lower body weight and regulate glucose homeostasis.[15,16,28–32] Thus, an imbalanced GLP-1R/GIPR co-agonist with potent GIP pharmacology and low but acceptable GLP-1R potency may be the next development direction for controlling blood glucose and body weight in patients with T2DM and obesity.

[1]Department of Oncology, NHC Key Laboratory of Cancer Proteomics, State Local Joint Engineering Laboratory for Anticancer Drugs, National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China
[2]Department of Oncology, NHC Key Laboratory of Cancer Proteomics, State Local Joint Engineering Laboratory for Anticancer Drugs, National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China
[3]Research Center for Molecular Metabolomics, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China
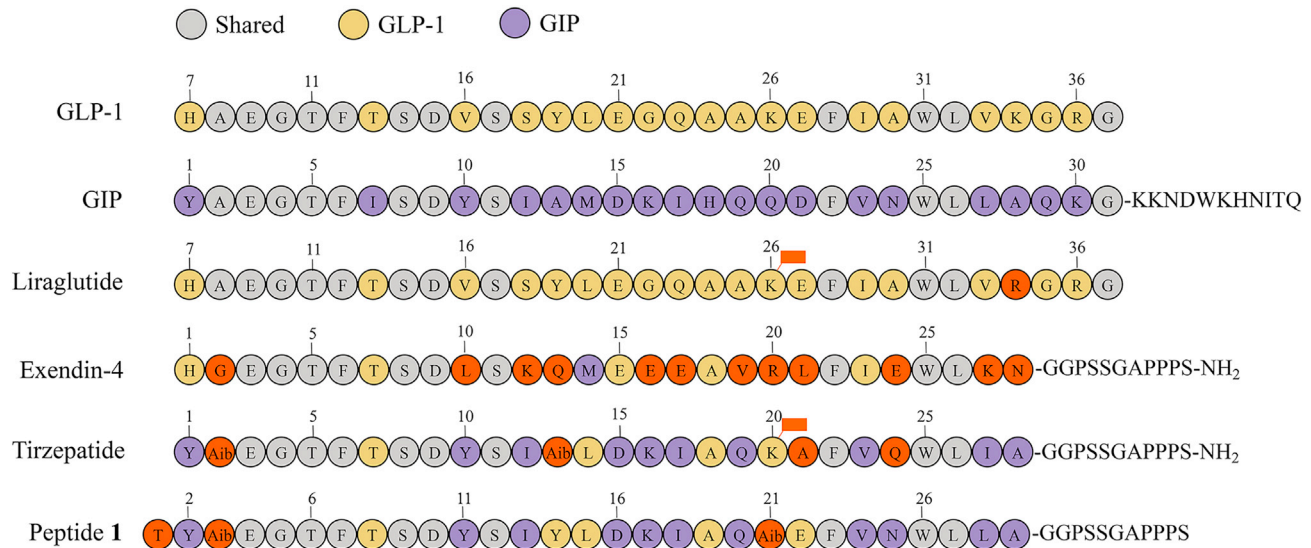[4]These authors contributed equally
[5]Lead contact
*Correspondence: yonghenc@163.com
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Figure 1. Amino acid sequence alignment of GLP-1, GIP, liraglutide, exendin-4, tirzepatide, and peptide 1
Amino acids common to GIP and GLP-1 are in gray, amino acids unique to GLP-1 and GIP are in yellow and purple, respectively, while amino acids not found in GIP and GLP-1 are in red. The red boxes linked to K in liraglutide and tirzepatide represent the conjugation of palmitic acid and carbon fatty diacid, respectively.

Herein, we prepared a potent and imbalanced GLP-1R/GIPR co-agonist peptide with potent GIP pharmacology and a low but acceptable GLP-1 response. Its half-life extension was achieved through a site-specific N-terminal PEGylation strategy to maximize glycemic efficacy and therapeutic index. Among the resulting long-acting PEGylated peptides, D-5K exhibited potent GLP-1R/GIPR co-activation potencies and amplified metabolic benefits, as indicated by sustained glycemic, HbA1c, body-weight, and food-intake control, outperforming GLP-1R mono-agonists. Our results demonstrated that D-5K may hold the therapeutic potential for treating T2DM and obesity, and may serve as a template for further polypeptide design and optimization to maximize therapeutic efficacy.

RESULTS

Generating potent and imbalanced GLP-1R and GIPR co-agonist peptides

We prepared the potent and imbalanced GLP-1R and GIPR co-agonist peptide **1** (Figure 1) (GLP-1R EC_{50} = 0.1004 nM; GIPR EC_{50} = 0.0125 nM), which has a potency comparable to that of GIP and is approximately 4-fold weaker than that of GLP-1, as confirmed by cAMP induction assays (Figure 2). Peptide **1** was then PEGylated to extend its action time *in vivo* for pharmacokinetic optimization, generating D-5K and D-10K (Figure S1). All the native, intermediary and resulting peptides are summarized in Table S1 and were characterized by RP-HPLC and MALDI-TOF MS (Figure S2). Although the N-terminal PEGylation may affect receptor binding and activation at the expense of improved pharmacokinetic profiles, D-5K efficacy remained acceptable with an EC_{50} of 2.189 nM for GLP-1R and 0.2271 nM for GIPR according to the cAMP induction assay (Figure 2), indicative of the potent insulinotropic potential of D-5K. As confirmed in Figure S3, D-5K produced a significant enhancement of insulin secretion in the presence of stimulating (16.8 mM) glucose in INS-1 cells. In contrast, the larger MW of PEG cannot be tolerated by D-10K, with a great reduced potency at the two receptors.

In vitro to *in vivo* translation of the GLP-1R and GIPR co-agonistic effects

Furthermore, we validated the *in vitro* GLP-1R/GIPR co-agonism potencies of the resulting peptides translated to *in vivo* activities by comparing the acute and long-acting hypoglycemic effects via oral glucose tolerance tests (OGTT) and hypoglycemic duration assays, respectively. The long-term hypoglycemic effects of peptide **1** and its PEGylated derivatives were evaluated in non-fasted db/db mice, with PBS injected as a control group. As shown in Figure 3A, the control group maintained a hyperglycemic state throughout the experiment, and the peptide **1** group exhibited marginal glucose control ability, as the blood glucose level rapidly attenuated in the first half an hour and returned to the baseline level within 2 h following injection. Compared with peptide **1** group, D-5K and D-10K injections exerted pronounced hypoglycemic effects, among which D-5K achieved a lower blood glucose level and a much later increase to the baseline level after more than 36 h, consistent with the *in vitro* cAMP induction assay and GSIS assay results. The calculated glucose AUC_{0-36h} values also revealed that D-5K had greater gluco-regulatory bioactivity than peptide **1** and D-10K (Figure 3B, p < 0.005 for D-5K vs. peptide **1**/D-10K). These results demonstrated that D-5K had preferred long-term hypoglycemic potency over other peptides and suggested that the improvement in glucose-lowering efficacy can be achieved by PEGylation but that a larger PEG molecule might affect the hypoglycemic effect.

The acute hypoglycemic effects of peptide **1**, D-5K and D-10K were also evaluated by OGTT in fasted db/db mice. As shown in Figure 3C, the blood glucose level of the control group (injected with PBS) rapidly increased to more than 20 mmol/L within 1 h and slowly returned to the
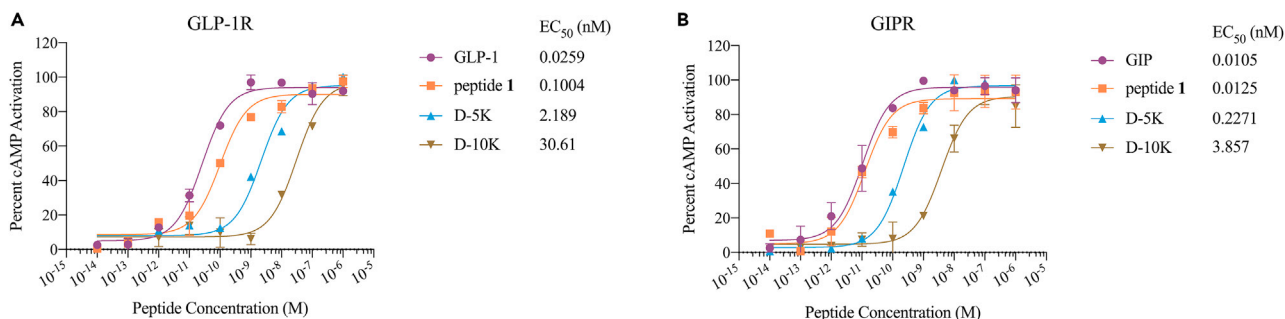
Figure 2. GLP-1R and GIPR activation potency of peptide 1 and its PEGylated derivatives
The potency and efficacy of GIP, GLP-1, peptide 1 and its PEGylated derivatives on the activation of GLP-1R (A) or GIPR (B) in HEK293 cells. The EC_{50} values represent the effective peptide concentrations (M) that stimulate half-maximal activation at the GLP-1R and GIPR. All the data are expressed as the mean $\pm$ SD, (n = 2).

baseline over 3 h. In contrast, peptide **1**, D-5K, and D-10K all similarly improved glucose tolerance with the maximal blood glucose concentrations much lower than that of PBS group, and maintained relatively hypoglycemic state as indicated by the significant reductions in the AUC $_{glucose\ 0-210\ min}$ compared with the control group (Figure 3D, $p < 0.01$ for peptide **1** vs. PBS, $p < 0.005$ for D-5K vs. PBS, and $p < 0.01$ for D-10K vs. PBS). Consistent with the hypoglycemic duration assay results, D-5K exhibited superior acute glucose stabilizing ability to peptide **1** and D-10K (Figure 3D, $p < 0.005$ for D-5K vs. peptide **1** and $p < 0.05$ for D-5K vs. D-10K), highlighting the importance of PEGylation for peptide stability *in vivo* and the impact of PEG molecules on GLP-1 and GIP receptor activation. Collectively, these results demonstrated that D-5K exhibited the best *in vivo* activity among the resulting peptides, and was therefore selected for further pharmacological investigations.
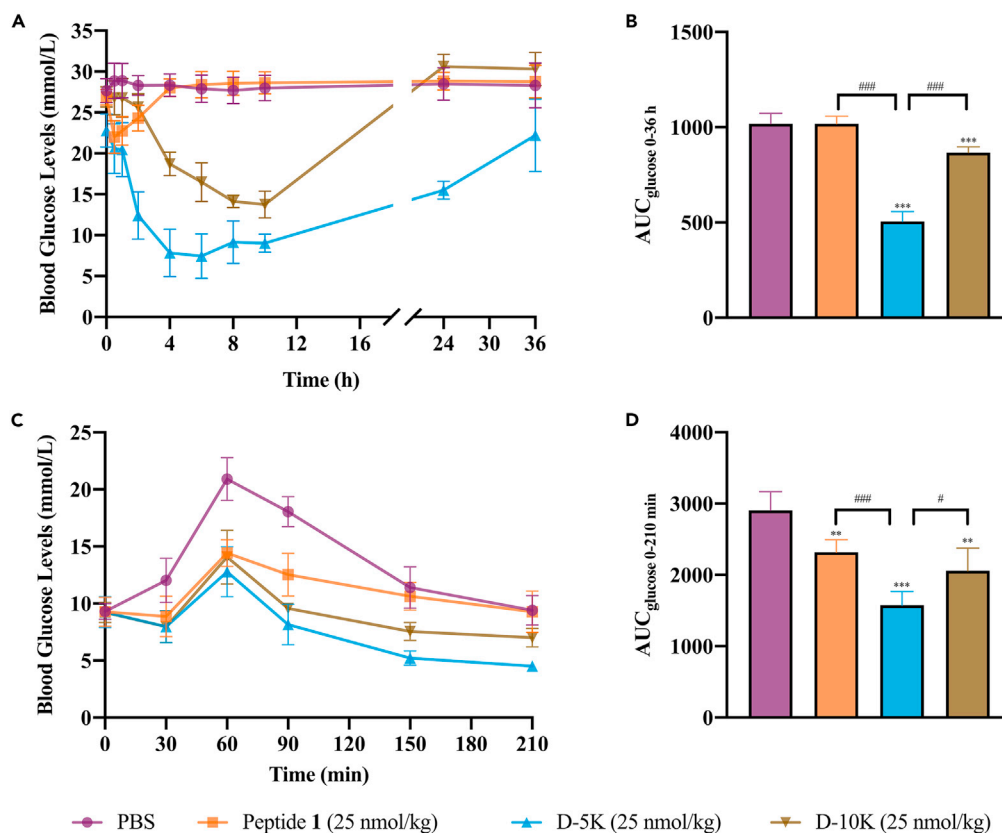
Figure 3. D-5K exhibits superior long-term and acute glucose-lowering effects compared with native peptide 1 and D-10K in db/db mice
Time-course blood glucose levels of db/db mice in response to treatment with PBS, peptide **1**, D-5K, or D-10K in hypoglycemic duration assay (A) and OGTT (C). The area under the curve (AUC) values of the time-course blood glucose levels (versus the 0% baseline) were calculated for the course from 0 to 36 h for the hypoglycemic duration assay (B), and from 0 to 210 min for the OGTT (D). All the data are expressed as the mean $\pm$ SD, (n = 5). P-values were determined using one-way ANOVA with Tukey's multiple comparisons test. **$p < 0.01$, ***$p < 0.005$, compared with the PBS group. #$p < 0.05$, ###$p < 0.005$, compared with the D-5K group.
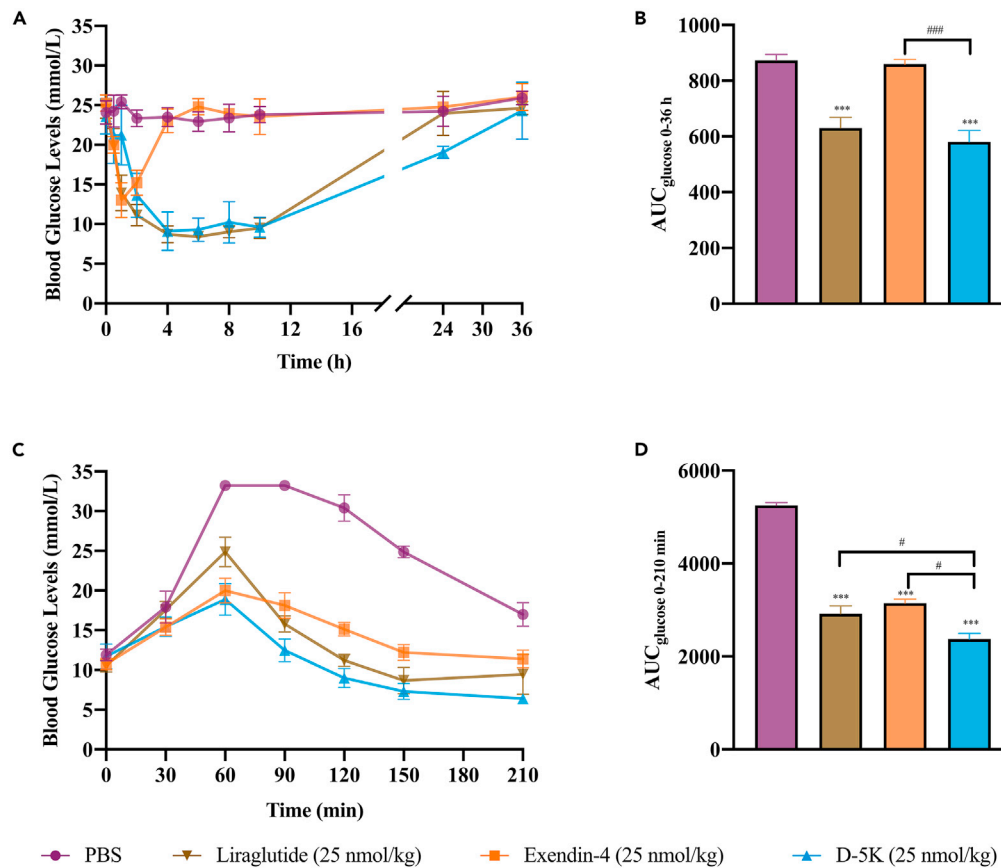
Figure 4. D-5K is more effective at glucose control compared with GLP-1R mono-agonists

Time-course blood glucose levels of db/db mice in response to treatment with PBS, liraglutide, exendin-4, or D-5K in hypoglycemic duration assay (A) and OGTT (C). The AUC values of the time-course blood glucose levels (versus the 0% baseline) were calculated for the course from 0 to 36 h for the hypoglycemic duration assay (B), and from 0 to 210 min for the OGTT (D). All the data are expressed as the mean ± SD, (n = 5). P-values were determined using one-way ANOVA with Tukey's multiple comparisons test. ***$p < 0.005$, compared with the PBS group. ###$p < 0.005$, compared with the D-5K group.

D-5K is effective at glucose control as supplemented with GIP pharmacology

To further confirm the enhanced hypoglycemic performance of the PEGylated GLP-1R/GIPR co-agonist, we compared D-5K with equimolar doses of the GLP-1R mono-agonists, exendin-4 and liraglutide, through OGTT and hypoglycemic duration assays in db/db mice. In the hypoglycemic duration assay, both D-5K and liraglutide exhibited excellent long-acting glucose-lowering effects to a similar extent, whereas exendin-4 failed to recapitulate glucose control with AUC $_{glucose\ 0-36\ h}$ value comparable to that of the PBS group (Figures 4A and 4B). In the context of acute glycemic control, the improvements in glucose tolerance were observed for all the tested peptides, among which D-5K provided greater hypoglycemic efficacy than exendin-4 and liraglutide with a significant decrease in the AUC $_{glucose\ 0-210\ min}$ (Figures 4C and 4D, $p < 0.05$ for D-5K vs. exendin-4/liraglutide).

To validate the hypoglycemic benefits of GIP pharmacology presented in D-5K, additional OGTT experiments were also conducted in db/db mice that were co-administered the GLP-1R antagonist Jant-4 to generate a GLP-1R loss-of-function model, mimicking the GLP-1R knockout mice.[16,33] Jant-4 is a human-based GLP-1R antagonist designed with an enhanced human GLP-1 sequence and is slightly more selective for GLP-1R antagonism than exendin-4 (9–39) that contains a nonhuman amino acid sequence.[33] Here, GLP-1R blockade abolished glucose tolerance induced by liraglutide as evidenced by the considerable difference in blood glucose levels and AUC $_{glucose\ 0-210\ min}$ values between the liraglutide group and the liraglutide + Jant-4 group; however, GLP-1R blockade had a negligible impact on the efficacy of D-5K, as indicated by the comparable blood glucose levels and AUC $_{glucose\ 0-210\ min}$ values in the presence or absence of Jant-4 (Figure 5). Taken together, these results revealed that D-5K is more effective at controlling glucose levels than GLP-1R mono-agonists due to the supplementation of GIP pharmacology.

Chronic treatment with D-5K provides amplified weight control and metabolic benefits

Encouraged by the remarkable hypoglycemic performance of the single-dose D-5K, we further explored the efficacy of GLP-1R/GIPR co-agonism by chronic D-5K treatment in db/db mice as compared with exendin-4 and liraglutide in terms of HbA1c, body weight and food
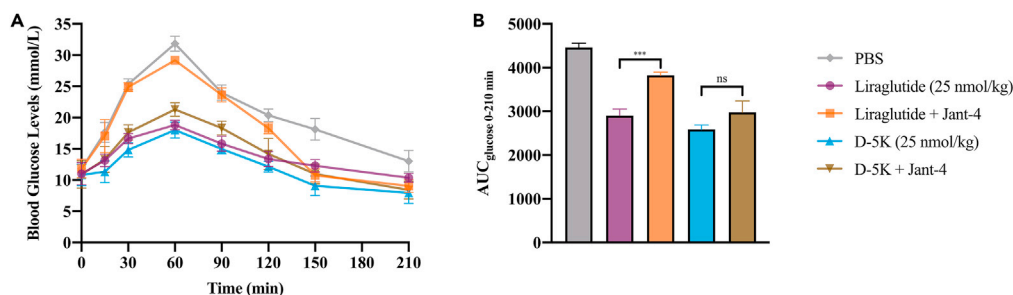
Figure 5. Validation of GIP pharmacology in hypoglycemic effects for D-5K

(A) Time-course blood glucose levels of db/db mice after acute treatment with D-5K or liraglutide with or without the GLP-1R antagonist Jant-4.

(B) The AUC values of the time-course blood glucose levels (versus the 0% baseline) of each group were calculated for the course from 0 to 210 min. All the data are expressed as the mean ± SD, (n = 5). P-values were determined using the Student's *t* test to compare the AUC values between two groups. ***p < 0.005. n.s., not significant.

consumption changes. After consecutive 4-week administration, the %HbA1c value of the PBS-treated group was dramatically increased by ∼45%. In contrast, the chronic treatments of exendin-4, liraglutide and D-5K all obviously improved the %HbA1c-lowering effects with HbA1c values attenuated from baseline by approximately 15%, 30% and 30%, respectively (Figure 6A). The body weights of the exendin-4- and liraglutide-treated groups increased relatively slowly by ∼6.9% and ∼7.4%, respectively (Figure 6B, p < 0.01 for PBS vs. exendin-4/liraglutide), compared with the PBS-treated group with an ∼14% increment. Notably, the chronic administration of D-5K significantly improved body weight control effect (p < 0.05 for D-5K vs. exendin-4/liraglutide) as a minor increment in body weight (∼2.1%) was observed (Figure 6B). Body weight changes were correlated with robust decreases in food intake. It appears that D-5K produced a greater and more prolonged reduction in food consumption than exendin-4 or liraglutide did (Figure 6C, p < 0.05 for D-5K vs. exendin-4/liraglutide).

Apart from HbA1c, body weight and food intake changes, we also assessed improvements in glucose metabolism via three rounds of OGTT on day 1, day 15 and day 29. On day 1, all the AUC $_{glucose\ 0-210\ min}$ values of the tested peptides were reduced by ∼25% relative to the PBS-treated group (Figures 6D and 6E). As shown by the results of the OGTT performed on day 15, D-5K demonstrated glucose-lowering effects with profiles similar to that of liraglutide (both AUC $_{glucose\ 0-210\ min}$ values were reduced by ∼40%; p > 0.05 for D-5K vs. liraglutide) but superior to that of exendin-4 (AUC $_{glucose\ 0-210\ min}$ value was reduced by ∼18%; p < 0.01 for D-5K vs. exendin-4) (Figures 6F and 6G). Furthermore, the results of the OGTT performed on day 29 revealed that D-5K exhibited greater and more sustained glucose tolerance than exendin-4 and liraglutide, of which the AUC $_{glucose\ 0-210\ min}$ value was reduced by ∼50% (p < 0.01 for D-5K vs. exendin-4/liraglutide) (Figures 6H and 6I). Overall, these results indicated that chronic administration of D-5K provided sustained metabolic efficacy comparable to or even superior to that of GLP-1R mono-agonists, thus offering a potential clinical benefit for the treatment of metabolic diseases such as T2DM and obesity.

DISCUSSION

Although there are many limiting factors associated with GLP-1 mimetics, dose-dependent gastrointestinal side effects, which are the most commonly observed adverse events in the clinic and usually lead to high discontinuation rates, play a dominant role.[6,8] In a phase III study (SURPASS-1), a high incidence (28–41%) of gastrointestinal disorders associated with tirzepatide treatment across all doses led to common study drug discontinuation (3–7%).[23] More recently, Pfizer announced topline phase 2b results of oral GLP-1R Agonist, Danuglipron, in adults with obesity (NCT04707313) on December 01, 2023, which was dropped due to high rates of side effects (73% nausea, 47% vomiting, and 25% diarrhea) and consequently high discontinuation rates (greater than 50%).[34] In addition, the effect on the weight control is well below what is needed for metabolic improvement.[5] Therefore, supplementation with GIP agonism may extensively broaden the therapeutic index of GLP-1RAs. Hyperglycemia seems to lead to a downregulation of GIPR, causing a decreased GIP response, whereas GLP-1 pharmacology could induce a reduction in blood glucose levels, which fosters GIP function to lower body weight and reduce GLP-1R-mediated gastrointestinal effects.[15,18,19,35,36] Therefore, imbalanced GLP-1R/GIPR co-agonism may maximize the GIP response to synergistically broaden the therapeutic range and amplify the metabolic benefits.

To achieve this goal, we prepared the potent and imbalanced GLP-1R/GIPR co-agonist peptide 1 (Figure 1), which was primarily derived from GLP-1 and GIP residues as well as some residues from exendin-4, to enhance receptor activation and structural stability. Peptide 1 has great GIPR stimulation potency and slightly lower but acceptable potency upon GLP-1R activation, as evidenced by accumulative cAMP production (Figure 2), underlying the potential to increase insulin secretion and regulate blood glucose balance. Consistent with the results of the cAMP induction assay, peptide 1 potently stimulated insulin secretion in a glucose-dependent manner (Figure S3). These in vitro evaluations indicated the potent hypoglycemic efficacy of D-5K in vivo. Unfortunately, the in vivo gluco-regulatory function of peptide 1 was unfavorable (Figure 3), presumably due to its short circulation duration, despite the Aib substitution being introduced to protect against proteolytic degradation, which indicated a requirement of half-life extension.

Given that the traditional PEGylation strategy has some concerns of cysteine-involved self-dimerization and non-selective modification, we have established a novel site-specific PEGylation strategy with high selectivity and efficiency to improve the pharmacokinetic profiles and
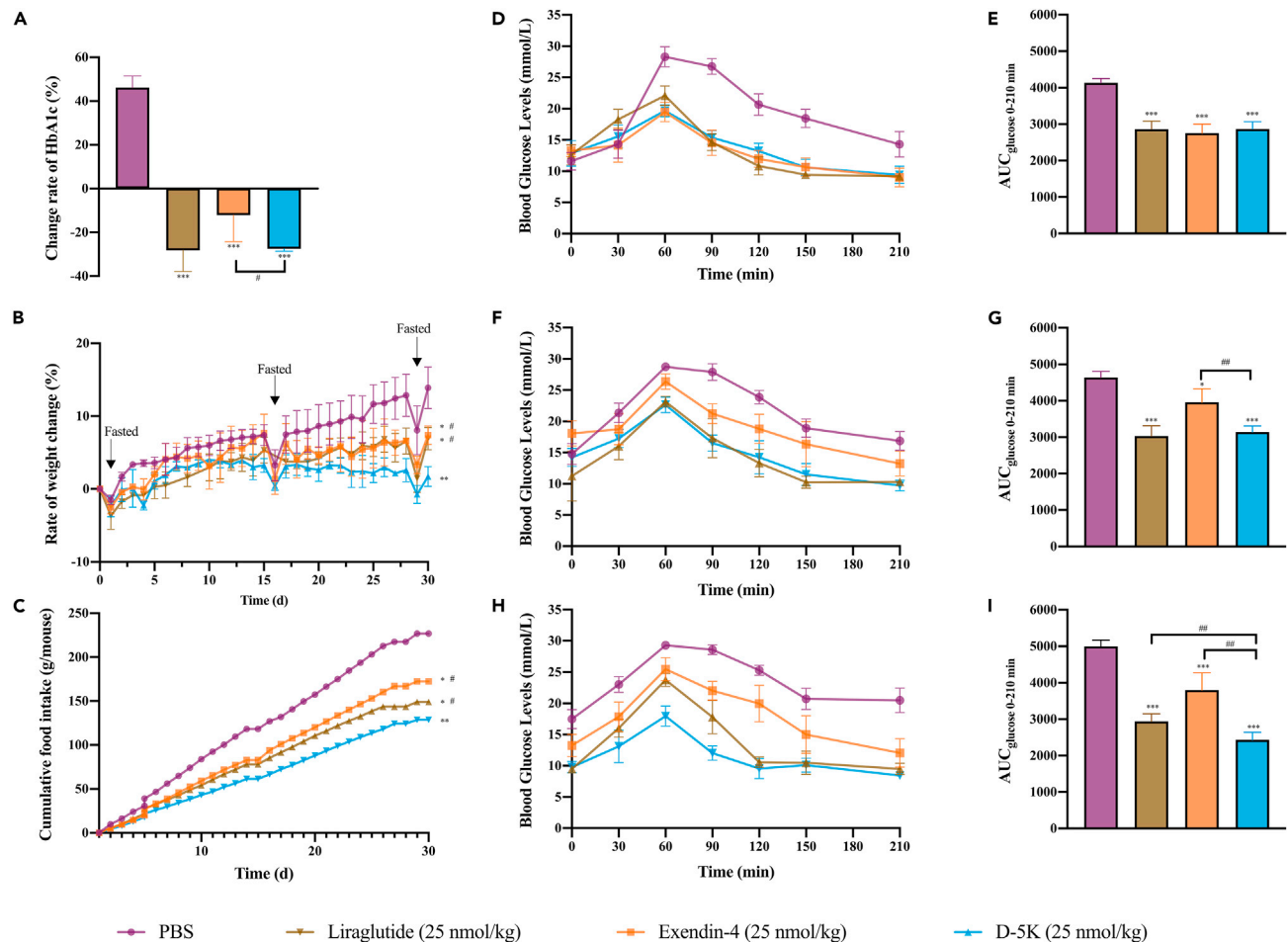
Figure 6. Chronic treatment with D-5K exhibits amplified and sustained metabolic benefits compared with GLP-1R mono-agonists

Effects on (A) HbA1c, (B) body weight, and (C) cumulative food intake after 4-week treatment with PBS, liraglutide, exendin-4, or D-5K. Time-course blood glucose levels of db/db mice in OGTT experiments on day 1 (D), day 15 (F), and day 29 (H). The AUC values were calculated based on the time-course blood glucose levels (versus the 0% baseline) in db/db mice during the OGTT on day 1 (E), day 15 (G), and day 29 (I). All the data are expressed as mean ± SD, (n = 5). P-values were determined using one-way ANOVA with Tukey's multiple comparisons test. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$, compared with the PBS group. $\#p < 0.05$, $\#\#p < 0.01$, compared with the D-5K group.

hypoglycemic effects for biotherapeutics.[37] To extend its application potential and prolong peptide **1**'s action time, we employed this strategy with the engagement of Thr[1] as a PEG anchor to synthesize two homogeneous derivatives following two simple chemical steps (Figure S1). The additional Thr[1] was oxidized in the first chemical step to form an aldehyde group (-CHO), which is an ideal and specific binding site for mPEG-HZ in the second chemical step, contributing to the high yield and purity of these two derivatives (Figure S2).

The pharmacological performances of these two derivatives were assessed both *in vitro* and *in vivo*. Owing to the steric hindrance of receptor recognition by the polymer chains, both PEGylated derivatives exhibited impaired GLP-1R and GIPR stimulating abilities compared to native peptide **1** (Figure 2). A larger MW of PEG conjugated to D-10K may provide a longer action time but had a greater impact on the GLP-1R and GIPR stimulation. Similarly, the ability of D-10K to stimulate insulin secretion was inferior to that of D-5K (Figure S3). There was a balance between receptor activation potency and pharmacokinetic profile during PEGylation. Although their receptor activation abilities were affected, the total efficacy could be counterbalanced by the extended circulation duration and enhanced stability from PEGylation,[37] as evidenced by the amplified glucose-lowering effects of the PEGylated derivatives *in vivo* (Figure 3), highlighting the important role of PEGylation in pharmacokinetic and pharmacological optimization. Among these derivatives, D-5K achieved preferred efficacy over D-10K owing to its relative advancement in stimulating GLP-1R and GIPR downstream cAMP signaling and increasing insulin secretion. Moreover, D-5K even outperformed exendin-4 and liraglutide in the control of blood glucose (Figure 4), via the synergism of GLP-1 and GIP pharmacology.

Recent works have shown that GIPR is widely expressed within the central nervous system (CNS). Its activation could improve insulin sensitivity to regulate glucose levels and reduce caloric intake to drive weight loss, especially when combined with GLP-1 agonism.[15,18,28–32] In our work, the pharmacological effects of GIPR agonism were confirmed in a GLP-1R loss-of-function model by pretreatment with Jant-4, a potent and selective GLP-1R antagonist.[16,33] In this context, liraglutide, a GLP-1R mono-agonist, exhibited a complete loss of acute glucose-lowering

efficacy (Figure 5). Conversely, a slight but not significant decrease in the hypoglycemic efficacy of D-5K was observed in the presence of Jant-4, which may be attributed to the insulinotropic properties of GIP pharmacology in D-5K. Hence, the addition of GIPR agonism could enhance the inherent efficacy of D-5K in lowering glucose. These results were consistent with the previous findings of the hypoglycemic function of GIP pharmacology,[15,28–32,38,39] proving its potentially therapeutic supplementation to GLP-1 pharmacology for therapeutic index extension.

The synergistic metabolic benefits from the imbalanced GLP-1R/GIPR co-agonist were further validated in long-term studies. Chronic treatment of db/db mice with D-5K resulted in a pronounced decrement in HbA1c levels, with an ∼30% decrease from the baseline (Figure 6A), indicating the excellent insulinotropic effect of D-5K, which was aligned with the results of the cAMP induction assay and GSIS assay. Improved managements of body weight and food intake were also observed in D-5K-treated mice, which were significantly greater than those of exendin-4 and liraglutide (Figures 6B and 6C), possibly derived from the supplementation of GIP activity to D-5K. Several lines of evidence support that GIP has the potential to inhibit food intake and increase energy expenditure to drive weight control.[15,32,40,41] In addition, GIP might enhance the appetite-suppressing function of GLP-1, thereby promoting food-intake and body-weight control.[15,41] Thus, these improved metabolic parameters of D-5K may attribute to synergistic GLP-1R and GIPR activation, which underlies the preferred gluco-regulatory behavior of D-5K in multiple OGTTs. Initially, D-5K behaved similarly to exendin-4 and liraglutide, reflecting the dominance of GLP-1 pharmacology in the early stage of treatment. However, the GLP-1 pharmacology of exendin-4 and liraglutide began to plateau after several days of treatment, probably caused by some degree of desensitization from receptor internalization. In contrast, the synergistic effects of GLP-1 and GIP on D-5K continued to improve glucose tolerance (Figures 6D–6I), where the GLP-1 response may be enhanced by GIP activation and meanwhile foster GIP activity.[15,35] Collectively, these results demonstrated the amplified pharmacological benefits of D-5K, which may hold potential against T2DM and obesity by improving glucose tolerance and maximizing weight control.

In this study, we prepared the potent and imbalanced GLP-1R/GIPR co-agonist peptide **1**, which favors GIP pharmacology to improve glucose homeostasis and weight control. The action time of peptide **1** was refined to maximize the therapeutic index and efficacy through a highly simple, efficient and site-specific N-terminal PEGylation strategy. The long-acting PEGylated co-agonist, D-5K, exhibited great glucose-lowering and metabolic improvements, more effectively than did selective GLP-1R agonists, which may hold the therapeutic potential for the treatment of T2DM and obesity. The co-agonist peptides provided in our study may serve as templates for our further polypeptide design and optimization to maximize therapeutic efficacy. Furthermore, our results highlighted the therapeutic feasibility of maximizing GIP pharmacology with an acceptable GLP-1 response to meet the increasing need for glycemic and body-weight control and demonstrated the high versatility of this site-specific N-terminal PEGylation approach for achieving improved pharmacokinetic and pharmacological properties.

Limitations of the study

An imbalance toward GIPR combined with bias at the GLP-1R to favor cAMP generation over β-arrestin recruitment, together may account for the effectiveness of tirzepatide.[42] β-arrestins play a canonical role in GPCR desensitization associated with receptor internalization.[43,44] There is accumulating evidence that agonist-induced cAMP signaling is prolonged and insulin secretion is improved in β-arrestin-knockout clonal β cell lines/mice, suggesting that β-arrestin deficiency favors sustained GLP-1R action.[45–47] Therefore, GLP-1R agonists biased away from β-arrestin recruitment are pursued by many researchers to sustain insulin secretion and improve anti-hyperglycemic efficacy. In this study, the effectiveness of D-5K may result from an imbalance in potency favoring GIP over GLP-1, allowing dosing schemes that maximize GIP effects while simultaneously achieving an efficacious and tolerable GLP-1 response to reduce dose-related gastrointestinal disorders. The bias at GLP-1R in favor of G protein-dependent cAMP signaling may be another possible mechanism for D-5K efficiency, but further validation is needed, which is a limitation of our study.

STAR★METHODS

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AUTHOR CONTRIBUTIONS

Q.L. performed *in vitro* studies, and X.X. performed *in vivo* studies. Z.Z. helped in the data analysis and supervised the experiment processes. Q.L. and X.X. wrote the manuscript. Y.C. conceived the study and supervised the paper writing and reviewing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Liraglutide	GL Biochem	N/A
Exendin-4	GL Biochem	N/A
Peptide 1	GL Biochem	N/A
Tirzepatide	Medchemexpress	CAS #72023788-19-2
GLP-1	GL Biochem	71122
GIP	GL Biochem	55416
Jant-4	Shanghai Apeptide Co, Ltd	https://doi.org/10.1021/cb1002015
Linear 5 kDa methoxy PEG Hydrazide	Jenkem Technology	N/A
Linear 10 kDa methoxy PEG Hydrazide	Jenkem Technology	N/A
Sodium periodate	Sigma−Aldrich	311448-5G
Ethylene glycol	Sigma−Aldrich	324558-2L
Sodium cyanoborohydride	Sigma−Aldrich	156159-10G
Acetonitrile	Merck	CAS #75-05-8
Formic acid	Merck	CAS #64-18-6
Trifluoroacetic acid	Macklin Biochemical	CAS #76-05-1
Critical commercial assays		
Superdex 75 10/30 GL column	GE Healthcare	29148721
Xbridge peptide BEH C18 column	Waters	186003613
Sephadex G-25 desalting column	GE Healthcare	17140801
cAMP dynamic 2 kit	Cisbio	62a.m.4PEB
Enzyme-linked Immunosorbent Assay Kit For Glycated Hemoglobin A1c (HbA1c)	Cloud-Clone	CEA190Mu
Wide area rat insulin test kit	EZassay	RT300
Deposited data		
Raw data	This paper	
Experimental models: Cell lines		
Human embryonic kidney 293 cells	Pricella	CL-0005
INS-1 cells	iCell Bioscience	iCell-r036
Experimental models: Organisms/strains		
Male mice (C57BL/6 db/db mice)	Slac Laboratory Animal Co., Ltd	430727221100607551
Software and algorithms		
GraphPad Prism 8.	GraphPad	https://www.graphpad.com/
Other		
Enzyme Labeling Instrument	BioTek	ELX808 I ULALXH
High-Performance Liquid Chromatography	RIGOL	L-3400
EnVision Multimode Plate Reader	PerkinElmer	2105–0010
MALDI-TOF/TOF	AB SCIEX	5800 MALDI TOF/TOFTM

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding contact, Yong-heng Chen (yonghenc@163.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animal materials

Male C57BL/6 db/db mice (8–10 weeks old, 25 ± 2 g) were purchased from Slac Laboratory Animal Co., Ltd (Shanghai, China). The animals were kept in a controlled environment (22 ± 2°C, 12 h light/12 h dark cycle) at the Experimental Animal Research Center of Central South University, with free access to food and water. All animal care and experimental procedures were approved by the CSUN Laboratory Animal Management Committee [No. 2020sydw1046].

Cell culture

human embryonic kidney 293 (HEK293) cells were purchased from the Pricella Biotchnology Co., Ltd. (Wuhan, China) and incubated in Dulbecco's modified Eagle's medium (DMEM, Corning), which is added with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin. INS-1 cells were purchased from the iCell Bioscience Inc (Shanghai, China) and incubated in RPMI 1640 medium which is added with 10% FBS, 1% penicillin, 1% streptomycin, 1% sodiumpyruvate, and 0.05 mM beta-mercaptoethanol. Cells were placed in an incubator with a humidified atmosphere containing 5% CO_2 at 37°C.

METHOD DETAILS

Peptide preparation

We constructed a dual receptor agonist peptide **1** by intermixing GLP-1 and GIP sequences and introducing C-terminal-extended residues of exendin-4. The residues at positions 2 and 21 were replaced by 2-aminoisobutyric acid (Aib) to improve the activity at the two receptors and prevent *in vivo* enzymatic degradation without interfering with GLP-1R or GIPR bindings.[17,37,48,49] An additional threonine (Thr1) was added at the N-terminus, serving as an anchor for subsequent modification with polyethylene glycol (PEG).[37] Peptide **1** was then PEGylated through a site-specific N-terminal PEGylation approach (Figure S1).[37] Briefly, peptide **1** was oxidized by sodium periodate ($NaIO_4$) in 20 mM sodium phosphate buffer (pH 7.4) at 4°C for 45 min, forming an oxidized peptide, named peptide **2,** with an aldehyde group (-CHO) that is a sole binding site for mPEG-HZ. Peptide **2** was purified by a Sephadex G-25 desalting column equilibrated with 20 mM acetic acid (pH 4.0), and then conjugated with different molecular weight (MW) of mPEG-HZ to produce peptide **3** and peptide **4**, which were named D-5K and D-10K, respectively. D-5K and D-10K were purified by a Superdex 75 10/30 GL column equilibrated with 20 mM sodium phosphate buffer (pH 7.4). The collected fractions were dialyzed against phosphate-buffered saline (PBS, pH 7.4) at 4°C overnight using a 3.5 kDa MW cutoff membrane, and analyzed by RP-HPLC for purity and MALDI-TOF MS for identity.[37] Purified peptides were lyophilized and stored at −80°C.

In vitro GLP-1R and GIPR-mediated cAMP induction assay

cAMP is an important messenger in the GLP-1R/GIPR downstream signaling pathway, reflecting the GLP-1R/GIPR activation.[50] The ability of each peptide was tested *in vitro* by detecting cAMP production in HEK293 cells that were stably overexpressed human GLP-1R or GIPR as previously described with minor modifications.[37,51–53] Briefly, cells were seeded at 5000 cells/well in a white solid 384-well plate and allowed to adhere overnight. On the day of the experiment, the culture medium was replaced with the stimulation buffer supplemented with 0.5 mM 3-isobutyl-1-methylxanthineto (IBMX) and various concentrations of peptides. After 40-min incubation at 37°C, cAMP responses were quantified following the manufacturer's protocol of cAMP dynamic 2 kit from Cisbio. The data were analyzed with GraphPad Prism 8.

Glucose-stimulated insulin secretion (GSIS) assay

The insulinotropic effect of these peptides was assessed in INS-1 cells. Cells were seeded in 12-well plates and cultured for 48 h at 80–90% confluence on the experimental day. After 2-h pre-incubation in KRBH buffer (129 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM HEPES, 5 mM $NaHCO_3$, and 0.1% BSA, pH 7.4) containing 2.8 mM glucose, the cells were treated with each peptide (25 nM) at basal (2.8 mM) and stimulant (16.8 mM) concentrations of glucose for 1 h. Insulin levels in the media were detected via ELISA (RT300, EZassay).

Hypoglycemic duration assay

To examine the long-term hypoglycemic efficacy of peptide **1** and its PEGylated derivatives, C57BL/6 db/db mice were randomly divided into 4 groups (n = 5) with free access to food and drinking water. On the day of the experiment, the non-fasted mice were subcutaneously

administered PBS, peptide **1**, D-5K, or D-10K at a dose of 25 nmol/kg. Blood glucose levels were measured via an Accu-Chek Performa single-touch glucose monitoring device (Roche, Germany) at 0, 0.5, 1, 2, 4, 6, 8, 10, 24 and 36 h after subcutaneous administration. Long-lasting hypoglycemic comparisons of D-5K with exendin-4 and liraglutide were conducted as described above.

Oral glucose tolerance test (OGTT)

To investigate the acute glucose-lowering effects of peptide **1** and its PEGylated derivatives, an OGTT was performed in C57BL/6 db/db mice (n = 5). The mice were fasted overnight (12–16 h). On the day of the experiment, these mice were administered an oral glucose load (1 g/kg) and then subcutaneously injected with PBS, peptides **1**, D-5K, or D-10K at a dose of 25 nmol/kg after half an hour. Blood glucose levels were quantified at time points of 0, 30, 60, 90, 150, and 210 min after the subcutaneous injection. The acute hypoglycemic potency of D-5K was compared with that of exendin-4 and liraglutide as described above. In some studies, mice were dosed subcutaneously with Jant-4 (0.5 nmol/kg) 15 min prior to glucose challenge.[33]

Chronic efficacy study

The long-term efficacy of chronic administration of D-5K was further evaluated. The diabetic db/db mice were subcutaneously administered with PBS, exendin-4, liraglutide or D-5K at a dose of 25 nmol/kg once daily for consecutive 4 weeks. The HbA1c values were measured by an HbA1c kit before and at the end of the treatment, while body weight and food consumption were recorded every day. These mice were subjected to three rounds of OGTT at weeks 0, 1 and 4 to assess the improvement in glucose control.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical method used for each dataset is indicated for each figure in the figure legend. All statistical comparisons were performed by unpaired Student's t test or one-way ANOVA with Tukey's multiple comparisons test using GraphPad Prism 8.0. Statistical significance is shown as: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.005$. All data were expressed as the mean $\pm$ standard deviation (SD).