PAPER

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Discovery of lixisenatide analogues as long-acting hypoglycemic agents using novel peptide half-life extension technology based on mycophenolic acid†

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Noncovalent binding of peptides to human serum albumin protects against renal clearance and enzymatic degradation. Herein, we investigated the effect of mycophenolic acid (MPA) albumin binders for improving the stability of peptides. For proof-of-principle, the short acting glucagon-like peptide-1 (GLP-1) receptor agonist lixisenatide was selected and functionalized with different MPA albumin binders. *In vitro*, all lixisenatide analogues showed well preserved GLP-1 receptor activation potency. High performance affinity chromatography (HPAC) and ultrafiltration analyses indicated that DiMPA was able to confer high albumin affinity to lixisenatide and revealed that affinity is increased for DiMPA modified lixisenatide analogues containing OEG spacers. In *db/db* mice, the selected peptide **2c** showed comparable efficacies to lixisenatide with respect to glucose-lowering and insulinotropic activities. Furthermore, the duration of action of glucose homeostasis of **2c** was comparable to semaglutide in *db/db* mice. Importantly, DiMPA albumin binder did not bring significant toxicity of lixisenatide, as reflected by the comparable toxicity indexes in **2c** and semaglutide groups after 2 weeks dosing in normal Kunming mice. Short-term study (21 days) conducted on *db/db* mice showed the better therapeutic efficacies of **2c** than semaglutide on pancreas islets protection. Importantly, in chronic studies (84 days) on *db/db* mice, **2c** exhibited a sustained improvement in glycaemic control, to a greater extent than that of semaglutide. Thus, we propose DiMPA modification as a novel and general method for development of long-acting GLP-1 receptor agonists for type 2 diabetes treatments, and **2c** as a promising antidiabetic candidate.

1. Introduction

Over 400 million people are living with type 2 diabetes mellitus (T2DM) which account for around 90% of diabetes cases all over the world.[1-3] Worryingly, over the past 30 years, the global prevalence of type 2 diabetes is estimated to be doubled.[4-6] T2DM is a complex and costly disease which can cause cardiovascular disorder, blindness, kidney failure, lower limb amputation, and some other long-term complications to decline substantially the quality of life with disability.[7-10] The eventual need for multiple medications due to T2DM and its complications in most patients promotes the development of new drugs. Glucagon-like peptide-1 (GLP-1) is a promising pharmacological target in T2DM due to its multiple biological activities including activation of GLP-1 receptor, stimulating the insulin secretion from pancreatic cells, increasing the appetite suppression and weight loss.[11,12] However, the half-life is short due to the quick degradation the two N-terminal amino acids of native GLP-1 by dipeptidyl peptidase IV (DPP-IV).[13,14] Researchers are committed themselves to utilize numerous approaches to prolong the half-life of GLP-1.[15-18]

Facilitating the physical interaction of the target peptides with human serum albumin (HSA) has been proved an effective way to prolong half-life.[19,20] Fatty acids are albumin bound

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molecules possessed high albumin affinities.[21] Therefore, peptide lipidation can be exploited to improve the pharmacokinetic profiles of peptide pharmaceuticals by promoting albumin interactions. This approach has been successfully used in the discovery of liraglutide and semaglutide with reduced proteolytic degradation and renal clearance due to the affinity to HSA.[22,23] However, lipidation can bring to a drastic reduction in solubility and is not generally applicable for peptides. Thus, alternative strategies for mediating serum albumin interactions with enhanced solubility, HSA affinity or additional therapeutic property continues to be attractive.[24,25] Herein, we explored an alternative methodology to conjugate of novel small molecule albumin binders to peptides.

Some anionic aromatic small molecules have high albumin binding affinities and can be used as an alternative to lipidation. Mycophenolic acid (MPA) has high albumin binding affinity and could extensively bound to HSA (97–98%). In our previous research, we have demonstrated that MPA albumin binders modified *Xenopus* GLP-1 analogues has longer hypoglycemic activities in *db/db* mice, as compared to liraglutide.[26] In nature, multivalent binding strategies are frequently used to increase the weak ligand–receptor interactions, and the affinity of a dimer ligand can be significantly higher than the sum of avidities for its inherent components.[27] Therefore, we hypothesized those small molecule albumin binders with two MPA moieties (DiMPA) could serve as an effective method to further improve the half-life of peptide pharmaceuticals.

Lixisenatide is a potent GLP-1 receptor agonist based on exendin-4 with 3–4 h *in vivo* half-life, and is clinical approved as once-daily administration.[28] To investigate our hypothesis, we selected lixisenatide as a model peptide and conducted a series of proof-of-principle studies on DiMPA modified lixisenatide analogues. First, to avoid serious biological activities lost and identify the suitable modification site on lixisenatide, three MPA albumin binders with different length of alkyl chain were site-specific linked to three lysine residues of lixisenatide (Lys$_{12}$, Lys$_{27}$ and Lys$_{39}$). Using this strategy, we prepared nine different MPA modified lixisenatide analogues at Lys$_{12}$, Lys$_{27}$ and Lys$_{39}$ (**1a–1i**, Fig. 1), and then explored their *in vitro* GLP-1 receptor activation potency, stabilities and *in vivo* glucose lowering activities. Compound **1f** with high *in vitro* receptor activation potency, plasma stability and *in vivo* hypoglycemic activity was selected for the next step DiMPA modification.

Next, by using the two amino groups on lysine, we designed a DiMPA albumin ligand with C11 alkyl chain and linked to Lys$_{27}$ of lixisenatide, affording **2a** (Fig. 3). Considering the DiMPA modification could bring solubility issues and also investigate of the importance of a spacer, different length of 8-amino-3,6-dioxa-octanoic acid (OEG) spacers were introduced between the DiMPA albumin binding ligand and lixisenatide, giving **2b–2d** (Fig. 3). MPA is an inhibitor of inosine monophosphate dehydrogenase (IMPDH) and has been used as an immunosuppressive agent.[29] We hypothesize that MPA conjugated to peptide hormones like lixisenatide will not generate immunosuppressive effects. Mycophenolate mofetil is a prodrug of MPA, which is normally administered in thousands of mg per day and with ~90% bioavailability,[30] it

pharmacologically relevant dose far exceed that of lixisenatide (recommended dose of lixisenatide is 10–20 μg per day).[31]

In the present study, we investigated the DiMPA albumin binders as a novel approach to improve peptide pharmacokinetics. For this purpose, we conducted proof-of-principle studies using short-acting GLP-1 receptor agonist lixisenatide and site-specific modified by DiMPA albumin ligands. Moreover, the physicochemical, stabilities, antidiabetic and insulinotropic characteristics, toxicities, and long-term treatment effects of these lixisenatide analogues were extensively investigated.

2. Materials and methods

2.1. Materials and animals

Rink Amide MBHA resin, DIC, HOBT, N-Fmoc-amino acids, Boc-His(Boc)-OH, Fmoc-Lys(Dde)-OH, Fmoc-Glu-O*t*Bu, Fmoc-Lys(Fmoc)-OH, lixisenatide, semaglutide were obtained from the GL Biochem (Shanghai, China) Ltd. Fmoc protected 6-aminocaproic acid (Fmoc-6-Ahx-OH) and 12-aminolauric acid (Fmoc-12-Ado-OH) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fmoc-8-amino-3,6-dioxa-octanoic acid (Fmoc-AEEAc-OH) was purchased from Bachem (Bachem AG, Switzerland). The cAMP kit was purchased from the CIS Bio International (Bedford, MA, USA) and the insulin kit was obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). All other reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise indicated.

Male Kunming mice (20–25 g) were purchased from Qinglongshan Animal Breeding Farm (Nanjing, China). Male *db/db* mice (BKS-Lepr^{db}/Nju, 8 weeks, 30–40 g) were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). All animals were housed six in a cage with a 12 : 12 h light–dark cycle and the temperature was set at 25 ± 2 °C. All mice had free access to food (standard chow) and water and all experiments were approved by the institutional committee at Jiangsu Normal University and were in compliance with the Laboratory Animal Management Regulations in China, the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (revised 2011).

2.2. General procedure for peptide synthesis

The peptide backbones of lixisenatide analogues (**1a–1i**, **2a–2d**) were carried out on Rink amide MBHA resin with a loading of 0.382 mmol g^{-1} using Fmoc synthesis strategy on a semi-automated peptide synthesizer (mode PSI-200, Peptide Scientific Inc., USA).[32] The N-terminal His was replaced with Boc-His(Trt)-OH and the Lys on the conjugation site was replaced with Fmoc-Lys(Dde)-OH to prepare peptides with MPA side chain. The Dde protection group was removed selectively by washing 5 times (10 min) with 2% (v/v) hydrazine hydrate in DMF. For **1a–1i**, Fmoc-Glu-O*t*Bu, Fmoc-6-Ahx-OH or Fmoc-12-Ado-OH, and MPA (4 equiv.) were coupling in order by using DIC/HOBT according to their structure. For **2a–2d**, Fmoc-Lys(Fmoc)-OH was used to construct DiMPA ligand. Specifically, the Dde protection group on the modification site was

|	
Lixisenatide	HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPSKK KKKK
1a	HGEGT FTSDL SX_1QME EEAVR LFIEW LKNGG PSSGA PPSKK KKKK
1b	HGEGT FTSDL SX_2QME EEAVR LFIEW LKNGG PSSGA PPSKK KKKK
1c	HGEGT FTSDL SX_3QME EEAVR LFIEW LKNGG PSSGA PPSKK KKKK
1d	HGEGT FTSDL SKQME EEAVR LFIEW LX_1NGG PSSGA PPSKK KKKK
1e	HGEGT FTSDL SKQME EEAVR LFIEW LX_2NGG PSSGA PPSKK KKKK
1f	HGEGT FTSDL SKQME EEAVR LFIEW LX_3NGG PSSGA PPSKK KKKK
1g	HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPSX_1K KKKK
1h	HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPSX_2K KKKK
1i	HGEGT FTSDL SKQME EEAVR LFIEW LKNGG PSSGA PPSX_3K KKKK
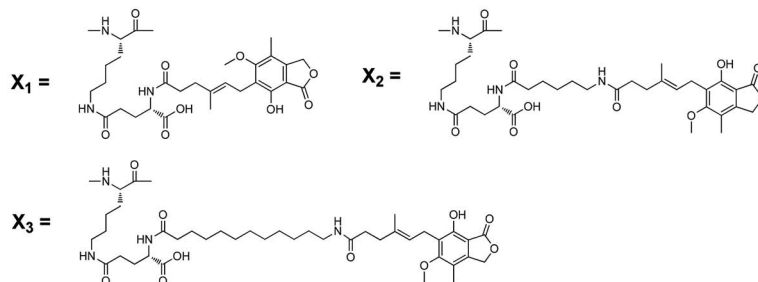
Fig. 1 Structures of mono-MPA lixisenatide conjugates **1a–1i**.

removed using the same method described above. Then Fmoc-AEEAc-OH, Fmoc-Glu-OtBu, Fmoc-12-Ado-OH, and Fmoc-Lys(Fmoc)-OH (4 equiv.) were added in order according to their structure and coupled using the same condition described above. The two Fmoc protected groups of Fmoc-Lys(Fmoc)-OH were removed, and MPA (8 equiv.) was dissolved with DIC (8 equiv.) and HOBt (8 equiv.) in DMF (5 mL), and coupled with the two amine groups on Lys for 3 h. The crude products were precipitated in cold ether and washed with cold ether for 4 times after cleaved from the resin with the usage of EDT/phenol/water/thioanisole/TFA (2.5 : 5:5 : 5 : 82.5). The crude analogues were further purified by RP-HPLC (LC-20AP, Shimadzu) with C18 column (Hypersil GOLD, 250 × 20 mm, 12 μM). For **1a–1i**, the isolated yields were between 45–55% due to unavoidable loss during purification. For **2a–2d**, the isolated yields decreased to 30–40% due to the more complicated synthetic process. All products were confirmed characterized by Agilent 6538 Q-TOF mass or Bruker MicroTOF Q2 with the application of ESI ionization method and the purity was confirmed by analytical HPLC (Agilent 1260 infinity).

2.3. *In vitro* GLP-1 receptor activation assay

HEK293 cells expressing the hGLP-1 receptor were used for functional assay as previously described.[33] Cells were suspended in DMEM growth medium containing 0.5% FBS, 20 mM HEPES, 1% penicillin–streptomycin and 2 mM L-glutamine. Cells were plated into 384-well microplates. The tested compounds were dissolved in DMSO, then diluted in assay buffer and transferred to the microplate to make the assay concentrations at 1×10^{-13} to 1×10^{-6} M. Then, the plate was incubated at 5% CO_2 for 30 min at 37 °C. The cAMP

concentrations was measured by Cisbio cAMP dynamic 2 kit with the use of Envision 2104 multilabel reader (PerkinElmer, U.K.). The cAMP data was exported and the EC_{50} values were calculated by sigmoidal curve fitting by GraphPad Prism 5.0.

2.4. *In vitro* stability assay

The *in vitro* stability of lixisenatide and **1a–1i** were determined using a previously described method with small modification.[34] In brief, plasma were obtained from SD rats (male, 200–250 g) and stored at −20 °C until use. Lixisenatide and **1a–1i** were dissolved in PBS (pH 7.4) and 0.5 mL of the compound were added to the 1 mL plasma to achieve concentration of 1000 ng mL^{-1}. The mixture was vortex mixed for 60 s and then incubated at 37 °C for 6, 12, 24, and 48 h. At each time point, 100 μL of sample was removed and mixed with acetonitrile (200 μL) containing 2% formic acid for the precipitation of plasma protein. Then the mixture was centrifuged at 14 000 rpm for 15 min, and the supernatant (50 μL) was analyzed *via* liquid chromatography-mass spectrometry (LC-MS/MS) system (AB SCIEX TripleTOF 4600, USA). The degradation curves of each tested compounds were determined in triplicate.

2.5. Intraperitoneal glucose tolerance test (IPGTT) in normal Kunming mice

IPGTT were carried out in normal male Kunming mice ($n = 6$) using a previously described method with small modification.[35] Mice were fasted overnight (12 h) prior to a 2 g kg^{-1} *i.p.* injected of glucose. The mice were administered a single *i.p.* injection of saline (control), lixisenatide, **1d**, **1e**, and **1f** (25 nmol kg^{-1}) 30 min prior to the *i.p.* glucose injection. Tail vein blood was drawn for glucose measurements 30 min before glucose load

and at $t = 0$, 15, 30, 45, 60, and 120 min. One-touch glucometer (GA-3, Sinocare, Changsha, China) was used to measure the blood glucose levels.

2.6. Relative and direct albumin binding assay

The relative serum albumin binding abilities of lixisenatide, **1f**, and **2a–2d** were measured by high-performance affinity chromatography (HPAC) analysis using CHIRALPAK-HSA column (Chiral Technologies Europe) according to previous described method with small modifications.[36] In brief, an Agilent 1260 HPLC equipped with an immobilized HSA column (2 mm × 50 mm, 5 μm) was used for chromatographic separation. Considering the possible high albumin binding rate of **2a–2d**, 20 mM phosphate buffer containing 25% 2-propanol was used as the isocratic mobile phase. The tested peptides were injected into the column at 25 °C and the detection wavelength was 214 nm. The retention times (t_r) of the tested compounds were the average of three independent measurements. The column void time (t_o) was determined by injecting a non-binding compound (acyclovir).

The direct serum albumin binding abilities of lixisenatide, semaglutide, **1f**, and **2a–2d** were measured using a modified ultra filtration method according to previous described method with small modifications.[26] In brief, test peptides were dissolved in 1 mL of PBS phosphate buffer (pH 7.4, 100 μg mL^{-1}), and 3 mL of 10 mg mL^{-1} of HSA in PBS (pH 7.4) was added. The solution was vortex mixed for 2 min. The mixture was incubated at 37 °C for 1 h, and 1 mL of the mixture was removed and loaded into Centricon centrifugal filter device (30 kDa molecular weight cutoff, Millipore, Bedford, USA). Then, the filter device was subjected to centrifuge for 40 min at 3500 rpm. The filtrate was determined using HPLC to analysis the content of tested peptides. The adsorption of each peptide to the filter membrane was also determined using the similar method. The albumin binding rates of tested peptides were determined in triplicate.

2.7. IPGTT in diabetic db/db mice

Male *db/db* mice were used to evaluate acute actions of peptide on blood glucose.[37] Briefly, *db/db* mice ($n = 6$) were subjected to 16 h of fasting and injected *i.p.* with glucose (1 g kg^{-1}). Saline (control), lixisenatide, **2a**, **2b**, **2c**, and **2d** (25 nmol kg^{-1}) were *i.p.* injected 30 min prior to the glucose load. Tail blood glucose levels were determined with a one-touch glucometer before (−30 min) and at 0, 15, 30, 60, and 120 min after the injection of glucose.

2.8. Glucoregulatory and insulinotropic assay

The effects of lixisenatide and **2c** on glucoregulatory and insulinotropic were tested by IPGTT on *db/db* mice as previous reported.[38] Mice ($n = 6$) were fasted overnight (16 h) and then *i.p.* injected with saline (control), lixisenatide and **2c** (25 nmol kg^{-1}) 30 min prior to the glucose load. The glucose (1 g kg^{-1}) was *i.p.* loaded at 0 min. Tail vein blood was drawn for blood glucose levels measurement at −30 and 0, 10, 15, 30, 45, 60, and 120 min by using one-touch glucometer, and for plasma insulin

measurement at 0, 15, 30, and 60 min by using mouse insulin ELISA kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

2.9. Glucose-stabilizing activity in db/db mice

The antidiabetic duration of lixisenatide, semaglutide, and **2c** were determined using two methods on *db/db* mice. In the first pre-oral glucose tolerance test (OGTT) experiment, glucose was administered orally to mimic the postprandial glycemic condition.[39] Male *db/db* mice ($n = 6$) were fasted overnight (16 h) and saline (control), lixisenatide, semaglutide, and **2c** (25 nmol kg^{-1}) were pre-injected (*i.p.*) at 4 and 8 hours before oral glucose administered ($t = 0$ h, 1.5 g kg^{-1}). Blood samples were obtained at −4 or −8, 0, 0.25, 0.5, 1, 1.5 and 2 h from the cut of the tip tail, and blood glucose concentrations were determined by one-touch glucometer. In the second experiment, the hypoglycemic activities of lixisenatide, semaglutide and **2c** were measured in *db/db* mice ($n = 6$) under non-fasted condition.[40] The mice were allowed to free access to food. On the experiment day, mice were *s.c.* injected with saline (control), lixisenatide, semaglutide, and **2c** (25 nmol kg^{-1}) at $t = 0$ h. The blood was obtained and the blood glucose levels were measured using the same above described method at 0, 2, 4, 6, 12, 24 and 48 h.

2.10. Acute toxicity assay of 2c

The toxicity test of **2c** was conducted on normal Kunming mice for two weeks using previously described method with small modifications.[41] Mice were randomly divided into three groups, and saline (control), semaglutide (100 nmol kg^{-1}) and **2c** (1000 nmol kg^{-1}) were once daily *s.c.* injected for 14 days. Water and food were provided ad libitum during the experiment. At day 15, each group of mice were sacrificed and the whole blood were collected, and the blood samples were centrifuged to obtain serum. Blood urea nitrogen (BUN), alanine aminotransferase (ALT), serum creatinine (SCr), and aspartate aminotransferase (AST) were measured using biochemical analyzer (Beckman Coulter AU5811, Japan).

2.11. Immunogenicity testing of 2c

The immunogenicity test of **2c** was conducted by evaluated the ability of **2c** to induce T cell proliferation in peripheral blood mononuclear cells (PBMC) from 50 Chinese individuals (obtained from LDEBiO, Guangzhou, China), using previous described method.[42] Briefly, PBMC were cultured in AIMV medium (Life Technologies, Carlsbad, CA) and added to the 24-well plates (2 mL) to reach a final concentration of ∼3 ×10^6 cells per mL, and then stimulated by addition of lixisenatide, semaglutide and **2c** in AIMV medium with a final concentration of 30 μg mL^{-1} of each tested peptides. The 24-well plates were incubated in CO$_2$ incubator (5%) at 37 °C for 8 days. The cells in each well of the plate were transferred to 96-well plate on days 5, 6, 7 and 8. The cultures were pulsed with [^{3}H]-Thymidine (PerkinElmer) and incubated for further 18 h and counts per minute (cpm) for each well were determined. The stimulation index (SI) was calculated by dividing the proliferative response of the test well (cpm) by the proliferative response of the medium-only

treated well (cpm) for each donor, and SI greater than 2.0 was considered positive. The % donors responding was calculated by taking the number of donors that had a positive response over the entire time course (5–8 days) as a percentage of the total number of donors that were tested.

2.12. Short-term effects of 2c on pancreas development by once daily administration

Seven days before the first dosing, tail vein blood was obtained from non-fasted animals and DCA 2000 chemistry analyzer (Siemens/Bayer, USA) was used to determine of baseline HbA1c levels using previous described method.[43] Based on the HbA1c levels, male *db/db* mice ($n = 6$) were stratified into the following groups: control (saline); semaglutide (25 nmol kg^{-1}); 2c (25 nmol kg^{-1}). The mice were once daily s.c. treated with saline, semaglutide and 2c. Food intake and body weight were determined daily in the morning. The treatment study was lasting 21 days and tail vein blood was collected again from non-fasted mice for determination of HbA1c levels. Finally, mice were sacrificed by cervical dislocation, and the pancreases were removed and stored in 4% paraformaldehyde. The insulin immunohistochemistry studies were performed using previously described method. The integrated optical density (IOD) values of insulin in each group were analyzed by software Image-pro plus 6.0 (Media Cybernetics Inc., USA).

2.13. Short-term effects of 2c on pancreas development by once-weekly administration

Male *db/db* mice ($n = 6$) were stratified into the following groups: control (saline); lixisenatide (25 nmol kg^{-1}); semaglutide (25 nmol kg^{-1}); 2c (25 nmol kg^{-1}) based on the HbA1c levels, using the same method described above. The mice were once weekly s.c. treated with saline, lixisenatide, semaglutide and 2c. Body weight were determined daily in the morning. Fasting blood glucose concentrations were measured before and at the end of the study from mice fasted for 6 h. The experiment was lasted for three weeks and HbA1c levels were determined again using the same method described above. Finally, each group of mice were sacrificed and the pancreases were removed to perform insulin immunohistochemistry studies using the same method described above.

2.14. Chronic treatment of 2c on glycaemic control

Male *db/db* mice were subjected to an OGTT performed as previously described. In brief, mice were fasted 16 h and glucose (1.5 g kg^{-1}) were loaded orally, and glucose tolerance was measured in relation to area under the curve (AUC) of blood glucose levels. Based on the OGTT results, mice exhibiting similar glucose tolerances were divided into 3 groups ($n = 6$). Three groups of mice were once daily s.c. injected with saline, semaglutide, and 2c (25 nmol kg^{-1}) based on their antidiabetic duration profiles for 42 days, and then followed by 42 days of vehicle (saline) treatment. Glucose tolerance was assessed on days 42, 56, 70 and 84 during the treatment period using the same method described above. Furthermore, fasting glucose levels were determined from 10 h fasted mice on days 0, 35, 49,

63 and 77 of the study. HbA1c levels were determined on days −1, 43 and 85 using the same method described above.[44]

2.15. Data analysis

Results are expressed as means ± SD unless otherwise stated. Statistical significances of the data were performed by one-way ANOVA followed by appropriate post hoc tests. All statistical calculations were conducted with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and $P < 0.05$ values were considered statistically significant.

3. Results

3.1. Design and synthesis of lixisenatide analogues 1a–1i

To investigate whether DiMPA small molecule albumin binders could be an effective methodology to improve peptide pharmacokinetics, DiMPA was linked to a short acting GLP-1 agonist, lixisenatide. However, to avoid the serious biological activities lost caused by DiMPA modification and identify the suitable modification site on lixisenatide, the three lysine residues on lixisenatide (Lys$_{12}$, Lys$_{27}$ and Lys$_{39}$) were selected and site-specific modified by three MPA albumin binders with one MPA moiety and different length of alkyl chain, affording 1a–1i (Fig. 1). Rink Amide MBHA resin was used as a polymer support to obtain an amidated C-terminus.

Site-specific MPA modification at the side chain of Lys$_{12}$, Lys$_{27}$ and Lys$_{39}$ was achieved by replacing the corresponding Lys to Fmoc-Lys(Dde)-OH, and the N-terminal His was replaced with Boc-His(Boc)-OH. The Dde group was specially removed by 2% hydrazine hydrate/DMF (v/v), and Fmoc-Glu-O*t*Bu was coupled with the side amino group of lysine. Then MPA was directly attached to the amine of a glutamyl spacer, affording 1a, 1d, 1g. For other compounds with fatty chain spacer, N-Fmoc-protected ω-amino acids (6-aminocaproic acid and 12-amino-lauric acid) were attached to the amine of a glutamyl spacer. Finally, after removal of Fmoc group, the ω-amino groups were attached to the carboxyl group of MPA. Using this method, the whole synthetic procedure of the crude products was conducted on solid support (Scheme S1, see ESI†). Crude products were characterized by HPLC and MS and further purified using semi-preparative RP-HPLC (see ESI†).

3.2. Biological activities and stabilities of 1a–1i

The *in vitro* GLP-1 receptor activation potency of 1a–1i was studied by a GLP-1 receptor cAMP activity assay to evaluate whether attachment of the MPA had changed the GLP-1 receptor potency of lixisenatide. The EC$_{50}$ values of 1a–1i are summarized in Table 1 and the concentration response curves of lixisenatide and 1a–1i were shown in Fig. 2A and B. In general, MPAs modification was well tolerated at Lys$_{27}$ (1d–1f), and the length of alkyl chain has no significant impact on potency. Meanwhile, the analogues with MPAs modification on Lys$_{12}$ (1a–1c) exhibited a more profound reduction in potency compared to lixisenatide and 1d–1f. The greatest impact on potency was found by introducing MPAs at Lys$_{39}$, as the serious reductions in receptor activation potency observed for 1g–1i.

Table 1 The *in vitro* bioactivities and stabilities of **1a–1i**

Peptide	EC_{50}^{a} (nM)	Plasma half-life^b (h)	Peptide	EC_{50}^{a} (nM)	Plasma half-life^b (h)
Lixisenatide	0.076 ± 0.013	7.1	1e	0.13 ± 0.03	21.2
1a	0.19 ± 0.02	13.2	1f	0.32 ± 0.13	33.4
1b	0.49 ± 0.11	19.1	1g	0.59 ± 0.16	11.9
1c	0.98 ± 0.24	30.0	1h	1.97 ± 0.53	17.8
1d	0.085 ± 0.021	13.1	1i	4.68 ± 0.85	31.6

^{a} Data are represented as EC_{50}. Values are the means $\pm$ SD from three independent experiments. ^{b} The plasma half-lives of lixisenatide and **1a–1i** were calculated after incubation of peptides in rat plasma over 48 h.

The *in vitro* stabilities of **1a–1i** were examined in rat blood plasma at 37 °C for 48 h in order to examine their stability against peptidases and proteases in a life-near system. The degradation profiles of **1a–1i** were shown in Fig. 2C and D. The stabilities of **1a–1i** were exhibited positive correlation with length of the alkyl chain and the modification site of MPA has
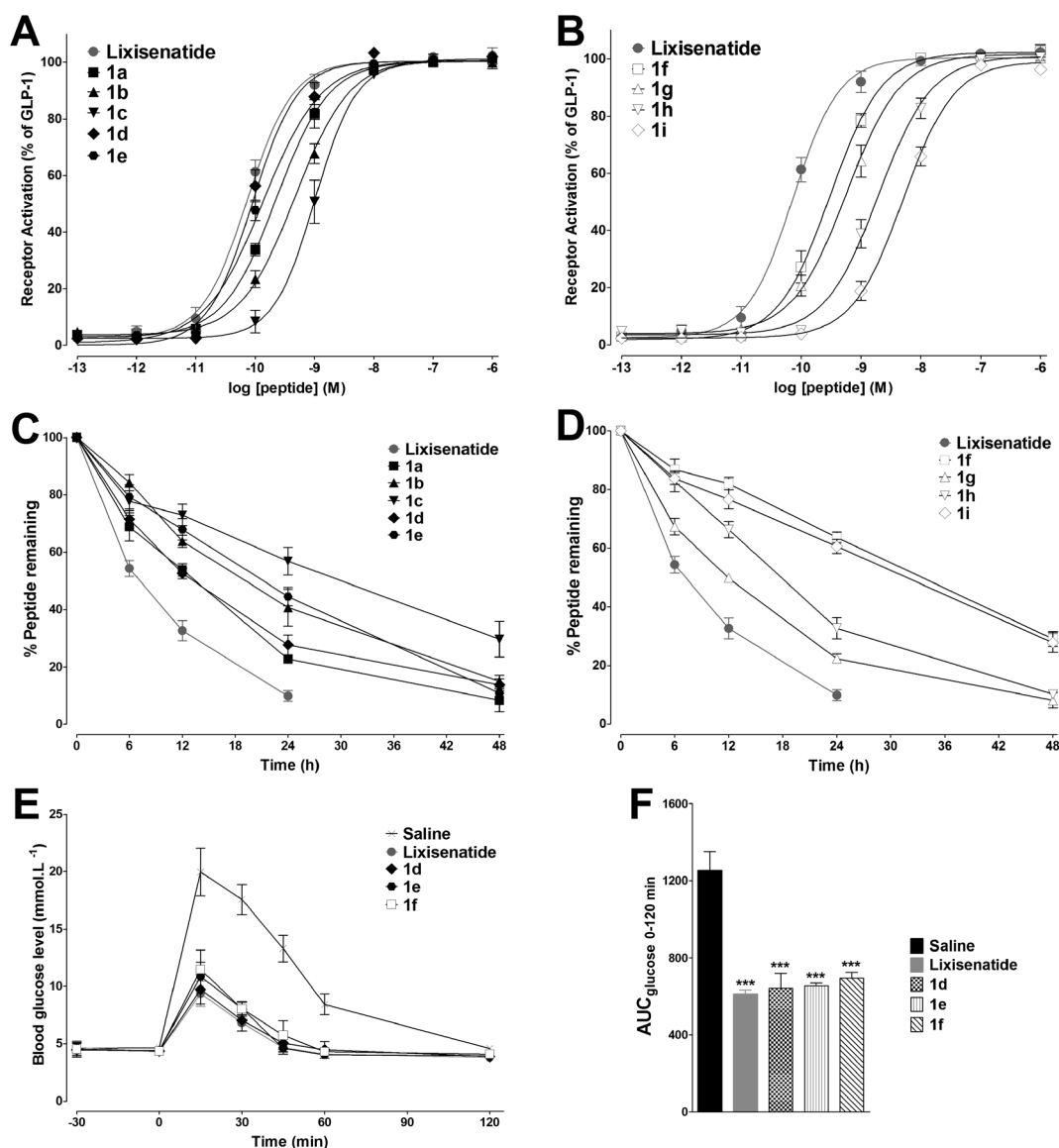

Fig. 2 *In vitro* bioactivity and stability tests of **1a–1i** and *in vivo* biological activities of **1d–1f**. (A and B) Dose–response curves of **1a–1i** for activation at the GLP-1 receptor. Means $\pm$ SD. Tests were performed in triplicate and conducted in three independent experiments. (C and D) Degradation profiles of **1a–1i** in rat plasma. Means $\pm$ SD, $n = 3$. (E) Acute effects on IPGTT in normal Kunming mice treated with saline, lixisenatide or **1d–1f**. (F) Hypoglycemic effects of lixisenatide and **1d–1f** expressed as $AUC_{glucose\ 0-120\ min}$ values. Means $\pm$ SD, $n = 6$. $^{***}P < 0.001$ *vs.* saline.

little impact on stabilities (Table 1). Considering the high potencies and stabilities of **1d–1f**, IPGTT was performed on Kunming mice to validate their *in vivo* antidiabetic activities. As shown in Fig. 2E and F, the administration of lixisenatide significantly blunted IPGTT-evoked blood glucose increases compared to the vehicle. The same applied to **1d–1f**, which reduced blood glucose excursion levels to similar level as lixisenatide. The hypoglycemic activity of **1f** was similar with **1d** and **1e**, and **1f** only showed slightly decreased activity toward glucose-lowering as compared with **1d** and **1e**. Moreover, the *in vitro* stability of **1f** (33.4 h) was significant longer than that of **1d** (13.1 h) and **1e** (21.2 h). Considering the high *in vitro* stability is essential for the *in vivo* long-acting glucose-lowering activity, together with the similar *in vivo* glucose-lowering activities of **1d–1f**, **1f** was finally selected for the next step DiMPA modification.

3.3. Design and synthesis of DiMPA modified lixisenatide analogues 2a–2d

The above results showed that MPA modification significant enhanced the *in vitro* half-life of **1f** to ~33 h. However, this duration is far shorter than the serum half-life of HSA, which is up to 3 weeks in humans.[15] Therefore, we envisioned that an increased affinity to HSA could increase the *in vitro* half-life beyond ~33 h seen for **1f**. We hypothesized that the HSA binding affinity for MPA albumin ligands with two MPA moieties (DiMPA) could be much higher than those with one MPA moiety. Based on the MPA side chain of **1f**, we conducted two steps structure optimization. First, to achieve DiMPA modification, an additional Lys was introduced to the ω-amino group of 12-aminolauric acid, and the carboxyl group of MPA was attached to the two amino groups of Lys to obtain DiMPA structure. Next, different length of 8-amino-3,6-dioxaoctanoic acid (O2Oc) spacers were introduced between Lys_{27} and DiMPA side chain to investigate the importance of a spacer and also minimize the high hydrophobicity brings by DiMPA modification. A total of four DiMPA modified lixisenatide analogues (**2a–2d**, Fig. 3) were designed and synthesized. The synthesis process of **2a–2d** was similar to **1a–1i**, and Fmoc-Lys(Fmoc)-OH was used to achieve DiMPA modification (Schemes S2 and S3, see ESI†). Crude products were characterized and purified using the same method described above (see ESI†).

3.4. MPA → DiMPA substitution to further improve the albumin binding affinity

Multivalent binding strategies such as dimeric interactions are frequently occurred in nature and used to increase the weak ligand–receptor interactions,[45] we predicted that DiMPA substitution could further increased the albumin binding affinities of **2a–2d** compared with **1f**. The albumin binding abilities of lixisenatide, **1f**, and **2a–2d** was firstly evaluated by HPAC analysis, using the retention factor (k') as the indicator. The value of k' was obtained according to the equation below: $k' = (t_r - t_o)/t_o$.[36] As shown in Fig. 4A, the k' values of lixisenatide and **1f** were ~1.1 and ~6.7, respectively. As expected, the albumin affinity of **2a** ($k' = $~13.3) was higher than that of lixisenatide and **1f**, for **2b–2d** with OEG

spacers, there is a clear trend that with increasing length of the OEG spacer, the compounds display an increased affinity to albumin binding relative to **2a** (**2b**, $k' = $~14.1; **2c**, $k' = $~15.2; **2d**, $k' = $~17.2). Next, the absolute albumin binding rates of lixisenatide, **1f**, and **2a–2d** were measured using an ultrafiltration method. As illustrated in Fig. 4B, the albumin binding rates of lixisenatide and **1f** were 21.3 ± 2.1% and 81.7 ± 3.5%, respectively, which were significant lower than those of **2a** (92.7 ± 2.5%), **2b** (95.0 ± 2.0%), **2c** (96.3 ± 1.5%) and **2d** (97.7 ± 0.6%). Similar with the results in relative albumin binding study, **2b–2d** with OEG spacers showed higher albumin binding rates than that of **2a**, indicated the favorable effect of OEG spacer on albumin binding.

3.5. Selection of a lead compound: 2c exhibited comparable efficacious with lixisenatide *in vivo*

The *in vitro* GLP-1 receptor activation potency of **2a–2d** was determined using the same method described above. As shown in Fig. 4C, compared with **1f**, **2a** ($EC_{50} = 0.53 ± 0.11$ nM) showed a slight reduction in potency as a consequence of DiMPA modification. For **2b–2d**, the OEG spacer containing two OEG moieties was more favorable for the potency, as reflected by the higher potency of **2c** ($EC_{50} = 0.36 ± 0.10$ nM) compared to **2a**, **2b** ($EC_{50} = 0.77 ± 0.22$ nM) and **2d** ($EC_{50} = 0.93 ± 0.25$ nM). Next, the *in vivo* glucose-lowering activities of **2a–2d** were studied in the IPGTT in type 2 diabetic *db/db* mice. Fig. 4D shows the time course for blood glucose changes during IPGTT. Consistent with the *in vitro* potency results, **2c** significantly reduced glucose excursion after glucose challenge, comparable with that observed in lixisenatide group, and glucose AUC values further proved that the antidiabetic effects of **2c** and lixisenatide were similar (Fig. 4E). The hypoglycemic activity of **2a** was less effective compared with lixisenatide and **2c**, while the hypoglycemic activities of **2b** and **2d** were much worse than **2c**. Based on the above *in vitro* and *in vivo* studies, we identified **2c** as a novel and potent GLP-1 receptor agonist with high albumin binding affinity and hypoglycemic activity, and **2c** was selected as lead compound and more biological evaluations were conducted to explore its therapeutic utilities.

3.6. 2c Exhibited potent glucose-lowering and insulinotropic activities in db/db mice

In the IPGTT, lixisenatide and **2c** significantly improved glucose tolerance after *i.p.* administration at doses of 25 nmol kg^{-1} relative to the control (saline). As shown in Fig. 5A and C, accompanied by the enhanced plasma insulin levels, the blood glucose levels were decreased. Both lixisenatide and **2c** resulted in increased plasma insulin concentrations at 15 min after the glucose load compared with control. The time courses and AUC values for blood glucose and plasma insulin concentrations were very similar following treatment with either of lixisenatide and **2c** (Fig. 5).

3.7. 2c displayed potent long-acting anti-hyperglycemic effect

Two different experiments were conducted on *db/db* mice to verify whether the high albumin binding affinity of **2c** could

2a HGEGT FTSDL SKQME EEAVR LFIEW L–NGG PSSGA PPSKK KKKK

2b HGEGT FTSDL SKQME EEAVR LFIEW L–NGG PSSGA PPSKK KKKK

2c HGEGT FTSDL SKQME EEAVR LFIEW L–NGG PSSGA PPSKK KKKK

2d HGEGT FTSDL SKQME EEAVR LFIEW L–NGG PSSGA PPSKK KKKK
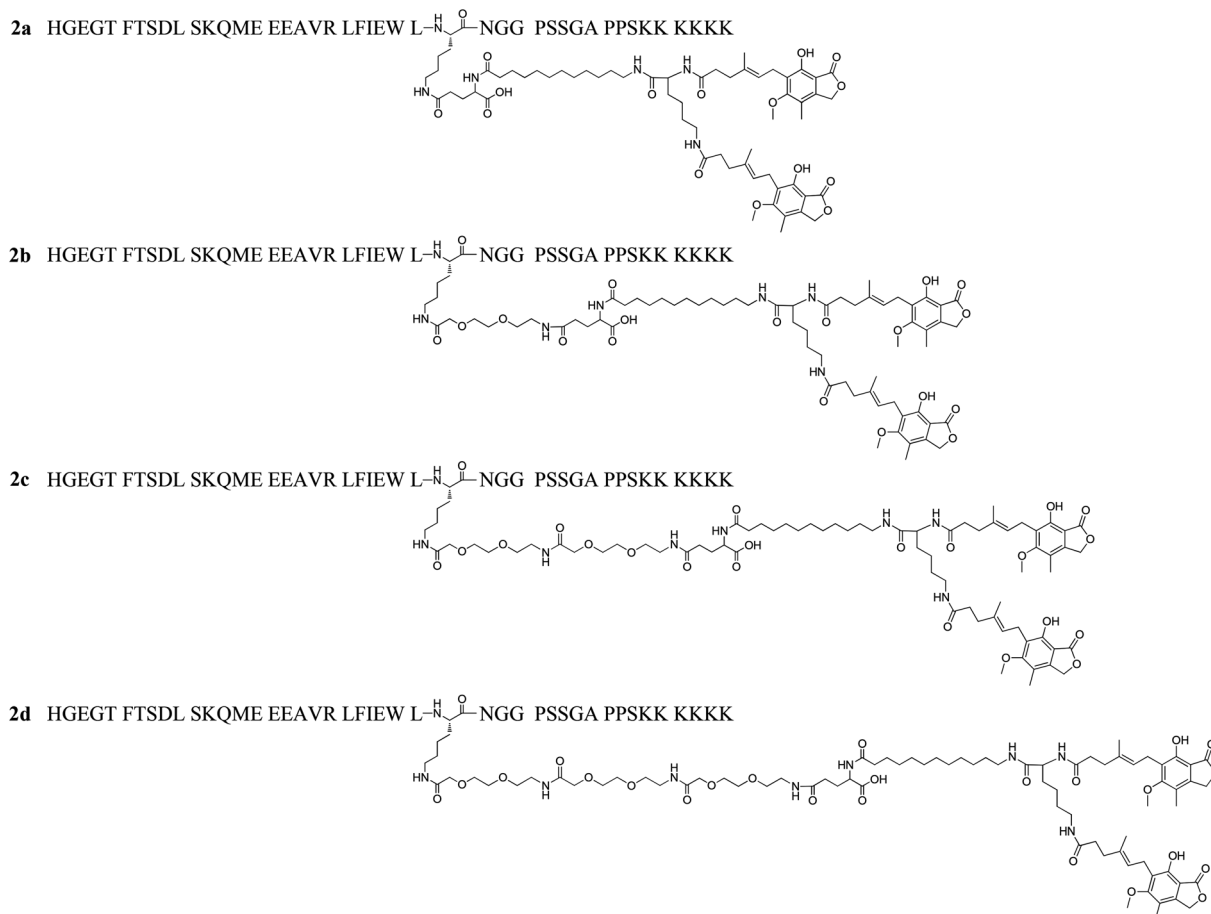

Fig. 3   Structures of dimeric-MPA lixisenatide conjugates **2a–2d**.

transfer to the long-acting hypoglycemic activity *in vivo*. In the pre-OGTT experiments, compared with control, both semaglutide and **2c** could potently reduce blood glucose when mice were pre-injected of tested compounds at 4 or 8 h pre-OGTT. However, the glucose-stabilizing effects of lixisenatide were significantly compromised when administered at 4 h pre-OGTT, and completely lost at 8 h pre-OGTT (Fig. 6A and B). Normally, the blood glucose levels in *db/db* mice were higher than 15 mmol L^{-1} under non-fasting condition. By using the hyperglycemic properties of *db/db* mice, we conducted the second experiment on non-fasting *db/db* mice. As shown in Fig. 6C, semaglutide and **2c** could maintain normoglycemia state up to 48 h, and lixisenatide clearly showed a significant shorter duration of action compared to semaglutide and **2c**. Importantly, semaglutide and **2c** exhibited similar glucose-lowering activities during 0–48 h. Blood glucose $AUC_{0-48\ h}$ values also verified that **2c** had similar antidiabetic activity compared with semaglutide.

3.8. Acute *in vivo* toxicity study and immunogenetic testing of 2c

The acute *in vivo* toxicity study of **2c** was studied by loading extreme high dose of **2c** (1000 nmol kg^{-1}) on Kunming mice for two weeks. As shown Fig. 6D–G, compared with saline and low dose of semaglutide (100 nmol kg^{-1}), no significant difference was observed for the key indexes of renal toxicity (SCr and BUN) and liver toxicity (AST and ALT) between saline, semaglutide and **2c** treated groups, preliminary indicating the safety of **2c**. The immunogenicity test of **2c** was conducted by evaluating the ability of **2c** to induce T cell proliferation in peripheral blood mononuclear cells (PBMC). As shown in Fig. 6H, the percentage of responding donors was 10.4% for **2c**, similar to that of lixisenatide (10.5%) and semaglutide (9.9%). Considering that lixisenatide and semaglutide were both approved pharmaceutics, the above results preliminarily suggest the low immunogenicity of **2c**.

3.9. Short-term effects of 2c on body weight, food intake, and pancreas by once daily administration

Twenty-one days of once daily treatment with semaglutide or **2c** potently reduced cumulated food intake and body weight gain (Fig. 7A–C). In addition, treatment with semaglutide or **2c** prohibited worsening of the HbA1c in *db/db* mice (Fig. 7D). The immunohistochemistry analysis of pancreata revealed that the staining of insulin in islets of semaglutide or **2c** treated mice were significantly improved compared with saline treated mice, as reflected by the significant enhanced IOD values of insulin in semaglutide or **2c** treatment groups (Fig. 7E). Representative
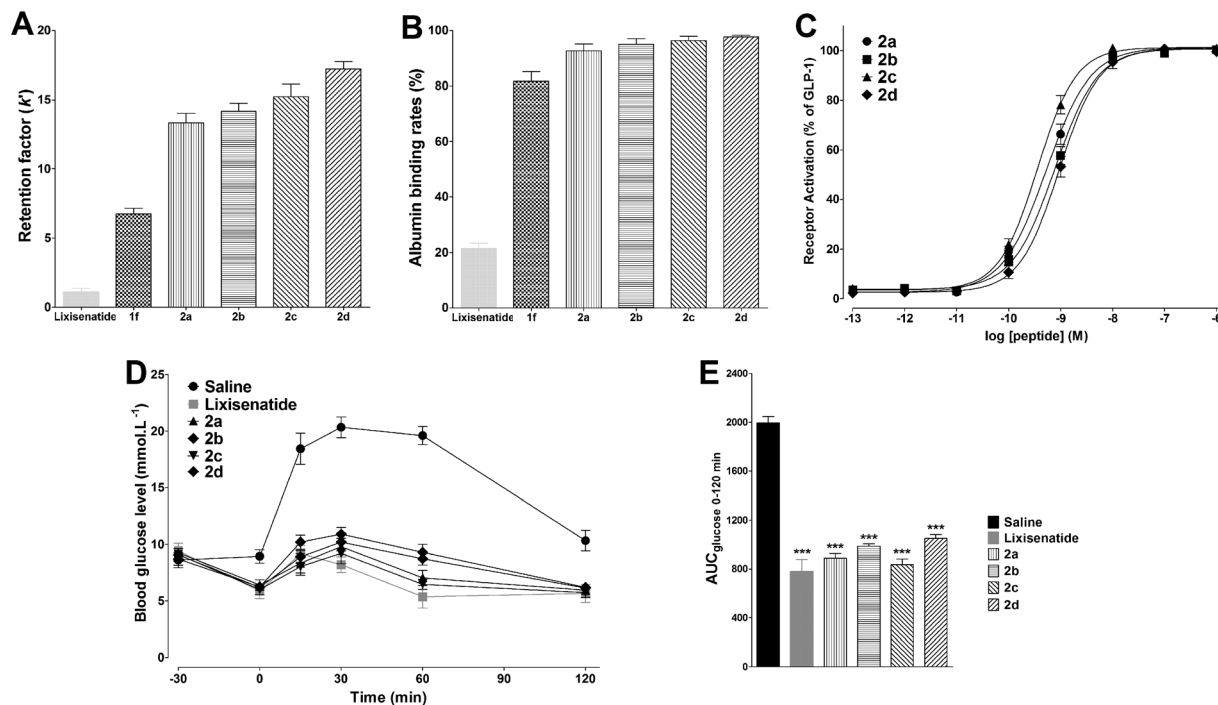
Fig. 4 Physicochemical and bioactivity profiles of **2a–2d**. (A) The relative albumin binding affinities of lixisenatide, **1f** and **2a–2d** were determined on an immobilized HSA column using retention factor (k') as the indicator. Means $\pm$ SD, $n = 3$. (B) The direct albumin binding rates of lixisenatide, **1f** and **2a–2d** were determined by ultrafiltration method. Means $\pm$ SD, $n = 3$. (C) Dose–response curves of **2a–2d** for activation at the GLP-1 receptor. Means $\pm$ SD. Tests were performed in triplicate and conducted in three independent experiments. (D) Acute effects on IPGTT in diabetic *db/db* mice treated with saline, lixisenatide or **2a–2d**. (E) Hypoglycemic effects of lixisenatide and **2a–2d** expressed as $AUC_{glucose\ 0-120\ min}$ values. Means $\pm$ SD, $n = 6$. ***$P < 0.001$ *vs.* saline.

images of insulin staining histologic samples are shown in Fig. 7F–H. Importantly, we did not observed significant difference of IOD values of insulin between semaglutide and **2c** treatment groups, indicating that DiMPA modification did not change the beneficial treatment effects of lixisenatide. The results of the immunohistochemistry also revealed the beneficial treatment effects of semaglutide and **2c** on amelioration of pancreas deficiencies.

3.10. Short-term effects of 2c on body weight, food intake, and pancreas by once weekly administration

Due to **2c** exhibited similar long-acting anti-hyperglycemic effect compared with semaglutide, and semaglutide was approved as once weekly administration GLP-1 agonist, we further tested the effect of weekly *s.c.* injections of lixisenatide, semaglutide or **2c** in *db/db* mice. As shown in Fig. 8, once weekly treatment with lixisenatide did not achieve prominent beneficial effects on body weight, HbA1c and pancreas function. However, both semaglutide and **2c** potently reduced body weight gain (Fig. 8A and B). In addition, once weekly treatment with semaglutide and **2c** prohibited worsening of the fasted blood glucose and HbA1c levels in *db/db* mice (Fig. 8C–F). The IOD values of insulin in semaglutide or **2c** treatment groups were significantly higher than that of in saline group (Fig. 8G). Unlike the similar IOD values of insulin between semaglutide and **2c** treatment groups in once daily administration

experiment, the IOD value of insulin in **2c** group was significant higher than that of in semaglutide group ($P < 0.05$). Representative images of insulin staining histologic samples are shown in Fig. 8H–K.

3.11. Long-term effects of 2c on glycaemic control

The time course of blood glucose and $AUC_{glucose}$ of repeated OGTTs in *db/db* mice were shown in Fig. 9A–F. During the 84 days of the study, there was progress impairment in glucose tolerance in saline treatment group. This phenomenon was expected, as the diabetic state in *db/db* mice is known to progress over time. During the 42 days treatment period in all groups of mice treated with semaglutide or **2c**, we observed significant improvement in glucose tolerance compared with control group. In the following 42 days saline treatment period, both semaglutide and **2c** caused long lasting improvement in glucose tolerance (Fig. 9F), and an effect that was most pronounced in mice treated with **2c**, as reflected by the significant lower glucose AUC values in **2c** group compared with that observed in semaglutide group at days 56 ($P < 0.001$), 70 ($P < 0.01$) and 84 ($P < 0.001$). Fasting blood glucose concentrations were also determined throughout the study on different weeks from the OGTT. The results of OGTT and fasting blood glucose levels showed a similar trend (Fig. 9G). HbA1c values were reduced in semaglutide and **2c** groups relative to control after 6 weeks treatment. Furthermore, HbA1c values remained lower in
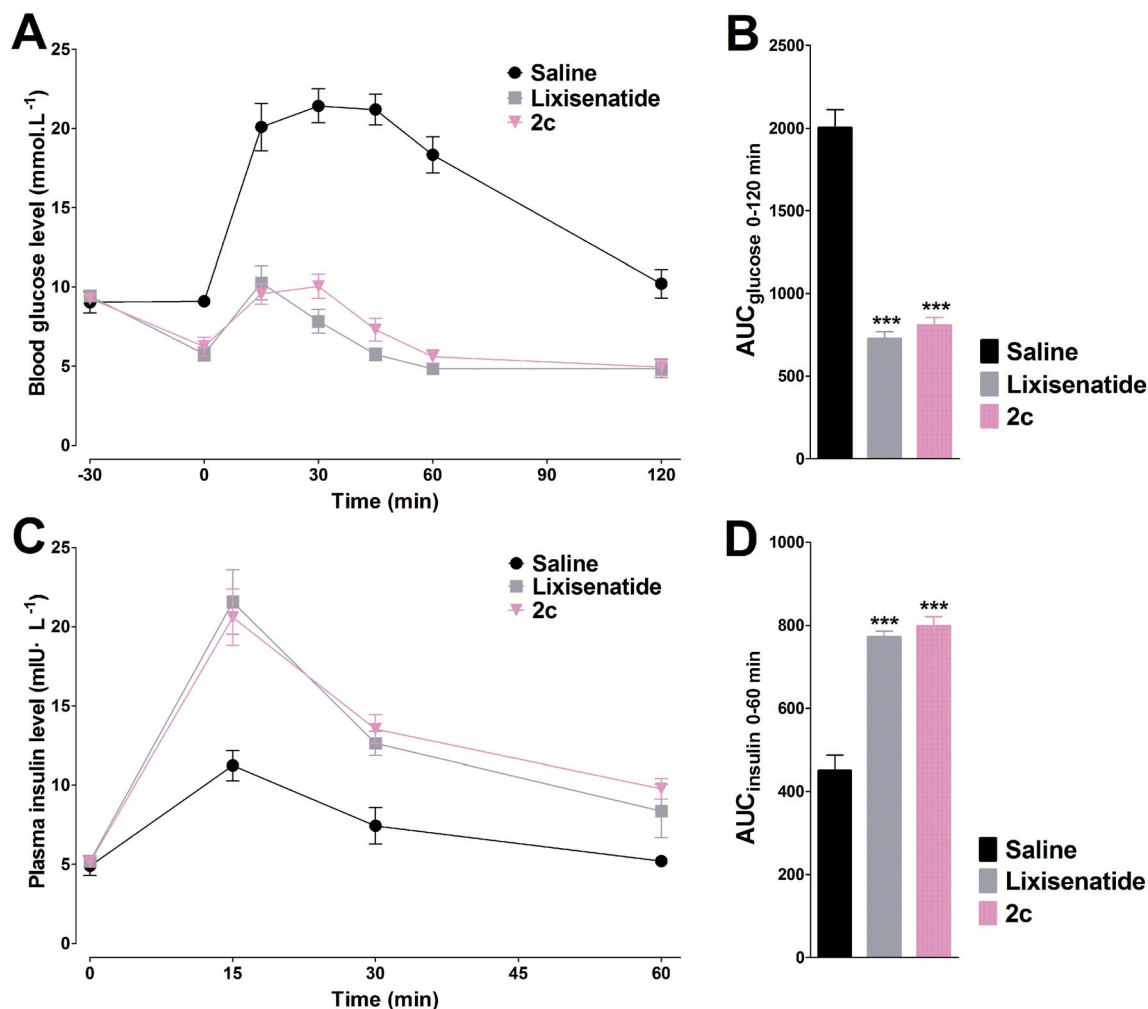
Fig. 5 Glucose–lowering and insulinotropic activities of lixisenatide and **2c** were determined by IPGTT in *db/db* mice. The time−response curves of blood glucose (A) and plasma insulin concentrations (C). The calculated AUC values of blood glucose (B) and plasma insulin (D). Means ± SD, *n* = 6. ***P < 0.001 *vs.* saline.

semaglutide and **2c** groups than control for a further 6 weeks after cessation of semaglutide and **2c** treatment (Fig. 9H).

4. Discussion

Peptides are subject to rapid renal clearance and extensive enzymatic degradation. Thus, half-life prolonging is a prerequisite for the development of peptide pharmaceuticals. In the present study, a novel method for prolonging peptide half-life using DiMPA small-molecule albumin ligands was explored and demonstrated for short acting GLP-1 receptor agonist lixisenatide. In the past decade, many research efforts were focused on chemical modification on exenatide to develop of long-acting incretin mimetics, however, little research has been done on lixisenatide.[12,46] Thus, the present research was using lixisenatide instead of exenatide as a model peptide. Although promote peptide to bind with albumin is a well-established method for peptide half-life extension, while the way to achieve it is variable and directly affect the potential therapeutic utility of peptide. The suitable albumin binders should possess

two important characteristics, which are low toxicity and high albumin binding affinity, and to discover the proper albumin binders from the approved therapeutic drugs should be a practical way due to the high safety of the approved therapeutic drugs.[24] As mentioned above, MPA is an effective immunosuppressive agent, and is extensively (∼98%) binds to HSA. The recommended dosage of mycophenolate mofetil (an ester prodrug of MPA) is 2 g per day, and this drug can be safely used as anti-rejection therapy in diabetic animal models, and in transplant recipients.[47,48] Considering the high safety and albumin binding affinity of MPA, we explored the possibility of using albumin binders based on MPA for half-life extension on lixisenatide analogues.

Lixisenatide had the same Lys_{12} and Lys_{27} residues compared with exenatide, and had additional six lysine residues on the C-terminal region. Previous researches have confirmed that both Lys_{12} and Lys_{27} on exenatide are suitable sites for chemical modification, and consequentially, Lys_{12} and Lys_{27} on lixisenatide were firstly selected for MPA modification.[49] Moreover, to further explore whether the C-terminal region of
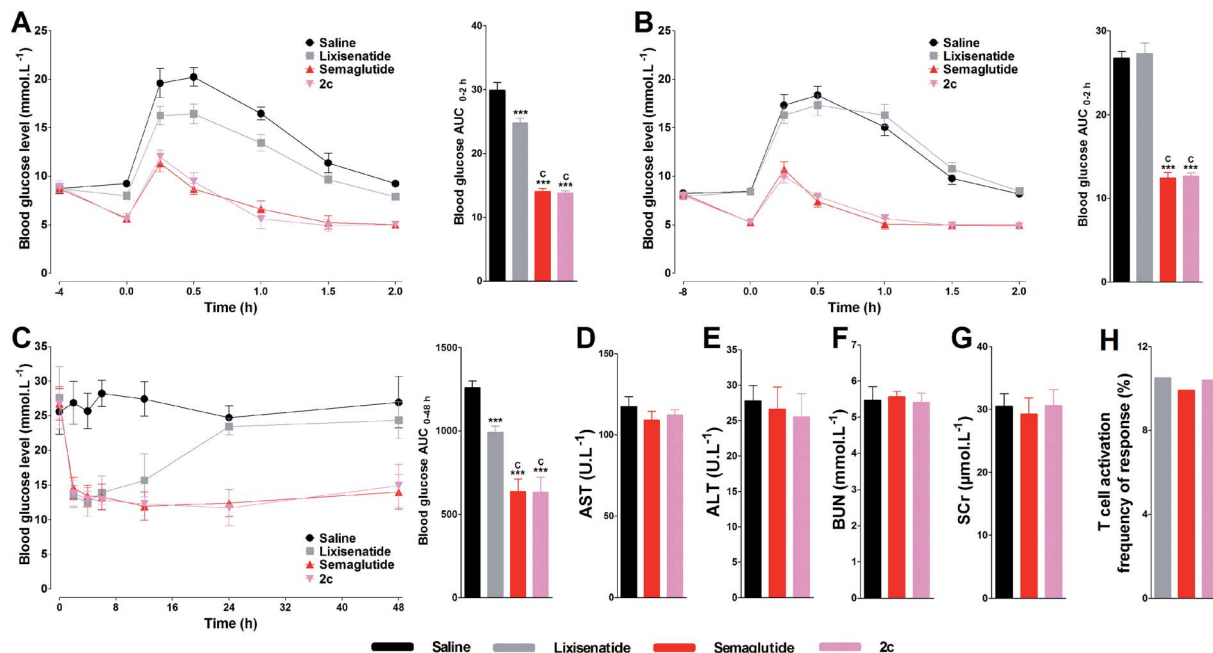
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Fig. 6 Glucose-stabilizing activity of **2c** on *db/db* mice and acute toxicity assay of **2c** on normal Kunming mice. Glucose-stabilizing profiles of lixisenatide, semaglutide and **2c** in *db/db* mice fasted for 16 h, after the administrations (*i.p.*) of these agents at 4 h (A) and 8 h (B) before OGTT. (C) Hypoglycemic activities of lixisenatide, semaglutide and **2c** on non-fasted *db/db* mice. Biochemical analysis results of **2c** on AST (D), ALT (E), BUN (F) and SCr (G) after two weeks dosing on normal Kunming mice. Means ± SD, $n = 6$. ***$P < 0.001$ *vs.* saline, $^{C}P < 0.001$ *vs.* lixisenatide. The immunogenicity testing of **2c** was evaluated by *in vitro* T cell proliferation assay and compared with lixisenatide and semaglutide (H). The frequency of response (%) that showed a positive T-cell proliferative response over the course of the entire study ($n = 50$).

lixisenatide was suitable for chemical modification, Lys39 was also selected and site-specific connected with MPA albumin binders, and a total of twelve MPA modified lixisenatide analogues (**1a–1i**) were firstly synthesized to identify the suitable modification site on lixisenatide.

The following *in vitro* biological studies showed direct evidence of the relevant structure–activity relationships. The MPAs modification on Lys27 has little impact on potency, while the modification on Lys12 exhibited a more profound reduction in potency and Lys39 was clearly not suitable for chemical modification.
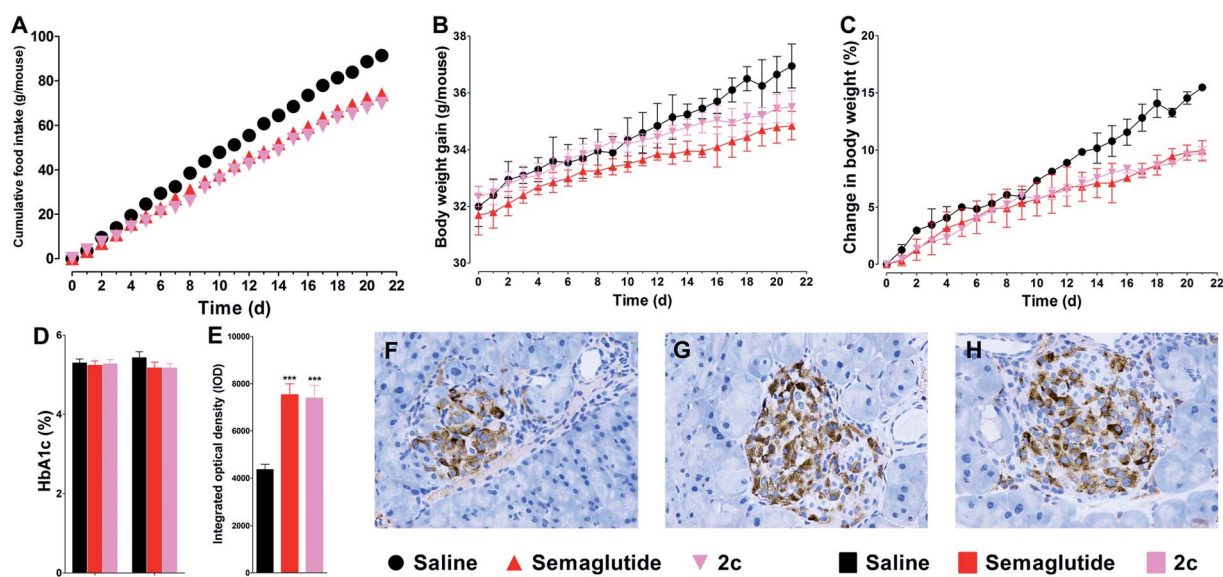

Fig. 7 Beneficial treatment effects of semaglutide and **2c** in *db/db* mice on food intake (A), body weight (B and C) and HbA1c (D). (E) Effects on pancreas development after 21 days treatment with saline, semaglutide and **2c**. Representative images of insulin staining in saline (F), semaglutide (G) and **2c** (H) groups. Means ± SD, $n = 6$. ***$P < 0.001$ *vs.* saline.

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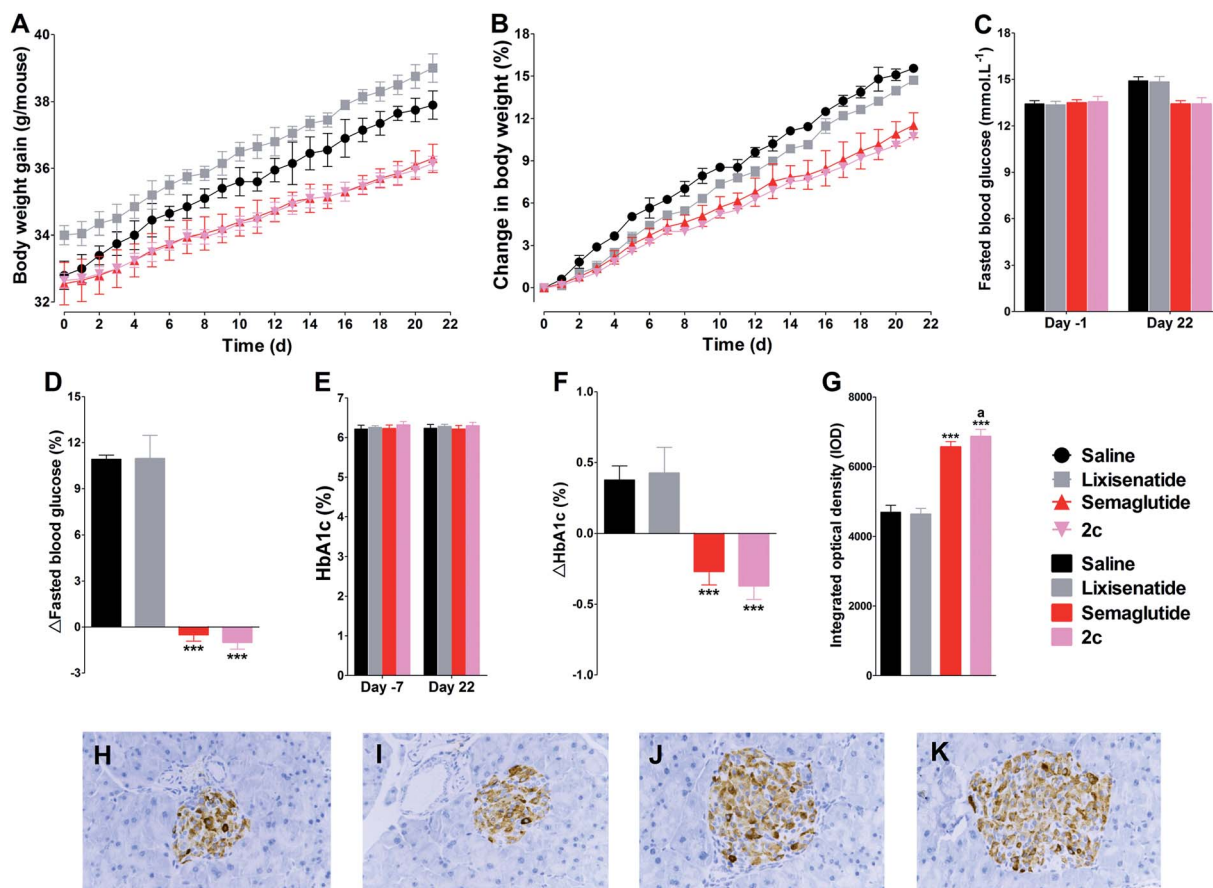*RSC Adv.*, 2020, **10**, 12089–12104 | **12099**

Fig. 8 Beneficial treatment effects of lixisenatide, semaglutide and **2c** by once weekly administration in *db/db* mice on body weight (A and B), fasted blood glucose (C and D) and HbA1c (E and F). Effects on immunohistochemistry for insulin after three weeks treatment with saline, lixisenatide, semaglutide and **2c** (G). Representative images of insulin staining in saline (H), lixisenatide (I), semaglutide (J) and **2c** (K) groups. Means ± SD, $n = 6$. ***$P < 0.001$ *vs.* saline, [a]$P < 0.05$ *vs.* semaglutide.

By using structure-inducing probe (SIP) technology, Zealand Pharma successfully discovered lixisenatide. The principle of the SIP technology is that a short sequence (5–10 amino acids) of certain charged amino acids (*e.g.*, asparagine or lysine) attached to the C-terminus of another peptide may constrain it into a more ordered conformation based on intermolecular hydrogen bonds.[50] The C-terminal hexalysine extension of lixisenatide is the main reason that lixisenatide could bind to GLP-1 receptor more tightly than exendin-4 (4-fold increase).[51] The serious potency lost observed for MPA modified lixisenatide analogues **1g–1i** (derivatized at Lys_{39}) might be attributed to two reasons. First, the chemical modification on Lys_{39} may interrupt the interaction between lixisenatide and GLP-1 receptor, thus causing serious biological activity lost. Second, the phenolic hydroxyl group on MPA may compete with other amino acid residues to form intermolecular hydrogen bonds with the C-terminus hexalysine, interfering the hexalysine-assisted formation of a more stable α-helix of the whole peptide. The N terminal portion of GLP-1 receptor agonists is crucial for mediating the cellular response to GLP-1 signaling. This explains the moderate potency lost observed for **1a–1c**, as the MPA was modified close to the N terminal of lixisenatide at Lys_{12}.

The protraction of **1a–1i** *in vitro*, however, was exhibited positive correlation with length of the alkyl chain between MPA and peptide backbone. These results provide direct evidence on the important role of the long alkyl chain linker on half-life extension. Next, the *in vivo* IPGTT study further proved that the higher GLP-1 receptor activation potencies of **1d–1f** successfully transferred to the potent *in vivo* hypoglycemic activities, and **1f** with balanced stability and biological activity was selected for the DiMPA modification. As was mentioned above, multivalent interaction commonly occurred in nature as a means to enhance the weak ligand–receptor interactions. Proper structure optimization on MPA to design DiMPA might further improve the HSA binding affinity. In the present study, DiMPA greatly improved the HSA binding affinity of lixisenatide and could further enhance when DiMPA combined with OEG spacer. We found that **2c** with proper length of OEG spacer (two OEG moieties) showed the best *in vitro* potency and *in vivo* hypoglycemic activity, this interesting finding was similar to the previous research of semaglutide, which OEG spacer played an important role in albumin binding and potency. The hypoglycemic and insulinotropic activities of **2c** were further evaluated by IPGTT on *db/db* mice, and lixisenatide and **2c** administration potent reduced blood
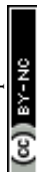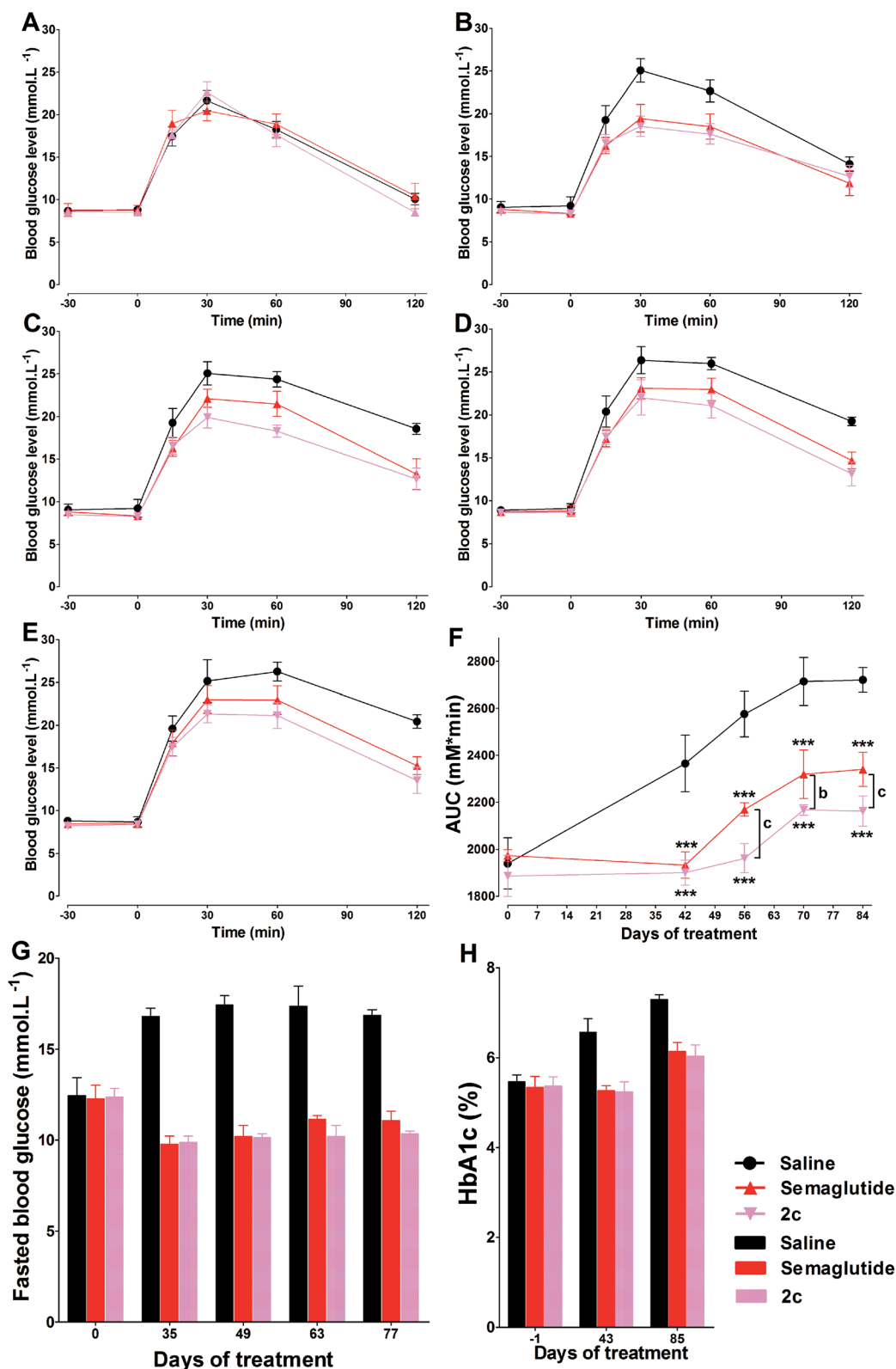
Fig. 9 Chronic treatment effects of semaglutide and **2c** in *db/db* mice. Effects of saline, semaglutide and **2c** on glucose tolerance at days 0 (A), 42 (B), 56 (C), 70 (D) and 84 (E) during the treatment period. (F) Effects of *s.c.* administration of saline, semaglutide and **2c** on glucose tolerance as measured by the glucose AUC in *db/db* mice. Effects of saline, semaglutide and **2c** on fasted blood glucose (G) and HbA1c (H) in *db/db* mice, see Methods for details. Means ± SD, $n = 6$. ***$P < 0.001$ *vs.* saline, [b]$P < 0.01$ *vs.* semaglutide, [c]$P < 0.001$ *vs.* semaglutide.

glucose levels and the insulin levels also significantly increased at 15 min. The decreasing in blood glucose levels in **2c** treated group was accompanied by the increasing plasma insulin levels which were in accordance with GLP-1R-dependent mechanism.

Subsequent long-acting hypoglycemic activity studies revealed that the duration of action of **2c** on glucose-lowering was similar to semaglutide, indicating that **2c** might has the potential to develop as a once-weekly administration antidiabetic agent. The acute toxicity test of **2c** demonstrated its low *in vivo* renal toxicity and hepatoxicity. Normally, MPA exhibited profound toxicity in mice when the administered dose over 1000 mg per kg per day,[52] which is thousands times higher than the dose of **2c** in acute toxicity test (1000 mg kg^{-1} of MPA corresponds to ∼19386.1 mg kg^{-1} of **2c**). Moreover, in clinical, the adverse effect profile of MPA is comparatively benign, and gastrointestinal adverse effects are a major concern.[53] These adverse effects are partially explained by the enhanced immune suppression. Considering the administration dose of **2c** is far lower (thousands times) than that of MPA, we envision that introduce of MPA to lixisenatide will not generate immunosuppressive effects, thus avoid potential toxicity brings by MPA modification. However, the systematic chronic toxicity tests of **2c** still need to be conducted to more carefully evaluate the toxicity of **2c**. The *in vitro* T cell proliferation test of **2c** revealed the similar immunogenic potential of **2c** with lixisenatide and semaglutide, preliminary indicated the low immunogenicity of **2c**.

The short-term and long-term anti-diabetic effects of **2c** were evaluated on *db/db* mice, in which the development of diabetes status was characterized by increased apoptosis and reduced proliferation in pancreatic β-cells.[54] A once daily injection of **2c** on *db/db* mice for 22 days achieved beneficial effects on HbA1c lowering and effectively suppressed food intake and body-weight gain. Thorough histological investigation of pancreas revealed a significant improved β-cell function following **2c** treatment. Importantly, the IOD value of insulin in **2c** group by once weekly administration was significant higher than that of in semaglutide group, indicated the better effect of **2c** on amelioration of pancreas deficiencies than semaglutide. The different results observed in once daily and once weekly treatment experiments might attribute to the different biological activities of **2c** and semaglutide, and further studies should be conducted to more carefully evaluate the stability, PK profiles and bioactivity of **2c**, and compared with semaglutide or other once weekly GLP-1 receptor agonists, such as albiglutide and dulaglutide. When we treated *db/db* mice for 42 days followed by 42 days of no treatment, compared with the benchmark of control, **2c** exhibited a sustained improvement in glycaemic control, and the beneficial treatment effects of **2c** on glucose tolerance clearly should be attributed to the restoration of pancreatic function of **2c**, and were significantly better than that of semaglutide.[55] Taken together, our studies indicated that a better sustainable effect on glycaemic control through improved pancreatic function is exerted by the DiMPA modified lixisenatide in comparison with the once-weekly GLP-1 agonist semaglutide.

5. Conclusion

Novel lixisenatide analogues with covalently attached MPA or DiMPA albumin binders were synthesized. *In vitro* studies proved the ability of MPA albumin binders to confer HSA affinity. Moreover, the unprecedented DiMPA albumin binders had significant increased affinity for HSA, which confirmed our original design. DiMPA modified lixisenatide analogues exhibited superior long-acting hypoglycemic activities *in vivo*, as a consequence of enhanced HSA affinity. We show that selected compound **2c** has a prominent preventive effect on the progressive pancreatic function deterioration seen in the *db/db* mice. In addition, we report that treatment of *db/db* mice with **2c**, improves glycaemic control greater than that of commercially available once-weekly GLP-1 agonist semaglutide. We expect that **2c** could be used for the treatment of diabetes with once-weekly administration. The present study also represents a novel method for half-life extension of peptide pharmaceuticals, which is how DiMPA albumin binders can promote longer-acting *in vivo* activity of GLP-1 analogs. We can envision that further customization of the DiMPA albumin binder in applications for specific proteins and peptides. This could include replacing the ligand moieties themselves, replacing functional groups of the ligands, changing the linker, and so forth. It can be anticipated that DiMPA albumin binder technology has potential as an alternative to traditional lipidation and PEGylation.

Abbreviations

GLP-1	Glucagon-like peptide-1
HPAC	High performance affinity chromatography
T2DM	Type 2 diabetes mellitus
DPP-IV	Dipeptidyl peptidase IV
HSA	Human serum albumin
MPA	Mycophenolic acid
IMPDH	Inosine monophosphate dehydrogenase
LC-MS/MS	Liquid chromatography-mass spectrometry
IPGTT	Intraperitoneal glucose tolerance test
OGTT	Oral glucose tolerance tests
BUN	Blood urea nitrogen
ALT	Alanine aminotransferase
SCr	Serum creatinine
AST	Aspartate aminotransferase
IOD	Integrated optical density
AUC	Area under the curve
SIP	Structure-inducing probe

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

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