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Biased agonists with less glucagon-like peptide-1 receptor-mediated endocytosis prolong hypoglycaemic effects

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ABSTRACT

Receptor endocytic trafficking entails targeting receptors and ligands to endocytic sites, followed by internalization and sorting to recycling or degradative compartments. Thus, membrane receptor-mediated signalling pathways not only contribute to the efficacy of the drugs but also play a crucial role in the metabolic elimination of peptide drugs. Glucagon-like peptide-1 (GLP-1) receptor is the crucial target for type 2 diabetes mellitus. We mainly focused on the characteristics, early evaluation of GLP-1 receptor endocytosis and effects of optimization for endocytosis on druggability. The GLP-1 receptor endocytosis characteristics of agonists were analysed by a multifunction microplate reader, flow cytometer and confocal microscope. The intracellular cyclic adenosine monophosphate (cAMP) activation of agonists was analysed based on a reporter gene assay, and intracellular β-arrestin recruitment detection was detected based on a Tango assay. We established quantitative evaluation methods of endocytosis based on fluorescently labelled agonist and receptor trafficking and used them to screen agonists with less endocytosis. Sprague-Dawley rats were used for pharmacokinetic analyses, and the hypoglycaemic activity was evaluated by intraperitoneal glucose tolerance tests (IPGTT). Our results showed that GLP-1 receptor-mediated endocytosis, as a manner of elimination, was clathrin-dependent. More importantly, we found that agonists biased towards the G protein pathway were less endocytosed by GLP-1 receptor. We screened an analogue of Exendin-4 M4, which was biased toward the G protein pathway with less endocytosis by the GLP-1 receptor. M4, which shows prolonged hypoglycaemic activities and a long half-life, can be used as a lead compound for type 2 diabetes mellitus treatment.

1. Introduction

With advances in recombinant DNA biotechnology, the number of biotech drugs, including peptides and proteins, available for clinical use has dramatically increased (Lin, 2009). However, the pharmacokinetic properties, including susceptibility to protease degradation and size-dependent renal elimination, of biotech drugs often seriously restrict their druggability (Ezan, 2013). With the development of understanding the physicochemical properties of biotech drugs, there is an emergence of biotech drugs being designed, which involves the physiological mechanisms responsible for their pharmacokinetics. Most optimizations that increase the half-life of drugs were modified using glycosylation engineering (Jung et al., 2011), polyethylene glycol (Harris and Chess, 2003) or Fc fusion (Liu, 2018). However, drug-targeted-mediated disposition, also known as receptor-mediated endocytosis, is often neglected because of incomplete research. Meanwhile, the lack of quantitative endocytosis evaluation methods makes research progress on receptor-mediated endocytosis less common.

G protein-coupled receptors (GPCRs) play an important role in many physiological and pathophysiological processes (Chan et al., 2019). Meanwhile, GPCRs are the family of protein targets most frequently targeted by approved drugs for diseases involving cancer, metabolic disorders, inflammatory diseases and neurodegenerative diseases (Sriram and Insel, 2018). Glucagon-like peptide-1 (GLP-1) receptor, as a member of the class B family of GPCRs, is the crucial target for type 2 diabetes mellitus (Hauser et al., 2017). The main effect of GLP-1 is the reduction of blood glucose, mediated by the regulation of hormonal pancreatic secretions. However, native GLP-1 has a very short half-life

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because of rapid degradation by the endogenous enzymes dipeptidyl-peptidase-IV and neutral endopeptidase. Thus, various GLP-1 receptor agonists have been developed and used, including exenatide, liraglutide, albiglutide, lixisenatide and semaglutide (Lee and Lee, 2017). These agonists are mainly targeted at protease degradation and size-dependent renal elimination without receptor-mediated endocytosis. Therefore, optimization of receptor-mediated endocytosis may be a new direction for the development of GLP-1 receptor agonists.

Physiological output receptor activation results from ligand binding, signal propagation, and endocytosis. Receptor endocytic trafficking entails targeting receptors and ligands to endocytic sites, followed by internalization and sorting to recycling or degradative compartments (Moore et al., 2007; Reiter and Lefkowitz, 2006). Thus, membrane receptor-mediated signalling pathways contribute to the efficacy of the drugs. Meanwhile, membrane receptor-mediated clearance pathways play a crucial role in the metabolic elimination of peptide drugs. However, our understanding of the characteristics of receptor-mediated endocytosis is far from complete because of the complexity of its process.

In the present study, we mainly focused on characteristics, early evaluation of GLP-1 receptor endocytosis and the effects of optimization for endocytosis on druggability. We established quantitative evaluation methods of endocytosis based on fluorescently labelled agonist and SNAP-labelled receptors. The relationship between signal bias and endocytosis and the molecular mechanism were analysed. As a result, after GLP-1 receptor endocytosis, ligands were degraded by lysosomes and transported by clathrin-dependent mechanisms. We used the evaluation methods and screened an analogue of Exendin-4 M4, which was biased toward the G protein pathway with less endocytosis by the GLP-1 receptor. As expected, M4, which shows prolonged hypoglycaemic activities and a long half-life, can be used as a lead compound in drug discovery.

2. Materials and methods

2.1. Peptides

GLP-1 (7–36)-NH_2, Exendin-4 and M1-4, analogues of Exendin-4, were synthesized by GL Biochem (Shanghai, China). GLP-1 receptor agonists P5 and No.8 were synthesized from GenScript (Nanjing, Jiangsu Province, China).

2.2. Animals

C57BL/6J mice (8 weeks old) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu Province, China), and male Sprague-Dawley (200 g) rats were obtained from Charles River (Beijing, China). All animals were housed under a 12 h:12 h light/dark cycle in a temperature- and humidity-controlled environment. Standard rodent chow (Purina 5001) and tap water were available *ad libitum*. All animal care and experimental procedures were approved by the Laboratory Animal Care and Use Committee of China Pharmaceutical University (No. SCXK [Su] 2012-0004).

2.3. Plasmids

The coding sequences of the human GLP-1 receptor (GLP-1 receptor transcript variant 1; GenBank accession no. NM_002062) were synthesized by GenScript. Then, the fragment was subcloned into pcDNA3.1, named pcDNA3.1-GLP-1R. The vector pGL4.29[luc2P/CRE/Hygro] (Promega, Madison, WI, USA) contained a cyclic adenosine monophosphate (cAMP) response element-driven luciferase reporter. The coding sequences of the SNAP tag and human GLP-1 receptor (GLP-1 receptor transcript variant 1; GenBank accession no. NM_002062) were synthesized by GenScript. The two fragments were ligated together and subcloned into pCL-neo, named pCL-SNAP-GLP-1R. The coding sequences of the human GLP-1 receptor (GLP-1 receptor transcript variant 1; GenBank accession no. NM_002062) were concatenated together with the tobacco etch virus (TEV) protease cleavage site ENLYFQL followed by a tetracycline-controlled transactivator (tTA). The recombinant sequences were synthesized by GenScript and subcloned into pCL-neo, named pCL-GLP-1R-tTA. The coding sequences of human β-arrestin 1 (ARRB1 transcript variant 1; GenBank accession no. NM_004041) were concatenated together with TEV Nla protease (amino acids 189–424 of the mature Nla protease, GenBank accession no. M15239). The recombinant sequences were synthesized by GenScript and subcloned into pcDNA3.1, named pcDNA3.1-β-arrestin 1-TEV. The coding sequences of human β-arrestin 2 (ARRB2 transcript variant 1; GenBank accession no. NM_004313) were concatenated together with TEV Nla protease (amino acids 189–424 of the mature Nla protease, GenBank accession no. M15239). The recombinant sequences were synthesized by GenScript and subcloned into pcDNA3.1, named pcDNA3.1-β-arrestin 2-TEV. The vector pTRE[Rluc/Pur] contained a tetracycline-controlled Renilla luciferase reporter.

2.4. Cell culture

MIN6 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific), 100 U/ml penicillin and 5 μg/ml streptomycin. CHO–K1 cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/ml penicillin and 5 μg/ml streptomycin. Stable CHO/GLP-1R-CRE cells were generated by pcDNA3.1-GLP-1R and pGL4.29-CRE-luc transfection and cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/ml penicillin and 5 μg/ml streptomycin. Then, the individual clone was screened with G418 and hygromycin B selection by luciferase assay.

2.5. Quenching agent investigation

MIN6 cells were plated in 35 mm glass base dishes at a density of 1×10^5 cells per dish for 18 h. For temperature investigation, cells were incubated with 1 μM FITC-labelled GLP-1 in Opti-MEM (Thermo Fisher Scientific) for 2 h at 37 °C or 4 °C, and then cells were washed with 10 mg/ml trypan blue and PBS successively. For trypan blue wash investigation, cells were incubated with 1 μM FITC-labelled GLP-1 in Opti-MEM (Thermo Fisher Scientific) for 2 h at 37 °C. Afterwards, the cells were washed with 10 mg/ml trypan blue or PBS. For energy investigation, cells were incubated with 1 μM FITC-labelled GLP-1 in Opti-MEM (Thermo Fisher Scientific) for 2 h at 37 °C after incubation with 100 μM dynasore for 30 min in advance. The nuclei were fixed with 4% paraformaldehyde and stained with DAPI (Beyotime). Fluorescent images were observed with a confocal laser scanning microscope (CLSM, LSM800, Zeiss) and processed using ZEN imaging software.

2.6. Transfections

Transient transfections were performed using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's guidelines. Cotransfection of pCL-GLP-1R-tTA, pcDNA3.1-β-arrestin 1-TEV or pcDNA3.1-β-arrestin 2-TEV and pTRE[Rluc/Pur] was performed with plasmids mixed at a ratio of 1:1:1 in CHO–K1, named β-arrestin 1 recruitment or β-arrestin 2 recruitment cells. CHO/SNAP-GLP-1R cells were generated by pCL-SNAP-GLP-1R transfection.

2.7. Western blotting

Total cell proteins were extracted using RIPA lysis buffer (Beyotime) with protease inhibitors (Thermo Fisher Scientific). The protein concentrations of cells were determined with the BCA Protein Assay Kit (Beyotime). Subsequently, the total cell proteins were electrophoresed

in a 10% SDS-PAGE gel followed by transfer to PVDF membranes (Merck Millipore). The membranes were blocked in TBST buffer with 3% bovine serum albumin for 2 h and incubated with primary antibody at 4 °C overnight. On the following day, the membranes were washed with TBST buffer five times and incubated in horseradish peroxidase conjugated goat anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA) for 1.5 h at room temperature. Then, the membranes were washed as described above. The signals were detected by chemiluminescence reagents and imaged by Tanon 5200 Multi Imaging Systems. The primary antibodies used in western blotting included anti-ACTB (1:1000, Cell Signaling Technology) and anti-SNAP (1:1000, GenScript).

2.8. LysoTracker colocalization assay

MIN6 cells were plated in 35 mm glass base dishes at a density of 1×10^5 cells per dish for 18 h. The cells were incubated with 1 μM FITC-labelled GLP-1 at 37 °C in Opti-MEM for 2 h. The cells were incubated with LysoTracker Red (1:10,000, Beyotime). The nuclei were stained with Hoechst (Beyotime). Fluorescent images were observed with confocal laser scanning microscope (Zeiss) and processed using ZEN imaging software.

2.9. Analysis of endocytic mechanism

The transport mechanism of GLP-1 receptor endocytosis was analysed by flow cytometry using inhibitors disturbing the fluorescence intensity of intracellular FITC-labelled GLP-1. MIN6 cells were pre-incubated with endocytosis inhibitors for 30 min at 37 °C. The inhibitors included dynasore (MedChemExpress, Shanghai, China), chlorpromazine (MedChemExpress), nystatin (MedChemExpress), amiloride (MedChemExpress), wortmannin (MedChemExpress), chloroquine (MedChemExpress), and deoxyglucose (MedChemExpress), whose concentrations were chosen based on the MTT assay to ensure cell vitality greater than 80%. Then the cells were incubated with 1 μM FITC-labelled GLP-1 for 2 h accompanied by inhibitors in Opti-MEM (Thermo Fisher Scientific) at 37 °C. After incubation, the cells were washed with 10 mg/ml trypan blue solution and PBS. Then, the cells were digested by trypsin, centrifuged (400 g, 5 min) to remove the medium and analysed by flow cytometry (BD Biosciences).

2.10. Endocytosis analysis based on a FITC-labelled agonist

MIN6 cells were plated in 96-well plates at a density of 1×10^4 cells per well for 12 h. Cells were incubated with agonists. Then, trypan blue solution (10 mg/ml) was used to quench surface-bound agonists. Cells were lysed and intracellular fluorescence was detected. The concentrations of endocytosed agonists were measured at 525 nm by a full wavelength multifunction microplate reader (Tecan). Known concentrations of FITC-labelled agonists were used to normalize the difference in fluorescence intensity.

2.11. Cell surface receptor internalization analysis

CHO/SNAP-GLP-1R cells were plated in 35 mm glass base dishes at a density of 1×10^5 cells per dish for 12 h. Then, the cells were labelled with 5 μM SNAP-Surface Alexa Fluor 546 (New England Biolabs) for 30 min at 37 °C, and the cells were washed with PBS. The cells were incubated with 10 nM agonists. The cells were fixed with 4% paraformaldehyde and stained with DAPI (Beyotime). Fluorescent images were observed by confocal laser scanning microscopy (Zeiss) and processed using ZEN imaging software.

CHO/SNAP-GLP-1R cells were plated in 96-well plates at a density of 1×10^4 cells per well for 12 h. Then, the cells were incubated with 1 μM BS-SS-rhodamine (Synthgene, Nanjing, Jiangsu Province, China) for 30 min to label the receptor with fluorescence. The cells were washed with PBS three times to remove redundant BS-SS-rhodamine. Then, the cells were incubated with 1 μM GLP-1 receptor agonists in Opti-MEN for 30 min at 37 °C. The cells were washed with PBS containing 100 mM MesNa three times to remove BS-SS-rhodamine that bound the cell surface or not. The cells were lysed, and the fluorescence was analysed at 550 nm by a full wavelength multifunction microplate reader (Tecan). The internalization rates of agonists were calculated according to the following equation:

$$\text{Internalization}(\%) = \frac{F(+\text{MesNa})}{F(-\text{MesNa})} \times 100$$

F(+MesNa) represents the amount of endocytosis with MesNa washing. F(-MesNa) represents the total amount binding the GLP-1 receptor without MesNa washing.

2.12. Measurement of cAMP by reporter gene assay

CHO/GLP-1R-CRE cells were plated in 96-well plates at a density of 1×10^5 cells per well for 4 h. Then, the cells were incubated with various concentrations of GLP-1 receptor agonists in Opti-MEM for 4 h at 37 °C. After incubation, the cells were lysed and quantified for luciferase activity by using the Steady-GLO luciferase assay system (Promega) using the manufacturers' protocols. Luciferase signals were analysed by a full wavelength multifunction microplate reader (Tecan).

2.13. β-arrestin recruitment analysis by Tango assay

β-arrestin 1 recruitment and β-arrestin 2 recruitment cells were plated in Opti-MEN 96-well plates at a density of 1×10^5 cells per well for 12 h. Then the cells were incubated with various concentrations of GLP-1 receptor agonists in Opti-MEM for 12 h at 37 °C. After incubation, the cells were lysed, and luciferase activity was quantified by using the Renilla luciferase assay system (Promega) using the manufacturers' protocols. Luminescence was measured by using a full wavelength multifunction microplate reader (Tecan).

2.14. Bias factors analysis

The concentration-response curves of cAMP activation were determined by nonlinear regression analysis using Prism software (GraphPad Software Inc., San Diego, CA, USA). The ligand signal bias was determined using the equiactive comparison as previously described (Rajagopal et al., 2011). The bias factors (β) were calculated according to the following equation:

$$\beta = \log\left(\left(\frac{Emax,1}{EC50,1}\frac{EC50,2}{Emax,2}\right)ligand \times \left(\frac{Emax,2}{EC50,2}\frac{EC50,1}{Emax,1}\right)reference\right),$$

where Emax was the maximal effect, EC_{50} was the half maximal concentration of the peptide, ligand was the peptide that needed to be tested, reference was the reference peptide (GLP-1); 1 was the pathway of cAMP activation; and 2 was the pathway of β-arrestin recruitment.

2.15. The intraperitoneal glucose tolerance tests

The hypoglycaemic activities of agonists in vivo were evaluated by the intraperitoneal glucose tolerance tests (IPGTT). Healthy C57BL/6J mice (n = 5/group) were randomly distributed and fasted overnight (12 h). Each group of mice were given vehicle (saline, s.c.), M4 (0.025 mg/kg, 0.05 mg/kg 0.10 mg/kg, s.c.) or Exendin-4 (0.10 mg/kg, s.c.), prior to glucose challenge (0.5 or 6 h). Mice were given glucose (2.0 g/kg, i.p.) at 0 min. Then, plasma glucose was monitored by a blood glucose metre (Sinocare Inc., Changsha, Hunan, China) at predetermined times (−30, 0, 15, 30, 60, 90 and 120 min, with the injection of glucose as time 0).

2.16. Stability test of peptides for hydrolase in blood

Exendin-4 or M4 (100 μl, 0.5 mg/ml) was incubated at 37 °C with 25% serum from Sprague-Dawley rats (diluted with PBS). Hydrolysate was collected at 0, 15, 30 and 60 min. The concentrations of Exendin-4 and M4 in the hydrolysate were measured by Exendin-4 ELISA kits (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA).

2.17. Pharmacokinetic analysis

The pharmacokinetic profiles of agonists were evaluated in male Sprague Dawley rats (n = 5/group). The rats were fasted overnight (12 h) and randomly divided into two groups. Either Exendin-4 (0.07 mg/kg, i.v.) or M4 (0.07 mg/kg, i.v.) was given to rats. Blood samples were collected from the lateral tail vein at 0, 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8 and 10 h. The concentrations of Exendin-4 and M4 in the plasma were measured by Exendin-4 ELISA kits (Phoenix Pharmaceuticals Inc.). The pharmacokinetic parameters were estimated by WinNonlin Professional Version 6.0 (Pharsight Corp., Mountain View, CA, USA).

2.18. Statistical analysis

All the data are expressed as the mean ± standard error of the mean (S.E.M.). All statistical analyses were performed via GraphPad Prism 5 (GraphPad Software Inc.). The statistical analysis was compared by Student's test or one-way ANOVA, followed by Tukey's test. Levels of ***P < 0.001, **P < 0.01 and *P < 0.05 were considered significant.

3. Results

3.1. Characterizations of GLP-1 receptor-mediated endocytosis

Endocytosis is a common phenomenon of G protein-coupled receptor activation (Sorkin and von Zastrow, 2009). However, the details of endocytosis of GLP-1 receptors, which belong to the B class G protein-coupled receptors, are still limited. Thus we attempted to investigate the character of GLP-1 receptor endocytosis via fluorescence-labelled agonists. To eliminate the effects of surface binding on the quantification analysis of endocytosed ligands, an approach to removing surface-bound and noninternalized fluorescence-labelled agonists was needed. Therefore, the effects of trypan blue solution (10 mg/ml) on quenching surface-bound agonists were first confirmed. After incubation with 1 μM FITC-labelled GLP-1 for 2 h at 37 °C, MIN6 cells were washed with trypan blue solution and PBS, and subsequently analysed by confocal microscopy (Fig. 1A). The results proved that trypan blue solution could quench surface-bound fluorescence.

To investigate the effects of temperature on GLP-1 receptor endocytosis, we performed an endocytosis assay at different temperatures. As shown in Fig. 1A, FITC-labelled GLP-1 bound to the cell surface at 4 °C, but intracellular FITC-labelled GLP-1 could be detected at 37 °C. To

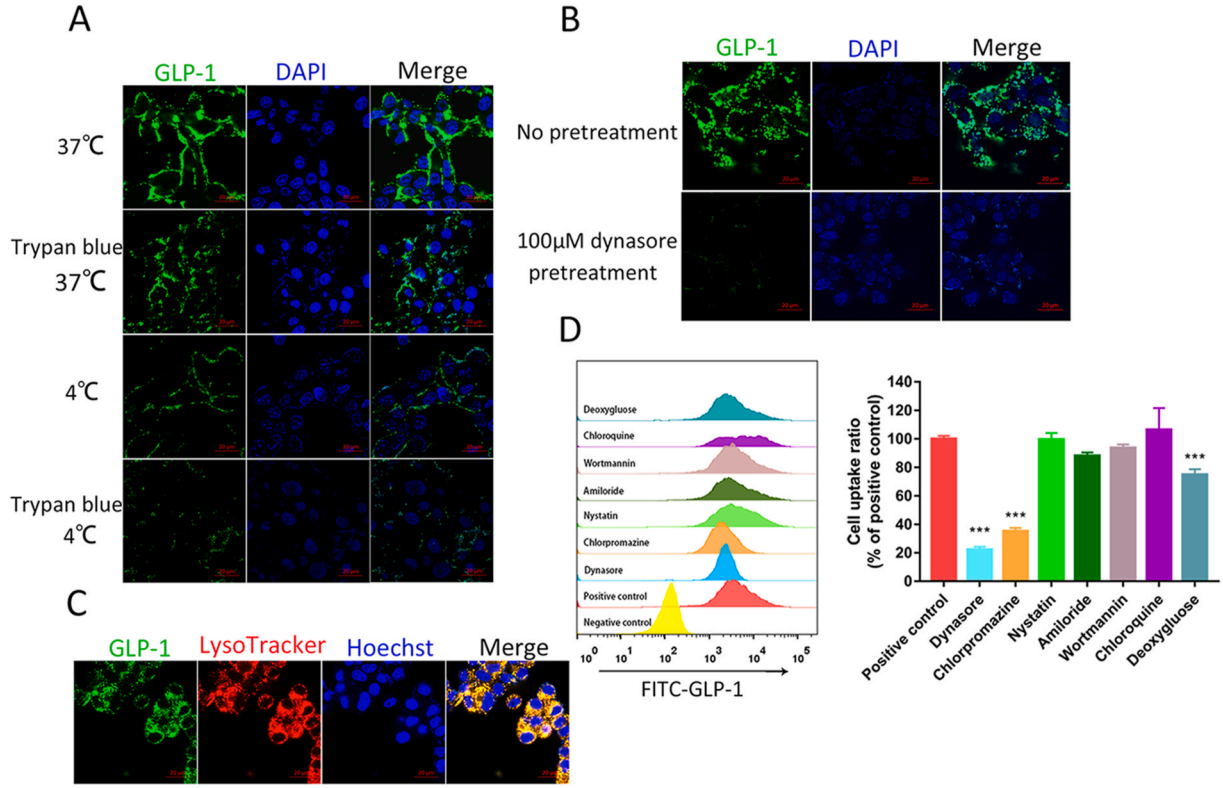

Fig. 1. Characterizations of GLP-1 receptor-mediated endocytosis. (A) Effects of trypan blue on quenching surface-bound FITC-labelled agonists and incubation at 4 °C on GLP-1 receptor endocytosis by confocal microscopy. MIN6 cells were incubated with 1 μM FITC-labelled GLP-1 in Opti-MEM for 2 h at 37 °C or 4 °C, and then the cells were washed with 10 mg/ml trypan blue and PBS. Endocytosis was analysed by confocal images and representative images from n = 3 experiments; scale bars, 20 μm. (B) Effects of dynasore on GLP-1 receptor endocytosis by confocal microscopy. MIN6 cells were incubated with 1 μM FITC-labelled GLP-1 for 2 h at 37 °C after incubation with 100 μM dynasore for 30 min in advance. Endocytosis was analysed by confocal images and representative images from n = 3 experiments; scale bars, 20 μm. (C) Colocation analysis of FITC-labelled GLP-1 with lysosomes after GLP-1 receptor endocytosis. MIN6 cells were incubated with 1 μM FITC-labelled GLP-1 at 37 °C in Opti-MEM for 2 h. The colocation of GLP-1 with LysoTracker red was analysed by confocal microscopy, representative images from n = 3 experiments; scale bars, 20 μm. (D) The traffic manner analysis of GLP-1 receptor endocytosis with inhibitors by flow cytometry. MIN6 cells were incubated with 1 μM FITC-labelled GLP-1 for 2 h preincubated by inhibitors, including 100 μM dynasore, 20 μg/ml chlorpromazine, 20 μM nystatin, 0.5 mM amiloride, 2 μM wortmannin, 100 μM chloroquine and 2 mM deoxyglucose. Quantification of the experiment normalized to the positive control. One-way ANOVA was performed vs positive control. Data are presented as the mean value ± S.E.M. ***P < 0.001, compared with the control, by the statistical test indicated above.

confirm further the role of energy in GLP-1 receptor-mediated endocytosis, we performed an endocytosis assay in the presence of energy inhibitors. As shown in Fig. 1B, FITC-labelled GLP-1 bound to the cell surface in the presence of dynasore, but intracellular FITC-labelled GLP-1 was hardly detected. The results confirmed that GLP-1 receptor endocytosis could be considered to belong to active transport with energy consumption.

To elucidate further the elimination pathway of GLP-1 receptor endocytosis, the colocalization of FITC-labelled GLP-1 and lysosomes was analysed. After MIN6 cells were incubated with 1 μM FITC-labelled GLP-1, the colocalization with lysosomes was detected by confocal microscopy. As shown in Fig. 1C, the results demonstrated that there was a colocation phenomenon between FITC-labelled GLP-1 and LysoTracker red.

To explore the transport mechanism of GLP-1 receptor endocytosis, we further investigated the endocytosis assay by inhibiting different endocytic pathways. The concentrations of the inhibitors used in the experiments were determined by MTT assay (Fig. S1). The results showed that when cells were treated with dynasore, chlorpromazine and deoxyglucose, the cellular uptake of FITC-labelled GLP-1 was significantly reduced by 78%, 65% and 25%, respectively (Fig. 1D). Chlorpromazine is a clathrin-mediated endocytosis inhibitor, and dynasore is a dynamin inhibitor that participates in clathrin-mediated endocytosis. Meanwhile, nystatin, a caveolin-mediated endocytosis inhibitor, was also used in this assay. Both chlorpromazine and dynasore but not nystatin could significantly suppress GLP-1 receptor endocytosis. Deoxyglucose, a glycolysis inhibitor, slightly depressed the fluorescence intensity increase, and chloroquine (an endosome form inhibitor) caused no significant decrease. Pre-treatment with amiloride and wortmannin, micropinocytosis inhibitors had almost no effect on the intracellular fluorescence increase. The above results suggested that GLP-1 was degraded by lysosomes after GLP-1 receptor endocytosis and that GLP-1 receptor endocytosis mainly belonged to active transport with energy consumption in the clathrin-dependent pathway.

3.2. Endocytosis analysis based on a FITC-labelled agonist and receptor trafficking

To explore the agonist-related differences in GLP-1 receptor endocytosis, endocytosis evaluation methods from the perspective of ligands and receptors were used because receptor endocytosis involved both agonists and receptors. Meanwhile, GLP-1, as a natural ligand of GLP-1 receptor, Exendin-4, as a commercial GLP-1 receptor agonist, and their derivative with more characteristic differences, No.8 (Hager et al., 2016) and P5 (Zhang et al., 2015), were assessed. A schematic diagram of the endocytosis evaluation method based on a FITC-labelled agonist is shown in Fig. 2A. The cellular uptake of agonists was quantified via known concentrations of FITC-labelled agonists. The EC_{50} of endocytosis curves indicated the half concentrations when the cellular uptake reached a plateau. The AUC of endocytosis curves reflected the intensities of endocytosis. As shown in Fig. 2B, the four agonists displayed different endocytosis characteristics. The AUC of No.8 was significantly higher than that of the other agonists. The AUC of P5 was lower than that of GLP-1 and No.8 (Fig. 2C). The AUC of P5 was slightly lower, but there was no significant difference from that of Exendin-4. On the other hand, the EC_{50} of Exendin-4 was less than that of P5, thus the cellular uptake of Exendin-4 was greater than that of P5 at low concentrations. Overall, these results suggested that the uptake of P5 was the least, and that of No.8 was the highest from the ligand perspective.

To confirm further the endocytosis characteristics of the four agonists from the perspective of receptor trafficking, a cleavable BS-SS-rhodamine probe (Fig. 3B) was used to label GLP-1 receptor with a SNAP tag via transfection. A schematic diagram of the endocytosis evaluation method based on receptor trafficking is shown in Fig. 3A. The intracellular fluorescence intensity responding to the levels of receptor internalization could be detected. Consistent with the results of ligand analysis, the internalization rate of P5 was lowest, while that of No.8 was the highest (Fig. 3C). To characterize better receptor trafficking with agonists stimulation, the receptor internalization was detected by confocal microscopy. We observed more internalized receptors when

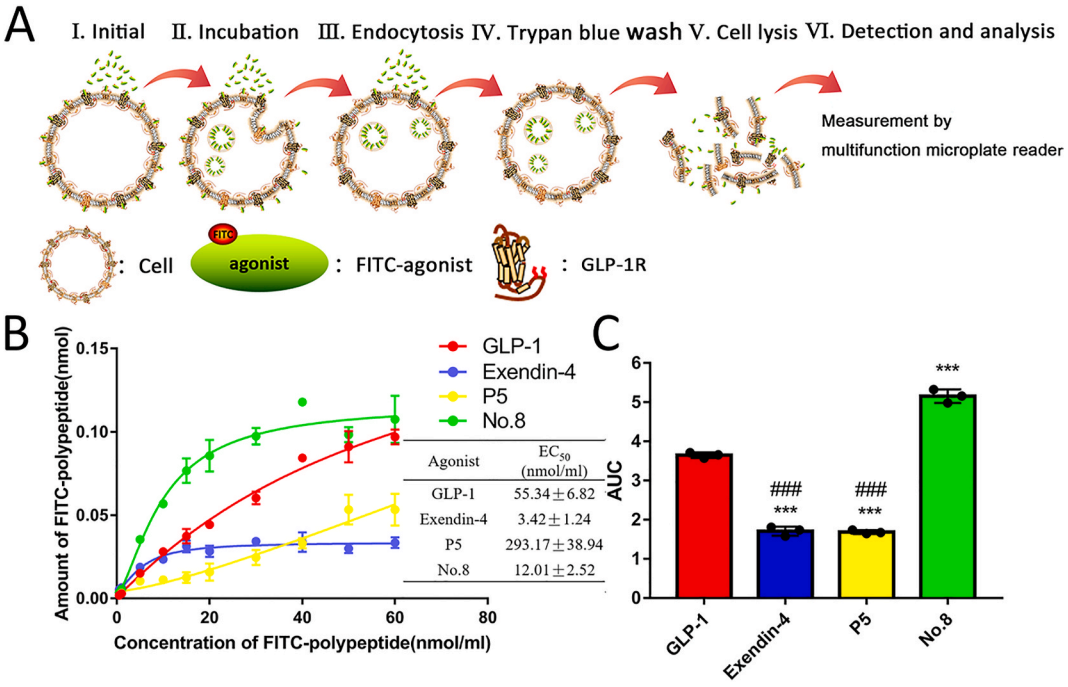

Fig. 2. Endocytosis analysis based on a FITC-labelled agonist. (A) Schematic for the evaluation method of endocytosis based on FITC-labelled agonist (B) Cells were incubated with GLP-1 receptor agonists, including GLP-1, Exendin-4, P5 and No.8, and then trypan blue solution (10 mg/ml) was used to remove surface-bound agonists. The cells were lysed, and intracellular fluorescence was detected. The endocytosis curves were formed by standard curves. (C) The AUC analysis of endocytosis curves. Data are presented as the mean value ± S.E.M. ***P < 0.001 compared with GLP-1, ###P < 0.001 compared with No.8, by the statistical test indicated above.

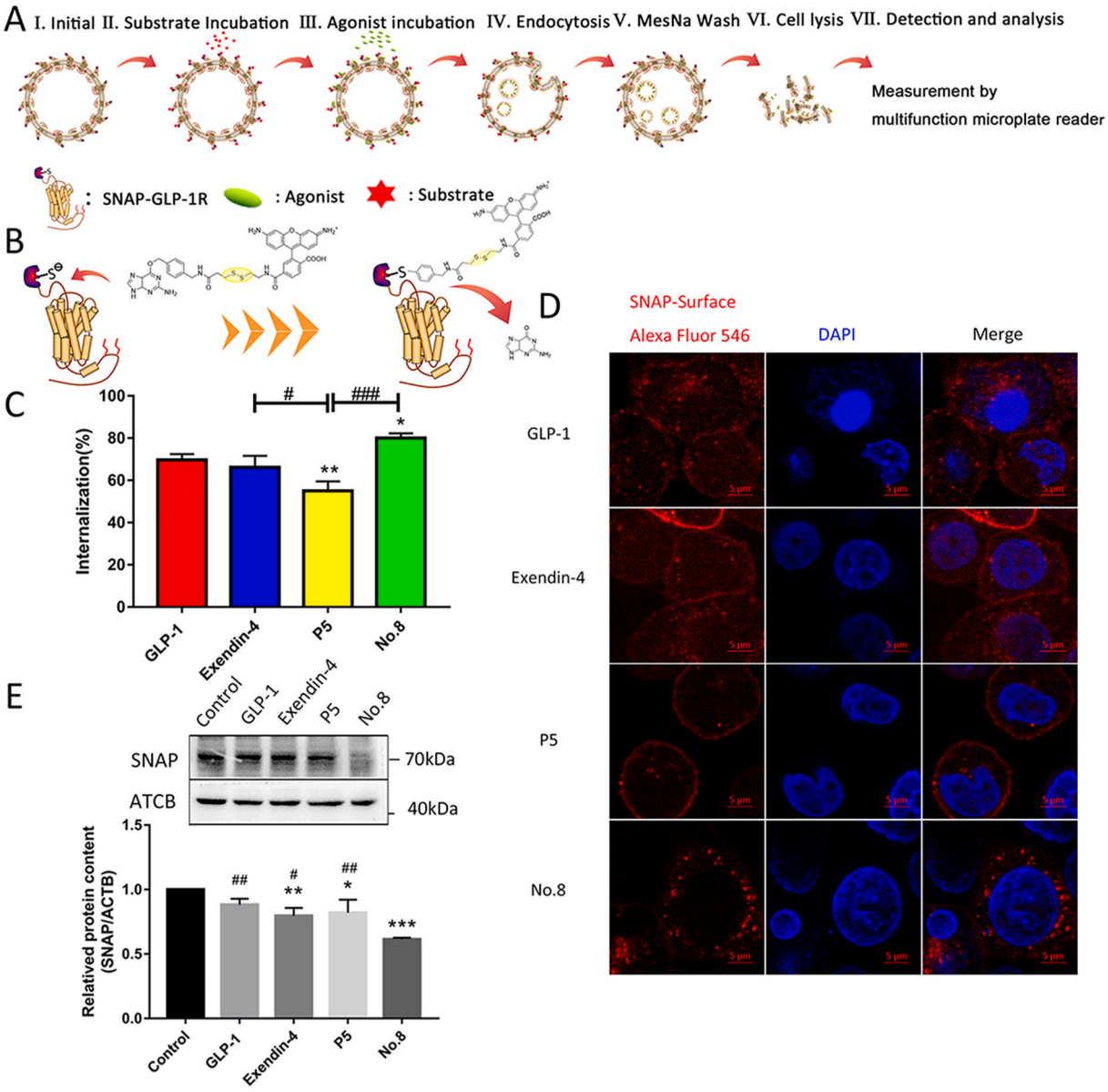

Fig. 3. Endocytosis analysis based on receptor trafficking. (A) Schematic for the evaluation method of endocytosis based on receptor trafficking. (B) Schematic for BS-SS-rhodamine (SNAP tag substrate). (C) The internalization rates of GLP-1 receptors were analysed based on receptor trafficking. CHO/SNAP-GLP-1R cells were incubated with 1 μM BS-SS-rhodamine and 1 μM GLP-1 receptor agonists (GLP-1, Exendin-4, P5 and No.8) in Opti-MEN for 30 min at 37 °C. PBS containing 100 mM MesNa was used to wash the cells three times to remove BS-SS-rhodamine that bound the cell surface or not. (D) Confocal images analysis of the SNAP-GLP-1R distribution in CHO/SNAP-GLP-1R cells treated with 1 μM GLP-1 receptor agonists (P5 and No.8) for 2 h. (E) Western blotting analysis of SNAP-GLP-1R in CHO/SNAP-GLP-1R cells treated with 1 μM GLP-1 receptor agonists (GLP-1, Exendin-4, P5 and No.8) for 6 h. Quantification of the experiment normalized to the control. Data are presented as the mean value ± S.E.M. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with GLP-1, #P < 0.05, ##P < 0.01, and ###P < 0.001 compared with No.8 or P5, by the statistical test indicated above.

exposed to No.8 and GLP-1, but much less internalization with P5 and Exendin-4 (Fig. 3D).

Because of receptor degradation as a manner of receptor trafficking, we measured receptor degradation after incubation with GLP-1 receptor agonists. As shown in Fig. 3E, there was a significant decrease in the quantity of SNAP-GLP-1R. The degradation of SNAP-GLP-1R incubated with No.8 was significantly greater than that of the others, indicating that increased degradation might be one of the reasons for the increase in internalization of the GLP-1 receptor.

All results showed that the uptake of ligands by endocytosis was mainly consistent with the level of receptor internalization. Meanwhile, the uptake of P5 was lowest by GLP-1 receptor-mediated endocytosis, and that of No.8 was the highest. To investigate the effects of endocytosis on the half-life of drugs, we chose to compare Exendin-4 and P5, because GLP-1 has an obvious hydrolysis site (Lee and Lee, 2017). It has been reported that the half-life of P5 was slightly longer than that of Exendin-4, which proved that less endocytosis was responsible for this phenomenon (Zhang et al., 2015). To investigate further the relationships of endocytosis, intracellular signals and pharmaceutical metabolism, the uptake parameters of agonists by endocytosis were analysed for subsequent study.

3.3. *The effects of intracellular signal and signal bias on GLP-1 receptor endocytosis*

For the purpose of investigating further factors to explain the

differences in endocytosis, we further explored the relationship between signalling bias and endocytosis. Therefore, we measured G protein-dependent cAMP activation and β-arrestin 1/2 recruitment of cells incubated with different agonists. The potency (EC_{50}) and maximal efficacy (Emax) of dose-response curves were determined according to cellular assays. As shown in Fig. 4B and Table 1, P5 showed slightly diminished potency and slightly increased efficacy (EC_{50} = 3.72 ± 1.78 nM; Emax = 113.90 ± 2.31%) compared with Exendin-4 (EC_{50} = 1.79 ± 0.47 nM; Emax = 99.79 ± 1.12%) in the cAMP assay. No.8 showed greatly diminished potency and steeply increased efficacy (EC_{50} = 1538.26 ± 522.23 nM; Emax = 141.60 ± 10.27%) compared with the endogenous peptide GLP-1 (EC_{50} = 25.17 ± 7.77 nM; Emax = 100%). The levels of cAMP usually reflect the pharmaceutical effects of ligands. Moreover, the binding conformation of ligands and receptors was associated with the levels of cAMP. The results suggested that all four agonists can activate the GLP-1 receptor.

The β-arrestin 1/2 recruitment assays were designed based on the Tango assay. P5 revealed greatly diminished potency and slightly diminished efficacy (EC_{50} = 42.34 ± 8.89 and 24.38 ± 2.77 nM; Emax = 89.87 ± 2.13% and 85.75 ± 2.25% for the β-arrestin 1 and 2 recruitment assays, respectively) compared with Exendin-4 (EC_{50} = 0.38 ± 0.12 and 0.82 ± 0.11 nM; Emax = 102.39 ± 2.16% and 104.59 ± 1.31% for β-arrestin 1 and 2 recruitment assays, respectively; Fig. 4B–C and Table 1). No.8 showed greatly diminished potency (EC_{50} = 271.21 ± 156.80 nM and 132.60 ± 47.63 nM for β-arrestin 1 and 2 recruitment assays, respectively) compared with the endogenous agonist GLP-1 (EC_{50} = 28.58 ± 11.22 and 11.49 ± 2.10 and for β-arrestin 1 and 2 recruitment assays, respectively). However, there was a slight decrease in efficacy between β-arrestin 1 and 2 recruitment assay when No.8 (Emax = 88.37 ± 1.78 and 92.46 ± 0.46% for β-arrestin 1 and 2 recruitment assay, respectively) compared with GLP-1 (Emax = 100 and 100% for β-arrestin 1 and 2 recruitment assay, respectively). These results showed that there were differences in β-arrestin recruitment between different agonists. GPCR endocytosis is dependent on the recruitment of β-arrestin in most cases. However, only β-arrestin recruitment makes it difficult to analyse fully the relationship between

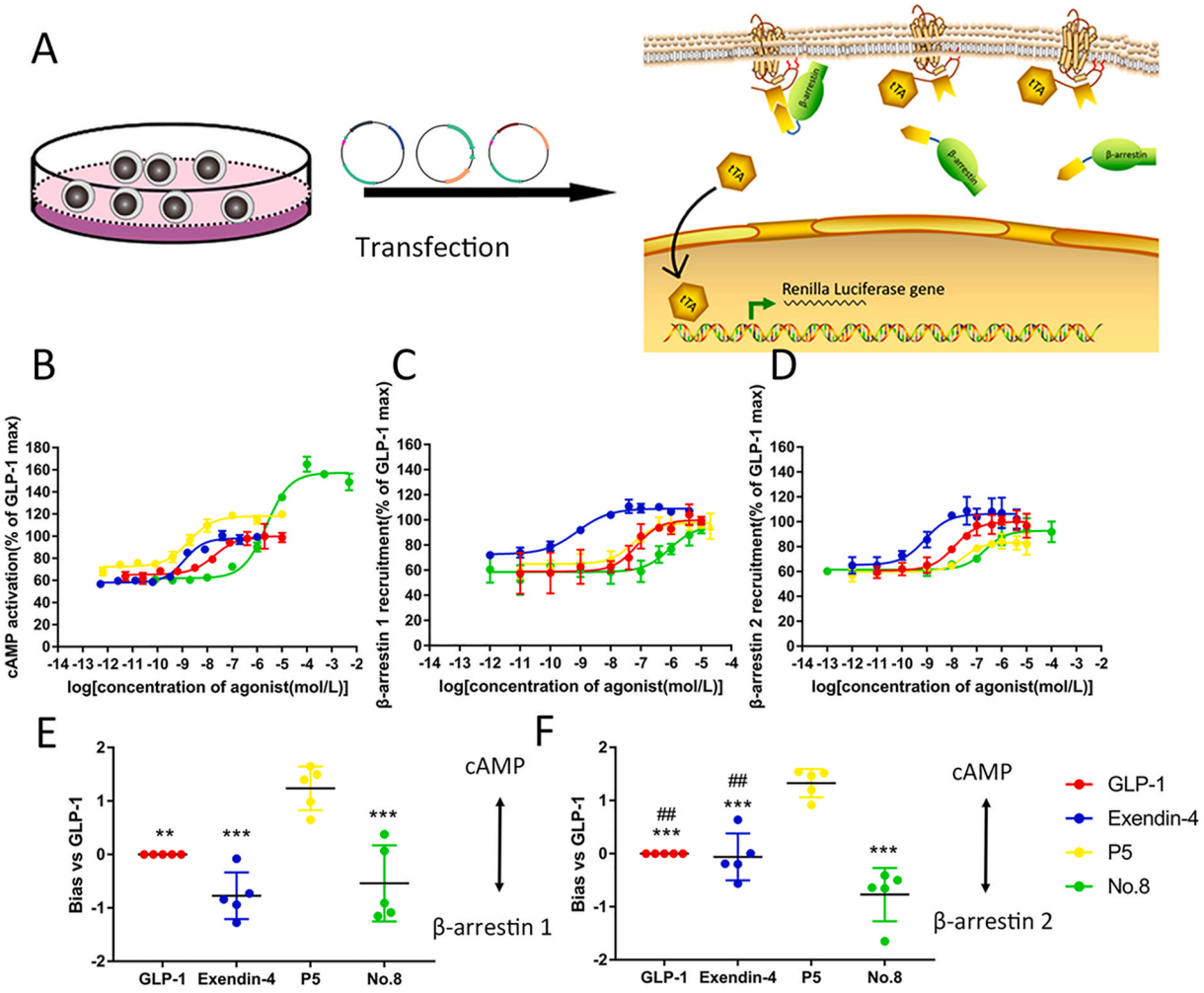

Fig. 4. Effects of intracellular signal and signal bias on GLP-1 receptor endocytosis. (A) Schematic for β-arrestin recruitment based on the Tango assay. (B) The cAMP activation of GLP-1 receptor agonists was analysed. CHO/GLP-1R-CRE cells were incubated with various concentrations of GLP-1 receptor agonists in Opti-MEM for 4 h at 37 °C. The cells were lysed and quantified for luciferase activity by using the Steady-GLO luciferase assay system. (C) β-arrestin 1 recruitment of GLP-1 receptor agonists was analysed. β-arrestin 1 recruited cells were incubated with various concentrations of GLP-1 receptor agonists in Opti-MEM for 12 h at 37 °C. After incubation, cells were lysed and quantified for luciferase activity by using the Renilla luciferase assay system. (D) β-arrestin 2 recruitment of GLP-1 receptor agonists was analysed. β-arrestin 2 recruited cells were incubated with various concentrations of GLP-1 receptor agonist in Opti-MEM for 12 h at 37 °C. After incubation, cells were lysed and quantified for luciferase activity by using the Renilla luciferase assay system. (E) Bias analysis demonstrating the relative pathway preference between cAMP activation and β-arrestin 1 recruitment of GLP-1 receptor agonists, n = 5. (F) Bias analysis demonstrating the relative pathway preference between cAMP activation and β-arrestin 2 recruitment of GLP-1 receptor agonists, n = 5. Data are presented as the mean value ± S.E.M. **P < 0.01, and ***P < 0.001 compared with P5, ##P < 0.01 compared with No.8, by the statistical test indicated above.

Table 1
Intracellular signal of GLP-1 receptor agonists.

Peptide	cAMP activation			β-arrestin 1 recruitment			β-arrestin 2 recruitment		
	EC50 (nM)	Emax (%GLP-1)	Hill slope	EC50 (nM)	Emax (%GLP-1)	Hill slope	EC50 (nM)	Emax (%GLP-1)	Hill slope
GLP-1	25.17 ± 7.77	100.00	0.96 ± 0.03	28.58 ± 11.22	100.00	0.96 ± 0.04	11.49 ± 2.10	100.00	1.01 ± 0.04
Exendin-4	1.79 ± 0.47	99.79 ± 1.12	1.05 ± 0.03	0.38 ± 0.12	102.39 ± 2.16	0.99 ± 0.04	0.82 ± 0.11	104.59 ± 1.31	0.97 ± 0.02
P5	3.72 ± 1.78	113.90 ± 2.31	1.00 ± 0.04	42.34 ± 8.89	89.87 ± 2.13	1.03 ± 0.05	24.38 ± 2.77	85.75 ± 2.25	1.04 ± 0.04
No.8	1538.26 ± 522.23	141.60 ± 10.27	0.96 ± 0.03	271.21 ± 156.80	88.37 ± 1.78	0.99 ± 0.03	132.60 ± 47.63	92.46 ± 0.46	0.99 ± 0.02
M1	38.49 ± 4.58	107.38 ± 2.14	0.97 ± 0.01	116.54 ± 58.73	110.79 ± 6.15	1.08 ± 0.06	23.18 ± 2.71	100.78 ± 2.70	0.96 ± 0.02
M2	434.86 ± 80.82	140.14 ± 7.01	1.05 ± 0.02	163.84 ± 17.43	101.87 ± 7.78	0.97 ± 0.07	102.37 ± 3.61	91.51 ± 3.62	0.99 ± 0.02
M3	7.32 ± 0.65	102.88 ± 1.17	1.01 ± 0.02	5.36 ± 3.86	148.26 ± 5.06	0.97 ± 0.02	2.42 ± 1.14	107.66 ± 3.15	0.95 ± 0.02
M4	3.02 ± 0.11	99.90 ± 3.22	0.99 ± 0.03	105.11 ± 32.62	135.50 ± 9.28	0.99 ± 0.04	47.99 ± 0.97	96.09 ± 5.38	1.07 ± 0.01

EC_{50} values represent the concentration (nM) of agonists to simulate half-maximum GLP-1 receptor activation. Emax values represent the maximum activation compared with GLP-1 maximum activation. Hill slope values represent the slope of curves. Data are presented as mean value ± S.E.M.

intracellular signals and endocytosis. Agonists with less β-arrestin recruitment may be due to their inadequate binding to the receptors. The binding conformation of receptors and agonists also affects β-arrestin recruitment, commonly reflected by G protein-dependent cAMP generation.

Biased agonists were proposed, which selectively activated different intracellular pathways, potentially regulating cellular responses including endocytosis. We therefore measured the pathway bias. Because the hill slopes of the dose-response curves were close to 1, the pathway bias factors were calculated using an 'equiactive' comparison (Rajagopal et al., 2011). The endogenous ligand GLP-1 was selected as the reference ligand. As shown in Fig. 4E and F, the results demonstrated that P5 favoured the G protein-dependent cAMP activation signalling pathway more than other agonists. However, No.8 preferred the β-arrestin recruitment signalling pathway. Exendin-4 yielded different biases in G protein-dependent cAMP activation over β-arrestin 1 recruitment or β-arrestin 2 recruitment. Summarizing the above endocytosis results, we speculated that the signalling bias of G protein-dependent cAMP activation over β-arrestin recruitment was inversely proportional to endocytosis. More samples were investigated to confirm this relationship in further studies.

3.4. Evaluation of GLP-1 receptor agonists based on endocytosis

GLP-1 receptor-mediated endocytosis is a manner of target receptor-mediated clearance. To obtain better metabolic peptides that were less endocytosed by the GLP-1 receptor, we further evaluated a series of GLP-1 receptor agonists, M1, M2, M3 and M4, which were derivatives of Exendin-4 designed as in a previous report to resist digestive enzymes in the intestinal tract (Fig. 5A) (Sai et al., 2017). First, endocytosis curves were obtained based on molarity-fluorescence intensity standard curves (Fig. 5B). M4 showed weaker endocytosis than Exendin-4 because of the lower AUC (Fig. 5C). Consistent with this result, M4 exhibited a lower receptor internalization rate than Exendin-4 (Fig. 5D). The above results suggested that M4 was less endocytosed by GLP-1 receptor than Exendin-4, which may have better pharmacokinetic characteristics and treatment effects. Consistent with the endocytosis results, there was a signal bias suggesting that M4 was more potent than Exendin-4 in G protein-dependent cAMP activation (Fig. 5E and F). In addition, more agonists were used to verify the correlation analysis of endocytosis and bias factors (Fig. 5G and H). There was good linearity between the AUC of endocytosis curves and bias factors of β-arrestin 2 ($R^2 = 0.548$, $P = 0.036$). However, the linearity between the AUC of endocytosis curves and bias factors of β-arrestin 1 was poor ($R^2 = 0.315$, $P = 0.148$).

Collectively, the above results indicated that M4 was a G protein signal-biased agonist with less metabolic elimination by GLP-1 receptor-mediated endocytosis. Furthermore, the signal bias was a good predictor of endocytosis, especially in terms of β-arrestin 2 recruitment.

3.5. Identification of M4 as a lead peptide with prolonged hypoglycaemic activities

To explore the effects of endocytosis on pharmacology, we therefore checked the hypoglycaemic activities of M4 by IPGTT. As shown in Fig. 6A, there was no significant difference in the hypoglycaemic activity of different doses of M4 and Exendin-4 in mice when agonists was injected, prior to glucose challenge for 0.5 h. However, the area under the blood glucose curve of the mice given high-dose (0.10 mg/kg) and medium-dose (0.05 mg/kg) M4 prior to glucose challenge for 6 h was significantly decreased compared to that of the Exendin-4 (0.10 mg/kg) group (Fig. 6B). There was no significant difference in the hypoglycaemic activity of low-dose (0.025 mg/kg) M4 and Exendin-4 (0.10 mg/kg) in mice (Fig. 6B). The results suggested that M4 had prolonged hypoglycaemic activities.

To investigate the effects of endocytosis on metabolism, the pharmacokinetics of M4 and Exendin-4 were measured in SD rats. As shown in Fig. 6C, the plasma concentration of Exendin-4 quickly declined to baseline 6 h postinjection with a circulating elimination half-life of 0.32 ± 0.04 h (Table 2). M4 exhibited a prolonged plasma duration with a half-life of 1.80 ± 0.23 h (Table 2). In addition, M4 resulted in an increased AUC value, which was 5.53-fold greater than that of Exendin-4. To confirm whether the difference in agonists in half-life was caused by the difference in enzymatic hydrolysis in the blood, we analysed the retention rates of M4 and Exendin-4 at different incubation times in the blood. The results suggested that there was no difference observed between the two in the retention rates (Fig. 6D).

All these results demonstrated that M4 had prolonged hypoglycaemic activities, because of its long half-life.

4. Discussion

The pharmaceutical effects of peptides, therapeutic proteins and monoclonal antibodies are directly related to their target membrane receptors (Hutchings et al., 2017). Accompanied by their efficacy, drugs are also affected by target receptor-mediated clearance. Thus, target receptor endocytosis contributes to not only pharmacodynamics but also pharmacokinetics (Lin, 2009). The glucagon-like peptide-1 receptor is a member of the G protein-coupled receptors, and it has been widely explored as a drug target in diabetes (Muller et al., 2019). However, our understanding of endocytosis induced by GLP-1 receptor agonists is still limited. Thus, in the present study, we mainly focused on characteristics, early evaluation of GLP-1 receptor endocytosis and the effects of optimization for endocytosis on druggability. We found that the agonists underwent lysosomal degradation after GLP-1 receptor endocytosis as an important metabolic elimination mechanism in a clathrin-dependent manner. The endocytic characteristics of different agonists were analysed by evaluation methods of endocytosis based on agonists and receptors. Our study indicated that GLP-1 receptor agonists with a G protein bias were weak stimulators of endocytosis whereas agonists with

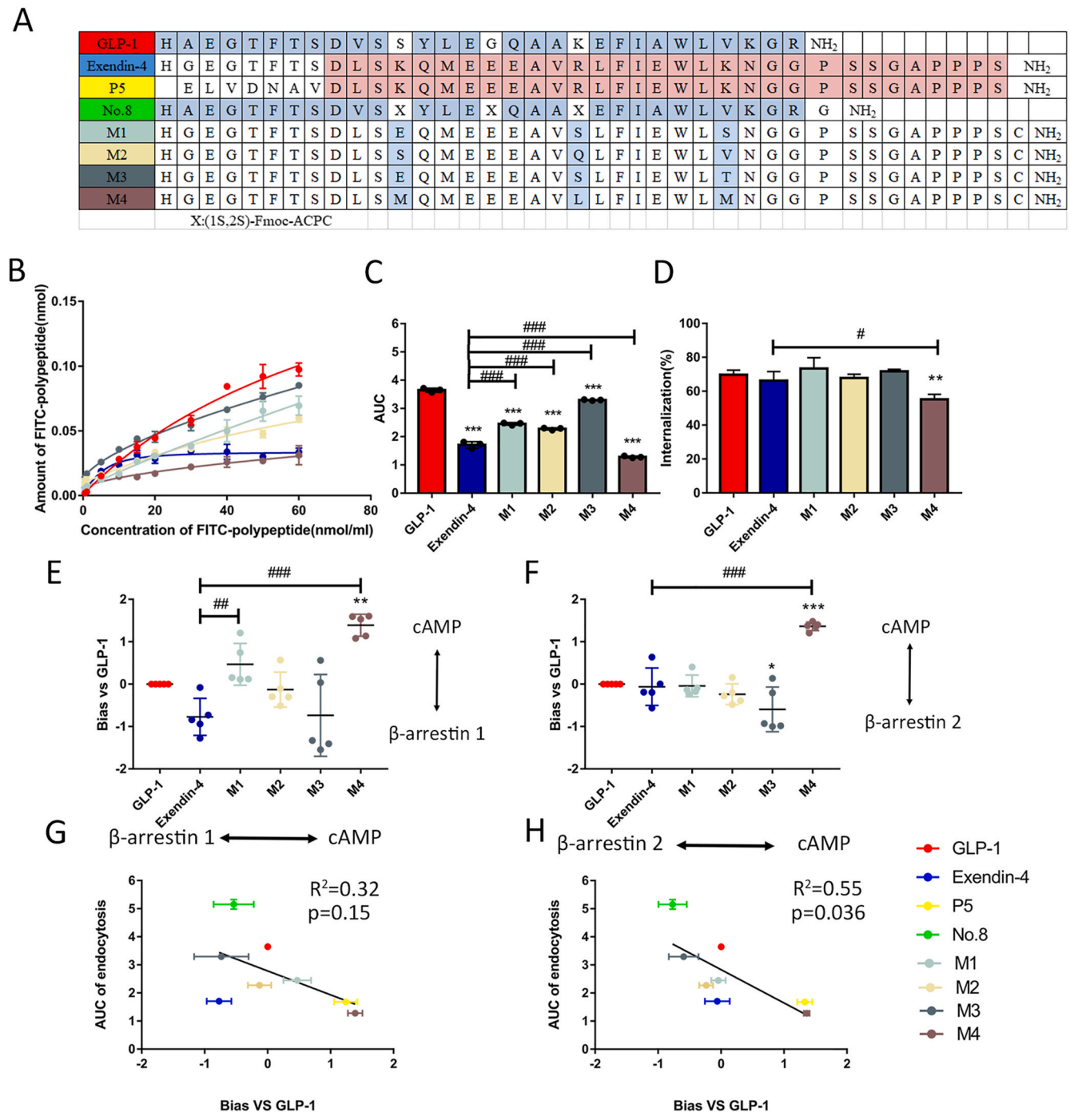

Fig. 5. Evaluation of GLP-1 receptor agonists based on endocytosis. (A) Sequences in single letter amino acid code for GLP-1 receptor agonists. (B) Analysis of the endocytic properties of agonists based on FITC-labelled agonists. (C) AUC analysis of endocytosis curves of agonists. (D) Analysis of the endocytic properties of agonists based on endocytosis based on receptor trafficking. (E) Bias analysis demonstrating the relative pathway preference between cAMP activation and β-arrestin 1 recruitment of GLP-1 receptor agonists, n = 5. (F) Bias analysis demonstrating the relative pathway preference between cAMP activation and β-arrestin 2 recruitment of GLP-1 receptor agonists, n = 5. (G) Correlation analysis between signal-biased factors (between cAMP and β-arrestin 1) and the AUC of the endocytosis curves of GLP-1 receptor agonists. (H) Correlation analysis between signal biased factors (between cAMP and β-arrestin 2) and the AUC of the endocytosis curves of GLP-1 receptor agonists. Data are presented as the mean value ± S.E.M. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with GLP-1, #P < 0.05, ##P < 0.01, and ###P < 0.001 compared with Exendin-4, by the statistical test indicated above.

a β-arrestin 2 bias were stronger inducers of endocytosis. Moreover, screening by endocytosis evaluation, we have described a novel GLP-1 receptor agonist peptide, M4, which has long-lasting hypoglycaemic effects with less metabolic elimination by GLP-1 receptor endocytosis.

Endocytosis, including clathrin-dependent endocytosis pathways and clathrin-independent endocytosis pathways, is a vital physiological process by which molecules at the cell surface enter internal membrane compartments. Many signal receptors are stimulated to endocytose via clathrin-coated pits. In addition, it was suggested that some receptors underwent clathrin-independent endocytosis, such as transforming growth factor-β receptors (Sorkin and von Zastrow, 2009). However, there are some controversies regarding whether the GLP-1 receptor uses clathrin- or caveolin-mediated endocytosis for agonist-induced internalization. In theory, GLP-1 receptor endocytosis occurs in a

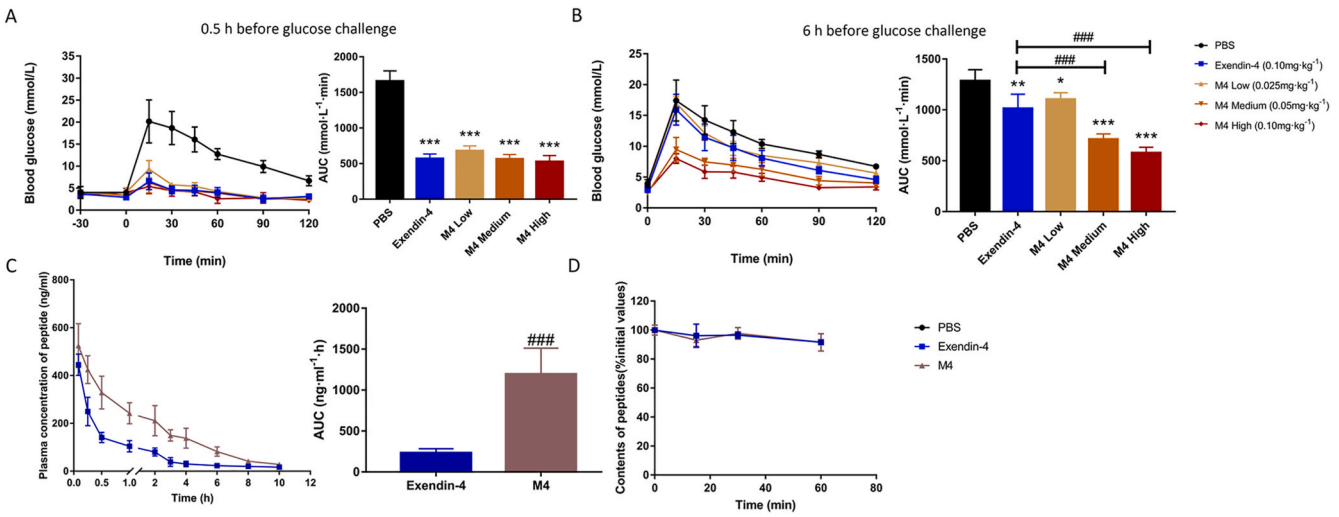

Fig. 6. Hypoglycaemic activities and pharmacokinetic profiles of M4. (A) IPGTT performed in C57BL/6J mice with high-dose M4 (0.10 mg/kg, s.c.) administration, medium-dose M4 (0.05 mg/kg, s.c.) administration, low-dose M4 (0.025 mg/kg, s.c.) administration, Exendin-4 (0.10 mg/kg, s.c.) administration and vehicle, respectively, prior to glucose challenge for 0.5 h, and the area under the glucose level curve (AUC) of the IPGTT. (B) The IPGTT performed in C57BL/6J mice with high-dose M4 (0.10 mg/kg, s.c.) administration, medium-dose M4 (0.05 mg/kg, s.c.) administration, low-dose M4 (0.025 mg/kg, s.c.) administration, Exendin-4 (0.10 mg/kg, s.c.) administration and vehicle, respectively, prior to glucose challenge for 6 h, and the area under the glucose level curve (AUC) of the IPGTT. (C) Pharmacokinetic profiles of M4 and Exendin-4 in Sprague-Dawley rats. Plasma concentrations versus time curves and AUC values of M4 and Exendin-4 were analysed. (D) Stability analysis of peptides for hydrolase in blood. Exendin-4 or M4 (100 μl, 0.5 mg/ml) was incubated at 37 °C with 25% serum from Sprague-Dawley rats (diluted with PBS). The hydrolysate was collected at 0, 15, 30 and 60 min. The concentrations of Exendin-4 and M4 in the hydrolysate were measured by Exendin-4 ELISA kits. Data are presented as the mean value ± S.E.M. ***P < 0.001 compared with PBS, ##P < 0.01 and ###P < 0.001 compared with Exendin-4, by the statistical test indicated above.

Table 2
Pharmacokinetic profiles of M4 and Exendin-4 in Sprague Dawley rats.

Group	*n*	Dosage (mg·kg^{-1})	$t_{1/2}$ (h)	C_{max} (ng·ml^{-1})	$AUC_{0\text{-}t}$ (h·ng^{-1}·ml^{-1})	CL_{obs} (ml·h^{-1}·kg^{-1})
Exendin-4	5	0.07	0.32 ± 0.04	500.38 ± 24.00	232.65 ± 23.03	314.16 ± 33.90
M4	5	0.07	1.80 ± 0.23	470.06 ± 33.21	1194.60 ± 142.50	61.51 ± 6.17

$AUC_{0\text{-}t}$ represent area under the plasma concentration versus time curve from time 0 to the last measurable concentration; CL_{obs} represent the volume of plasma that is completely cleared of drugs by all routes of elimination; C_{max} represent peak plasma concentration of drugs; n represent number of animals. Data are presented as mean value ± S.E.M.

clathrin-dependent manner, as GLP-1 receptors are GPCRs. Buenaventura et al. also reported that clathrin was mainly involved in GLP-1 receptor internalization, and it was indicated that Huntingtin-interacting protein 1, a clathrin-dependent endocytosis modulator, regulated GLP-1 receptor trafficking and signalling (Buenaventura et al., 2018). However, some researchers took different views that GLP-1 receptor interacting with caveolin-1 was the mechanism that regulated receptor trafficking by colocalization (Syme et al., 2006). In the latest research, it was indicated that the main pathway of GLP-1 receptor entry into cells was clathrin-mediated by colocalization of GLP-1 receptor and AP2 (Buenaventura et al., 2019). In this study, we used different transport inhibitors to investigate the effects on endocytosis. The results demonstrated that GLP-1 receptor endocytosis occurred in a clathrin-dependent manner, while caveolin was involved in other forms of endocytosis accompanied by GLP-1 receptor endocytosis or rarely.

Target-mediated disposition is the interaction of drugs with their targets, and the targets are receptors in most cases. Once bound, drugs subsequently experience endocytosis and lysosomal degradation (Ezan, 2013). To analyse further the influence of GLP-1 receptor endocytosis on pharmacokinetics, the postendocytosis traces were analysed. The colocalized fluorescent signals indicated that the agonists underwent lysosomal degradation after endocytosis as an important metabolic elimination. ING-1, an engineered monoclonal antibody against epithelial cell adhesion molecules, was eliminated faster in mice with human HT-29 colon tumours than in healthy mice (Ammons et al., 2003). Recombinant human macrophage colony stimulating factor had a similar phenomenon (Bauer et al., 1994). These results strongly supported that target-mediated clearance had important effects on drug metabolism.

To explore endocytosis and its influencing factors, quantitative evaluation methods of endocytosis are required. As a result of agonist-receptor interactions, endocytosis should be explored from two aspects: ligands and receptors. The endocytosis evaluation method of the ligand was based on a FITC-labelled agonist. Thus, surface-bound and noninternalized agonists seriously affected the accurate quantification of endocytosis. The effective quenching dye should have an absorbance spectrum that significantly overlaps the fluorescence emission of others. The peak absorbance of trypan blue is 607 nm. The emission of FITC is 520–530 nm, so trypan blue was used as a quencher with green emitters, since it interfered with the fluorescent signal at a longer wavelength of the spectrum (Shilova et al., 2017). Using this method, we found that GLP-1, Exendin-4, P5 and No.8 exhibited different endocytic properties, mainly expressed in the EC_{50} and AUC of endocytosis. Moreover, No.8 was more endocytosed and P5 was less endocytosed. Based on an engineered variant of the DNA repair protein O^6-alkylguanine-DNA-alkyltransferase that covalently reacts with O^6-benzylguanine, the SNAP tag was a powerful tool to label the receptors in the cells (Cole and Donaldson, 2012). Using the SNAP tag, the purpose of fluorescence-labelled receptors was achieved. Furthermore, quantitative analysis of receptor internalization confirmed the results obtained by the ligand evaluation method. Although there was a slight difference

between the two results, it was probably due to the amount change of surface receptors and receptor recycling.

GPCR signalling is mainly regulated by interactions with three proteins families, including G proteins, receptor kinases and β-arrestins. Classically, when agonists bind receptors, they cause conformational changes, guanine exchange factors catalyse the G protein, and the subunits of the G protein promote the second-messenger effectors such as cAMP. Nonclassically, receptors are generally phosphorylated on their cytoplasmic loops and C-terminus, which stimulate β-arrestins binding to the receptors (Wootten et al., 2018). Moreover, β-arrestin also couples many signal mediators, such as mitogen-activated protein kinases. This situation is named signal bias in which the agonists stimulate the unique receptor conformation that results in differential coupling to the signal transduction cascades. These ligands that selectively trigger either G protein or β-arrestin signalling are named biased ligands. In our study, the relationship between signal bias and endocytosis was explored. According to the reporter gene assay and Tango assay, the capacities of agonists in cAMP activation and β-arrestin recruitment were measured. Meanwhile, the bias factors were determined using the equiactive comparison. P5 showed a relative preference of the cAMP signal compared to the β-arrestin signal. No.8 showed a relative preference of the β-arrestin signal compared to the cAMP signal. The results were basically consistent with those reported in previous papers (Hager et al., 2016; Zhang et al., 2015). Only for β-arrestin recruitment was it difficult to analyse fully the relationship between intracellular signals and endocytosis. Agonists with less β-arrestin recruitment may be due to their inadequate binding to the receptors. The binding conformation of receptors and agonists also affects β-arrestin recruitment, commonly reflected by G protein-dependent cAMP generation. Thus, we explored the effects of signal bias on GLP-1 receptor endocytosis. We discovered that agonists were less endocytosed and were more biased towards the G protein signalling pathway. Biased factors of G protein-dependent cAMP activation over β-arrestin 2 recruitment were highly predictive of endocytosis, but biased factors of G protein-dependent cAMP activation over β-arrestin 1 recruitment were not. The reason for this phenomenon was that β-arrestin 1 did not affect the internalization of receptors or less. The main functions of β-arrestin 1 are in terms of signal transduction (Tocci et al., 2019). Nevertheless, β-arrestin 2 enhanced receptor endocytosis by G protein receptor kinase 5. Furthermore, it was indicated that β-arrestin 2 was involved in trafficking of other receptors (Cerniello et al., 2017).

To explore further the effects of endocytosis on druggability, we screened and analysed a series of GLP-1 receptor agonists. It was shown that M4 showed a relative preference for cAMP signalling compared to Exendin-4. Mainly consistent results were obtained based on two evaluation methods of endocytosis: M4 was less endocytosed than Exendin-4. To investigate the effects of endocytosis, as a metabolic elimination mechanism, on the efficacy and half-life of the drug, we explored the hypoglycaemic effect of M4 by IPGTT. When the drugs were added 30 min prior to glucose, there was no significant difference in hypoglycaemic activity. However, M4 showed a better hypoglycaemic effect than Exendin-4 prior to glucose 6 h. As expected, the pharmacokinetic results showed that M4 had a longer half-life, causing a longer hypoglycaemic effect. For most peptides, their molecular weight is generally well below 10 kDa. Therefore, they would be filtered by the glomerulus in the kidney, depending on their molecular size (Deen et al., 2001). As a result of the small difference in amino acids and molecular weight between M4 and Exendin-4, their renal elimination was basically similar. Peptide drugs undergo proteolytic degradation and proteolytic enzymes in the blood play a more important role in their metabolism (Werle and Bernkop-Schnürch, 2006). Their retention rates in the blood proved that there was no significant difference in enzymatic hydrolysis between the two agonists. Exendin-4 and M4 have no definite glycosylation site, and both are chemically synthesized. Thus, they are not cleared by glycoprotein receptors. Receptor-mediated endocytosis may be responsible for better hypoglycaemic effects, excluding other metabolic elimination mechanisms. However, this effect may not be exclusively through modulation of receptor-mediated endocytosis in an elimination manner. Jones et al. reported that a set of agents with specific GLP-1 receptor trafficking profiles, including internalization, recycling and receptor desensitization, had greater efficacy and tolerability as a T2D treatment (Jones et al., 2018). Thus, the advantages in GLP-1 receptor trafficking and desensitization may be the reasons for the better hypoglycaemic effects of M4. However, the effects of endocytosis on drug metabolism can not be ignored.

5. Conclusion

In summary, our results showed that GLP-1 receptor-mediated endocytosis, as a manner of elimination, was clathrin-dependent. More importantly, our research found that agonists biased towards the G protein pathway were less endocytosed by the GLP-1 receptor. We established quantitative evaluation methods of endocytosis based on fluorescently labelled agonists and SNAP-labelled receptors. We used the evaluation methods and screened M4, which was biased towards the G protein pathway with less endocytosis by the GLP-1 receptor. M4, which shows prolonged hypoglycaemic activities and a long half-life, can be used as a lead compound for type 2 diabetes mellitus treatment.

Author contributions

Q.W. and S.C. designed experiments and wrote the main manuscript text, Q.W., H.Z., N.X. and Q.Y. performed experiments and gathered the data. W.Y. and X.G. reviewed and edited the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174203.

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