Azapeptide-based GLP-1 receptor agonist improves glucose metabolism in a diet-induced obesity model

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Abstract:

Peptidomimetics, synthetically modified peptides which have been engineered to eliminate certain undesirable features of native therapeutic peptides including short half-lives, immunogenicity, and delivery challenges, are a growing class of peptide-based therapeutics. Azapeptides (αC to N substitution(s)), a type of peptidomimetic with several examples in clinical use, are known to exhibit increased resistance to endogenous proteases, extending plasma half-life, while potentially retaining or enhancing biological activities and therefore therapeutic efficacy. Previously, we have developed a unique synthesis platform that enables rapid and efficient solid-phase synthesis of custom azapeptides using standard peptide synthesizer technology. To test the capabilities of our azapeptide synthesis platform for a therapeutically relevant peptide of longer length, we designed and synthesized several 31 amino-acid long analogs with aza-amino acid substitutions at critical sites of semaglutide, a popular type 2 diabetes and weight loss, glucagon-like peptide-1 (GLP-1) receptor agonist. The leading receptor agonist analogue (AzaA^{8}/R^{34}-GLP-1) was identified based on its protease resistance, ex vivo whole blood stability, in vitro receptor potency (cAMP signaling), , and efficacy in a high fat diet-induced obesity murine model. This AzaGLP-1 analogue displays significant biological activity and therapeutic efficacy on glucose metabolism which demonstrates its potential for further development as a therapeutic agent.

Introduction:

Worldwide, the rate of metabolic syndrome and obesity-related type 2 diabetes (T2D) in all ethnic groups has steadily increased. Poor dietary choices, combined with lack of exercise and sedentary lifestyle promote an increased adipose tissue mass and deposition of fat in other organs which results in both insulin and leptin resistance, chronic inflammation, and the disruption of multiple downstream pathways. The state of obesity increases the possibility of progression to end-organ damage and diseases such as diabetes, atherosclerosis, non-alcoholic fatty liver disease (NAFLD) (aka- metabolic dysfunction-associated steatotic liver disease (MASLD)), and others.(1-3) Just one of the new line of treatments for metabolic disease (obesity/T2D) includes the incretin peptides modeled around endogenous glucagon-like peptide-1 (GLP-1), which bind and activate the GLP-1 receptor.(4) GLP-1 receptor agonism mediated by endogenous GLP-1 has been shown to increase pancreatic β-cell insulin secretion, stimulate peripheral and central GLP-1 receptors augmenting satiety, delay gastric emptying, inhibit beta-cell apoptosis, promote beta-cell neogenesis, reduce glucagon secretion, and also increase peripheral glucose disposal (reviewed in (5)). New GLP-1 receptor agonists are continuing to be developed not only for type 2 diabetes and obesity, but also in other conditions and diseases including, metabolic dysfunction-associated steatohepatitis (MASH) , neurodegenerative diseases, musculoskeletal inflammation, osteoporosis(6), and some types of cancer (reviewed in (7)).

The active GLP-1 peptide(s) (7-36$_{NH2}$, 7-37) have an extremely short half-life, rendering them unsuitable as viable therapies. Therefore, peptide modifications to the GLP-1 sequence have been engineered to enhance therapeutic features.(8) The popular diabetes and weight loss drug Ozempic® (semaglutide) is the most well-known of this class and was designed to overcome the rapid catabolism and clearance of the native peptide. Its structure is highly similar to native GLP-1(7-37) but substitutes α-aminoisobutyric acid (Aib) in place of alanine (A) at position 8, arginine (R) replaces lysine (K) at position 34, and it includes a spacer linked to an 18-carbon fatty diacid bridged at lysine 26, which allows reversible binding to serum albumin increasing blood half-life time and slowing clearance. The alanine at position 8 is a well-known target of the main protease dipeptidyl peptidase-4 (DPP-4), and catabolism at this peptide bond is accountable for a significant degree of native GLP-1's (7-37)) brief in vivo half-life. Many studies have investigated GLP-1 analogues with substitutions/modifications at protease/peptide cleavage sites, but until recently there had been no investigations of the efficacy of the GLP-1 (7-37) peptide substituted with aza-amino acid residues (azapeptides). Compared with natural peptides, azapeptides have the potential advantage of being more resistant to endogenous proteases and peptidases due to a nitrogen substitution of the α-carbon of one or more amino acids in the peptide chain. This conservative atomic substitution in aza-amino acid(s) may preserve more of the peptide's 3-D structure, thus maintaining or even enhancing key features of ligand/receptor binding and downstream signaling, creating preferred therapeutic peptide characteristics.(9, 10)

Previously, our group has engineered a platform method using patented pre-activated aza-amino acid building blocks that can be incorporated into most peptide sequences using standard solid-phase peptide synthesis instruments.(11, 12) This biochemical engineering advancement was initially tested using two therapeutic peptide examples, namely an high mobility group box-1/myeloid differentiation group-2/toll-like receptor-4 (HMGB1/MD2/TLR4) antagonist (4 amino acids) and bradykinin (9 amino acids). In both instances, the aza-analogues not only displayed comparable or preferred therapeutic activity in vitro and in vivo, but also possessed significantly increased half-lives, relative to the natural peptides.(13) Using this patented azapeptide synthesis platform, libraries of aza-substituted therapeutic peptide candidates can be conveniently and efficiently synthesized for screening and selection in relevant biological assays. We synthesized a number of aza GLP-1 receptor agonist analogues and compared them in in vitro assays of stability (dipeptidyl peptidase-4 (DPP-4) resistance, ex vivo whole blood half-life($t_{1/2}$)) and biological activity including (GLP-1 receptor-activated cAMP). The lead AzaA8/R34 GLP-1 (AzaA8) was selected based on its in vitro pharmacological properties and then assessed for its effects on glucose metabolism in an acute study (single treatment dose) in lean (chow-fed) animals, and a chronic study (4wks daily injection) in a high fat diet induced obesity model.

Results:

For these studies, we compared semaglutide and exendin-4 (53% homology to GLP-1(14)), two clinically approved GLP-1 receptor agonists, and in some cases unlipidated semaglutide, the root peptide of semaglutide (Aib^8/R^{34}-GLP-1), with select aza-GLP-1 analogues. The structure/sequence and purity of test articles was confirmed using LC-MS. Our initial structure-activity relationship and screening assays were designed to study the effects of site-directed insertion of aza-amino acids at DPP-4 cleavage sites on both stability (DPP-4 resistance ($t_{1/2}$), ex vivo whole blood half-life) and in vitro receptor potency (cAMP signaling). Figure 1 illustrates the sequence/structures of glp-1 peptides and leading aza-analogue (Fig.1a) with DPP-4 cleavage site indicated by an arrow, and optimized platform synthesis methods used for generating peptides/azapeptides (Fig. 1b).

In vitro dipeptidyl peptidase-4 (*DPP-4) stability*

DPP-4 is an abundant prolyl oligopeptidase that exists as both an integral membrane protein, and in a soluble form which when cleaved, can circulate in plasma. It has a widespread tissue distribution and catalyzes the hydrolysis of the first two N-terminal amino acids (aa) of various(less than 80 residue) peptides (Xaa, Pro) (where Pro, Ala, Gly, Ser, Val, Leu can occupy the second aa position and third aa is not Pro), including endogenous GLP-1 (7-37), leading to its inactivation.(15) This enzyme was incubated for up to 24 hours with R34-GLP-1(7-37), unlipidated semaglutide (Aib8/R34-GLP-1), and the aza-GLP-1 analogues AzaA8 (azaA^{8}/R^{34}-GLP-1), AzaH7/R34, AzaH7/Aib8/R34, and AzaH7/azaE9/R34 yielding half-life values of 0.3+/-0.07, 6, and greater than 24 hours for the remaining aza-GLP-1 analogues respectively (calculated based on recovery/quantitation of test articles using HPLC). We did not detect significant catabolism of any Aza-GLP-1 analogues, even up to 48hrs. These results indicated that the aza-substitutions around the N-terminal region more efficiently prevented DPP-4 cleavage as compared with the Aib8 substitution of semaglutide (unlipidated semaglutide). (Table 1)

Ex vivo stability in human whole blood

Anonymous donor human blood was incubated with AzaA8, in addition to positive (unlipidated semaglutide) and negative (vehicle-saline) controls to investigate stability in an a more complex ex vivo environment as compared with in vitro DPP-4 assays. We found that AzaA8 was extremely stable for up to 3 hours at 37°C, with 96% recovery of the intact peptide, while unlipidated semaglutide demonstrated 78% recovery of full peptide. Future experiments will be extended in order to more accurately assess the catabolic rate in ex vivo whole blood assays using additional donors.

In vivo pharmacokinetic study

The standard pharmacokinetic parameters (t1/2, C0, AUClast, AUCInf, AUC/D, AUC Extr, MRT, Vss and CL) of AzaH7/R34 and AzaA8/R34 were determined and compared to both native GLP-1 and the unlipidated semaglutide (Aib8/R34-GLP-1)). The results show that AzaA8 extended half-life (t1/2-a measure of clearance and volume of distribution) to 30min, while AzaH7/R34 extended t1/2 to 149min, as compared with unlipidated semaglutide (14min), and native GLP-1 (4min) (Table 1). The plasma clearance rate (CL) of AzaA8 was 42 ml/min/kg, compared with 23 ml/min/kg and 128 ml/min/kg of unlipidated semaglutide, and GLP-1 respectively (Figure 1. Supplemental). The approximately 2-fold faster plasma clearance rate (CL) of AzaA8 contrasts with the blood half-life findings, indicating either a more rapid renal clearance or a reduced volume of distribution (likely the former). In sum, the half-life results confirm the significance of the AzaA8 substitution in slowing endogenous catabolism.

In vitro receptor potency (cAMP signaling)

In order to assess the in vitro potency of the Aza-GLP1 analogues, assays were performed in baby hamster kidney (BHK) fibroblast cells stably overexpressing the human GLP-1 receptor and containing a reporter gene (cAMP response element -Firefly luciferase). While other aza-GLP-1 analogues presented EC50 values in the high pM to nM range, and were therefore triaged from in vivo analyses, AzaA8 and unlipidated semaglutide had EC50 values of 22.2pM and 8.77pM respectively, providing strong evidence that the AzaA8 analogue would show biological activity in vivo (Table 1, Supplemental Figure 2).

In vivo efficacy-acute treatment- oral glucose tolerance test after single dose in lean animals

We next sought to study glucose responses for AzaA8 compared with controls (unlipidated semaglutide, exendin-4) after a one-time administration in c57/Bl6N lean male mice at 21 weeks of age (age matched to

the high fat diet-diet induced obesity model we studied next). A standard oral glucose tolerance (oGTT) was performed after a six-hour fast (water ad libitum) where the test article/control was given at 2 hours before oGTT start. AzaA8 significantly and dose-dependently lowered blood glucose levels when given at 125ug/kg and 400ug/kg and showed near equivalence with the activity of the unlipidated semaglutide. The positive control, exendin-4 (125ug/kg) was shown to be the most effective at lowering blood glucose levels after the oral glucose challenge of the oGTT and was lowered by more than half, compared with vehicle control after the single subcutaneous dose. (Figure 2)

In vivo efficacy-chronic treatment – oral glucose tolerance test after a 29-day daily dose in a HFD-DIO model
To further extend our findings from the acute treatment in lean animals, we designed a chronic administration study with 4 weeks of daily subcutaneous injections in a high fat diet induced obesity model in c57/Bl6N mice. We used a standard 60% fat diet started at 7 weeks of age and continued the diet throughout age 21 weeks until the terminal oGTT. As compared with vehicle control, AzaA8 and exendin-4 inhibited weight gain, while semaglutide induced weight loss (Figure 3). The AzaA8 treated animals demonstrated significant reductions in body weight gain (versus vehicle group) from day 19 through the end of study (day 29) (Figure 3). Furthermore, in the oGTT, AzaA8 treated animals showed the largest reduction in blood glucose after challenge, even greater than for semaglutide or exendin-4 (Figure 4a,b). No changes in basal (unfasted) blood glucose levels were noted in AzaA8 treated animals over the 29-day treatment when doses were given after daily blood glucose measurement, however when test articles were given 2 hours prior to measurement of basal (fed-state) blood glucose, results mirrored the oGTT blood glucose results (Supplemental Figure 6a, b). There were also no significant differences noted in HbA1c values between any of the experimental groups as compared with vehicle control (Supplemental Figure 5), indicating our HFD model did not progress to diabetes.

Discussion:

Our abridged SAR investigations determined that the top lead analogue of an unlipidated semaglutide peptide surrogate, containing an aza-alanine substitution at position 8 (azaA^{8}/R^{34} GLP-1) was effective in extending in vitro half-life in protease (DPP-4) and ex vivo whole blood half-life assays, as well as improving pharmacokinetic parameters (t1/2, CL) in mice. This AzaA8 analogue was chosen for further in vivo investigations. Herein, we report that lead azapeptide, AzaA8 (aza^{8}/R^{34})-GLP-1) demonstrated significant activities in both in vitro and in vivo models including receptor-mediated signaling, reductions in weight gain during high fat diet, and modulation of glucose metabolism measured after acute glucose challenge in the oGTT, as compared with vehicle and positive controls (semaglutide, unlipidated semaglutide, exendin-4)). Concurrently with our investigations, it was discovered that another group had reported utilization of aza-amino acids in a small structure activity relationship (SAR) investigation with GLP-1 and semaglutide peptides. Analogous to our findings, Dinsmore et al. have discerned that the top leading aza-analogues contained residue 8 replaced with aza-alanine (AzaAla) in either the endogenous GLP-1 (A8/K34) sequence or semaglutide (Aib8,lipidated-K26,R34-GLP-1) and that both A8-AzaA analogues (one unlipidated/K34, one lipidated-K26/Aib8/R34) significantly limited the peptidase/protease degradation and extended the half-life of the modified peptides, while retaining in vitro efficacy in a receptor-mediated downstream signaling assay.(16)

In contrast to Dinsmore et al., our studies on the AzaA8 analogue extended to in vivo models, where we measured a protracted reduction in glucose levels as compared with unlipidated semaglutide in an acute

oGTT study, and a reduction in weight gain in a HFD model. This suggests that AzaA8 may provide extended benefits possibly through its longer blood half-life. Furthermore, with additional modifications (lipidation), AzaA8 could be used an alternative to semaglutide or other GLP-1 agonists. Taken together, our results strongly support the continued study and development of AzaA8 and other azapeptide analogues as type 2 diabetes and/or obesity therapeutics.

Materials and Methods

Reagents and chemicals

All chemicals and reagents were purchased from Fisher Scientific (Hampton, NH) or from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. GLP-1 (7-37) was obtained from Tocris Bioscience™ (Minneapolis, MN), while semaglutide and exendin-4 were obtained from MedChemExpress LLC (Monmouth Junction, NJ).

Azapeptide analogue and peptide synthesis

Peptide controls and azapeptides were synthesized using standard reagents and methods on a Liberty Blue™ 2.0 Microwave Peptide Synthesizer (CEM Corporation, Matthews, NC). Aza-amino acids and azapeptides were synthesized according to Altiti et al. (13), using our patented platform reagents and methods. Briefly, our previous method was adapted using microwave-assisted heating to optimize reaction speed (17).

Structure verification and purity methods

Flash Chromatography and HPLC were used to purify all synthesized peptides and aza-analogues, and high-resolution mass spectrometry instrument (HRMS) was used to confirm sequence/structures *(*Agilent 6550 iFunnel QTOF LC/MS*)*. Detailed support for characterization and structural confirmation of the GLP-1 azapeptide analogues are provided in preprint published in bioRxiv (17).

In vitro protease stability: DPP-4 half-life assay- Methods were adapted from (18).

In summary, stock solutions (5 mg/ml) of test articles and positive controls were made in sterile ddH2O. Reactions were prepared by incubating 16 nM DPP-4 (D3446, Aldrich) at 37°C, with a final test article concentration of 0.1mM in sterile Tris HCl buffer (50 mM, pH 7.4). The vials were kept in a 37°C incubator for the duration of the experiment. Aliquots (30 µL) were taken at various timepoints (0, 1, 4, 8, 24 hours) and added to vials which had been pre-filled with 20 µL of 0.05% TFA in water, and gently mixed. The samples were injected directly into the HPLC to monitor the recovery of intact peptide. The HPLC (Waters (Breeze QS System) was equipped with a 1525 binary pump, and a Phenomenex kinetex 2.6 mm EVO C18 analytical column (100Å 150x4.6mm). Chromatography was performed at ambient temperature with flow rate of 0.7 mL/min with linear gradient from water (0.05% TFA): MeOH (0.05% TFA) [95:5] to water (0.05% TFA): MeOH (0.05% TFA) [5:95] over 15 mins and resolved peaks were detected by a 2998 photodiode array (PDA) detector at 215 nm. The peak area response ratio (PARR) was compared to the PARR at time 0 to determine the percent of test article remaining at each time point. Half-lives of samples in DPP-4 were calculated using Excel.

Ex vivo stability- *Human blood half-life studies*

Anonymous blood donors were recruited by the Feinstein Clinical Research Center using anonymous donor standard operating procedures (SOPs) and not subject to IRB approval. Donor requirements were adults 18-60 in general good health and on no maintenance medications. Blood was drawn into sodium heparin vacutainer tubes (BD 367874), and half-life study was initiated within 20 minutes of collection. Sterile stock solutions (5 mg/ml in ddH2O) were prepared for samples (peptide test articles) and added to 2mL of whole blood (kept at

37°C throughout) to achieve a final test article concentration of 0.1mg/mL. Aliquots (200 µL) were taken at each time point (0, 5, 10, 20, 30, 60, 120, 180 minutes) and added to vials which had been pre-filled with 400 µL of mixture of acetonitrile (ACN) and MeOH (1:1). All samples were stored at 4°C until the end of the experiment. After the final time point was removed, the vials were mixed and then centrifuged at 18,200xg for 10 minutes. Aliquots of the supernatant were removed, diluted 1:1 into 2% acetonitrile in 0.1% formic acid water, filtered, and analyzed by LCMS/MS.

The peak area response ratio (PARR) to calibration curve was compared to the PARR at time 0 to determine the percent of test article remaining at each time point and the t1/2 (min) results were calculated and averaged. Statistical significance was determined using student T tests.

Ex vivo stability-murine pharmacokinetic (pK) studies*- (Pharmacology Discovery Services Taiwan, Ltd.-Eurofin-partner lab) (CRO))*

A plasma pK study was performed in male ICR mice (n=6) following single intravenous (IV) administration of test articles at 1 mg/kg. The semi-serial plasma samples were collected from three alternative animals at each time point at 0.05, 0.167, 0.5, 1, 2, 4, 6 and 8 hours (h) after IV administration. The body weight of each animal was recorded and exposure levels (ng/mL) of test articles in plasma samples determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS(19)). The exposure levels (ng/mL) of test articles in plasma samples were determined by LC-MS/MS. Results were plotted for plasma concentrations versus (vs.) time (mean ± SD). The fundamental PK parameters after IV administration were obtained from the NCA of the plasma data using WinNonlin (manual mode). The lower limit values of quantification (LLOQ) in plasma samples was 2 ng/mL, and the exposure levels below the 75% of LLOQ (1.5 ng/mL) were determined as below the limit of quantification (BLOQ). The fundamental pK parameters of test articles after IV (t1/2, C0, AUClast, AUCInf, AUC/D, AUC Extr, MRT, Vss and CL) administration were obtained from the non-compartmental analysis (NCA) of the plasma data using WinNonlin.

In vitro GLP-1 receptor potency- Human GLP-1 receptor activated cAMP signaling

The in vitro potency assay relied on a reporter gene assay (CRE-luc) and was carried out in baby hamster kidney (BHK-21) cells overexpressing the hGLP-1 receptor. For these experiments, the compounds were tested in four technical pseudo-replicates. The combined results from three independent experiments used to determine EC50 values with the respective 95% confidence interval.

Acute in vivo efficacy- Glucose metabolism- *Acute treatment with AzaA8 and oral glucose tolerance test (oGTT) in lean mice (Pharmaron)*

The contract research organization (CRO) Pharmaron (Beijing, China) was employed to perform in vivo studies. All the procedures related to animal handling, care, and the treatment were performed according to guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Pharmaron following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Based on the statistical power calculations, eight animals per group were sufficient. C57BL/6N male mice (Supplier: SPF (Beijing) Laboratory Animal Technology Co., Ltd) that were 21 weeks (end of week 21) of age on a standard chow diet were used for the acute treatment oral glucose tolerance test (oGTT). Groups of weight matched mice (n=8) were fasted for 6 hours with free access to water and then at -2hours prior to oGTT start blood glucose was measured, followed by a single dose (400ug/kg) of test article or control (positive (semaglutide) and negative (saline)) via subcutaneous (s.c) injection in back of neck/scruff. Test articles and positive control samples were blinded to the CRO, and mice were observed clinically for any obvious external pathology or behavior issues after injections. For the oGTT, body weights were recorded and then at time zero mice were give 2g/kg of

100mg/mL glucose solution (in sterile water) by oral gavage. Blood glucose was monitored at time 0 (as close as possible), 15, 30, 60, 90, and 120 minutes using an Accu-Chek Guide blood glucose meter from tail cut/bleed.

Chronic in vivo efficacy-Obesity endpoints, glucose metabolism*- Measures of obesity, basal blood glucose levels and oGTT after chronic treatment with AzaA8 in high fat diet-induced obesity mouse model. (Pharmaron)*

The contract research organization (CRO) Pharmaron (Beijing, China) performed in vivo studies, and matched relevant parameters of the acute treatment study as closely as possible. All the procedures related to animal handling, care, and the experimental treatment were performed according to guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Pharmaron following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). C57BL/6N male mice (Supplier: SPF (Beijing) Laboratory Animal Technology Co., Ltd) were weighed and assigned to weight-matched groups and then started on a high fat diet (HFD (Research Diets, Inc. -D12492) at 7 weeks of age. The HFD continued for an additional 15 weeks through the end of 21 weeks. Based on the statistical power calculations, groups with n=8 animals were used.

Test article treatment was given for 4 weeks (18-21 weeks of age), by subcutaneous (s.c.) route of administration via an injection in back of neck/scruff. For the test article AzaA8, 125µg/kg was injected daily for week 1, then at 400µg/kg daily for weeks 2-4 (1x/day for week 2, and then given 2x/day (1/2 dose each injection) for week 3 and 4 (s.c. week 3, i.p. week 4). For semaglutide positive control 40µg/kg was given once daily for all 4 weeks. Saline vehicle control was given daily (s.c.) for 4 weeks. Test articles and positive control samples were blinded to the CRO, and during the 4-week treatment, mice were observed clinically for any obvious external pathology or behavior issues. Parameters measured included, twice weekly body weights, food intake, and basal blood glucose (unfasted) and were recorded at the same time of day throughout the study. At the end of 4 weeks of daily treatment (s.c. injections) an oral glucose tolerance test (oGTT) was performed. Briefly, for the oGTT, during early light cycle mice underwent a 6 hr. fast (-6h), blood glucose was recorded at -2h and then test article/controls were given -2h (just after glc. measurement) at specified concentrations (doses listed above), body weights were recorded and then at time zero mice were give 2g/kg of 100mg/mL glucose solution (in sterile water) by oral gavage. Blood glucose was monitored at minutes 0 (as close as possible), 15, 30, 60, 90, 120 using a standard glucose meter from tail cut/bleed. Mice were euthanized humanely after 120min and blood was obtained by cardiac puncture for measurement of insulin, leptin, total cholesterol, total triglycerides, ALT/AST (liver function), and creatine/urea (kidney function).

Figure 1**. Schematic of Glucagon-like peptide-1 (GLP-1) analogues and their synthesis.** a) Sequence and descriptive (abbreviated names) information for GLP-1 analogues, b) An experimental application of the optimized azapeptide synthesis platform -Schematic of synthesis with standard microwave-assisted solid phase peptide synthesis (SPPS) instrument using AzaA8 as model reaction.

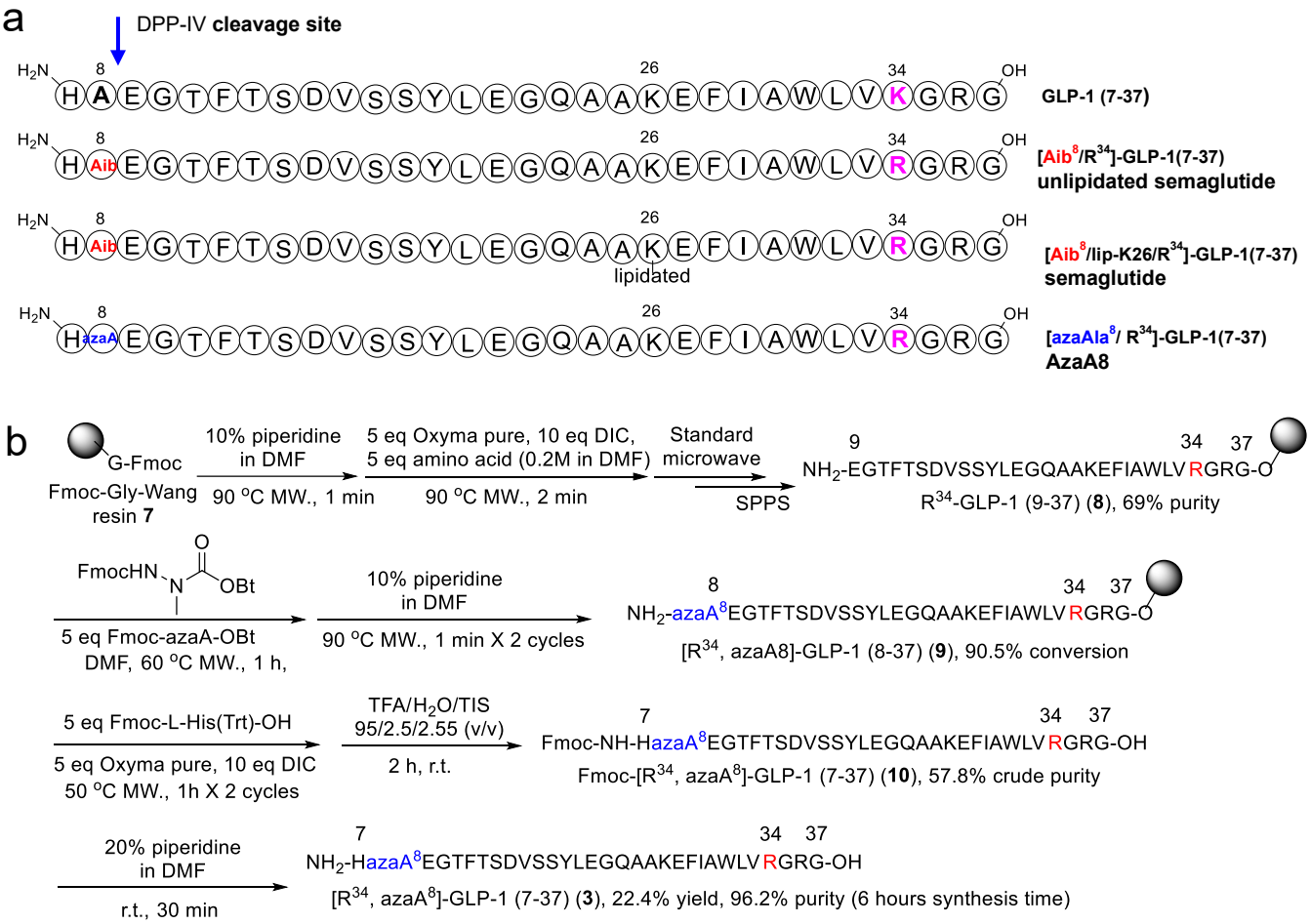


Table 1. **Descriptions, abbreviations and activity profiles of GLP-1 analogues for signaling (cAMP) and stability (in vitro DPP-4, in vivo pK t1/2).**

Aza-GLP-1 analogues		In vitro receptor activity cAMP assay (EC50, pM)	In vitro DPP-4 t ½ (h)	In vivo plasma t ½ (pK) (min)
Description	**Abbreviation**			
[R^{34}]-GLP-1(7-37)	**R34-GLP-1**	11.3 ± 1.2	0.3 ± 0.07 h	4.2
[Aib^{8}/R^{34}]-GLP-1(7-37)	**unlipidated semaglutide**	8.84 ± 1.2	6 h	13.8
[$azaAla^{8}/R^{34}$]-GLP-1(7-37)	**AzaA8**	22.3 ± 2.4	>24h	30
[$azaHis^{7}/R^{34}$]-GLP-1(7-37)	**AzaH7/R34**	743 ± 164	>24 h	149.4
[$azaHis^{7}/Aib^{8}/R^{34}$]-GLP-1(7-37)	**AzaH7/Aib8/R34**	306 ± 44	>24 h	-
[$azaHis^{7}/azaGlu^{9}/R^{34}$]-GLP-1(7-37)	**AzaH7/AzaE9/R34**	>15 nM	>24 h	-

Figure 2. **Acute treatment-Oral glucose tolerance test (oGTT) in lean mice**. a) Mean blood glucose responses over time (min) and b) total average area under curve of blood glucose levels – in male 21 week old chow-fed lean mice (c57Bl/6N) after single injection (s.c) of test articles, controls, or vehicle (saline) given at -2hrs prior to glucose bolus, n = 8 animals per group, *p<0.05 vs. vehicle for all test article groups, **p<0.01 vs vehicle

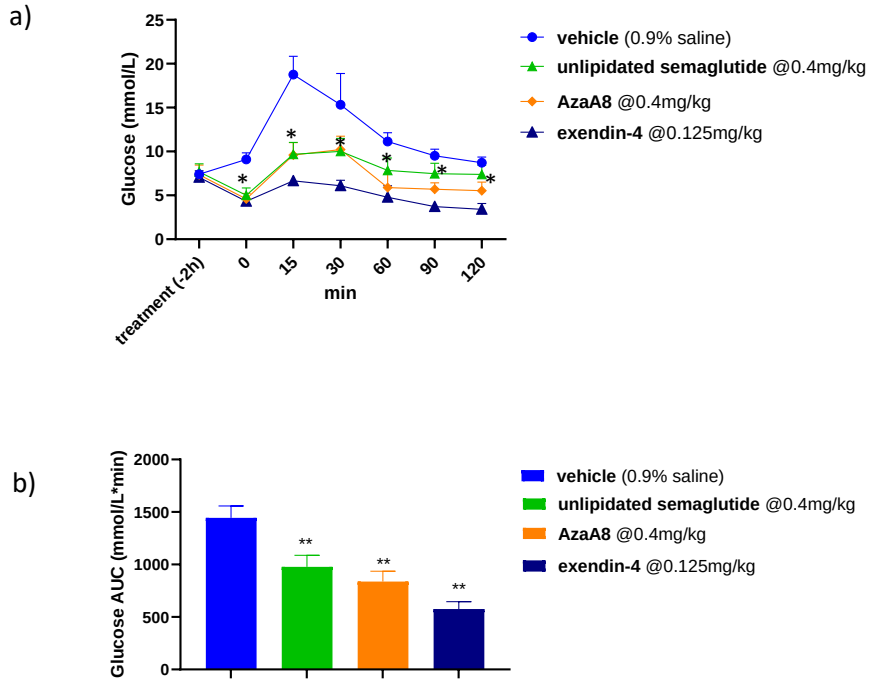


Figure 3. **Biweekly body weights in HFD-DIO mice with chronic treatment.** In vivo effects of 4 weeks (18-21wks) azaA8-GLP-1 analogue treatment versus positive and negative controls in DIO mice (high fat diet-fed C57BL/6N mice). The weight of male C57BL/6N mice was monitored on a twice-weekly basis over a period of four weeks with daily subcutaneous injections of saline vehicle, azaA8-GLP-1, exendin-4, and semaglutide, n = 8 animals/group. All treatment groups, *p<0.05 vs. vehicle, **p<0.01 vs. vehicle.

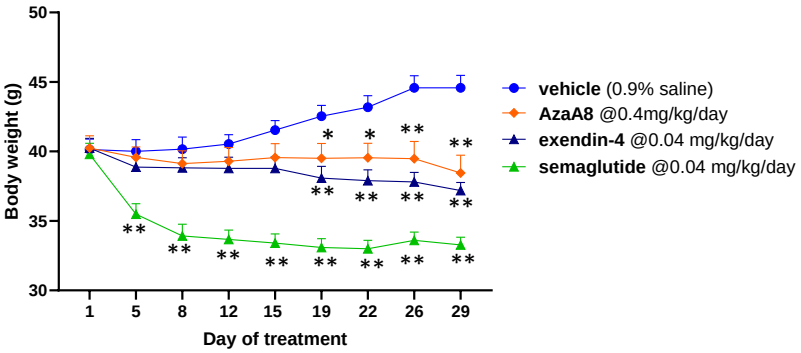
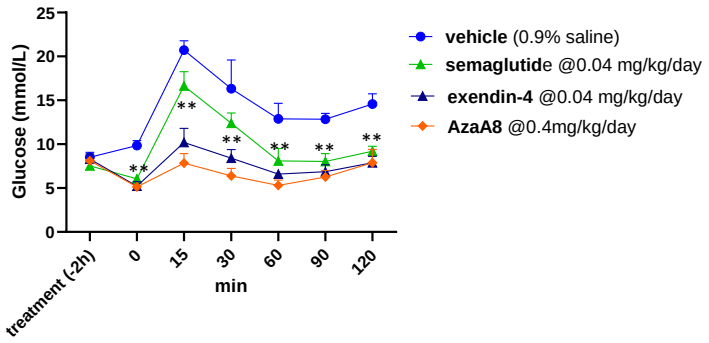


Figure 4. **Oral glucose tolerance test after 29-day treatment in DIO model**. Effects of 4 weeks of daily azaA8 treatment (0.4mg/kg/day) on glucose levels after challenge (oral glucose bolus) in DIO mice. a) Mean blood glucose responses over time (min) and b) total average area under curve of blood glucose levels. OGTTs performed after 4 weeks treatment in DIO mice, 2 hours after administration of test articles or vehicle, n = 8 per group, **p<0.01 for all groups vs. vehicle

a)



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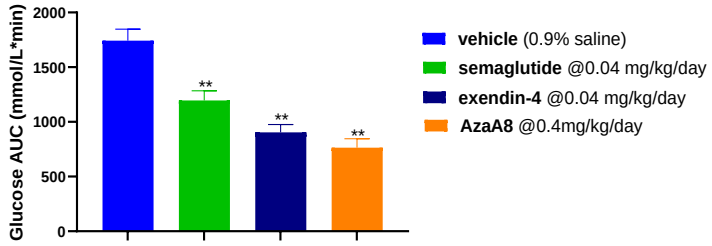

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Supplemental Table 1. Peptide name, MW (QC), purity was reported in (17).

Peptide	MW/ QC verification technique	purity
GLP-1 (7-37)	3355.71 (purchased)	95.0%
unlipidated semaglutide	3398.68 (HPLC/MS)	95.9%
AzaA8	3384.68 (HPLC/MS)	96.2%
semaglutide	4113.64 (purchased)	99.0%
exendin-4	4186.57 (purchased)	99.0%

Supplemental Figure 1. Pharmacokinetic parameters in mice performed in ICR mice by Pharmacology Discovery Systems, Eurofins (CRO).

Male ICR mice weighing 30+/-5 g were provided by BioLASCO Taiwan Co., Ltd. (under Charles River Laboratories Licensee). Animals were acclimated for 3 days prior to use and were confirmed to be in good health. The animals were housed in animal cages with a space allocation of 30 x 19 x 13 cm. All animals were maintained in a controlled temperature (20 - 24°C) and humidity (30% - 70%) environment with 12-h light/dark cycles. Free access to standard lab diet [MFG (Oriental Yeast Co., Ltd., Japan)] and autoclaved tap water were granted. All aspects of this work including housing, experimentation, and animal disposal were performed in general accordance with the "Guide for the Care and Use of Laboratory Animals: Eighth Edition" (National Academies Press, Washington, D.C., 2011) in the AAALAC-accredited laboratory animal facility. In addition, the animal care and use protocol was reviewed and approved by the IACUC at Pharmacology Discovery Services Taiwan, Ltd. (PDS). On the testing day, test articles (GLP-1 (7-37), Aib8/R34-GLP-1, azaA8/R34-GLP-1 and azaH7/R34-GLP-1), were administered at 1 mg/kg through IV injection. Plasma Sample Collection from Mice (Semi-serial Sampling) after the administrations of GLP-1 via IV injections, blood aliquots were collected via facial vein (~0.15 mL) for the first three (1st to 3rd) time points and cardiac puncture (~0.3 mL) for the last (4th) time point from mice. The blood samples were loaded in tubes coated with lithium heparin, mixed gently, and centrifuged at 2,500 ×g for 15 minutes (min) at 4°C, within 1 h of collection. The plasma samples were then harvested and kept frozen at ≤-70°C until further bioanalysis.

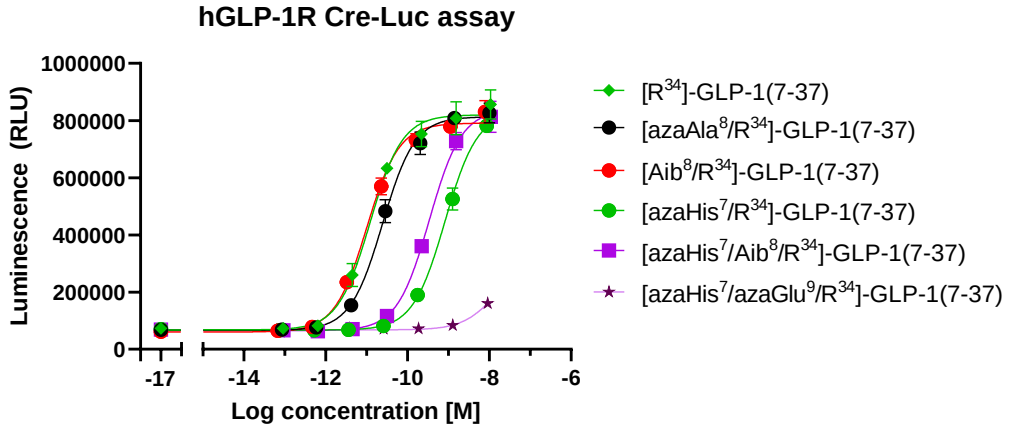
GLP-1 (7-37)	$t_{1/2}$	C_0	AUClast	AUCInf	AUC/D	AUC Extr	MRT	Vss	CL
	(h)	(ng/mL)	(h*ng/mL)	(h*ng/mL)	(h*kg*ng/mL/mg)	(%)	(h)	(L/kg)	(mL/min/kg)
1 mg/kg, IV	0.07	1795	130	131	131	0.16	0.55	4.20	127.56

Aib8/R34-GLP-1	$t_{1/2}$	C_0	AUClast	AUCInf	AUC/D	AUC Extr	MRT	Vss	CL
	(h)	(ng/mL)	(h*ng/mL)	(h*ng/mL)	(h*kg*ng/mL/mg)	(%)	(h)	(L/kg)	(mL/min/kg)
1 mg/kg, IV	0.23	8044	740	742	742	0.26	0.11	0.15	22.45

azaA8/R34-GLP-1	$t_{1/2}$	C_0	AUClast	AUCInf	AUC/D	AUC Extr	MRT	Vss	CL
	(h)	(ng/mL)	(h*ng/mL)	(h*ng/mL)	(h*kg*ng/mL/mg)	(%)	(h)	(L/kg)	(mL/min/kg)
1 mg/kg, IV	0.50	4500	394	397	397	0.90	0.14	0.34	41.94

azaH7/ R34-GLP-1	$t_{1/2}$	C_0	AUClast	AUCInf	AUC/D	AUC Extr	MRT	Vss	CL
	(h)	(ng/mL)	(h*ng/mL)	(h*ng/mL)	(h*kg*ng/mL/mg)	(%)	(h)	(L/kg)	(mL/min/kg)
1 mg/kg, IV	2.48	36401	2365	2376	2376	0.45	0.18	0.07	7.02

Supplemental Figure 2. Human GLP-1 receptor potency - data from Novo Nordisk (Måløv, Denmark)



Representative concentration response agonism curve

Compound	Mean EC50 (95% CI), [M]
[R^{34}]-GLP-1(7-37)	1.13E-11 (8.58E-12 to 1.48E-11)
[Aib^{8}/R^{34}]-GLP-1(7-37)	8.84E-12 (6.43E-12 to 1.20E-11)
[$azaAla^{8}/R^{34}$]-GLP-1(7-37)	2.23E-11 (1.73E-11 to 2.85E-11)
[$azaHis^{7}/R^{34}$]-GLP-1(7-37)	7.43E-10 (4.01E-10 to 1.32E-09)
[$azaHis^{7}/Aib^{8}/R^{34}$]-GLP-1(7-37)	3.06E-10 (2.11E-10 to 4.35E-10)
[$azaHis^{7}/azaGlu^{9}/R^{34}$]-GLP-1(7-37)	> 1E-8

Supplemental Methods. Pharmaron (CRO) Animal Maintenance protocols

1.2.1 Quarantine: Animals were quarantined for at least 14 days before the study. The general health of the animals was evaluated by a veterinarian, and complete health checks were performed. Animals with abnormalities were excluded prior the study.

1.2.2 Housing: General procedures for animal care and housing were in accordance with the standard, Commission on Life Sciences, National Research Council, Standard operating procedures (SOPs) of Pharmaron, Inc. The mice were kept in laminar flow rooms at constant temperature and humidity with three animals in each cage. Animals were housed in polycarbonate cages and in an environmentally monitored, well-ventilated room maintained at a temperature of (22 ± 3°C) and a relative humidity of 40 %-80 %. Fluorescent lighting provided illumination approximately 12 hours per day. The bedding material were soft wood, which were changed once per week. Analysis of bedding contamination were conducted annually per facility SOPs and the results of the analyses were retained in the facility records.

1.2.3 Animal ID: Each animal was assigned an identification number and marked in the tail; the following identification method was applied. Each cage card was labeled with such information as study number, group, sex, dose, animal number, initiation date, study director.

1.2.4 Diet: All animals have free access to standard chow (single dose, lean animals study) or HFD diet (D12492) (chronic doses, DIO study) during the entire study period except for time periods specified by the oGTT protocol (pre-test 6hr fast).

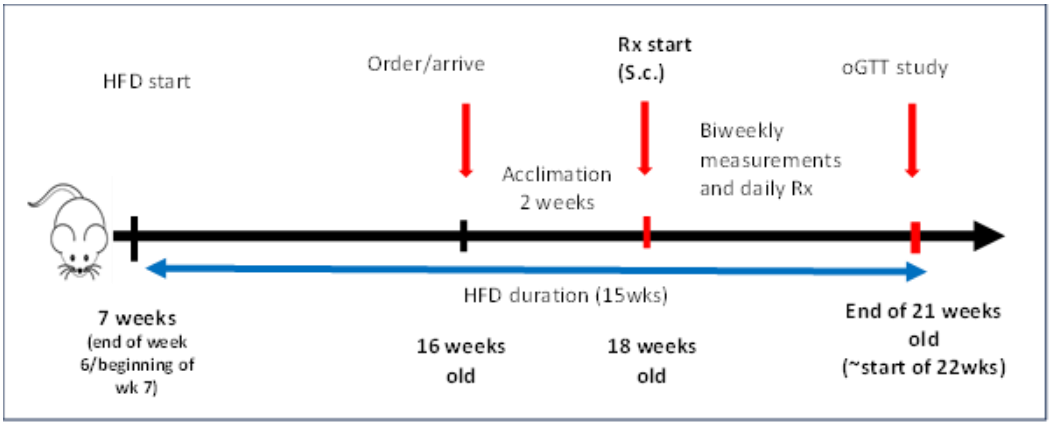
1.2.5 Water: Sterile drinking water in a bottle was available to all animals' ad libitum during the quarantine and study periods. The bottle and the stopper with attached sipper tube were autoclaved prior to use. Samples of

water from the animal facility were analyzed and results of water analysis were retained in the facility records and were reviewed by the veterinarian, or designee, to assure that no known contaminants were present that could interfere with or affect the outcome of studies.

Supplemental Figure 3. Oral glucose tolerance test result values (mean+/-SD) in 21 week lean c57/BL6N male mice, chow fed with single test article administration. TA-1: Aib8/R34-GLP-1, TA-2: azaA8/R34-GLP-1, TA-3: azaH7/R35-GLP-1, TA-4: exendin-4, *p<0.05, **p<0.01

Group	Treatment	Glucose (mmol/L)						
		pre-dose	0 min	15 min	30 min	60 min	90 min	120 min
1- vehicle	Vehicle (0.9% NaCl, saline)	7.4±0.5	9.1±0.8	18.8±2.1	15.3±3.6	11.1±1.0	9.5±0.8	8.7±0.6
2- Aib8/R34-GLP-1	TA-1 @0.125mg/kg	7.5±0.9	6.0±0.8**	12.6±2.4**	12.8±2.3	10.2±1.5	9.2±1.4	8.7±1.4
3- Aib8/R34-GLP-1	TA-1 @0.4mg/kg	7.7±0.9	5.0±0.8**	9.7±1.4**	10.0±1.5**	7.8±1.4**	7.5±1.2**	7.4±1.0*
4- azaA8/R34-GLP-1	TA-2 @0.125mg/kg	8.1±0.6	5.4±0.4**	12.3±1.9**	11.0±1.5**	8.5±0.6**	7.9±0.6**	7.9±0.8
5- azaA8/R34-GLP-1	TA-2 @0.4mg/kg	7.3±1.1	4.6±0.6**	9.6±1.4**	10.2±1.5**	5.9±1.5**	5.7±0.7**	5.5±1.0**
6- azaH7/R35-GLP-1	TA-3 @0.125mg/kg	7.4±1.1	6.2±1.1**	15.1±1.4**	14.6±1.5	9.3±1.3*	9.0±0.9	8.7±1.2
7- azaH7/R35-GLP-1	TA-3 @0.4mg/kg	7.7±0.7	5.6±0.6**	13.1±2.8**	12.1±1.5**	8.9±1.0**	8.1±0.6*	7.8±0.8
8- exendin-4	TA-4 @0.125mg/kg	7.1±0.8	4.3±0.5**	6.7±0.5**	6.1±0.6**	4.8±1.3**	3.7±0.5**	3.4±0.7**

Supplemental Figure 4. Schematic of chronic administration study design.

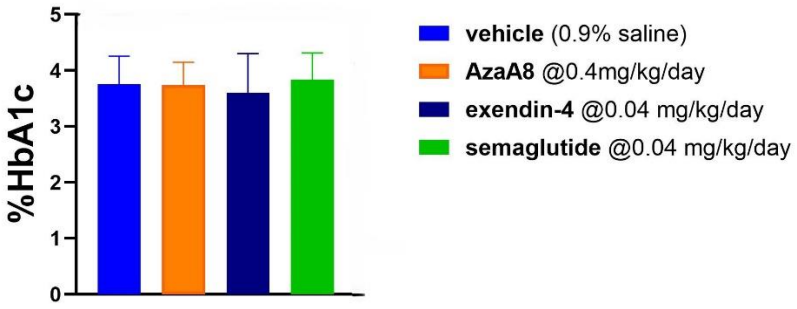


Supplemental Data. Clinical Observation parameters after 4-week daily treatment in chronically dosed mice c57/BL6N). Routine observation of all animals were performed daily during the study by animal facility staff to monitor for any sick or dying animals. The detailed clinical observation items were listed below. No clinically relevant signs were noted in animals over the 4-week test article administration.

Observation	Abnormality
Vital signs	Death
	Dying
Respiration	Difficulty breathing
	Shortness of breath
	Respite
	Apnea
	Abdominal
Nutritional state	Abnormal Weight loss
Mental status	Lassitude
	Sleepy
	Excited / irritable
Behavior	Ataxia

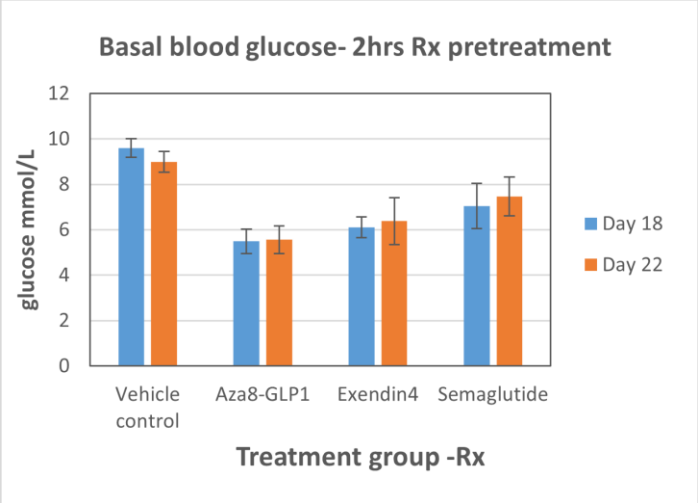
	Circling
	Tilted
	Catalepsy
	Prone
	Quiver/ tremble
	Startle roll
	Self-mutilation
	Convulsions
Muscle	Spasm
	Opisthotonos
	Forelimb and / or hind limb weakness
	Muscle tremors
Reflection	Paralysis
	Righting reflex
	Weakening or disappearance of the pupillary light reflex
Eye	Tears
	Secretions
	Miosis
	Mydriasis
	Blood tears
	Ptosis
	Eyelid swelling/ redness
	Abnormal conjunctiva
	Corneal abnormalities
Nose	Nasal secretions
Oral cavity	Excessive saliva
	Saliva white foam
	Malocclusion
Gastrointestinal tract	Loss of appetite
	Little or no stool
	Dry hard stool
	Diarrhea
	Soft stool
	Blood in the stool / bloody stool
Abdomen status	Abdominal swelling
	Abdominal collapse
Urine	Anuria
	Abnormal Appearance
Clothing hair	Bristle
	Rough coat
	Epilation
Skin/mucous membrane	Swelling
	Skin erythema
	Scleroma
	Color
	Flushing
	Wound
	Scab
	Loose skin
Perineum	Anal dirty

Supplemental Figure 5. Hemoglobin A1c percentage at terminal endpoint in chronic treatment, HFD-DIO model. C57/BL6N mice treated daily for 4 weeks were sacrificed after oGTT study and blood levels of HbA1c were measured (Pharmaron, Beijing, China) by Sjodax HbA1C test kit.



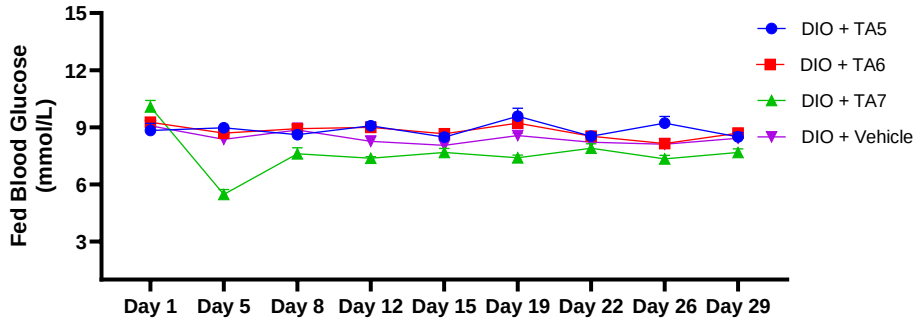
Supplemental Figure 6. Basal blood glucose levels in fed state, measured a) 2 hours after dosing (test article injection), or b) before daily dosing, in HFD-DIO chronically (29 days) dosed mice. (TA5: AzaA8, TA-6: exendin-4, TA-7: semaglutide) c) food consumption over 4 week daily test article administration. *p<0.05, **p<0.01

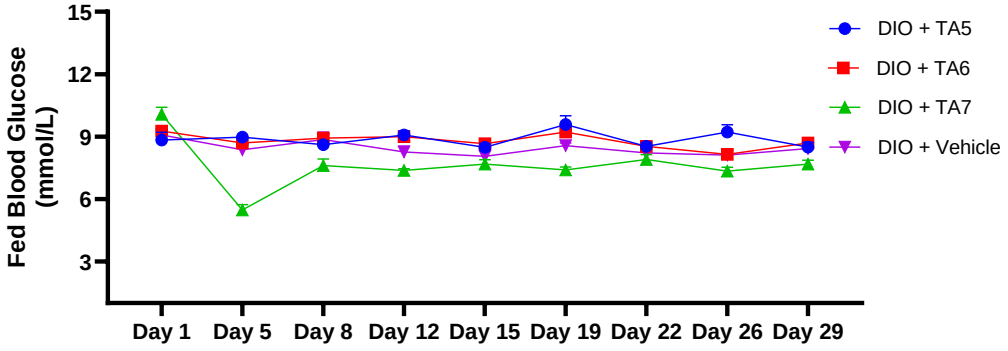
a)



b)

Group	Treatment	Fed Blood Glucose(mmol/L)								
		Day 1	Day 5	Day 8	Day 12	Day 15	Day 19	Day 22	Day 26	Day 29
1	DIO + TA5@ week 1 of Rx 125ug/kg, then for weeks 2-4 @ 400 ug/kg	8.8±1.1	9.0±0.7	8.6±0.5	9.1±0.6*	8.5±0.5	9.6±1.2*	8.5±0.5	9.2±1.0**	8.5±0.6
2	DIO + TA6@40 ug/kg for all 4 weeks	9.3±0.6	8.7±0.8	8.9±0.6	9.0±0.7	8.7±0.6	9.2±0.7	8.5±0.7	8.2±0.6	8.7±0.4
3	DIO + TA7@40 ug/kg for all 4 weeks	10.1±0.9	5.5±0.7**	7.6±0.9**	7.4±0.2*	7.7±0.6	7.4±0.4*	7.9±0.6	7.3±0.5	7.7±0.6*
4	DIO + Vehicle	9.1±0.6	8.4±0.6	8.9±1.0	8.3±0.8	8.1±0.8	8.6±0.4	8.2±1.2	8.1±0.6	8.4±0.6



c)

Group	Treatment	Food Consumption(g/day)							
		Day 1-4	Day 5-7	Day 8-11	Day 12-14	Day 15-18	Day 19-21	Day 22-25	Day 26-28
1	DIO + TA5@ week 1 of Rx 125ug/kg, then for weeks 2-4 @400 ug/kg	2.2±0.3	2.2±0.3	2.4±0.3	2.6±0.4	2.4±0.2*	2.3±0.2**	2.3±0.6**	2.3±0.6*
2	DIO + TA6@40 ug/kg for all 4 weeks	1.9±0.2**	2.3±0.2	2.3±0.2	2.4±0.2*	2.2±0.2**	2.1±0.2**	2.4±0.2**	2.2±0.2**
3	DIO + TA7@40 ug/kg for all 4 weeks	1.0±0.4**	1.7±0.6**	2.1±0.5**	2.2±0.2**	2.1±0.2**	2.0±0.2**	2.3±0.1**	2.2±0.2*
4	DIO + Vehicle	2.5±0.3	2.6±0.2	2.7±0.5	2.9±0.4	2.8±0.4	2.8±0.3	3.0±0.3	2.7±0.2