The activity of sulfono-γ-AApeptide helical foldamers that mimic GLP-1

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Existing long α-helix mimicking necessitates the retention of most natural amino acid residues to maintain their biological activity. Here, we report the exploration of helical sulfono-γ-AApeptides with entire unnatural backbones for their ability to structurally and functionally mimic glucagon-like peptide 1 (GLP-1). Our findings suggest that efficient construction of novel GLP-1 receptor (GLP-1R) agonists could be achieved with nanomolar potencies. In addition, the resulting sulfono-γ-AApeptides were also proved to display remarkable stability against enzymatic degradation compared to GLP-1, augmenting their biological potential. This alternative strategy of α-helix mimicking, as a proof of concept, could provide a new paradigm to prepare GLP-1R agonists.

INTRODUCTION

The glucagon-like peptide 1 receptor (GLP-1R) (1–4) belongs to the class B family of G protein–coupled receptors, and its incretin helical peptide ligand GLP-1 (5–7) analogs are a promising drug candidate for treating type 2 diabetes and obesity. However, the half-life of GLP-1 is very short owing to rapid degradation by proteases (8–12). Stabilizing GLP-1 is critical in the development of drugs to treat diabetes. Side-chain cross-linking strategies have been used for conformational and metabolic stabilization of GLP-1 (13–15). However, unintended contacts between the cross-links and GLP-1R may occur, and the extent of proteolytic stabilization may be limited. It is conceivable that helical foldamers may provide an alternative strategy to develop proteolytically stable GLP-1R agonists. Gellman et al. (16, 17) reported beautiful work mimicking endogenous GLP-1 using conformationally constrained β-amino acid residues to replace some native α residues. The properly designed α/β-peptides displayed potent and prolonged activity in vivo. Goudreau et al. recently reported the use of oligourea moieties in GLP-1 sequence to improve the pharmaceutical properties of GLP-1 (18). Very recently, Levine et al. (19) used the O-GlcNAcylation to GLP-1 to improve the stability and in vivo activity. However, to the best of our knowledge, there is no report on mimicking GLP-1 using entire unnatural backbones. Mimicking a long α-helix by unnatural peptidomimetics is highly challenging owing to the difference in helicity between α-helix and helical foldamers yet appealing because complete unnatural backbone could be more resistant to proteolysis than peptide hybrids.

Sulfono-γ-AApeptides are recently introduced as a new class of helical mimetics to address some enduring challenges in disrupting α-helix–mediated protein–protein interactions (20–27). As proteolytically stable peptidomimetics, sulfono-γ-AApeptides exhibit unusual folding stability by adopting a series of helical structures with well-defined hydrogen bonding patterns (21). Half of the residues are introduced by sulfonyl chlorides in sulfono-γ-AApeptides (Fig. 1A), providing enormous chemical diversity. Furthermore, the single crystal of the homogeneous sulfono-γ-AA foldamers reveals a precise three-dimensional (3D) arrangement of their side functional groups and the helical pitch (5.1 versus 5.4 Å of α-helix) (21), leading to helical mimetics for targeting various α-helix–interacting proteins (Fig. 1, B and C) (23, 24). We were intrigued whether this α-helix–mimicking strategy could be used to mimic GLP-1, a very long helical peptide. Functionally, it was unclear whether native GLP-1 could be mimicked by sulfono-γ-AApeptide-based helical foldamers to form potent GLP-1R agonists. Could the critical residues in native GLP-1 still play an important role in sulfono-γ-AApeptides? Additional questions could arise from the monolithic helix structure of sulfono-γ-AApeptides instead of peptide hybrids that may pose extra challenges in interacting with GLP-1R. Without mimicking the exact structure of GLP-1, would the sulfono-γ-AApeptide be capable of mimicking residues on multiple faces of GLP-1 helix? If successful, it would provide a general strategy for the development of GLP-1R agonists with chemically diverse functional groups based on sulfono-γ-AApeptides. This mode of α-helix mimicking would offer a new paradigm for mimicking a myriad of long and complex helical peptides. Here, as a proof of concept, we report the first example of foldameric peptidomimetics with entire unnatural residues for GLP-1 mimicking.

RESULTS AND DISCUSSION

Design and biological activity of sulfono-γ-AApeptides

Analysis of GLP-1/GLP-1R interaction reveals the critical residues on GLP-1 that are responsible for tight binding to GLP-1R (4, 28). Briefly, GLP-1 engages both its N-terminal domain and C-terminal domain to interact with GLP-1R (Fig. 2A). Of the N-terminal domain, interaction of the polar residues H7, E9, T11, T13, and S17 played potent and prolonged activity in vivo. Goudreau et al. recently introduced the O-GlcNAcylation to GLP-1 to improve the stability and in vivo activity. However, to the best of our knowledge, there is no report on mimicking GLP-1 using entire unnatural backbones. Mimicking a long α-helix by unnatural peptidomimetics is highly challenging owing to the difference in helicity between α-helix and helical foldamers yet appealing because complete unnatural backbone could be more resistant to proteolysis than peptide hybrids.

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packing, which alleviates the challenge to mimic both N- and C-terminal domains of GLP-1 simultaneously. A close view of the critical residues on GLP-1 (Fig. 2, B and C) reveals that H7 and T11 are on the same face (X) of the helix, whereas F28 and L32 are on face Y; E9, T13, and T11 are on face Z (X, Y, and Z are designated arbitrarily).

The preliminary analysis prompted our design of helical sulfono-γ-ＡApeptides that potentially mimic GLP-1. As shown in Fig. 1 (D and E), chiral side chains and sulfonamide side chains are distributed perfectly on four faces of helical scaffold, which could be used to mimic critical residues of a native GLP-1 helical domain that interact with GLP-1R. An intimate comparison suggests that side chains 1a and 3a could mimic residues on the X face of GLP-1, 11b and 13b could potentially reproduce the functionality of residues on the Y face of GLP-1, whereas 2a, 4a, and 6a were speculated to capture the ability of E9, T13, and S17. This strategy led to the synthesis of series of sulfono-γ-ＡApeptides (Table 1, 3 to 16). For comparison purposes, the native GLP-1 peptides were also synthesized (Table 1 and Fig. 3, 1a and 2). The agonistic activity of these peptides was obtained by measuring the receptor-mediated cAMP production in the Chinese hamster ovary (Choki) cells overexpressed with the hGLP-1R (14, 32). The N-terminal capped and uncapped sulfono-γ-ＡApeptides 3 and 4 contain desired side chains shown in Fig. 2C. The potency of sulfono-γ-ＡApeptide 3 is only two orders of magnitude lower than that of GLP-1 1 (39.79 nM versus 0.11 nM) and ~10-fold lower than that of the capped GLP-1 2 (39.79 nM versus 3.33 nM, respectively).

Our findings demonstrate that the design of full-length unnatural peptidomimetics of GLP-1 is viable. The acetyl capped sulfono-γ-ＡApeptides displayed more potent agonistic effects than those with an unmodified N terminus. The opposite effect was observed for the regular GLP-1 peptides 1 and 2 (Table 1) where acetylation of the N terminus reduced the agonistic potency of GLP-1 (16, 17). These observations are consistent with different helical scaffolds in GLP-1 and the sulfono-γ-ＡApeptides.

The functional group on position 6b is not highly critical, as the change of methyl group (3 and 4) to a bulky anisole group only resulted in a fourfold decrease in binding affinity (Table 1 and Fig. 3, 5 and 6). Consistent with previous findings, the C-terminal domain is less important than the N-terminal domain, as replacement of the benzyl group on position 11b with a methyl group (7 and 8) only resulted in a twofold decrease in activity compared with 5 and 6. It is known that H7 is a critical residue for both receptor binding and activation, and replacement of H7 with other aromatic groups, such as Phe, virtually retains the same biological function of GLP-1, implying that aromaticity is critical at the N-terminal end (5). This is similarly observed in sulfono-γ-ＡApeptides (3 and 4). Substitution of phenyl group for Lys side chain resulted in complete loss of activity (9 and 10). As expected, the side chain on position 4a is highly critical. Replacement of the 2-hydroxypropyl group (the side chain to mimic T13) with a benzyl group led to completely inactive sequences 11 and 12 (Table 1). It is intriguing that the first side chain preferred is the aromatic group, as the longer sequences (13 to 16) completely abolished the activity to activate cAMP release. Overall, these studies revealed excellent structure-activity relationship for sulfono-γ-ＡApeptides to mimic GLP-1.

Circular dichroism measurements
To obtain further structural information and assess any conformational changes resulting from chemical modifications, we investigated the secondary structure of homogeneous sulfono-γ-ＡApeptides 3 to 16 and compared it with that of regular GLP-1 peptides 1 and 2. Circular dichroism (CD) spectra were recorded between 190 and 270 nm in phosphate-buffered saline (PBS). As anticipated, the regular GLP-1 peptides 1 and 2 adopted a right-handed helical conformation,
Table 1. Structures of GLP-1(7-36) 1 and 2 and sulfono-γ-AApeptides 3 to 16.

| Peptide | Sequence |
|---------|----------|
| 1       | H-HAEGTFTSDVSSYLEGQAAKEFIAWLKVGR-NH₂ |
| 2       | Ac-HAEGTFTSDVSSYLEGQAAKEFIAWLKVGR-NH₂ |
| 3       | ![Structure 3](image1) |
| 4       | ![Structure 4](image2) |
| 5       | ![Structure 5](image3) |
| 6       | ![Structure 6](image4) |
| 7       | ![Structure 7](image5) |
| 8       | ![Structure 8](image6) |
| 9       | ![Structure 9](image7) |
| 10      | ![Structure 10](image8) |
| 11      | ![Structure 11](image9) |
| 12      | ![Structure 12](image10) |
| 13      | ![Structure 13](image11) |
| 14      | ![Structure 14](image12) |
| 15      | ![Structure 15](image13) |
| 16      | ![Structure 16](image14) |
whereas sulfono-γ-AApeptides 3 to 16 reveal a strong cotton effect with a positive maximum at around 208 nm, which is a characteristic of a well-defined left-handed 414 helix (Fig. 4) (21) similar to previously reported homogeneous sulfono-γ-AApeptides.

**Stability study**
A major problem limiting the use of GLP-1 analogs is their instability due to the rapid degradation by proteases. Having found peptides 3 and 5 as the lead candidates based on their in vitro potency, we next examined their enzymatic stability in comparison to the regular GLP-1 peptides. The assays were performed by incubating 0.1 mg/ml of peptides 3 and 5 and the regular peptides (1 and 2) with pronase (0.1 mg/ml) in 100 mM ammonium bicarbonate buffer (pH 7.8) at 37°C for 24 hours. High-performance liquid chromatography (HPLC)–mass spectrometry was then used to analyze the stability of the examined peptides. Unlike the regular GLP-1 peptides 1 and 2, which degraded completely with no intact peptide remaining, sulfono-γ-AApeptides 3 and 5 showed no detectable degradation (Fig. 5A). These results display notable enhancement in the stability of the sulfono-γ-AApeptides against enzymatic degradation, augmenting their potential in therapeutic application. In addition, peptides 3 and 5 also showed remarkable stability in the presence of serum for 24 hours (Fig. 5B), whereas peptides 1 and 2 had more than 60% degradation under the same condition.

**Oral glucose tolerance test**
Next, we examined the blood glucose–lowering effect of the lead sulfono-γ-A Apostle 3 in comparison to native GLP-1 (Fig. 5, C and D). The peptides were administered as single doses intraperitoneally to C57BL/6 mice (overnight fasted; n = 6 per group) 60 min before a glucose challenge. Two doses of sulfono-γ-AApeptide 3 (4 and 40 mg/kg) were tested, and their efficacies were compared with native GLP-1 (1 mg/kg) and vehicle control. At both doses, sulfono-γ-AApeptide 3 markedly decreased blood glucose levels at the time points of 30 and 45 min with the dose of 40 mg/kg showing a more pronounced effect, suggesting good pharmacodynamic effect in vivo (Fig. 5C). A dose-related decrease in glucose clearance was observed for 3 at both 4 and 40 mg/kg doses (Fig. 5D), consistent with the abovementioned results of the cell-based assay.

**CONCLUSION**
In conclusion, we have developed a series of helical sulfono-γ-AApeptides that can structurally and functionally mimic residues on the multiple faces of the α-helical domain of GLP-1. These unnatural helical peptidomimetics display potent GLP-1R agonistic activity in cell-based assay and oral glucose tolerance test (OGTT). To the best of our knowledge, this work represents the first example of foldameric peptidomimetics based on an entire unnatural backbone for GLP-1 mimicking. The excellent proteolytic stability of these helical sulfono-γ-AApeptides augments their biological potential. This alternative strategy of α-helix mimicking based on sulfono-γ-AApeptides provides a new paradigm for the preparation of GLP-1R agonists. Exploration of this new strategy for the development of more potent GLP-1R agonists is currently underway.

**MATERIALS AND METHODS**

**Synthetic route of sulfono-γ-AApeptides**
The resin was swelled in N,N′-dimethylformamide (DMF) for 5 min before use, followed by treatment with 20% piperidine/DMF solution (2 ml) for 15 min (×2) to remove an Fmoc (9-fluorenyl methoxy-carbonyl) protecting group, and washed with DCM (×3) and DMF (×3) afterward. A premixed solution of the Fmoc protected regular amino acid/sulfono-γ-AApeptide building block (2 eq), HOBt (4 eq), and dissolved inorganic carbon (4 eq) in 2 ml of DMF was added to the resin and shaken for 4 hours to complete the coupling reaction. After washing with DCM and DMF, the resin was treated with 20% piperidine/DMF solution for 15 min (×2). Another Fmoc protected regular amino acid/sulfono-γ-AApeptide building block (2 eq) was attached on the resin following the procedure in the first coupling step, and the Fmoc protecting group was removed after the coupling reaction was done. The reaction cycles were repeated until the desired sulfono-γ-AApeptides were synthesized. For the capped sequence, the N terminus was treated with acetic anhydride (1 ml) in pyridine.
Fig. 5. Stability study and oral glucose tolerance test. (A) Analytic HPLC traces of 1, 2, 3, and 5 before and after incubation with Pronase (0.1 mg/ml). (B) The serum stability of 1, 2, 3, and 5 was determined in 25% serum (v/v) at 37°C for 24 hours. (C and D) Pharmacodynamics of the GLP-1 mimic peptides in mice. A single dose of peptides was intraperitoneally administered into mice 1 hour before the oral glucose tolerance test (OGTT) (2 g/kg glucose). (C) Blood glucose concentrations were monitored for up to 120 min after oral glucose challenge. (D) Average area under the curve (AUC) calculated from OGTT data. Results show mean ± SEM of six mice per treatment group; *P < 0.05 versus vehicle; t test.
and Milan, Italy) (33) The serum stabilities of peptides were determined in 25% (v/v) Serum stability assays ultraviolet detector was set to 215 nm. The final sequence was cleaved using trifluoroacetic acid (TFA)/DCM (3 ml, 1:1, v/v) for 3 hours. The cleavage solution was collected, and the beads were washed with DCM (3 ml × 2). The solution was combined and evaporated under air flow to give the crude product, which was analyzed and purified by the Water Alliance HPLC System, at flow rates of 1 and 16 ml/min for analytic and preparative HPLC, respectively. The gradient elution method of 5 to 100% of solvent B [0.1% TFA in acetonitrile (MeCN)] in A (0.1% TFA in H2O) over 50 min was performed. All the sulfono-γ-AA peptides were obtained with a purity >95% after prep-HPLC purification.

Circular dichroism CD spectra were measured on an Aviv 215 CD spectrometer using a 1-mm path length quartz cuvette, and compound solutions in PBS buffer were prepared using dry weight of the lyophilized solid followed by dilution to give the desired concentration (100 μM) and solvent combination (23, 24). Ten scans were averaged for each sample, three times of independent experiments were conducted, and the spectra were averaged. The final spectra were normalized by subtracting the average blank spectra. Molar ellipticity \([\theta] = \theta_{\text{obs}}/(n \times l \times c \times 10)\) was calculated using the equation

\[ \theta_{\text{obs}} = \frac{\theta}{n \times l \times c \times 10} \]

where \(\theta_{\text{obs}}\) is the measured ellipticity in millidegrees, \(n\) is the number of side groups, \(l\) is path length in centimeters (0.1 cm), and \(c\) is the concentration of the sulfono-γ-AA peptide in molar units.

In vitro GLP-1R activation assay Chokl cells overexpressed with GLP-1R were incubated with increased concentration of tested compounds for 30 min at 37°C. The dose response is plotted as the HTRF ratio (EM665/615 nm). Data points are the mean ± SEM of ≥3 independent experiments with duplicate measurements for each experiment. Half maximal effective concentration (EC_{50}) values were calculated for each duplicate and the mean values for cAMP EC_{50} ± SEM are reported in the table (15).

Enzymatic stability study Lead compounds (3 and 5) and regular GLP-1 peptides (1 and 2) (0.1 mg/ml) were incubated with protease (0.1 mg/ml) in 100 mM ammonium bicarbonate buffer (pH 7.8) at 37°C for 24 hours (23, 24). Then, the reaction mixtures were concentrated in a speed vacuum at medium temperature to remove water and ammonium bicarbonate. The resulting residues were redissolved in H2O/MeCN and analyzed on a Waters Alliance HPLC system with a flow rate of 1 ml/min and 5 to 100% linear gradient of solvent B (0.1% TFA in MeCN) in A (0.1% TFA in H2O) over a duration of 50 min. The ultraviolet detector was set to 215 nm.

Serum stability assays The serum stabilities of peptides were determined in 25% (v/v) aqueous pooled serum from human male AB plasma (Sigma-Aldrich, Milan, Italy) (33). Peptides were dissolved in H2O/MeCN (70:30 v/v) and then diluted in serum and incubated at 37°C for 24 hours. Fifty microliters of solution was added to 50 μl of MeCN on ice for 20 min and then was centrifuged at 4°C for 15 min. The supernatant was diluted with H2O (0.1% TFA) at a final concentration of 0.1 mg/ml analyzed by reversed-phase HPLC. The amount of intact peptide was estimated by integrating the area under the corresponding elution peak monitored at 215 nm.

OGTT and intraperitoneal glucose tolerance test All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of California Institute for Biomedical Research (Calibr) and strictly followed the NIH guidelines for humane treatment of animals. Female Charles River C57BL/6 mice were fasted overnight and then intraperitoneally administered with 100 μl of each peptide in PBS (pH 8.2) containing 1% dimethyl sulfoxide. After 60 min, mice were orally or intraperitoneally administered with 2 g of glucose solution per kilogram of body weight and their tail blood glucose levels were measured before (0 min) and after glucose challenge for 2 to 3 hours.

Supplementary materials Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/20/eaaz4988/DC1

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