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Data-Driven Synthesis of Proteolysis-Resistant Peptide Hormones

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Supporting Information

ABSTRACT: Peptide hormones are key physiological regulators, and many would make terrific drugs; however, the therapeutic use of peptides is limited by poor metabolism including rapid proteolysis. To develop novel proteolysis-resistant peptide hormone analogs, we utilize a strategy that relies on data from simple mass spectrometry experiments to guide the chemical synthesis of proteolysis-resistant analogs (i.e., data-driven synthesis). Application of this strategy to oxyntomodulin (OXM), a peptide hormone that stimulates insulin secretion from islets and lowers blood glucose *in vivo*, defined the OXM cleavage site in serum, and this information was used to synthesize a proteolysis-resistant OXM analog (prOXM). prOXM and OXM have similar activity in binding and glucose stimulated-insulin secretion assays. Furthermore, prOXM is also active *in vivo.* prOXM reduces basal glucose levels and improves glucose tolerance in mice. The discovery of prOXM suggests that proteolysis-resistant variants of other important peptide hormones can also be found using this strategy to increase the number of candidate therapeutic peptides.

The use of insulin, a prototypical peptide hormone, to treat diabetes is one of the landmark achievements of medical research.[1] Many additional peptide hormones regulate physiology, and this fact has not gone unnoticed by the pharmaceutical industry. Today, in addition to insulin, there are several other peptide hormones or peptide hormone analogs that are used in the clinic[2−4] to treat human disease. Analogs are employed because they have improved pharmacological properties, such as an increased half-life. Exenatide, for example, is a naturally occurring proteolysis-resistant glucagon-like peptide 1 (GLP1) analog that is used clinically because of its longer half-life.[5,6]

In theory, other peptide hormones could become candidate biologic drugs if proteolysis-resistant analogs could be developed. The design of proteolysis-resistant analogs requires the natural cleavage site of a peptide to be known so that it can be modified. For most peptide hormones, however, the cleavage sites are currently unknown or incorrectly assigned. We report that mass spectrometry data can be used to identify relevant peptide cleavage sites,[7] followed by the use of this information to design a proteolysis-resistant peptide hormone analog. We demonstrate this strategy with the peptide hormone oxyntomodulin (OXM).[8]

OXM is a gut-secreted 37-amino acid peptide hormone.[9] Pharmacological administration of OXM[8] has been shown to lower blood glucose levels and suppress feeding. OXM is achieves these effects by binding to the glucagon-like peptide 1 receptor (GLP1R),[10] though other receptors might also be involved in its activity.[11] OXM is an attractive therapeutic hormone to treat diabetes and obesity.

Proteolysis of OXM has been reported to occur at the penultimate serine amino acid on its N-terminus by dipeptidyl peptidase 4 (DPP4).[12,13] This assignment was made *in vitro* using recombinant enzyme. We have previously demonstrated that some *in vitro* DPP4 substrates are not cleaved by the enzyme *in vivo* because of substrate competition at the DPP4 active site.[14] Since penultimate serine-containing peptides are not preferred DPP4 substrates,[15] we wondered whether OXM is actually cleaved by DPP4 in serum. Earlier work has reported endogenous OXM_{19-37} and OXM_{30-37} fragments,[16] suggesting that there might be several endogenous OXM fragments. Due to this discordance between reported OXM cleavage sites, we used an unbiased mass spectrometry assay to identify OXM cleavage site(s) in serum.

Addition of OXM to freshly prepared mouse serum resulted in the proteolysis of this peptide over the course of 15 min (Figure 1A), as measured by MALDI-TOF. (Note: different serum preps result in different absolute rates, but the same major fragments are detected in each experimental replication). Analysis of the data revealed that the predominant fragments are OXM_{1-17}, OXM_{1-18}, OXM_{18-37}, and OXM_{19-37} (Figure 1B). The identification of a previously reported endogenous fragment, OXM_{19-37},[16] bolstered our confidence in this data. Based on this information OXM cleavage proceeds between amino acids 17−18 and 18−19 (Figure 1B). A heat-denatured sample abolished all OXM-cleaving activity, which validates that these fragments are produced by proteolytic activity in the sample (Figure 1A).

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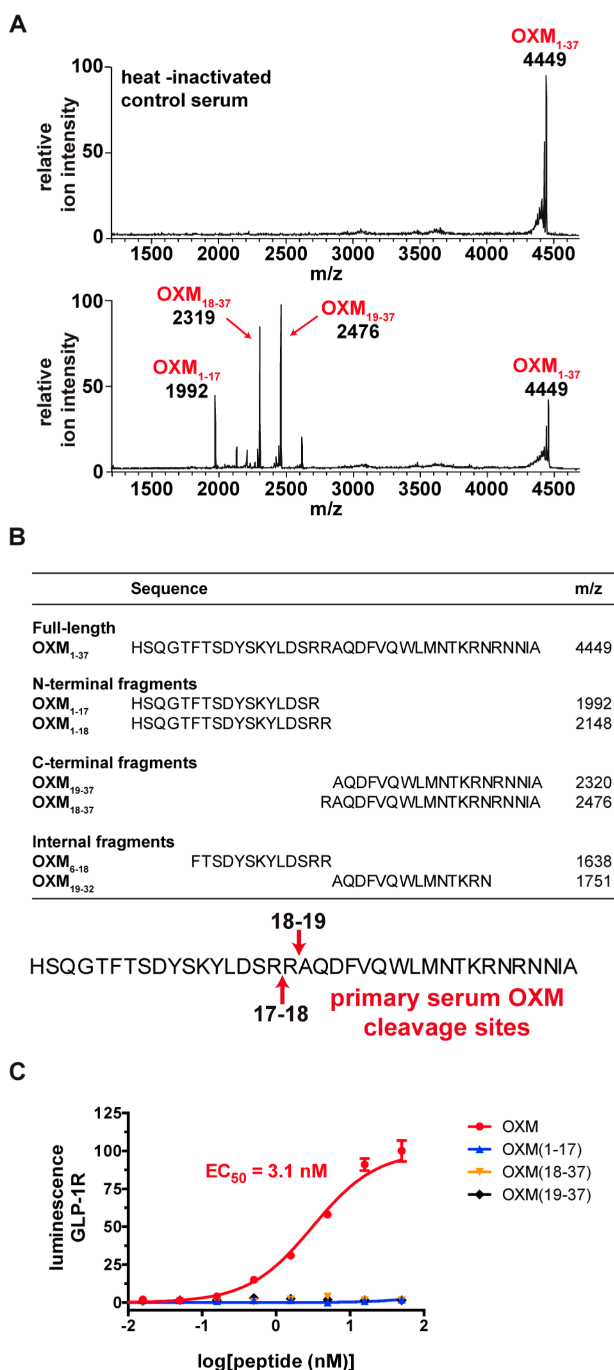

	Sequence	m/z
Full-length		
OXM_{1-37}	HSQGTFTSDYSKYLDSRRAQDFVQWLMNTKRNRNNIA	4449
N-terminal fragments		
OXM_{1-17}	HSQGTFTSDYSKYLDSR	1992
OXM_{1-18}	HSQGTFTSDYSKYLDSRR	2148
C-terminal fragments		
OXM_{19-37}	AQDFVQWLMNTKRNRNNIA	2320
OXM_{18-37}	RAQDFVQWLMNTKRNRNNIA	2476
Internal fragments		
OXM_{6-18}	FTSDYSKYLDSRR	1638
OXM_{19-32}	AQDFVQWLMNTKRN	1751

Figure 1. Serum proteolysis of OXM and activity of OXM fragments. (A) MALDI analysis of OXM (100 μM) incubated with freshly prepared mouse serum (5 mg/mL) for 15 min revealed the generation of numerous OXM fragments that are not present in the heat-inactivated control reaction. (B) The list of serum-generated OXM fragments identifies the primary OXM cleavage sites between amino acids 17−18 and 18−19, which both contain arginine. (C) OXM fragments are inactive as agonists of GLP1R, while the full-length peptide OXM_{1-37} has an EC_{50} of 3.1 nM against this receptor.

We note that DPP4 is present in these serum samples as a DPP4 substrate was added and cleaved by DPP4 (Figure S1). Furthermore, the addition of the DPP4 inhibitor sitagliptin prevented this cleavage (Figure S1). We did not observe any evidence that full-length OXM is cleaved by DPP4, and the addition of sitagliptin did not affect the degradation rate of OXM (Figure S2). In aggregate, the data indicate that the primary OXM cleavage site is between amino acids 17−18 and 18−19 (Figure 1B), and in serum DPP4 does not have a role in OXM proteolysis.

Having determined the predominant OXM cleavage site in serum, we needed to understand what impact OXM cleavage at this site has on the activity of this peptide hormone. As mentioned, OXM is an agonist for the GLP1R,[10] and we used a GLP1R reporter assay to determine the activity, if any, of these OXM fragments (Figure 1C). Titration of full-length OXM, OXM_{1-37}, into this reporter assay resulted in a dose-dependent increase in activation of GLP1R with an EC_{50} of 3.1 nM, which is consistent with previous reports[17,18] (Figure 1C). None of the three OXM fragments we tested (OXM_{1-17}, OXM_{18-37}, and OXM_{19-37}) were active in this assay. Thus, OXM proteolysis in serum results in the loss of OXM GLP1R agonist activity.

Using the data from the cleavage experiments that revealed the two primary cleavage sites, we designed a prOXM analog by mutating Arg17 and Arg18 to alanine (Figure 2A). Previous modifications at or near these sites indicate that peptides can still retain activity,[18,19] which suggested that we might be able to reduce cleavage of OXM while maintaining activity. There were no appreciable differences in the physicochemical

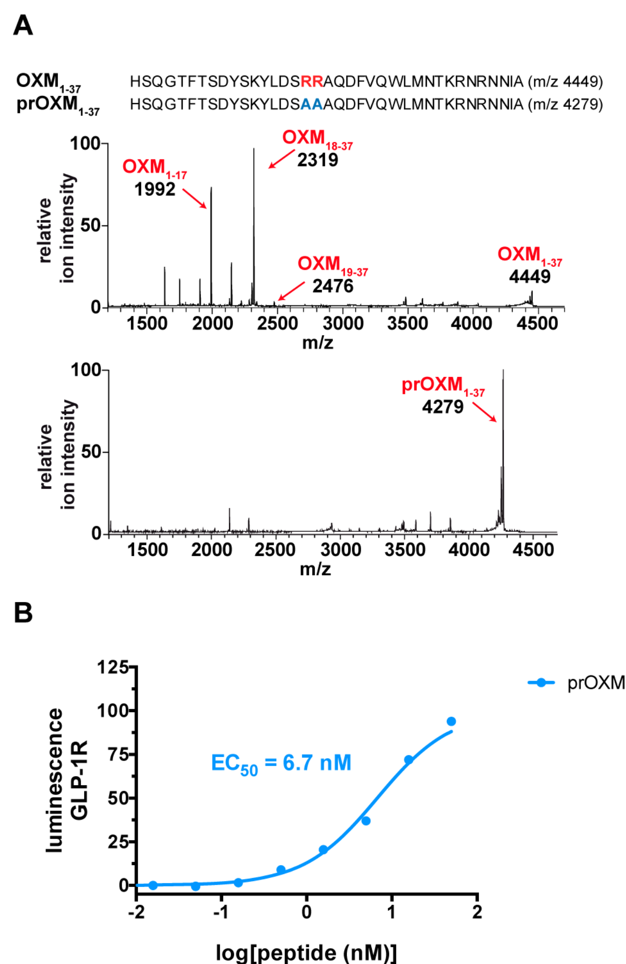

Figure 2. prOXM stability and activity. (A) MALDI-MS analysis of OXM and prOXM (100 μM each) in serum (5 mg/mL) for 15 min reveals that prOXM is significantly more stable than OXM. (B) prOXM is a GLP1R agonst with an EC_{50} of 6.7 nM, similar to the activity of OXM.

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properties between prOXM and OXM that we could observe (i.e., solubility). We repeated our serum assays with prOXM and no longer observed cleavage at amino acid positions 17−18 or 18−19 (Figures 2A, S3 and S4). prOXM is more stable in these assays indicating that we identified the primary OXM cleavage site. This experiment is highly reproducible, and similar results are seen with longer incubation times (30 min) (Figure S3). This validates the second step of the overall strategy by demonstrating that mutation of the cleavage site results in a more stable analog.

Next, we needed to test the activity of prOXM. Two cell-based assays were employed to test whether this prOXM retains activity. First, prOXM was tested in a GLP1R assay. Addition of prOXM between 2 pM to 50 nM resulted in a dose-dependent increase in GLP1R activation, resulting in an EC_{50} of 6.7 nM (Figure 2B). In this case, the amino acid sequences required for efficient proteolysis of OXM are not involved in OXM activation of GLP1R, which made the preparation of an active peptide-hormone analog much easier. In other situations, it is likely that additional mutants will have to be prepared to maintain activity while limiting proteolysis.

We also tested OXM and prOXM in a glucose-stimulated insulin secretion (GSIS) assay using primary mouse pancreatic islets.[20] Molecules that promote GSIS are of therapeutic interest because they can raise endogenous insulin levels to treat diabetes. prOXM raises insulin levels higher than glucose alone indicting that this peptide is active and can promote GSIS (Figure 3A).

The fact that prOXM shows similar activity to OXM in these *in vitro* assays demonstrates that the identification and mutation of key cleavage sites is an efficient method for designing proteolysis-resistant hormone analogs. We also appreciate that simply mutating cleavage sites to alanine will not work in every case because this may disrupt the activity of the peptide and may not inhibit cleavage. Fortunately, chemists have provided a number of alternative solutions to developing proteolysis-resistant peptides. In particular, unnatural amino acids, such as β-amino acids[21] or D-amino acids,[22] that are not substrates for endogenous proteases can be used to overcome limitations of only using natural amino acids.

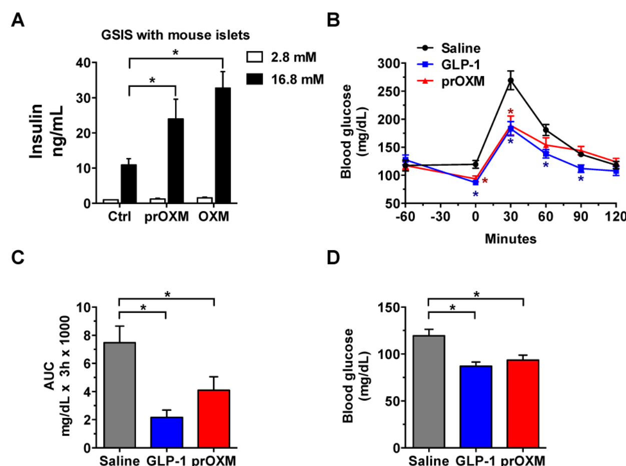

Figure 3. prOXM promotes GSIS with primary mouse islets and glucose tolerance *in vivo*. (A) Mouse pancreatic islets were treated with control, prOXM, or OXM, at low (2.8 mM) or high (16.8 mM) glucose concentrations. prOXM stimulates insulin secretion in the presence of high concentrations of glucose, but not low concentrations (Student's *t* test versus control; *, *p*-value <0.05). (B) Intraperitoneal GTT in mice with saline (vehicle), GLP-1, or prOXM. prOXM improved glucose tolerance indicating that the peptide is active *in vivo* (Student's *t* test versus control; *, *p*-value <0.05). (C) Area under the curve for the saline, GLP-1, and prOXM treated animals between −60 and 120 min. (D) Blood glucose levels prior to glucose challenge (t = 0 min) demonstrates that prOXM reduces basal blood glucose levels.

The *in vitro* activity of prOXM prompted us to explore whether this peptide is active *in vivo*. prOXM was tested *in vivo* using a glucose tolerance test (GTT). We used GLP-1 as a positive control in these assays. At an equivalent dose, prOXM and GLP-1 improved glucose tolerance to a similar extent during the GTT (Figure 3B,C). In addition, prOXM also lowered basal glycemia (Figure 3D). These experiments highlight the therapeutic potential of prOXM for the treatment diabetes.

In aggregate, we demonstrate a simple strategy that identifies cleavage site of a peptide hormone in combination with chemical synthesis can produce novel proteolysis-resistant hormone analogs. We were able to prepare the prOXM and show that this compound is a candidate biologic that should be investigated further. Since nothing in this approach is specific to OXM, we think that this approach should be applicable to the preparation of numerous peptide hormone analogs with improved pharmacological properties. Filling the pipeline with more candidates will increase the chances of identifying novel next-generation biologic therapeutics based on peptide hormones.

■ ASSOCIATED CONTENT

Supporting Information

Additional data and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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