

Structure-Based Design of Glycosylated Oxytocin Analogues with Improved Selectivity and Antinociceptive Activity

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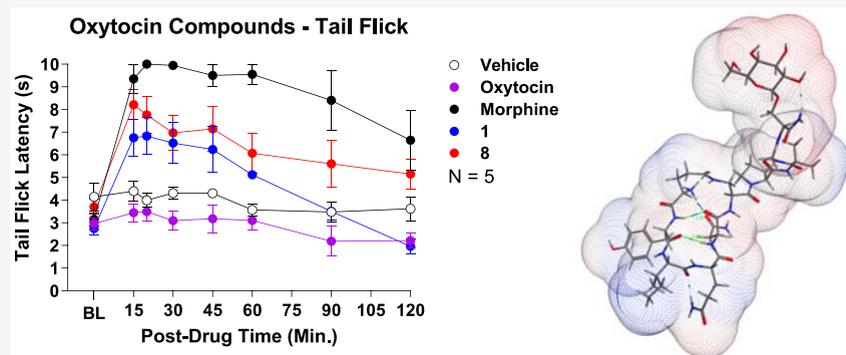
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ABSTRACT: Acute and chronic pain is often treated with opioids despite the negative side effects of constipation, physical dependence, respiratory depression, and overdose. The misuse of opioid analgesics has given rise to the opioid crisis/epidemic, and alternate nonaddictive analgesics are urgently needed. Oxytocin, a pituitary hormone, is an alternative to the small molecule treatments available and has been used as an analgesic as well as for the treatment and prevention of opioid use disorder (OUD). Clinical implementation is limited by its poor pharmacokinetic profile, a result of the labile disulfide bond between two cysteine residues in the native sequence. Stable brain penetrant oxytocin analogues have been synthesized by replacement of the disulfide bond with a stable lactam and glycosidation of the C-terminus. These analogues show exquisite selectivity for the oxytocin receptor and potent *in vivo* antinociception in mice following peripheral (i.v.) administration, supporting further study of their clinical potential.

KEYWORDS: glycopeptide, neurohormone, pain, cyclic, oxytocin agonist

Opioid analgesics are at the center of the current opioid crisis. However, they are legitimately prescribed for otherwise intractable cases of pain. In the United States, pain is the leading cause of distress and comorbidity.¹ Opioid analgesics are the most effective prescribed treatments for moderate to severe pain.^{2,3} They relieve pain but can also cause physical dependency and fatal overdose. Even when medically appropriate, prolonged use can cause physical dependency, tolerance, and withdrawal when discontinued, which can lead to opioid-induced hyperalgesia.⁴ The prevalence of prescription opioid misuse across the United States has increased more than 3-fold over the past two decades, leading to a public health crisis.³ Prescription opioids are now among the most misused drugs in the U.S.⁵

There are currently no opioid medications without risk of misuse, abuse, and addiction.¹ Opioids are responsible for 80% of healthy lives lost from disability and premature death from substance use disorders (SUD).⁵ At least 50% of these deaths

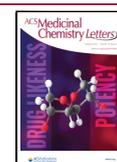
involve fentanyl, a μ opioid receptor agonist (MOR) that is 50–100 times more potent than morphine whether alone or added to illicit drugs.⁵ Fentanyl has become an increasingly significant contributor to the opioid epidemic and is directly linked to opioid-induced respiratory depression (OIRD), the principal cause of death in most overdoses.⁶ Opioid overdose deaths are responsible for over 75% of the drug overdose deaths reported in 2021 through April,^{7,8} indicating that the COVID-19 pandemic exacerbated these problems.

An important consideration of novel pain therapeutics is avoidance of negative opioid side effects, including reinforcing

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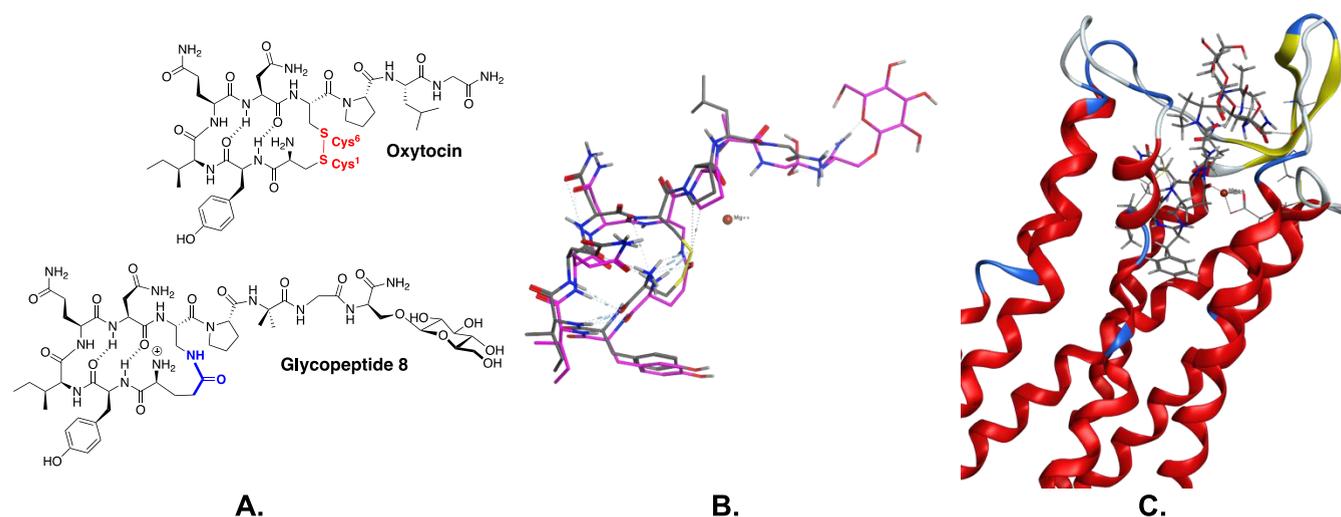


Figure 1. Oxytocin β -turn structures are retained by replacement of the labile S–S bond by amides. (A) Type-I β -turns show conserved intramolecular hydrogen bonds between Tyr²–C=O and N–H with Asn⁵–N–H and C=O backbone atoms. The stabilized lactam ring gains flexibility upon increasing the ring size from a 20- to 21- or even 22-membered ring size but largely retains the intramolecular hydrogen bonds and β -turn structure observed in aqueous solution. (B) Oxytocin and glycopeptide 8 (in Table 1) show strong homogeneity in the receptor-bound (OTR) conformation. (C) The resulting scaffold presents the amino acid side chains toward the OTR. Structural models were determined *in silico* using published cryo-EM coordinates^{22,23} and the Flexible Alignment feature on MOE.

behavior, and oxytocin (OT) has been investigated as a potential OUD treatment in animals. Exogenous OT administration has been shown to mitigate classical addiction metrics, including self-administration, withdrawal, and tolerance.^{9–13} More recently, clinical trials have lacked consistency and yielded mixed results,¹⁰ though strong evidence indicates that intranasal administration of OT leads to increased compliance with therapeutic interventions, addressing a well-established barrier to effective OUD/SUD treatment. Thus, OT was found to be a safe, effective attenuation for nonopioid respiratory depression in patients at increased risk for OIRD.^{6,14} Cardiorespiratory depression caused by fentanyl in rats was dose-dependently reversed by OTR activation, both with OT and with a nonpeptide partial agonist in a recent study by Brackley et al.⁶

Oxytocin has been investigated for treating pain for three decades in both animals and humans.^{10,15–17} Most of these studies showed administration-independent antinociceptive effects¹⁶ (i.e., systemic, peripheral, intrathecal, intracerebroventricular, intranasal, i.v., or inhaled vapor administration); fewer studies showed no significant antinociception.¹¹ OT has also shown mixed results for analgesia in humans,^{9,16} at least partly due to design inconsistencies.^{12,13,18,19}

Unfortunately, OT itself remains a poor drug candidate.^{14,20} Debate over its effective BBB penetration has persisted, although recent evidence indicates success.⁶ Yet its half-life in blood circulation is estimated at 5 min in humans and rats, and about 20 min in rat cerebrospinal fluid (CSF).¹⁴ OT is not completely selective for the oxytocin receptor (OTR), with similar binding affinities for V1a vasopressin receptor (V1a) and transient receptor potential vanilloid type-1 (TRPV1)¹⁴ and moderate binding affinities for other arginine vasopressin receptor subtypes (V1b and V2).¹⁸ Until recently, there was little known about the active conformation of OTR until its crystal structure was determined by cryo-electron microscopy (cryo-EM),²¹ and within the past year, two crystal structures

were published of OT bound to OTR via cryo-EM, giving insight into the OTR:OT activation mechanism.^{22,23}

Synthetic modifications to increase the peripheral bioavailability of OT or its active fragments are well studied, leading researchers to the discovery of carbetocin in 1974.²⁴ Frič et al. modified OT to yield a long-acting uterotonic octapeptide analogue, simultaneously addressing aminopeptidase cleavage and disulfide bond breakage. The peripheral half-life of carbetocin increased from 5 min (OT)¹⁴ to 85–100 min.²⁵ Despite increased metabolic stability, carbetocin has about 10-fold lower affinity for OTR than OT has, and there have been no studies into its CNS effects. To date, many synthetic strategies have been employed to improve OT's pharmaceutical profile.^{14,26,27}

OT, a neurohormone conserved across species for 700 million years,^{15,28} plays a major role in many fundamental physiological processes in both the central and peripheral nervous system.^{15,18,26,29} Its primary role in behavior is “...to facilitate stability in changing environments, rooted in its evolutionary origins as a system to promote survival.”³⁰ This biological significance led to sustained interest in its pharmaceutical application to a range of disorders,²⁷ including pain. Its extensive conservation suggests that at least some of its native structure is advantageous. We made *minimal* structural changes (Figure 1) to address the pharmacological limitations of oxytocin: a lack of receptor specificity and poor bioavailability. We aimed to optimize its druggability and elicit antinociception in mice.

Specifically, we modified the native amino acid sequence and glycosylated the C-terminus of the peptide chain. Replacing the labile disulfide bond with a more robust amide bond produced highly potent glycopeptides, which are exquisitely selective for the OT receptor, brain penetrant, and antinociceptive after intravenous administration in mice. The amide bond was also used to vary the size of the resultant macrocyclic ring. Our synthetic modifications drew on established design strategies in peptide drug design, such as

Table 1. *In Vitro* Molecular Pharmacology of Novel Oxytocin Derivatives^a

	structure	MW/RT _{HPLC}	OTR EC ₅₀ (nM)	AVP1a EC ₅₀ (nM)	AVP1b EC ₅₀ (nM)
OT	C[Cys-Tyr-Ile-Gln-Asn-Cys]-Pro-Leu-Gly-NH ₂	1007.19/—	2.3 ± 1.7		
VP	C[Cys-Tyr-Phe-Gln-Asn-Cys]-Pro-Arg-Gly-NH ₂	1008.2		32 ± 23	11 ± 8
1	C[Asp-Tyr-Ile-Gln-Asn-Dap]-Pro-Leu-Gly-NH ₂	986.61/9.81	3800 ± 3100	NA	NA
2	C[Glu-Tyr-Ile-Gln-Asn-Dap]-Pro-Leu-Gly-NH ₂	1000.12/10.00	7000 ± 3000	NA	NA
3	C[Asp-Tyr-Ile-Gln-Asn-Dap]-Pro-Leu-Ser(Lact)-NH ₂	1340.76/9.37	0.54 ± 0.48	NA	NA
4	C[Asp-Tyr-Ile-Gln-Asn-Dap]-Pro-Leu-Gly-Ser(β-Lact)-NH ₂	1397.76/9.49	160 ± 160	NA	NA
5	C[Asp-Tyr-Ile-Gln-Asn-Dap]-Pro-Aib-Ser(β-Glc)-NH ₂	1150.64/8.84	0.28 ± 0.17	NA	NA
6	C[Glu-Tyr-Ile-Gln-Asn-Dap]-Pro-Leu-Ser(β-Glc)-NH ₂	1192.72/9.65	0.73 ± 0.49	NA	NA
7	C[Glu-Tyr-Ile-Gln-Asn-Dap]-Pro-Aib-Ser(β-Glc)-NH ₂	1164.67/8.50	220 ± 110	NA	NA
8	C[Glu-Tyr-Ile-Gln-Asn-Dap]-Pro-Aib-Gly-Ser(β-Glc)-NH ₂	1221.67/8.73	0.06 ± 0.01	NA	NA

^aBold amino acids indicate cyclization site. Dap = L-1,3-diaminopropionic acid, Aib = α -amino-isobutyric acid. NA = no activity observed. Potency values at the 3 receptors reported as the mean \pm SEM of $N = 3$ independent experiments.

macrocyclization and glycosylation. Macrocyclization has been used for increasing stability toward proteolysis^{31,32} and for providing an entropic advantage in receptor binding (as compared to more flexible linear peptides).³³ Glycosylation typically slows the metabolism of peptide drugs, increasing their bioavailability, and promotes BBB penetration, in addition to increasing receptor selectivity.^{34–40} The sugar serves to modulate membrane affinity of the molecule (nM binding \rightarrow μ M binding),⁴¹ allowing it to “hop” from membrane to membrane and still retain enough membrane affinity to promote transcytosis to cross the BBB.⁴²

Insertions and deletions of the “tail portion” of the lactam analogues were explored in an attempt to increase potency and selectivity of the glycosides. “Extension” of the tail by adding the serine glycoside residue instead of “replacement” of glycine(9) with the serine glycoside provides some additional distance between the receptor binding site and the bulky and well-hydrated sugar. Retention of an aliphatic residue at position 8 seemed to be essential for good activity at the receptor. The non-natural residue α -aminoisobutyric acid (Aib) served as a good replacement for leucine(8), retaining the hydrophobicity without the steric bias of leucine. Further discussion of the modeling and docking process used is discussed in the [Supporting Information](#).

All novel oxytocin derivatives were tested for their potency and selectivity at the oxytocin receptor (OTR) and closely related arginine-vasopressin 1a/1b receptors (AVP1a/b). They were evaluated using an in cell Western assay for ERK MAPK activation in CHO cells expressing human OTR/AVP. We found that oxytocin (OT) and vasopressin (VP) control peptides activated the cells with high potency, as expected (Table 1). The eight OT derivatives showed a range of receptor potencies at the OTR, from the very high (8) to low (1 and 2) with ligands performing in between. Several compounds improved the potency of OT, demonstrating that our modifications can enhance both pharmacodynamic and pharmacokinetic performance (3, 5, 6, and 8). Notably, all derivatives showed no activity at the AVP1a/1b receptors, suggesting that they may be selective *in vivo* and avoid blood-pressure-related side effects caused by AVP activation (Table 1).

Based on their activity in cells, we selected oxytocin derivatives to evaluate their antinociceptive ability *in vivo* (Figure 2). We chose 1 as a comparison since it is not glycosylated and only contains the stabilized amide bond and selected 8 as the most potent glycoside (38-fold more potent

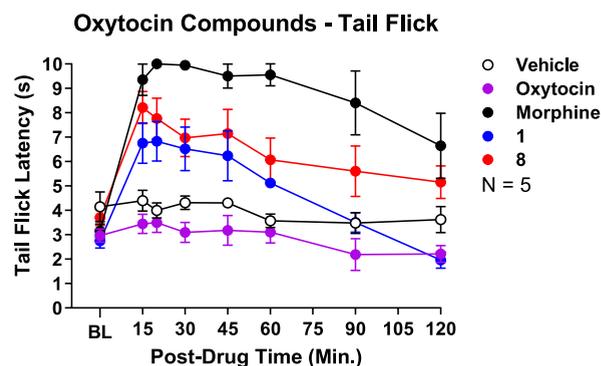


Figure 2. Oxytocin derivatives produce tail flick antinociception in mice with systemic delivery. Male CD-1 mice injected with 10 mg/kg of vehicle control, OT, morphine, 1, or 8 by the intravenous route. Data shown as the mean \pm SEM of $N = 5$ mice/group. Antinociception tested by the tail flick assay, 52 °C water, 10 s cutoff. Vehicle and OT produced no response, while 1 and 8 produced progressive antinociception. Morphine produced expected levels of pain relief, validating the assay.

than OT). All drugs were delivered by the intravenous route (tail vein) to mice. When we tested antinociceptive activity by the tail flick test, we found robust and efficacious pain relief for both 1 and 8, with 8 slightly higher than 1. Vehicle and oxytocin control produced no effect, while morphine produced robust antinociception, validating the assay. These results strongly suggest that our stabilized oxytocin derivatives can penetrate the brain to produce pain relief.

Following the cell-based efficacy data from *in vitro* assays, we selected compound 8 for extensive NMR characterization. Importantly, researchers in the past have found the 20-membered ring of OT to be essential for its binding and activity.^{43,44} To our knowledge, only one other 21-membered analogue has produced OTR agonism, and this alkene-containing derivative required much higher doses to elicit antinociceptive effects.⁴⁵ The results from analogue 8 suggest that the 20-membered ring motif is not essential, since 8 has greater specificity for and activity at OTR despite its 21-membered ring. We obtained NMR spectra to unambiguously confirm the structure of the compound and obtain sequence specific resonance assignments. Sequential NOEs (nuclear Overhauser effects) between the HG/HG' of Glu¹ and HG in Dap⁶ confirm the cyclization between those amino acids (Figure 3). Sequential HN-HA NOEs were not significantly different in strength as compared to the sequential HN-HN

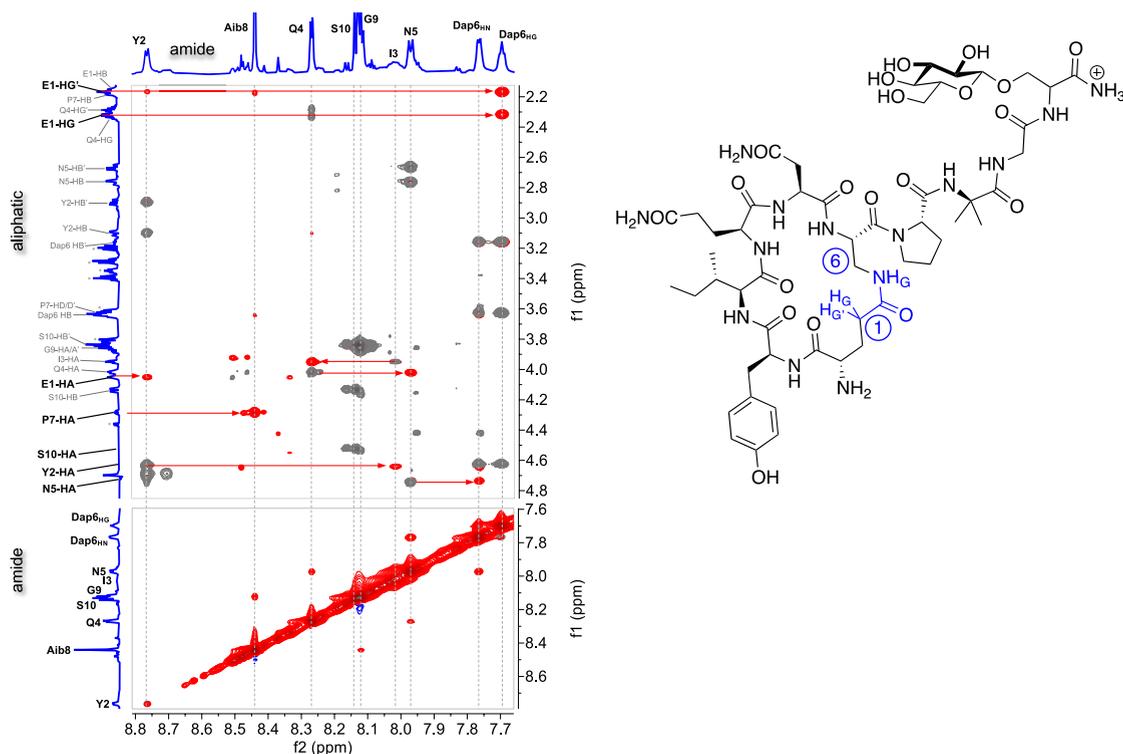


Figure 3. TOCSY (gray) and NOESY (red) spectral overlay of **8**. Asterisks along the f1 trace denote protons in the b-D-glucose moiety. Sequential NOEs between HG/HG' of Glu¹ and Dap⁶ confirm the cyclization between those amino acids, indicated in the structure above in blue. HA signals for Ser¹⁰, Tyr², and Asn⁵ are suppressed by the NMR pulse sequence.

NOEs. Chemical shifts for H α , C α , and backbone carbonyl carbons were compared to Wishart's Chemical-Shift Index (CSI) to predict secondary structure.^{46,47} Variable temperature ¹H NMR (VT NMR) data collected at five temperatures was used to quantify chemical shift changes of the amide protons (temperature coefficient, TC, $\Delta\delta\text{NH}/\Delta T$), which are used to predict the presence of intramolecular hydrogen bonding (IMHB).^{48,49} TC calculations showed evidence of intramolecular hydrogen bonding between residues in the cyclic moiety, although that evidence is weakened by the CSI and NOESY analysis. Preliminary TC data in ionic (SDS) micelles provides much greater evidence for these interactions, although the extent of the contribution from the charge has yet to be determined (manuscript in preparation). Additional spectra, assignments, TC calculations, and CSI analysis can be found in the [Supporting Information](#).

Published studies based on molecular modeling, photo-affinity labeling, and site-directed mutagenesis indicate that the oxytocin macrocycle Cys¹-Tyr²-Ile³-Gln⁴-Asn⁵-Cys⁶ binds into the upper third of the oxytocin receptor pocket at the transmembrane helices TM3, TM4, and TM6. The linear tail Pro-Leu-Gly-amide is bound closer to the surface and interacts with TM2 and TM3.^{50,51} Oxytocin-like biological activity (e.g., selectivity for OT vs AVP) is marked by the presence of two conserved intramolecular hydrogen bonds in the ligand backbone. This bonding system produces a type I β -turn within the macrocyclic ring.^{52,53} In addition, the activity of oxytocin is also associated with a more compact structure as indicated with a radius of gyration (Rg) centered at 5 Å and below. In our studies, all analogues were modeled *in silico*⁵⁴ by building the structures of the compounds guided by either crystal structure of deamino-oxytocin,⁵² oxytocin,²² or MOE-based energy calculations of each amino acid sequence. Each

structure was subjected to charge correction, energy minimization, and conformational search using the Low-ModeMD method available in MOE. Analysis of each ensemble for the presence of β -turn conformers, Rg and dE (i.e., strain energy of the conformation relative to the lowest energy conformation) across all the conformers showed that they all retained an accessible type I β -turn. Descriptors of solubility, lipophilicity, and total polar surface area were calculated for all analogues under evaluation. See the [Supporting Information](#) for details of the calculations.

The stability of these compounds was assessed *in vitro* in artificial cerebrospinal fluid (aCSF) to verify the utility of these structural modifications (see [Supporting Information](#) for data). Substitution of the disulfide cyclization with the amide linker shows a significant increase in tested compounds as compared to oxytocin. Interestingly, unlike most linear (glyco)peptides assessed by us, the half-lives of the cyclic glycosides were not statistically different than their unglycosylated "partners," which indicates that the amide cyclization substitution is a greater contributing factor to stability *in vitro*. The candidates were also studied *in vivo* to assess their serum stability and BBB penetration. The *in vivo* results are also different than most comparisons of glycosylated/unglycosylated peptides we have studied.^{34,37,38,41,42} In the case of the studied OT analogues, the glycosylated peptide was slightly less stable *in vivo*, degrading initially to the unglycosylated peptide. However, in every case, the glycosylated "partner" showed greater CSF concentration than the corresponding unglycosylated peptide, but this improvement was not always statistically significant. BBB penetration was probed by measuring CSF concentration using microdialysis and MS². The tested OT compounds showed a peak in concentration around 10–20 min, and the

CSF levels were sufficiently high to suggest BBB penetration, consistent with the *in vivo* antinociception studies.

Unmodified OT remains a poor drug candidate; however, 700 million years of evolutionary conservation across species and almost 100 years of clinical applications in humans support the merit of much of its structure. Following this logic and guidance from Sir Francis Bacon who taught, “*Nature to be commanded must be obeyed*,”⁵⁵ we made minimal synthetic modifications. OT is known for its short *in vivo* half-life, primarily due to its unstable disulfide bond. Replacement of the labile S–S bond with the more robust lactam (HN–C=O) increased the *in vitro* serum stability from 1.4 ± 0.6 min to 25.8 ± 0.9 min, and glycosylation increased the *in vitro* stability to 34.5 ± 0.9 min. The increased BBB penetration observed with the OT glycosides seems largely due to the increased stability of the lactam rings (see SI for details). In contrast, the antinociception observed with the nonglycosylated peptide **1** may be due to peripheral OT receptor stimulation by the stabilized peptide,⁵⁶ leading to antinociception, despite the poor OTR potency observed in Table 1.

One of the most significant pharmacodynamic shortcomings of OT is its lack of receptor selectivity. It is well-known that promiscuous binding can lead to unwanted and potentially dangerous side effects.^{57,58} Since OT binds to a wide range of receptors, including those involved in physiological regulatory mechanisms we have no interest in modulating (e.g., V1a receptor binding has been linked to bradycardia and body temperature changes after peripheral OT administration),⁴¹ we aim to design analogues that are selective for OTR. Additional support for this aim comes from Brackley et al., who found that V1aR cross-activation from high doses of their OT agonist compounds compromised OIRD reversal in their study of OTR agonism for fentanyl-induced OIRD.⁶

NMR data of **8** in aqueous solution do not indicate a preference for any specific secondary structure. Increasing the intensity of the spectra produces signals that indicate conformational isomers, which is expected for peptides in solution and especially for those containing proline residues.⁵⁹ Glycopeptide **8** showed changes in TC ($\Delta\delta\text{NH}/\Delta T$) that indicate the potential for some structural preference, but this evidence is challenged by the CSI analysis for both proton and carbon spectra (see Supporting Information). Conformational data of OT in aqueous solution has been published, and the receptor-bound conformation of OT•OTR was recently determined via cryo-EM.²³ The crystal structure of OT bound to OTR has been recently determined via cryo-EM.^{22,23} Further NMR studies in micelles (both ionic and neutral) will be used and compared with previous literature examining OT in nonpolar solvents (e.g., CDCl_3 , DMSO)^{60,61} and in ionic (e.g., SDS) micelles.⁶²

Several analogues showed increased potency compared to OT, demonstrating that relatively minor modifications can enhance both pharmacodynamic and pharmacokinetic performance. All compounds described here show no activity at the AVP1a/1b receptors, implying that they will be selective *in vivo* and avoid side effects caused by AVP activation. Antinociception was evaluated using the 52 °C tail flick test in analogue **1** and its glycosylated “partner” **8**, which showed robust, efficacious pain relief. Intravenous delivery in mice requires BBB penetration, and the antinociceptive effects *in vivo* suggest that penetration was successful. Serum stability *in vivo* supports this notion from increased brain dialysate levels 10–20 min after administration. These results strongly suggest

that our stabilized oxytocin derivatives can penetrate the brain to produce antinociceptive effects. The half-lives of several glycopeptides were measured both *in vitro* and *in vivo* and showed no statistically significant differences between the glycosylated and unglycosylated peptides of the same sequence, indicating that the lactam substitution is much more significant in stabilizing the compounds than the glycosylation state.

Preliminary data will guide our future work generating additional efficacious analogues by consideration of conformational analysis, cell-based potencies, and extent of BBB penetration to guide our design efforts. These data suggest that the 20-membered ring in OT is **not** required for its activity at OTR, contributing to the growing insights into binding and conformation, and will inform our future drug design efforts. We will modify the structure of **8** and then study its receptor binding affinity, antinociceptive activity, and serum stability. NMR studies (both solution and micellar conditions) will be used to provide additional insight into *in silico* modeling efforts. Ultimately, *in vivo* studies will assess the addictive potential of these compounds to confirm that their administration yields no reward that might lead to overuse and/or misuse analogous to that which is associated with opioids. It is possible that these antiaddictive properties could be retained in OT analogues, making them an even more promising candidate for pain treatment. Further studies looking into this potential must be completed.

Despite decades of efforts, chronic pain treatment remains challenging to researchers and clinicians alike.⁶³ Growing insight into pain perception mechanisms is aiding the search for effective treatments. However, prescription opioids remain the primary approach to pain treatment, and their use is irrefutably dangerous. It is imperative for researchers to discover alternative nonopioid analgesics to address the persistent problem of pain without exacerbating the opioid epidemic. We anticipate that drugs such as **8** can be delivered intranasally, which would be an attractive form of patient-controlled analgesia.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.2c00455>.

Chemical structures, methodology for synthesis and biological evaluations, conformational analysis, compound characterization, and NMR data (PDF)

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Author Contributions

#These authors contributed equally to the study.

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P.T. created the cell lines, measured *in vitro* pharmacology, and performed mouse tail-flick data. L.Z.S. synthesized and purified peptides and glycopeptides. S.S. synthesized and purified glycopeptides. H.J.G. collected and analyzed NMR data and drafted the manuscript. C.L. collected MS data and performed stability studies. F.A.-H. performed *in silico* structural calculations. V.K.K. modified pulse sequences for NMR data acquisition and aided with data analysis. M.L.H. directed microdialysis and MS² studies. M.J.B. and T.F. provided *in vivo* sampling. J.M.S. directed cell and mouse studies, and R.P. designed drug candidates and provided overall project direction. All authors had editorial input into the manuscript.

Notes

The authors declare the following competing financial interest(s): J.M.S., M.L.H., T.F., and R.P. are co-founders of Teleport Pharmaceuticals, LLC, a company for the development and commercialization of glycosylated peptides for neurodegenerative disease and other neurological conditions. J.M.S. is an equity holder in Botanical Results, LLC, a local cannabidiol company; no company products or interests were tested here.

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ABBREVIATIONS:

OUD, opioid use disorder; OIRD, opioid-induced respiratory depression; SUD, substance use disorder; OT, oxytocin; OTR, oxytocin receptor; V1a, V1a vasopressin receptor; TRVP1, transient receptor potential vanilloid type-1; cryo-EM, cryogenic electron microscopy; Cl-HOBT, 6-chloro-1-hydroxybenzotriazole; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; SPPS, solid phase peptide synthesis; PyClock, 6-chloro-benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate; DIPEA, N,N-diisopropylethylamine; Dap, L-1,3-diaminopropionic acid; Aib, α -amino-isobutyric acid; AVP1a/b, arginine-vasopressin 1a/1b receptors; CHO, Chinese hamster ovary; Lact, lactose; Glc, glucose; CD-1, cluster of differentiation 1; HG/HG', gamma proton/gamma proton'; HA, α proton; CA, α carbon; CSI, chemical shift index; VT, variable temp; TC, temperature coefficient; IMHB, intramolecular hydrogen bond; TOCSY, total correlation spectroscopy; TM3, transmembrane helix 3; TM4, transmembrane helix 4; MOE, Molecular Operating Environment; aCSF, artificial cerebrospinal fluid; CDCl₃, deuterated chloroform; MHBA, methylbenzhydryl amine; NMM, N-methylmorpholine; Alloc, allyloxycarbonyl; Oall, allyl ester; Et₂O, diethyl ether; ARRIVE, Association for the Study of Pain or Animal Research: Reporting of In Vivo Experiments; D₂O, deuterium oxide; d₂₅-SDS, deuterated SDS; TSP, 3-(trimethylsilyl)propionic-2,2,3,3 acid; Trt, trityl; TES, triethylsilane; AAALAC, Association and Accreditation of Laboratory Animal Care, International; DMEM/F-12, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; G418, Geneticin; FBS, fetal bovine serum; AP, anterior-posterior; ML, medial-lateral; USP, United States Pharmacopeia; DADLE, [D-Ala², D-Leu⁵]-enkephalin; Rg, radius of gyration

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