Design and Synthesis of Brain Penetrant Glycopeptide Analogues of Pituitary Adenylate Cyclase Activating Peptide (PACAP) for the Treatment of Parkinson's Disease

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Abstract

The pituitary adenylate cyclase activating polypeptide (PACAP) is an endogenous neuropeptide closely related to the two vasoactive intestinal peptides (VIPs). These peptides are members of the secretin family of peptide hormones that activate Class B GPCRs. PACAP binds and agonizes PAC1, VPAC1, and VPAC2 and inhibits neuronal apoptosis. It is considered to be neuroprotective in various pathological conditions in the CNS; making it a potential drug candidate for treating various neurodegenerative disorders. However, native PACAP exhibits poor pharmacokinetics as it is rapidly degraded by several proteases and peptidases; showing low bioavailability. Furthermore, activation of the VPAC2 receptor can lead to undesired peripheral side effects such as vasodilation and water retention. Therefore, it is critical that more stable and PAC1/VPAC1-selective agonists be developed, and that they can be targeted toward the CNS. One strategy that has not been extensively explored in the context of PACAP agonists is glycosylation. In other contexts, glycosylation of peptides has been shown to improve stability, enhance their original biological activities, and modulate their ability to cross cellular barriers like the BBB. To this end, we have designed and synthesized several PACAP glycopeptides containing C-terminal serine glycosides and additional amino acid substitutions. These glycopeptides were evaluated for their ability to stimulate cAMP production *in vitro* using individual CHO cell lines expressing PAC1, VPAC1, and VPAC2 receptors. A select number of the examined glycopeptides exhibited the desired pharmacological profiles. These compounds will be used as leads to further optimize their receptor selectivity, stability, and transport properties *in vivo*.

Background: Advantages of Glycosylation and Applications in Opioid Peptides and Angiotensin

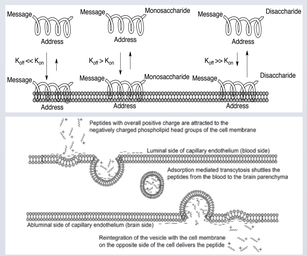


Figure 1. Proposed mechanism of BBB penetration. Glycosylation induces membrane "hopping", which in turn can lead to reversible interactions with the membrane surface. This "hopping" can possibly induce negative membrane curvature and promote adsorptive endocytosis similar to positively charged peptides.

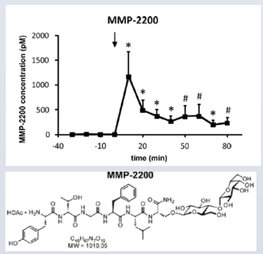


Figure 2. Demonstration of BBB penetration of the Enkephalin-derived glycopeptide MMP2200 via *in vivo* microdialysis.[1]

Table 1. Structures of Angiotensin Glycopeptides

	1	Asp	Arg	Val	Tyr	Ile	His	Pro	COOH
	2	Asp	Arg	Val	Tyr	Ile	His	Pro	$CONH_2$
	3	Asp	Arg	Val	Tyr	Ile	His	Ser	$CONH_2$
	4	Asp	Arg	Val	Tyr	Ile	His	Ser	$CONH_2$
								Glc	
	5	Asp	Arg	Val	Tyr	Ile	His	Ser	$CONH_2$
								Glc-β1,4-Glc	
	6	Asp	Arg	Val	Tyr	Ile	His	Ser	$CONH_2$
								Glc-β1,4-Gal	

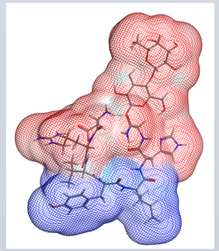


Figure 3. Angiotensin (1-6) lactoside highlighting hydrophobic surface area (red) and hydrophilic surface area (blue).[2]

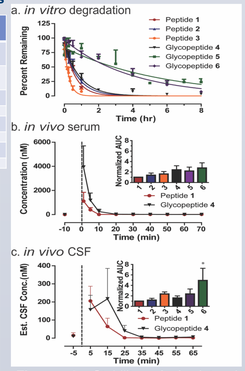


Figure 4. Stability of Angiotensin glycopeptides *in vitro* (a), and *in vivo* in rat serum (b) and in rat CSF (c).[2]

Table 1. Structures of β-Endorphin-Inspired Glycopeptides

S^n = S OH	S^n = S* β-glucose	S^n = S** β-lactose	helix determinant	message~Pro$_n$~helix-amide
U1	G1	L1	~B~B~	YtGFL-P^n-NL$^B_8$EK$^B_{12}$LKS*L-NH_2
U2	G2	L2	~A~B~	YtGFL-P^n-NL$^A_8$EK$^B_{12}$LKS*L-NH_2
U3	G3	L3	~B~A~	YtGFL-P^n-NL$^B_8$EK$^A_{12}$LKS*L-NH_2
U4	G4	L4	~A~A~	YtGFL-P^n-NL$^A_8$EK$^A_{12}$LKS*L-NH_2
U5	G5	L5	~A~G~	YtGFL-P^n-NL$^A_8$EK$^G_{12}$LKS*L-NH_2
U6	G6	L6	~G~A~	YtGFL-P^n-NL$^G_8$EK$^A_{12}$LKS*L-NH_2
U7	G7	L7	~G~G~	YtGFL-P^n-NL$^G_8$EK$^G_{12}$LKS*L-NH_2

S = L-serine, S = β-*O*-glucosyl-L-serine, S** = β-*O*-lactosyl-L-serine, and B = α-aminoisobutyric acid (Aib).

Linker Address		A_{50} Values, μmol/Kg (95% Confidence Intervals)		
		R = OLact	R = OGlc	R = OH
Helicity (Low → High)	BB	3.89 (3.0-5.1)	3.39 (2.6-4.5)	>14.9 (n/a)
	BA	0.96 (0.67-1.4)	0.32 (0.24-4.2)	>4.72 (n/a)
	AB	1.11 (0.93-1.33)	3.86 (3.0-5.0)	>4.75 (n/a)
	AA	3.71 (2.6-5.3)	4.30 (3.3-5.7)	>4.75 (n/a)
	AG	7.91 (5.3-11.8)	4.07 (2.9-5.8)	>4.78 (n/a)
	GA	1.19 (0.88-1.6)	3.43 (2.6-4.5)	>4.78 (n/a)
	GG	>4.16 (n/a)	4.21 (3.0-5.9)	>4.81 (n/a)

Figure 5. Potency values in mouse tail flick assay following i.v. administration. The glycopeptides were more potent than the unglycosylated controls, suggesting penetration through the BBB.[3]

Glycosylation and Structural Modifications of PACAP: Improving BBB Transport and Receptor Selectivity

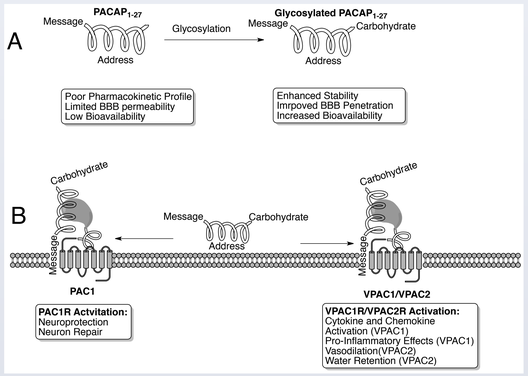
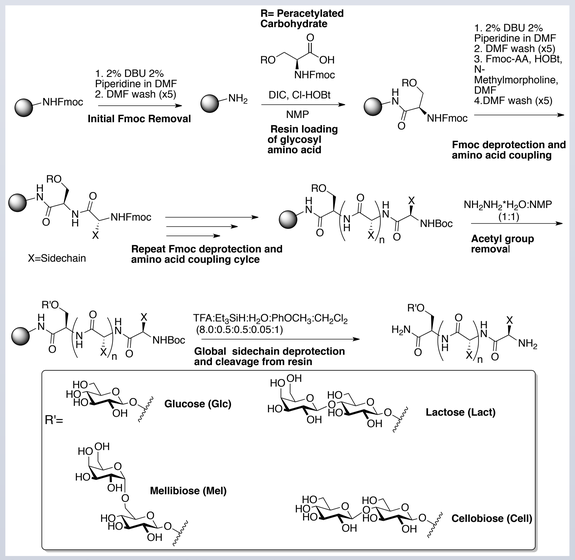


Figure 4. A. Native PACAP is rapidly degraded *in vivo* and exhibits limited BBB penetration. Glycosylation should effectively address these concerns. **B.** Another aspect of the PACAP glycopeptide design of is shifting selectivity towards the PAC1 receptor to reduce side effects mediated through the VPA1 and VPAC2 receptors.[4]

Materials and Methods



Scheme 1. Synthesis of PACAP glycopeptides. The glycopeptides were synthesized on a Rink amide resin using standard Fmoc SPPS protocols. Prior to cleavage from the solid support, the acetyl groups on the carbohydrate moiety were removed using a 50% solution of $NH_2NH_2{\cdot}H_2O$ in *N*-methyl-2-pyrrolidone. The crude peptides were washed with cold ether and purified using reversed-phase HPLC. The fractions collected from HPLC purification were then lypohlilized to afford the pure glycopeptides in moderate yields.

Results

Table 2. Functional activity of PACAP glycopeptides at PAC1, VPAC1 & VPAC2.

		PAC1		VPAC1		VPAC2	
Compound	Sequence	EC_{50} (nM)	E_{MAX} (%)	EC_{50} (nM)	E_{MAX} (%)	EC_{50} (nM)	E_{MAX} (%)
$PACAP_{1-27}$	HSDGIFTDSY$_{10}$SRYRKQMAVK$_{20}$KYLAAVL-$CONH_2$	0.4, 0.13, 0.34	100	14.8 ± 1.6	100	0.35 ± 0.16	100
CRA3000	HsDGIFTDSY$_{10}$SRYRKQÑAVK$_{20}$KYLAAVL-Ser(Glc)-$CONH_2$	25.5	85	1.3	90	241	104
CRA3001	HsDGIFTDSY$_{10}$SRYRKQÑAVK$_{20}$KYLAAV-Ser(Glc)-$CONH_2$	54.5	86	4.8	90	654	107
CRA3002	HsDGIFTDSY$_{10}$SRYRKQÑAVK$_{20}$KYLAALL-Ser(Glc)-L-$CONH_2$	>250	-79	5.9	93	>2500	-95
CRA3003	HsDGIFADSY$_{10}$SRYRKQÑAVK$_{20}$KYLAAVL-Ser(Glc)-$CONH_2$	>250	-71	78.8	93	>2500	-42
CRA3004	HsDGIFADSY$_{10}$SRYRKQÑAVK$_{20}$KYLAAV-Ser(Glc)-$CONH_2$	NC	NC	1366	85	NC	NC
CRA3005	HsDGIFADSY$_{10}$SRYRKQÑAVK$_{20}$KYLAAV-Ser(Glc)-L-$CONH_2$	NC	NC	1623	78	NC	NC
2ls98Cell	HSDGIFTDSY$_{10}$SRYRKQLAVK$_{20}$KYLAAV$_{d8}$L-Ser(Cell)-$CONH_2$	0.84	92	0.52	93	55.6	91
2ls98Lact	HSDGIFTDSY$_{10}$SRYRKQLAV$_{d8}$K$_{20}$KYLAAV$_{d8}$L-Ser(Lact)-$CONH_2$	0.72	93	0.45	101	193	100
2ls98Mel	HSDGIFTDSY$_{10}$SRYRKQLAVK$_{20}$KYLAAVL-Ser(Mel)-$CONH_2$	0.57	99	0.55	102	9.4	86

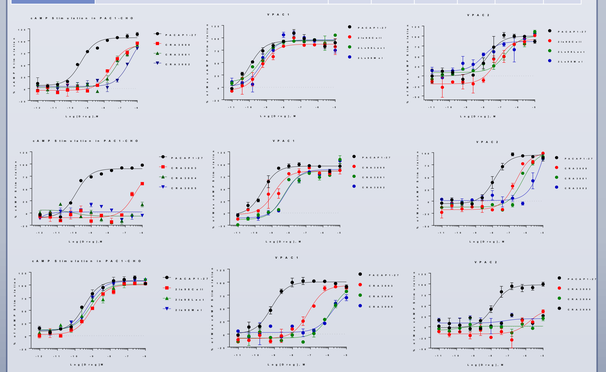


Figure 5. Summary of PACAP glycopeptide functional activity. Compounds were evaluated for their ability to stimulate cAMP production in CHO cells expressing the PAC1 (far left column), VPAC1 (center column), and VPAC2 (far right column) receptors. These data were obtained in Dr. John Streicher's laboratory.

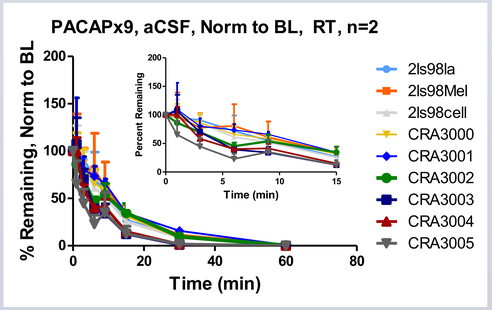


Figure 6. Stability of PACAP glycopeptide analogues in artificial CSF. CRA3000-CRA3002 and the 2ls disaccharide compounds exhibit slightly longer half-life values compared to CRA3003-CRA3005. These data were obtained in Dr. Michael Heien's laboratory.

Conclusions

- The introduction of a carbohydrate residue into the C-terminus does not have a drastic effect on functional activity.
- Substitution of Methionine in position 17 with Norvaline or Leucine is tolerated.
- Substitution of Threonine 7 with Alanine (CRA3003, CRA3004, and CRA3005) is detrimental to functional activity and leads to a slight reduction in half-life compared to the other analogues.
- CRA series of compounds are relatively more VPAC1 receptor selective compared to the PAC1 receptor and VPAC2 receptor.
- 2ls98Cell, 2ls98Lact, and 2lsMel exhibited higher selectively for the PAC1 and VPAC1 receptors over the VPAC2 receptor.
- The half-lives of all the PACAP glycopeptides were roughly around 15 minutes except for CRA3003, CRA3004, and CRA3005, which exhibited half-life values closer to 10 minutes.

Future Directions

- N-acylation of the N-terminus will be explored to improve half-life.
- Amino acid substitutions will be performed in positions 4 and 5. This region may be to be involved with receptor selectivity.[5,6]
  - Flexible amino acids or amino acid-like linkers will be placed in position 4.
  - Amino acids with alkyl side chains of varying steric bulk will be placed in position 5.
  - Simultaneous substitution at positions 4 and 5 containing α-helix and β-turn inducing motifs will be explored.
- Analogues containing extended C-termini will be explored to regain PAC1 receptor selectivity

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Acknowledgements

Research reported in this publication was supported by the National Institute Of Neurological Disorders and Stroke of the National Institutes of Health under Award Number R01NS091238. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional funding has been granted by the Migraine Research Foundation, the Michael J. Fox Foundation and the RDI Accelerate for Success program.