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Stapled Vasoactive Intestinal Peptide (VIP) Derivatives Improve $VPAC_2$ Agonism and Glucose-Dependent Insulin Secretion

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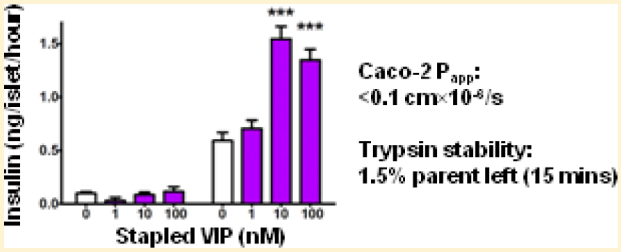
Supporting Information

ABSTRACT: Agonists of vasoactive intestinal peptide receptor 2 ($VPAC_2$) stimulate glucose-dependent insulin secretion, making them attractive candidates for the treatment of hyperglycaemia and type-II diabetes. Vasoactive intestinal peptide (VIP) is an endogenous peptide hormone that potently agonizes $VPAC_2$. However, VIP has a short serum half-life and poor pharmacokinetics in vivo and is susceptible to proteolytic degradation, making its development as a therapeutic agent challenging. Here, we investigated two peptide cyclization strategies, lactamisation and olefin-metathesis stapling, and their effects on $VPAC_2$ agonism, peptide secondary structure, protease stability, and cell membrane permeability. VIP analogues showing significantly enhanced $VPAC_2$ agonist potency, glucose-dependent insulin secretion activity, and increased helical content were discovered; however, neither cyclization strategy appeared to effect proteolytic stability or cell permeability of the resulting peptides.



KEYWORDS: *VIP, vasoactive intestinal peptide, VPAC, peptide agonist, insulin secretion, glucose tolerance, stapled peptide, lactamized peptide, ring-closing metathesis*

Vasoactive intestinal peptide (VIP) is a 28 residue linear neuropeptide initially isolated from porcine intestine.[1] VIP is expressed in the central nervous system and peripheral tissues and has been shown to mediate numerous physiological processes (e.g., vasodilation, inflammation,[2] and neuroprotection[3,4]) as recently reviewed.[5] VIP belongs to the secretin/glucagon class, a group of structurally related peptides encompassing several regulatory hormones, including glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP). In common with other secretin-like peptides, VIP exerts its physiological actions by regulating the B1 family of G-protein-coupled receptors (GPCR). VIP binds to and activates two receptor subtypes: $VPAC_1$ and $VPAC_2$.[6,7] The manifold functions mediated through these receptors have been recently summarized.[8]

Interestingly, VIP was shown to elicit insulin secretion in insulinoma cells, mouse pancreatic islets, and perfused rat pancreas in a glucose-dependent manner.[9−11] This effect is largely mediated through its action on $VPAC_2$, as suggested by the receptor expression profile in insulin-secreting cells.[12] Briefly, agonism of $VPAC_2$ enhances glucose-induced insulin secretion, while $VPAC_1$ activation increases glucose production by the liver through glycogenolysis. Hence, a $VPAC_2$-selective agonist may counter the hyperglycaemia commonly associated with type-II diabetes through increased insulin release, without a $VPAC_1$-mediated increase in hepatic glucose production.[11] Accordingly, a selective peptide agonist of $VPAC_2$, BAY55-9837, stimulated glucose-dependent insulin secretion in vitro, resulting in a dose-dependent increase in plasma insulin and improved glucose tolerance in fasted rats.[11] In contrast, Asnicar et al. showed that $VPAC_2$ knockout mice demonstrate improved insulin sensitivity after glucose challenge.[13] On the basis of these results, we became interested in the development of a peptide-based $VPAC_2$ agonist to further explore the role of this receptor in insulin regulation.

Major challenges facing development of therapeutic peptides generally include their often high metabolic instability, rapid renal clearance, and limited cellular absorption. For instance,

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proteolytic degradation and renal clearance account mainly for the fact that the in vivo half-life of VIP is less than one minute,[14] making its chronic use problematic. Therefore, limiting or preventing proteolytic degradation would be of value in developing VIP as a therapeutic.

Conformational-restriction by peptide cyclization are valuable in certain instances, as reviewed by Jiang et al.[15] Given the α-helical nature of VIP, we felt that side chain-to-side chain stapling using either olefin metathesis[16−20] or lactamization could be applied here.[21] Both techniques are known to stabilize and enhance native α-helicity, which may increase plasma stability and potentially enable in vivo dosing. Both approaches are also likely to increase proteolytic stability by shielding otherwise exposed scissile bonds from the relevant degrading enzymes. Olefin stapling of BH3 helix mimetics,[22,23] NOTCH transcription factor complex inhibitor[24] and HIV-1 fusion inhibitor,[25] and lactamization of GLP-1[21] and glucagon[26] have been shown to impart significantly improved therapeutic properties. When cyclization retains the bioactive conformation, both an increase in target affinity and selectivity may result, however, such advantages cannot be taken for granted.[27]

Within this work, we set out to explore the effects of side chain-to-side chain cyclization on enhancing potency, proteolytic stability, and cell membrane permeability of VIP. Although a number of structure−activity relationships studies on VIP have been reported,[28,29] our design rationale was based on the hypothesis that VIP binds the $VPAC_2$ receptor in an α-helical conformation, in an analogous manner to that demonstrated for GIP binding the extracellular domain of its receptor.[30] While structural information for the VIP-$VPAC_2$ receptor complex is not yet available, superimposition of the extracellular domains of the GIP and $VPAC_2$ receptor,[31] coupled with sequence alignment of VIP and GIP, identified those VIP residues likely to interact with $VPAC_2$ (V^{19}, Y^{22}, L^{23}, I^{26}, L^{27}). Also indicated were those residues that could likely be mutated without significantly disturbing these interactions (L^{13}, M^{17}, K^{21}, N^{24}, S^{25}, N^{28}). With the latter null residues in mind, we felt that functionalization in these positions would retain both the active structure and original potency/selectivity profile, while shielding potentially susceptible protease cleavage sites. Hence, we designed, synthesized, and characterized stapled VIP-based peptides to verify their potential as $VPAC_2$ agonists and insulinotropic agents. Specifically, both lactam- and olefin-based staples were incorporated in identical positions, and the corresponding unstapled peptides from each class served as linear, noncyclic internal controls. Accordingly, residues L^{13}, M^{17}, K^{21}, N^{24}, S^{25}, N^{28} were stapled using an i, i + 4 pattern, as summarized in Figure 1. We then transposed M^{17} to I^{17},

```
                1.....5.....10.....15.....20.....25...28
VIP             HSDAVFTDNYTRLRKQMAVKKYLNSILN
I17-VIP-GK      HSDAVFTDNYTRLRKQIAVKKYLNSILNGK
St1*            HSDAVFTDNYTR*RKQ*AVKKYLNSILNGK
St2*            HSDAVFTDNYTRLRKQ*AVK*YLNSILNGK
St3*            HSDAVFTDNYTRLRKQIAVK*YLN*ILNGK
St4*            HSDAVFTDNYTRLRKQIAVKKYL*SIL*GK
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Figure 1. Design hypothesis for VIP-based stapled peptides. Red and green residues highlight chosen residues for stapling and additional chemical modification, respectively. Asterisks indicate the position of residues, which may be reacted to form the corresponding lactam or olefin staple or left unreacted to act as the unstapled linear peptide control.

facilitating synthesis and purification of the native peptide, a well tolerated modification, which has been exploited in half-life extension strategies.[32] Incorporation of a *C*-terminal extension motif G^{29} K^{30} provided not only a potential reactive handle, but also acts to neutralize the macrodipole and offers helix-capping hydrogen bonds, based on the structure of receptor-bound GIP (Figure 1).

Table 1 shows that the initial modifications made to wild-type VIP did not compromise functional $VPAC_2$ agonism, and

Table 1. $VPAC_2$ Receptor Functional Agonism for Compounds 1−14

	staple position[a]	i,i + 4	stapled	$VPAC_2$ EC_{50} (nM)[b]
VIP			no	0.19 (±0.04)
I^{17}-VIP-GK			no	0.14 (±0.09)
1	13−17	E,K	no	6.6 (±1.8)
2	17−21	E,K	no	0.38 (±0.11)
3	21−25	E,K	no	0.21 (±0.11)
4	13−17	E,K	yes	1.16 (±0.41)
5	17−21	E,K	yes	0.10 (±0.03)
6	21−25	E,K	yes	0.11 (±0.04)
7	13−17	X,X[c]	no	1.37 (±0.57)
8	17−21	X,X[c]	no	1.49 (±0.35)
9	21−25	X,X[c]	no	3.8 (±2.5)
10	24−28	X,X[c]	no	0.21 (±0.23)
11	13−17	X,X[c]	yes	0.049 (±0.02)
12	17−21	X,X[c]	yes	0.33 (±0.10)
13	21−25	X,X[c]	yes	1.52 (±0.54)
14	24−28	X,X[c]	yes	0.39 (±0.28)

[a]Residues numbered as shown for VIP (Figure 1). [b]Means (±SE) (n = 9) (Supporting Information). [c](S)-pentenylalanine.

therefore, **I^{17}-VIP-GK** serves as a good reference peptide against which other analogues described here may be compared. In addition to in vitro potency data recorded on $VPAC_2$, far-UV CD spectra (190−260 nm) were also recorded for the linear reference peptide **I^{17}-VIP-GK** and derivatives **1**−**14** to show secondary structural changes resulting from functionalization and/or cyclization. When lactams were introduced in peptides **4**−**6**, the most effective staples were those bridging positions 17−21 and 21−25 in peptides **5** and **6**, with $VPAC_2$ EC_{50} values of 0.10 (±0.03) and 0.11 (±0.04) nM, respectively. Although the potency increase is small, both peptides are more potent than either wild-type VIP or **I^{17}-VIP-GK** (Table 1). While peptide **5** offered a notable potency improvement over its "unstapled" counterpart **2** (EC_{50} = 0.10 (±0.03) vs 0.38 (±0.11) nM, respectively), functional activity for lactam **6** and its linear analogue **3** were more similar (EC_{50} = 0.11 (±0.04) vs 0.21 (±0.11) nM, respectively). Interestingly, polar charged mutations L13E and I17K, while remaining unlactamized in peptide **1**, drastically reduce $VPAC_2$ agonism in comparison with **I^{17}-VIP-GK** (EC_{50} = 6.6 (±1.8) vs 0.14 (±0.09) nM, respectively) representing a potency loss of ∼50-fold. Lactamization of **1** between residues 13−17 effectively removes charged E^{13} and K^{17} side chains in peptide **4**; restoring some functional activity (EC_{50} = 6.6 (±1.8) vs 1.16 (±0.41) nM, respectively) though not to the original level of **I^{17}-VIP-GK**. Figure 2a shows that CD spectra produced by open lactam precursors **1**−**3** closely resemble that of the linear model peptide and that their lactamized variants **4**−**6** exhibit far greater α-helical content as expected (Supporting Information). However, while it is true that in all cases lactamized peptides

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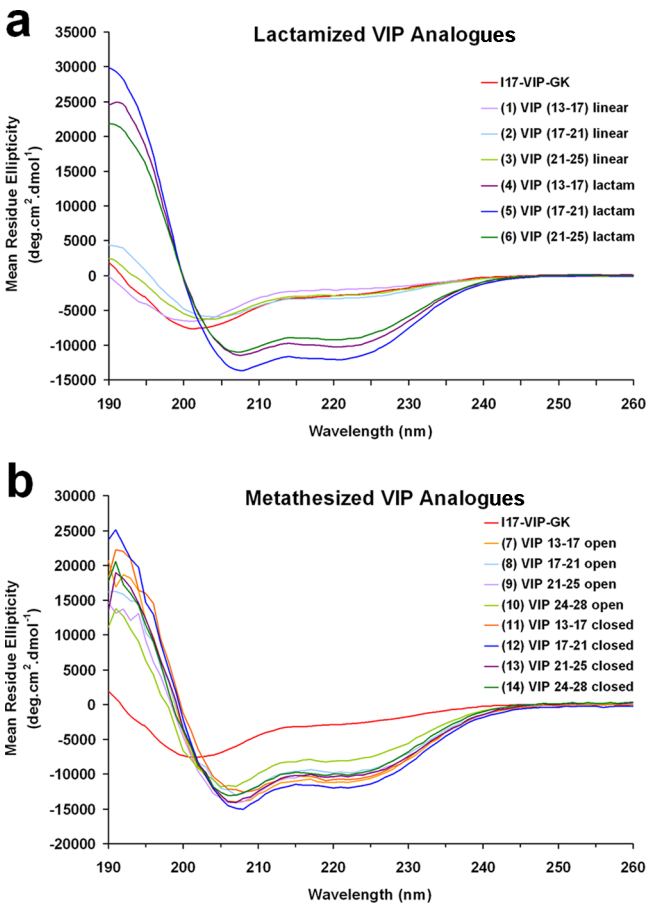

Figure 2. Circular dichroism spectra for lactamized (a) (**4**–**6**) and metathesis stapled (b) (**11**–**14**) VIP peptides. The corresponding linear analogues (**1**–**3** and **7**–**10**, respectively) and the reference **I^{17}-VIP-GK** (shown in red) peptide are included for structural comparison.

show increased in vitro potency over their linear counterparts, the fact that lactam **4** is significantly less potent than the linear model compound indicates that potency at $VPAC_2$ does not correlate with α-helicity. In the case of peptides **1** and **4**, this finding indicates that functionalization of VIP in positions 13–17 with polar residues proves deleterious to receptor binding, even though the overall α-helical content of peptide **4** is increased significantly through lactamization (Figure 2 and Supporting Information). The ∼50-fold potency loss observed with peptide **1** is perhaps not surprising since we are mutating native hydrophobic L^{13} and M^{17} to polar charged E^{13} and K^{17}, respectively, a mutation likely to strongly disfavor any hydrophobic interactions potentially required for $VPAC_2$ receptor binding. Further corroborating this hypothesis, the removal of the charge-interaction likely to occur between E^{13} and K^{17} in peptide **1** by lactamization affords an uncharged (though still polar) cyclic amide in peptide **4**. Although **4** gains ∼8-fold potency over its unlactamized analogue **1**, it does not fully restore the original potency of **I^{17}-VIP-GK**.

Unlactamized peptides **2** and **3** are only marginally less potent than **I^{17}-VIP-GK**, indicating that the chosen substitutions in positions 17, 21, and 25 are well tolerated. In **2**, native K^{21} is retained, and the only effective mutation is therefore $L^{17}E$. Since the potency difference between **2** (**E^{17}-VIP-GK**) and **I^{17}-VIP-GK** is small (∼2-fold), mutation of hydrophobic L^{17} to polar charged E^{17} appears insignificant. This implies that it is mutation $L^{13}E$ in unlactamized **1** that accounts for the majority of potency lost; supporting reports suggesting that M^{17} mutations are relatively benign and well tolerated.[32] Incorporation of (*S*)-pentenylalanine in positions analogous to the linear and lactamized peptides **1**–**3** and **4**–**6**, respectively (Figure 1), are allowed direct comparison of both open (bis-olefinic) and closed (metathesized) peptides **7**–**10** and **11**–**14** respectively. The open linear peptides that retain their terminal olefin functions could be considered almost isosteres of the hydrophobic unnatural amino acid norleucine. In addition to the olefin side chain of pentenylalanine, the residue also bears an α-methyl substituent, which alone appears to significantly increase the α-helicity of all peptides containing the residue (**7**–**14**), stapled or otherwise, as shown in the CD spectra (Figure 2b) and calculated α-helical content (Supporting Information). Given the topological similarities between cyclization using either amide-based lactams, or hydrocarbon staples, we were surprised to have observed such large differences in potency of the resulting peptides; particularly in light of the fact that the relative α-helicity of peptides **4**–**14** is so similar.

Peptides prepared using olefin staples generally showed a similar trend to those incorporating lactams, and cyclization between the positions explored generally increased $VPAC_2$ agonism. Interestingly, the only exception to this was observed between stapled peptide **14** and its unstapled variant **10**, the latter (bis-olefin) proving to be the more potent $VPAC_2$ agonist. The mutations involved are $N^{24}X$ and $N^{28}X$, implying that the polarity of N^{24} and N^{28} are useful in the random coiled region of the peptide at its native *C*-terminus.[33] This result also indicates that constraining the peptide in this region disturbs its ability to agonize $VPAC_2$. Overall, hydrocarbon-stapled peptide **11** afforded the most potent $VPAC_2$ receptor agonist evaluated in this study (EC_{50}, 0.049 (±0.02) nM), a significant improvement over both **I^{17}-VIP-GK** (∼3-fold) and wild-type VIP (∼4-fold) (Table 1). This potency gain is especially noteworthy considering that (a) its linear analogue **7** showed a significant drop in activity (∼10-fold) in comparison to **I^{17}-VIP-GK** and that (b) the corresponding lactam **4** was >20 times less potent. Again, this indicates that not only does positioning of the staple have an impact on binding and $VPAC_2$ potentiation but also that hydrophobic interaction plays an important role here. In the case of the lactamized peptides, we previously hypothesized that mutation L13E in unlactamized **1** accounts for the majority of potency lost from the otherwise identical peptide **2**. Here, in peptide **11**, mutations $L^{13}X$ and $I^{17}X$ do not imply that hydrophobicity is paramount, rather that hydrophobic constraints are more effective than polar lactam constraints in positions 13–17. The ∼30-fold increase in potency between corresponding hydrocarbon stapled and unstapled variants **11** and **7** (EC_{50} = 0.049 (±0.02) vs 1.37 (±0.57) nM, respectively) is surprising and difficult to fully rationalize; particularly in light of their almost identical CD spectra. A possible explanation could be the formation of potency-enhancing hydrophobic interactions between the hydrophobic staple of **11** and the $VPAC_2$ receptor, as previously observed in the case of a stapled p53 peptide bound to Mdm2.[34] Generally, the alpha helical content of peptide hormones, e.g., glucagon, correlates well with their functional potency; however, comparison of CD spectra recorded for peptides **1**–**14** failed to provide a clear trend between helicity and potency.

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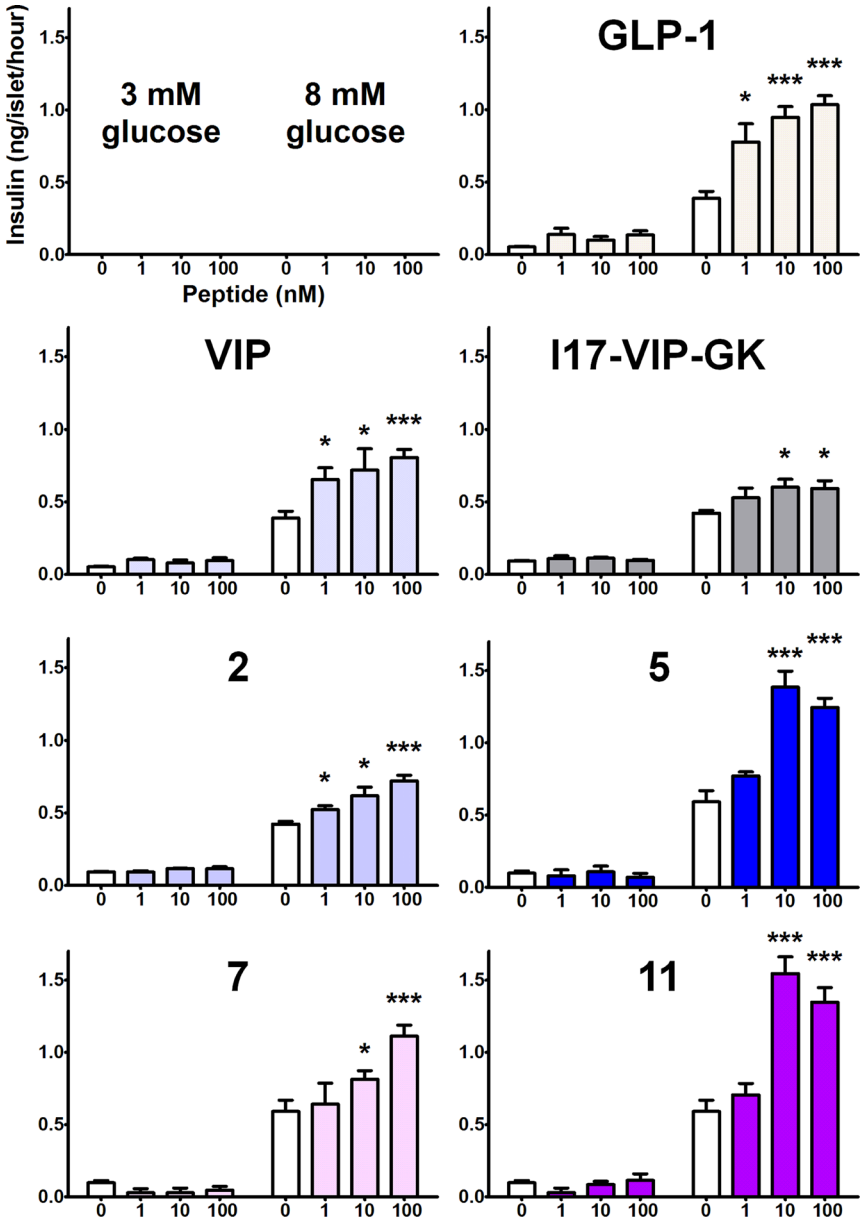

Figure 3. Insulin secretion in isolated rat pancreatic islets following glucose infusion (3 and 8 mM) and incubation after administrating selected peptides (numbered per chart). Bars represent the mean (±SE, $n > 3$). ***$p < 0.001$, **$p < 0.01$, and * $p < 0.05$ compared to vehicle-treated samples.

On the basis of these results, the most potent cyclic peptides, **5** and **11**, were evaluated for their ability to increase glucose-stimulated insulin secretion (GSIS) in pancreatic rat islets. Their linear analogues **2** and **7**, respectively, albeit less active, were also tested to verify that any differences observed in the recombinant system translated to a more native and relevant setting. The results are summarized in Figure 3. GLP-1 and VIP elicited a concentration-dependent increase in insulin secretion from isolated rat islets when incubated with 8 mM glucose. This is consistent with earlier reports highlighting their glucose-dependent insulin secretagogue properties.[9−11] **I^{17}-VIP-GK** also induced insulin secretion although to a significantly lower extent than VIP and GLP-1 (Figure 3). At the highest concentration tested, the linear VIP analogues **2** and **7** offered an improvement in insulin secretion over **I^{17}-VIP-GK**, with stapled variant **7** being superior to linear analogue **2**. Intriguingly, cyclization of **2** and **7** to yield the corresponding lactam- and olefin-based analogues **5** and **11**, resulted in significantly higher glucose-dependent insulin secretion with a plateau effect starting at 10 nM concentration, as depicted in Figure 3. While no significant differences between **5** and **11** were apparent, both **5** and **11** provided tangible improvements over VIP ($p < 0.01$) and GLP-1 ($p < 0.05$) standards when compared at 10 and 100 nM. Having confirmed the $VPAC_2$ functional agonism of the most potent stapled analogues in a physiologically relevant medium, we next examined two key optimization parameters for peptide-based research: protease stability and membrane permeability, as presented in Table 2.

To assess stability to proteolytic degradation, trypsin was selected due to its digestive role in the duodenum and gastrointestinal (GI) tract and previously published results using VIP and phosphor-lipidated-VIP derivatives.[35] In the development of an orally bioavailable peptide, minimal proteolytic degradation by enzymes abundant in the GI tract is paramount.

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Table 2. Trypsin Stability and Caco-2 Permeability for Selected Peptides

	staple position[a]	*i*,*i* + 4	stapled	% parent remaning[b]	P_{app}[d]
VIP			no	0.5	<0.1
I^{17}-VIP-GK			no	0.3	<0.1
2	17−21	E,K	no	0.9	<0.1
5	17−21	E,K	yes	0.4	<0.1
7	13−17	X,X[c]	no	0.7	<0.1
11	13−17	X,X[c]	yes	1.5	<0.1

[a]Residues numbered as shown for VIP (Figure 1). [b]After 15 min incubation with trypsin (n = 2) (Supporting Information). [c](*S*)-pentenylalanine. [d]Permeability (10^{-6} cm/s) measured in Caco-2 cells in the A to B direction, pH 6.5 (Supporting Information).

After 15 min of incubation with trypsin, minimal quantities (0.5%) of in-tact VIP remained, as expected considering its short in vivo.[14] Modification of wild-type VIP to the reference system **I^{17}-VIP-GK** did not substantially alter its metabolic profile (0.3% parent remaining), rendering it an adequate reference system for the present study. The lactam staple introduced in **5** did not offer any advantage in terms of resistance to trypsin degradation over the corresponding linear peptide **2** (cf. 0.9 and 0.4% remaining, Table 2). A similar trend was observed for the olefin equivalent pair **8** and **12**, and stability to trypsin digestion for the cyclized peptide **11** was also not significantly better (p = 0.06) than its linear analogue **7** (Table 2). It is still possible that significant differences in protease degradation profiles between these peptides would emerge using shorter incubation times. Nevertheless, based on pharmacokinetic considerations using VIP, 15 min represented a pragmatic trade-off at which significant differences would have warranted further characterization and development.

Unfortunately, evaluation of passive membrane permeability did not reveal any difference across the peptides tested. Regardless of the chemical nature of the staple and its position within the sequence, linear and cyclized peptides appeared to be equally poor at passively diffusing through the Caco-2 monolayer (P_{app} < 0.1 × 10^{-6} cm/s, Table 2), as assessed using a widely used, industrial protocol (Supporting Information).

The metabolic stability and membrane permeability results presented here may not be surprising considering the chemical structure and size of the peptides studied (30 residues). For instance, the cyclization elements in the most potent cyclized peptide from each class **5** (lactam) and **11** (hydrocarbon staple) would still leave substantial room for protease action at the peptide termini, while molecular size is inversely related to a molecule's diffusion coefficient. It has been shown, for example, that multiple staples have increased resistance to specific proteases in the case of GLP-1[21] and enfuvirtide[25] analogues. Several cell-penetrant stapled peptides have previously been described, although this property is normally assessed using flow cytometry and confocal fluorescence microscopy, requiring the need for fluorescently labeled peptides.[22−25,36,37] Nevertheless, the results presented here highlight just how critical the studied system is to the properties of interest and how complex it is to generalize and transpose observations in medicinal chemistry.[27]

Starting from native VIP, a number of stapled analogues were designed and synthesized to evaluate the effect of their helical content on the interaction with the $VPAC_2$ receptor. A number of VIP derivatives showed significant improvement in their ability to functionally activate the $VPAC_2$ receptor, although no clear structure−activity relationships (in terms of (a) presence or absence of the staple, (b) chemical nature of the staple, and (c) staple location on the amino acid sequence) could be derived. There also appeared to be no clear trend between the secondary structure shown by far UV CD spectroscopy and the functional potency of the analogues generated. Improved $VPAC_2$ functional agonism was confirmed in rat islets where the best stapled VIP derivatives significantly increased glucose-dependent insulin secretion compared to wild-type VIP and GLP-1 standards, as well as their linear analogues. Further profiling for increased metabolic stability and membrane permeability failed to reveal the sought-after improvements normally ascribed to stapled peptides, indicating opportunities for further medicinal chemistry optimization.

■ ASSOCIATED CONTENT

Supporting Information

Detailed description of peptide synthesis, purification and analysis, HPLC and mass spectral data, circular dichroism and calculated α-helical content, $VPAC_2$, insulin secretion, trypsin stability, and Caco-2 permeability assays. 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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■ REFERENCES

(1) Said, S. I.; Mutt, V. Isolation from porcine-intestinal wall of a vasoactive octacosapeptide related to secretin and to glucagon. *Eur. J. Biochem.* **1972**, *13*, 199−204.

(2) Delgado, M.; Abad, C.; Martinez, C.; Leceta, J.; Gomariz, R. P. Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease. *Nat. Med.* **2001**, *7*, 563−568.

(3) Offen, D.; Sherki, Y.; Melamed, E.; et al. Vasoactive intestinal peptide (VIP) prevents neurotoxicity in neuronal cultures: relevance to neuroprotection in Parkinson's disease. *Brain Res.* **2000**, *854* (1), 257−262.

(4) Delgado, M.; Ganea, D. Neuroprotective effect of vasoactive intestinal peptide (VIP) in a mouse model of Parkinson's disease by blocking microglial activation. *FASEB J.* **2003**, *17*, 944−946.

(5) Igarashi, H.; Fujimori, N.; Ito, T.; Nakamura, T.; Oono, T.; Nakamure, K.; Suzuki, K.; Jensen, R. T.; Takayanagi, R. Vasoactive Intestinal peptide (VIP) and VIP receptors—elucidation of structure and function for therapeutic applications. *Intl. J. Clin. Med.* **2011**, *2*, 500−508.

(6) Ishihara, T.; Shigemoto, R.; Mori, K.; Takahashi, K.; Nagata, S. Functional expression and tissue distribution of a novel receptor for vasoactive intestinal polypeptide. *Neuron* **1992**, *8*, 811−819.

(7) Lutz, E. M.; Sheward, W. J.; West, K. M.; Morrow, J. A.; Fink, G.; Harmar, A. J. The VIP2 receptor: molecular characterisation of a cDNA encoding a novel receptor for vasoactive intestinal peptide. *FEBS Lett.* **1993**, *334*, 3−8.
(8) Harmar, A. J.; Fahrenkrug, J.; Gozes, I.; Laburthe, M.; May, V.; Pisegna, J. R.; Vaudry, D.; Vaudry, H.; Waschek, J. A.; Said, S. I. Pharmacology and functions of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide: IUPHAR Review 1. *Br. J. Pharmacol.* **2012**, *166*, 4−17.
(9) Bertrand, G.; Puech, R.; Maisonnasse, Y.; Bockaert, J.; Loubatieres-Mariani, M. M. Comparative effects of PACAP and VIP on pancreatic endocrine secretions and vascular resistance in rat. *Br. J. Pharmacol.* **1996**, *117*, 764−770.
(10) Winzell, M. S.; Ahren, B. Role of VIP and PACAP in Islet Function. *Peptides* **2007**, *28*, 1805−1813.
(11) Tsutsumi, M.; Claus, T. H.; Liang, Y.; Li, Y.; Yang, L.; Zhu, J.; et al. A potent and highly selective $VPAC_2$ agonist enhances glucose-induced insulin release and glucose disposal: a potential therapy for type 2 diabetes. *Diabetes* **2002**, *51*, 1453−1460.
(12) Inagaki, N.; Yoshida, H.; Mizuta, M.; Mizuno, N.; Fujii, Y.; Gonoi, T.; Miyazaki, J.; Seino, S. Cloning and functional characterization of a third pituitary adenylate cyclase-activating polypeptide receptor subtype expressed in insulin-secreting cells. *Proc. Natl. Acad. Sci.* **1994**, *91*, 2679−2683.
(13) Asnicar, M. A.; Koster, A.; Heiman, M. L.; Tinsley, F.; Smith, D. P.; Galbreath, E.; et al. Vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor 2 deficiency in mice results in growth retardation and increased basal metabolic rate. *Endocrinology* **2002**, *143*, 3994−4006.
(14) Domschke, S.; Domschke, W.; Bloom, S. R.; et al. Vasoactive Intestinal Peptide in Man: Pharmacokinetics, Metabolic and Circulatory Effects. *Gut* **1978**, *19*, 1049−1053.
(15) Jiang, S.; Li, Z.; Ding, K.; Roller, P. P. Recent progress of synthetic studies to peptide and peptidomimetic cyclization. *Curr. Org. Chem* **2008**, *12*, 1502−1542.
(16) Blackwell, H. E.; Grubbs, R. H. Highly efficient synthesis of covalently cross-linked peptide helices by ring-closing metathesis. *Angew. Chem., Int. Ed.* **1998**, *37*, 3281−3284.
(17) Schafmeister, C. E.; Po, J.; Verdine, G. L. An all-hydrocarbon cross-linking system for enhancing the helicity and metabolic stability of peptides. *J. Am. Chem. Soc.* **2000**, *122*, 5891−5892.
(18) Blackwell, H. E.; Sadowsky, J. D.; Howard, R. J.; Sampson, J. N.; Chao, J. A.; Steinmetz, W. E.; O'Leary, D. J.; Grubbs, R. H. Ring-closing metathesis of olefinic peptides: design, synthesis, and structural characterization of macrocyclic helical peptides. *J. Org. Chem.* **2001**, *66*, 5291−5302.
(19) Kim, Y.-W.; Grossmann, T. N.; Verdine, G. L. Synthesis of all-hydrocarbon stapled a-helical peptides by ring-closing olefin metathesis. *Nat. Protoc.* **2011**, *6*, 761−771.
(20) Verdine, G. L.; Hilinski, G. J. All-hydrocarbon stapled peptides as synthetic cell-accessible mini-proteins. *Drug Discovery Today* **2012**, *9*, e41−e47.
(21) Murage, E. N.; Gao, G.; Bisello, A.; Ahn, J.-M. Development of potent glucagon-like peptide-1 agonists with high enzyme stability via introduction of multiple lactam bridges. *J. Med. Chem.* **2010**, *53*, 6412−6420.
(22) Walensky, L. D.; Kung, A. L.; Escher, I.; Malia, T. J.; Barbuto, S.; Wright, R. D.; Wagner, G.; Verdine, G. L.; Korsmeyer, S. J. Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. *Science* **2004**, *305*, 1466−1470.
(23) Danial, N. N.; Walensky, L. D.; Zhang, C. Y.; Choi, C. S.; Fisher, J. K.; Molina, A. J.; Datta, S. R.; Pitter, K. L.; Bird, G. H.; Wikstrom, J. D.; Deeney, J. T.; Robertson, K.; Morash, J.; Kulkarni, A.; Neschen, S.; Kim, S.; Greenberg, M. E.; Corkey, B. E.; Shirihai, O. S.; Shulman, G. I.; Lowell, B. B.; Korsmeyer, S. J. Dual role of proapoptotic BAD in insulin secretion and beta cell survival. *Nat. Med.* **2008**, *14*, 144−153.
(24) Moellering, R. E.; Cornejo, M.; Davis, T. N.; Del Bianco, C.; Aster, J. C.; Blacklow, S. C.; Kung, A. L.; Gilliland, D. G.; Verdine, G. L.; Bradner, J. E. Direct inhibition of the NOTCH transcription factor complex. *Nature* **2009**, *462*, 182−188.
(25) Bird, G. H.; Madani, N.; Perry, A. F.; Princiotto, A. M.; Supko, J. G.; He, X.; Gavathiotis, E.; Sodroski, J. G.; Walensky, L. D. Hydrocarbon double-stapling remedies the proteolytic instability of a lengthy peptide therapeutic. *Proc. Natl. Acad. Sci.* **2010**, *107*, 14093−14098.
(26) Day, J. W.; Ottaway, N.; Patterson, J. T.; Gelfanov, V.; Smiley, D.; Gidda, J.; Findeisen, H.; Bruemmer, D.; Drucker, D. J.; Chaudhary, N.; Holland, J.; Hembree, J.; Abplanalp, W.; Grant, E.; Ruehl, J.; Wilson, H.; Kirchner, H.; Lockie, S. H.; Hofmann, S.; Woods, S. C.; Nogueiras, R.; Pfluger, P. T.; Perez-Tilve, D.; DiMarchi, R.; Tschöp, M. H. A new glucagon and GLP-1 co-agonist eliminates obesity in rodents. *Nat. Chem. Biol.* **2009**, *10*, 749−757.
(27) Okamoto, T.; Zobel, K.; Fedorova, A.; Quan, C.; Yang, H.; Fairbrother, W. J.; Huang, D. C. S.; Smith, B. J.; Deshayes, K.; Czabotar, P. E. Stabilizing the pro-apoptotic BimBH3 helix (BimSAHB) does not necessarily enhance affinity or biological activity. *ACS Chem. Biol.* **2013**, *8*, 297−302.
(28) Moreno, D.; Gourlet, P.; De Neef, P.; Cnudde, J.; Waelbroeck, C. M.; Robberecht, P. Development of selective agonists and antagonists for the human vasoactive intestinal polypeptide VPAC(2) receptor. *Peptides* **2000**, *21*, 1543−1549.
(29) Yung, S. L.; Dela Cruz, F.; Hamren, S.; Zhu, J.; Tsutsumi, M.; Bloom, J. W.; Caudle, M.; Roczniak, S.; Todd, T.; Lemoine, L.; MacDougal, M.; Shanafelt, A. B.; Pan, C. Q. Generation of highly selective VPAC2 receptor agonists by high throughput mutagenesis of vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide. *J. Biol. Chem.* **2003**, *278*, 10273−10281.
(30) Parthier, C.; Kleinschmidt, M.; Neumann, P.; Rudolph, R.; Manhart, S.; Schlenzig, D.; Fanghanel, J.; Rahfeld, J.-U.; Demuth, H.-U.; Stubbs, M. T. Crystal structure of the incretin-bound extracellular domain of a G protein-coupled receptor. *Proc. Natl. Acad. Sci* **2007**, *104*, 13942−13947.
(31) Crystal Structure of the Extracellular Domain of Human Vasoactive Intestinal Polypeptide Receptor 2.http://www.pdb.org/pdb/explore/explore.do?structureId=2X57.
(32) Gozes, I.; Bardea, A.; Reshef, A.; Zamostiano, R.; Zhukovsky, S.; Rubinraut, S.; Fridkin, M.; Brenneman, D. E. Neuroprotective strategy for Alzheimer disease: Intranasal administration of a fatty neuropeptide. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 427−432.
(33) Nicole, P.; Lins, L.; Rouyer-Fessard, C.; Drouot, C.; Fulcrand, P.; Thomas, A.; Couvineau, A.; Martinez, A.; Brasseur, R.; Laburthe, M. Identification of key residues for interaction of vasoactive intestinal peptide with human VPAC1 and VPAC2 receptors and development of a highly selective VPAC1 receptor agonist: alanine scanning and molecular modeling of the peptide. *J. Biol. Chem.* **2000**, *275*, 24003−24012.
(34) Baek, S.; Kutchukian, P. S.; Verdine, G. L.; Huber, R.; Holak, T. A.; Lee, K. W.; Popowicz, G. M. Structure of the stapled p53 peptide bound to Mdm2. *J. Am. Chem. Soc.* **2012**, *134*, 103−106.
(35) Golobov, G.; Noda, Y.; Sherman, S.; Rubinstein, I.; Baranowska-Kortylewicz, J.; Paul, S. Stabilization of vasoactive intestinal peptide by lipids. *J. Pharmacol. Exp. Ther.* **1998**, *285*, 753−758.
(36) Verdine, G. L.; Hilinski, G. J. Stapled peptides for intracellular drug targets. *Methods Enzymol.* **2012**, *503*, 3−33.
(37) Spokoyny, A. M.; Zou, Y.; Ling, J. J.; Yu, H.; Lin, Y.-S.; Pentelute, B. L. A perfluoroaryl-cysteine S_NAr chemistry approach to unprotected peptide stapling. *J. Am. Chem. Soc.* **2013**, *135*, 5946−5949.