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Structure−Activity Studies on Antiproliferative Factor (APF) Glycooctapeptide Derivatives

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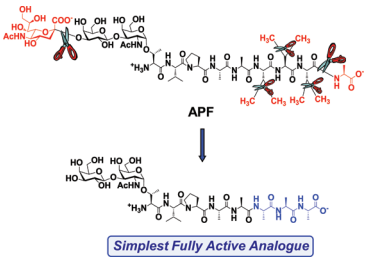
ABSTRACT Antiproliferative factor (APF), a sialylated glycopeptide secreted by explanted bladder epithelial cells from interstitial cystitis/painful bladder syndrome (IC/PBS) patients, and its unsialylated analogue (*as*-APF) significantly decrease proliferation of bladder epithelial cells and/or certain carcinoma cell lines in vitro. We recently reported a structure−activity relationship profile for the peptide portion of *as*-APF and revealed that truncation of the C-terminal alanine did not significantly affect antiproliferative activity. To better understand the structural basis for the maintenance of activity of this truncated eight amino acid *as*-APF (*as*-APF8), we synthesized several amino acid-substituted derivatives and studied their ability to inhibit bladder epithelial cell proliferation in vitro as well as their solution conformations by CD and NMR spectroscopy. While single amino acid changes to *as*-APF8 often strongly reduced activity, full potency was retained when the trivaline tail was replaced with three alanines. The Ala$^{6-8}$ derivative **9** is the simplest, fully potent APF analogue synthesized to date.

KEYWORDS Interstitial cystitis/painful bladder syndrome, antiproliferative, glycopeptide, hydrophobicity, peptide conformation



Interstitial cystitis/painful bladder syndrome (IC/PBS) is a devastating disease of the bladder that is characterized by thinning or even focal obliteration of the bladder epithelium.[1−3] Approximately 1 million Americans suffer from IC/PBS, and it occurs 8−9 times more frequently in women than in men.[1,3] Although a bladder condition resembling IC/PBS was first described about 200 years ago,[2] the cause of this disease has remained unknown. However, urine from approximately 95% of IC/PBS patients who fulfill modified NIDDK criteria for IC[4] contains an antiproliferative factor (APF) that potently decreases the proliferation of human bladder epithelial cells in vitro[5] and elicits changes in normal bladder epithelium also seen in bladder biopsy specimens and/or explanted bladder epithelial cells from IC/PBS patients (including specific changes in growth factor expression,[6] increased paracellular permeability, and aberrations in tight junction protein expression and tight junction formation).[7−16] APF has been identified as a short trisaccharide-containing glycopeptide—viz., Neu5Acα2-3Galβ1-3GalNAcα-*O*-TVPAAVVVA[17] (**1**, Figure 1) with an intriguing structure−activity relationship (SAR).[18] Although the peptide sequence is identical to a segment of the sixth transmembrane domain of the frizzled-8 protein,[17,19] a Wnt ligand receptor, the details of its biosynthesis and mechanism of action in IC/PBS patients remain unknown. An important advance into understanding the mechanism of APF function was identification of cytoskeletal-associated protein 4 (CKAP4/ERGIC-63/CLIMP-63) as a cellular receptor for APF in bladder epithelium.[20]

A preliminary analysis of APF showed that the trisaccharide is not an absolute requirement as the desialylated native APF as well as the asialo synthetic derivative (*as*-APF, **2**, Figure 1) maintained full potency; however, two proximal sugar residues are required for activity[17,18] (the unglycosylated peptide as well as the α-GalNAc monosaccharide derivatives were both inactive). A recent SAR study[18] of the peptide portion of APF revealed certain amino acid requirements for maintenance of *as*-APF activity. Greater understanding of the SAR for active APF derivatives potentially will allow for more rational design of APF mimics/inhibitors.

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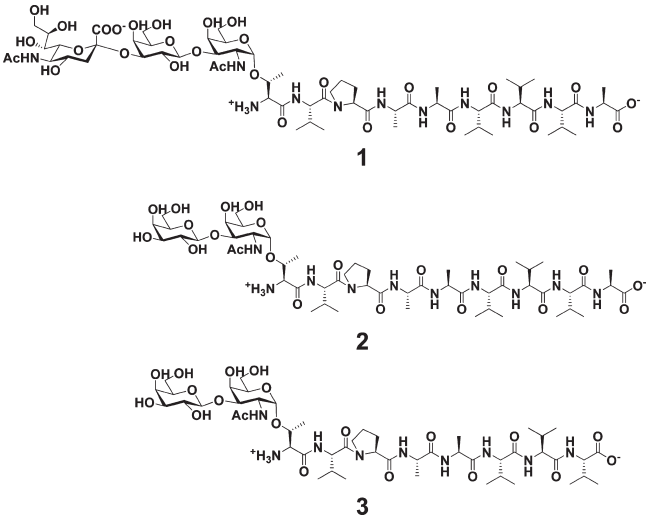

Figure 1. Structures of APF (**1**), *as*-APF (**2**), and *as*-APF8 (**3**).

As the 8-residue derivative (*as*-APF8, **3**, Figure 1) was equipotent to *as*-APF, we synthesized several analogues of *as*-APF8 and qualitatively compared their activity and solution structures as determined from circular dichroism (CD) and NMR spectroscopies. We concentrated primarily on C-terminal amino acid substitutions, because our previous data indicated that the C-terminal A-XXX motif was important for activity, and modifications to this segment tended to have negative effects on potency (with some exceptions). During initial[17] structural characterization and subsequent SAR[18] work on APF, it was discovered that compounds **2** and **3** (Figure 1) were essentially equipotent in antiproliferation assays with normal bladder epithelial cells; further truncation to a 7-mer yielded a completely inactive compound. On the basis of these results, the potency of each *as*-APF8 analogue described in this report was compared simultaneously to that of **2**.

General procedures for glycopeptide synthesis, their structures, and activity data are outlined in the Supporting Information (Table S1). Generally, Fmoc-protected amino acids were coupled using HATU and HOAt in the presence of DIPEA in dry NMP. Incorporation of the valine residue in position 2 was affected by two rounds of coupling (double coupling) in the synthesis of all glycopeptides due to the lower reactivity of the secondary prolyl amino group. Coupling of Fmoc-Thr(Ac_4Galβ1-3Ac_2GalNAcα-*O*)-OH (1.1 equiv) was performed without any added base to prevent epimerization. All compounds were determined to be >95% pure by analytical high-performance liquid chromatography (HPLC) and electrospray ionization liquid chromatography/mass spectrometry (LC/MS) analysis (Supporting Information).

Inhibition of [^{3}H]thymidine incorporation was used to evaluate antiproliferative activity.[5] The activity data for the synthetic APF derivative glycopeptides described herein along with previously prepared compounds **1**−**4** are listed in Table S1 in the Supporting Information. We were aware that a disaccharide was a requirement for antiproliferative activity of the *as*-APF glycopeptide;[17,18] unglycosylated peptide **5** was also devoid of activity. A representative set of assay curves for compounds **2**, **7**, **9**, and **14** are shown in Figure 2.

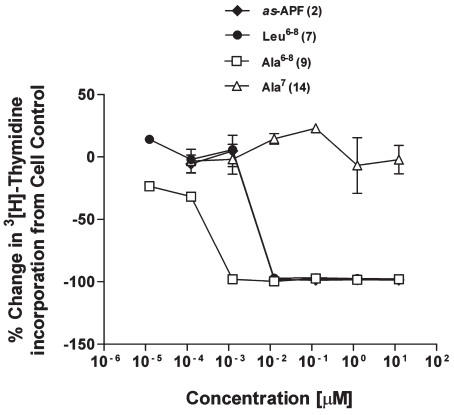

Figure 2. Antiproliferative activity of compounds **2**, **7**, **9**, and **14** in normal bladder epithelial cell explant cultures. Explanted normal bladder epithelial cells were treated with varying concentrations of *as*-APF **2** (♦), trileucine analogue **7** (●), trialanine analogue **9** (□), or Val-Ala-Val analogue **14** (△) for 48 h prior to the determination of ^{3}[H]-thymidine incorporation. The assay was performed in triplicate twice; data are expressed as percent inhibition of thymidine incorporation as compared to control cells incubated with medium alone.

The modifications made to the three C-terminal amino acids were either "single" (compounds **6**, **13**, and **14**) or "global" involving all three C-terminal amino acids (compounds **7**−**12**). It was previously determined that ionization at either the C- or the N-amino acid terminus of *as*-APF was important for full potency as "end-capping" by N-acetylation or carboxamidation caused a 2.5 order of magnitude drop in potency.[18] This behavior was even more dramatic for *as*-APF8 derivatives as the C-terminal carboxamide analogue **6** was completely inactive (acetylation of the N-terminal amino group also resulted in inactivation, data not shown).

Compounds **7**−**12** were prepared to evaluate the importance of the hydrophobicity and β-branching in the A-XXX motif of APF. Previous data showed that this unit might allow the C-terminal tail of active APF analogues to populate some form of defined secondary structure.[18,21] Because *as*-APF8 maintained full antiproliferative potency, we systematically tested *as*-APF8 analogues incorporating amino acids with varying hydrophobicities, by globally changing the trivaline segment to one with amino acids with one added methylene unit (trileucine replacement, designated Leu$^{6-8}$, compound **7**) to one with amino acids having no carbon-containing side chains (Gly$^{6-8}$, compound **10**). The octanol/water partition coefficient (logP) values of each new compound were calculated[22] and are listed in Table S1 in the Supporting Information to compare with *as*-APF8. The slightly more hydrophobic Leu$^{6-8}$ analogue **7** maintained a high degree of potency (10% of full activity), while removal of one methyl group from valines 6−8 by substitution with unnatural aminobutyric acid (Abu, **8**) resulted in inactivation. The even less hydrophobic Ala$^{6-8}$ derivative **9** yielded a compound that retained full potency. This stands in contrast to replacement of the valines 6−8 in the 9-mer *as*-APF with alanines, as this

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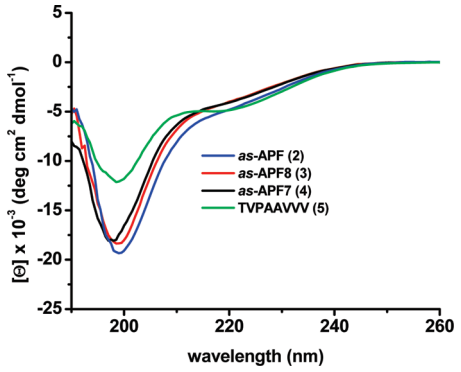

Figure 3. CD spectra of a 50 μM solution of **2** (blue line), **3** (red line), **4** (black line), and **5** (green line) in H_2O:TFE (1:1) recorded at 25 °C.

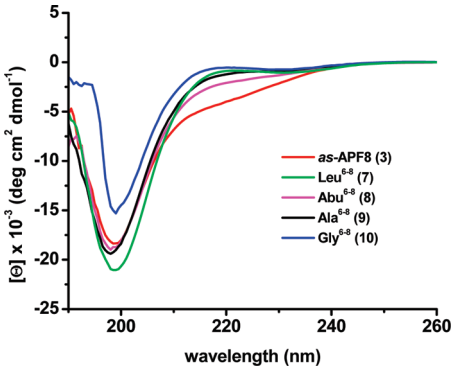

Figure 4. CD spectra of 50 μM solutions of **3** (red line), **7** (green line), **8** (pink line), **9** (black line), and **10** (blue line) in H_2O:TFE (1:1) mixture recorded at 25 °C.

congener showed a reduction in potency of 2 orders of magnitude as compared to *as*-APF.[18] This result made it difficult to rationalize the lack of the activity of Galβ1-3GalNAcα-*O*-TVPAA-Abu-Abu-Abu analogue **8**, as its ethyl side chain is "between" alanine and valine in both hydrophobicity and steric bulk. Lyu et al.[23] found that alanine and Abu possess equal (but greater than valine) ability to stabilize helical content. Assuming that stabilization of helical content is also important in the C terminus of APF, it would seem reasonable to predict that **8** should show some potency in our assay. However, other factors may complicate this hypothesis (see below). As expected, compound **10** (Gly$^{6-8}$ substitution) resulted in complete loss of activity. Potency was partially recovered (0.1 %) when Val$^{6-8}$ was substituted with β-alanine (compound **11**), a replacement used in many instances to induce helical content in small peptides.[24] Adding back an α-methyl group by way of β^3-homoalanine replacements (compound **12**) once more inactivated the glycopeptides.

Two single replacements used previously in *as*-APF were also used in *as*-APF8, viz. replacement of Val^7 with D-valine or alanine. As it had for *as*-APF, D-valine substitution inactivated *as*-APF8, illustrating that non-natural amino acid derivatives (compounds **8**, **12**, and **13**) are not tolerated. The single Ala^7-substituted analogue **14** was also prepared to offer a compound with a logP that was midway between the two active derivatives **3** and **9**. However, this also resulted in complete inactivation of *as*-APF8.

CD spectroscopy[25] was initially used to study each analogue with comparisons to the spectra for either **2** and/or **3**. The CD spectrum of **3** revealed no organized structure of the glycopeptide in water (similar to previous data for **2**[18]), as was the case for all other new analogues. However, **3** appeared to adopt some secondary structure in a 1:1 H_2O:TFE mixture, (Figure 3) the milieu that was used for all subsequent CD spectra. The spectrum, with two minima centered at ca.199 and 220 nm, resembled that previously obtained for **2** in neat TFE (unpublished results). In 1:1 H_2O:TFE, nearly identical CD spectra for the three derivatives **2**, **3**, and **4** suggested that truncation does not significantly affect conformation in this milieu. Surprisingly, the structure of unglycosylated 8-mer **5** appeared to be significantly more organized than the structure of **3** (Figure 3). Other notable CD results (see Figure 4) showed that (1) compound **7** (10 % potency) was significantly less organized than structure **3**, (2) compounds **8** (inactive) and **9** (full activity) had almost superimposable spectra, and (3) substitution with non-natural amino acids resulted in no organized structures (compounds **11**, **12**, and **13**; Figure S1 in the Supporting Information).

The glycopeptides synthesized in this study were also examined by NMR spectroscopy in aqueous solution. All 8-mer peptides from Table S1 in the Supporting Information were fully assigned by exhaustive NMR experiments (a representative comparison of chemical shifts and temperature coefficients data for compounds **3** and **5** can be found in Table S2 and Figure S3 in the Supporting Information). Spectra of APF derivatives in 9:1 H_2O:D_2O, in general, gave well-dispersed and sharp signals, suggesting little if any slow chemical exchange on the NMR time scale. Buffered solutions at pH 4.6 gave the highest quality spectra as compared with several pH values tested. A stacked plot of the amide region of several 8-residue analogues synthesized here is shown in Figure 5. Most NH protons were fully resolved in several derivatives, suggesting preferred conformations in solution. Some more notable observations were (1) consistent chemical shift values across all derivatives for amide protons for GalNAc, Val^2, Ala^4, and Ala^5, indicating, little "cross-talk" between the N and the C termini; (2) very distinct chemical shift differences for the two C-terminal carboxamide protons for "inactive" derivative **6**; and (3) a higher population of the cis amide bond for Pro^3 when the sugar is removed. In addition, amide temperature coefficient data showed that the $\Delta\delta/\Delta T$ value for Val^2 decreased from -7.3 to -6.0 ppb/K, indicating a possible shielding or H-bonding effect upon glycosylation. Our data thus far (refs 17 and 18 and unpublished results) have shown that a distinct sugar disaccharide profile is important for the maintenance of activity. The sugars have important and also remote conformational effects, which in turn can be closely related to changes in antiproliferative activity.

With the exception of values for alanine residues, three-bond amide-Hα ($^3J_{NH\text{-}\alpha}$) coupling constants were found to be between 7.0 and 8.8 Hz, indicating an extended conformation in the backbone (representative $^3J_{NH\text{-}\alpha}$ values for

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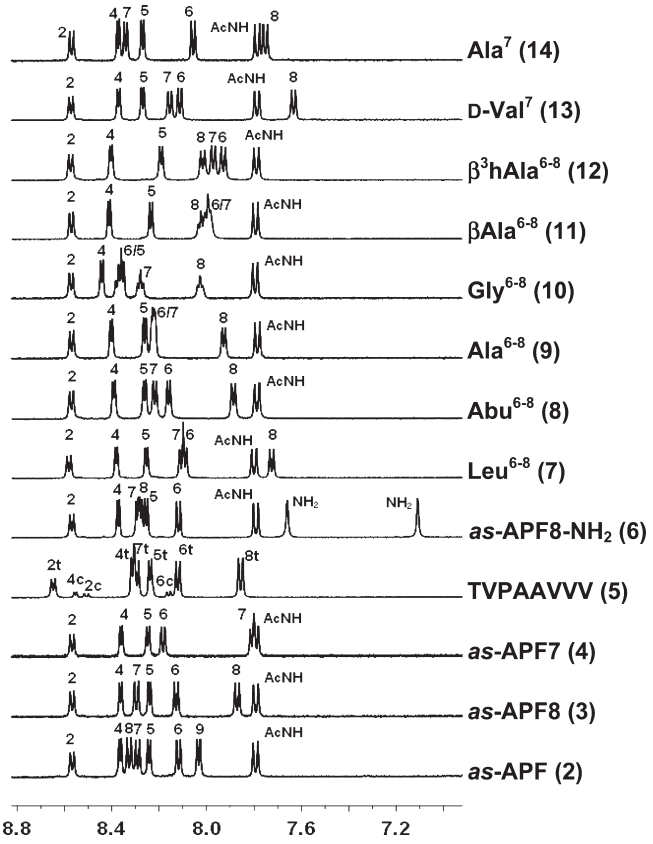

Figure 5. One-dimensional NMR spectra of the amide region of 8-mer APF derivatives in 9:1 H_2O:D_2O in acetate buffer, pH 4.6. Amino acid assignments are labeled over the peaks. Compound labels for each spectrum (right of spectrum) identify the substitution employed and the compound number from Table S1 in the Supporting Information.

compounds **3** and **5** are listed in the Supporting Information). However, $^3J_{NH\text{-}\alpha}$ values were between 4.8 and 6.0 Hz for nearly all alanine residues, consistent with backbone angles that resemble twisted (helical) motifs. Interestingly, values of 5.2−5.9 Hz were observed for residues Ala^4-Ala^7 for the fully active trialanine derivative **9**, suggesting an "alanine-extended" short stretch of folded type secondary structure. In contrast, the $^3J_{NH\text{-}\alpha}$ values for Val^6-Val^8 in both **2** and **3** were between 7.9 and 8.7 Hz. Further analysis showed that chemical shift indices[26,27] for backbone carbon, nitrogen, and Hα protons (data not shown) suggested that, contrary to our original hypothesis,[18] the C-terminal tail may be structured more closely to a β-motif and not α-disposed. Looking at these combined data lead us to conclude that there is little correlation between NMR observables in the C-terminal residues and activity.

The only receptor known for *as*-APF is CKAP4, a reversibly palmitoylated protein of ∼600 residues that connects the endoplasmic reticulum to microtubules.[20,28] Structural studies on CKAP4 reported that the large extracellular/luminal domain of this protein (putatively where APF binds) is a helical coiled coil that forms large aggregates.[29] Interestingly, the only other protein isolated from solubilized bladder epithelial cell membrane proteins with APF was vimentin, an intermediate filament protein that also forms helical coiled coils. It is possible that *as*-APF disrupts protein−protein interactions during multimerization of CKAP4. Alternatively, APF could function by disruption of CKAP4/vimentin coiled−coil interactions. These disruptions may prevent subsequent signaling or internalization of the CKAP4 chaperone protein. The varied "conformations" of active APF analogues also suggest that APF may act at more than one motif on the protein's surface, a tentative explanation for the confounding activity swings with minor changes to the peptide backbone.

In summary, our lead compound, *as*-APF (**2**), has a unique and distinct SAR profile that is maintained in Ala^9-truncated derivatives. In the current study, we identified two quite potent derivatives of *as*-APF and determined that Ala$^{6-8}$ analogue **9** is the "simplest" fully potent derivative of APF synthesized to date. NMR data are beginning to uncover some important clues to the structural implications of specific features of the APF molecule. These data will assist in designing glycopeptidemimetics that could be useful tools in the therapy of IC/PBS and cancer.

SUPPORTING INFORMATION AVAILABLE

Experimental details, structures, activity, purity, mass spectroscopy, HPLC, and selected CD and NMR data for new glycopeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes: ▽ Deceased in 2007.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on July 19, 2010 with errors in Table S3 of the Supporting Information file. The revised version was published on July 28, 2010.

REFERENCES

(1) Hanno, P. M. *Interstitial Cystitis*; Springer-Verlag: London, New York, 1990; p xvi, 218 pp.
(2) Parsons, J. K.; Parsons, C. L. The historical origins of interstitial cystitis. *J. Urol.* **2004,** *171*, 20–22.
(3) Parsons, C. L. Interstitial cystitis: A chronic disease with ever increasing recognition and prevalence. *J. Urol.* **2005,** *173*, 98–102.

DOI: 10.1021/ml100087a | *ACS Med. Chem. Lett.* **2010,** *1*, 390–394

(4) Gillenwater, J. Y.; Wein, A. J. Summary of the National Institute of Arthritis Diabetes Digestive and Kidney Diseases workshop on Interstitial Cystitis, National Institutes of Health, Bethesda, Maryland, August 28−29, 1987. *J. Urol.* **1988,** *140*, 203–206.
(5) Keay, S.; Zhang, C. O.; Trifillis, A. L.; Hise, M. K.; Hebel, J. R.; Jacobs, S. C.; Warren, J. W. Decreased ^{3}[H]-thymidine incorporation by human bladder epithelial cells following exposure to urine from interstitial cystitis patients. *J. Urol.* **1996,** *156*, 2073–2078.
(6) Keay, S.; Zhang, C. O.; Kagan, D.; Hise, M. K.; Jacobs, S. C.; Hebel, R. J.; Gordon, D.; Whitmore, K.; Bodison, S.; Warren, J. W. Alterations in HB-EGF, EGF, IGF1 and IGFBP3 levels in the urine of interstitial cystitis patients. *J. Urol.* **1998,** *159*, 307–307.
(7) Zhang, C. O.; Wang, J. Y.; Koch, K.; Keay, S. Antiproliferative factor (APF) decreases occludin and ZO-1 production and increases paracellular permeability in bladder epithelial cell monolayers. *J. Urol.* **2005,** *173*, 81–81.
(8) Zhang, C. O.; Wang, J. Y.; Koch, K. R.; Keay, S. Regulation of tight junction proteins and bladder epithelial paracellular permeability by an antiproliferative factor from patients with interstitial cystitis. *J. Urol.* **2005,** *174*, 2382–2387.
(9) Slobodov, G.; Feloney, M.; Gran, C.; Kyker, K. D.; Hurst, R. E.; Culkin, D. J. Abnormal expression of molecular markers for bladder impermeability and differentiation in the urothelium of patients with interstitial cystitis. *J. Urol.* **2004,** *171*, 1554–1558.
(10) Johansson, S. L.; Fall, M. Clinical-Features and Spectrum of Light Microscopic Changes in Interstitial Cystitis. *J. Urol.* **1990,** *143*, 1118–1124.
(11) Southgate, J.; Varley, C. L.; Garthwaite, M. A. E.; Hinley, J.; Marsh, F.; Stahlschmidt, J.; Trejdosiewicz, L. K.; Eardley, I. Differentiation potential of urothelium from patients with benign bladder dysfunction. *BJU Int.* **2007,** *99*, 1506–1516.
(12) Smith, B. H.; Dehner, L. P. Chronic Ulcerating Interstitial Cystitis (Hunners Ulcer)—Study of 28 Cases. *Arch. Pathol.* **1972,** *93*, 76–81.
(13) Parsons, C. L.; Lilly, J. D.; Stein, P. Epithelial Dysfunction in Nonbacterial Cystitis (Interstitial Cystitis). *J. Urol.* **1991,** *145*, 732–735.
(14) Fowler, J. E.; Lynes, W. L.; Lau, J. L. T.; Ghosh, L.; Mounzer, A. Interstitial Cystitis Is Associated with Intraurothelial Tamm-Horsfall Protein. *J. Urol.* **1988,** *140*, 1385–1389.
(15) Tomaszewski, J. E.; Landis, J. R.; Russack, V.; Williams, T. M.; Wang, L. P.; Hardy, C.; Brensinger, C.; Matthews, Y. L.; Abele, S. T.; Kusek, J. W.; Nyberg, L. M.; Stu, I. C. D. Biopsy features are associated with primary symptoms in interstitial cystitis: Results from the Interstitial Cystitis Database Study. *Urology* **2001,** *57*, 67–81.
(16) Eldrup, J.; Thorup, J.; Nielsen, S. L.; Hald, T.; Hainau, B. Permeability and Ultrastructure of Human Bladder Epithelium. *Br. J. Urol.* **1983,** *55*, 488–492.
(17) Keay, S. K.; Szekely, Z.; Conrads, T. P.; Veenstra, T. D.; Barchi, J. J.; Zhang, C. O.; Koch, K. R.; Michejda, C. J. An antiproliferative factor from interstitial cystitis patients is a frizzled 8 protein-related sialoglycopeptide. *Proc. Natl. Acad. Sci. U.S.A.* **2004,** *101*, 11803–11808.
(18) Kaczmarek, P.; Keay, S. K.; Tocci, G. M.; Koch, K. R.; Zhang, C. O.; Barchi, J. J.; Grkovic, D.; Guo, L.; Michejda, C. J. Structure-activity relationship studies for the peptide portion of the bladder epithelial cell antiproliferative factor from interstitial cystitis patients. *J. Med. Chem.* **2008,** *51*, 5974–5983.
(19) Saitoh, T.; Hirai, M.; Katoh, M. Molecular cloning and characterization of human Frizzled-8 gene on chromosome 10p11.2. *Int. J. Oncol.* **2001,** *18*, 991–996.
(20) Conrads, T. P.; Tocci, G. M.; Hood, B. L.; Zhang, C. O.; Guo, L.; Koch, K. R.; Michejda, C. J.; Veenstra, T. D.; Keay, S. K. CKAP4/p63 is a receptor for the frizzled-8 protein-related antiproliferative factor from interstitial cystitis patients. *J. Biol. Chem.* **2006,** *281*, 37836–37843.
(21) Liff, M. I.; Lyu, P. C.; Kallenbach, N. R. Analysis of Asymmetry in the Distribution of Helical Residues in Peptides by H-1 Nuclear-Magnetic-Resonance. *J. Am. Chem. Soc.* **1991,** *113*, 1014–1019.
(22) Cheng, T. J.; Zhao, Y.; Li, X.; Lin, F.; Xu, Y.; Zhang, X. L.; Li, Y.; Wang, R. X.; Lai, L. H. Computation of octanol-water partition coefficients by guiding an additive model with knowledge. *J. Chem. Inf. Model.* **2007,** *47*, 2140–2148.
(23) Lyu, P. C.; Sherman, J. C.; Chen, A.; Kallenbach, N. R. Alpha-Helix Stabilization by Natural and Unnatural Amino-Acids with Alkyl Side-Chains. *Proc. Natl. Acad. Sci. U.S.A.* **1991,** *88*, 5317–5320.
(24) Seebach, D.; Beck, A. K.; Bierbaum, D. J. The world of beta- and gamma-peptides comprised of homologated proteinogenic amino acids and other components. *Chem. Biodiversity* **2004,** *1*, 1111–1239.
(25) Greenfield, N. J. Analysis of the kinetics of folding of proteins and peptides using circular dichroism. *Nat. Protoc.* **2006,** *1*, 2891–2899.
(26) Berjanskii, M. V.; Wishart, D. S. A simple method to predict protein flexibility using secondary chemical shifts. *J. Am. Chem. Soc.* **2005,** *127*, 14970–14971.
(27) Shen, Y.; Delaglio, F.; Cornilescu, G.; Bax, A. TALOS plus: A hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J. Biomol. NMR* **2009,** *44*, 213–223.
(28) Planey, S. L.; Keay, S. K.; Zhang, C. O.; Zacharias, D. A. Palmitoylation of Cytoskeleton Associated Protein 4 by DHHC2 Regulates Antiproliferative Factor-mediated Signaling. *Mol. Biol. Cell* **2009,** *20*, 1454–1463.
(29) Klopfenstein, D. R.; Klumperman, J.; Lustig, A.; Kammerer, R. A.; Oorschot, V.; Hauri, H. P. Subdomain-specific localization of CLIMP-63 (p63) in the endoplasmic reticulum is mediated by its luminal alpha-helical segment. *J. Cell Biol.* **2001,** *153*, 1287–1299.

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