Journal of Medicinal Chemistry

Article

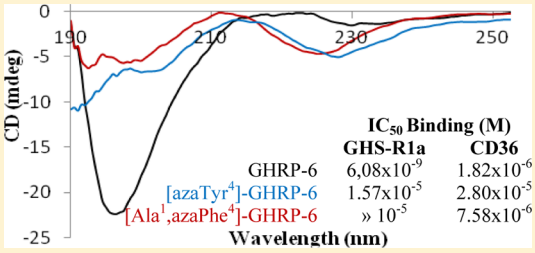
pubs.acs.org/jmc

Azapeptide Analogues of the Growth Hormone Releasing Peptide 6 as Cluster of Differentiation 36 Receptor Ligands with Reduced Affinity for the Growth Hormone Secretagogue Receptor 1a

Caroline Proulx,[†] Émilie Picard,[‡] Damien Boeglin,[†] Petra Pohankova,[§] Sylvain Chemtob,[‡] Huy Ong,*[,§] and William D. Lubell*[,†]

[†]Département de Chimie, [‡]Département de Pédiatrie and [§]Faculté de Pharmacie, Université de Montréal, C.P. 6128, Succursale Centre-Ville, Montréal, Québec H3C 3J7, Canada

ABSTRACT: The synthetic hexapeptide growth hormone releasing peptide-6 (GHRP-6) exhibits dual affinity for the growth hormone secretagogue receptor 1a (GHS-R1a) and the cluster of differentiation 36 (CD36) receptor. Azapeptide GHRP-6 analogues have been synthesized, exhibiting micromolar affinity to the CD36 receptor with reduced affinity toward the GHS-R1a. A combinatorial split-and-mix approach furnished aza-GHRP-6 leads, which were further examined by alanine scanning. Incorporation of an aza-amino acid residue respectively at the D-Trp[2], Ala[3], or Trp[4] position gave aza-GHRP-6 analogues with reduced affinity toward the GHS-R1a by at least a factor of 100 and in certain cases retained affinity for the CD36 receptor. In the latter cases, the D-Trp[2] residue proved important for CD36 receptor affinity; however, His[1] could be replaced by Ala[1] without considerable loss of binding. In a microvascular sprouting assay using a choroid explant, [azaTyr[4]]-GHRP-6 (**15**), [Ala[1], azaPhe[2]]-GHRP-6 (**16**), and [azaLeu[3], Ala[6]]-GHRP-6 (**33**) all exhibited antiangiogenic activity.



INTRODUCTION

Growth hormone releasing peptide-6 (GHRP-6, His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2)[1] is a synthetic hexapeptide exhibiting affinity for the ghrelin receptor (GHS-R1a) and for the type B scavenger receptor CD36. Initially, GHRP-6 was found to exhibit high affinity to the former G-protein coupled receptor, which is expressed predominantly in the hypothalamic–pituitary axis, causing secretion of growth hormone (GH) by an independent pathway from that regulated by way of the growth hormone releasing hormone (GHRH) receptor.[2] The 2-methyl-tryptophan analogue of GHRP-6, hexarelin (His-D-2-Me-Trp-Ala-Trp-D-Phe-Lys-NH_2),[3] was subsequently shown to be more potent than GHRP-6, exhibiting reproducible, dose-dependent growth-hormone-releasing activity after administration. Growth hormone secretagogues (GHS), such as GHRP-6 and hexarelin, have since attracted interest for diagnosing and countering physiological imbalances associated with impaired GH secretion[4] such as obesity,[5] acromegaly, short stature, and aging.[6]

GHRP-6 and hexarelin were later shown to exhibit pleiotropic effects. In the cardiovascular system, hexarelin exhibited antiatherosclerotic activity, which resulted likely from inhibition of the binding of oxidized low density lipoproteins (oxLDL) by the type B scavenger receptor CD36,[7,8] which plays a critical role in foam cell formation and atherogenesis by mediating recognition and internalization of oxLDL.[9] For example, CD36-apoE double-null mice exhibited a significant decrease in aortic atheromatous plaques in animals fed on a western diet.[10] The CD36 binding sites of hexarelin and (1-palmitoyl 2-(5′-oxovaleroyl) phosphatidylcholine (POVPC), the active fragment of oxidized low-density lipoprotein, have been shown to overlap.[11] OxLDL has been shown to induce an antiangiogenic effect in hyperlipidemic states such as atherosclerosis and diabetes.[12] Moreover, this antiangiogenic effect was found to be activated upon the binding of the extracellular matrix protein thrombospondin-1(TSP-1) to CD36 as endothelial cell receptor.[13,14] The CD36 receptor has thus emerged as an interesting target with potential therapeutic implications in the treatment of atherosclerotic conditions as well as those exhibiting marked angiogenesis (tumors, diabetic retinopathy, age-related macular degeneration) because its effects may be modulated both at the thrombospondin-1 binding site[13] as well as at the distinct oxLDL binding site.

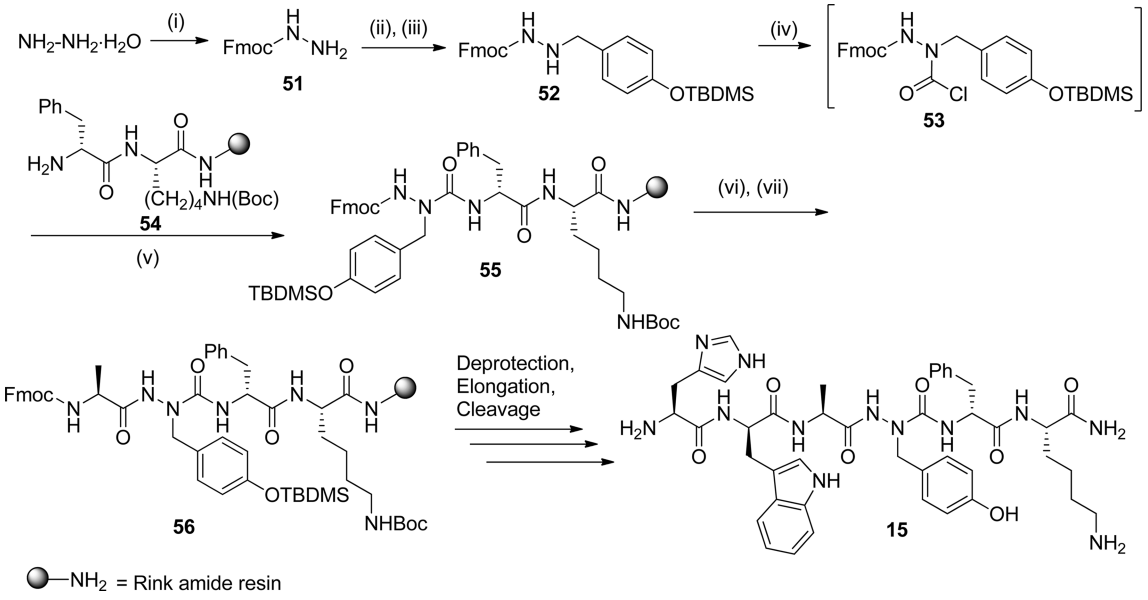
Considering that GHRP-6 analogues exhibit dual affinity for both the GHS-R1a and CD36 receptors, selective analogues are desired in order to chemically probe their specific functions. Moreover, the discovery of compounds with selectivity for the CD36 receptor, as opposed to the GHS-R1a, may afford novel therapeutic advantages. With the goal to dial-in specificity, azapeptide analogues of GHRP-6 were pursued to probe the biologically active conformations responsible for affinity at the GHS-R1a and CD36 receptors.

Azapeptides possess one or more aza-amino acid residues in which the α-CH is replaced by a nitrogen atom.[15] In this

Received: April 18, 2012
Published: June 19, 2012

   dx.doi.org/10.1021/jm300557t | J. Med. Chem. 2012, 55, 6502–6511

Scheme 1. Representative Synthesis of 15[a]



[a](i) FmocOSu (0.1 equiv), CH_3CN/H_2O (1:1); (ii) *p*-OTBDMS-benzaldehyde (1 equiv), EtOH, reflux; (iii) $Pd(OH)_2$, H_2; (iv) phosgene (20% in toluene, 2 equiv), DCM, 0 °C, 15 min; (v) DIEA (6 equiv); (vi) 20% piperidine in DMF; (vii) Fmoc-Ala-OH, BTC (1 equiv), collidine (12 equiv).

semicarbazide analogue of a natural peptide, the planar urea and lone pair repulsion between adjacent hydrazine nitrogen impose conformational restrictions, which favor a β-turn conformation about the position of the aza-residue, as predicted by computation and validated by spectroscopic and crystallographic studies.[16] Moreover, azapeptide analogues may exhibit greater metabolic stability and longer duration of action because semicarbazides are more difficult to hydrolyze than the corresponding amides.[17] Aza-amino acids have previously been introduced into peptide sequences to yield compounds with useful biological activity. Specifically, improved biological activity has been observed in aza-analogues of peptide hormones[18] such as luteinizing-hormone-releasing hormone (LHRH),[19] and enzyme inhibitors.[20−22] For example, the antagonist activity of [Asp[31], Pro[34], Phe[35]]-$CGRP_{27-37}$ at the calcitonin gene-related peptide (CGRP) receptor was found to be contingent on a type II′ β-turn conformer centered at Gly^{33}-Pro^{34} by using an aza-amino acid scan of the undecapeptide.[23] Introduction of aza-residues has also improved selectivity of cysteine and serine protease inhibitors.[24] Azapeptides have thus proven effective for development of analogues exhibiting increased biological activity and improved selectivity.

Previously, we reported an azapeptide GHRP-6 analogue, [azaPhe[4]]-GHRP-6, with improved receptor selectivity for the CD36 versus the GHS-R1a receptor.[25] This aza-GHRP-6 analogue was later proposed to adopt a turn conformation based on NMR studies, suggesting that the improved selectivity may be due to preorganization of an active conformation for binding to the CD36 receptor.[26] An aza-amino acid scan of the D-Trp^2-Ala^3-Trp^4 portion of the GHRP-6 peptide has now been performed to explore in more detail the importance of a biologically active turn conformation. Employing solid-phase synthesis using an Fmoc protection strategy, azapeptide analogues of GHRP-6 were generated in which the nature of each aza-amino acid was varied. Moreover, the structural requirements for selective CD36 binding of promising candidates was explored by alanine scans and *N*-terminal modifications using a combinatorial split-and-mix approach for making azapeptides. The IC_{50} binding values for both the GHS-R1a and CD36 receptors have been ascertained and azapeptides have been identified exhibiting significantly reduced affinity to the former with maintained potency for the latter receptor. Finally, the antiangiogenic properties of a set of azapeptides were assessed in an ex vivo assay in microvascular choroid explants.[27]

RESULTS AND DISCUSSION

Chemistry. Sequential replacement of the amino acid residues of the biologically active peptide with the corresponding aza-amino acids, a so-called "aza-amino acid scan",[28] was used to probe for active turn conformations of GHRP-6. The aza-amino acid scan of GHRP-6 was accomplished by an Fmoc protection strategy on Rink amide resin.[18] Azapeptides **1**−**49** were synthesized by employing *N*′-substituted fluorenylmethyl carbazates that were activated with phosgene prior to coupling to the peptide linked to the solid-phase as illustrated in the synthesis of [azaTyr[4]]GHRP-6 (**15**, Scheme 1). *N*′-Substituted fluorenylmethyl carbazates were prepared in solution in a two-step procedure from 9-*H*-fluoren-9-ylmethyl carbazate (**51**), which was heated at reflux with 1 equiv of the respective aldehyde to prepare the acyl hydrazones. Reduction of hydrazones was accomplished using either hydrogenation in the presence of palladium hydroxide or sodium cyanoborohydride reduction in the presence of acetic acid.[18] For the synthesis of the aza-proline analogues, fluoren-9-yl-methyl pyrazolidine-1-carboxylate hydrochloride was synthesized by acylation of *tert*-butyl pyrazolidine-1-carboxylate with Fmoc succinimide, followed by Boc group deprotection using a 1:1 TFA:DCM mixture.[29,30] After Fmoc cleavage, the aza-residue was acylated using Fmoc-amino acid chloride, generated by the bis-(trichloromethyl) carbonate (BTC) activated coupling procedure.[18] Notably, epimerization of the trityl-protected histidine residue was found to occur in some cases as observed by the presence of two overlapping peaks with identical mass by LCMS analysis. The epimerization likely occurred during

dx.doi.org/10.1021/jm300557t | J. Med. Chem. 2012, 55, 6502−6511

Table 1. Retention Times, Purity, and Mass of GHRP-6 Analogues

compound	RT (min) in MeCN[a]	RT (min) in MeOH	HPLC purity at 214 nm[d] (%)	MS [M + 1] or [M + 23] ions	
				m/z (calcd)	*m/z* (obsd)
1 (D/L)His-AzaPhe-Ala-Trp-D-Phe-Lys-NH_2	14.08	22.64	>99	835.4	835.5
2 (D/L)His-AzaTyr-Ala-Trp-D-Phe-Lys-NH_2	13.41	20.21	>99	851.4	851.3
3 (D/L)His-AzaNal-1-Ala-Trp-D-Phe-Lys-NH_2	15.41	25.23	>99	885.4	885.5
4 (D/L)His-AzahomoPhe-Ala-Trp-D-Phe-Lys-NH_2	14.80	23.37	>99	849.4	849.5
5 (D/L)His-AzaBip-Ala-Trp-D-Phe-Lys-NH_2	16.88	26.80	>99	911.5	911.5
6 His-D-Trp-AzaGly-Trp-D-Phe-Lys-NH_2	14.48	21.84	>99	860.4	860.3
7 His-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	16.24	25.33	>99	916.5	916.5
8 His-D-Trp-Ala-AzaPhe-D-Phe-Lys-NH_2	14.96	23.87	>99	835.4	835.5
9 His-D-Trp-Ala-AzaLeu-D-Phe-Lys-NH_2	14.27	22.83	>99	801.4	801.5
10 His-D-Trp-Ala-AzaBip-D-Phe-Lys-NH_2	17.81	27.68	>99	911.5	911.5
11 His-D-Trp-Ala-AzahomoPhe-D-Phe-Lys-NH_2	15.44	24.69	>99	849.4	849.5
12 His-D-Trp-Ala-AzaNal-1-D-Phe-Lys-NH_2	16.53	26.08	>99	885.4	885.5
13 His-D-Trp-Ala-Tyr-D-Phe-Lys-NH_2	12.59	20.64	>99	850.4	850.5
14 His-D-Trp-Ala-D-Tyr-D-Phe-Lys-NH_2	13.17	20.99	>99	850.4	850.5
15 His-D-Trp-Ala-AzaTyr-D-Phe-Lys-NH_2	13.57	20.59	>99	851.4	851.3
16 Ala-AzaPhe-Ala-Trp-D-Phe-Lys-NH_2	15.67	7.80[c]	93	769.4	769.4
17 (D/L)His-AzaPhe-Ala-Ala-D-Phe-Lys-NH_2	9.99	11.93	>99[e]	720.4	720.4
18 His-AzaPhe-Ala-Trp-D-Ala-Lys-NH_2	9.08	9.11	>99	759.4	759.4
19 His-AzaPhe-Ala-Trp-D-Phe-Ala-NH_2	15.54	11.05	97	778.4	778.4
20 Ala-AzaTyr-Ala-Trp-D-Phe-Lys-NH_2	13.19	19.02	>99	785.4	785.4
21 (D/L)His-AzaTyr-Ala-Ala-D-Phe-Lys-NH_2	8.13	8.18	>99[e]	736.4	736.4
22 His-AzaTyr-Ala-Trp-D-Ala-Lys-NH_2	8.79	10.79	97	775.4	775.4
23 His-AzaTyr-Ala-Trp-D-Phe-Ala-NH_2	14.89	21.34	98	794.4	794.4
24 Ala-D-Trp-AzaGly-Trp-D-Phe-Lys-NH_2	15.61	17.95[b]	99	794.4	794.4
25 His-D-Ala-AzaGly-Trp-D-Phe-Lys-NH_2	9.64	9.80	>99	745.4	745.4
26 His-D-Trp-AzaGly-Ala-D-Phe-Lys-NH_2	8.88	8.95	>99	745.4	745.4
27 His-D-Trp-AzaGly-Trp-D-Ala-Lys-NH_2	9.95	12.54	>99	784.4	784.4
28 His-D-Trp-AzaGly-Trp-D-Phe-Ala-NH_2	11.01[b]	18.56[b]	99	803.4	803.4
29 Ala-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	17.08	12.08[c]	>99	850.5	850.5
30 His-D-Ala-AzaLeu-Trp-D-Phe-Lys-NH_2	11.16	10.60[b]	>99	801.4	801.4
31 His-D-Trp-AzaLeu-Ala-D-Phe-Lys-NH_2	14.55	12.10	>99	801.5	801.5
32 His-D-Trp-AzaLeu-Trp-D-Ala-Lys-NH_2	11.65	11.73	>99	840.5	840.5
33 His-D-Trp-AzaLeu-Trp-D-Phe-Ala-NH_2	17.54	13.64[c]	>99	859.4	859.4
34 Ala-D-Trp-Ala-AzaPhe-D-Phe-Lys-NH_2	15.86	10.62[c]	>99	791.4	791.4
35 His-D-Ala-Ala-AzaPhe-D-Phe-Lys-NH_2	8.43	9.97	>99	720.4	720.4
36 His-D-Trp-Ala-AzaPhe-D-Ala-Lys-NH_2	10.00	10.10	>99	759.4	759.4
37 His-D-Trp-Ala-AzaPhe-D-Phe-Ala-NH_2	16.55	11.96	>99	778.4	778.4
38 His-D-Trp-AzaGly-Pro-D-Phe-Lys-NH_2	8.90	11.04	>99	771.4	771.4
39 His-AzaPro-Ala-Trp-D-Phe-Lys-NH_2	9.79	9.82	>99[e]	785.4	785.4
40 His-D-Trp-AzaPro-Trp-D-Phe-Lys-NH_2	12.85	17.67	>99	900.5	900.5
41 His-D-Trp-Ala-AzaPro-D-Phe-Lys-NH_2	10.94	10.95	>99	785.4	785.4
42 His-D-Trp-Ala-Trp-AzaPro-Lys-NH_2	9.96	12.61	>99	824.4	824.4
43 Ppa-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	22.63[c]	17.88[c]	>99	857.4	857.4
44 D-Ala-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	17.69	11.86[c]	>99	872.5	872.5
45 Gly-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	17.65	11.72[c]	>99	858.4	858.4
46 Pro-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	18.37	12.58[c]	>99	898.5	898.5
47 His-D-Trp-AzaLeu-Trp-D-Phe-NH_2	18.14	13.98[c]	>99	788.4	788.4
48 His-D-Trp-AzaLeu-Trp-NH_2	14.62	10.75[c]	>99	641.3	641.3
49 D-Trp-AzaLeu-Trp-NH_2	18.65	14.06[c]	>99	504.3	504.3

[a]Unless otherwise noted, analytical HPLC analyses were performed on a TARGA column from Higgins Analytical, Inc. (4.6 mm × 250 mm, 5 μm, C18) with a flow rate of 1.5 mL/min using a 40 min linear gradient from water (0.1% TFA) to CH_3CN (0.1% TFA) (method 1) or to MeOH (0.1% TFA) (method 2) for compound **1**–**15** and on a GEMINI column from Phenomenex (4.6 mm × 150 mm, 5 μM, C18) with a flow rate of 0.5 mL/min using a 2–40% gradient of water (0.1% FA) in CH_3CN (0.1% FA) or in MeOH (0.1% FA) for compounds **15**–**49**. [b]Analytical HPLC analyses were performed using the same GEMINI column as in (a), with a 10–50% gradient of water (0.1% FA) in CH_3CN (0.1% FA) or in MeOH (0.1% FA). [c]Analytical HPLC analyses were performed using the same GEMINI column as in (a), with a 20–80% gradient of water (0.1% FA) in CH_3CN (0.1% FA) or in MeOH (0.1% FA). [d]HPLC purity at 214 nm of the purified peptide. [e]A mixture of isomers was observed as two overlapping peaks with identical masses. [f]Observed masses corresponding to the H^+ and Na^+ adducts.

dx.doi.org/10.1021/jm300557t | J. Med. Chem. 2012, 55, 6502–6511

Table 2. IC_{50} Binding Values for GHS-R1a and CD36 Receptors of GHRP-6 Analogues

compd no.	compd	IC_{50} binding GHS-R1a (M)	IC_{50} binding CD36 (M)
GHRP-6	His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2	6.08×10^{-9}	1.82×10^{-6}
hexarelin	His-D-Trp(2-Me)-Ala-Trp-D-Phe-Lys-NH_2	1.59×10^{-8}	2.08×10^{-6}
57 (EP80317)	Haic-D-Trp(2-Me)-D-Lys-Trp-D-Phe-Lys-NH_2	7.50×10^{-7}	1.11×10^{-6}
1	(D/L)His-AzaPhe-Ala-Trp-D-Phe-Lys-NH_2	1.61×10^{-5}	7.24×10^{-5}
2	(D/L)His-AzaTyr-Ala-Trp-D-Phe-Lys-NH_2	8.53×10^{-6}	1.80×10^{-6}
3	(D/L)His-AzaNal-1-Ala-Trp-D-Phe-Lys-NH_2	4.65×10^{-7}	$\gg 10^{-5}$
4	(D/L)His-AzahomoPhe-Ala-Trp-D-Phe-Lys-NH_2	7.29×10^{-7}	3.68×10^{-5}
5	(D/L)His-AzaBip-Ala-Trp-D-Phe-Lys-NH_2	1.64×10^{-6}	2.32×10^{-5}
6	His-D-Trp-AzaGly-Trp-D-Phe-Lys-NH_2	8.08×10^{-7}	9.61×10^{-6}
7	His-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	1.20×10^{-6}	2.89×10^{-6}
8	His-D-Trp-Ala-AzaPhe-D-Phe-Lys-NH_2	2.77×10^{-6}	1.34×10^{-6}
9	His-D-Trp-Ala-AzaLeu-D-Phe-Lys-NH_2	1.95×10^{-5}	$\gg 10^{-5}$
10	His-D-Trp-Ala-AzaBip-D-Phe-Lys-NH_2	1.34×10^{-6}	1.35×10^{-5}
11	His-D-Trp-Ala-AzahomoPhe-D-Phe-Lys-NH_2	3.74×10^{-6}	$\gg 10^{-5}$
12	His-D-Trp-Ala-AzaNal-1-D-Phe-Lys-NH_2	7.23×10^{-7}	3.62×10^{-5}
13	His-D-Trp-Ala-Tyr-D-Phe-Lys-NH_2	7.71×10^{-7}	1.32×10^{-5}
14	His-D-Trp-Ala-D-Tyr-D-Phe-Lys-NH_2	3.25×10^{-6}	1.20×10^{-5}
15	His-D-Trp-Ala-AzaTyr-D-Phe-Lys-NH_2	1.57×10^{-5}	2.80×10^{-5}
16	Ala-AzaPhe-Ala-Trp-D-Phe-Lys-NH_2	$\gg 10^{-5}$	4.27×10^{-5}
17	(D/L)His-AzaPhe-Ala-Ala-D-Phe-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
18	His-AzaPhe-Ala-Trp-D-Ala-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
19	His-AzaPhe-Ala-Trp-D-Phe-Ala-NH_2	5.10×10^{-5}	$\gg 10^{-5}$
20	Ala-AzaTyr-Ala-Trp-D-Phe-Lys-NH_2	$\gg 10^{-5}$	3.69×10^{-5}
21	(D/L)His-AzaTyr-Ala-Ala-D-Phe-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
22	His-AzaTyr-Ala-Trp-D-Ala-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
23	His-AzaTyr-Ala-Trp-D-Phe-Ala-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
24	Ala-D-Trp-AzaGly-Trp-D-Phe-Lys-NH_2	5.68×10^{-7}	6.06×10^{-6}
25	His-D-Ala-AzaGly-Trp-D-Phe-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
26	His-D-Trp-AzaGly-Ala-D-Phe-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
27	His-D-Trp-AzaGly-Trp-D-Ala-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
28	His-D-Trp-AzaGly-Trp-D-Phe-Ala-NH_2	4.50×10^{-5}	$\gg 10^{-5}$
29	Ala-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	1.12×10^{-5}	6.66×10^{-6}
30	His-D-Ala-AzaLeu-Trp-D-Phe-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
31	His-D-Trp-AzaLeu-Ala-D-Phe-Lys-NH_2	$\gg 10^{-5}$	2.59×10^{-5}
32	His-D-Trp-AzaLeu-Trp-D-Ala-Lys-NH_2	$\gg 10^{-5}$	9.48×10^{-6}
33	His-D-Trp-AzaLeu-Trp-D-Phe-Ala-NH_2	8.17×10^{-6}	7.59×10^{-6}
34	Ala-D-Trp-Ala-AzaPhe-D-Phe-Lys-NH_2	$\gg 10^{-5}$	7.58×10^{-6}
35	His-D-Ala-Ala-AzaPhe-D-Phe-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
36	His-D-Phe-Ala-AzaPhe-D-Ala-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
37	His-D-Phe-Ala-AzaPhe-D-Phe-Ala-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
38	His-D-Trp-AzaGly-Pro-D-Phe-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
39	His-AzaPro-Ala-Trp-D-Phe-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
40	His-D-Trp-AzaPro-Trp-D-Phe-Lys-NH_2	$\gg 10^{-5}$	9.81×10^{-6}
41	His-D-Trp-Ala-AzaPro-D-Phe-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
42	His-D-Trp-Ala-Trp-AzaPro-Lys-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
43	Ppa-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	3.86×10^{-6}	$\gg 10^{-5}$
44	D-Ala-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	3.61×10^{-6}	1.33×10^{-5}
45	Gly-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	5.41×10^{-6}	1.05×10^{-5}
46	Pro-D-Trp-AzaLeu-Trp-D-Phe-Lys-NH_2	1.17×10^{-6}	7.97×10^{-6}
47	His-D-Trp-AzaLeu-Trp-D-Phe-NH_2	4.55×10^{-6}	9.01×10^{-6}
48	His-D-Trp-AzaLeu-Trp-NH_2	8.56×10^{-5}	$\gg 10^{-5}$
49	D-Trp-AzaLeu-Trp-NH_2	$\gg 10^{-5}$	$\gg 10^{-5}$
50	Aib-D-Trp(2-Me)-D-Trp(2-Me)-NH_2	6.29×10^{-8}	$\gg 10^{-5}$[a]

[a]Value was reported earlier in ref 7.

coupling to the aza-residue using BTC. Such azapeptides were examined as inseparable diastereoisomeric mixtures. Peptide chain elongation, cleavage, and purification were conducted according to general solid-phase peptide synthesis protocols.[31] Loss of the side-chain of the aza-tryptophan residue was found to occur during the acidic cleavage of the azapeptide derivatives such that the azaGly analogue was recovered.[18] Instead of azaTrp analogues, a series of aza-amino acid chlorides were

dx.doi.org/10.1021/jm300557t | J. Med. Chem. 2012, 55, 6502−6511

made on exposing phosgene to different *N*′-substituted fluorenyl-methyl carbazates: benzyl,[18] 4-(*t*-butyldimethyl-silyloxy)benzyl,[18] 2-phenylethyl, 1-naphthylmethyl,[32] and bi-phenyl-4-ylmethyl.[32]

A library of azapeptides was prepared in parallel for the alanine scan. Azapeptides **15**−**49** were constructed on resin in IRORI Kans.[33] MacroKans were respectively filled with 75−100 mesh Rink resin and a radiofrequency (Rf) tag associated to a unique ID number. In a split-and-mix approach, Kans were pooled together and exposed to identical conditions in a normal glass vessel. Upon completion of the reaction, MacroKans were washed, separated, sorted, and pooled accordingly for next reactions. Cleavage of the final peptide from the resin was performed by treating each individual MacroKan with TFA:H_2O:TES (95: 2.5: 2.5 v/v/v) for 2 h in a 20 mL glass vial. The MacroKans were washed with TFA, and the filtrate and washings were concentrated to an approximate volume of 1 mL before dilution with Et_2O for precipitation of the azapeptides. The ether layer was decanted after centrifugation, and the resulting peptide was dissolved in MeCN:H_2O (1:1) and freeze-dried to yield a white foam in average yields of 44% for peptides **1**−**15** and 5.1% for peptides **15**−**49** after RP-HPLC purification. The retention times, HPLC purity, and exact mass for each azapeptide are reported in Table 1.

Biological Results. Azapeptides **1**−**49** were examined for binding to the GHS-R1a and CD36 receptors using previously reported protocols. In short, the affinity for the ghrelin receptor was assessed in a competition binding study using [I^{125}] radiolabeled ghrelin, membranes from GHS-R1a transfected cells, and aza-GHRP-6 analogues as the competitive ligands in increasing concentrations ranging from 10^{-12} to 10^{-5} M.[34] CD36 receptor binding was assessed by a covalent competition binding study using [I^{125}] radiolabeled photoactivatable hexarelin derivative as a radioligand and rat cardiac membranes as a source of CD36 receptor, as previously reported.[35] Azapeptide GHRP-6 analogues were used as competitive ligands in concentrations ranging from 0.1 to 50 μM. According to the test results, certain azapeptides were found to retain affinity for the CD36 receptor; however, their binding was considerably reduced at the GHS-R1a receptor (Table 2).

Azapeptides were made by placement of an aza-residue at either the D-Trp^2, Ala^3, or Trp^4 positions. A variety of aromatic side chains were examined at the 2- and 4-positions due to the instability of aza-tryptophan under acidic conditions.[18] Moreover, [Tyr^4]- and [D-Tyr^4]-GHRP-6 (**13** and **14**) were prepared for direct comparison with **15**. Many azapeptides exhibited significant losses of activity at both receptors. Azapeptides **2**, **6**, **7**, and **8**, having respectively azaTyr^2, azaGly^3, azaLeu^3, and azaPhe^4, retained affinity to the CD36 receptor in the micromolar range, yet lost affinity to GHS-R1a by a factor of 10^2−10^4. Loss of affinity to the CD36 receptor by about a factor of 10 was observed on going from [azaTyr^2]-GHRP-6 (**2**) to [azaPhe^2]-GHRP-6 (**1**) and from [azaPhe^4]-GHRP-6 (**8**) to [azaTyr^4]-GHRP-6 (**15**), indicating that the tyrosine side chain was better tolerated at the D-Trp^2 position. Furthermore, a 10-fold loss of affinity toward the CD36 receptor was observed upon substitution of the Trp^4 residue for Tyr (**13**) or D-Tyr (**14**). Replacement of D-Trp^2 and Trp^4, respectively, with azanaphthalene-1 (azaNal-1), azaHomoPhe, and azaBiphenyl (azaBip) decreased affinity to the CD36 receptor by a factor of 10−10^3. From the preliminary results, azapeptides **1**, **2**, **6**, **7**, and **8** were selected as leads for alanine scans and further structure−activity relationship studies.

Replacement of the amino acid residues by an alanine residue was performed systematically on leads **1**, **2**, **6**, **7**, and **8** to provide azapeptides **16**−**37**, and their affinity was ascertained at both the GHS-R1a and CD36 receptors (Table 2). Notably, the His^1 residue could be replaced with alanine without significant loss of affinity. For example, relative to the CD36 receptor binding of their respective His^1 azapeptide counterparts, [Ala^1, azaGly^3]-GHRP-6 (**24**), [Ala^1, azaLeu^3]-GHRP-6 (**29**), and [Ala^1, azaPhe^4]-GHRP-6 (**34**), all retained affinity, and [Ala^1, azaPhe^2]-GHRP-6 (**16**) and [Ala^1, azaTyr^2]-GHRP-6 (**20**) exhibited only a 10-fold loss in affinity. The replacement of D-Trp^2 by D-Ala caused a significant drop in affinity to both receptors in all cases (e.g., **27**, **30**, and **35**), indicating the importance of the aromatic side chain at this position. Except for [azaLeu^3, Ala^4]-GHRP-6 (**31**), which exhibited a 10-fold loss of binding for the CD36 receptor relative to its Trp^4 counterpart, azapeptides (e.g., **17**, **21**, and **26**) suffered >10-fold losses in affinity on replacement of Trp^4 by an alanine. Similarly, [azaLeu^3, D-Ala^5]- and [azaLeu^3, Ala^6]-GHRP-6 (**32** and **33**) retained micromolar affinity for the CD36 receptor; however, replacement of D-Phe^5 and Lys^6 respectively by D-Ala and Ala caused >10-fold losses of affinity toward both the GHS-R1a and CD36 receptors in [Ala^5]-azapeptides **18**, **22**, **27**, and **36** and [Ala^6]-azapeptide **19**, **23**, **28**, and **37**.

The results of the alanine scan identified [Ala^1, azaLeu^3]-GHRP-6 (**29**), [azaLeu^3, D-Ala^5]-GHRP-6 (**32**), and [Ala^1, azaPhe^4]-GHRP-6 (**34**), which retained micromolar affinity for the CD36 receptor, yet lost by >10^4-fold ability to bind GHS-R1a. Moreover, [Ala^1, azaGly^3]-GHRP-6 (**24**) and [azaLeu^3, Ala^6]-GHRP-6 (**33**) maintained micromolar affinity for the CD36 receptor and lost by ≥100-fold affinity for the GHS-R1a. Notably, replacement of Ala^3 by azaLeu^3 in GHRP-6 provided several analogues with good affinity, including [Ala^1, azaLeu^3]-GHRP-6 (**29**), [azaLeu^3, D-Ala^5]-GHRP-6 (**32**), and [azaLeu^3, Ala^6]-GHRP-6 (**33**). On the basis of these findings, along with the discovery that replacement of His^1 by Ala was well tolerated, a third generation of analogues were designed and synthesized. The His^1 residue of [azaLeu^3]-GHRP-6 (**7**) was replaced with propionic acid (Ppa), D-Ala, Gly, and Pro to afford aza-GHRP-6 analogues **43**−**46**, and truncation of **7** afforded penta-, tetra-, and triazapeptides **47**−**49**.

Replacement of His^1 by proline in **46** maintained micromolar affinity for the CD36 receptor. A 10-fold loss of affinity ensued on replacement of His^1 respectively by D-alanine **44** and glycine **45** and a >10-fold loss of affinity for the CD36 receptor resulted from substituting a propionyl moiety **43** for His^1. In all cases, a 10^3 fold loss of affinity was observed with respect to the GHS-R1a. Results from the affinity studies of truncated azapeptides **47**−**49** toward both receptors revealed that only loss of Lys^6 was tolerated and penta-azapeptide **47** maintained micromolar affinity for the CD36 receptor. This result and the maintained affinity of [azaLeu^3, Ala^6]-GHRP-6 (**33**) demonstrated that the Lys^6 residue was unnecessary for [azaLeu^3]-GHRP-6 to bind to the CD36 receptor, in sharp contrast to the decreased affinity exhibited by [azaPhe^2, Ala^6]-GHRP-6 (**19**), [azaTyr^2, Ala^6]-GHRP-6 (**23**), [azaGly^3, Ala^6]-GHRP-6 (**28**), and [azaPhe^4, Ala^6]-GHRP-6 (**37**).

In parallel to the alanine scan of azapeptides **1**, **2**, **6**, **7**, and **8**, azapeptides **39**−**42** containing an aza-proline residue were synthesized to further explore the conformational requirements for binding to GHS-R1a and CD36 receptors. Systematic

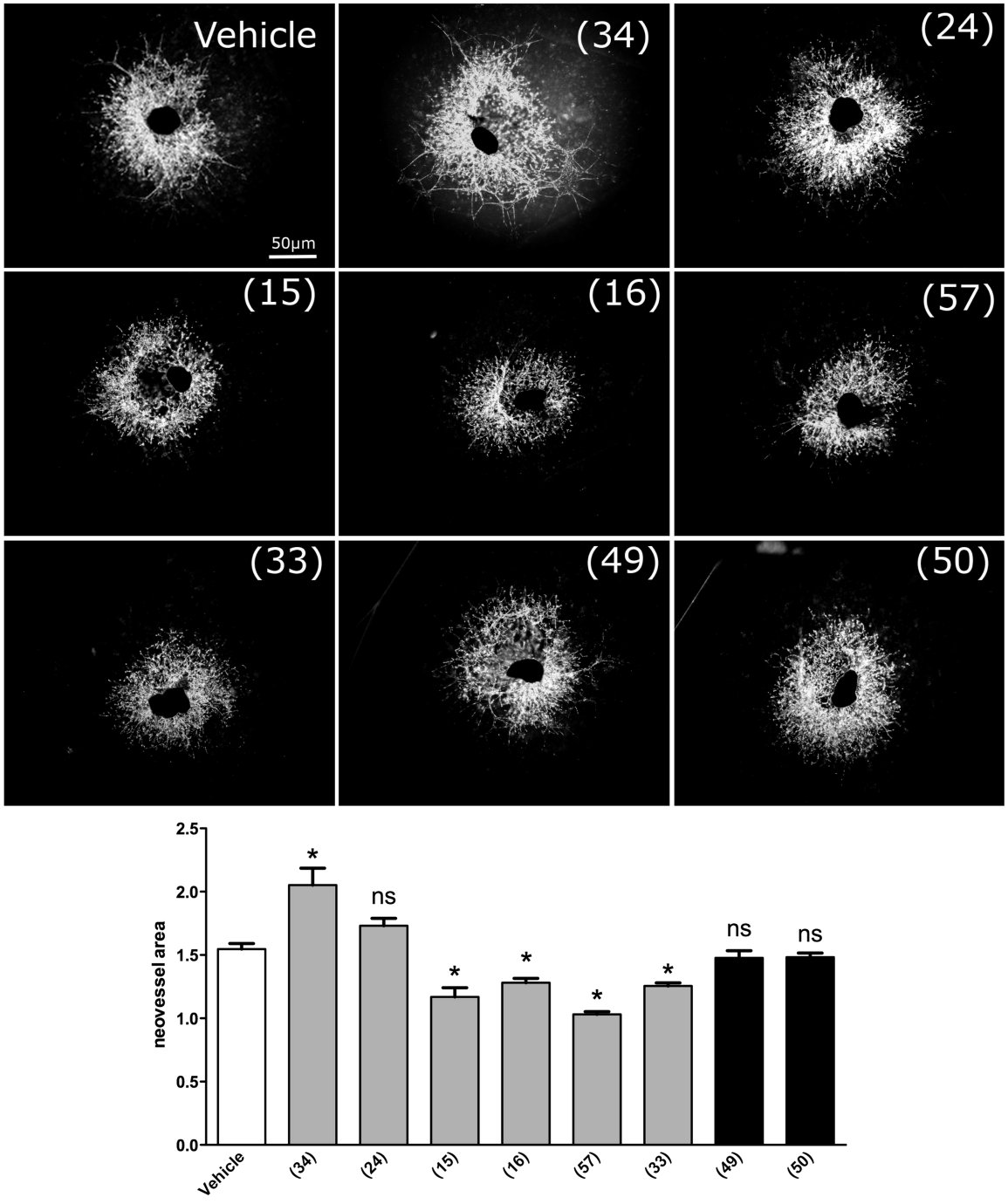


Figure 1. Antiangiogenic effect of CD36 mediators. (A) Representative microvascular sprouting from Matrigel-embedded choroidal explants treated 4 days with azapeptides (10^{-5} M, scale bar 50 μm). (B) The corresponding histogram values of quantification of choroidal sprouting areas in (A). Values are mean ± SEM *P < 0.05 compared to corresponding control values. Values are mean ± SEM choroidal area sprouting (J4) compared to corresponding control values (J0) *P < 0.05. The units are arbitrary. The values correspond to the subtraction of total area minus area of explant.

substitution of azaPro for each residue in the D-Trp^2-Ala^3-Trp^4-D-Phe^5 portion of GHRP-6 gave [azaPro^3]-GHRP-6 (**40**), which retained micromolar affinity for the CD36 receptor and lost affinity for the GHS-R1a receptor. The other azaPro analogues (**39**, **41**, and **42**) lost affinity to both receptors. Considering the conformational preference for azaPro to adopt the i + 2 position of a type VI β turn,[36] such a conformation may be responsible for [azaPro^3]-GHRP-6 (**40**) retaining CD36 receptor affinity without binding to GHS-R1a.

A subset of azapeptides containing [azaTyr^4]-GHRP-6 (**15**), [Ala^1, azaPhe^2]-GHRP-6 (**16**), [Ala^1, azaGly^3]-GHRP-6 (**24**), [azaLeu^3, Ala^6]-GHRP-6 (**33**), and [Ala^1, azaPhe^4]-GHRP-6 (**34**) was selected for investigation of potential antiangiogenic properties. Relative to hexarelin, all five analogues displayed a ≥100-fold decrease in affinity for GHS-R1a, azapeptides **24**, **33**, and **34** retained micromolar affinity to the CD36 receptor and azapeptides **15** and **16** exhibited a 10-fold drop in CD36 receptor affinity. In addition, azapeptides **16**, **24**, and **33** were found to feature relatively lower differential binding selectivity toward both ghrelin and CD36 receptors. Antiangiogenic properties were examined by means of a microvascular sprouting assay using mouse choroidal explants (Figure 1).

dx.doi.org/10.1021/jm300557t | J. Med. Chem. 2012, 55, 6502−6511

The tripeptides D-Trp-AzaLeu-Trp-NH_2 (**49**) and Aib-D-Trp(2-Me)-D-Trp(2-Me)-NH_2 (**50**), which had demonstrated a ≥10 loss of affinity toward both receptors, were used as negative controls. On the other hand, Haic-D-Trp(2-Me)-D-Lys-Trp-D-Phe-Lys-NH_2 (**57**, Haic = 5-amino-1,2,4,5,6,7-tetrahydroazepino[3,2,1-*hi*]indol-4-one-2-carboxylate),[35] a peptide known to bind to the CD36 receptor (Table 2, entry 3), was used as a positive control.

Quantification of microvascular sprouting after a 4-day treatment (J4 vs J0) with azapeptides **15**, **16**, and **33** revealed respectively 17, 28, and 25% of choroid sprouting, respectively, compared to the control condition of 53% of neovascularization, thereby underlining their antiangiogenic properties. Positive control **57** featured 3% of choroid sprouting. On the other hand, [Ala[1], azaGly[3]]-GHRP-6 (**24**) and D-Trp-AzaLeu-Trp-NH_2 (**49**), showing 72 and 47% of choroid sprouting respectively as compared to the control condition of 53% of neovascularization, did not feature any angiogenic activity. A reverse pharmacologic profile was displayed by [Ala[1], azaPhe[4]]-GHRP-6 (**34**), which increased neovascularization to 105% after the 4-day treatment, an effect which may be due to activation of Src recruitment and a VEGF-driven Akt phosphorylation pathway.[37] Azapeptide analogues, which bind selectively the CD36 receptor without affinity for GHS-R1a, may thus induce different biological responses, contingent on the nature and position of the aza-amino acid residue.

The conformations of the azapeptides [azaTyr[4]]-GHRP-6 (**15**) and [Ala[1], azaPhe[4]]-GHRP-6 (**34**), which respectively caused a reduction or an increase in neovascularization relative to control, were examined by circular dichroism spectroscopy in water (Figure 2). The CD signature of GHRP-6 in water was

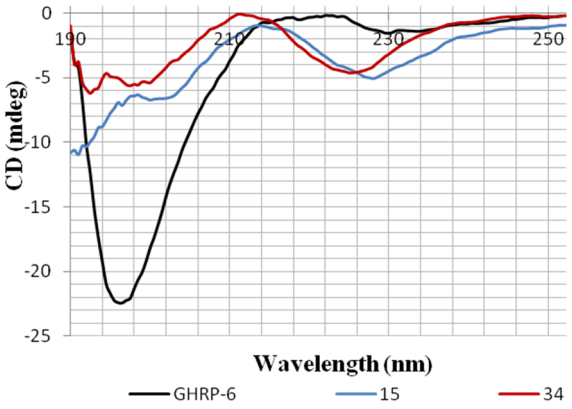


Figure 2. Circular dichroism spectra in water of azapeptides **15** and **34** compared with GHRP-6.

characteristic of a random coil, exhibiting a negative maximum around 190 nm. On the other hand, the CD curves of azapeptides **15** and **34** were indicative of β-turn conformations, with distinctive negative maxima at 230 and 190 nm and a positive maximum at 215 nm.[38] Previously, [azaPhe[4]]-GHRP-6 (**8**) was found to exhibit a similar CD curve shape indicative of a β-turn conformation, which was supported by NMR spectroscopic studies.[25] The similar conformations exhibited by [azaTyr[4]]-GHRP-6 (**15**) and [Ala[1], azaPhe[4]]-GHRP-6 (**34**) in water does not correlate with their divergent biological responses arising from the binding to the CD36 receptor. Further studies are in progress to elucidate the structural and conformational requirements, which are responsible for azapeptides exhibiting differential responses on angiogenesis upon binding to the CD36 receptor.

CONCLUSION

In contrast to natural GHRP-6, which exhibits affinity for the GHS-R1a and CD36 receptor, in nanomolar and micromolar range, respectively, certain azapeptide analogues of GHRP-6 have been developed that maintain binding to the CD36 receptor while losing affinity for the GHS-R1a receptor. Additionally, certain analogues were found to reduce microvascular sprouting, displaying antiangiogenic properties. Aza-amino acid residues are known to induce turn conformations in peptides, and such conformational constraints may likely prevent binding toward one receptor. In particular, aza-residues at positions 2–4 (D-Trp-Ala-Trp) of GHRP-6 may induce a β-turn geometry responsible for affinity to the CD36 receptor. In this geometry, His[1] appears to be less important for binding than D-Trp[2] and Lys[6]. Setting a foundation for ongoing research, this study is positioned to further the elucidation of the structural features for selectivity and affinity for the CD36 receptor and modulation of its function.

EXPERIMENTAL SECTION

Azapeptides **1**–**49** were prepared on solid support using an acid labile Rink resin and a Fmoc/*t*Bu protocol previously reported in which the syntheses of **2**, **8**, and **15** were described.[18] Incorporation of aza-amino acid into peptide was performed using either 1,3,4-oxadiazol-2(3*H*)-one for the aza-Gly residue[39] or suitable *N*′-alkyl fluoren-9-ylmethyl carbazates which provided the corresponding *N*-(Fmoc)aza-amino acid chlorides for coupling onto the growing chain of the resin-bound peptide.[18] Acylation of the aza-amino residue was performed using BTC as an activating agent.[18] Coupling to normal amino acid residues with *N*-(Fmoc)amino acids (300 mol %) was performed according to general solid-phase peptide synthesis protocols using HBTU (300 mol %), DIEA (600 mol %), and DMF for 3 h.[31] *N*-(Fmoc)Amino acids were purchased from GL Biochem. The side-chains of lysine and tryptophan were protected with Boc groups, the histine imidazole was protected with a trityl group, and the phenol of tyrosine was protected as a *tert*-butyldimethylsilyl ether.

Rink resin (0.65 mmol/g) was purchased from Advanced Chemtech Inc., and the manufacturer's reported loading of the resin was used in the calculation of the yields of the final products. Phosgene (20% in toluene) was purchased from Fluka. Melting points were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded respectively at 400 and 100 MHz in $CDCl_3$ or DMSO as the solvent and internal reference. Thin-layer chromatography was performed on silica gel 60 F_{254} plates from Merck. Flash chromatography was performed on silica gel 60 (230–400 mesh ASTM) from Merck.

Azapeptides 1–15. Analytical HPLC analyses were performed on a TARGA column from Higgins Analytical, Inc. (4.6 mm × 250 mm, 5 μm, C_{18}) with a flow rate of 1.5 mL/min using a 40 min linear gradient from water (0.1% TFA) to CH_3CN (0.1% TFA) (method 1) or MeOH (0.1% TFA) (method 2). Azapeptides were purified using semipreparative LC-MS (Previal C18 column, 22 mm × 250 mm, particle size 5 μm) with solvent A, H_2O (0.1% TFA), and solvent B, acetonitrile (0.1% TFA) using a gradient of 20–40% of A over 20 min at a flow rate of 15 mL/min.

Purity of azapeptides was assessed using analytical HPLC analyses performed on a TARGA column from Higgins Analytical, Inc. (4.6 mm × 250 mm, 5 μm, C18) with a flow rate of 1.5 mL/min using a 40 min linear gradient from water (0.1% TFA) to CH_3CN (0.1% TFA) (method 1) or to MeOH (0.1% TFA) (method 2), and high resolution mass spectrometry. Azapeptides were purified to >99% purity.

Azapeptides 15–49. Azapeptides **15**–**49** were synthesized using IRORI Kan technology. MacroKans were respectively filled with 130 mg (0.0845 mmol) of 75–100 mesh Rink Resin SS and a

dx.doi.org/10.1021/jm300557t | J. Med. Chem. 2012, 55, 6502–6511

radiofrequency (Rf) tag associated to a unique ID number. In a split-and-mix approach, Kans undergoing identical reactions were pooled together in a normal glass vessel that was filled with solvent and reagents. Upon completion of the reaction, MacroKans were washed three times sequentially with DMF, MeOH, and DCM, separated, sorted, and pooled accordingly for next reactions. Cleavage of the final peptide from the resin with simultaneous removal of protection from amino acid side chains was performed by treatment with TFA:H_2O:TES (95:2.5:2.5 v/v/v) for 2 h. The resin was filtered and washed with TFA, and the filtrate and washings were concentrated to an approximate volume of 1 mL before dilution with Et_2O for precipitation of the azapeptides. The ether layer was decanted after centrifugation, and the resulting peptide was dissolved in MeCN:H_2O (1:1) and freeze-dried to yield a white foam in average yields of 44% for peptides **1**−**15** and 5.1% for peptides **15**−**49** after purification using RP-HPLC.

Analytical HPLC analyses were performed on a Gemini column (4.6 mm × 150 mm, 5 μM, C_{18}) with a flow rate of 0.5 mL/min using either a 2−40%, 10−50%, or 20−80% gradient from water (0.1% FA) to CH_3CN (0.1% FA) or MeOH (0.1% FA). Azapeptides were purified using semipreparative LC-MS (Previal C18 column, 22 mm × 250 mm, particle size 5 μm) with solvent A, H_2O (0.1% FA), and solvent B, acetonitrile (0.1% FA) using a gradient over 20 min at a flow rate of 10.6 mL/min.

Purity of azapeptides was assessed using a GEMINI column from Phenomenex (4.6 mm × 150 mm, 5 μM, C18) with a flow rate of 0.5 mL/min using a 2−40%, 10−50%, or 20−80% gradient of water (0.1% FA) in CH_3CN (0.1% FA) or in MeOH using (0.1% FA), and high resolution mass spectrometry. Azapeptides were purified to 93−99% purity.

General Procedure for the Synthesis of 9*H*-Fluoren-9-ylmethyl carbazate and *N*′-Alkyl-Fluorenylmethyl carbazates. 9*H*-Fluoren-9-ylmethyl carbazate (**51**),[40] *N*′-2-isobutyl-fluorenyl methyl carbazate,[18] *N*′-benzyl-fluorenylmethyl carbazate,[18] *N*′-(4-(*t*-butyldimethylsilyloxy)-benzyl)-fluorenyl-methyl carbazate,[18] *N*′-(1-naphthylmethyl)-fluorenylmethyl carbazate,[32] and *N*′-(biphenyl-4-ylmethyl)-fluorenylmethyl carbazate[32] all were synthesized according to literature procedures.

Synthesis of *N*′-2-Phenylethyl-fluorenylmethyl Carbazate. A suspension of 9*H*-fluoren-9-ylmethyl carbazate in EtOH (0.2 M, 2.5 mmol) was treated with 100 mol % of phenylacetaldehyde (2.5 mmol), heated at reflux for 2 h, and concentrated in vacuo. The hydrazone was dissolved in THF (0.2 M) and treated successively with 110 mol % of AcOH and 110 mol % of $NaBH_3CN$, stirred for 1 h, and treated with additional $NaBH_3CN$ if necessary until completion of the reaction was observed by TLC. The mixture was concentrated in vacuo. The residue was dissolved in EtOAc, and the organic solution was washed with aqueous $KHSO_4$ (1M) and brine, dried over Na_2SO_4, and concentrated under reduced pressure to yield a white solid, which was dissolved in EtOH and heated at reflux for 1 h. The mixture was concentrated under reduced pressure to yield a residue that was purified by flash chromatography using 50% EtOAc in hexane as eluant and isolated as white foam in 76% yield: R_f = 0.26 (50% EtOAc in hexanes). ^{1}H NMR (DMSO) δ 2.67 (t, J = 7.2 Hz, 2H), 2.94 (m, 2H), 4.24 (t, J = 6.8 Hz, 1H), 4.34 (d, J = 6.8 Hz, 2H), 4.63 (m, 1H), 7.14−7.36 (m, 7H), 7.42 (t, J = 7.6 Hz, 2H), 7.71 (d, J = 7.2 Hz, 2H), 7.90 (d, J = 7.6 Hz, 2H), 8.78 (brs, 1H). ^{13}C NMR (DMSO) δ 33.9, 46.8, 52.5, 65.6, 120.2 (2C), 125.3 (2C), 125.9, 127.2 (2C), 127.8 (2C), 128.3 (2C), 128.8 (2C), 140.2, 140.8 (2C), 143.9 (2C), 157.0. LRMS (EI) 359.1 $(M + H)^+$, 381.2 $(M + Na)^+$. HRMS (EI) m/e for $C_{23}H_{23}N_2O_2$ $(M + H)^+$, calcd 396.1918, found 396.1919.

Synthesis of Fluoren-9-yl-methyl pyrazolidine-1-carboxylate hydrochloride. *tert*-Butyl pyrazolidine-1-carboxylate[41] (1.39 g, 8.08 mmol) was treated with Fmoc succinimide (3.27 g, 9.69 mmol, 1.2 equiv) in dry dichloromethane (20 mL) with stirring overnight. The volatiles were removed using a rotary evaporator. The residue was dissolved in EtOAc. The organic solution was washed three times, respectively, with 5% citric acid, 5% $NaHCO_3$ and brine, dried over Na_2SO_4, filtered, and concentrated to a white foam, which was purified by column chromatography using 7:3 EtOAc:hexane as eluant. Evaporation of the collected fractions afforded 1-(9*H*-fluoren-9-yl)methyl 2-*tert*-butylpyrazolidine-1,2-dicarboxylate as a low melting white solid in a 94% yield. Pyrazolidine-1,2-dicarboxylate (1.34 g, 3.4 mmol) was treated with 25 mL of a 1:1 TFA:DCM solution and stirred for 1 h. Removal of the volatiles by rotary evaporation gave a residue, which was dissolved in 1N HCl, stirred for 1 h, and freeze-dried to yield (9*H*-fluoren-9-pyrazolidine-1-yl)methyl-1-carboxylate hydrochloride: mp 143.2−148.1 °C, lit. mp 142 °C.[30] Spectral characterization data was identical with that in ref 29.

Membrane Preparation for CD36. Animal use was in accordance with the Institutional Animal Ethics Committee and the Canadian Council on Animal Care guidelines for the use of experimental animals. Sprague−Dawley (275−350 g) rats were necrotized with CO_2 until complete loss of consciousness, and their hearts were promptly removed in ice-cold saline and the cardiac membranes were prepared according to Harigaya and Schwartz.[42]

Competitive Covalent CD36 Binding Assay Using Photoactivatable [^{125}I]-Tyr-Bpa-Ala-Hexarelin as Radioligand. The radioiodination procedure to prepare the photoactivatable ligand and the receptor binding assays were performed as previously described by Bodart et al.[35] Briefly, the rat cardiac membranes (200 μg) as source of CD36 were incubated in the dark in 525 μL of 50 mM Tris-HCl pH 7.4 containing 2 mM EGTA (buffer A) in the presence of a fixed concentration of [^{125}I]-Tyr-Bpa-Ala-hexarelin (750 000 cpm) in buffer B (50 mM Tris-HCl pH 7.4 containing 2 mM EGTA and 0.05% bacitracin) and increasing concentrations of competitive ligand (ranging from 0.1 to 50 μM). Nonspecific binding was defined as binding not displaced by 50 μM peptide. After an incubation period of 60 min at 22 °C, membranes were submitted to UV irradiation at 365 nm for 15 min at 4 °C. After centrifugation at 12000g for 15 min, the pellets were suspended in 100 μL of sample buffer consisting of 62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 15% 2-mercaptoethanol, and 0.05% bromophenol blue and boiled for 5 min prior to be subjected to electrophoresis on 7.5% SDS-PAGE. The SDS/PAGE gels were fixed, colored in Coomassie Brilliant Blue R-250, dried, exposed to a storage phosphor intensifying screen (Amersham Biosciences), and analyzed by using a Typhoon PhosphorImager (Amersham Biosciences) and ImageQuant 5.0 software to establish competition curves. The band corresponding to the specifically labeled protein of 87 kDa was quantified by densitometry analysis.

Membrane Preparation for GHS-R1a Receptor. *Transfection.* LLC-PK1 cells were seeded at 1.5 × 10^6 cells/10 cm in Petri dishes and grown for 24 h in DMEM high-glucose (4.5 g/L) with 10% fetal bovine serum supplemented with penicillin (10000 units/mL) and streptomycin (10000 μg/mL), and cultured at 37 °C, under 5% of CO_2. The medium was then replaced for another 4−5 h, before $CaPO_4$ calcium phosphate transfection. The DNA solution consisted of 40 μg of DNA in a volume of 500 μL in which was added 500 μL of 2 mM Tris-HCl pH 8.0, and 0.2 mM EDTA pH 8.0 containing 500 mM $CaCl_2$, to a final volume of 1 mL. Then 1 mL of 50 mM Hepes, 280 mM NaCl, 1.5 mM Na_2HPO_4 pH to 7.1 (HBSS) was added by alternating 1 drop/2 air bubbles. The transfection mixture was incubated at RT for 30 min. After the incubation period, 1 mL of the mix was added to each plate and distributed evenly for incubation. The media was then replaced with standard DMEM-high glucose media for another 24 h, and cells were collected for membrane preparation.

Membrane Preparation. The experiment was carried out at 4 °C unless specified. Cells were washed twice with PBS and with the homogenization buffer (HB) consisting of 50 mM Tris, 5 mM $MgCl_2$, 2.5 mM EDTA, and 30 μg/mL bacitracin at pH 7.3, and were scraped into Eppendorf tubes. Cells were lysed with two cycles of freeze/thawing using liquid nitrogen and were then centrifuged at 4 °C for 20 min at 10000g to collect the membranes. The membranes were suspended in a small volume of HB, aliquoted, and stored at −80 °C.

GHS-R1a Receptor Binding Assay. The competitive binding assay employed 200 μL HB, 100 μL ^{125}I-ghrelin (40,000 cpm), 100 μL competitive ligand (from 10^{-12} to 10^{-5} M), and 100 μL of GHS-R1a transiently transfected in LLC-PK1 cells as source of binding sites (10 μg protein/tube). The nonspecific binding was determined using excess of competitive ligand at 10^{-5} M. The reaction was performed at

RT for 1 h. After the incubation period, the separation of bound from free fractions was performed by filtration over a GF/C filter presoaked with 0.5% polyethyleneimine, and the filters were washed with 4 mL of HB consisting of 50 mM Tris, 10 mM $MgCl_2$, 2.5 mM EDTA, and 3 mL of wash buffer consisting of 50 mM Tris-10 mM $MgCl_2$ and 20 mM EDTA, 0.015% Triton X100 (pH 7.3) and collected for radioactivity counting using a gamma counter (LKB Wallac 1277, Turku Finland).

Microvascular Sprouting from Choroidal Explants. Explants from choroidal capillaries of adult CB57BL/6 mice were prepared as previously reported, with some modifications to the protocol.[27] After enucleation, eyes were collected and incubated in 2% dispase for 1 h at 37 °C in EBM free medium. The anterior segment, vitreous, and neurosensory retina were removed and an eyecup was made. The retinal pigment epithelium (RPE) were removed from choroid-sclera by gently scraping. Choroid-sclera were cut into 4−5 pieces, and sides closed to the optic nerve and the cornea were recut. Each piece was cultured in growth factor-reduced Matrigel (BD Biosciences) in 24-well plates with EBM + EGM 0.4% of serum without hydrocortisone (RPE side up). Plates were then incubated at 37 °C and 5% CO_2 for 6 days before incubation in the absence (control) or in presence of different peptides at final concentration of 10^{-7} M in EBM without serum. Photographs of individual explants were taken before (J0) and after 4-day exposure (J4). The sprouting covered area was quantified with ImageJ 1.42q (Wayne Rasband), and the % of differential sprouting (J4 vs J0) was determined for tested peptide and azapeptide candidates.

AUTHOR INFORMATION

Corresponding Author

*For W.D.L.: phone, 514-343-7339; fax, 514-343-7586; E-mail, william.lubell@umontreal.ca. For H.O.: phone, 514-343-6460; E-mail, huy.ong@umontreal.ca.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the Fonds Québecois de la Recherche sur la Nature et les Technologies (FQRNT), and the Canadian Institutes of Health Research. C.P. is grateful to NSERC and Boehringer Ingelheim for graduate student fellowships. We thank Dr. A. Fürtös, K. Venne, and M-C. Tang for assistance with mass spectrometry.

ABBREVIATIONS USED

GHRP-6, growth hormone releasing peptide 6; GHS-R1a, growth hormone secretagogue receptor 1a; CD36, cluster of differentiation 36; GH, growth hormone; GHRH, growth hormone releasing hormone; oxLDL, oxidized low density lipoproteins; POVPC, (1-palmitoyl 2-(5′-oxovaleroyl) phosphatidylcholine; TSP-1, thrombospondin-1; TFA, trifluoroacetic acid; BTC, bis-(trichloromethyl) carbonate; azaNal-1, azanaphthalene-1; azaBip, azabiphenyl

REFERENCES

(1) Momany, F.; Bowers, C. Y.; Reynolds, G. A.; Hong, A.; Newlander, K. Conformational Energy Studies and in Vitro and in Vivo Activity Data on Growth Hormone-Releasing Peptides. *Endocrinology* **1984**, *114* (5), 1531−1536.

(2) Popovic, V.; Damjanovic, S.; Micic, D.; Djurovic, M.; Dieguez, C.; Casanueva, F. F. Blocked Growth Hormone-Releasing Peptide (GHRP-6)-Induced GH Secretion and Absence of the Synergic Action of GHRP-6 Plus GH-Releasing Hormone in Patients with Hypothalamopituitary Disconnection: Evidence That GHRP-6 Main Action Is Exerted at the Hypothalamic Level. *J. Clin. Endocrinol. Metab.* **1995**, *80* (3), 942−947.

(3) Ghigo, E.; Arvat, E.; Gianotti, L.; Imbimbo, B. P.; Lenaerts, V.; Deghenghi, R.; Camanni, F. Growth Hormone-Releasing Activity of Hexarelin, a New Synthetic Hexapeptide, after Intravenous, Subcutaneous, Intranasal, and Oral Administration in Man. *J. Clin. Endocrinol. Metab.* **1994**, *78* (3), 693−698.

(4) Imbimbo, B. P.; Mant, T.; Edwards, M.; Amin, D.; Dalton, N.; Boutignon, E.; Lenaerts, V.; Wiithrich, P.; Deghenghi, R. Growth hormone-releasing activity of hexarelin in humans. A dose−response study. *Eur. J. Clin. Pharmacol.* **1994**, *46*, 421−425.

(5) Cordido, F.; Peñalva, A.; Dieguez, C.; Casanueva, F. F. Massive growth hormone (GH) discharge in obese subjects after the combined administration of GH-releasing hormone and GHRP-6: evidence for a marked somatotroph secretory capability in obesity. *J. Clin. Endocrinol. Metab.* **1993**, *76*, 819−23.

(6) Frutos, M. G-S.; Cacicedo, L.; Fernandez, C.; Vicent, D.; Velasco, B.; Zapatero, H.; Sanchez-Franco, F. Insights into a role of GH secretagogues in reversing the age-related decline in the GH/IGF-1 axis. *Am. J. Physiol. Endocrinol. Metab.* **2007**, *293*, E1140−E1152.

(7) Marleau, S.; Harb, D.; Bujold, K.; Avallone, R.; Iken, K.; Wang, Y.; Demers, A.; Sirois, M. G.; Febbraio, M.; Silverstein, R. L.; Tremblay, A.; Ong, H. EP 80317, a ligand of the CD36 scavenger receptor, protects apolipoprotein E-deficient mice from developing atherosclerotic lesions. *Fed. Am. Soc. Exp. Biol. J.* **2005**, *19*, 1869−1871.

(8) Bodart, V.; Bouchard, J. F.; McNicoll, N.; Escher, E.; Carriere, P.; Ghigo, E.; Sejlitz, T.; Sirois, M. G. Identification and characterization of a new growth hormone-releasing peptide receptor in the heart. *Circ. Res.* **1999**, *85*, 796−802.

(9) Endemann, G.; Stanton, L. W.; Madden, K. S.; Bryant, C. M.; White, R. T.; Protter, A. A. CD36 is a receptor for oxidized low density lipoprotein. *J. Biol. Chem.* **1993**, *268*, 11811−11816.

(10) Febbraio, M.; Podrez, E. A.; Smith, J. D.; Hajjar, D. P.; Hazen, S. T.; Hoff, H. F.; Sharma, K.; Silverstein1, R. L Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. *J. Clin. Invest.* **2000**, *105*, 1049−1056.

(11) Demers, A.; McNicoll, N.; Febbraio, M.; Servant, M.; Marleau, S.; Silverstein, R.; Ong, H. Identification of the growth hormone-releasing peptide binding site in CD36: a photoaffinity cross-linking study. *Biochem. J.* **2004**, *382*, 417−424.

(12) Febbraio, M.; Silverstein, R. L. CD36: Implications in Cardiovascular Disease. *Int. J. Biochem. Cell Biol.* **2007**, *39* (11), 2012−2030.

(13) Jiménez, B.; Volpert, O. V.; Crawford, S. E.; Febbraio, M.; Silverstein, R. L.; Bouck, N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nature Med.* **2000**, *6*, 41−48.

(14) Sun, J.; Hopkins, B. D.; Tsujikawa, K.; Perruzi, C.; Adini, I.; Swerlick, R.; Bornstein, P.; Lawler, J.; Benjamin, L. E. Thrombospondin-1 modulates VEGF-A mediated Akt signaling and capillary survival in the developing retina. *Am. J. Physiol.* **2009**, *296*, H1344−H1351.

(15) Proulx, C.; Sabatino, D.; Hopewell, R.; Spiegel, J.; García Ramos, Y.; Lubell, W. D. Azapeptides and their therapeutic potential. *Future Med. Chem.* **2011**, *3* (9), 1139−1164.

(16) (a) Reynolds, C. H.; Hormann, R. E. Theoretical study of the structure and rotational flexibility of diacylhydrazines: implications for the structure of nonsteroidal ecdysone agonists and azapeptides. *J. Am. Chem. Soc.* **1996**, *118*, 9395−9401. (b) Lee, H.-J.; Song, J.-W.; Choi, Y.-S.; Park, H.-M.; Lee, K.-B. A theoretical study of conformational properties of *N*-methyl azapeptide derivatives. *J. Am. Chem. Soc.* **2002**, *124*, 11881−11893. (c) André, F.; Boussard, G.; Bayeul, D.; Didierjean, C.; Aubry, A.; Marraud, M.; Aza-peptides., I. I. X-Ray structures of aza-alanine and aza-asparagine-containing peptides. *J. Peptide Res.* **1997**, *49*, 556−562. (d) André, F.; Vicherat, A.; Boussard, G.; Aubry, A.; Marraud, M. Aza-peptides. III. Experimental structural analysis of aza-alanine and aza-asparagine-containing peptides. *J. Peptide Res.* **1997**, *50*, 372−381.

(17) Gassman, J. M.; Magrath, J. An active-site titrant for chymotrypsin, and evidence that azapeptide esters are less susceptible to nucleophilic attack than ordinary esters. *Bioorg. Med. Chem. Lett.* **1996**, *6* (15), 1771−1774.
(18) Boeglin, D.; Lubell, W. D. Aza-amino acid scanning of secondary structure suited for solid-phase peptide synthesis with Fmoc chemistry and aza-amino acids with heteroatomic side chains. *J. Comb. Chem.* **2005**, *7*, 864−878.
(19) Dutta, A. S.; Furr, B. J. A.; Giles, M. B.; Valcaccia, B. Synthesis and biological activity of highly active α-aza analogues of luliberin. *J. Med. Chem.* **1978**, *21* (10), 1018−1024.
(20) Patchett, A. A.; Harris, E.; Tristram, E. W.; Wyvratt, M. J.; Wu, M. T.; Taub, D.; Peterson, E. R.; Ikeler, T. J.; ten Broeke, J.; Payne, L. G.; Ondeyka, D. L.; Thorsett, E. D.; Greenlee, W. J.; Lohr, N. S.; Hoffsommer, R. D.; Joshua, H.; Ruyle, W. V.; Rothrock, J. W.; Aster, S. D.; Maycock, A. L.; Robinson, F. M.; Hirschmann, R.; Sweet, C. S.; Ulm, E. H.; Gross, D. M.; Vassil, T. C.; Stone, C. A. A new class of angiotensin-converting enzyme inhibitors. *Nature* **1980**, *288*, 280−283.
(21) Baggio, R.; Shi, Y.-Q.; Wu, Y.-Q.; Abeles, R. H. From Poor Substrates to Good Inhibitors: Design of Inhibitors for Serine and Thiol Proteases. *Biochemistry* **1996**, *35* (11), 3351−3353.
(22) Magrath, J.; Abeles, R. H. Cysteine Protease Inhibition by Azapeptide Esters. *J. Med. Chem.* **1992**, *35*, 4279−4283.
(23) Boeglin, D.; Hamdan, F. F.; Melendez, R. E.; Cluzeau, J.; Laperriere, A.; Héroux, M.; Bouvier, M.; Lubell, W. D. Calcitonin gene-related peptide analogues with aza and indolizidinone amino acid residues reveal conformational requirements for antagonist activity at the human calcitonin gene-related peptide 1 receptor. *J. Med. Chem.* **2007**, *50*, 1401−1408.
(24) Ekici, Ö. D.; Götz, M. G.; James, K. E.; Li, Z. Z.; Rukamp, B. J.; Asgian, J. L.; Caffrey, C. R.; Hansell, E.; Dvoràk, J.; McKerrow, J. H.; Potempa, J.; Travis, J.; Mikolajczyk, J.; Salvesen, G. S.; Powers, J. C. Aza-Peptide Michael Acceptors: A New Class of Inhibitors Specific for Caspases and Other Clan CD Cysteine Proteases. *J. Med. Chem.* **2004**, *47*, 1889−1892.
(25) Sabatino, D.; Proulx, C.; Klocek, S.; Bourguet, C. B.; Boeglin, D.; Ong, H.; Lubell, W. D. Exploring Side-Chain Diversity by Submonomer Solid-Phase Aza-Peptide Synthesis. *Org. Lett.* **2009**, *11*, 3650−3653.
(26) Sabatino, D.; Proulx, C.; Pohankova, P.; Ong, H.; Lubell, W. D. Structure−Activity Relationships of GHRP-6 Azapeptide Ligands of the CD36 Scavenger Receptor by Solid-Phase Submonomer Azapeptide Synthesis. *J. Am. Chem. Soc.* **2011**, *133*, 12493−12506.
(27) Kobayashi, S.; Fukuta, M.; Kontani, H.; Yanagita, S.; Kimura, I. A Quantitative Assay for Angiogenesis of Cultured Choroidal Tissues in Streptozotocin-Diabetic Wistar and Spontaneously Diabetic GK Rats. *Jpn. J. Pharmacol.* **1998**, *78*, 471−478.
(28) Melendez, R. E.; Lubell, W. D. Aza-Amino Acid Scan for Rapid Identification of Secondary Structure Based on the Application of *N*-Boc-Aza^1-Dipeptides in Peptide Synthesis. *J. Am. Chem. Soc.* **2004**, *126* (21), 6759−6764.
(29) Proulx, C.; Lubell, W. D. Solid-Phase Synthesis of Aza-Proline Analogs of GHRP-6. In *Peptides: Breaking Away. Proceedings of the 21st American Peptide Symposium*; Lebl, M., Ed.; Prompt Scientific Publishing: San Diego, CA, 2009; pp 56−57.
(30) Freeman, N. S.; Tal-Gan, Y.; Klein, S.; Levitzki, A.; Gilon, C. Microwave-Assisted Solid-Phase Aza-peptide Synthesis: Aza Scan of a PKB/Akt Inhibitor Using Aza-arginine and Aza-proline Precursors. *J. Org. Chem.* **2011**, *76*, 3078−3085.
(31) Lubell, W. D.; Blankenship, J. W.; Fridkin, G.; Kaul, R. Peptides. *Science of Synthesis 21.11, Chemistry of Amides*; Thieme: Stuttgart, 2005; pp 713−809.
(32) Boeglin, D.; Xiang, Z.; Sorenson, N. B.; Wood, M. S.; Haskell-Luevano, C.; Lubell, W. D. Aza-scanning of the potent melanocortin receptor agonist Ac-His-D-Phe-Arg-Trp-NH_2. *Chem. Biol. Drug Des.* **2006**, *67*, 275−283.
(33) *Nexus Biosystems*; http://www.irori.com/.
(34) Demange, L.; Boeglin, D.; Moulin, A.; Mousseaux, D.; Ryan, J.; Bergé, G.; Gagne, D.; Heitz, A.; Perrissoud, D.; Locatelli, V.; Torsello, T.; Galleyrand, J.-C.; Fehrentz, J.-A.; Martinez, J. Synthesis and Pharmacological in Vitro and in Vivo Evaluations of Novel Triazole Derivatives as Ligands of the Ghrelin Receptor. *J. Med. Chem.* **2007**, *50*, 1939−1957.
(35) Bodart, V.; Febbraio, M.; Demers, A.; McNicoll, N.; Pohankova, P.; Perreault, A.; Sejlitz, T.; Escher, E.; Silverstein, R. L.; Lamontagne, D.; Ong, H. CD36 mediates the cardiovascular action of Growth Hormone-Releasing Peptides in the heart. *Circ. Res.* **2002**, *90*, 844−849.
(36) (a) Didierjean, C.; Del Duca, V.; Benedetti, E.; Aubry, A.; Zouikri, M.; Marraud, M.; Boussard, G. X-ray structures of aza-proline-containing peptides. *J. Pept. Res.* **1997**, *50*, 451−457. (b) Zouikri, Vicherat, A.; Aubry, A.; Marraud, M.; Boussard, G. Azaproline as a β-turn-inducer residue opposed to proline. *J. Pept. Res.* **1998**, *52*, 19−26. (c) Lecoq, A.; Boussard, G.; Marraud, M.; Aubry, A. Crystal state conformation of three azapeptides containing the azaproline residue, a β-turn regulator. *Biopolymers* **1993**, *33*, 1051−1059. (d) Lecoq, A.; Boussard, G.; Marraud, M. The couple Pro/AzaPro: a means of β-turn formation control synthesis and conformation of two aza-pro-containing dipeptides. *Tetrahedron Lett.* **1992**, *33* (36), 5209−5212.
(37) Sun, J.; Hopkins, B. D.; Tsujikawa, K.; Perruzzi, C.; Adini, I.; Swerlick, R.; Bornstein, P.; Lawler, J.; Benjamin, L. E. Thrombospondin-1 modulates VEGF-A-mediated Akt signaling and capillary survival in the developing retina. *Am. J. Physiol. Heart Circ. Physiol.* **2009**, *296* (5), H1344−51.
(38) Bush, C. A.; Sarkar, S. K.; Kopple, K. D. Circular dichroism of β turns in peptides and proteins. *Biochemistry* **1978**, *17*, 4951−4954.
(39) Gibson, C.; Goodman, S. L.; Hahn, D.; Hölzemann, G.; Kessler, H. Novel solid-phase synthesis of azapeptides and azapeptoides via Fmoc-strategy and its application in the synthesis of RGD-mimetics. *J. Org. Chem.* **1999**, *64*, 7388−7394.
(40) Carpino, L. A.; Han, J. Y. The 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. *J. Org. Chem.* **1972**, *37*, 3404−3409.
(41) Zhang, W.-J.; Berglund, A.; Kao, J. L.-F.; Couty, J.-P.; Gershengorn, M. C.; Marshall, G. R. Impact of Azaproline on Amide Cis−Trans Isomerism: Conformational Analyses and NMR Studies of Model Peptides Including TRH Analogues. *J. Am. Chem. Soc.* **2003**, *125*, 1221−1235.
(42) Harigaya, S.; Schwartz, A. Rate of calcium binding and uptake in normal animal and failing human cardiac muscle: membrane vesicles (relaxing system) and mitochondria. *Circ. Res.* **1969**, *25*, 781−794.