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Discovery of potent, cyclic calcitonin gene-related peptide receptor antagonists‡

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Calcitonin gene-related peptide (CGRP), a potent dilator of cerebral and dural vasculature, is known to be elevated in plasma and cerebral spinal fluid during migraine attacks. Selective blockade of the CGRP receptor offers the promise of controlling migraine headache more effectively and without the side-effects associated with the use of triptans. Our efforts to develop a novel, peptide-based CGRP antagonist focused on the C-terminal portion of the peptide which is known to bind the receptor but lack agonist properties. Extensive SAR studies of the C-terminal CGRP (27–37) region identified a novel cyclic structure: Bz-Val-Tyr-cyclo[Cys-Thr-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH_2 (23) with a kb value of 0.126 nM against the cloned human CGRP receptor. Additional SAR studies directed at enhancement of potency and improvement of physicochemical properties yielded a series of analogs with kb values in the 0.05–0.10 nM range. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: CGRP; migraine; peptide antagonists; solubility

Introduction

Migraine is a common neurovascular disorder characterized by episodes of severe headache, nausea, and increased sensitivity to light and sound lasting from 4 to 72 h [1,2]. Conventional therapeutic agents include disease nonspecific remedies such as aspirin, acetaminophen, and nonsteroidal anti-inflammatory drugs, which are sometimes used in combination with ergotamine or caffeine. The $5HT_{1B/1D}$ selective agonist class of 'triptans' such as sumatriptan and rizatriptan revolutionized migraine therapy and continue to offer an important therapeutic option for migraine sufferers [3]. The triptans, however, fail to provide pain relief in more than 50% of patients and are in addition contraindicated in individuals with cardiovascular disease [4]. This deficiency has stimulated extensive investigation of alternative mechanism-based approaches for both prevention and treatment of migraine headache [5]. One of the most promising is based on the selective blockade of calcitonin gene-related peptide (CGRP) interaction with its receptor. CGRP is a neuropeptide which is expressed in both the central and peripheral nervous systems and is thought to be a likely causative factor in migraine pathogenesis [6,7]. This was demonstrated in a double-blind crossover study of migraine sufferers who experienced attacks following intravenous administration of human CGRP (hCGRP) [8]. Validation of antagonism came from a clinical study of BIBN 4096, a potent non-peptide CGRP antagonist which delivered rapid symptomatic relief with a high overall response rate following intravenous administration to migraineurs [9]. Despite these favorable clinical results, however, the therapeutic utility of BIBN 4096 is limited by intravenous administration. Structurally, α-CGRP (**1**) is a 37 amino acid polypeptide with a disulfide bridge spanning residues Cys^2 and Cys^7 [10]. Structure–activity relationship studies have determined that the N-terminal, disulfide bond-containing region is primarily involved in receptor activation, while the central and C-terminal regions are thought to be responsible for receptor binding. Deletion of the N-terminal heptapeptide from the native CGRP sequence yields the competitive antagonist CGRP 8–37 reported by Chiba [11,12] as well as more potent analogs disclosed recently by Miranda [13]. Further truncations have produced fragments with substantially diminished receptor affinity [14]. Our efforts to develop a novel CGRP antagonist focused on the C-terminal region encompassing residues 27–37, which had previously been shown to be the minimal fragment necessary for receptor binding [15,16]. High *in vitro* potency was a primary goal for this series, driven partly by the need to achieve high selectivity in order to minimize off-target activity and facilitate alternatives to administration by injection. The relatively low bioavailability associated with delivery methods such as intranasal inhalation elevates the need for high potency to achieve an acceptable commercial cost in peptide manufacture.

Methods

Peptide Synthesis

All peptide sequences were assembled by automated α-Fmoc chemistry starting from 4-(2′,4′-dimethoxyphenyl-Fmoc-

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aminomethyl)-phenoxy 'Rink' resin on an ABI 433 Peptide Synthesizer (PE Applied Biosystems Inc., Foster City, CA, USA) using the manufacturer's standard FastMoc HBTU/DIEA protocols. The *N*-α-Fmoc-protected amino acids utilized the following protecting group scheme: Arg(Pbf), Asn(Trt), Asp(OtBu), Cys(Trt), Agp(Boc)$_2$ (Agp: α-amino-3-guanidino-propionic acid), Dap(Boc) (Dap: 1,3 diamino-propionic acid), His(Trt), Hyp(tBu) (Hyp: hydroxy proline), Lys(Boc), Orn(Boc), Ser(tBu), Thr(tBu), Tyr(tBu). Other building blocks, including Fmoc-Cit and Fmoc-3Pal (3Pal: 3-pyridyl-alanine), were used without side chain protection. Following peptide chain assembly, the N-terminal Fmoc group was removed using 20% piperidine in DMF. N-terminal modification was carried out using five equivalents of either the appropriate anhydride or activated carboxylic acid in DMF or *N*-methyl-2-pyrrolidinone for 1 h at room temperature. The linear peptides were simultaneously deprotected and cleaved from the resin using a scavenger cocktail of TFA/H_2O/triisopropylsilane (TIS)/1,2-ethanedithiol (EDT) (95/2/1/2, v/v/v/v) for 2 h at room temperature. The solvents were removed under reduced pressure and the crude peptides precipitated and washed with cold diethyl ether three times to remove scavengers, then dried under vacuum. The oxidation of the free cysteine sulfhydryl groups was carried out in 0.2 M ammonium acetate buffer containing 20% DMSO overnight followed by purification using preparative HPLC techniques. Final characterization was performed using analytical HPLC and mass spectral analysis with the data summarized in Table 1.

In vitro activity was assessed by measurement of cyclic adenosine monophosphate (cAMP) using SK-N-MC cells, a human neuroepithelioma cell line endogenously expressing the hCGRP receptor 1 purchased from Receptor Biology (Beltsville, MD, USA). SK-N-MC cells were grown in minimal essential medium(Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin at 37 °C incubator with 95% humidity and 5% CO_2. For cAMP measurement, SK-N-MC cells were dissociated from the flask with enzyme-free cell dissociation solution (Specialty Media, S-014-B, Billerica, MA, USA), then suspended in the cAMP assay stimulation buffer (100 ml of Hank's balanced salt solution supplemented with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.1% BSA, 100 µM ascorbic acid + 200 ml of Dulbecco's phosphate buffered saline + 300 µl of 500 mM 3-isobutyl-1-methylxanthine). The reaction was performed in low volume (0.5 ml) black polystyrene 96 well plates (Costar, Lowell, MA, USA). Each well contained approximately 15 000 SK-N-MC cells and 2 nM hCGRP for the hCGRP receptor cAMP assay. The reaction was carried out in the presence of various concentrations of CGRP receptor antagonists. One hour after incubation at room temperature, cAMP levels were measured using an homogeneous time-resolved fluorescence cAMP assay kit (Dynamic 2 Bulk, Cisbio, Bedford, MA, USA) according to the manufacturer's instructions. EC50 or IC50 values were calculated by fitting the competition curves with Graphpad Prism 4.01. (GraphPad Software, Inc., La Jolla, CA 92037 USA) kb values were calculated by using the Cheng–Prusoff equation:

$$kb = IC50/(1 + ([agonist]/agonist\ EC50))$$

and summarized in Table 2.

Table 1. Characterization of new calcitonin gene-related peptide antagonist peptides

No.	Theoretical MW (Da)	Observed MW (Da)	HPLC purity (%)	HPLC[a] RT (min)	Saline solution (mg/ml)[b]
2	1165.35	1165.40	96.75	20.61	>50.00
3	1195.38	1195.40	97.61	30.82	<0.10
4	1211.39	1211.76	96.43	30.58	n/a
5	1261.45	1261.79	99.62	34.23	n/a
6	1273.47	1273.79	99.9	33.83	n/a
7	1259.48	1259.82	99.79	32.49	n/a
8	1275.44	1275.78	99.77	30.43	n/a
9	1317.52	1317.82	98.7	31.81	n/a
10	1277.45	1277.78	98.32	30.65	n/a
11	1227.39	1227.8	99.16	25.77	n/a
12	1303.49	1303.83	99.66	31.59	n/a
13	1277.39	1227.79	97.58	26.43	n/a
14	1089.27	1089.58	91.61	37.98	n/a
15	1089.27	1089.58	99.038	40.2	n/a
16	1089.27	1089.33	99.02	37.87	n/a
17	1089.27	1089.69	96.93	37.95	n/a
18	1063.23	1063.63	98.71	36.27	n/a
19	1013.17	1013.64	99.81	29.73	n/a
20	1344.59	1344.67	97.90	36.52	n/a
21	1394.65	1394.76	95.34	38.70	n/a
22	1247.47	1246.91	94.93	34.55	n/a
23	1351.58	1351.71	97.36	41.73	0.02
24	1332.57	1332.76	94.76	33.13	4.67
25	1380.62	1380.77	98.91	38.20	n/a
26	1378.65	1378.78	94.85	39.43	0.17
27	1406.66	1405.96	90.01	39.82	0.05
28	1047.64	1406.75	96.72	40.10	0.03
29	1364.62	1364.10	91.99	39.43	0.04
30	1336.57	1336.45	95.73	39.88	0.05
31	1337.55	1337.94	98.55	29.52	4.57
32	1337.55	1338.06	97.6	31.07	6.70
33	1337.55	1337.19	98.7	31.12	10.00
34	1275.48	1275.30	97.08	26.72	15.40
35	1355.54	1355.19	98.80	32.20	4.30
36	1397.58	1397.47	98.58	32.10	1.00

MW, molecular weight.
[a] HPLC conditions: mobile phase: buffer A, water with 0.1% TFA; buffer B, acetonitrile with 0.1% TFA. Flow rate: 1.0 ml/min; gradient: 10% B 5 min, 10–55% B 45 min, 90% B 5 min, 10% B 5 min. Column: Phenomenex, Jupiter C18 (0.46 × 25 cm), 5 µm, 300 Å.
[b] Solubility measurement: weighted out 1.0 mg of peptide material and dissolved in 0.5 ml of saline (0.9% sodium chloride), then removed any non-dissolved material by centrifugation. Concentration was calculated based on the measurement of the UV absorption (extinction coefficients: 2200 M^{-1} cm^{-1} for 3-pyridyl-alanine).

Results

Our lead optimization strategy focused on the C-terminal CGRP 27–37 fragment (**2**) previously explored by Beck-Sickinger and coworkers who reported several potent antagonists including Asp^{31}, Pro^{34}, Phe^{35}-CGRP 27–37 (**3**), and Hyp^{29}, Asp^{31}, Pro^{34}, Phe^{35} CGRP 27–37 (**4**) [15–17]. The discovery of Tyr^{0}CGRP 28–37 by Chakder and Rattan [18,19] resulting from the substitution of Tyr for Phe at position 27, prompted us to conduct a Tyr scan of peptide analog **4** in order to optimize the position of this residue (peptides **5–13**). *In vitro* evaluation of this series

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Table 2. Antagonistic data of CGRP peptides in cAMP assay

No.	Structure	hCGRP1 cAMP Antag kb(nM)
	H-Ala-cyclo[Cys-Asp-Thr-Ala-Thr-Cys]-Val-Thr-His-Arg-Leu-Ala-Gly	
1	Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2	n/a
2	Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2	>100
3	Phe-Val-Pro-Thr-Asp-Val-Gly-Pro-Phe-Ala-Phe-NH_2	1.72 ± 1.19
4	Phe-Val-Hyp-Thr-Asp-Val-Gly-Pro-Phe-Ala-Phe-NH2	1.12 ± 1.51
5	Phe-Val-Tyr-Thr-Asp-Val-Gly-Pro-Phe-Ala-Phe-NH2	13.3 ± 13.7
6	Phe-Val-Hyp-Tyr-Asp-Val-Gly-Pro-Phe-Ala-Phe-NH2	>100
7	Phe-Val-Hyp-Thr-Tyr-Val-Gly-Pro-Phe-Ala-Phe-NH2	1.73 ± 0.49
8	Phe-Val-Hyp-Thr-Asp-Tyr-Gly-Pro-Phe-Ala-Phe-NH2	>100
9	Phe-Val-Hyp-Thr-Asp-Val-Tyr-Pro-Phe-Ala-Phe-NH2	>100
10	Phe-Val-Hyp-Thr-Asp-Val-Gly-Tyr-Phe-Ala-Phe-NH2	>100
11	Phe-Val-Hyp-Thr-Asp-Val-Gly-Pro-Tyr-Ala-Phe-NH2	7.09 ± 0.00
12	Phe-Val-Hyp-Thr-Asp-Val-Gly-Pro-Phe-Tyr-Phe-NH2	>100
13	Phe-Val-Hyp-Thr-Asp-Val-Gly-Pro-Phe-Ala-Tyr-NH2	3.20 ± 0.35
14	Bz-Thr-Asp-Val-cyclo[Cys-Gly-Pro-Phe-Cys]-Phe-NH2	>100
15	Bz-Thr-Asp-cyclo[Cys-Val-Gly-Pro-Phe-Cys]-Phe-NH2	>100
16	Bz-Thr-cyclo[Cys-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	>100
17	Bz-cyclo[Cys-Thr-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	9.14 ± 6.68
18	Bz-cyclo[Cys-Thr-Asp-Val-Gly-Cys]-Phe-Ala-Phe-NH2	>100
19	Bz-cyclo[Cys-Thr-Asp-Val-Gly-Pro-Cys]-Ala-Phe-NH2	>100
20	Phe-Val-Hyp-cyclo[Cys-Thr-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	59.4 ± 16.4
21	Phe-Val-Tyr-cyclo[Cys-Thr-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	4.73 ± 3.29
22	Val-Tyr-cyclo[Cys-Thr-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	1.72 ± 0.00
23	Bz-Val-Tyr-cyclo[Cys-Thr-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	0.126 ± 0.042
24	Bz-Val-Tyr-cyclo[Cys-Thr-Asp-Val-Gly-Pro-Lys-Cys]-Phe-NH2	0.761 ± 0.388
25	Bz-Val-Tyr-cyclo[Cys-Thr-Asp-Lys-Gly-Pro-Phe-Cys]-Phe-NH2	51.0 ± 5.6
26	Bz-Val-Tyr-cyclo[Cys-Lys-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	0.184 ± 0.093
27	Bz-Val-Tyr-cyclo[Cys-Arg-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	0.122 ± 0.079
28	Bz-Val-Tyr-cyclo[Cys-Cit-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	0.277 ± 0.202
29	Bz-Val-Tyr-cyclo[Cys-Orn-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	0.305 ± 0.068
30	Bz-Val-Tyr-cyclo[Cys-Dap-Asp-Val-Gly-Pro-Phe-Cys]-Phe-NH2	0.106 ± 0.068
31	Bz-Val-Tyr-cyclo[Cys-Dap-Asp-Val-Gly-Pro-3Pal-Cys]-Phe-NH2	0.351 ± 0.042
32	Bz-Val-Tyr-cyclo[Cys-Dap-Asp-Val-Gly-Pro-Phe-Cys]-3Pal-NH2	0.290 ± 0.230
33	Bz-(D-Val)-Tyr-cyclo[Cys-Dap-Asp-Val-Gly-Pro-Phe-Cys]-3Pal-NH2	0.0436 ± 0.0149
34	Ac-(D-Val)-Tyr-cyclo[Cys-Dap-Asp-Val-Gly-Pro-Phe-Cys]-3Pal-NH2	0.103 ± 0.042
35	(4-F-Bz)-(D-Val)-Tyr-cyclo[Cys-Dap-Asp-Val-Gly-Pro-Phe-Cys]-3Pal-NH2	0.0491 ± 0.0032
36	(4-F-Bz)-(D-Val)-Tyr-cyclo[Cys-Agp-Asp-Val-Gly-Pro-Phe-Cys]-3Pal-NH2	0.0286 ± 0.0230

CGRP, calcitonin gene-related peptide; hCGRP, human CGRP; cAMP, cyclic adenosine monophosphate; 3Pal, 3-pyridyl-alanine.

revealed that Tyr could be tolerated at positions 27, 29, 35, and 37 (peptides **5, 7, 11, 13**) with only modest attenuation of activity. We next applied disulfide constraint in order to stabilize the β-turn in this region as noted by Carpenter [20], which produced mainly inactive peptides (**14–19**), except analog **17** with a respectable potency of 9.14 nM. We attempted to boost potency further by modifying the N-terminus of cyclic analog **17** with the N-terminal tripeptide from the linear series. The addition of the Phe-Val-Hyp (Hyp: 4-hydroxy proline) tripeptide resulted in disappointing potency (peptide **20,** kb = 59.4 nM), however the substitution of Tyr for Hyp (shown to be tolerated in the linear series) resulted in an improvement to 4.73 nM (**21**).

Insertion of the disulfide bridge necessitated a renumbering from the linear CGRP convention to the following: Phe^{1}-Val^{2}-Tyr^{3}-Cys^{4}-Thr^{5}-Asp^{6}-Val^{7}-Gly^{8}-Pro^{9}-Phe^{10}-Cys^{11}-Phe^{12}-NH_2 (**21**). This numbering applies to all cyclic peptides in the series discussed hereinafter. Deletion of Phe^{1} gave a still better kb value of 1.72 nM (**22**), while *N*-benzoylation of the free N-terminus resulted in the highly potent analog **23** with a kb value of 0.126 nM. While gratifying in terms of receptor affinity, we noted that this peptide exhibited unacceptably low aqueous solubility (0.02 mg/ml in saline) which complicated plans for *in vivo* studies.

Attempts to enhance solubility of this peptide included substitution of Lys at several positions which revealed that while this residue could not be tolerated at position 10 (analog **24**), it could be introduced at position 7 and 5 without significant loss of potency (analog **25** and **26**). Analog **26** exhibited a potency of 0.184 nM as well as a slightly improved solubility of 0.17 mg/ml. At this point, we sought to optimize the side chain at position 5 in terms of steric parameters and charge. The Arg (**27**), Cit (**28**), Orn (**29**), and Dap (**30**) analogs demonstrated excellent potency but uniformly low solubility. Next, we turned to the replacement of one or both Phe residues with a functionally equivalent but

more polar side chain in order to enhance solubility. Substitution of 3Pal for Phe[10] (analog **31)** resulted in a kb value of 0.351 nM, but a much improved solubility of 4.57 mg/ml. Substitution of 3Pal for the exocyclic Phe[12] gave a similar potency of 0.290 nM with a solubility of 6.70 mg/ml (analog **32**). In an attempt to restore the higher potency of the earlier, less soluble analogs, we carried out a series of iterative modification to the N-terminus. Inversion of configuration at Val[2] produced analog **33** with a surprising boost in potency to 0.0436 nM, along with further improvement in solubility of 10.0 mg/ml. The choice of the N-terminal capping group was noted to be an important factor in achieving a balance between potency and solubility. Additional improvement in solubility to 15.4 mg/ml was achieved with replacement of the hydrophobic *N*-benzoyl group with an *N*-acetyl group (analog **34**, kb = 0.103 nM). Conversely, the 4-fluorobenzoyl group (analog **35**, kb = 0.0491 nM) gave better potency at the cost of lower solubility of 4.30 mg/ml. The best absolute potency of the series was obtained by incorporating the strongly basic Agp (α-Amino-3-guanidino-propionic acid) group in place of Dap at position 5 which resulted in remarkable potency of 0.0286 nM, but diminished solubility of 1.0 mg/ml (analog **36)**.

Discussion

Our ligand optimization strategy which combined positional scanning and use of disulfide-induced constraint yielded a number of potent cyclic peptides with subnanomolar kb values at the hCGRP receptor. An unexpected challenge that surfaced in this peptide series pertained to the generally low solubility observed among linear and cyclic analogs. We attribute this phenomenon to the substitution of $Ser^{34}Lys^{35}$ with $Pro^{34}Phe^{35}$, a modification which significantly enhanced potency but caused a dramatic drop in solubility when comparing analogs **2** and **3**. Literature precedent suggests that the presence of proline in a β-turn conformation can increase the propensity for aggregation. Studies by Morimoto *et al*. noted extensive aggregation when proline was introduced into the β-turn region of amyloid peptides [21]. Since Pro^{34} was a prerequisite for high potency in our series, we investigated alternative methods of restoring solubility. Modification of the aromatic side chains at positions 10 and 12 with a 3-pyridyl group offered a practical solution to this problem. These substitutions improved the aqueous solubility from less than 0.1 mg/ml to a range of 5–15 mg/ml while maintaining potencies in the 0.050–0.10 nM range.

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