Brief Articles

Combinatorial Lead Optimization of a Neuropeptide FF Antagonist

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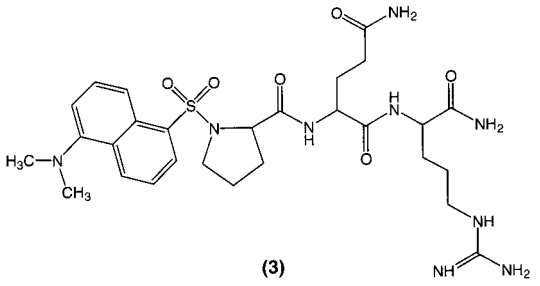
The tripeptide Pro-Gln-Arg-NH_2, derivatized at the secondary amino group of the proline residue with 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl-PQR-NH_2), antagonizes the central anti-opioid action of neuropeptide FF in animals after systemic administration and, therefore, is a therapeutic lead to treat opiate withdrawal. For a combinatorial optimization to improve potency, libraries focused on the possible replacement of the proline and glutamine residues of this lead compound were obtained by a solid-phase split-and-mix method using coded amino acids (excluding cysteine) as building blocks. After screening for competitive binding against a radioiodinated neuropeptide FF analogue, 5-(dimethylamino)-1-naphthalenesulfonyl-Gly-Ser-Arg-NH_2 (dansyl-GSR-NH_2) has emerged as one of the compounds in the library with high affinity to the NPFF receptor and even with a moderate increase compared to dansyl-PQR-NH_2 in its predicted ability to penetrate the central nervous system.

Introduction

Opiate tolerance, dependence, and abuse represent major medical and social problems. Neuropeptide FF (**1**, Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2 or F8F-amide), together with the related mammalian neuropeptides NPAF and the N-terminally extended (Ser-Gln-Ala-) **1**, has been identified as a high-affinity endogenous ligand for a novel neuropeptide Y-like human orphan G-protein coupled receptor HLWAR77.[1] Receptors activated by **1** have also been isolated from human and rat central nervous system (CNS) tissue recently.[2] Neuropeptide FF is an anti-opioid and has been implicated in pain modulation, morphine tolerance, and morphine abstinence.[3] Intracerebroventricular (icv) pretreatment with immunoglobulin G (IgG) from antiserum of **1** restored the analgesic effect of icv morphine in morphine-tolerant rats[4] and potentiated the anti-opioid effect of **1**.[5] Centrally administered neuropeptide FF also has been known to precipitate quasi-morphine abstinence syndrome (QMAS) in opiate-naive animals. Therefore, antagonists of **1** (besides their importance as pharmacological agents helpful in defining the physiological/pharmacological role of the endogenous neuropeptide) may allow for the management of withdrawal symptoms that adversely affect the treatment of opiate abuse.

Desaminotyrosyl-Phe-Leu-Phe-Gln-Pro-Gln-Arg-NH_2 (**2**), the first putative antagonist of **1** discovered, has indeed attenuated abstinence-like signs induced by **1** in opiate-naive rats and upon naloxone challenge in morphine-dependent animals after icv administration.[6] To date, **2** showed the highest potency upon icv administration in blunting behavioral effects precipitated by **1**. However, this peptide analogue did not show any CNS bioavailability after systemic administration and, thus, could not be considered a potential therapeutic lead compound.[7]

Derivatization with 5-(dimethylamino)-1-naphthalenesulfonyl (dansyl) at the secondary NH group of the N-terminal proline residue of the tripeptide Pro-Gln-Arg-NH_2, obtained from the sequence (residues 5−7) of **1**, has afforded an antagonist with significant lipid solubility to cross the blood−brain barrier (BBB).[8,9] Dansyl-Pro-Gln-Arg-NH_2 (**3**) dose-dependently antagonized QMAS induced by **1**, and it also blunted naloxone-precipitated withdrawal symptoms in morphine-dependent rats when administered subcutaneously. In the meantime, **3** was also expected to improve resistance compared to **2** against proteolytic enzymes.[8] Considering competitive binding against a radioiodinated analogue of **1** in a CNS membrane preparation, the micromolar-range inhibition constant (K_i) of **3** has remained the sole "benchmark" measuring antagonism of the endogenous octapeptide at the receptor level. Although a recent study has identified Pro-Phe-Arg(Tic)-NH_2 (Tic = L-1,2,3,4-tetrahydroisoquinoline-3-carboxyl) as a puta-



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tive antagonist of **1** that attenuated naloxone-precipitated withdrawal symptoms in morphine-dependent rats after systemic administration,[10] the potency (or efficacy) of this compound was less than that of **3** in the pharmacological tests employed.

Herein, we report our search for novel antagonists of **1** that show improved potency while retaining the ability to cross the BBB. Our studies included the validation of the receptor-based approach (competitive binding against a radiolabeled analogue of **1**) chosen and finally focused on a combinatorial optimization based on the CNS-bioavailable antagonist **3** to obtain a new therapeutic lead for potential management of opiate abstinence.

Results and Discussion

The affinity of the lead compound **3** to the receptor labeled by the radioiodinated analogue of **1** (**4**, [^{125}I]-YLFQPQRF-NH_2 or [^{125}I]Y8F-amide) was confirmed by using the assay adapted, and the measured K_i of 13.6 ± 2.5 μM was in good agreement with the value reported before.[7] On the other hand, K_i = 840 ± 180 nM was obtained for **2** that showed the highest efficacy upon icv administration in blunting opiate abstinence in animals. These observations demonstrated a correlation between the binding affinity to the receptors labeled by **4** and the desired pharmacological effect as an antagonist of **1**.

In the search for an antagonist of **1** with improved affinity to the receptor under inquiry, we considered the structure−binding affinity study of the endogenous neuropeptide and its synthetic analogues.[11] On the basis of the results of this study, we did not manipulate the arginine (R) residue of **3**, because its replacement by any other amino acid residue in the N-terminal region of **1** had been shown to yield a significant loss of affinity. A preliminary study was also done to investigate the effect of substituting the dansyl moiety of **3** with a group that would allow for the application of chemical-enzymatic CNS-targeting strategies.[12] However, our synthesis and competitive binding experiments (against **4**) of compounds involving the replacement of dansyl with a 3-(4-hydroxyphenyl)propionyl group (desaminotyrosyl) that permits the attachment of a dihydropyridine ↔ pyridinium redox targeting moiety removable by enzymatic hydrolysis or with the hydrolytically nonremovable redox residue 3-(3-carboxamidopyrid-1-yl)propionyl yielded inactive analogues. Therefore, we did not modify the dansyl group of **3** in this study. These considerations have simplified our task to a search for improved antagonists of **1** by manipulating the remaining two variable residues (proline and glutamine). An approach involving mixture-based synthetic combinatorial libraries and positional scanning[13] appeared to be rapid and economical for this purpose.

Dansyl-OXR-NH_2 and dansyl-XOR-NH_2 combinatorial libraries were prepared by solid-phase synthesis using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and the split-and-mix method.[14] In the sublibraries, position O defined each of the 19 coded amino acid residues (excluding cysteine), whereas X represented an equimolar mixture of these (19) residues. The peptide mixtures were cleaved from the resin, precipitated and washed with diethyl ether, and freeze-dried from water. Electrospray ionization (ESI) mass spectrometric characterization and correlation with the simulated mass distribution confirmed the presence and practically equimolar concentration of the expected compounds in the mixtures.

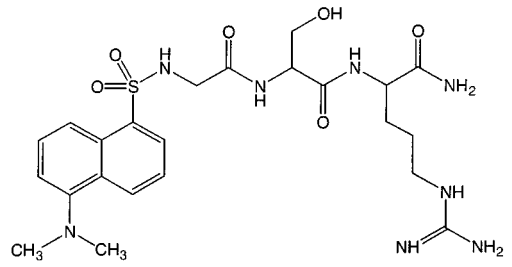
Table 1. Displacement of [^{125}I]YLFQPQRF-NH_2 (**4**) by the Combinatorial Mixtures at 50 μM Total Binding Concentration in Rat Spinal Cord Membrane Preparation[a]

mixture	displacement of **4** (%, ±SE)	mixture	displacement of **4** (%, ±SE)
dansyl-GXR-NH_2	97.7 ± 4.7	dansyl-XSR-NH_2	91.6 ± 4.3
dansyl-KXR-NH_2	97.3 ± 2.6	dansyl-XQR-NH_2	86.3 ± 6.9
dansyl-QXR-NH_2	94.8 ± 5.2	dansyl-XWR-NH_2	82.7 ± 2.4
dansyl-HXR-NH_2	91.1 ± 2.3	dansyl-XIR-NH_2	80.9 ± 3.9
dansyl-EXR-NH_2	90.9 ± 7.3	dansyl-XTR-NH_2	80.3 ± 4.5
dansyl-SXR-NH_2	90.8 ± 0.9	dansyl-XGR-NH_2	80.1 ± 3.2
dansyl-NXR-NH_2	87.9 ± 2.2	dansyl-XPR-NH_2	78.5 ± 0.7
dansyl-AXR-NH_2	87.5 ± 6.2	dansyl-XNR-NH_2	77.0 ± 4.5
dansyl-FXR-NH_2	85.2 ± 7.8	dansyl-XER-NH_2	76.1 ± 1.2
dansyl-WXR-NH_2	84.7 ± 2.1	dansyl-XMR-NH_2	76.1 ± 1.3
dansyl-YXR-NH_2	83.4 ± 4.4	dansyl-XYR-NH_2	75.2 ± 1.6
dansyl-RXR-NH_2	83.2 ± 4.9	dansyl-XFR-NH_2	75.1 ± 1.4
dansyl-VXR-NH_2	74.4 ± 2.9	dansyl-XRR-NH_2	75.1 ± 1.2
dansyl-DXR-NH_2	73.4 ± 4.5	dansyl-XKR-NH_2	74.3 ± 0.6
dansyl-TXR-NH_2	71.1 ± 5.9	dansyl-XVR-NH_2	72.5 ± 5.3
dansyl-IXR-NH_2	69.1 ± 4.2	dansyl-XHR-NH_2	71.8 ± 2.5
dansyl-LXR-NH_2	52.6 ± 9.5	dansyl-XLR-NH_2	69.8 ± 0.5
dansyl-MXR-NH_2	43.2 ± 7.8	dansyl-XDR-NH_2	62.6 ± 3.1
dansyl-PXR-NH_2	40.0 ± 6.4	dansyl-XAR-NH_2	39.4 ± 4.7
dansyl-XXR-NH_2	*75.6 ± 6.4*		

[a] The one-letter abbreviations are used to denote coded amino acid residues (19, cysteine excluded) and X indicates the mixture thereof. Values are the average of 3 measurements.

Screening results of the combinatorial mixtures in the radioligand-binding assay are given in Table 1. Mixtures containing glycine (G), lysine (L), and glutamine (Q) showed the highest increase in the percentage displacement of **4** upon screening for residues that could replace proline in **3**. In fact, the dansyl-PXR-NH_2 sublibrary showed the weakest binding to the receptor labeled by **4** among the sublibraries tested. Because the goal of our combinatorial lead optimization has been to improve affinity while retaining CNS bioavailability, we ignored the potential replacement of proline in **3** with lysine, which would add an additional basic moiety expected to significantly reduce the ability of the molecule to cross the BBB. Upon screening for residues to replace the glutamine of **3**, serine (S) was the only building block in this position that increased the displacement of **4** in the radioligand-binding assay. Therefore, dansyl-Gly-Ser-Arg-NH_2, dansyl-Gly-Gln-Arg-NH_2, dansyl-Gln-Ser-Arg-NH_2, and dansyl-Gln-Gln-Arg-NH_2 were chosen based on their affinity to the cognate receptor for further consideration.

A simple, rule-based reasoning analogous to that of Lipinski's "rule of 5"[15] was employed to select an improved lead compound as an antagonist of **1** for further studies. Among the compounds considered, dansyl-Gly-Ser-Arg-NH_2 (**5**) had the smallest number of heteroatoms to serve as H-bond donors or acceptors and had the lowest molecular weight. While the *n*-octanol/water partitioning of **5** also was essentially identical to that of **3** upon considering the predicted log *P* values,[16] the other compounds emerged from the receptor-based screening showed a decrease in lipophilicity compared to **3**. Therefore, **5** was synthesized (by the Fmoc strategy identical to that of the preparation of the mixtures) and purified as an individual analogue for further characterization. The purity of the target



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compound was confirmed by combustion analysis (data were within ±0.4% of calculated values) and ESI mass spectrometry (no impurities exceeding 1% based on relative ion abundance). The measured K_i value of **5** in the radioligand-binding assay was 1.4 ± 0.5 μM that was equivalent to an approximately 10-fold increase in binding affinity to the intended receptor compared to **3** and approached the K_i of the most active but not CNS-bioavailable antagonist (**2**) known to date. We have also characterized the compounds by immobilized artificial membrane (IAM) chromatography, a method that furnishes capacity factors whose logarithm generally correlates with in vivo absorption[17] including penetration across the BBB.[18] On the basis of IAM retention, **5** (k_{IAM}' = 8.0, log k_{IAM}' = 0.90) is, indeed, expected to show better CNS bioavailability than **3** (k_{IAM}' = 5.9, log k_{IAM}' = 0.77).

In conclusion, **5** has emerged from our combinatorial lead optimization as one of the compounds with high affinity to the receptor of **1** and without an apparent decrease in its ability compared to **3** to cross the BBB. On the basis of physicochemical properties, structural features, and IAM chromatography, this improved agent even afforded a moderate increase compared to **3** in its predicted CNS bioavailability.

Experimental Section

Instruments and Materials. All chemicals used were of reagent or peptide synthesis grade. Fmoc-protected amino acids were obtained from Bachem Bioscience, Inc. (Philadelphia, PA). Solvents and other chemicals were purchased from Fisher Scientific, Inc. (Pittsburgh, PA). Fmoc-Arg(Pbf)-Rink amide-methoxybenzylhydrazine (MBHA) resin (0.46 mequiv/g, 100−200 mesh) was purchased from AnaSpec, Inc. (San Jose, CA). The combinatorial libraries were prepared on a SynPep (Dublin, CA) multiple peptide synthesizer. A Synthor 2000, Peptide International (Louisville, KY) instrument was used for the preparation of the individual peptides by Fmoc chemistry. Immobilized artificial membrane (IAM) chromatography was performed on a system that included a model SP 8810 precision isocratic pump, an SP 8880 autosampler with a 20-μL injection loop, an SP 8450 variable wavelength UV/VIS detector operated at 254 nm, and SP 4290 computing integrator (all from ThermoSeparation/SpectraPhysics, Fremont, CA). RP-HPLC purification was done on a system composed of an SP 200 binary gradient pump (ThermoSeparation), a Rheodyne (Cotati, CA) model 7125 injector valve equipped with a 5-mL sample loop, and an SP 100 UV/VIS detector (ThermoSeparation) operated at 210 nm. Electrospray ionization (ESI) and tandem mass spectra were obtained on a quadrupole ion trap instrument (LCQ, Finnigan MAT, San Jose, CA). NMR spectra were recorded on Bruker AVANCE instruments (Bremen, Germany). Resonance frequencies were 500 and 600 MHz for ^{1}H and 127 MHz for ^{13}C. The samples were dissolved in H_2O/D_2O (8/2, v/v). Atlantic Microlab, Inc. (Norcross, GA) performed the combustion analyses.

Synthesis. The combinatorial libraries were prepared on Fmoc-Arg(Pbf)-Rink amide-MBHA resin by using a split-and-mix method. The peptide mixtures were cleaved from the resin using TFA:dithiothreitol:diisopropylsilane:water mixture (88:5:2:5, v/v) and precipitated with ether. The peptides were further washed with ether several times, then freeze-dried. Individual peptides (**2**, **3** and **5**) were synthesized similarly and purified by semipreprarative RP-HPLC. A 25-cm × 10-mm i.d. Econoprep octadecylsilica (C18) column (Phenomenex, Torrance, CA) was used at a flow rate of 5.0 mL/min, and the solvent gradient (from 5 to 45% organic solvent in 20 min) was mixed from 0.1% (v/v) trifluoroacetic acid (TFA) in water as an aqueous component and 0.1% (v/v) TFA in ethanol/1-propanol (5/2, v/v) as an organic component.

Dansyl-Gly-Ser-Arg-NH$_2$ (5): ESI-MS *m/z* 551.2 $[M + H]^+$, 276.1 $[M + 2H]^{2+}$; MS/MS (product ions of *m/z* 551.2) *m/z* 534.1, 517.1, 499.1, 378.1, 350.1, 332.1, 317.1, 298.1, 268.1, 234.0; ^{1}H NMR (H_2O/D_2O) δ 8.75 (1H, d, J = 8.7 Hz, dansyl 2-CH), 8.50 (1H, d, J = 8.7 Hz, dansyl 6-CH), 8.41 (1H, d, J = 7.4 Hz, dansyl 8-CH), 8.33 (2H × 0.8, d, J = 6.9 Hz, Arg-NH/Ser-NH), 8.04 (1H, d, J = 7.7 Hz, dansyl 4-CH), 7.92 (1H, t, J = 8.3 Hz, dansyl 3-CH), 7.91 (1H, t, J = 7.6 Hz, dansyl 7-CH), 7.58 (1H × 0.8, s, NH), 7.18 (2H × 0.8, bs, NH, Arg-ϵNH), 6.6 (3H × 0.8, b, N^+H_3), 4.32 (1H, m, Arg-αCH), 4.29 (1H, m, Ser-αCH), 3.78 (2H, dd, J = 17.5 Hz, Gly-CH_2), 3.72 (2H, ddd, J = 11.5, 5.5 and 5.3 Hz, Ser-βCH_2), 3.45 (6H, s, dansyl N-CH_3), 3.2 (2H, q, J = 6.2 Hz, Arg-δCH_2), 1.90 (1H, m, Arg-βCH_2), 1.77 (1H, m, Arg-βCH_2), 1.65 (2H, m, Arg-δCH_2); ^{13}C NMR (H_2O/D_2O) δ 179.2 (C═O), 174.68 (C═O), 174.23 (C═O), 166.04 and 165.75 ($^2J_{CF}$ = 35 Hz, C═O of TFA), 159.8 and 159.76 (C═N of Arg), 143.28 and 137.21 (dansyl C-5 and C-1), 133.58, 131.62 (dansyl C-4a or C-8a), 131.28, 129.59, 129.29, 129.14 (dansyl C-4a or C-8a), 128.29, 121.99, 120.38 and 118.06 ($^1J_{CF}$ = 292 Hz, CF_3 of TFA), 63.78 (Ser-βC), 58.42 and 56.24 (Arg-αC or Ser-αC), 49.45 (dansyl N-CH_3), 47.79 (Gly-αC), 43.51 (Arg-δC), 30.90 (Arg-βC), 27.34 (Arg-γC). Anal. ($C_{23}H_{34}N_8O_6S \cdot 2.5CF_3COOH \cdot 1.5H_2O$) C, H, N.

Receptor Binding. The radioligand **4** (specific activity = 2000 Ci/mmol) was a gift from Nycomed Amersham plc (Little Chalfont, Buckinghamshire, U.K.). Whole spinal cords from adult rats were obtained frozen from Hilltop Lab Animals (Scottdale, PA). Bestatin and **1** (both from Sigma, St. Louis, MO) were dissolved at 30 mM concentration in 10% acetic acid in ethanol. Aprotinin and bovine serum albumin (BSA) also were from Sigma.

Membrane Preparation. The membrane preparation and assay for NPFF receptor binding were modified from those reported before.[19,21] Frozen spinal cord tissue was thawed and homogenized with an Ultra-Turrax apparatus for 30 s in 20−50 mL/g cold buffer (50 mM Tris-HCl, 5 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, pH 7.4). The suspension was centrifuged at 1100g_{max} for 10 min at 4 °C. The supernatants were stored on ice, and the pellets were resuspended and centrifuged as before. The supernatants from both spins were then combined and centrifuged at 46000g_{max} for 10 min. The pellets were resuspended in 100 mL of cold Tris-buffer (50 mM Tris-HCl, pH 7.4) and the centrifugation was repeated. After another resuspension and centrifugation, the pellets were suspended in 50 mL of Tris buffer at 37 °C and incubated at this temperature for 10 min, followed by dilution to 100 mL. After keeping on ice for an additional hour, the centrifugation−resuspension (cold Tris buffer)−centrifugation cycle was repeated. The final pellet was suspended in membrane buffer (50 mM Tris, 200 μM EDTA, and 0.5 mM phenylmethanesulfonyl fluoride, pH 7.4) and aliquots in polypropylene tubes were frozen in dry ice/ethanol. The protein concentrations were determined by the method of Lowry et al.[22] using BSA as the standard.

Radioligand Binding Assay. Membranes were thawed, cooled on ice, passed three times through a 25-gauge needle, and diluted to about 1 mg/mL in the membrane buffer described above. Aliquots of 50 μL (generally ca. 50 μg of protein) were added on ice to 12 × 75-mm glass tubes containing the buffer (50 mM Tris, 3 μL/mL aprotinin, 30 μM

bestatin, 120 mM NaCl, and 7.5 g/L BSA, pH 7.4) and various concentrations of tested compounds. The final volume of each sample was 250 μL. The experiment was started by adding **4** at a final concentration of 0.1 nM, a value close to the dissociation constant (K_D) of the ligand.[20] The tubes were vortexed and incubated at 25 °C for 30 min, after which the reaction was ended by the addition of 1 mL of ice-cold wash buffer (50 mM Tris, 120 mM NaCl, and 5 g/L BSA, pH 7.3). The contents of the tubes were rapidly (within 6 s) vacuum-filtered and washed at 0 °C through presoaked (0.5% polyethylenimine) CF/B filters (Whatman, Maidstone, U.K.) four times each with 4 mL of wash buffer. The radioactivity retained on the filters was measured with a model 5500B γ-counter (Beckman Instruments, Fullerton, CA) at 80% efficiency. Total and nonspecific bindings were determined in the absence and presence of 1 μM **1**, respectively. The displacement of the receptor-bound **4** in the presence of the test compounds or the combinatorial mixtures was expressed as a percentage value calculated by relating the measured specific binding to a control value (total minus nonspecific binding). Each experiment was performed in triplicate. IC_{50} values (concentrations producing half-maximal inhibition of specific binding) were calculated by nonlinear fitting to the binding data. K_i values were calculated from IC_{50} by the Cheng−Prusoff equation.[23]

IAM Chromatography. The IAM capacity factors were measured on a 3-cm × 4.6-mm i.d. IAM.PC.DD column (Regis Technologies, Morton Grove, IL) eluted at 1.0 mL/min with 0.01 M Dulbeco's phosphate-buffered saline (DPBS) that was adjusted to pH 5.4 with phosphoric acid and also contained 5% (v/v) acetonitrile. The void volume marker was citric acid and the k_{IAM}' capacity factor was calculated as follows: $k_{IAM}' = (t_{R(X)} - t_{R(citric\ acid)})/t_{R(citric\ acid)}$, where $t_{R(X)}$ and $t_{R(citric\ acid)}$ are the retention times for the analyte and the void volume marker, respectively.

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Supporting Information Available: Detailed analytical and spectroscopic characterization of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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