

SYNTHESIS OF POTENT ANTAGONISTS FOR THE NATRIURETIC PEPTIDE CLEARANCE RECEPTOR (NPR-C)

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ABSTRACT

Objectives: The objective of this research was to design and prepare natriuretic peptide clearance receptor (NPR-C) antagonists with potential therapy for achondroplasia, an autosomal dominant disorder that interferes with the synthesis of the cartilage growth plate of long bones.

Methods: Peptides were synthesized by the standard solid-phase peptide synthesis (SPPS) protocol on Rink resin using the N-Fmoc/t-butyl protection methodology. Biological activity of NPR-C antagonists was assessed using ATDC5 cells.

Results: SPPS rapidly generated many crude compounds with purities exceeding 80%. The synthesized ligands were further purified by liquid chromatography-mass spectrometry (LC-MS), and their identities were confirmed by MS and nuclear magnetic resonance. Ligands with nanomolar potencies were obtained.

Conclusion: Structure-activity relationship studies resulted in a good selection of stable, low nanomolar, and linear NPR-C antagonists.

Keywords: Natriuretic peptides, Clearance receptor, Achondroplasia, Osteocrin, Structure-activity relationship, Antagonists.

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INTRODUCTION

The natriuretic peptide (NP) family comprises four structurally related bioactive peptides. Atrial NP (ANP) (also known as ANF), brain NP (BNP), and C-type NP (CNP) are important regulators of mammalian physiology while dendroaspis NP (DNP) is found in snake venom. The mammalian peptides arise from the processing of precursors and consist of 28 (ANP), 31 (BNP), and 22 (CNP) residues. A conserved disulfide bridge creates a ring structure of 17 amino acid residues with a D-R-I conserved sequence (Fig. 1). Although many minor sites of expression have been detected, ANP is mainly synthesized by atrial cardiomyocytes, whereas BNP is secreted by ventricular cardiomyocytes under hypertensive conditions. CNP, initially found in brain, is most abundant in bone, kidney, and vasculature [1,2].

The natriuretic peptides ANP, BNP, and CNP demonstrate a number of actions on the cardiovascular system including: Natriuresis, diuresis, and relaxation of vascular smooth muscle. They bind two classes of cell surface receptors: The guanylyl cyclase-linked A and B receptors (natriuretic peptide clearance receptor [NPR]-A and NPR-B) and the C receptor (NPR-C) which serves as a clearance receptor for the three peptides [2,3]. The recently cloned bone peptide osteocrin binds specifically to NPR-C and attenuates its inhibitory effect on natriuretic peptide signaling. Osteocrin was shown to augment CNP action in bone by blocking the NPR-C-mediated clearance [4]. CNP and osteocrin bind NPR-C by an almost identical sequence. Recently, the potential skeletal therapeutic benefit of the manipulation of the natriuretic system was demonstrated in the rescue of achondroplasia in a mouse model by CNP overexpression [5]. Achondroplasia, the most common form of short-limbed dwarfism, is an autosomal dominant disorder that interferes with the synthesis of the cartilage growth plate of long bones. The role of the natriuretic system in the human skeleton has also been demonstrated by the severe dwarfism exhibited by patients carrying inactivating mutations in the guanylyl cyclase-B (GC-B) gene [6].

Osteocrin and CNP are not drug candidates due to their size and short half-life in circulation, and therefore, we envisioned that small molecules that potentiate endogenous CNP by antagonizing the CNP clearance receptor (NPR-C) would constitute a potential therapy for achondroplasia.

METHODS

Reagents and solvents

All commercially available reagents were purchased and used without further purification. Commercially available highly purified grade dimethylformamide (DMF, >99.0% purity) and piperidine (redistilled) were purchased from Acros Organics, NJ, USA. Diisopropylethylamine (DIPEA), O-(7-azabenzotriazole)-N,N,N',N'-tetramethyluronium-hexafluoro-phosphate (HATU), and all protected amino acids were purchased from ChemImpex International, Wood Dale, IL, USA. Fmoc-Ile-Rink resin, used for making the peptides, was purchased from NovaBiochem, (Merck), Germany. Readily available acylating aromatic building blocks, and all other reagents and solvents were purchased from Aldrich, USA.

Reactions and purifications

Solid-phase reactions were conducted according to the standard solid-phase peptide synthesis (SPPS) protocols [7,8] and were monitored, for coupling completion, using the Kaiser Test. High-performance liquid chromatography (HPLC) was performed on a water semi-preparative HPLC system (Alliance 2695) equipped with PDA detector (2996). When using liquid chromatography-mass spectrometry (LC-MS), the Waters Alliance LC system was integrated with the ZQ Waters Mass detector system. The columns used were Vydac C₁₈ (5 μm, 4.6 × 250 mm), Vydac C₈ (5 μm, 4.6 × 250 mm), Vydac C₁₈ (5 μm, 10 × 250 mm), Phenomenex C₁₈ (5 μm, 21.2 × 250 mm), and Zorbax C₈ (5 μm, 21.2 × 250 mm). All HPLC solvents were filtered with a Millipore filtration system under vacuum before use.

SPPS

SPPS was performed using glass or polypropylene (PP) tubes fitted with 20 µ PE frits and a capped leuc bottoms (Aldrich/Supelco: product no. 57176). The reaction is shaken using an orbital shaker or simply stirred gently using a magnetic stirrer. All the peptide sequences were synthesized on the *N*-(9-Fluorenylmethoxycarbonyl)-L-isoleucine-Rink (Fmoc-Ile-Rink) resin (loading 0.62 mmol/g, 100-200 mesh) purchased from NovaBiochem, Germany. The sequential coupling of protected amino acids was performed using the HATU/DIPEA protocol. The diisopropylcarbodiimide/hydroxybenzotriazole (DIC/HOBt) protocol proved also successful [9].

General procedure for Fmoc deprotection

The Fmoc-protected peptidyl-resin was treated with 20% piperidine in DMF for 3 minutes. The resin was drained using a stream of Argon and the piperidine treatment was repeated twice, for 3 and 10 minutes. The resin was washed using the following standard washing cycle: DMF (4× 4 ml), dichloromethane (DCM, 4× 4 ml), methanol (MeOH, 4× 4 ml), DCM (2× 4 ml), and MeOH (2× 4 ml).

Coupling of amino acids

0.05 mmol of Fmoc-Ile-Rink resin was placed in the fritted PE tube and 2 ml of DMF was added. The resin was then left for 10 minutes before being filtered. The Fmoc group was then deprotected using the Fmoc deprotection protocol mentioned above, and the resin was prepared for coupling to the next required amino acid residue as follows: The next Fmoc-amino acid (5 equivalents relative to the resin loading) and HATU (4.9 equivalents relative to the resin loading) were dissolved in DMF. The activation was then initiated by adding DIPEA (10 equivalents relative to the resin loading) to the amino acid solution. The resulting solution was then stirred and transferred immediately to the resin (preswelled in 1 ml of DMF). The reaction tube was gently shaken or stirred for 90 minutes, and then, the coupling was terminated by filtering the solution from the resin. The resin was washed thoroughly as mentioned above. After drying the resin, the resin was analyzed by Kaiser Test to determine the completeness of the reaction and was then carried through the next cycle.

Capping (final coupling) with aromatic tether

The *N*-acetylation reaction on the Freidinger lactam (FL) was run for 2 hrs with 10 equivalents (relative to the resin loading) of the aromatic acid building block, 9.8 equivalents of HATU, and 20 equivalents of DIPEA.

Cleavage of peptides

Cleavage from the resin with simultaneous removal of side chains protecting groups was done by adding a solution of trifluoroacetic acid (TFA)/triisopropylsilane/H₂O (95:2.5:2.5) to the dry resin with stirring for 2 hrs. The resin was then removed by filtration and was washed twice with TFA. The combined filtrate was concentrated by removing most of the TFA and then was precipitated with cold ether. The precipitated peptide was isolated by centrifugation and the crude peptide was purified by LC-MS. Pure product (>95%) containing fractions were pooled, concentrated, frozen, and lyophilized to yield the product as a white powder. Peptide structures were confirmed using HRMS and nuclear magnetic resonance (NMR). Peptides showed similar spectroscopic data for the peptide portion, including the FL moiety.

Compound 1

¹H NMR (D₂O, 400 MHz): δ 9.36 (s, 1H, Quinoxaline ring), 8.25 (m, 1H, Ar-H), 8.11 (m, 1H, Ar-H), 7.92 (m, 2H, Ar-H), 7.76 (d, 2H, J = 8.5 Hz, Ar-H), 7.21 (d, 2H, J = 8.5 Hz, Ar-H), 4.72 (m, 2H, Asp-Hα), 4.54-4.34 (m, 3H, 2xIle-Hα + D-Arg-Hα), 4.21 (m, 1H, lactam-Hα), 3.40 (s, 2H, CH₂-Ar), 3.21 (m, 2H, lactam CH₂-N), 2.65 (t, D-Arg CH₂-N), 2.55 (dd, 2H, Asp-Hβ), 2.17-2.02 (m, 4H, lactam CH₂ + 2x Ile-Hβ), 1.71-1.45 (m, 8H, D-Arg -(CH₂)₂ + 2x Ile-2Hγ), 1.2 (6H, 2x Ile-CH₃γ), and 0.95-0.81 (m, 6H, 2x Ile-Hδ); high resolution (ESI-MS) calculated for C₄₃H₅₈N₁₂O₉, 886.4450, found 886.4445. Anal. Calcd for C₄₃H₅₈N₁₂O₉: C, 58.23; H, 6.59; and N, 18.95. Found: C, 58.12; H, 6.68; and N, 18.86.

Compound 2

¹H NMR (D₂O, 400 MHz): δ 7.26-7.09 (m, 5H, Ar ring) and 3.45 (s, 2H, CH₂-Ar); high resolution (ESI-MS) calculated for C₃₄H₅₃N₉O₈, 715.4017, found 715.4014.

Compound 3

¹H NMR (D₂O, 400 MHz): δ 7.49-7.43 (m + d, 4H, terminal Ar-H), 7.39 (m, 1H, terminal Ar-H), 7.29-7.21 (dd, 4H, Ar-H), and 3.36 (s, 2H, CH₂-Ar); high resolution (ESI-MS) calculated for C₄₀H₅₇N₉O₈, 791.4330, found 791.4319.

Compound 4

¹H NMR (D₂O, 400 MHz): 9.56 (s, 1H, Pyrazine ring), 8.92 (d, 1H, Pyrazine ring), 8.71 (d, 1H, Pyrazine ring), 7.46 (dd, 2H, Ar-H), 7.19 (dd, 2H, Ar-H), and 3.36 (s, 2H, CH₂-Ar); high resolution (ESI-MS) calculated for C₃₉H₅₆N₁₂O₉, 836.4293, found 836.4288.

Compound 5

¹H NMR (D₂O + few drops of CD₃OD, 400 MHz): 8.09 (m, 1H, Ar-H), 7.75-7.58 (m, 3H, Ar-H), 7.41 (dt, 2H, Ar-H), 7.32 (dt, 1H, Ar-H), and 3.94 (s, 2H, Gly-CH₂); (ESI- MS)846.7 (MNa⁺).

Compound 6

¹H NMR (D₂O, 400 MHz): 7.49 (br d, 3H, Indol-H + Ar-H), 7.41 (br s, 1H, Indol-H), 7.21 (d, 1H, Indol-H), 7.18 (d, 2H, Ar-H), 6.9 (dd, 1H, Indol-H), 3.81 (s, 3H, CH₃O), and 3.40 (s, 2H, CH₂-Ar); high resolution (ESI-MS) calculated for C₄₄H₆₁N₁₁O₁₀, 903.4603, found 903.4598.

Compound 7

¹H NMR (D₂O, 400 MHz): 7.86 (dt, 1H, Benzofuran-H), 7.61 (dd, 1H, Benzofuran-H), 7.56 (m, 1H, Benzofuran-H), 7.50 (d, 2H, Ar-H), 7.39-7.26 (m, 2H, Benzofuran-H), 7.21 (d, 2H, Ar-H), and 3.39 (s, 2H, CH₂-Ar); high resolution (ESI-MS) calculated for C₄₃H₅₈N₁₀O₁₀, 874.4337, found 874.4334.

Compound 8

¹H NMR (D₂O, 400 MHz): 7.56 (d, 2H, Ar-H), 7.31 (d, 1H, Furan-H), 7.11 (d, 1H, Furan-H), 7.01 (d, 2H, Ar-H), and 3.80 (s, 3H, CH₃O); high resolution (ESI-MS) calculated for C₃₈H₅₅N₉O₁₀, 797.4072, found 797.4049.

Compound 9

¹H NMR (D₂O, 400 MHz): 7.55 (d, 2H, Ar-H), 7.32 (d, 1H, Furan-H), 7.11 (d, 1H, Furan-H), 7.00 (d, 2H, Ar-H), 3.84 (s, 3H, CH₃O), and 3.85 (s, 2H, Gly-CH₂); (ESI-MS)855.9 (MH⁺).

Compound 10

¹H NMR (D₂O, 400 MHz): 7.58 (d, 1H, Ar-H), 7.51 (d, 2H, Ar-H), 7.41 (dd, 1H, Ar-H), 7.21 (d, 2H, Ar-H), 7.16 (d, 1H, Ar-H), 5.95 (s, 2H, OCH₂O), and 3.39 (s, 2H, CH₂-Ar); high resolution (ESI-MS) calculated for C₄₂H₅₈N₁₀O₁₁, 878.4287, found 878.4283.

Compound 11

¹H NMR (D₂O, 400 MHz): 8.75 (dd, 1H, Naphthyl-H), 8.39 (dd, 1H, Naphthyl-H), 8.29 (dd, 1H, Naphthyl-H), 8.12-7.91 (m, 2H, Naphthyl-H), 7.91 (m, 2H, Naphthyl-H), 7.31 (d, 2H, Ar-H), 6.99 (d, 2H, Ar-H), and 3.36 (s, 2H, CH₂-Ar); (ESI- MS)921.7 (MH⁺).

Compound 12

¹H NMR (D₂O, 400 MHz): 8.34 (m, 1H, Naphthyl-H), 8.04 (dd, 1H, Naphthyl-H), 7.86 (dd, 1H, Naphthyl-H), 7.76 (dd, 1H, Naphthyl-H), 7.48 (d, 2H, Ar-H), 7.31 (m, 1H, Naphthyl -H), 7.21-7.12 (m+dd, 3H, Naphthyl + Ar-H), and 3.38 (s, 2H, CH₂-Ar); high resolution (ESI-MS) calculated for C₄₅H₆₀N₁₀O₁₀, 900.4494, found 900.4487.

Compound 13

¹H NMR (D₂O, 400 MHz): 8.84 (m, 1H, Quinoline-H), 8.44 (m, 1H, Quinoline-H), 8.17-7.88 (m, 2H, Ar-H), 7.73 (dt, 1H, Ar-H), 7.52 (dt, 1H, Ar-H), 3.21-3.04 (m, 4H, Pip ring), 2.31 (m, 1H, Pip ring), and 1.61-1.42 (m, 4H, Pip ring); (ESI- MS)864.9 (MH⁺).

Compound 14

^1H NMR (D_2O , 400 MHz): 8.82 (m, 1H, Quinoline-H), 8.43 (m, 1H, Quinoline-H), 8.15-7.84 (m, 2H, Ar-H), 7.71 (dt, 1H, Ar-H), 7.52 (dt, 1H, Ar-H), 3.08 (m, 2H, $\text{NH-CH}_2\text{-Cyclohexyl}$), 2.32 (m, 1H, $\alpha\text{H}(\text{C=O-Cyclohexyl})$), 2.01 (m, 1H, Cyclohexyl ring), 1.78-1.55 (m, 4H, Cyclohexyl ring), and 1.38-1.21 (m, 4H, Cyclohexyl ring); high resolution (ESI-MS) calculated for $\text{C}_{44}\text{H}_{65}\text{N}_{11}\text{O}_9$, 891.4967, found 891.4961.

Compound 15

^1H NMR (D_2O , 400 MHz): 8.82 (m, 1H, Quinoline-H), 8.44 (m, 1H, Quinoline-H), 8.15-7.84 (m, 2H, Ar-H), 7.72 (dt, 1H, Ar-H), 7.50 (dt, 1H, Ar-H), 3.01 (t, 2H, Hexyl chain- $\text{CH}_2\text{-NH}$), and chemical shifts of Hexyl chain (C2-C5) are overlapping with Ile and D-Arg side chains; high resolution (ESI-MS) calculated for $\text{C}_{42}\text{H}_{63}\text{N}_{11}\text{O}_9$, 865.4810, found 865.4798.

Compound 16

^1H NMR (D_2O , 400 MHz): 8.19 (d, 1H, Quinoline-H), 8.12 (dd, 1H, Quinoline-H), 7.83 (m, 1H, Ar-H), 7.63 (dd, 1H, Ar-H), 7.55 (m, 1H, Ar-H), 7.27 (dt, 1H, Ar-H), 2.51-2.55 (2 overlapping t, 4H, Succinimide), and 3.95 (s, 2H, Gly- CH_2); high resolution (ESI-MS) calculated for $\text{C}_{41}\text{H}_{60}\text{N}_{12}\text{O}_{10}$, 880.4555, found 880.4551.

Compound 17

^1H NMR (D_2O , 400 MHz): 8.01 (m, 1H, Benzothiophene-H), 7.55-7.48 (d overlapping m, 3H, Benzothiophene-H + Ar-H), 7.26-7.19 (d overlapping m, 3H, Benzothiophene-H + Ar-H), and 3.39 (s, 2H, $\text{CH}_2\text{-Ar}$); (ESI-MS) 943.4 (MH^+).

Cellular assay

ATDC5 cells [10] expressing endogenous CNP clearance receptor at the cell surface were used. Briefly, the IC_{50} of the synthesized peptides was determined in a competition assay using ^{125}I -CNP as the tracer. ATDC5 cells were seeded in 24-well plates for 3 days and then were incubated in ^{125}I -CNP and varying concentrations of cold ligands for 90 minutes at 4°C .

RESULTS AND DISCUSSION

Recently, the bone peptide osteocrin was cloned and it was demonstrated that it specifically binds NPR-C with low nanomolar affinity and potentiates CNP action on cyclic guanosine monophosphate. Osteocrin and CNP are not drug candidates due to their short half-life in circulation, and thus, we looked at smaller molecules that are more stable and can augment CNP action in bone by blocking the NPR-C-mediated clearance. Small peptides were synthesized on Rink resin using the Fmoc SPPS strategy. The FL building block was synthesized using the literature procedure [11,12]. The subsequent *N*-acylation with the appropriate aromatic acid (aromatic tether) was done on resin. Using the same coupling protocol as for the amino acid sequence, it was found that the coupling was never complete. The *N*-acetylation reaction on the FL moiety gave the best results when it was run for 2 hrs with 10 equivalents (relative to the resin loading) of the aromatic acid building block, 9.8 equivalents of HATU, and 20 equivalents of DIPEA.

The synthesized peptides were cleaved from the solid support and were purified using LC-MS before biological testing. Our SPPS of compound 1, a known NPR-C antagonist [13], is presented below (Fig. 2) and the same synthetic protocol was adopted throughout the preparation of all final compounds.

Initially, we synthesized and tested compound 1 for a confirmatory study of its NPR-C antagonistic activity which was determined to be 5 nM. Furthermore, we tested the stability of this compound for further optimization studies. Compound 1 showed no degradation after 2 hrs (at 4°C or 37°C) during an *in vitro* stability study. However, the *in vivo* half-life was <10 minutes which prompted us to undertake a structure-activity relationship (SAR) study on this compound in search for molecules with better PK profiles. This paper reports on the influence of the aromatic tether on the biological activity. Table 1 summarizes a representative SAR study on the effect of varying the structure of the aromatic tether.

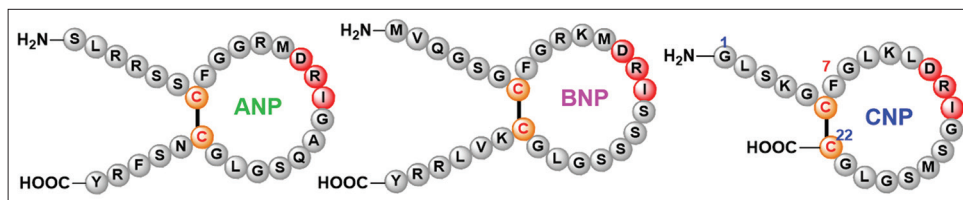


Fig. 1: Structures of mammalian natriuretic peptides

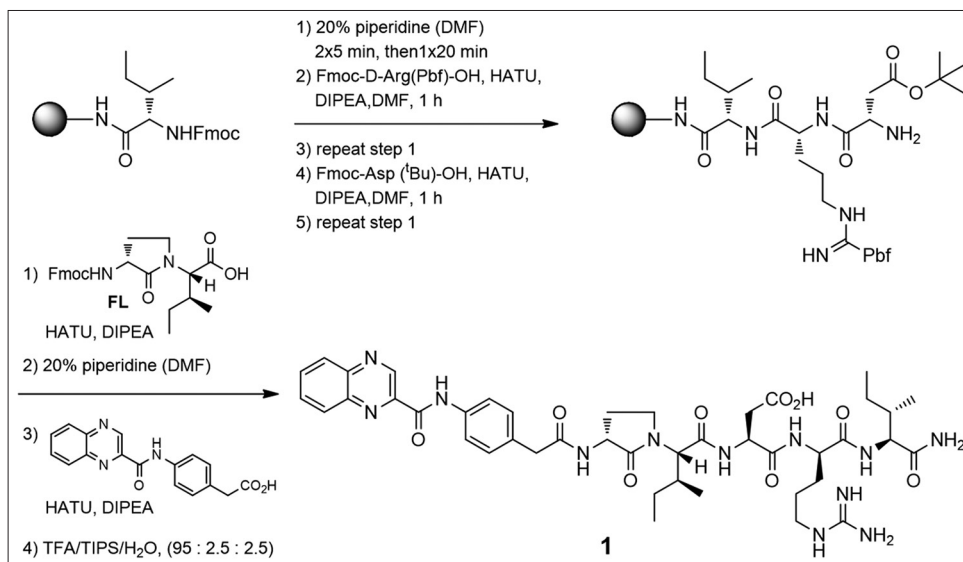


Fig. 2: Representative solid-phase peptide synthesis of natriuretic peptide clearance receptor antagonists

Table 1: Influence of varying the structure of the aromatic tether moiety on receptor affinity

Compound	Aromatic tether	IC ₅₀ (nM)*	Compound	Aromatic tether	IC ₅₀ (nM)*
2		1400	10		25
3		1000	11		110
4		210	12		11
5		11000	13		31
6		11	14		15
7		5	15		12
8		9700	16		17
9		680	17		38

*Values are based on mean of three replicates

It seems that the length of the tether is important to dip into the Phe-7 pocket of the receptor (see CNP structure, Fig. 1). For example, compound 4 lacking the second aromatic ring is much less potent than compound 1. A similar observation can be made when comparing compounds 8 and 9 in that the aromatic moiety must be reaching the Phe-7 pocket for better potency (compound 9). The aminophenylacetic acid moiety of compound 1 can be replaced with aminomethyl cyclohexyl carboxylic acid or with more flexible straight chain aminohexyl moieties (compounds 14 and 15, respectively). Heteroatoms within or on the aromatic nucleus also seem to contribute to potency; possibly through hydrogen bonding. Incorporating urea (compound 5) or sulfonamide (compound 11) at the quinoxaline amide position in compound 1 drastically reduced potency. The quinoxaline nucleus can successfully be replaced with indol, benzofuran, naphthyl, and benzothiophene moieties (compounds 6, 7, 12, and 17, respectively).

To access novelty in the design, we investigated the replacement of the FL moiety in compound 1 with readily available amino acids. We envisioned that Pro and its mimics may induce a similar β-turn that the FL is known to induce. Successfully, this replacement led to equally potent compounds. These binding results, as well as stability studies on these compounds, will be communicated in future publications.

CONCLUSION

NPR-C antagonists with low nanomolar potency were rapidly prepared by SPPS that is amenable to scale-up. SAR studies indicated the right aromaticity nucleus needed at the Phe-7 pocket of the receptor. Once the PK properties are established, the potential application of some of these compounds in treating achondroplasia will be investigated.

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