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

ELAREF RATEMI*
Department of Chemical and Process Engineering Technology, Jubail Industrial College, P. O. Box No - 10099, Jubail 31961, Saudi Arabia.
Email: ratemi_e@jic.edu.sa

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.

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 cardiomycocytes, whereas BNP is secreted by ventricular cardiomycocytes 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, dieresis, 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-II (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 piperdine (redistilled) were purchased from Acros Organics, NJ, USA. Dicypropylethylamine (DPEA), 0-(7-azabenzotriazole)-N,N,N,N-tetramethyluroniumhexafluorophosphate (HATU), and all protected amino acids were 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 C8 (5 µm, 4.6 × 250 mm), Vydac C18 (5 µm, 4.6 × 250 mm), Phenomenex C18 (5 µm, 21.2 × 250 mm), and Zorbax C18 (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 tweezer 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-Fluorenlymethylxycarbonyl)-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 DIPEA (10 equivalents relative to the resin loading) were added. The activation was then initiated by adding DIPEA (10 equivalents relative to the resin loading) to the amino acid solution. The resulting DMF was added. The resin was then left for 10 minutes before being filtered. The resin was then removed by filtration and was stirred gently using a magnetic stirrer. All the peptide sequences were washed twice with DCM (2× 4 ml), and MeOH (2× 4 ml). The resin was then left for 10 minutes before being filtered. The resin was then removed by filtration and was stirred gently using a magnetic stirrer. All the peptide sequences were washed twice with DCM (2× 4 ml), and MeOH (2× 4 ml).

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)/trisopropylsilane/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 filtering the solution from the resin. The resin was washed thoroughly with 20 µ PE frits and a capped leur 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-Fluorenlymethylxycarbonyl)-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].

### Compound 1

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 2

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 3

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 4

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 5

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 6

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 7

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 8

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 9

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 10

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 11

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 12

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.

### Compound 13

°C NMR (D_2O, 400 MHz): δ 7.93 (m, 5H, Ar-H), 7.73 (s, 2H, CH-Ar); high resolution (ESI-MS) calculated for C_{39}H_{56}N_{12}O_{9} 836.4293, found 836.4288.
**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-acylation 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.

| Compound | 1H NMR (D2O, 400 MHz) | Chemical Shifts | High Resolution (ESI-MS) |
|----------|-----------------------|-----------------|-------------------------|
| 14       | 8.82 (m, 1H, Quinoline-H), 8.43 (m, 1H, Quinoline-H), 7.71 (dt, 1H, Ar-H), 7.52 (dt, 1H, Ar-H), 3.08 (m, 2H, NH-CH2-Cyclohexyl), 2.32 (m, 1H, αH(C=O)-Cyclohexyl), 2.01 (m, 1H, Cyclohexyl ring), 1.78-1.55 (m, 4H, Cyclohexyl ring), and 1.30-1.21 (m, 4H, Cyclohexyl ring); high resolution (ESI-MS) calculated for C44H65N11O9 891.4967, found 891.4961. |
| 15       | 8.82 (m, 1H, Quinoline-H), 8.44 (m, 1H, Quinoline-H), 7.72 (dt, 1H, Ar-H), 7.50 (dt, 1H, Ar-H), 3.01 (t, 2H, Hexyl chain-CH2-NH), and chemical shifts of Hexyl chain (C2-C5) are overlapping with Ile and D-Arg side chains; high resolution (ESI-MS) calculated for C42H63N11O9 865.4810, found 865.4798. |
| 16       | 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-CH2); high resolution (ESI-MS) calculated for C41H60N12O10 880.4555, found 880.4551. |
| 17       | 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, CH2-Ar); (ESI-MS) 943.4 (MH+) |

**Cellular assay**

ATDC5 cells [10] expressing endogenous CNP clearance receptor at the cell surface were used. Briefly, the IC50 of the synthesized peptides was determined in a competition assay using 125I-CNP as the tracer. ATDC5 cells were seeded in 24-well plates for 3 days and then were incubated in 125I-CNP and varying concentrations of cold ligands for 90 minutes at 4°C.

![Fig. 1: Structures of mammalian natriuretic peptides](image1)

**Fig. 1:** Structures of mammalian natriuretic peptides

![Fig. 2: Representative solid-phase peptide synthesis of natriuretic peptide clearance receptor antagonists](image2)

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

**ACKNOWLEDGMENT**

The author is thankful to Jubail Industrial College, Saudi Arabia, for support in preparing and writing this manuscript. The assistance of Dr. Isabelle Lemire with biological testing is greatly appreciated.

**REFERENCES**

1. Vasile VC, Jaffe AS. Natriuretic peptides and analytical barriers. Clin Chem 2017;63:50-8.
2. Baxter GF. The natriuretic peptides. Basic Res Cardiol 2004;99(2):71-5.
3. Rose RA, Giles WR. Natriuretic peptide C receptor signalling in the heart and vasculature. J Physiol 2008;586(2):553-66.
4. Thomas G, Moffatt P, Salois P, Gaumond MH, Gingras R, Godin E, et al. Osteocrin, a novel bone-specific secreted protein that modulates the osteoblast phenotype. J Biol Chem 2003;278(50):50563-71.
5. Yasoda A, Komatsu Y, Chusho H, Miyazawa T, Ozasa A, Miura M, et al. Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. Nat Med 2004;10(1):80-6.
6. Bartels CF, Büklüméz H, Padayatti P, Rhee DK, van Ravenswaaij-Arts C, Pauli RM, et al. Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux. Am J Hum Genet 2004;75(1):27-34.
7. Amblard M, Fehrentz JA, Martinez J, Subra G. Methods and protocols of modern solid phase Peptide synthesis. Mol Biotechnol 2006;33(3):239-54.
8. Behrendt R, White P, Offer J. Advances in Fmoc solid-phase peptide synthesis. J Pept Sci 2015;21(2):22-27.
9. Saxena R, Manjan MJ. A thermally responsive short elastin like polypeptide-drug conjugate: Synthesis, characterization and biological evaluation for targeted delivery of anticancer drugs. Int J Pharm 2014;462:326-33.
10. Anbarasi G, Kumar SK, Naradhipa P, Srinivasan N. Effect of Vitamin C on mRNA expression of bmps during fracture healing in the femur callus of Sprague-Dawley female rats. Int J Pharm Sci 2014;6:208-12.
11. Freidinger RM. Synthesis of γ-lactam-constrained tryptophyllysine
derivatives. J Org Chem 1985;50:3631-3.
12. Wolfe MS, Dutta D, Aubé J. Stereoselective synthesis of freidinger lactams using oxaziridines derived from amino acids. J Org Chem 1997;62(3):654-63.
13. Veale CA, Alford VC, Aharony D, Banville DL, Bialecki RA, Brown FJ, et al. The discovery of non-basic atrial natriuretic peptide clearance receptor antagonists. Part 1. Bioorg Med Chem Lett 2000;10(17):1949-52.