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Cyclic biphalin analogues with a novel linker lead to potent agonist activities at mu, delta, and kappa opioid receptors

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ABSTRACT

In an effort to improve biphalin's potency and efficacy at the μ-(MOR) and δ-opioid receptors (DOR), a series of cyclic biphalin analogues **1–5** with a cystamine or piperazine linker at the C-terminus were designed and synthesized by solution phase synthesis using Boc-chemistry. Interestingly, all of the analogues showed balanced opioid agonist activities at all opioid receptor subtypes due to enhanced κ-opioid receptor (KOR) activity. Our results indicate that C-terminal flexible linkers play an important role in KOR activity compared to that of the other cyclic biphalin analogues with a hydrazine linker. Among them, analogue **5** is a potent (*Ki* = 0.27, 0.46, and 0.87 nM; EC_{50} = 3.47, 1.45, and 13.5 nM at MOR, DOR, and KOR, respectively) opioid agonist with high efficacy. Based on the high potency and efficacy at the three opioid receptor subtypes, the ligand is expected to have a potential synergistic effect on relieving pain and further studies including in vivo tests are worthwhile.

1. Introduction

Multifunctional ligands for the μ-opioid receptor (MOR) and δ-opioid receptor (DOR) have gained notoriety due to their promise in the realm of pain therapeutics.[1] It has been previously demonstrated that co-administration of MOR and DOR agonists lead to an attenuation of serious side effects caused by MOR agonists, such as tolerance, while augmenting potency and efficacy for the receptors.[2–4] Synergistic effects from MOR and DOR agonists have also been observed which increase analgesic efficacy and thereby allow the usage of lesser doses of MOR agonists and decrease unwanted MOR-related side effects.[5]

Biphalin (Fig. 1) is a homodimer of an enkephalin-based tetrapeptide linked by hydrazine that shows high binding affinities in the nanomolar range at the MOR and DOR, and potent antinociceptive effects with promising bioavailability including potential blood brain barrier (BBB) penetration.[6–10] For these reasons, numerous structure-activity relationship (SAR) studies have been performed on biphalin to develop efficacious therapeutics for the treatment of pain, but most studies have been focused on modifications of the C-terminal linker and 2,2′- and/or 4,4′-amino acid residues.[9,11–13] Peptide cyclization is a very efficient tool to improve receptor specificity (lower off-target toxicity), potency, and efficacy of peptides along with proteolytic stability and bioavailability by limiting the peptide's dynamic nature. Limiting conformational variants by constraints induced by formation of covalent bonds such as lactones, lactams, and disulfides, enhances their ability to interact with target receptors. Despite the benefits of incorporating cycles into peptides, few attempts have been made to cyclize biphalin's structure.[9,14–17] In those cyclic biphalin studies, L-chirality of Cys and Pen residues at positions 2 and 2′ resulted in a significant loss in affinity and efficacy at the opioid receptors (OR), whereas D-chirality enhanced affinity and efficacy, similar to previous SAR studies on linear analogues.[15,16]

On the basis of these data, we sought to design and investigate a series of cyclic biphalin derivatives of which the homodimeric opioid pharmacophores are linked through a flexible cystamine or piperazine and 2,2′ positions are utilized for cyclization via a disulfide bond

Abbreviations: Acm, acetamidomethyl; ACN, acetonitrile; BBB, blood brain barrier; Boc, *t*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate); DAMGO, [DAla[2], NMePhe[4], Glyol[5]]-enkephalin; DADLE, [DAla[2], DLeu[5]]-enkephalin; DMF, *N*,*N*-dimethylformamide; Dmt, 2,6-dimethyl tyrosine; DOR, δ-opioid receptor; EtOAc, ethyl acetate; FAB-MS, fast-atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, hexafluorophosphate benzotriazole tetramethyl uronium; HEK, human embryonic kidney; HOBt, *N*-hydroxybenzotriazole; KOR, κ-opioid receptor; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; MeOH, methanol; MOR, μ-opioid receptor; NMM, *N*-methyl morpholine; Pen, penicillamine; RP-HPLC, reversed-phase high performance liquid chromatography; SAR, structure-activity relationship; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TLC, thin-layer chromatography

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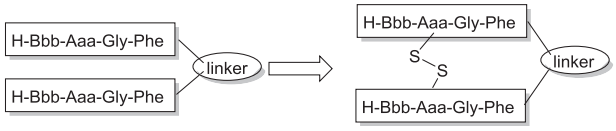


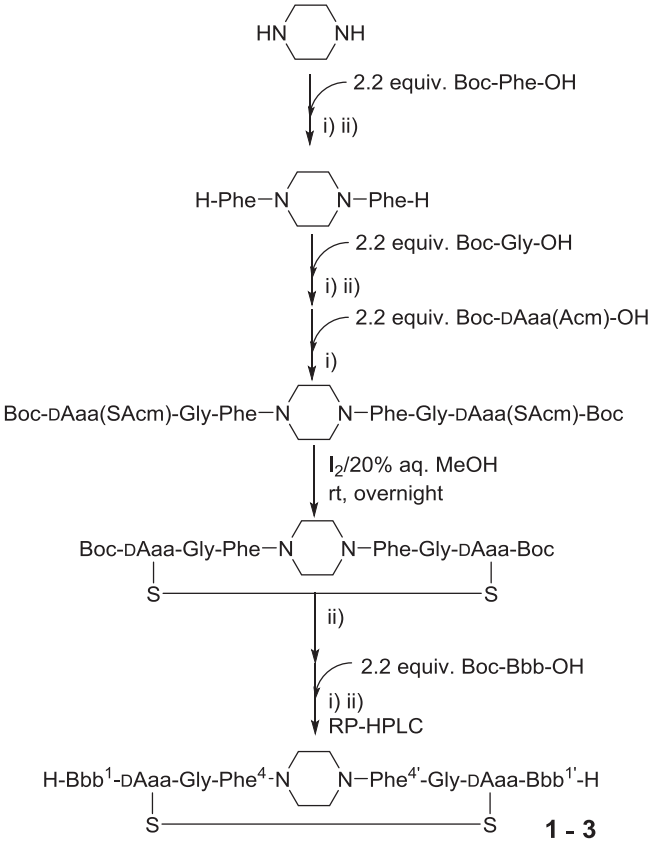
	biphalin	1	2	3	4	5
linker	-NH-NH-					
Aaa	DAla	DCys	DCys	DPen	DCys	DCys
Bbb	Tyr	Tyr	Dmt	Tyr	Tyr	Dmt

Fig. 1. Design of new cyclic biphalin analogues.

between DPen or DCys residues. Compared to constrained aromatic linkers, the flexible piperazine linker was shown to be more potent at the MOR and DOR, and therefore, another flexible linker, cystamine, was also chosen and utilized in this study. Additionally, Tyr residues at positions 1,1′ were substituted with 2,6-dimethyl-tyrosine (Dmt) residues in an effort to increase potency and efficacy at the ORs.[18,19] As a result, a series of nonselective MOR/DOR/κ-opioid receptor (KOR) agonists were designed and synthesized (Fig. 1).

2. Results

The designed cyclic biphalin analogues were prepared by stepwise liquid phase peptide synthesis using the N^{α}-Boc-chemistry approach (Scheme 1, Table 1). During the stepwise chain elongations, intermediate peptides were isolated by routine precipitation using common organic solvents, typically diethyl ether. A work-up process using basic



Scheme 1. Synthesis of cyclic biphalin analogues **1**–**3**. Aaa: Cys for **1** & **2**, Pen for **3**; Bbb: Tyr for **1** & **3**; Dmt for **2**. i) 2.2 equiv Bop/2.2 equiv HOBt/4.4 equiv NMM, 0 °C for 30 min & rt for 2–4 h ii) 95% TFA/5% TIS, 0 °C for 20–30 min.

(5% $NaHCO_3$) and subsequent acidic aqueous solutions (5% citric acid) removed excess amounts of unreacted N^{α}-Boc-amino acids and coupling reagents from the reaction mixture and allowed intermediate peptides to be isolated by precipitation. Cyclization of Acm-protected linear hexapeptides were performed using an I_2 solution prior to N^{α}-Boc-group deprotection due to sluggishness caused by N^{α}-free amino groups. After cyclization, the cyclic N^{α}-Boc-hexapeptides were deprotected and coupled with N^{α}-Boc-Tyr-OH or N^{α}-Boc-Dmt-OH. By following the synthetic scheme, we were able to accelerate disulfide bond formation and avoid serious side reactions caused by iodination of tyrosine or Dmt residues. In our first trial to cyclize a linear octapeptide using I_2, the iodinated cyclic octapeptide was obtained as a major product. To avoid the unwanted side reaction, an N^{α}-Boc-protected linear hexapeptide was cyclized first, and then coupled with N^{α}-Boc-Tyr-OH or N^{α}-Boc-Dmt-OH. After the final chain elongation and deprotection, crude products were isolated by preparative RP-HPLC to afford more than 98% purity of cyclic analogues **1**–**5** in overall 20–40% yields.

To evaluate an analogue's biological activity, in vitro binding and functional assays were performed at the MOR, DOR, and KOR. *K*i determinations, receptor binding profiles, and agonist functional data (EC_{50} and E_{max}) were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # HHSN-271-2013-00017-C (NIMH PDSP).[20–22]

Binding affinities at the ORs were determined for ligands **1**–**5** using membranes prepared from transient human embryonic kidney (HEK) cells. Competition binding assays were performed against radiolabeled ligands [^{3}H] DAMGO, [^{3}H]DADLE, and [^{3}H]U69593 for MOR, DOR, and KOR, respectively (Table 2). In these assays, interestingly, all ligands showed balanced binding affinities (selectivity < 8-fold) at the three subtype receptors due to enhanced KOR affinity (*K*i: 0.87–19 nM). Biphalin interacts with MOR and DOR strongly but not with KOR (*K*i = 270 nM), and its cyclic analogue **6** with a disulfide bond at positions 2 and 2′ was shown to retain the same biological profile as biphalin.[16] Based on this previous SAR, the enhanced KOR binding affinity is considered to be attributed to linker modifications from a hydrazine to a piperazine or a cystamine.

Overall, ligands linked via a cystamine (**4** and **5**), which is a more flexible and dynamic motif, showed slight affinity improvements relative to ligands with a piperazine linker (**1** and **2**). Modification of Tyr residues at positions 1 and 1′ with Dmt also improved binding affinities at the ORs in ligands **2** and **5**. Ligand **3** substituted DPen for DCys, a more constrained residue due to the presence of geminal methyl groups on C_{β}, and enhanced DOR binding affinity more so than that of MOR and KOR (14-fold at DOR vs 3-fold for both MOR and KOR). It was shown that a bulky disulfide bond and the geminal methyl groups of a Pen residue limit backbone flexibility and increase DOR activity in DPDPE. Ligand **3** might resemble the DPDPE structure and thus its increased DOR activity. It turned out that ligand **5** with Dmt at positions 1 and 1′ with a cystamine linker is the most potent lead, which displayed a subnanomolar range of binding affinities at the three ORs.

Opioid functional activity was explored by employing a split luciferase cAMP assay using HEK cells that stably express the respective OR (Table 3). For agonist modes, efficacies were compared to DAMGO, DADLE, and Salvinorin A at MOR, DOR, and KOR, respectively. Antagonist mode was run using naltrexone, naltrindole, and GNTI as reference ligands for the MOR, DOR, and KOR, respectively. Ligands **1**–**5** did not show antagonist activities at the ORs. Overall, ligands' functional activities correlated with their binding affinities at the ORs, and most ligands exhibited high efficacy at the MOR, DOR, and KOR. Ligand **5**, which showed binding affinities in the subnanomolar range at the ORs, was the most potent MOR/DOR/KOR agonist with the same high efficacies (E_{max} = 119%, 106%, and 98% for MOR, DOR, and KOR, respectively) relative to the reference ligands. In these functional assays, it was again observed that the linkers piperazine and cystamine aided ligands **1**–**5** to interact with the KOR, and therefore, all ligands