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Proposed Bioactive Conformations of Opiorphin, an Endogenous Dual APN/NEP Inhibitor

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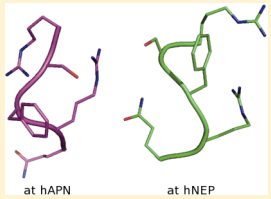
S *Supporting Information*

ABSTRACT: The conformational profiles for the endogenous peptide Opiorphin and a set of seven analogues exhibiting different inhibitory activities toward human aminopeptidase N (hAPN) and human neprilysin (hNEP) were independently computed to deduce a bioactive conformation that Opiorphin may adopt when binding these two enzymes. The conformational space was thoroughly sampled using an iterative simulated annealing protocol, and a library of low-energy conformers was generated for each peptide. Bioactive Opiorphin conformations fitting our experimental structure−activity relationship data were identified for hAPN and hNEP using computational pairwise comparisons between each of the unique low-energy conformations of Opiorphin and its analogues. The obtained results provide a structural explanation for the dual hAPN and hNEP inhibitory activity of Opiorphin and show that the inborn flexibility of Opiorphin is essential for its analgesic activity.



KEYWORDS: *Opiorphin, bioactive conformation, conformational analysis*

One approach in the search of new analgesic compounds relies on the potentiation of the enkephalins' action by using small-molecule inhibitors of their catabolism. The targets of this long-pursued drug discovery concept are the enkephalin-degrading ectoenzymes aminopeptidase N (APN, EC 3.4.11.2) and neprilysin (NEP, EC 3.4.24.11), and up to now, several dual inhibitors have been developed.[1] A new opportunity has arisen after the discovery and characterization of Opiorphin, a human endogenous pentapeptide (QRFSR) secreted in saliva, which is able to bind and inhibit both human aminopeptidase N (hAPN) and human neprilysin (hNEP) in vitro. The compound is also active in vivo, showing a pain-suppressive potency similar to morphine in the formalin pin-pain and tail-flick behavioral rat pain models.[2,3]

As a preliminary step toward the design of potential small-molecule peptidomimetic drugs based on the Opiorphin structure, we have undertaken a computational study to deduce the conformation(s) that enable Opiorphin to inhibit two different enzymes such as hAPN and hNEP. Depending on the availability of the structural information about the target and/or the ligand, computer-assisted drug design studies currently rely on either "structure" or "ligand-based" approaches.[4] In our case, the structure of the extracellular domain of hNEP complexed with six different inhibitors is available (PDB entries: 1DMT, 1R1H, 1R1I, 1R1J, 1Y8J, and 2QPJ)[5−8] and has led to the development of novel nonpeptidic hNEP inhibitors.[9−11] Conversely, structural information on APN is constrained to the *Escherichia coli* APN structure complexed with three inhibitors (PDB entries: 2DQM, 2ZXG, and 3KED).[12,13] Unfortunately, hAPN has an overall sequence identity of only 13.6% with its *E. coli* orthologue,[12] thus limiting its usefulness in structure-based approaches.[14] Being a small peptide, Opiorphin is a highly flexible molecule, whose conformations have not been previously studied in either solution or the solid state. Because of this lack of structural information on Opiorphin, we have decided to use a ligand-based approach to computationally characterize its bioactive conformation. Our approach involves two steps: (i) sampling the conformational space of Opiorphin and a set of analogues, including active and inactive compounds, and (ii) a comparative conformational analysis among the low-energy conformation libraries of the parent compound and of this set of analogues, taking into account their activity at each target (hAPN and hNEP).

Given the high number of allowed bond rotations in peptides, efficient sampling methods are always needed to compute a library of accessible conformations. Here, we have

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Table 1. In Vitro Activity of Opiorphin and a Set of Different Analogues against hNEP and hAPN[a]

								$IC_{50}(\mu M)$	
peptide	name	sequence						hAPN	hNEP
1	*Opiorphin*		*Q*	*R*	*F*	*S*	*R*	*8 ± 1*	*30 ± 3*
2	Opiorphin-(3−5)				F	S	R	120[b]	7[b]
3	Opiorphin-(2−5)			R	F	S	R	27[b]	~180[b]
4	Opiorphin-(1−4)		Q	R	F	S		75[b]	≫100[b]
5	*[Asn^1]-Opiorphin*		*N*	*R*	*F*	*S*	*R*	*≫100*	*~130*
6	*[Thr^4]-Opiorphin*		*Q*	*R*	*F*	*T*	*R*	*≫100*	*≫100*
7	*[Phe^0]-Opiorphin*	*F*	*Q*	*R*	*F*	*S*	*R*	*122*	*51 ± 7*
8	[Cys^0]-Opiorphin	C	Q	R	F	S	R	0.8 ± 0.1[b]	7 ± 3[b]

[a]Peptides in *italics* are new measurements (see the text for details). [b]Ref 25.

used an iterative simulated annealing protocol, which was originally developed by one of us,[15] and has been tested in various peptides ranging from 2 to 11 residues.[16−19] The protocol has been recently upgraded to enhance efficiency and automation by an in-house-developed program (see the Supporting Information for details).

Briefly, starting zwitterionic structures were generated in their extended conformation using the all-atom ff03 force field with an implicit solvent as implemented in AMBER10. For each peptide, the initial structure was energy minimized and subsequently heated to 900 K using a fast heat rate of 100 K/ps to force the structure to jump among different regions of the conformational space. The resulting high-energy structure was then slowly cooled to 200 K at a cool rate of 7 K/ps. The so-obtained structure was used as a starting point for a new SA cycle. After each cycle, low-temperature structures were minimized before being added to a library of low-energy conformations for further analysis. The procedure is repeated until no new conformations, excluding those that are local reoptimizations of the side chains, appear after 300 cycles within a 5 kcal/mol energy range with respect to the lowest energy structure already found. A conformation was considered unique if at least one of the backbone dihedral angles (except the first and the last) differs by more than 60° as compared to any of the previous obtained structures (see the Supporting Information for more details).

The subsequent comparative conformational analysis of the peptide low-energy conformation libraries thus obtained uses a methodology that we and others have previously used satisfactorily to elucidate the bioactive conformations of peptides.[20−22] This analysis is based on the paradigm that the bioactive conformer is one among the set of thermodynamically accessible conformations,[23] which will show up in the group of conformations common to all of the analogues that are efficient binders. Furthermore, inactive analogues will not be able to attain the bioactive conformation or have/lack some structural feature(s) that inactivate them.

In the present paper, we have applied this methodology to Opiorphin and a set of analogues (Table 1); some of them have already been described in two patents.[24,25] To do so, peptides **1** and **5**−**7** were synthesized, and their in vitro inhibitory activity against hAPN and hNEP was assessed (see the Supporting Information for experimental details). For hAPN, peptides **1**, **3**, and **8** make the group of active compounds (IC_{50} < 50 μM), while **2** and **4**−**7** are considered inactive (IC_{50} > 70 μM). In the case of hNEP, good binders are peptides **1**, **2**, **7**, and **8**, while nonbinders are **3**−**6**. Only two compounds, Opiorphin and analogue **8**, are considered true in vitro dual inhibitors of hAPN and hNEP.

The conformational landscape of Opiorphin and seven analogues was first sampled using the iterative simulated annealing protocol (see the Supporting Information for details). For Opiorphin, this required 9251 cycles of iterative simulated annealing, yielding a library of 28 unique low-energy conformations. Next, each of the seven low-energy conformation libraries obtained for the analogues were compared with the one of Opiorphin. The analysis was independently performed for the two targets since the activity displayed by the different analogues against the two enzymes is different (see Figure S1 in the Supporting Information). Low-energy conformation libraries of active or good binding analogues were first compared to reduce the number of conformation candidates from 28 to 6 in the case of hAPN and from 28 to 3 for hNEP. Further comparison with the inactive or nonbinding analogue libraries allowed arrival at one and two candidate conformations for hAPN and hNEP, respectively.

By the above comparative conformational analysis, it was found that Opiorphin shares six very close low-energy conformations with hAPN active analogues **3** and **8**. However, only one of them is able to explain the inactivity of peptide **5** and was therefore selected as our proposed bioactive conformation for hAPN.

The proposed bioactive conformation of Opiorphin for hAPN is depicted in Figure 1. It displays a distorted C shape

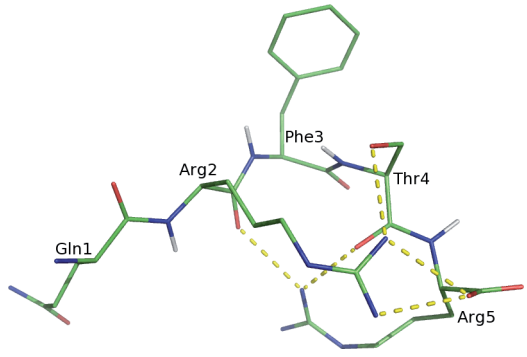

Figure 1. Representation of the 3D structure of our proposed bioactive conformation for Opiorphin hAPN inhibition. Intramolecular hydrogen bonds are indicated by dashes. Hydrogen atoms of side chains have been omitted for clarity.

and is highly stabilized by five intramolecular hydrogen bonds. All backbone atoms, except those of Ser^4 and the carbonyl oxygen of Arg^2, are surface-exposed, and they can freely interact with hAPN. Conversely, it seems that the main role of the Arg^2, Ser^4, and Arg^5 side chains may be to maintain the bioactive

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conformation. The Gln[1] side chain does not have a role in the stabilization of the bioactive conformation since it is exposed to the surface, but also, it does not make any key interaction with APN, since the activity is still retained by deletion of this residue (peptide **3**). The substitution of Gln[1] by Asn produced peptide **5**, which is not able to adopt the bioactive conformation, probably because changes in this side chain may influence the Arg[2] backbone, which seems to be a key residue, as suggested by the inactivity of peptide **2**. From this proposed bioactive conformation, it can be hypothesized that Phe[3] may mediate crucial interactions with hAPN because it is completely surface-exposed and therefore arranged to make appropriate interactions with the enzyme. The substitution of the Ser[4] by Thr also yields an inactive compound (peptide **6**), which is able to adopt the bioactive conformation. However, the close vicinity of the Thr methyl group with the side chain of Phe[3] induces conformational changes (see Figure S2 in the Supporting Information), which could prevent its interaction with the enzyme, thus reinforcing the hypothesis of the key role of Phe[3] side chain for APN inhibition. As mentioned before, the main role of Arg[5] may be to maintain the bioactive conformation. Accordingly, the bioactive conformation was not found in the low-energy conformation library of peptide **4**. Although the RFS sequence looks very important for activity, the existence of extra space in the binding pocket makes it possible to accommodate longer peptides such as active peptide **8**. The nature and volume of this additional residue are also important determinants of activity (peptide **8** vs **7**).

In the case of hNEP, we found three common conformations for active peptides **2**, **7**, and **8** and the parent compound. One of them was eliminated because it was also common to inactive analogue peptide **3**. Further comparisons with inactive analogues **5** and **6** did not allow us to unambiguously select which of the two remaining candidate conformations (1.1 and 1.17) is the bioactive one (1 stands for Opiorphin, and the second number is its position in the low-energy conformation library). One of the main differences between these candidates is the dihedral angle φ for Ser[4] (175.84° vs −14.38°), which produces a change in the shape of the FSR part of the peptide (see Figure S3 in the Supporting Information).

At this stage, we decided to take into account the structural data available for other hNEP inhibitors. Of particular interest were the bioactive conformations of five dipeptide derivatives (RDF[5], BIR[6], OIR[6], TI1[6], and I20[8]) that had been obtained by X-ray crystallography. All of them exhibited the same conformation at the C-terminal moiety, which is very close to the FSR fragment of conformation 1.17, and accordingly, we selected it as our proposed bioactive conformation for hNEP (see the Supporting Information for more details).

This conformation has a distorted S shape, and it is highly stabilized by three hydrogen bonds, plus a salt bridge formed between the C-terminal carboxylate and the side chain of Arg[2] (Figure 2). In this conformation, most of the backbone atoms, as well as the side chains of Phe[3], Ser[4], and Arg[5], are exposed to the surface, where they become available to make appropriate interactions with the enzyme. On the other hand, the side chains of Gln[1], Arg[2], and Arg[5] are involved in intrapeptide hydrogen bonds, which stabilize the bioactive conformation. Nevertheless, the role of Arg[2] is not essential, as demonstrated by the fact that the FSR peptide retains activity (peptide **2**). The hydrogen bond between the nitrogen side chain of Gln[1] and the carbonyl oxygen of Phe[3] seems to play a critical role in the 3D architecture of the bioactive conformation for active

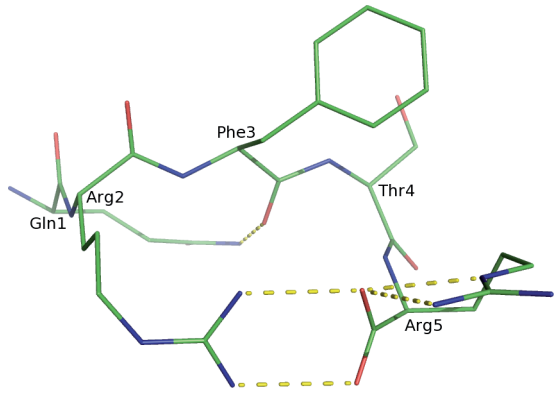

Figure 2. Representation of the 3D structure of our proposed bioactive conformation for Opiorphin hNEP inhibition. Intramolecular hydrogen bonds are indicated by dashes. Hydrogen atoms of side chains have been omitted for clarity.

peptides **1**, **7**, and **8**, stabilizing the backbone conformation of the FSR moiety. Accordingly, the deletion of the Gln[1] (peptide **3**) and even the substitution of Gln by Asn (peptide **5**) yield inactive compounds, which are not able to adopt the bioactive conformation. The hydroxyl group of the Ser[4] residue does not play any critical role in the stabilization of the putative bioactive conformation of Opiorphin for hNEP. However, replacement of this residue by Thr results in inactivity (peptide **6**). Because our proposed bioactive conformation was not found in its low-energy conformation library, it seems that the presence of an additional methyl group destabilizes the backbone of the bioactive conformation. Removal of the Arg[5] residue also yields an inactive analogue (peptide **4**). The inability of this peptide to reach the bioactive conformation shows the critical role of this C-terminal residue in the Opiorphin 3D spatial arrangement when interacting with hNEP. In addition, taking into account the carboxydipeptidase nature of NEP, it is likely that this may be due to its backbone atoms, rather than to its side chain. Accordingly, the most important structural feature in the bioactive conformation of Opiorphin looks like the backbone conformation of FSR sequence. Therefore, the activity of deletion peptide analogues seems to depend primarily on their ability to preserve the backbone conformation of these three C-terminal residues.

The C- and S-shaped conformations of Opiorphin that we propose as bioactive conformations for hAPN and hNEP, respectively, look rather unrelated. Their superimposition along the peptide backbone shows an rmsd of 2.24 Å that confirms their poor similarities (Figure 3). Such differences between them reflect the distinct three-dimensional arrangements of catalytic functions at both binding sites.

hNEP and hAPN are membrane-bound zinc metallopeptidases that belong to different families: hNEP is an M13 peptidase, while hAPN is an M1 peptidase. Although they share a common catalytic pattern, differences in the spatial arrangement of the binding site make NEP function as a carboxydipeptidase that cleaves substrates at the N-terminal side of hydrophobic residues such as Phe, Leu, and Met,[26] while making APN release the N-terminal amino acid from peptide substrates that are not substituted α-amino acids.[27] Our results are in accordance with the logical fact that a single conformation of Opiorphin could not simultaneously fulfill the requirements of both enzymes. Being an endogenous peptide, Opiorphin has most probably been naturally optimized

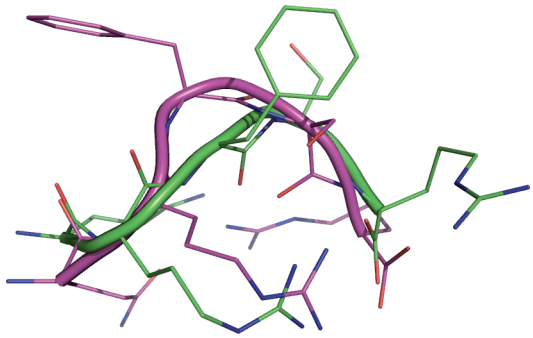

Figure 3. Superposition of backbone atoms of hAPN (magenta) and hNEP (green) proposed bioactive conformations. To highlight the overall shape differences, backbone atoms are shown as cartoons. Hydrogen atoms of side chains have been omitted for clarity.

to fulfill the spatial requirements of such structurally different binding sites. This is well reflected in the series presented in this study, where only $[Cys]^0$-Opiorphin retains dual activity, and any other modification abolishes at least one inhibitory activity.

In conclusion, our study reveals that instead of a common conformation of Opiorphin for the inhibition of both targets, two different bioactive conformations, one for each target, provide an adequate explanation of the SAR data observed in this series of Opiorphin's analogues. Such conformations explain the dual inhibitory activity of Opiorphin at a molecular level, which is the result of its inborn flexibility, and emphasizes the complexity of designing dual-acting NEP/APN nonpeptide inhibitors based on Opiorphin. Further investigation into the pharmacophore model based on these proposed bioactive conformations of Opiorphin is ongoing and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Experimental and computational methodological details, conformational search data (Table S2), workflow used to elucidate the bioactive conformations for hANP and hNEP (Figure S1), comparison of bioactive conformation for APN common to Opiorphin and $[Thr]^4$-Opiorphin (Figure S2), candidates for NEP bioactive conformation found by comparative conformational analysis (Figure S3), and details of the peptide−NEP complexes comparison with putative Opiorphin bioactive conformation candidates as a tool for choosing the most likely Opiorphin bioactive conformation for NEP (with Scheme S1 and Figures S4−S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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