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Rational design of novel neurotensin mimetics: Discovery of a pharmacologically unprecedented agent exhibiting concentration-dependent dual effects as antagonist and full agonist

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SUMMARY

We report the rational design of novel neurotensin mimetics through use of the Multiple Template Approach. This approach is based on our notion that a flexible peptide can be replaced by a partially flexible molecule, identified through testing a comparatively small number of molecules possessing a different intrinsic availability of conformations of the native peptide. The Multiple Template Approach has culminated in the discovery of a pharmacologically unprecedented agent, which behaves as a neurotensin antagonist at low concentration and as a full neurotensin agonist at high concentration.

INTRODUCTION

Neurotensin (NT) is a tridecapeptide (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu), distributed in the central and peripheral nervous systems [1–3]. It acts as a neuromodulator by stimulating the formation of intracellular cyclic guanosine monophosphate (*c*GMP) [4] and the turnover of phosphatidylinositol (PI) [5]. It is associated with numerous other actions in the central and peripheral systems [6]. Clinical studies implicate the involvement of NT in the pathophysiology of schizophrenia and suggest a possible role for NT agonists as therapeutics in the treatment of this disease [7].

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Due to the important role of NT in biology, we set out to design, construct and assay partial NT mimetics to arrive at full NT mimetics, i.e., organic molecules with improved stability and bioavailability that can mimic the action of the native peptide and that may possess improved selectivity, affinity and degrees of agonism or antagonism. The realization of these goals may lead us in turn to identify an approach for developing nonpeptidic peptide mimetics at a stage when information on the topography of the receptor is not available.

To design mimetics of NT at such a stage, application of the scaffolding approach or use of hierarchical approaches [8–15] would require that we deduce the receptor-bound conformation of NT or at least have knowledge of its backbone conformation, so that a rigid organic molecule can be designed to mimic this conformation. However, various conformational studies on NT reveal that the peptide is considerably flexible, so that numerous low-energy conformations have been identified [16–22]; this flexibility is a feature that greatly hampers the deduction of its receptor-bound conformation. Structure–activity studies employing fragments of NT have revealed that the NT(8–13) fragment is more potent than or equipotent to the parent peptide in its biological functions [23]. While conformational studies of NT(8–13) would reduce the amount of effort required for the conformational analysis, such studies will not eliminate the problems of conformational flexibility. Although the crystal structure of free NT(10–13) is available [24], the conformational dependence of small peptides on various environmental parameters in theory precludes the use of this crystal-structure information in a rational approach [25]. The costly screening approach thus appeared to be the only feasible route to the discovery of NT mimetics [26,27].

THE MULTIPLE TEMPLATE APPROACH

For the rational design of mimetics of a peptide like NT, we propose an approach that we term the Multiple Template Approach (MTA). This approach converts a vast number of conformers of a peptide to a comparatively small number of partially flexible molecules that can individually mimic a different portion of the conformations available to the native peptide. By testing all of these flexible molecules, one should arrive at a molecule that covers the receptor-bound conformation of the native peptide and that fits to the receptor. Each of these partially flexible molecules consists of a template of a different size, together with its appendant functional groups. The template is a structural counterpart of the spacer residues of the peptide, and the appendant functional groups are identical to the functional groups of the peptide. A functional group is defined as a group that interacts directly with the recognition site; a spacer residue is defined as a residue in the peptide that governs the conformation of the peptide and that can be replaced by an appropriate molecular unit without affecting the activity of the peptide. In some cases, a spacer residue can also serve as a functional group, or vice versa. As a caveat, the MTA is no longer suitable in such cases when the interaction of the dualistic residue with its receptor plays a decisive role in binding and receptor functions (these cases are not common and can be detected with relative ease), since treating the dualistic residue as a spacer residue will lead to weak mimetics, due to the loss of a functional group caused by substitution.

The partially flexible molecules are constructed by determining the functional groups and the spacer residues of the peptide, and by calculating the range of distance variables between the functional groups for all available conformations of the peptide. The range of distance variables and the characteristics of the functional groups determine the size of each template and the total

number of templates required to mimic all available conformations of the peptide. In order to introduce a minimal number of unknown parameters (i.e., the putative functional groups) at one time, it is better to choose at first a larger peptide fragment as the functional group, and then to iterate this process to shorten the peptide fragment. For example, we can first assign residues A to D of the peptide ABCDEFGH as a functional group, residues G and H as another functional group, and residues E and F as a spacer. Next, we can assign residues A to C as a new functional group and residue D as a new spacer. In some special cases, three functional groups can be chosen to expedite the iteration process, which is exemplified in the application to the NT mimetics.

To elaborate the MTA, we give an example of a peptide comprising a proline residue serving as spacer and two charged functional groups, designated as R^1 and R^2, attached to carbons C^1 and C^2, respectively (Fig. 1). In an actual case, assignment of the functional groups and spacer residues can generally be deduced and verified from an analysis of structure–activity relationships of a peptide and its analogues. The assignment is based on the well-accepted fact that more than one interacting point normally exists between a recognition site and its ligand. If substitution of one residue by another causes only a partial loss of activity, this residue is a functional group, since the substitution removes only one interacting point, and at least one remains. However, the substitution of a spacer residue with an improper residue is likely to remove all interacting points due to significant conformational changes, thus resulting in a total or nearly total loss in activity.

The key to the design of a mimetic for this peptide is to determine the distance D. Once D is known, an appropriate template can be chosen to fix the two functional groups. One immediate approach is to determine the receptor-bound conformation of the peptide and then to measure D in that conformation. However, this fails if the receptor-bound conformation is unknown.

To avoid the necessity of determining the receptor-bound conformation, the MTA focuses on determining the range of D, which can be easily obtained by a systematic conformational search. In this example, D was found to vary from 5.5 to 6.6 Å (using the MM2 force field in Macro-Model and assuming that all the amide bonds are trans) [28]. The maximum distance variation is 1.1 Å, which assures that if either R^1 or R^2 resides six consecutive sp^3 atoms or seven consecutive single bonds away from the template, only one template with D between 5.5 and 6.6 Å is required to mimic the peptide, despite the fact that a number of conformations are available to it.

The reason for this is that a chain with three consecutive single bonds between R^1 and C^1 or between R^2 and C^2 allows the group in question to compress or stretch by a maximum of 1.2 Å (vide infra). However, the distance C^2–R^1 does not normally equal the sum of the distances C^2–C^1 and C^1–R^1, since the orientations of these distances are normally different. Three more consecutive single bonds need to be incorporated into the chain of R^1 in order to adjust the orientation of the distance of C^1–R^1 equal to the orientation of the distance of C^1–C^2 around the x, y and

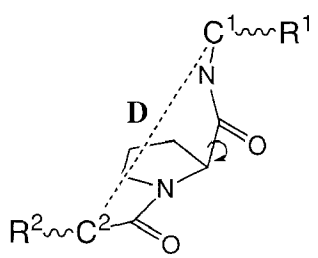

Fig. 1. Model of a proline-containing peptide.

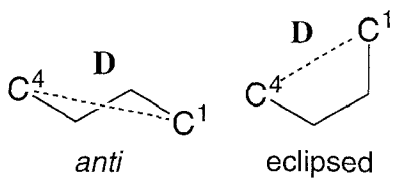

Fig. 2. The compressibility of *n*-butane.

z axes, so that these distances become additive. To compensate for the distance variation of C^1–R^1 (maximum 1.2 Å), caused by the orientational adjustment, one more single bond is required in the side chain of R^1, so that the first three bonds are used for orientational adjustment and the last four bonds for distance adjustment by a maximum of 2.4 Å. Consequently, to allow the C^1–C^2 distance change, caused by alteration of ψ^1, to be compensated by the compressibility of the side chain R^1, six consecutive sp^3 atoms or seven consecutive single bonds are required to be incorporated into the side chain of R^1.

The 'compressibility' factor can be perceived by using *n*-butane as an example (Fig. 2): the C^1–C^4 distance is 3.9 Å in the anti conformation (2.2 kcal/mol) and 2.7 Å in the eclipsed conformation (10.2 kcal/mol) [28]. The energy cost of 8.0 kcal/mol for compressing the C^1–C^4 distance by 1.2 Å can be compensated by the energy gained from the consequent interaction of a charged functional group attached to C^1 or C^4 with its recognition site, since the average intrinsic binding energy of a charged functional group is 9.9 kcal/mol [29]. This reveals that the compressibility of *n*-butane allows the C^1–C^4 distance to vary 'freely' by 1.2 Å when this chain bears a charged group. If it bears an uncharged group, more C–C bonds will be required to achieve compression by the same distance. Consequently, the MTA converts a number of conformations of the peptide to one molecule, which can assume all the distances between two functional groups in the peptide.

On a conceptual level, the MTA is different from the scaffolding approach, although both use linking templates. The MTA searches for a partially flexible molecule, which covers the receptor-bound conformation of the native peptide, through devising a small set of partially flexible molecules. These molecules individually mimic different subsets of conformations accessible to the peptide, but together mimic all the conformers of the peptide. Comparatively, the scaffolding approach searches for a rigid molecule that mimics one particular receptor-bound conformation through testing a large number of different scaffolds, and each scaffold mimics only one conformer of the native peptide. The MTA is proposed for flexible peptides such as NT(8–13), whose receptor-bound conformation is unknown. In contrast, the scaffolding approach can apply only to peptides of known conformation, such as the equine angiotensinogen fragment (H-Leu-Leu-Val-Tyr-OMe) [11] and the Gly-Ala-Ala-Gly fragment [8], or to relatively rigid peptides such as the cyclic hexapeptide SRIF [9].

APPLICATION OF THE MTA

In the application of the MTA to the design of NT(8–13) mimetics, Pro^{10} was assigned as a spacer residue, since an NT analog possessing D-proline substitution for Pro^{10} gave no response at 1 μM concentrations in binding and functional assays [30]. This role for Pro^{10} is also reasonable because its five-membered ring fixes the dihedral angle between C^α and the ring nitrogen to a small range of about ±20°. The side chains of Arg^8 and Arg^9 were assigned as charged functional groups, based on the fact that replacement of Arg^8 or Arg^9 with a D-arginine or a D-lysine or the absence of Arg^8 resulted only in a partial loss of activities [31]. In addition, the distance variation between the two arginine side chains is relatively small, since Arg^8 and Arg^9 are neighbors, and the distance variation between C^1 and C^2 (Fig. 1) is also relatively small, as shown above. For these reasons, we chose the last three residues as the functional group R^1 and the side chains of Arg^9 and Arg^8 as the functional groups R^2 and R^3, respectively (Fig. 3A). These functional groups form a triangle at the attachment points of these groups (i.e., C^1, C^2 and C^3, Fig. 3A), whose size is governed by the backbone conformations of NT(8–13).

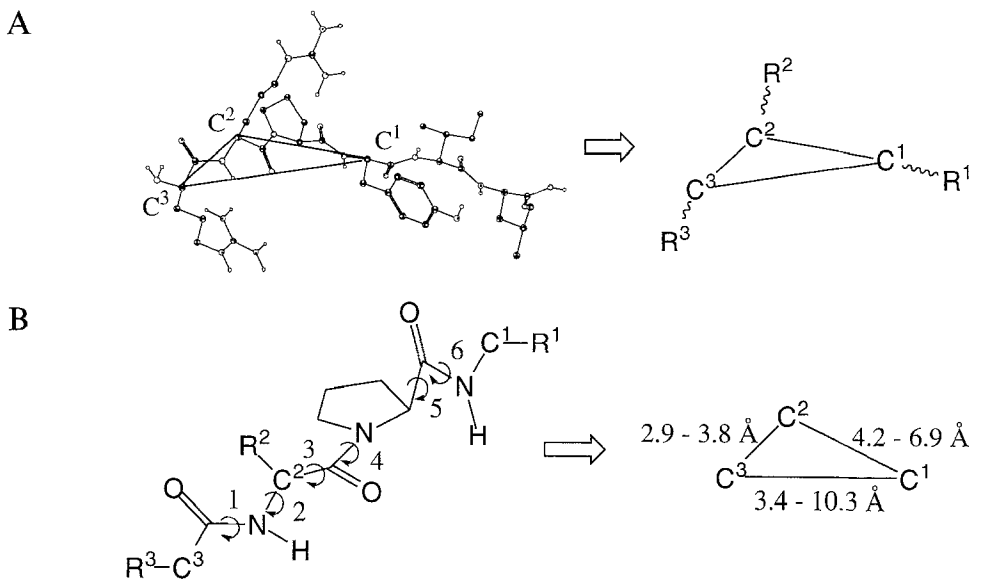

Fig. 3. (A) Triangle model of NT(8–13). (B) Definition of torsions affecting the size of the triangle and ranges of the triangle size.

A vast number of conformations of NT(8–13) were thus converted to a comparatively small range of sizes of the triangle, which was obtained by measuring the distances among the three functional groups in 306 energy-minimized conformers of the structure shown in Fig. 3B, where R^1, R^2 and R^3 are hydrogen atoms. These conformers were systematically generated without the trans amide bond constraint, by rotating six rotatable bonds as indicated in Fig. 3B at 30° increment and a van der Waals factor of 0.8, followed by energy minimization with the Tripos force field employing Sybyl and SysSearch [32]. In view of the range of the triangle size shown in Fig. 3B, 12 templates must be tested to cover the entire range of the triangle size. Because R^3 (Fig. 3A) allows for 1.2 Å contraction or expansion, six templates are required to cover the range of distances between C^1 and C^3 (3.4 to 10.3 Å), i.e., the first template covers the range from 3.4 to 4.6 Å, the second template from 4.6 to 5.8 Å, and so on. Similarly, two templates are required to cover the range between C^1 and C^2 (4.2 to 6.9 Å), and one template is required to cover the range between C^2 and C^3 (2.9 to 3.8 Å). Consequently, a total number of 12 (6×2×1) templates is required.

Selection of an indole-2-carboxylic acid as the basis for two molecular templates (Fig. 4A) from the set of 12 possible templates was based on synthetic expediency, in that either of the regioisomeric C5- or C7-substituted indole intermediates could be readily obtained from a single indole precursor. The functional groups R^1 and R^2 initially contain four C–C bonds from arginine, and these bonds can adjust themselves around the x, y and z axes to adopt the necessary orientations of the guanidino groups. To ensure the compressibility of these functional groups, we introduced three additional sp^3 atoms to these appendages (vide supra). To facilitate the synthesis, a methylene group was replaced by an oxygen atom (ether linkage) in the attachment of the guanidine appendage to C5 or C7 of the indole ring. The steric hindrance resulting from these extra atoms may be tolerated by the receptor topography, but in cases where it is not, use of a

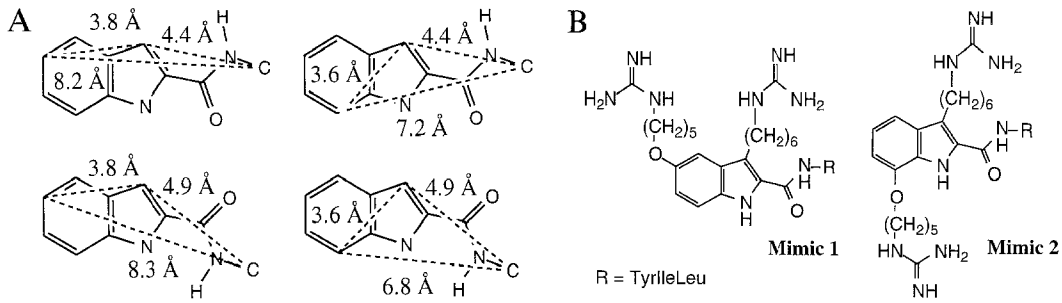

Fig. 4. (A) Different sizes represented by the indole-2-carboxylic acid nucleus. (B) Partial mimetics of NT(8–13).

smaller template will be required. The foregoing considerations led to the partial NT mimetics (mimics **1** and **2**) shown in Fig. 4B.

BIOLOGICAL RESULTS

The mimics **1** and **2** and the mimetics of the Arg-Arg-Pro portion, 3-(6-guanidinohexyl)-5(or 7)-[(5-guanidinopentyl)oxy]indole-2-carboxylic acids, were synthesized according to the procedure of Kozikowski et al. (manuscript in preparation) and tested for their ability to compete for [^{3}H]NT binding in N1E-115 cells [33]. While the Arg-Arg-Pro mimetics were found to be inactive at concentrations as high as 0.1 mM, both mimics **1** and **2** interacted with the NT receptor, with K_d values for mimics **1** and **2** and NT(8-13) of 3.3 μM, 1.9 μM and 0.61 nM, respectively [31,33].

The biological function of these mimetics was evaluated by their action on the intracellular *c*GMP production in N1E-115 cells [33]. Mimic **1** was a competitive antagonist of NT in this experiment. Interestingly, mimic **2** exhibited unprecedented dual effects, depending on concentration. When tested as an antagonist under the same conditions as used for mimic **1**, mimic **2** acted as a competitive antagonist of NT. At concentrations of 10 to 100 μM, it acted as a full agonist at the NT receptor, with an EC_{50} of about 19 μM. The literature contains examples of nonpeptidic mimetics (e.g., an SRIF mimetic [9]) which behave as agonist and antagonist at different concentration levels. However, these compounds are *partial* agonists, not *full* agonists like mimic **2** [33].

Additionally, mimic **2** was found to stimulate PI turnover as measured by the accumulation of inositol monophosphate. Using mimic **2** at 100 μM concentration, PI turnover was increased by 140% of basal levels, while maximal stimulation by NT was 180% of the basal value [33]. More conclusively, mimic **2** was tested for its ability to stimulate *c*GMP formation in cells on which the NT receptors were specifically desensitized or deactivated by [D-Lys^8]neurotensin(8–13), abbreviated as NT2. This NT2 analog has been reported to desensitize and downregulate the NT receptors [34]. Additionally, it has been reported that this desensitization is homologous and specific to the NT receptors [35]. Others have shown that under desensitization conditions the NT receptor is internalized beneath the cell membrane and therefore not available for ligand–receptor interactions [36]. After desensitizing the NT receptors with NT2, we found that both mimic **2**- and NT-stimulated *c*GMP production was dramatically decreased as compared to the untreated cells [33]. This result indicates that the observed stimulation by mimic **2** in the untreated cells stems from the *specific* interaction of mimic **2** with the NT receptors [33].

DISCUSSION AND CONCLUSIONS

As evident from the biological results, mimics **1** and **2** interact with the NT receptors. According to the desensitization experiment, mimic **2** *specifically* activates the NT receptors, but not other receptors such as G-protein-coupled receptors. Although the two partial NT mimetics exhibit only micromolar potencies, these compounds are nonetheless intriguing from the standpoint of their different functional activities. In particular, mimic **2** may serve as an important tool to further characterize the NT receptor and its diverse binding sites in N1E-115 cells [33].

It is important to note that the replacement of Pro^{10} in NT(8–13) with D-proline leads to a structure that fails to exhibit either binding or functional activities in the micromolar range and that the synthesized Arg-Arg-Pro mimetics and the tetrapeptide fragment Pro-Tyr-Ile-Leu were found to be inactive [24,30]. These findings rule out the possibility that even major structural changes to the Arg-Arg-Pro portion may be inconsequential to receptor recognition, and exclude the speculation that equally potent compounds could have just as easily been found by randomly preparing structures with two charged functional groups. Furthermore, the different biological functions (agonism and antagonism) of mimics **1** and **2** imply a relationship between the template size and the degree of activation of the NT receptors. The necessity for the guanidino side-chain appendages at position 5 or 7 of the indole moiety of the mimics is also underscored, since mimic **1** is different from mimic **2** only in that the surrogate of the functional group of residue 8 of NT(8–13) is located in a different region of 3D space. Thus, it is likely that the observed activities of these mimetics result from their entire structures, which rules out the possibility that a template of arbitrary size will lead to the observed activity.

As a further caveat, we note that the present work does not imply that the study of only two templates will be sufficient to achieve success in every case, nor does it exclude the possibility that other templates from the set of 12 possessing different geometries (or, for that matter, even completely flexible linking templates) could do just as well or better than the two templates reported herein. In theory, 12 templates must be tested to cover the entire conformational space of the peptide. Although the MTA is random over these 12 molecules, it is rational since it reduces 306 conformations of the structure shown in Fig. 3B to just 12 templates.

The information gleaned from the present study thus offers important structural insights into how to develop an NT agonist or antagonist, although the MTA itself provides no structural insights into the agonism and antagonism of the designed compounds. The efficiency of developing pharmacologically useful agents guided by the MTA can best be appreciated by noting that, prior to our work, 'lead' NT mimetics of only micromolar potency were identified after randomly screening thousands of compounds [26,27]. By further exploring the optimal size of the templates from the set of 12 and by applying the MTA to residues 11 to 13, we may be able to arrive at fully nonpeptidic, high-affinity agonists and antagonists of NT and to verify further the practicality of the MTA.

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