Bioorganic & Medicinal Chemistry Letters, Vol.3, No.3, pp. 405-412, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

THE USE OF SYNTHETIC PEPTIDE COMBINATORIAL LIBRARIES FOR THE DETERMINATION OF PEPTIDE LIGANDS IN RADIO-RECEPTOR ASSAYS: OPIOID PEPTIDES

Richard A. Houghten* and Colette T. Dooley
Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, CA 92121 USA

Abstract

A synthetic peptide combinatorial library (SPCL) was prepared, composed of 52,128,400 L-amino acid hexapeptides, which was used with an iterative selection process to determine peptides capable of inhibiting binding of [^{3}H] [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO) to crude rat brain homogenates. The first five residues corresponded exactly to the naturally occurring opioid peptide sequences of methionine and leucine enkephalin.

Introduction

The naturally occurring opioid peptides methionine and leucine enkephalin were isolated and identified from biological sources following years of laborious effort[1]. The past seventeen years have seen the synthesis and screening of thousands of analogues of the original biologically active sequences in an attempt to optimize their activity[2,3]. These analogues, however, represent only a small fraction of the possible pentapeptides (20^5 if one considers only the L-amino acids). Existing methods for the synthesis and screening of large numbers of peptides are severely limited in their ability to generate such numbers, i.e., millions[4-6], and/or in their inability to generate unmodified free peptides in quantities enabling solution interaction at concentrations appropriate for relevant *in vitro* assays[7-12].

We have developed synthetic peptide combinatorial libraries (SPCLs) composed of mixtures of free peptides, each of which in total exceed 50 million hexapeptides[13]. These libraries have been prepared in quantities which can be used directly in virtually all existing assay systems. We have employed SPCLs in a variety of assay systems, including the use of competitive ELISA to study the binding of synthetic peptide antigens to monoclonal antibodies in which the known antigenic determinant was precisely identified[13,14], in microdilution assays for the development of novel antimicrobial peptides (*S. aureus, P. aeruginosa, C. albicans*)[13,15], and for the development of potentially useful antiviral peptides in plaque inhibition assays (HIV-1 and HSV, work in progress). Thus, in the specific example presented here, a library composed of 52,128,400 nonacetylated hexapeptides, along with an iterative selection process, was used to confirm the utility of the SPCL approach for the identification of specific sequences capable of inhibiting the binding of a radiolabeled ligand to its receptor.

We chose to use the opioid receptor system since the enkephalins, known to be the endogenous ligands, are pentapeptides and would thus be expected to be among the active peptides detected using the aforementioned iterative process. There are at least three known subclasses of opioid receptors: mu, delta and kappa[3]. Enkephalins bind to each of these binding sites with differing affinities. For simplicity, we used an analogue of met-enkephalin ([^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin, DAGO) which is known to bind specifically and with high affinity to the mu binding site as the standard ligand.

Methods

Preparation of synthetic peptide combinatorial libraries

An SPCL consisting of six-residue peptide sequences having free N-terminals and amidated C-terminals was synthesized in which the first two amino acids in each peptide were individually and specifically defined, while the last four amino acids consisted of equimolar mixtures of 19 of the 20 natural L-amino acids (cysteine was omitted from this library). This generated 400 different peptide mixtures, each represented by the formula O_1O_2XXXX-NH_2 [O_1O_2 = AA, AC, AD, AE, etc., through YV, YW, YY; each X represents an equimolar mixture of the 19 amino acids] and containing a total of 130,321 combinations (19^4). This library, in total, contains 400 x 130,321 = 52,128,400 hexapeptides.

The SPCL was assembled using the solid phase approach[4] on methylbenzhydrylamine (MBHA) polystyrene resin in combination with simultaneous multiple peptide synthesis[6] using t-Boc protected amino acids. The XXXX-resin was prepared using a process of division, coupling, and recombination (DCR) of individual resins, which ensures the equimolarity of each peptide within the XXXX-resin as described previously[13]. Briefly, 19 equal portions of resin (and, therefore, equal numbers of milliequivalents) were placed into porous polypropylene packets and then coupled to each of the protected N-α-t-Boc amino acids. Coupling completion can be determined using either Kaiser's ninhydrin test[16] or Gisin's picric acid procedure[17]. The resins were removed from their packets, combined, and thoroughly mixed. The resulting mixture (X-resin) was then divided into 19 equal portions, the N-α-t-Boc protecting groups were removed with trifluoroacetic acid (TFA), and the resulting TFA salts neutralized. The DCR process was repeated three more times to yield XXXX-resin, representing an equimolar mixture of 130,321 tetramers (19^4). This XXXX-resin was then divided into 400 equal portions and the two individual defined positions, O_1 and O_2, were coupled using the SMPS method[6]. Amino acid analysis confirmed the expected equimolarity (± 10%). Following deprotection and cleavage from the resins, each of the 400 peptide mixtures was extracted with water to yield a final peptide concentration of 1-3mg/ml. This ensures that there is a sufficient concentration of each individual peptide within a mixture for use in standard *in vitro* receptor assays. Thus, at 1.0mg/ml each of the 130,321 (19^4) peptides within each peptide mixture is present at a concentration of approximately 10.3nM.

Radio-receptor assay

Crude membrane homogenates were prepared using a modification of the method described by Pasternak[18]. Rat brains frozen in liquid nitrogen were obtained from Rockland, Inc. (Gilbertsville, PA). The brains were thawed, the cerebella removed, and the remaining tissue weighed. Each brain was individually homogenized in 40 ml Tris-HCl buffer (50mM, pH 7.4, 4°C) and centrifuged (Sorvall RC5C SA-600 16000rpm) for 10 minutes. The pellets were resuspended in fresh Tris-HCl buffer and incubated at 37°C for 40 minutes. Following incubation, the suspensions were centrifuged as before, the resulting pellets resuspended in 100 volumes of Tris buffer, and the suspensions combined. Membrane suspensions were prepared and used in the same day. Protein content of the crude homogenates ranged from 0.15 - 0.2mg/ml as determined using the method described by Bradford[19].

Binding assays were carried out in polypropylene tubes. Each tube contained 0.5ml of membrane suspension, 8nM [^{3}H]-[D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGO) (specific activity 36 Ci/mmole, 160,000 cpm), 0.08mg/ml peptide mixture and Tris-HCl buffer in a total volume of 0.65ml. Assay tubes were incubated for 60 minutes at 25°C. The reaction was terminated by filtration through GF-B filters. The filters were subsequently washed with 6ml Tris-HCl buffer, at 4°C. Bound radioactivity was counted on an LKB Beta-plate Liquid Scintillation Counter and expressed in counts per minute (cpm). Inter- and intra-assay variation standard curves were determined by incubation of [^{3}H]-DAGO in the presence of a range of concentrations of unlabeled DAGO (0.13 - 3900nM). Both the tritiated and non-tritiated forms of DAGO were obtained from the National Insitute of Drug Abuse (NIDA) repository, as prepared by Multiple Peptide Systems (San Diego, CA). A control curve was included on each plate for each assay (using a 96-well format). Competitive inhibition assays were performed as above using serial dilutions of the peptide mixtures. IC_{50} values (the concentration necessary to inhibit 50% of [^{3}H]-DAGO binding) were then calculated using the software GRAPHPAD (ISI, San Diego, CA) and were found to be consistent in three determinations.

Results

Screening and selection of peptide ligands

Each of the 400 different peptide mixtures of the SPCL (O_1O_2XXXX-NH_2) was assayed to determine its ability to inhibit binding of [^{3}H]-DAGO. The concentrations of the individual peptides within each mixture (≈10nM of each at 1mg/ml) ensured that all of the most active sequences were determined in the first screening. Following the initial screening, IC_{50}s were determined for each of the most effective peptide mixtures. YGXXXX-NH_2, with an IC_{50} = 3452nM, was found to be one of the two most effective inhibiting peptide mixtures (Figure 1, Table 1) with WWXXXX-NH_2 having similar activity (the iterative process for WWXXXX-NH_2 has not been completed and will be reported elsewhere). An iterative process was begun in which the subsequent X positions of YGXXXX-NH_2 were defined with each of the 20 natural L-amino acids.

Twenty new peptide mixtures were synthesized in which the third position of the peptide mixture YGXXXX-NH_2 was defined (YGOXXX-NH_2, i.e., YGAXXX-NH_2, YGCXXX-NH_2, YGDXXX-NH_2, etc.). The peptide mixture sequences at each step of the screening and selection process, along with their respective IC_{50}s, are illustrated in Table 1. Of the 20 new peptide mixtures synthesized, YGGXXX-NH_2 (IC_{50} = 3254nM) was found to be the most effective inhibiting peptide mixture. The next best case, YGFXXX-NH_2, was approximately 50% less effective. All other substitutions in the third position were found to have activities at least two times less than YGGXXX-NH_2 (Table 1). The iterative process was then carried out in an identical manner for the remaining three positions.

On defining YGGOXX-NH_2, the two most active cases YGGFXX-NH_2 (IC_{50}=153nM) and YGGWXX-NH_2 (IC_{50}=292nM) were substantially more active than the other 18 cases, being 34- and 18-fold times more effective than the third most active case YGGYXX-NH_2 (IC_{50}=5190nM), and 21- and 11-fold times more effective than YGGXXX-NH_2. This illustrates the specificity of the fourth position in the enkephalin sequence. When the fifth position of YGGFXX-NH_2 was defined (YGGFOX-NH_2), it was found that YGGFMX-NH_2 was the most effective inhibiting mixture (IC_{50} = 28nM) and YGGFLX-NH_2 was the fourth most effective inhibitor (IC_{50}=74nM). The first five residues of the peptide sequences in YGGFMX-NH_2 and YGGFLX-NH_2 exactly match the sequence of naturally occurring methionine- and leucine-enkephalin which are known to recognize this receptor. The second and third most effective cases at this position were YGGFFX-NH_2 (IC_{50}=57nM) and YGGFIX-NH_2 (IC_{50}=67nM). Upon defining the final position (YGGFMO-NH_2) little improvement was found. YGGFMA-NH_2 was the most effective peptide (IC_{50}=28nM), but was only 10-fold greater than the least effective peptide YGGFMF-NH_2 (IC_{50}=223nM). These data illustrate the relative redundancy of this position. The sixth position of YGGFLO-NH_2 was also defined and tested. The most effective peptide was YGGFLG-NH_2 (IC_{50}=59nM), however, there is less than a two-fold improvement over YGGFLX-NH_2. The IC_{50} values for YGGFM-NH_2 and YGGFL-NH_2 were 34nM and 41nM respectively.

Conclusion

Using an SPCL composed of more than 52 million hexapeptides, combined with an iterative selection and enhancement process, we were readily able to determine a series of specific peptide sequences that inhibited binding of [^{3}H]-DAGO to the mu receptor. The equimolar representation of all possible individual peptide sequences in the SPCL enables precise identification of peptide ligands. It should be noted that no information about the sequence of the binding ligand is required to carry out determinations of this kind. While this example yielded the expected sequences of leucine and methionine enkephalin as the first five residues in the hexapeptide sequence, the same process carried out with an N-acetylated hexapeptide library yielded sequences with no obvious relationship to the known enkephalins. (The latter work is in progress and will be reported elsewhere.)

Unlike other peptide library approaches[8-12], SPCLs, as described in this and earlier examples[13-15], appear to be readily applicable to the identification of peptide ligands in the majority of existing assay systems. Since large numbers of highly active, defined peptide sequences can be readily generated through the use of SPCLs for any receptor system of interest, we believe that information derived from SPCLs will complement existing methods currently used for the detailed study of peptide/receptor interactions. We have found that SPCLs are of broad, general utility and greatly facilitate basic research and drug discovery involving peptides.

Acknowlegements

The authors wish to thank Ann Marie Prochera for technical assistance and Eileen Silva for assistance in preparing this manuscript.

Table 1: Binding inhibition of [^{3}H]–DAGO by peptide mixtures in the iterative process

Peptide mixture	IC_{50} (nM)
(a)	
YGXXXX–NH_2	3452
YPXXXX–NH_2	6005
YCXXXX–NH_2	15365
YLXXXX–NH_2	16055
YWXXXX–NH_2	16400
YHXXXX–NH_2	16770
(b)	
YGGXXX–NH_2	3254
YGXXXX–NH_2	3452
YGFXXX–NH_2	4037
YGNXXX–NH_2	7265
YGTXXX–NH_2	8689
YGSXXX–NH_2	8718
(c)	
YGGFXX–NH_2	153
YGGWXX–NH_2	292
YGGXXX–NH_2	3254
YGGYXX–NH_2	5190
YGGRXX–NH_2	11860
YGGCXX–NH_2	13415
(d)	
YGGFMX–NH_2	28
YGGFFX–NH_2	57
YGGFIX–NH_2	67
YGGFLX–NH_2	74
YGGFYX–NH_2	119
YGGFXX–NH_2	153

Peptide	IC_{50} (nM)
(e)	
YGGFMA–NH_2	28
YGGFMN–NH_2	28
YGGFMX–NH_2	28
YGGFMM–NH_2	30
YGGFMS–NH_2	34
YGGFMG–NH_2	35
YGGFML–NH_2	35
YGGFMH–NH_2	37
YGGFMY–NH_2	39
YGGFMQ–NH_2	40
YGGFMT–NH_2	41
YGGFMK–NH_2	41
YGGFMR–NH_2	46
YGGFMP–NH_2	57
YGGFMI–NH_2	80
YGGFMV–NH_2	107
YGGFMD–NH_2	167
YGGFME–NH_2	204
YGGFMF–NH_2	223

Peptide	IC_{50} (nM)
(f)	
YGGFLG–NH_2	59
YGGFLA–NH_2	60
YGGFLT–NH_2	65
YGGFLS–NH_2	65
YGGFLX–NH_2	74
YGGFLQ–NH_2	75
YGGFLY–NH_2	77
YGGFLN–NH_2	78
YGGFLL–NH_2	98
YGGFLP–NH_2	108
YGGFLM–NH_2	112
YGGFLH–NH_2	141
YGGFLR–NH_2	158
YGGFLI–NH_2	168
YGGFLV–NH_2	189
YGGFLK–NH_2	201
YGGFLD–NH_2	214
YGGFLE–NH_2	315
YGGFLF–NH_2	381

Table 1: IC_{50} values for the most effective inhibitory peptide mixtures at each iterative step are illustrated for a) peptide mixtures from the initial screening of the SPCL; b) the third position (YGOXXX-NH_2); c) the fourth position (YGGOXX-NH_2); d) the fifth position (YGGFOX-NH_2); e) the sixth position (YGGFMO-NH_2); and f) the sixth position (YGGFLO-NH_2). The IC_{50} value of the peptide mixture of the previous iterative step is boxed for comparison.

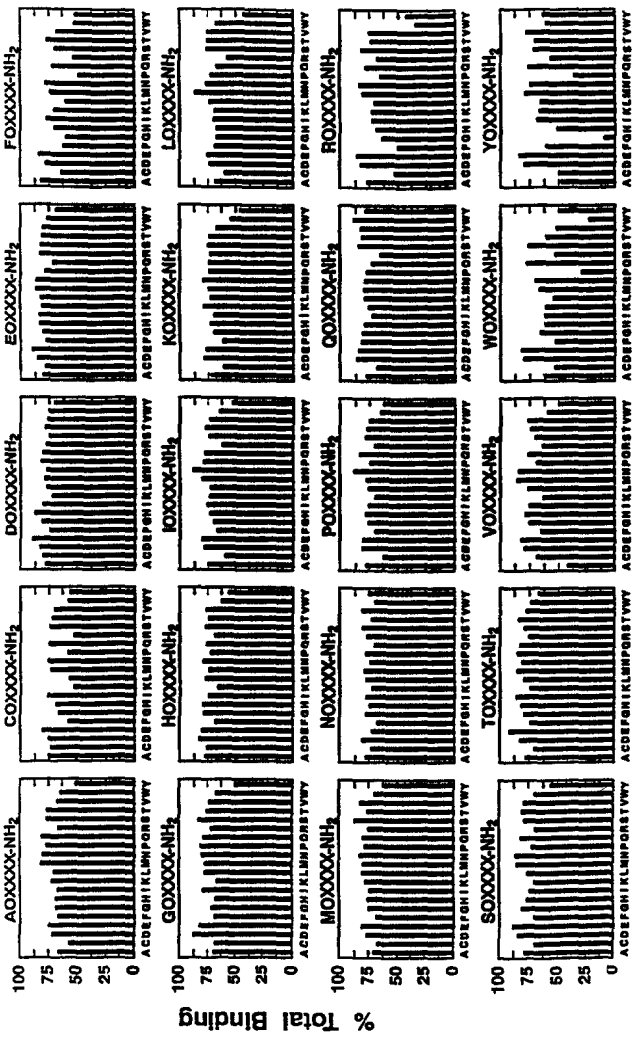

Figure 1: Initial screening of the SPCL ($O_1O_2XXXX\text{-}NH_2$) for ability to inhibit binding of [^{3}H]-DAGO to crude rat brain homogenates. Individual bar graphs are segregated by the first amino acid O_1, with individual bars in each graph representing the 20 amino acids making up the second position (O_2). The Y-axis represents the % Total Bound. Peptide mixtures (0.08 mg/ml) were incubated with [^{3}H]-DAGO (8nM), 0.5 ml rat brain homogenate (0.15-0.2mg/ml protein) and Tris-HCl buffer pH 7.4 at 25 °C for 60 minutes.

References and Notes

1. Hughes, J.; Smith, T. W.; Kosterlitz, H. W.; Fothergill, L. A.; Morgan, B. A.; Morris, H. R. *Nature* **1975**, *258*, 577-579.

2. Hruby, V. J.; Gehrig, C. A.; *Med. Res. Rev.* **1989**, *9*, 343-401.

3. Schiller P. W. *Progress in Medicinal Chemistry*; Ellis, G. P.; West, G. B., Eds.; Elsevier Science Publishers B.V.: Sweden, 1990; Vol.27, pp. 301-340.

4. Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149-2154.

5. Geysen, H. M.; Meloen, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 3998-4002.

6. Houghten, R. A. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 5131-5135.

7. Fodor, S. P. A.; Read, J. L.; Pirrung, M. C.; Stryer, L.; Lu, A. L.; Solas, D. *Science* **1991**, *251*, 767-773.

8. Geysen, H. M.; Rodda, S. J.; Mason, T. J. *Mol. Immunol.* **1986**, *23*, 709-715.

9. Scott, J. K.; Smith, G. P. *Science* **1990**, *249*, 386-380.

10. Devlin, J. J.; Panganiban, L. C.; Devlin, P. E. *Science* **1990**, *249*, 404-406.

11. Cwirla, S. E.; Peters, E. A.; Barrett, R. W.; Dower, W. J. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 6378-6382.

12. Lam, K. S.; Salmon, S. E.; Hersh, E. V.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *345*, 82-84.

13. Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84-86.

14. Pinilla, C.; Appel, J. R.; Houghten, R. A. *Vaccines 92*; Brown, F.; Chanock, R.M.; Ginsberg, H.S.; Lerner, R.A., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1992; p. 25.

15. Houghten, R. A.; Blondelle, S. E.; Cuervo, J. H. *Innovation and Perspectives in Solid Phase Synthesis. Canterbury*; Epton, R., Ed.; Solid Phase Conference Coordination, Ltd.: Canterbury, 1992; in press.

16. Kaiser, E. T.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595-598.

17. Gisin, B. F. *Anal. Chim. Acta* **1972**, *58*, 248.

18. Pasternak, G. W.; Alexander-Wilson, H.; and Snyder, S. H. *Mol. Pharmacol.*, **1975**, *11*, 340-351.

19. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.