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In Vitro and Direct In Vivo Testing of Mixture-Based Combinatorial Libraries for the Identification of Highly Active and Specific Opiate Ligands

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ABSTRACT

The use of combinatorial libraries for the identification of novel opiate and related ligands in opioid receptor assays is reviewed. Case studies involving opioid assays used to demonstrate the viability of combinatorial libraries are described. The identification of new opioid peptides composed of L-amino acids, D-amino acids, or L-, D-, and unnatural amino acids is reviewed. New opioid compounds have also been identified from peptidomimetic libraries, such as peptoids and alkylated dipeptides, and those identified from acyclic (eg, polyamine, urea) and heterocyclic (eg, bicyclic guanidine) libraries are reviewed.

KEYWORDS: positional scanning libraries, opioid receptor, in vivo screening, mixtures, peptides, peptidomimetics

INTRODUCTION

Binding to opioid receptors was first demonstrated in 1973¹⁻³; the endogenous peptides were first discovered in 1975.⁴ Since then, the search for new opioid ligands with better selectivity has continued. The search for the “holy grail” of opiates—that is, an opiate ligand without the traditional side effects (addiction, tolerance, respiratory depression, etc)—is ongoing, though with diminished expectations. Thousands of analogs of the natural opioid peptides enkephalin, dynorphin, endorphin, dermenkephalin, and dermorphin have been synthesized over the past 20 years and used to determine the workings of the opioid receptor family. More recently, highly selective compounds (in most cases structurally related to the classic opiates, eg, morphine) have been identified and used as research tools. Why then would one want to identify additional compounds that interact with opioid receptors? First, the question of the existence, number, and nature of subtypes of the 3 receptors remains unanswered. Two subtypes have been proposed for the mu receptor (μ_1 , μ_2); delta receptor subtypes have been

classified by 2 different schools of thought, into δ_1 and δ_2 by one school and δ_{cx} and δ_{ncx} by another; and subtypes reported for the kappa receptor include κ_1 , κ_2 , κ_3 , κ_{1a} , κ_{1b} , κ_{2a} , and κ_{2b} .⁵ To date, only a single gene has been identified for each of the 3 opioid receptors, mu,^{6,7} delta,^{8,9} and kappa.¹⁰ While alternate splicing and posttranslational modifications may prove to be responsible for receptor subtype expression, they have yet to be verified with pharmacological data. New ligands specific for, rather than selective for, the receptor subtypes would greatly facilitate binding studies. Structurally distinct new ligands may cover the binding spectrum of a particular receptor more completely and indicate whether ligands are binding to slightly different positions on the same receptor, thus giving rise to different inhibition profiles. The inability to determine specific ligands for the receptor subtypes may indicate that the differences observed in vivo are due to postbinding events (signaling). Second, new ligands may prove more useful as tools for in vivo study. Structurally diverse ligands will aid in the study of analgesic efficacy, addiction, and tolerance and will delineate central and peripheral antinociception, thus leading to effective new analgesics or treatments for drug abuse.

This review covers the use of combinatorial libraries in opioid binding assays, from an early demonstration of the concept to recently identified peptide and nonpeptide ligands. In our own research we have used combinatorial libraries to study opioids and, conversely, have used opioid receptors to study combinatorial libraries. This approach has demonstrated that mixture-based libraries can be used with a wide range of compound classes. It has also led to the development of a more rapid means of deconvolution for the identification of a wide variety of new ligands for the opioid receptors, and as first reported herein, a proof of concept illustration of its direct use in selected in vivo assays for the identification of inherently more advanced “hits” than those identified via traditional target-based in vitro assays.

SYNTHETIC COMBINATORIAL LIBRARIES

Synthetic combinatorial libraries were originally described for peptides but are now used with a wide variety of compound types; such libraries are responsible for the remarkable transformation of the manner in which new therapeutics and diagnostics are identified. The term *combinatorial* was

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originally applied to describe collections of compounds containing all possible combinations of the building blocks used in their preparation. "Combinatorial library" is a term now more generally used to describe collections of compounds ranging in number from a few dozen to more than 10^{12} . The first libraries described truly were combinatorial, since they represented a complete set of the compounds possible with the building blocks used and were generated either biologically (using molecular biology techniques) or synthetically. In the former, the peptide or protein diversity was presented on the surface of filamentous phage particles or plasmids.¹¹⁻¹³ In the latter, synthetic chemistry was used to generate a diversity of peptides attached to a solid support. The first synthetic combinatorial library described in the literature was composed of mixtures of tens to hundreds of thousands of octapeptides on plastic pins.¹⁴ This was followed by 2 simultaneous reports in 1991, the first of which described a second immobilized method, referred to as the one bead/one peptide approach.¹⁵ Immobilized libraries can be assayed for binding to soluble receptors (eg, antibodies). However, the vast majority of receptors are membrane-bound and cannot be readily screened with libraries attached to a support. Chemical synthesis of libraries on a solid support, which are subsequently cleaved and screened in solution, allows for a wider range of assays to be screened. This approach was first presented in the second of the 2 reports from 1991.^{16,17} The ability to screen mixtures that are free in solution confers decided advantages, in that a broad spectrum of assays can be used without alteration, and the concentration of the library mixtures being screened can be varied to adjust to the needs of the particular assay.

There remain 2 schools of thought regarding the screening of combinatorial libraries: (1) screen the greatest diversity possible, primarily through the use of mixtures, or (2) screen smaller diversities, primarily through the use of parallel arrays in conjunction with high-throughput screening. Although the successful use of mixture-based libraries was presented more than a decade ago, until recently the trend in the pharmaceutical industry has been to synthesize libraries as large parallel arrays of individual compounds, which are then screened individually or in small pools. Studies performed by our own and other laboratories, however, have shown that mixture-based libraries are a rapid, cost-effective means for the identification of extremely active, highly specific individual compounds.¹⁸

Combinatorial libraries are synthesized using solid-phase chemistry involving the consecutive incorporation of multifunctional building blocks with orthogonal protecting groups^{19,20} and contain a varied number of diversity positions. The solid support is typically polystyrene, but libraries have also been synthesized on plastic pins¹⁴ paper,²¹ cotton,²² and silicon chips.²³ Since amide bond formation on the solid phase has been optimized through 30 years of

solid-phase peptide synthesis, it has been used extensively in combinatorial synthesis.

One of 2 methods is used to generate the approximate equimolarity required for mixture-based libraries. In the first, equimolarity is achieved by mixing precoupled resins (a process termed divide-couple-recombine,¹⁶ split synthesis,¹⁵ or portioning-mixing²⁴). In the second, mixtures of incoming reagents are used during coupling.²⁵ The latter method also enables incorporation of mixture positions after the defined position has been incorporated, which is critical for most positional scanning libraries.

The initial combinatorial libraries described were all peptide libraries. Because of well-developed synthetic methods, amino acids and short peptides can be used as starting materials for the synthesis of low-molecular-weight heterocyclic libraries. The ability to exhaustively transform whole libraries of compounds to libraries of entirely different pharmacophores has been developed in our laboratory.²⁶⁻³¹ With this approach (termed libraries from libraries), the original diversity in a library may be expanded via chemical modification of the entire library. For example, peptide libraries may be modified by exhaustive methylation of their amide bonds to yield peptidomimetics,³¹ by complete reduction of the backbone amide carbonyl groups to yield polyamines,³² or by a combination of both to obtain alkylated polyamines.

Unlike parallel array libraries, mixture-based libraries must be deconvoluted to identify active compounds. While several mixture deconvolution methods have been described, including direct sequencing, encoded tags, high-performance liquid chromatography, mass spectrometry, and orthogonal synthesis, they have not been applied to the identification of opioid ligands and thus will not be discussed further in this review. The 2 most widely used deconvolution strategies for soluble combinatorial libraries are iterative^{16,17} and positional scanning.^{33,34} The iterative deconvolution approach uses a step-by-step selection and enhancement process to identify individual compounds. The most active sequence in this process is thus identified by the systematic reduction in the number of compounds in the most active mixture. The positional scanning deconvolution approach is a more rapid means to gather information about all possible variable positions in a soluble mixture library and was first presented by our laboratory in 1992.³³ As an illustration of the method for a simple tetrapeptide combinatorial library, envision 4 different amino acids (D, F, I, N) incorporated at each of 4 different variable positions, resulting in 256 (4^4) possible peptides. When this diversity is arranged as a positional scanning synthetic combinatorial library (PS-SCL), 16 peptide mixtures (4 separate mixtures for each of the 4 positions) are synthesized. The 4 positional sublibraries, namely $O_1XXX-NH_2$, XO_2XX-NH_2 ,

XXO₃X-NH₂, and XXXO₄-NH₂, contain all 256 peptides but differ in the arrangement of peptides within the mixtures and hence the location of the position defined. Thus, the 4 mixtures in O₁XXX-NH₂ are DXXX-NH₂, FXXX-NH₂, IXXX-NH₂, and NXXX-NH₂. Mixture DXXX-NH₂ contains the 64 peptides containing D at position 1. In sublibrary XO₂XX-NH₂, mixture XDXX-NH₂ contains the 64 peptides with D at position 2. The only peptides that mixtures DXXX-NH₂ and XDXX-NH₂ share are the 16 that have D in both positions (DDXX-NH₂). In this example, assume that the sequence FIND-NH₂ is the sole tetrapeptide having activity, with the other 255 being completely inactive. Since each positional sublibrary contains all 256 peptides, the individual tetrapeptide is present in only 1 mixture in each of the 4 positional sublibraries. Thus, the mixtures having activity will be FXXX-NH₂, XIXX-NH₂, XXNX-NH₂, and XXXD-NH₂ because of activity of FIND-NH₂. These 4 amino acids in their respective positions yield the sequence FIND in the initial screening. The corresponding peptide is then synthesized and tested for activity. It should be noted that the activity observed for each of the 4 mixtures is due to the presence of the tetrapeptide FIND-NH₂ in each mixture and is not due to the individual amino acids (F, I, N, and D) that occupy the defined positions. Often, more than 1 mixture is found to have activity at each position. Building block selection for the synthesis of individual compounds is based first on the activity of the mixture, but if there are too many possibilities, then similarities in chemical character may be used to reduce the number of individual compounds to be made. Freier and coworkers have provided an excellent discussion of the theoretical and experimental aspects of iterative and positional scanning deconvolution.^{35,36}

IDENTIFICATION OF OPIOID LIGANDS: CASE STUDIES USING COMBINATORIAL LIBRARIES

The monoclonal antibody (mAb) 3E7 raised against β -endorphin was used in many of the first screens of combinatorial libraries as a receptor of convenience. The antigenic determinant recognized by this antibody is well understood and represents the 6 residues of the N-terminal of β -endorphin, YGGFMT. This antibody is also known to bind to a variety of other related endogenous opioid ligands having similar N-terminal residues. This antigen/antibody system proved to be a good model for the early testing of library feasibility.³⁷ mAb 3E7 was first used in conjunction with the molecular biology approach, in which a library of peptide sequences was expressed on the N terminus of pIII, a fusion protein.¹¹ When a process of affinity purification termed *biopanning* was used, the enkephalin sequences YGGFM and YGGFL were not initially found, but peptides that bound with high affinity to mAb 3E7 (YGAFMQ, 13 nM) were reported. Using a related approach with mAb

3E7, McLafferty et al³⁸ tested a phage display library. Five of the 6 clones derived from the library contained sequences related to YGGF boarded by cysteine residues. mAb 3E7 was also used to demonstrate the principle of an approach in which thousands of individual peptides were synthesized on the silica surfaces of microchips using light-directed synthesis.²³ In this study, 1024 individual peptides were synthesized and screened to determine their binding to mAb 3E7. Active sequences (15 peptides) were found after visualization using a fluorescein-labeled antibody. The one bead/one peptide approach¹⁵ was also examined using mAb 3E7, in which binding was monitored by an alkaline phosphatase assay to produce a blue color in active beads. Specific peptides were identified by Edman degradation microsequencing. Six sequences were identified, the most active of which was found to be YGGFQ ($K_i = 15$ nM). Kassarian et al³⁹ used a similar process with I¹²⁵-labeled anti- β -endorphin mAb 3E7. Salmon et al⁴⁰ used an improved version of this approach, in which small quantities of peptide were released into and tested in solution. mAb 3E7 was again employed to verify the use of this library approach—3 peptide sequences were identified, 1 of which corresponded with the first 4 positions of methionine- and leucine-enkephalin. Although screening membrane-bound receptors is now possible, one bead/one peptide libraries have not been screened in membrane-bound opioid receptor assays.

Use of libraries with membrane-bound receptors has so far been limited to those that can be screened with compounds free in solution. Such a library was used in a case study our laboratory performed employing a μ opioid receptor binding assay and a hexapeptide synthetic combinatorial library composed of 400 different mixtures, each of which contained an equimolar mixture of 130 321 hexapeptides, for a library total of 52 128 400 L-amino acid hexapeptides. Two of the 6 positions were specifically defined using 20 naturally occurring L-amino acids (O); the remaining 4 positions were made up of mixtures of 19 amino acids (X; cysteine excluded). In the initial screening all 400 mixtures were tested at a single concentration for their ability to inhibit binding of tritiated DAMGO. IC₅₀ values were subsequently determined for the most effective peptide mixtures. YGXXXX-NH₂ was found to be the most effective inhibiting mixture. An iterative process was performed in which the subsequent X positions of YGXXXX-NH₂ were successively defined. For each of the 4 iterations, 20 peptide mixtures were synthesized. The final deconvolution yielded sequences in which the first 5 residues corresponded to the naturally occurring opioid peptide sequences of methionine and leucine enkephalin (YGGFMA-NH₂ IC₅₀ = 28 nM and YGGFLG-NH₂ IC₅₀ = 59 nM⁴¹).

Positional scanning deconvolution was also demonstrated using the μ opioid receptor binding assay as a case study.³⁴ A hexapeptide PS-SCL consisting of 6 separate sublibraries,

each having a single position defined with the remaining 5 positions as mixtures ($O_1XXXXX-NH_2$, $XO_2XXXX-NH_2$, $XXO_3XXX-NH_2$, $XXXO_4XX-NH_2$, $XXXXO_5X-NH_2$, and $XXXXXO_6-NH_2$; 108 separate peptide mixtures), was screened. Each mixture was composed of $\sim 18^5 = 1\,889\,568$ hexapeptides (cysteine and tryptophan were omitted). The 18 mixtures making up each of the 6 separate sublibraries addressed a single position in the hexapeptide. Each of the 6 positional sublibraries were different arrangements of the same 34 012 224 hexapeptides ($18 \times 18^5 = 34\,012\,224$). Used in concert, the data derived from the 108 mixtures yielded information about the most important amino acids for every position in the hexapeptide. This library was tested to determine the most active amino acids at each position. Peptides comprising all possible combinations of the amino acids active at each position were synthesized (position 1: tyrosine; position 2: glycine; position 3: glycine and phenylalanine; position 4: phenylalanine; position 5: phenylalanine, tyrosine, methionine, and leucine; and position 6: phenylalanine, arginine, and tyrosine). This generated $1 \times 1 \times 2 \times 1 \times 4 \times 3 = 24$ individual hexapeptides. The IC_{50} values obtained for these peptides ranged from 17 nM to 3276 nM. Nine peptides had activities below 200 nM, 5 of which possessed the sequences of methionine- or leucine-enkephalin within the first 5 residues.

IDENTIFICATION OF NOVEL OPIOID PEPTIDES

L-Amino Acid Libraries

Screening of an N-acetylated library, Ac-OOXXXXX-NH₂, in a μ binding assay yielded new opioid antagonists with no homology to known opioid peptides.^{42,43} The individual peptides derived from this library have been termed acetalins. Three peptides, Ac-RFMWMR-NH₂, Ac-RFMWMK-NH₂, and Ac-RFMWMT-NH₂, were tested for their selectivity among opioid receptors in μ , δ , κ_1 , κ_2 , and κ_3 receptor binding assays. These peptides showed high affinity for μ and κ_3 opioid receptors, somewhat lower affinity for δ receptors, weak affinity for κ_1 receptors, and no affinity for κ_2 receptors. They were found to be potent μ receptor antagonists in the guinea pig ileum assay and relatively weak antagonists in the mouse vas deferens assay. These peptides represent a new class of opioid receptor ligands. This study exemplifies the power of mixture-based combinatorial libraries for the identification of new ligands; such ligands would not have been found using classical structure-activity relationship (SAR) techniques.

Iterations were completed for additional mixtures found to have activity in the initial screening of the nonacetylated and acetylated hexapeptide libraries OOXXXXX-NH₂ and Ac-OOXXXXX-NH₂.⁴⁴ Two new series of nonacetylated hexapeptides not related to the enkephalins were identified, YPFGFO-NH₂ and WWP KHO-NH₂ (where O = 1 of 20

L-amino acids). Both possessed high affinity (IC_{50} values of the most active peptides were 10-15 nM) and selectivity for the μ receptor, individuals of which were found to be μ agonists. Two additional series were identified from the acetylated library, Ac-FRWWYO-NH₂ and Ac-RWIGWO-NH₂ (IC_{50} values of the most active peptides were 5-10 nM). The peptide Ac-FRWWYM-NH₂ was determined to be an agonist for the μ receptor, whereas the peptide Ac-RWIGWR-NH₂ was found to be an antagonist at this receptor. In total, 6 different families of sequences were identified from the 2 libraries (Table 1).

A positional scanning format of the hexapeptide library, in which 2 positions are defined for each sublibrary, was used to identify ligands for the delta receptor. The 3 related L-amino acid sublibraries (composed of identical hexapeptides but differing in the location of their defined positions, OOX XXXX-NH₂, XXOOXX-NH₂, and XXXXOO-NH₂) were used to identify a new ligand for the delta receptor, YGFDLV-NH₂ ($IC_{50} = 15$ nM). This peptide is similar to the sequences found when a single-position-defined positional scanning library was used in an earlier study, YHGWLV-NH₂ and YGMHLV-NH₂.⁴⁷ YGFDLV-NH₂ was found to be an agonist at the delta receptor.

An N-acetylated positional scanning decapeptide library was screened in binding assays specific for the kappa receptor. The library was made up of ~ 3 trillion decapeptides, with each of the 200 mixtures of these 2 separate libraries composed of ~ 200 billion decapeptides per mixture. From this extremely large pool of compounds, 2 peptide sequences having high affinities for the kappa receptor were identified from the N-acetylated library: Ac-YRTRYRYYYR-NH₂ ($IC_{50} = 28$ nM) and Ac-RGWFFHYKPKR-NH₂ ($IC_{50} = 30$ nM).

Burgess et al synthesized a library made up of a total of 96 peptides based on the sequences YGGFL-NH₂ and YGGFLRF-NH₂ to explore the effects of incorporating 2,3 methanoamino acids, in particular stereoisomers of 2,3 methanoleucine.⁵² None of the compounds, however, were found to show improved activity over the native peptides. The authors concluded that binding in mu and delta receptors is governed by amino acids other than the leu⁵ residue.

D-Amino Acid Libraries

A mixture-based combinatorial library containing all D-amino acid hexapeptides (Ac-ooxxxx-NH₂) was used to identify a novel ligand for the μ opioid receptor, Ac-rfwink-NH₂ ($IC_{50} = 18$ nM).⁴⁹ While the first 2 amino acid side chains (Arg and Phe) and the presence of the N-terminal acetyl is similar to that found in the acetalins, the D form of the amino acids was found to be essential. The systematic replacement of each of the 6 positions with each of the 20 L-amino acids yielded inactive peptides. The all-D-amino

Table 1. Opioid Ligands Identified From Combinatorial Libraries

Library	Receptor	Total No. of Compounds	Deconvolution	Individual Compound	Activity (nM)	Agonist/Antagonist	Ref
Tetrapeptide OXXX-NH ₂	Mu	6 × 10 ⁶	Iterative	YmFA-NH ₂	IC ₅₀ = 2	Agonist	45
Tetrapeptide OXXX-NH ₂	Mu	6 × 10 ⁶	PosScan	Y(DNve)G(L-Nal)-NH ₂	K _i = 0.4	Agonist	46
Tetrapeptide OXXX-NH ₂	Mu	6 × 10 ⁶	PosScan	YrAW-NH ₂	K _i = 1.8	Agonist	46
	Delta	6 × 10 ⁶	PosScan	Wy(aAba)R-NH ₂	K _i = 7	Agonist	46
	Kappa	6 × 10 ⁶	PosScan	ff(DNle)r-NH ₂	K _i = 1	Agonist	46
Hexapeptide OXXXXX-NH ₂	Mu	52 × 10 ⁶	Iterative	YGGFMA-NH ₂	IC ₅₀ = 28	Agonist	41
	Mu	52 × 10 ⁶	Iterative	YPFGFR-NH ₂	IC ₅₀ = 13	Agonist	44
	Mu	52 × 10 ⁶	Iterative	WWPR-NH ₂	IC ₅₀ = 10	Agonist	44
	Delta	52 × 10 ⁶	Iterative	YGGFLV-NH ₂	IC ₅₀ = 10	Agonist	
Hexapeptide Ac-OXXXXX-NH ₂	Mu	52 × 10 ⁶	Iterative	Ac-RFMWMK-NH ₂	IC ₅₀ = 5	Antagonist	42
	Mu	52 × 10 ⁶	Iterative	Ac-RWIGWR-NH ₂	IC ₅₀ = 5	Antagonist	44
	Mu	52 × 10 ⁶	Iterative	Ac-FRWWYM-NH ₂	IC ₅₀ = 33	Agonist	44
Hexapeptide OXXXXX-NH ₂	Mu	34 × 10 ⁶	PosScan	YGGFMY-NH ₂	IC ₅₀ = 17	Agonist	34
	Delta	34 × 10 ⁶	PosScan	YHGWLW-NH ₂	IC ₅₀ = 61	Not reported	47
	Delta	34 × 10 ⁶	Pos Scan	YGMHLV-NH ₂	IC ₅₀ = 45	Not reported	47
Hexapeptide OXXXXX-NH ₂	Delta	52 × 10 ⁶	PosScan	YGFDLV-NH ₂	IC ₅₀ = 15	Agonist	47
Decapeptide Ac-OXXXXXXXXX-NH ₂	Kappa	3.2 × 10 ¹²	PosScan	Ac-YRTRYRRYRR-NH ₂	IC ₅₀ = 28	none	
Hexapeptide Ac-OXXXXX-NH ₂	Orphanin	52 × 10 ⁶	PosScan	Ac-RYYRWK-NH ₂	IC ₅₀ = 23	Agonist	48
Hexapeptide D-amino acids Ac-OXXXXX-NH ₂	Mu	52 × 10 ⁶	Iterative	Ac-rfwink-NH ₂	IC ₅₀ = 18	Agonist	49
Hexapeptide D-amino acids OXXXXX-NH ₂	Mu	52 × 10 ⁶	Iterative	Iftwyr-NH ₂	IC ₅₀ = 5	Not reported	
Rhodamine-OXXX-NH ₂	Kappa	7.3 × 10 ⁶	PosScan	Rh-aOrn-R-7Aha-r-NH ₂	K _i = 5.7	Agonist	50
Trimer peptoid	Mu	3672	Iterative	CHIR 4531	K _i = 6	Not reported	51
Peptide/peptidomimetic	Mu and delta	96	Parallel	YGGF(2,3 methanoleucine)-NH ₂	Not reported	Not reported	52
N-alkylated dipeptide	Mu	57 500	Iterative	TPI 418-1	IC ₅₀ = 3	Agonist	53
Heptamine	Mu	37 791 360	PosScan	TPI 27-11	IC ₅₀ = 14	Antagonist	54
Urea	Mu	126 000	Iterative	TPI 632-4	IC ₅₀ = 100	Not reported	28
Hydantoins	Sigma	38 880	PosScan	TPI 610-2	IC ₅₀ = 62	Not reported	29
Tetrahydroisoquinoline	Sigma	43 472	Iterative	TPI 462-4	IC ₅₀ = 56	Not reported	55
Bicyclic guanidine	Kappa	102 459	PosScan	TPI 614-1	IC ₅₀ = 37	Not reported	18
Piperidine	Kappa	288	Parallel	RTI 5989-29	K _i = 6.9	Antagonist	56

acid peptide exhibits high selectivity for μ receptors (K_i values: $\mu_1 = 16 \pm 0.5$ nM, $\mu_2 = 41 \pm 4.8$ nM). Ac-rfwink-NH₂ had a K_i value of greater than 1500 nM for delta receptors, had very low affinity in assays for the κ_1 ($K_i > 2000$ nM) and κ_2 ($K_i > 5000$ nM) receptor subtypes, and had only modest affinity in assays for κ_3 receptors ($K_i = 288 \pm 71$ nM). Ac-rfwink-NH₂ was shown to be a full agonist in the guinea pig ileum assay ($EC_{50} = 433 \pm 43$ nM). This activity was antagonized by naloxone at a low concentration ($K_e =$

3.80 nM), indicating that it was an opioid effect mediated through interaction with μ opioid receptors. Ac-rfwink-NH₂ is a potent agonist at the μ receptor and induces long-lasting analgesia in mice. The *in vivo* potency of Ac-rfwink-NH₂ ($ED_{50} = 0.6$ nmol, intracerebroventricular) was found to be approximately twice that obtained for morphine ($ED_{50} = 1.29$ nmol, *icv*). Ac-rfwink-NH₂ produced antinociception equal to that of morphine following intraperitoneal (*ip*) administration. Since analgesia produced by *ip*-administered

Ac-rfwink-NH₂ is blocked by icv administration of naloxone, this peptide is most likely able to cross the blood-brain barrier.

A nonacetylated library made up entirely of D-amino acid hexapeptides (ooxxxx-NH₂) was also found to have activity at the mu receptor. Four iterative syntheses (20 mixtures for each iteration) were performed on each of the 2 mixtures found to be most active: imxxxx-NH₂ and itxxxx-NH₂. Upon completion of the iterative deconvolution process, the 2 peptides found to bind with the highest affinity to the mu receptor were iftwyr-NH₂ (IC₅₀ = 5 nM) and imswwg-NH₂ (IC₅₀ = 10 nM) (Table 1). These peptides are yet another class of opioid peptide, in that they do not have the typical N-terminal free tyrosine of endogenous ligands or the N-terminal arginine of the acetalins or Ac-rfwink-NH₂.

Mixed L- and D-Amino Acid Libraries

A synthetic peptide combinatorial library composed of mixtures of 50 different L-, D-, and unnatural amino acids (OXXX-NH₂) was screened in the 3 opioid binding assays. The tetrapeptide library contained 6.5 million peptides and required iterative deconvolution. Iterations were completed for the most active mixture at the mu receptor. After 3 iterative syntheses (50 mixtures each), the most active individual peptide found was YmFA-NH₂ (IC₅₀ = 2 nM). This compound was found to be a highly mu-specific receptor agonist.⁴⁵ A tetrapeptide positional scanning library that differed slightly in the amino acid making up the library was also synthesized and screened. Following an extensive study,⁴⁶ individual compounds were identified for all 3 opioid receptors, mu, delta, and kappa. The most active peptide for the mu receptor was Y(D-Nve-)G(L-Nal)-NH₂ (K_i = 0.4 nM), while the most mu-selective peptide found was YrAW-NH₂, with μ : δ and μ : κ ratios of greater than 1000 (Table 1). The most selective peptide identified for the delta receptor was Wy(aAba)R-NH₂ (K_i = 7 nM). The most active peptide identified for the kappa receptor was ff(D-Nle)r-NH₂ (K_i = 1 nM). The tetrapeptides identified for the kappa receptor were highly selective for this receptor, with κ : μ and κ : δ ratios of greater than 10 000.⁴⁶ The kappa-selective peptide kaffiralin (ffir-NH₂) is a full agonist in the GTP γ S binding assay (EC₅₀ = 10 nM hKOR-CHO cells). Kaffiralin generated a full dose-response curve in the mouse 55°C warm water tail flick assay and in the acetic acid writhing test after icv administration. It was ~10-fold more potent in the writhing test than in the tail flick test. After ip administration, kaffiralin did not produce a full dose-response curve in the tail flick assay, but it did in the writhing test. It was antagonized by an ip injection of nor-binaltorphimine (nor-BNI) but not an icv injection of nor-BNI. When given by ip administration, ffir-NH₂ may produce antinociception at spinal or peripheral receptors instead of supraspinal recep-

tors. There was no evidence of the peptide crossing the blood-brain barrier (J. Bidlack and B. Urban, oral communication, 1999).

Fluorescent-Labeled Libraries

In a recent study, kappa-selective peptides having intrinsic fluorescent properties were identified from a rhodamine-labeled tetrapeptide PS-SCL.⁵⁰ From a library of ~7.3 million rhodamine-labeled tetrapeptides, more than 250 individual peptides were synthesized from the most active mixtures. Eight individual rhodamine-labeled peptides were identified that were specific for the kappa opioid receptor, having binding affinities ranging from 5 to 20 nM (Table 1). The majority of the most active peptides identified contained at least 2 positively charged residues. Such strongly positively charged compounds have the potential for non-specific binding to the receptor through ionic interactions. This was found not to be the case since the same peptides lacking a rhodamine label did not inhibit binding of the radioligand to the kappa receptor (K_i >10 μ M). As rhodamine and rhodamine sulfonyl amide had no inhibitory activity, the peptide sequence together with the rhodamine moiety were necessary components of a single molecule responsible for receptor binding. We are currently pursuing a range of other fluorescent groups in a similar manner with both peptide and nonpeptide libraries for the identification of opioid receptor-specific ligands.

Orphanin FQ/Nociceptin

In October and November 1995, 2 separate reports were published^{57,58} on the identification of the endogenous ligand for the orphan receptor ORL1, a potential member of the opioid family of receptors. After establishing a binding assay in rat brain using tritiated orphanin FQ,⁴⁸ a hexapeptide positional scanning library containing 2 defined positions (1200 mixtures) was used to identify hexapeptide ligands for this receptor. The most active peptide found was Ac-RYYRWK-NH₂. The 5 most active compounds were found to be agonists using 3 separate biochemical bioassays: inhibition of cyclic adenosine monophosphate (cAMP) accumulation, stimulation of [³⁵S]GTP γ S binding, and inhibition of electrically induced contractions in the mouse vas deferens.⁵⁹

Peptidomimetic Libraries

The first use of a peptidomimetic library for the identification of opioids was described by Zuckermann et al,⁵¹ who screened a library of 5000 peptoids (oligo-N-substituted glycines). The library, composed of 3 sets of monomers (22 different monomers) from commercially available amines, and with the amino terminus either as a free amine,

N-acetylated or capped with cyclohexylurea, contained 204 peptoid trimers in 18 different mixtures. This library was screened in a μ -specific radioreceptor assay with ^3H -DAMGO as the radioligand. Through use of an iterative process in which the most active mixture from the first screening was deconvoluted by individually defining the first, second, and third positions in sequential order, 3 highly active compounds were found. The most active compound, CHIR 4531, had a K_i value of 6 nM.

A dipeptidomimetic library based on a dipeptide scaffold with the C-terminal and middle amide hydrogens replaced separately with 5 different alkyl groups (methyl, ethyl, allyl, benzyl, or naphthylmethyl) was also used to identify opioid

ligands.⁵³ The 2 variable amino acid positions of this library were made up of 50 different L-, D-, and nonproteinogenic amino acids. The library, which contained 57 500 different compounds and was screened against all 3 opioid receptors, required 2 iterative syntheses to identify individual compounds. Highly active individual compounds were identified for each of the 3 receptors: μ (TPI 418-1 K_i = 6 nM), δ (TPI 452-19 K_i = 39 nM), and κ (TPI 419-19 K_i = 1 nM) (Figure 1). All 3 were found to be agonists at their particular receptors. The selectivity of the μ and κ compounds was high, while that of the δ ligand was poor, with the compound binding equally to κ receptors.

Figure 1. Structures of nonpeptide compounds identified for opioid receptors.

Nonpeptide Libraries

In contrast to the stepwise synthesis of peptidomimetics mentioned above, the transformation of peptide libraries to libraries of entirely different pharmacophores has been reported.³¹ The approach, termed libraries from libraries, extends the diversity obtained with peptide libraries via chemical modification. Peptide libraries have been successfully modified by exhaustive methylation of their amide bonds to yield peptidomimetics³¹ and/or by reduction of the backbone amide carbonyl groups to yield polyamines.³²

A chemically modified peptide library in which all of the peptide amide carbonyls were reduced to methylenes was screened for opioid ligands. Mu-selective opioid compounds were identified from a library made up of 52 million different heptamines.⁵⁴ The library was obtained from the chemical transformation of a C-terminal amide hexapeptide positional scanning library. Combinations of the most active residues found at each position were synthesized as peptides, then reduced, and the activities of the resulting heptamines were determined. The activities of the individual compounds identified ranged from 14 to 345 nM. A truncation analog of the most active individual heptamine, red[YYFPTM-NH₂], was found to be equally active (red[YYFP-NH₂] IC₅₀ = 16 nM, Figure 1). It was found to be a selective mu antagonist in the guinea pig ileum assay. Preliminary results indicated that this compound was toxic to mice at high doses. A series of derivatives were then synthesized in which the backbone nitrogens of the compounds red[YYFP] and red[YYF] were per-alkylated with methyl, ethyl, allyl, benzyl, or naphthylmethyl. The most active compound found was TPI-gh-137 (IC₅₀ = 0.5 nM, Figure 1), which was found to be a highly specific, pure antagonist at the mu receptor. It was also found to be much less toxic than the unmodified pentamine from which it was derived. This example illustrates how compounds may be further refined after initial identification from a library.

A bicyclic guanidine library containing more than 100 000 different heterocyclic compounds was screened in the opiate assays. Twenty-four (3 × 4 × 2) individual bicyclic guanidines were then synthesized and tested for activity; they were found to range from 37 to 10 000 nM. The most active compound found was TPI 614-1 (IC₅₀ = 37 nM, Figure 1).

Even when libraries are not derived from peptide precursors, diversity in many libraries is achieved through the use of amino acid side chains. A library of over 125 000 linear N,N'-di-substituted ureas was used to identify ligands for mu and sigma receptors. The 276 separate mixtures in this library were each made up of 452 different linear ureas. Two separate iterative deconvolutions were performed for the most active mixtures found for the mu and sigma receptors. The most active compound found for the mu receptor was TPI 632-4 (IC₅₀ = 100 nM). Two active compounds were

identified having activity at the sigma receptor, TPI 634-6 (IC₅₀ = 5 nM) and TPI 632-9 (IC₅₀ = 30 nM).²⁸ Similarly, individual compounds were identified for the sigma receptor from a dialkylated hydantoin library. The 2 most active compounds found were TPI 610-2 (IC₅₀ = 62 nM) and TPI 610-4 (IC₅₀ = 64 nM).²⁹ A library of 43 472 isoquinolines was also used to identify new sigma receptor ligands. The most active compound identified from this library was TPI 462-4 (IC₅₀ = 56 nM).⁵⁵

A parallel synthetic strategy of 288 compounds was used by Thomas et al to identify kappa antagonists. The library was designed around N-substituted (+)-(3R,4R)-dimethyl-4-(-3-hydroxyphenyl)piperidine, a compound known to have nonselective opioid antagonist activity.⁵⁶ The most active compound identified was RTI 5989-29 (Figure 1), which was found to have a K_i of 7 nM. The compound was found to have antagonist activity at both the kappa and mu receptors (Table 1).

IN VIVO SCREENING OF MIXTURE-BASED LIBRARIES

Our laboratory has more than 15 years of experience in the synthesis and screening of mixture-based combinatorial libraries in target-based in vitro assays. It is now imperative to explore the enormous potential of the direct in vivo testing of the many mixture-based chemically diverse libraries as a superior approach to drug discovery. Our future studies will seek to demonstrate that the direct in vivo screening and deconvolution of mixture-based combinatorial libraries is a better approach to the identification of fundamentally more "advanced" therapeutic candidates. This new approach should prove faster and cheaper than the typical target-based drug discovery process.

A series of preliminary experiments have been conducted as proof of concept studies to validate the use of mixture-based libraries reported to exhibit opioid activity in an in vivo model. In this study we compared the activity of mixtures of tetrapeptides each containing 125 000 tetrapeptides. These peptide mixtures were based on the known active dermorphin analog 2,6-dimethyltyrosine-DALDA ([Dmt]-DALDA = H-Dmt-D-Arg-Phe-Lys-NH₂). Dmt-DALDA was identified through the outstanding classical synthetic medicinal chemistry effort by Schiller's group.⁶⁰ These compounds have high mu opioid receptor affinity^{60,61} with proven in vivo antinociceptive properties in mice.⁶² The Dmt-tyrosine group of [Dmt]-DALDA has been shown to be an essential component, as demonstrated by both in vitro and in vivo data. We therefore synthesized a positively biased mixture of 125 000 tetrapeptide compounds in which Dmt-tyrosine was fixed at the first position (Dmt-XXX). This mixture was examined for its activity and compared with the antinociceptive effect of the known active individual Dmt-DALDA

and 2 negatively biased tetrapeptide mixtures (F-XXX and k-XXX, which are 800 and 3580 times less active in vitro, respectively; Table 2).

To assess the antinociceptive effects of these mixtures, we used the mouse tail flick assay since it is well established, yields clear and reproducible end points, and can differentiate between mu and kappa activity. Dmt-XXX, at a dose of 100 mg/kg, was active and had duration of action comparable to the individual compound [Dmt]-DALDA at 10 mg/kg (Figure 2). This effect appears to be specific since F-XXX-NH₂ was inactive at comparable doses. It was also observed that Dmt-XXX was longer acting than morphine (ie, 5 hours vs 1 hour), a property already reported for [Dmt]-DALDA,⁶² and was maintained for the mixtures containing Dmt on the N-terminal. As anticipated, the mixtures required greater absolute doses than the individual compounds.

After achieving these results, we performed a single iteration by synthesizing the anticipated next Dmt-r-XX mixture; the second position was defined with D-arginine, which is the amino acid found in DALDA. This iteration decreases the complexity of the mixtures from 125 000 compounds (OXXX) to 2500 compounds (OOXX). The antinociceptive effects of these mixtures were tested in the tail flick assay as before. As the tetrapeptide mixture becomes more defined, one would expect an increase in activity when testing at the same concentration. It is of note that when decreasing the mixture size from 125 000 for Dmt-XXX to 2500 for Dmt-r-XX the increase in activity was modest (4-fold in vitro) while the decrease in mixture size was 50-fold. This discrepancy can be explained by several different mechanisms. Clearly, more than 1 substitution at the second position can be accepted and activity retained (lysine may be an acceptable replacement for arginine). Additionally, it is important to point out that we fixed D-arginine at the second position

simply as a test study to expand this proof of concept for the use of mixture-based combinatorial libraries directly in vivo. There is in fact no reason to assume that D-arginine is the most functionally useful amino acid at the second position, especially in vivo. At 5 hours postinjection at a dose of 25 mg/kg this peptide mixture of 2500 tetrapeptides was clearly more active than morphine (Figure 2). Six others were tested in vivo (Y-XXX, F-XXX, p-XXX, K-XXX, k-XXX, and D-XXX, data not shown), and the activities of these mixtures in binding and cAMP assays are shown in Table 2. This expected correlation between the in vitro and in vivo activities was found for all except k-XXX, which had significant tail flick activity from 30 minutes all the way through the 5-hour cutoff point. Of clear significance in this study was that k-XXX, while having very poor activity in vitro, was clearly active in the tail flick test. This may be due to several factors, including the interaction at an unknown nonopiate receptor, the inhibition or stimulation of an enzyme, or a translational factor or signaling event. This result illustrates perhaps the central most exciting aspect of the proposed studies, namely the direct in vivo identification of mixtures that may be active as pain-modulating agents at sites other than the expected 3 opiate receptors. These preliminary results are encouraging, and testing will be continued to determine individual compounds.

CONCLUSIONS

A wide range of combinatorial libraries, from peptides to low-molecular-weight heterocyclic compounds, have been successfully screened in assays specific for the opioid receptors and have enabled the identification of new ligands for these receptors. New opioid peptides found from combinatorial libraries range in length from tetramers to decamers. The compounds described in this review are the most active

Table 2. Mu Binding (K_i) and cAMP (IC₅₀) Data for Selected Individual and Tetrapeptide Mixtures*

				Binding K _i (nM)	cAMP IC ₅₀ (μM)	No of Compounds
L-(Dmt)-Tyr	X	X	X	16.2	6.8	125 000
L-Tyr	X	X	X	592.2	651.2	125 000
L-Phe	X	X	X	13 000	1027	125 000
D-Pro	X	X	X	41 238	ND	125 000
L-Lys	X	X	X	55 920	ND	125 000
D-Lys	X	X	X	58 030	ND	125 000
D-Asp	X	X	X	84 380	ND	125 000
L-(Dmt)-Tyr	D-Arg	X	X	4.5	0.4	2500
L-Tyr	D-Arg	X	X	136.0	91.3	2500
Dmt-DALDA: L-(Dmt)-Tyr	D-Arg	L-Phe	L-Lys	0.2	0.002	1
DALDA: L-Tyr	D-Arg	L-Phe	L-Lys	5.5	0.8	1

*cAMP, cyclic adenosine monophosphate; ND, not determined.

Figure 2. Comparison of in vivo activity of tetrapeptide mixtures and individual compounds using tail flick assay. Tail flick latency is the average of 2 readings per animal, 10 animals per compound. Vehicle is 10% Trappsol. Light bars = 30 minutes postinjection, dark bars = 5 hours postinjection.

found and represent only a fraction of the number of individual peptides and nonpeptidic compounds synthesized and tested. All the peptides found have an amide at the carboxyl terminus, as all libraries screened were in this format. Some of the peptides resemble the classical opioids—they possess a tyrosine at position 1 and a tertiary amino group on the N terminus. The peptide YPWFPO-NH₂ is a hexapeptide analog of the recently described tetrapeptide endomorphin-1 (YPWF-NH₂), thought to be the endogenous ligand for the mu receptor.⁶³ The delta-selective hexapeptides identified possess both Tyr¹ and Leu⁵ but differ from each other and the enkephalins at the remaining positions. YHGWLNV-NH₂ has a conservative replacement of Phe⁴ by Trp⁴; thus, it differs from the enkephalin mainly at the His². It is assumed that Val⁶ is redundant, although this has not yet been tested. The 2 remaining delta peptides have replacements at the fourth and fifth positions (YGMHLV-NH₂ and YGFDLV-NH₂). Novel peptides identified for the opioid receptors were found to contain basic residues (arginine and lysine), and many also contain tryptophan. Basic residues are found in position 1 in the acetalins and in the all D-amino acid peptide, Ac-rfwink-NH₂. They are also found on the C-terminal residue in peptides selective for mu, delta, or kappa (WWPR-NH₂, Wy(Nve)R-NH₂, and f(D-nal)(D-nle)r-NH₂, respectively). The highly active and receptor-specific tetrapeptide ligands have been fully developed and are now in phase I human trials.

It is still unclear what is required for a compound to bind to opioid receptors and what the effect of binding will be. It was assumed that compounds identified from very large mixture-based libraries would in virtually all instances be identified as antagonists since it was reasoned that there must be many more ways to block a binding interaction with one of many thousands of compounds than to bind

and affect a secondary signaling event. Antagonists need only bind to a receptor in order to block another ligand from binding, whereas an agonist must not only bind but also induce a conformational change in the receptor in order to achieve signal transduction. Thus, the mu agonist activity of Ac-rfwink-NH₂ was unexpected. Furthermore, the acetalins (Ac-RFMWMK-NH₂), which have sequence similarities to Ac-rfwink-NH₂ although they are composed entirely of L-amino acids, were found to be antagonists. Certainly the identification of the opioid peptide Ac-rfwink-NH₂, which does not require a phenolic hydroxyl or a tertiary amino group to be an agonist, challenges the preconception that these pharmacophores are required for agonist activity. Since then we have found that greater than 80% of the new sequences identified are agonists at the opioid receptors. Whether the high percentage of agonists found is due to a bias resulting from the specific radioligand used in the initial library screen has yet to be determined.

The high numbers of different compounds identified raises the question of the plasticity of the opioid receptors. Why are new ligands of widely varying structures so readily identified for the opioid receptors, particularly the mu receptor? This is not the case for other G-coupled receptors, even for the orphanin FQ/nociceptin receptor, at which few libraries are found to have activity. The plasticity of the mu receptor may account for the high numbers of novel agonists identified. The data obtained from in vivo studies on these new opioid ligands should generate interesting insights into this question.

Finally, the use of mixture-based libraries for direct in vivo testing for the identification of inherently more advanced “hits” was demonstrated here in a proof of concept illustration. This process, found to work well in preliminary studies with peptides and classic low molecular weight heterocyclic mixtures, will make the drug discovery process much faster than it has been with traditional target-based in vitro assays. Future studies will explore the enormous potential of the direct in vivo testing of the many mixture-based chemical of diverse libraries in an effort to reduce the time and resources required for drug discovery. This new approach is expected to lead to the identification of novel pain-modulating agents for the 3 opiate receptors and the identification of ligands to a variety of other orphan receptors that may play a role in pain regulation.

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