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De novo identification of highly active fluorescent kappa opioid ligands from a rhodamine labeled tetrapeptide positional scanning library

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Abstract—Highly active fluorescent compounds having kappa opioid activity were identified following the screening in a kappa-specific radioligand binding assay of a positional scanning tetrapeptide combinatorial library in which every tetrapeptide was fluorescently labeled. Lissamine rhodamine B sulfonyl chloride was coupled to the N terminal of a mixture-based tetrapeptide positional scanning library made up of over 7.3 million tetrapeptides. Upon determination of the most active mixtures for each position of the library in the kappa binding assay, individual rhodamine labeled tetrapeptides were then synthesized and tested to determine their activities. Eight individual rhodamine labeled peptides were identified that were specific for the kappa opioid receptor, having binding affinities ranging from 5–20 nM. These peptides were poor inhibitors at the mu and delta receptors ($K_i > 5{,}000$ nM). Furthermore, neither rhodamine itself nor these same tetrapeptides lacking the N-terminal rhodamine had any significant activity at the kappa receptor, indicating that both the tetrapeptide sequence and the rhodamine moiety are required for receptor binding. This study has demonstrated that novel fluorescent compounds with intrinsic activity can be identified through the use of combinatorial chemistry.

With the advent of high throughput drug discovery assays, strict environmental codes, and the need for higher detection efficiencies, there is a need to develop highly receptor-specific ligands with fluorescent properties. Fluorescent ligands typically have substantially longer shelf lives than radioligands, and due to the steadily increasing costs of disposing of radioactive compounds, new fluorescent ligands represent potentially important replacements for existing radioligands. Fluorescent labeled probes have been synthesized for both opioid peptides.[1–3] and non-peptide opiates.[4–9] The traditional process for the preparation of fluorescent compounds is to add a fluorescent label to a known ligand.[10] In some instances this can be synthetically difficult, and since fluorescent compounds are usually large and hydrophobic, the resulting labeled compounds often have significantly altered binding properties relative to the non-labeled ligand. While useful fluorescent compounds clearly have been identified in this manner, the increasing use of high throughput assay systems and the need for studies under physiological conditions using live cells have created a need for highly receptor-specific ligands with fluorescent properties.

The success of mixture-based combinatorial library approaches for the identification of novel, highly active and specific receptor ligands[11,12] prompts a different approach: to make and screen a mixture-based combinatorial library in which every compound making up the library has a fixed fluorescent moiety. Thus, our working hypothesis was that the binding activity of individual compounds identified would possess both the desired binding and fluorescent properties, with the fluorescent group inherently part of the binding interaction with the receptor. We tested this hypothesis by incorporating a fluorophore on the N-terminal of a mixture-based positional scanning library made up of 7.3 million tetrapeptides. Lissamine rhodamine B sulfonyl chloride was chosen as the fluorophore since it readily reacts with amino functionalities in peptides prepared by solid-phase synthetic chemistry and is stable to the conditions used for peptide synthesis. This rhodamine dye is excited in the visible part of the spectrum, has a large molar extinction coefficient (88,000 cm^{-1} M^{-1}), a quantum

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yield that approaches 1, and is quite photostable (i.e., compared to fluorescein). A recent study has described a combinatorial method for assembling fluorescently labeled compounds that can be screened in direct binding assays to identify small molecule mimics.[23]

The rhodamine labeled positional scanning library is composed of four sublibraries, in which each of the four positions are defined with a single amino acid (O) with the three remaining positions made up of a mixture of 52 different L-, D- and unnatural amino acids (X).[11,13] Thus, there are 52 tetrapeptide mixtures per positional sublibrary totaling 208 tetrapeptide mixtures, each made up of 140,608 labeled tetrapeptides, with the library containing a diversity of 7,311,616 tetrapeptides. The 208 tetrapeptide mixtures were synthesized using the solid-phase simultaneous multiple peptide synthesis (SMPS) approach[14] on methylbenzhydrylamine (MBHA) polystyrene resin. Mixture resins (X) were prepared using mixtures of *t*-Boc protected amino acids yielding close to equimolar coupling of each amino acid as previously described.[15] Lissamine rhodamine B sulfonyl chloride (L-20, Molecular Probes), which is a mixture of *ortho*- and *para*-isomeric sulfonyl chlorides, was coupled to the N-terminus of each mixture resin of the library. Side-chain deprotection and cleavage from the resin support were achieved using low-HF[16] and high-HF[17] procedures. The 208 mixtures were individually extracted with 10% acetic acid in water, lyophilized and resuspended in water at a final concentration of 10 mg/mL. Individual rhodamine-labeled peptides identified from the results of the library screening were synthesized using the SMPS approach, and the purity and identity of each compound were confirmed by reverse-phase HPLC (RP-HPLC) and mass spectrometry.

The rhodamine labeled positional scanning tetrapeptide library was screened in a competitive κ opioid radio receptor binding assay as described elsewhere.[11] Each assay tube contained 0.5 mL of membrane suspension, 3 nM tritiated competitor U69,593, and 0.05 mg/mL library mixture. The screening results for the kappa selective binding assay are shown in Figure 1. Mixtures yielding greater than 70% inhibition in the library screening assay were retested in a dose–response assay to determine the most active mixtures at each position of the library (Table 1). For position 1, the most active amino acids were alpha L-ornithine, L-phenylglycine, and D-arginine. At position 2, the amino acids defined in the most active mixtures were alpha L-ornithine, L- and D-arginine, D-naphthylalanine, and L-proline. Amino acids alpha L-ornithine, 7-amino hexanoic acid, D-arginine, D-cyclohexylalanine, L-proline, and epsilon aminocaproic acid were the amino acids defined in the most active mixtures at position 3. At position 4, amino acids D-arginine, 7-amino hexanoic acid, D-leucine, D-phenylglycine, and L-cyclohexylalanine were the most active mixtures.

Individual rhodamine-labeled tetrapeptides were identified by synthesizing all the combinations of selected amino acids defined in each position of the most active mixtures from the library. This resulted in the synthesis

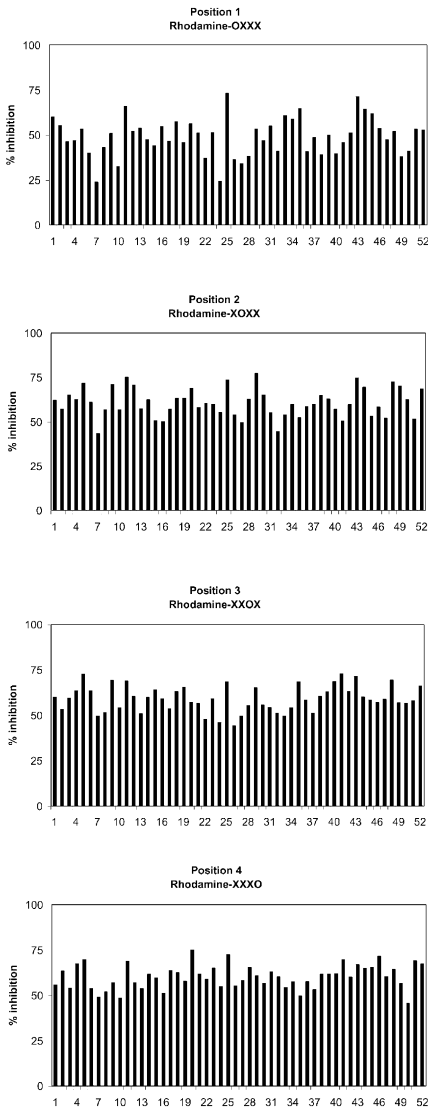


Figure 1. Screening profile of rhodamine library in kappa receptor binding assay. Each bar represents percent inhibition by a peptide mixture (0.05 mg/mL) defined in the O position with one of 52 amino acids. At least three independent experiments were carried out for each sample, each done in duplicate, and variation between screenings was less than 10%. The protected amino acids used to define each position of the library and the single letter or abbreviated codes are the following: 1, A, Boc-L-Alanine; 2, F, Boc-L-Phenylalanine; 3, G, Boc-Glycine; 4, I, Boc-L-Isoleucine; 5, K, Boc-L-Lysine; 6, L, Boc-L-Leucine; 7, M, Boc-L-Methionine; 8, N, Boc-L-Asparagine; 9, P, Boc-L-Proline; 10, Q, Boc-L-Glutamine; 11, R, Boc-L-Arginine; 12, S, Boc-L-Serine; 13, T, Boc-L-Threonine; 14, V, Boc-L-Valine; 15, W, Boc-L-Tryptophan; 16, Y, Boc-L-Tyrosine; 17, a, Boc-D-Alanine; 18, f, Boc-D-Phenylalanine; 19, i, Boc-D-Isoleucine; 20, k, Boc-D-Lysine; 21, l, Boc-D-Leucine; 22, n, Boc-D-Asparagine; 23, p, Boc-D-Proline; 24, q, Boc-D-Glutamine; 25, r, Boc-D-Arginine; 26, s, Boc-D-Serine; 27, t, Boc-D-Threonine; 28, v, Boc-D-Valine; 29, w, Boc-D-Tryptophan; 30, y, Boc-D-Tyrosine; 31, Y, Boc-L-Tyrosine diclbzl; 32, y, Boc-D-Tyrosine diclbzl; 33, (aAba), Boc-L-α-Aminobutyric acid; 34, (gAba), Boc-L-γ-Aminobutyric acid; 35, (aAib), Boc-α-Aminoisobutyric acid; 36, (D-Nve), Boc-D-Norvaline; 37, (L-Nve), Boc-L-Norvaline; 38, (L-NLe), Boc-L-Norleucine; 39, (D-Nle), Boc-D-Norleucine; 40, (eAca), Boc-ε-Aminocaproic acid; 41, (7Aha), Boc-7-Aminoheptanoic acid; 42, (eLys), N-ε-Boc-N-α-CBZ-L-Lysine; 43, (aOrn), N-α-Boc-N-δ-CBZ-L-Ornithine; 44, (dOrn), N-δ-Boc-N-α-CBZ-L-Ornithine; 45, (L-Phg), Boc-L-Phenylglycine; 46, (D-Phg), Boc-D-Phenylglycine; 47, (L-Nal), Boc-2-L-Naphthylalanine; 48, (D-Nal), Nboc-2-D-Naphthylalanine; 49, (B-Ala), Boc-β-Alanine; 50, (HyP-Bzl), Boc-*O*-BenzyL-L-Hydroxyproline; 51, (L-Cha), Boc-L-Cyclohexylalanine; 52, (D-Cha), Boc-D-Cyclohexylalanine.

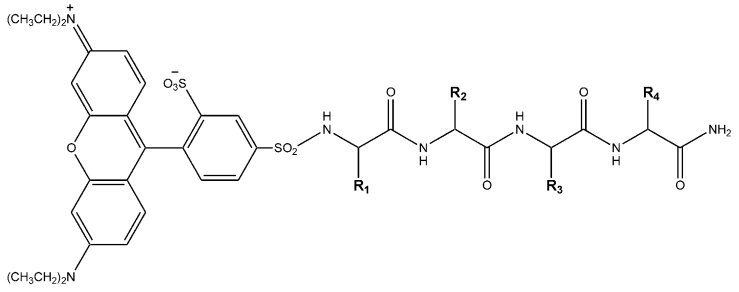
Table 1. K_i values for the most active mixtures for each positional sublibrary of the rhodamine labeled tetrapeptide library

Number		Pos 1	Pos 2	Pos 3	Pos 4	K_i, nM
43	Rhodamine	aOrn	X	X	X	674
45	Rhodamine	L-Phg	X	X	X	1010
25	Rhodamine	r	X	X	X	1266
44	Rhodamine	dOrn	X	X	X	1750
35	Rhodamine	aAib	X	X	X	2105
11	Rhodamine	R	X	X	X	4458
43	Rhodamine	X	aOrn	X	X	550
11	Rhodamine	X	R	X	X	778
25	Rhodamine	X	r	X	X	1074
48	Rhodamine	X	D-Nal	X	X	1084
9	Rhodamine	X	P	X	X	1139
18	Rhodamine	X	f	X	X	1252
29	Rhodamine	X	w	X	X	1322
5	Rhodamine	X	K	X	X	2136
43	Rhodamine	X	X	aOrn	X	775
41	Rhodamine	X	X	7Aha	X	863
25	Rhodamine	X	X	r	X	1074
52	Rhodamine	X	X	D-Cha	X	1105
9	Rhodamine	X	X	P	X	1139
40	Rhodamine	X	X	eAca	X	1238
11	Rhodamine	X	X	R	X	1556
5	Rhodamine	X	X	K	X	2136
48	Rhodamine	X	X	D-Nal	X	2374
35	Rhodamine	X	X	aAib	X	4202
25	Rhodamine	X	X	X	r	655
41	Rhodamine	X	X	X	7Aha	843
20	Rhodamine	X	X	X	l	862
46	Rhodamine	X	X	X	D-Phg	1015
51	Rhodamine	X	X	X	L-Cha	1557
11	Rhodamine	X	X	X	R	3731
5	Rhodamine	X	X	X	K	11,469

of 270 rhodamine-labeled peptides, and these peptides were then tested against the kappa receptor. The most active peptides are shown in Table 2. Of the 13 active peptides listed in Table 2, seven contained at least three positively charged residues, namely L- or D-arginine, or L-ornithine, and the remaining six peptides contained at least two positively charged residues. These same kappa active peptides had poor activity at the mu and delta receptors. Previous work identified several active peptides [most active ff(D-Nle)r, $K_i = 1.2$ nM] from a similar tetrapeptide positional scanning library but lacking the rhodamine moiety that was screened against the kappa opioid receptor.[11] To demonstrate that receptor binding was not due to nonspecific interactions between the positively charged residues and the receptor, a series of rhodamine-labeled analogues were prepared. Rhodamine labeled D-arginine, rhodamine labeled L-ornithine, and rhodamine tetra D-arginine, as well as free rhodamine having a sulfonamide group, had no significant activity at the kappa receptor ($K_i > 10$ μM, data not shown). Finally, the most active tetrapeptides were prepared without the rhodamine moiety and were all found to have poor activity ($K_i > 10$ μM, data not shown), demonstrating that it is combination of both the rhodamine moiety and the tetrapeptide that were responsible for the activity found.

The sulforhodamine dye used for the synthesis of the library and the individual peptides contains *ortho*- and *para*-isomeric monosulfonyl chlorides, which results in sulfonamides that are both *ortho* and *para* to the xan-

Table 2. Most active kappa-specific peptides identified from rhodamine-labeled positional scanning library



						K_i (nM)			
						Kappa		Mu	Delta
Peptide #		Pos 1	Pos 2	Pos 3	Pos 4	*para* isomer	*ortho* isomer		
98	Rhodamine	aOrn	R	7Aha	r	5.7	156	4186	> 10,000
86	Rhodamine	aOrn	aOrn	7Aha	r	6.7	260	> 5000	> 10,000
26	Rhodamine	aOrn	aOrn	7Aha	l	7.2	287	> 5000	> 10,000
18	Rhodamine	aOrn	aOrn	eAca	D-Phg	11.1	1003	> 5000	> 10,000
101	Rhodamine	r	r	D-Cha	r	14.3	971	> 5000	> 10,000
82	Rhodamine	aOrn	r	7Aha	r	14.9	493	4532	> 10,000
20	Rhodamine	aOrn	aOrn	eAca	L-Cha	16.2	1171	> 5000	> 10,000
38	Rhodamine	aOrn	R	7Aha	l	18.0	532	4486	> 10,000
16	Rhodamine	aOrn	aOrn	eAca	l	26.4	223	> 5000	> 10,000
17	Rhodamine	aOrn	aOrn	eAca	r	37.0	555	> 5000	> 10,000
2	Rhodamine	aOrn	r	eAca	r	63.3	439	> 5000	> 10,000
54	Rhodamine	L-Phg	aOrn	D-Cha	r	69.8	754	> 5000	> 10,000
69	Rhodamine	r	P	aOrn	r	75.9	771	> 5000	> 10,000

Table 3. IC-50 values of rhodamine peptides (*para*-isomer) for inhibition of cAMP in CHN-KOR cells

Peptide #	Compd					IC-50 (μM)
cAMP						6.7 ng
Forskolin						1.5
U50488						0.03
DynA (1–17)						0.3
86	Rhodamine	aOrn	aOrn	7Aha	r	1.4
20	Rhodamine	aOrn	aOrn	eAca	Cha	1.8
98	Rhodamine	aOrn	R	7Aha	r	1.9
82	Rhodamine	aOrn	r	7Aha	r	4.3
17	Rhodamine	aOrn	aOrn	eAca	r	4.6
101	Rhodamine	r	r	D-cha	r	22.1
69	Rhodamine	r	P	aOrn	r	37.3

thylium ring system. Rhodamine peptides with the sulfonamide *ortho* to the xanthylium ring eluted earlier on RP-HPLC than the equivalent peptides with the sulfonamide in the *para* position and permitted separation of the two isomers. The second peak was found to be the *para*-isomer both by comparison with published NMR and by stability of its color at elevated pH.[18] The peak eluting first (*ortho*-isomer) forms internal sulfatam ring closed products at pH above 10.5, resulting in complete loss of absorbance at 572 nm. Kappa-specific active rhodamine peptides were purified by RP-HPLC to separate each isomeric compound and were tested for their activity. The *para* isomers were found to be on average 10-fold more active for kappa than the *ortho* isomers (Table 2). The specificity of orientation of the rhodamine also supports the observation that both the peptide and the rhodamine are required for activity.

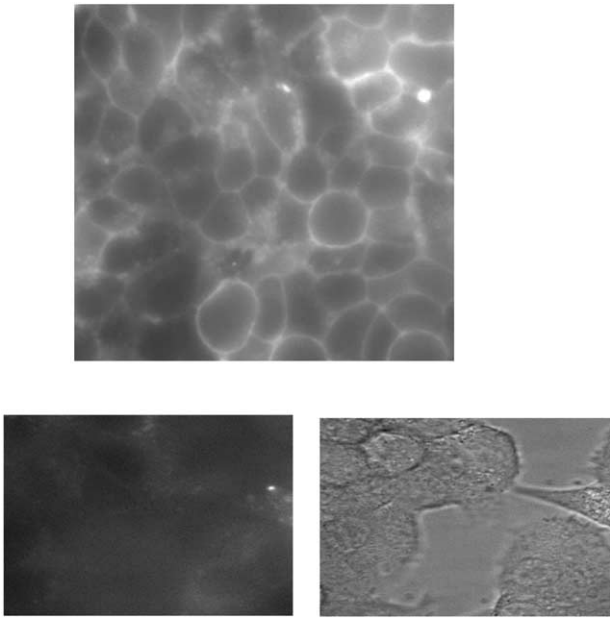


Figure 2. Top, CHN-KOR cells were incubated with TPI 1381-98 *para*-isomer (4.5 μM). Cells were visualized using 540 nm excitation and 630 nm emission wavelengths, 600 nm long pass dichroic and 100 msec exposure time. Bottom left, Fluorescent image of CHN-KOR cells incubated with TPI 1381-98 *para*-isomer (4.5 μM) and U50,488 (100 mM) for 5 min. Bottom right: Light image of left photo.

Opioid agonists inhibit adenylate cyclase activity, resulting in reduced levels of cyclic AMP (cAMP). Seven of the most active kappa specific peptides (*para*-isomer) identified from the rhodamine tetrapeptide library were tested in a cAMP assay using kappa receptor-expressing CHN cells to determine agonist or antagonist activity.[19] cAMP production was induced by forskolin, which directly activates adenylate cyclase, and the dose-dependent inhibition of cAMP resulting from peptide binding was measured. For the seven rhodamine labeled tetrapeptides tested, cAMP production was moderately inhibited, displaying opioid agonist behavior (Table 3). The IC-50 values for the most active peptides were approximately 1 μM, and were partial agonists since they did not inhibit cAMP 100% at the highest doses as compared to dynorphin A, a standard kappa receptor peptide agonist.

The most active peptide identified from the rhodamine tetrapeptide library (TPI 1381-98, *para* isomer) was tested for receptor binding using CHN cells expressing kappa receptor.[20] Following 5 min incubation in the presence of the fluorescent peptide, the surface of the cells were clearly labeled with the peptide; incubation for longer periods (15 min) resulted in punctate fluorescent images consistent with peptide being endocytosed (Fig. 2). The labeled peptide was completely inhibited by the kappa ligand U50,488, demonstrating specific binding to the kappa receptor. Cells not expressing the kappa receptor did not exhibit any fluorescent binding, indicating minimal nonspecific binding.

In this study, fluorescent peptides having intrinsic kappa opioid receptor selectivity were identified from a tetrapeptide positional scanning library in which every tetrapeptide was labeled on its N-terminal amino with rhodamine. From a library of approximately 7.3 million rhodamine labeled tetrapeptides, 270 individual peptides were synthesized from the most active mixtures. The most active peptides identified contained at least two positively charged residues. Such strongly positively charged compounds have the potential for nonspecific 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 radio-ligand to the kappa receptor ($K_i > 10$ μM). As rhodamine, rhodamine sulfonyl amide and rhodamine tetra-arginine had no inhibitory activity (data not shown), the peptide sequence combined with the rhodamine moiety are required components for receptor binding. It is known that rhodamine labeled compounds can aggregate and form complexes that can precipitate from solution.[21,22] It was observed that the rhodamine-labeled mixtures did indeed complex and formed precipitates upon storage in solution at 4 °C. This was found to be highly dependent on the defined residue in the mixtures, in which hydrophobic amino acids in the defined positions resulted in mixtures having noticeable precipitates. 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.

Acknowledgements

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19. cAMP assay-The amount of adenosine-3′,5′-cyclic monophosphate (cAMP) in cells expressing the human kappa receptor was determined using a cAMP DELFIA assay (PerkinElmer Life Sciences kit #CR89-102). cAMP formation was induced by the addition of forskolin, and the dose dependent inhibition of this cAMP production resulting from kappa specific rhodamine tetrapeptide binding was measured. Anti-cAMP antibody was added to a 96-well microtiter plate and incubated at 25 °C for 30 min. 3 Isobuyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, was added (10 μL, 60 μM final concentration) to each well. Rhodamine tetrapeptides (10 μL) were serially diluted (100 μM to 10 nM) and added to each well, followed by the addition of forskolin (10 μL, 50 μM final concentration). CHN cells expressing the human kappa opioid receptor (CHN-KOR, 125,000/well, 40 μL) were added to each well and the plate was incubated at 37 °C for 45 min. Cells were lysed and shaken slowly at room temperature for 5 min. Europium-labeled cAMP (100 μL, 1/150 dilution of stock tracer solution) was added to the plate and shaken at room temperature for 30 min. Wells were washed four times with buffer to remove unbound cAMP. Enhancement solution (200 μL) was added to each well and incubated for 15 min. Europium-chelated fluorescence (excitation 340 nm, emission 615 nm) was measured on a Wallac 1420 VICTOR multilabel plate reader. The standard kappa receptor agonists U50488 and dynorphin were tested as positive controls.
20. Fluorescence microscopy-CHN cells expressing kappa opioid receptor (CHN-KOR) were grown on glass coverslips in 2-cm dishes in DMEM (10% FBS) at 37 °C in 6% CO_2. After 24 h cells were washed with Hank's Balanced Salt Solution (HBSS). Cells were incubated with 4.5 μM of peptide TPI 1381-98 (*para* isomer) for 5 min in 2 mL HBSS and subsequently washed four times with 2 mL HBSS. Cells were imaged on a Zeiss Axiovert microscope using a cooled charge coupled device camera (Photometrics Tuscon, AZ) using Metafluor 2.75 as controlling software (Universal Imaging, Westchester, PA). Images were obtained using 535DF25 excitation filter, 630DF55 emission wavelengths, 600DRLP dichroic mirror and 100 ms exposure time. For the competition experiment cells were incubated with 4.5 μM of peptide TPI 1381-98 (*para* isomer) and 100 mM U50,488 for 5 min fluorescent image.
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