Use of peptide combinatorial libraries in drug design: the identification of a potent serotonin reuptake inhibitor derived from a tripeptide cassette library

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Background: Medicinal chemistry traditionally requires the identification of biologically active molecules by synthesizing and screening each purified substrate. Further progress in drug discovery then requires definition of the structure–activity relationship of the lead compound. More recently, combinatorial chemistry has emerged as a way to examine structure–activity relationships by screening a large mixture of compounds synthesized in a predictably random manner, without the labor-intensive costs of molecular isolation and purification. We set out to use this approach to examine the structural requirements for peptide binding to serotonin and dopamine transporters.

Results: We screened a tripeptide cassette library for serotonin and dopamine reuptake inhibition using cloned transporter assay systems. The method has afforded a number of tripeptide pharmacophores with inhibitory IC_{50} values ranging from 10 μM to < 1 μM in the dopamine and serotonin reuptake systems. The conformation of one of these tripeptides, N-acetyl-D-Trp-L-Phe-D-Lys-$CONH_2$ (which inhibits serotonin uptake with an IC_{50} of 10 μM) was compared to that of the serotonin uptake inhibitor s-fluoxetine, and was shown to be more similar in conformation to fluoxetine than was an analogous tripeptide containing L-Lys (IC_{50} > 50 μM).

Conclusions: We have identified five tripeptides with inhibitory IC_{50} values of < 10 μM in the serotonin reuptake system. One tripeptide was predicted to have pharmacophore features similar to that of fluoxetine, a selective and potent non-peptide serotonin reuptake inhibitor. Our results suggest that tripeptides derived from combinatorial libraries will help to define the important structural elements of pharmacophores.

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Introduction

Combinatorial molecular libraries have been used to allow the screening of large numbers of compounds without the cumbersome and time-consuming requirement of isolating and purifying each substituent [1]. Solid-phase synthesis of a predictable pattern of a mixture of peptides, followed by cleavage to the free peptides, could, in theory, be used in screening for functional and/or binding activity in enzyme and receptor systems. Our strategy was to assemble a 10 000 compound tripeptide cassette library via solid-phase synthesis and to investigate: (1) the ability to identify inhibitors of serotonin and dopamine cotransporter proteins that are active in the μmolar range from this library, and (2) the utility of a tripeptide inhibitor(s) as a 'molecular template' for identifying the important structural features of known serotonin non-peptide reuptake inhibitors [2–4].

Results and discussion

We selected 22 different amino acids, using both the D and L configurations, to construct the peptide library (Table 1). Each protected amino acid was separately bound to resin through a standard solid-phase peptide-synthesis protocol. The individual resin-bound amino acids were physically mixed to form an equimolar mixture of the 22 various amino acids. This mixture was subsequently redivided into 22 portions (each containing the equimolar ratio of the 22 amino acids). A second coupling of all protected amino

Table 1. Amino acids used to construct the peptide library.

D-Ala	L-Ile	D-Pro
L-Arg	D-Leu	L-Ser
D-Asn	L-Lys	L-Trp
L-Asp	D-Lys	D-Trp
L-Gln	L-Met	L-Tyr
D-Gln	L-Phe	D-Tyr
L-His	D-Phe	L-Val
	L-Pro	

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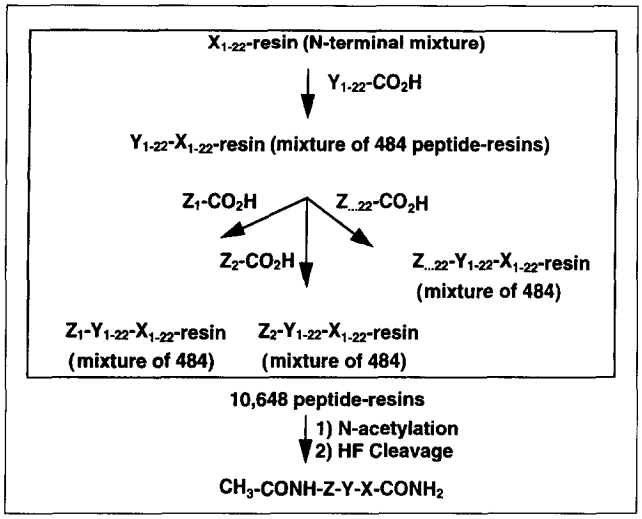


Fig. 1. Scheme for constructing a combinatorial peptide library. Each of the 22 amino acids shown in Table 1 was separately bound to resin, then all 22 were mixed in an equimolar ratio (X_{1-22}-resin). The mixture was divided into 22 aliquots, and a second coupling reaction was performed, again using all 22 amino acids, resulting in a mixture of all possible dipeptides (484 in all) coupled to the resin (Y_{1-22}–X_{1-22}–resin). The mixture was split again, and the third coupling reaction was performed, followed by N-acetylation and cleavage without mixing. This results in 22 mixtures of 484 tripeptides, in each of which the amino-terminal amino acid (Z_n) is known while the other two are not.

acids was performed on the mixtures and these were again mixed and redivided. The final coupling was done on all of the resin-bound dipeptide mixtures with all of the 22 protected amino acids. The identity of the final protected amino acid in each reaction vessel was noted so that after amino-terminal capping with acetic anhydride, followed by cleavage from the resin to form the carboxy-terminal amide, the first preparation of the 22 separate mixtures yielded a schematic generic structure depicted as: acetyl-[known amino acid]-[unknown amino $acid_{(1-22)}$]-[unknown amino $carboxamide_{(1-22)}$] (Fig. 1). Model

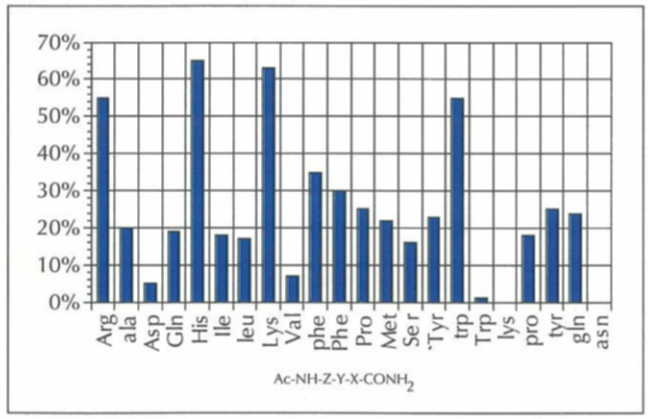


Fig. 2. Inhibition of the uptake of tritiated serotonin by first-round library pools. The histogram shows the percent inhibition of the uptake of serotonin into rat basophil leukemia (RBL) cells by a 500 nM concentration of peptides from the pools identified by their amino-terminal amino acid. First letter lower case denotes the D amino acid; upper case denotes the L amino acid.

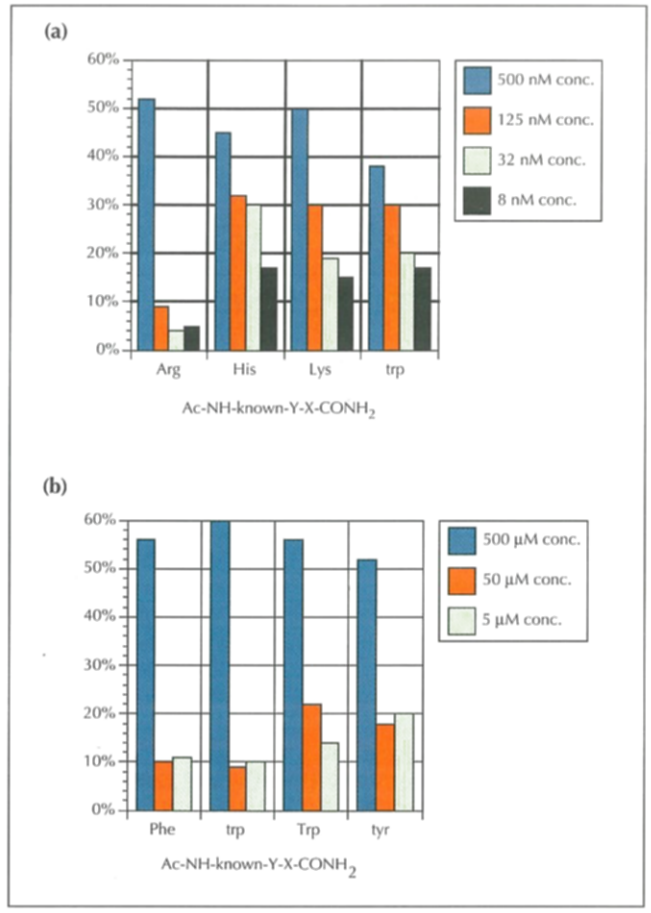


Fig. 3. Comparison of dose dependence of inhibition of serotonin uptake with inhibition of dopamine uptake for four peptide pools. **(a)** Inhibition of serotonin uptake. **(b)** Inhibition of dopamine uptake. First letter lower case denotes the D amino acid; upper case denotes the L amino acid.

peptides made using this process gave good amino-acid analysis (see [1]).

The first round of screening in both the dopamine and serotonin reuptake assays afforded a number of known-$Y_{(1-22)}$-$X_{(1-22)}$ tripeptides with comparable activity at 500 nM concentrations (Fig. 2) [5,6]. We next examined the dose response for selected tripeptide pools with both the serotonin and dopamine reuptake assays, using doses ranging from 8 nM to 500 nM and from 5 µM to 500 µM, respectively (Fig. 3). Relative inhibitory activity in both assays was comparable (compare Fig. 3a with Fig. 3b), and we therefore selected D-Trp for the amino-terminal position, that is, Z = D-Trp for the continued iterative processes. For the second iteration, tripeptide mixtures of the form: acetyl-D-Trp-[known amino acid]-$X_{(1-22)}$ were used (Fig. 4). The activity of the acetyl-D-Lys-[known amino acid]-$X_{(1-22)}$ was comparable to the acetyl-D-Trp-[known amino acid]-$X_{(1-22)}$ mixture up to this iteration and is shown in Figure 4a. The tripeptide mixture, acetyl-D-Trp-L-Phe-$X_{(1-22)}$ appeared to have the best overall activity in assays of dopamine (Fig. 4a) and serotonin (Fig. 4b) reuptake inhibition, and this mixture was therefore used for the final iteration, the synthesis of

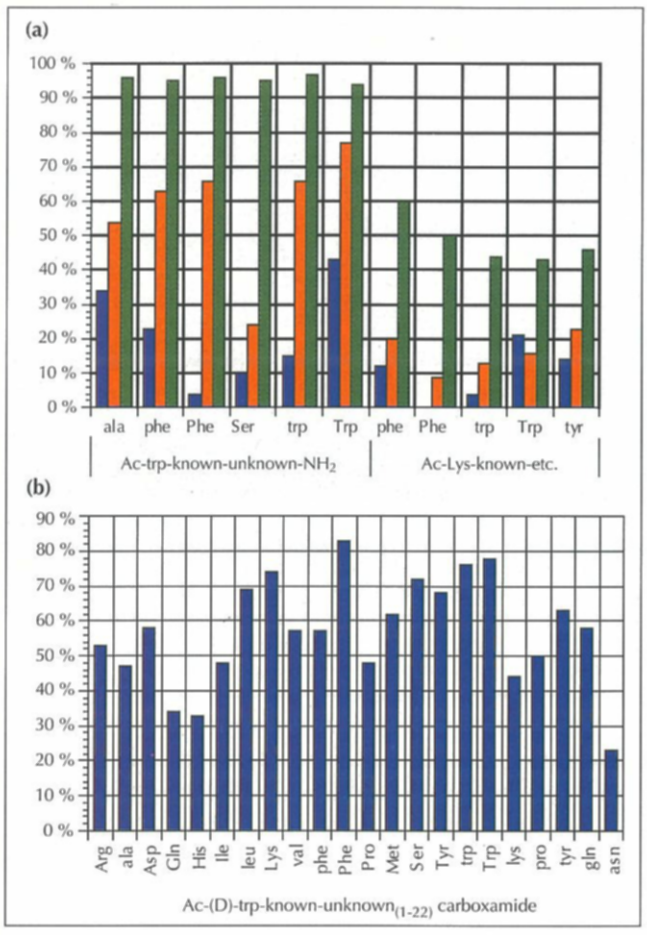


Fig. 4. Inhibition of dopamine and serotonin uptake by tripeptide pools in which the amino-terminal two amino acid residues are identified. **(a)** Dose dependence of inhibition of dopamine uptake by selected tripeptides of the form Ac-D-Trp-[known amino acid]-unknown$_{(1-22)}$-$CONH_2$ and Ac-L-Lys-[known amino acid]-unknown$_{(1-22)}$-$CONH_2$. Mixtures were selected on the basis of dopamine and serotonin inhibition. Concentrations used: blue, 1.1 μM; red, 3.3 μM ; green, 10 μM. **(b)** Inhibition of serotonin uptake by tripeptides of of the form Ac-D-Trp-(known amino acid)-unknown$_{(1-22)}$-$CONH_2$ at a concentration of 500 nM.

pure tripeptides. A second reason for choosing this peptide mixture was the prediction (by M.C.) that acetyl-D-Trp-L-Phe-Lys-$CONH_2$ would show good fit to s-fluoxetine (see below).

We synthesized 22 tripeptides derived from the family of mixtures N-acetyl-D-Trp-L-Phe-X-$CONH_2$ and nine tripeptides with an unacetylated amino terminus, and evaluated their individual activities in the assay systems [8]. Tripeptides having the carboxy-terminal D-Tyr and D-Trp had IC_{50} values of 3.2 μM and 4.5 μM, respectively, in the serotonin reuptake assay (Fig. 5). Most importantly, the carboxy-terminal D-Lys peptide, marked as [I] in Figure 5, had an IC_{50} of 10.0 μM, whereas the corresponding L-Lys peptide, marked as [II] in Figure 5, was inactive, with an $IC_{50} > 50$ μM. Moreover, the free NH_2, amino-terminus derivative of the carboxy-terminal D-Lys peptide, [III], had an IC_{50} of 5.3 μM in the serotonin reuptake assay and an $IC_{50} < 1$ μM in the dopamine reuptake assay.

At this stage of our investigation, we were intrigued by the idea of using peptide combinatorial libraries to identify three-dimensional structural features of successful neurotransmitter reuptake inhibitors, using the structure–activity relationships of selected known inhibitors and peptides selected from our libraries. To test this approach, comparisons were made between the active tripeptides and fluoxetine, a potent serotonin reuptake inhibitor, using molecular modeling methods. The Cartesian forcing algorithm within the molecular similarity module of QUANTA/CHARMm, Version 4.0, was used to determine the degree of best-fit between s-fluoxetine and each of the two library tripeptides, acetyl-D-Trp-L-Phe-L-Lys-amide, [I], and acetyl-D-Trp-D-Phe-D-Lys-amide, [II], which differ only in stereochemistry at the Lys position. Setup parameters used in these calculations were a 15 Å non-bonded cutoff distance, a root-mean square force of 0.01 kcal $mole^{-1}$ $Å^{-1}$

Compound	IC_{50} (μM)
NH_2-D-Trp-L-Phe-D-Tyr-$CONH_2$	3.2
Ac-NH-D-Trp-L-Phe-D-Trp-$CONH_2$	4.5
NH_2-D-Trp-L-Phe-D-Lys-$CONH_2$ [III]	5.3
NH_2-D-Trp-L-Phe-L-His-$CONH_2$	8.9
NH_2-D-Trp-L-Phe-L-Arg-$CONH_2$	8.9
Ac-NH-D-Trp-L-Phe-D-Lys-$CONH_2$ [I]	10.0
Ac-NH-D-Trp-L-Phe-L-Trp-$CONH_2$	11.2
Ac-NH-D-Trp-L-Phe-L-Tyr-$CONH_2$	12.6
Ac-NH-D-Trp-L-Phe-D-Tyr-$CONH_2$	14.0
NH_2-D-Trp-L-Phe-L-Ser-$CONH_2$	14.1
Ac-NH-D-Trp-L-Phe-D-Val-$CONH_2$	17.8
Ac-NH-D-Trp-L-Phe-L-Phe-$CONH_2$	25.0
Ac-NH-D-Trp-L-Phe-D-Leu-$CONH_2$	25.1
Ac-NH-D-Trp-L-Phe-D-Phe-$CONH_2$	28.2
Ac-NH-D-Trp-L-Phe-D-Pro-$CONH_2$	31.6

Compounds with IC_{50} >50 μM
Ac-NH-D-Trp-L-Phe-D-Ala-$CONH_2$
Ac-NH-D-Trp-L-Phe-L-Pro-$CONH_2$
Ac-NH-D-Trp-L-Phe-L-Ser-$CONH_2$
Ac-NH-D-Trp-L-Phe-D-Gln-$CONH_2$
Ac-NH-D-Trp-L-Phe-L-Asp-$CONH_2$
Ac-NH-D-Trp-L-Phe-L-Arg-$CONH_2$
Ac-NH-D-Trp-L-Phe-L-His-$CONH_2$
Ac-NH-D-Trp-L-Phe-L-Gln-$CONH_2$
Ac-NH-D-Trp-L-Phe-D-Asn-$CONH_2$
Ac-NH-D-Trp-L-Phe-L-Ile-$CONH_2$
Ac-NH-D-Trp-L-Phe-L-Met-$CONH_2$
Ac-NH-D-Trp-L-Phe-L-Lys-$CONH_2$ [II]
NH_2-D-Trp-L-Phe-D-Gln-$CONH_2$
NH_2-D-Trp-L-Phe-L-Asp-$CONH_2$
NH_2-D-Trp-L-Phe-L-Gln-$CONH_2$
NH_2-D-Trp-L-Phe-D-Asn-$CONH_2$

Fig. 5. Inhibition of serotonin reuptake by single tripeptides. The activity of fluoxetine is ~5 nM. The compound NH_2-D-Trp-L-Phe-D-Lys-$CONH_2$ was the only one to have notable activity in the dopamine inhibition assay (IC_{50} <1μM). All others were ineffective at 50 μM.

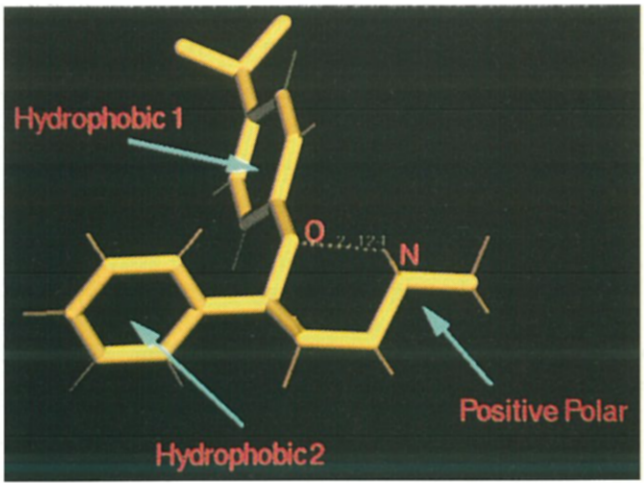
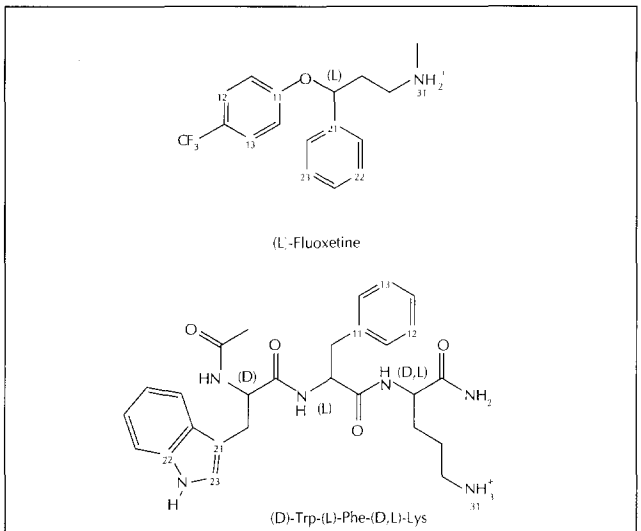


Fig. 6. Minimum energy conformation of s-fluoxetine. The conformation of the propylamine side chain is observed to be stabilized by an intramolecular hydrogen bond (2.1 Å) between the amine nitrogen and phenoxy oxygen atoms. Hydrophobic and positive ionizable regions that define the ligand pharmacophore are indicated.

and 0.001 energy tolerance for determining minimization convergence, and dielectric constance of 4.0, since discrete waters were not included. The global low energy



	ΔE_{Relax} (Kcal mol^{-1})	RMS fit (Å)	IC_{50} (μM)
Ac-NH-D-Trp-L-Phe-D-Lys-$CONH_2$	−10.4	2.1	5.3
Ac-NH-D-Trp-L-Phe-L-Lys-$CONH_2$	$>10^4$	2.3	>50

Fig. 7. Cartesian fit of D-Trp-L-Phe-D,L-Lys to s-fluoxetine. Equivalent atoms on different molecules, which were forced to occupy the same space during Cartesian fitting have corresponding numbers. The relaxation energy (ΔE_{relax}), root-mean square fit (RMS fit) and IC_{50} values calculated for the two peptides are shown below.

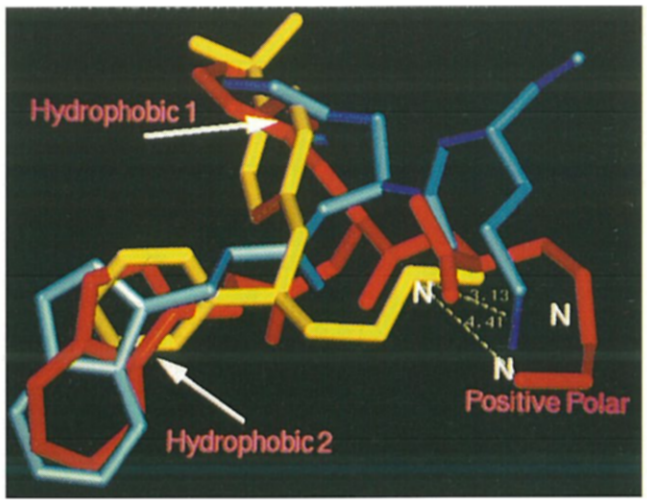


Fig. 8. Superposition of the two isomeric tripeptides (D-Lys isomer, blue, and L-Lys isomer, red), with s-fluoxetine, yellow. These structures have been "relaxed" by energy minimization after Cartesian fitting. The greatest deviation of fit is observable at the positive polarizable region of the pharmacophore. The nitrogen–nitrogen distance for the D-Lys tripeptide is measured at 3.13 Å, compared to 4.14 Å, for the L-Lys tripeptide.

conformation of s-fluoxetine was determined by the grid scan procedure, calculating molecular energies at 30° intervals about all sp^3–sp^3 bonds. The resulting structure is shown in Figure 6, with the propanamine side chain forming a weak intramolecular hydrogen bond between the phenoxy oxygen and amine nitrogen atoms. It was this conformation which gave the best fit for the two isomeric tripeptides.

The serotonin reuptake pharmacophore was modeled by fluoxetine, which can be represented by two hydrophobic (aromatic) regions and one positive ionizable region. As shown in Figure 7, seven atoms from each molecule were selected to represent these three regions of the pharmacophore. The fluoxetine molecule was frozen to its low energy conformation and equivalent atoms from each molecule were then constrained to the same space. As a result, each tripeptide was forced to adapt to that of the fluoxetine pharmacophore model. As a measure of the tripeptide's degree of similarity to the fluoxetine pharmacophore, a 'relaxation energy' (ΔE_{relax}) and root-mean square deviation of fit for the two peptide molecules were calculated and are tabulated along with measured IC_{50} values in Figure 7. The relaxation energy was defined by the difference in energy values between the best-fit and the fully minimized structures. The root-mean square deviation of fit is defined in this case as the root-mean square distance between the seven atoms of fluoxetine and each of the two tripeptides. Figure 8 shows the best-fit superposition of the three molecules, after each has been energy minimized.

Based on these values, the above procedure was able account for the difference in activity between the two tripeptides, [I] and [II]. The lower ΔE_{relax} calculated for

the D-Lys peptide, [I], is in agreement with the observed data, suggesting that this isomer can more readily adapt and bind to a serotonin reuptake active site than the L-Lys isomer, [II]. The root-mean square deviation of fit values appear to be less sensitive, but are still in agreement with the observed activity data.

The two tripeptides [I] and [II] were selected as examples for this exercise because they would be expected to differ only in their three-dimensional structures. As a consequence, other variables that could influence binding would be eliminated. If our pharmacophore model was accurate enough to account for all the molecular features that were representative of the entire peptide library, we would expect a trend of ΔE_{relax} and root-mean square deviation of fit proportional to inhibitory activity. This does indeed appear to be the case, at least in the two examples tested.

Significance

We have shown here that a 10 000 tripeptide cassette library can be screened as mixtures of compounds, allowing the identification of serotonin and dopamine reuptake inhibitors with respectable inhibitory activity. The peptides identified in this study had IC_{50} values ranging from 3 μM for inhibition of serotonin reuptake to less than 1 μM for inhibition of dopamine reuptake. Moreover, two of the peptides identified here, peptides [I] and [II], have pharmacophore features (namely, hydrophobicity and polarizability) similar to that of fluoxetine, which is known to be a selective and potent serotonin reuptake inhibitor. We conclude that peptide libraries will be useful in defining the important elements of a pharmacophore similar to active small non-peptide molecules such as fluoxetine. Because peptide libraries are more cost-effective to synthesize and are robust in spacial diversity, we suggest that this approach will be useful to 'jump-start' programs that aim to investigate structure–activity relationships.

Materials and methods

Molecular modeling

The construction, energy minimization and display of all molecules were performed with Molecular Simulations Incorporated (MSI) molecular modeling package QUANTA/CHARMm, version 4.0, on a Silicon Graphics Indigo 2 workstation. The Cartesian forcing algorithm within the molecular similarity module was used to determine the degree of similarity or best-fit. Setup parameters that were used in these calculations were a 15.0 Å non-bonded cutoff distance, a 4.0 dielectric constant, and a 0.1 Å rms force for determining energy convergence.

Preparation of peptide library

Peptide synthesis

t-Boc-strategy with diisopropylcarbodiimide/hydroxybenzotriazole (DIC/HOBT) activated esters was employed using RAMPS apparatus (Dupont) and a MBHA resin (Advanced Chem Tech) to generate carboxy-terminally amidated peptides. Boc-amino acids used in syntheses were D-Ala, L-Asp (OBzl), D-Glu (OBzl), Phe, D-Phe, L-His (Bom), L-Ile, L-Lys (2Cl-Z), D-Lys (2Cl-Z), D-Leu, L-Met, D-Asn, L-Pro, D-Pro, L-Gln, L-Arg (Tos), L-Ser (Bzl), D-Val, L-Trp, D-Trp, L-Tyr (2Br-Z) and D-Tyr (2,6Cl-Z).

HF cleavage

The peptide amides were removed from the resin and side-chain deprotected in HF/*p*-thiocresol/*m*-cresol(90:5:5) at 0°C for 60 min using a multiple peptide cleavage apparatus (multiple peptide systems). The HF was vacuum-distilled and the peptides were precipitated with 50 ml of diethylether. After collection by filtration, the peptides were washed extensively with diethylether, solubilized in aqueous 50% acetic acid containing 30% acetonitrile, concentrated by distillation and then lyophilized. The recovered peptides were weighed and redisolved in aqueous acetonitrile/acetic acid to prepared lyophilized aliquots. The final 31 purified tripeptides were prepared in a similar manner.

Dopamine and serotonin reuptake assays

Serotonin uptake assay

Rat basophil leukemia (RBL) cells were trypsinized, counted and plated at a density of 1 x 10^5 cells per 0.5 ml of growth medium in 24-well plates. For the uptake assay, cells were washed with uptake buffer (25 mM Hepes, pH 7.4/125 mM NaCl/4.8 mM KCl/1.2 mM KH_2PO_4/1.3 mM $CaCl_2$/1.2 mM $MgSO_4$/5.6 mM glucose/1 mM sodium ascorbate/10 mM pargyline) and preincubated with the tripeptides or other antagonists for 10 min at 37 °C. Uptake was initiated by the addition of ^{3}H-serotonin for 15 min at 37 °C followed by three washes with ice-cold uptake buffer on ice. Cells were solubilized in 0.5 N NaOH and the amount of radioactivity was determined by liquid scintillation spectrometry.

Dopamine uptake assay

CVSVDAT cells (CV-1 cells stably transfected with dopamine transporter (DAT) driven by the SV40 promoter) were plated as above. The uptake assay was performed as above, except that ^{3}H-dopamine was incubated with cells for 10 min and the uptake was terminated by the additon of 0.25 ml of 1 mM dopamine followed by three washes with ice-cold uptake buffer on ice. Cells were solubilized with 0.5% Triton X-100 and the amount of radioactivity was determined by liquid scintillation spectroscopy.

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