Combinatorial synthesis of ω-conotoxin MVIIC analogues and their binding with N- and P/Q-type calcium channels

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Abstract ω-Conotoxin MVIIC (MVIIC) blocks P/Q-type calcium channels with high affinity and N-type calcium channels with low affinity, while the highly homologous ω-conotoxin MVIIA blocks only N-type calcium channels. We wished to obtain MVIIC analogues more selective for P/Q-type calcium channels than MVIIC to elucidate structural differences among the channels, which discriminate the ω-conotoxins. To prepare a number of MVIIC analogues efficiently, we developed a combinatorial method which includes a random air oxidation step. Forty-seven analogues were prepared in six runs and some of them exhibited higher selectivity for P/Q-type calcium channels than MVIIC in binding assays.

Key words: ω-Conotoxin MVIIC; Calcium channel; Disulfide bond; Combinatorial synthesis

1. Introduction

Voltage-sensitive calcium channels control various biological processes such as neurotransmitter release and muscle contraction, and are classified on the basis of electrophysiological properties as L-, N-, P/Q-, R-, or T-type calcium channels [1]. While their molecular basis has been established in terms of diversity of the α_1 subunit [2], the contribution of each channel to a total calcium current is often dissected pharmacologically by using specific blockers. ω-Conotoxins, derived from the venom of marine *Conus* snails, are one of the most prominent among various classes of blockers, particularly for blocking of N- and P/Q-type calcium channels [3]. ω-Conotoxin GVIA and ω-conotoxin MVIIA bind to N-type channels and ω-conotoxin MVIIC binds to P/Q-type channels with high affinity and to N-type channels with low affinity. The N- and P/Q-type calcium channels regulate neurotransmitter release in presynaptic nerve terminals and play distinct roles in the nervous system, so that discrimination of these channels by the use of ω-conotoxins is important for understanding their contribution.

The three-dimensional structures of GVIA [4–7], MVIIA [8–10], and MVIIC [11,12] have been studied by nuclear magnetic resonance (NMR) analysis, revealing that these conotoxins have the same structural motif. The key residues for binding were identified as Tyr13 and Lys2 as a result of single Ala substitutions [13–18], but the determinants of the specificity have not been elucidated so far. The fact that MVIIC, which has a high sequence similarity to MVIIA, bind to P/Q-type channels more efficiently than to N-type channels indicates that MVIIC can be used as a promising tool to elucidate structural differences in the pore-forming region between N-type and P/Q-type channels [19,20].

Difficulty in preparing MVIIC analogues has been an obstacle to the thorough investigation of structure–activity relationships, so that effective methods for preparing many analogues are required. In this study we tried to prepare a number of MVIIC analogues at the same time by means of a combinatorial method, aiming to obtain an MVIIC analogue with improved specificity for P/Q-type channels. Linear precursors of MVIIC analogues were synthesized on a peptide synthesizer using cartridges which contained a mixture of 9-fluorenylmethoxycarbonyl (Fmoc)-amino acids and then subjected to random air oxidation. The resultant crude analogues were separated and purified by successive chromatographies. Forty-seven analogues were successfully prepared in six runs and some of them exhibited high affinity and selectivity for P/Q-type calcium channels.

2. Materials and methods

2.1. Materials

Fmoc-amino acids, Fmoc-amide-resin, and other reagents used on a synthesizer were obtained from Perkin-Elmer Applied Biosystems Japan (Chiba, Japan). [^{125}I]GVIA and [^{125}I]MVIIC were purchased from NEN (Boston, MA, USA).

2.2. Synthesis and purification of peptides

Solid phase peptide synthesis was conducted on a Perkin-Elmer Applied Biosystems 433A peptide synthesizer. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was measured on a PerSeptive Biosystems Voyager Linear DE mass spectrometer using α-cyano-4-hydroxycinnamic acid as a matrix. Analytical HPLC was conducted on a Shimadzu LC-6A system with an octadecylsilane (ODS) column (4.6×250 mm). Preparative HPLC was performed with a Shimadzu LC-8A system with an ODS column (20×250 mm).

Fmoc-amino acids to be introduced into a substitution site were packed into a regular cartridge in equimolar amounts such that the sum of them was the appropriate amount based on the program. The other steps of synthesis and purification were performed in the same way as described previously for preparation of MVIIC analogues [19]. Briefly, linear precursors of MVIIC analogues assembled by normal operation of Fmoc solid-phase synthesis were deprotected and subjected to air oxidation. The oxidized peptides were concentrated on an ODS column with a medium-pressure pump, and then the monomeric

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Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; GVIA, ω-conotoxin GVIA; HPLC, high-performance liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MVIIA, ω-conotoxin MVIIA; MVIIC, ω-conotoxin MVIIC; NMR, nuclear magnetic resonance; ODS, octadecylsilane

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peptides were isolated from the crude peptides by chromatography with Sephadex G-50F and separated into fractions by chromatography on CM-cellulose CM-52. The analogue was finally purified from each fraction by preparative HPLC with an ODS column and identified by MALDI-TOF-MS measurement. Among the obtained analogues, analogue 34 (in Table 1) was resynthesized on a larger scale for spectroscopic analysis.

2.3. Binding assay

Rat cerebellar P_2 membranes were prepared as previously described [21], and filter binding assays were performed according to the previous method with some modifications. For competition with GVIA, the analogue and [^{125}I]GVIA were incubated at 4°C for 1.5 h with P_2 membranes (10 μg) in 50 μl of 25 mM Tris–HEPES buffer (pH 7.4) containing 0.04% bovine serum albumin (BSA). The mixture was diluted with ice-cold 0.8 mM Tris–HEPES buffer containing 0.16 M choline chloride and 0.1% BSA and then rapidly filtered under vacuum with a GF/C filter (Whatman) soaked in the same buffer. The filter was washed with the ice-cold buffer twice and its radioactivity was measured with a γ-counter. For competition with MVIIC, the analogue and [^{125}I]MVIIC were incubated at 4°C for 1.5 h with P_2 membranes (10 μg) in 50 μl Tris-buffered saline: 150 mM NaCl, 25 mM Tris–HCl adjusted to pH 7.4 (TBS), containing 0.1% BSA. The mixture was diluted with ice-cold TBS containing 1.5 mM $CaCl_2$, 0.1% BSA, and 0.05% Tween-20 and then rapidly filtered under vacuum with a GF/C filter pre-treated with 0.03% polyethyleneimine. The

Table 1
Numbers, yields, and effects on radioligand binding of MVIIC and its analogues

Run	No.	Peptide[a]	Yield (%)[b]	Radioligand binding (%)[c]	
				GVIA	MVIIC
1	1	MVIIC	6.4	20 ± 3	18 ± 3
	2	K2Q	3.0	46 ± 8	61 ± 9
	3	M12T	0.5	17 ± 0	66 ± 8
	4	M12Y	1.7	22 ± 2	39 ± 6
	5	Y13W	4.4	101 ± 4	47 ± 4
	6	K2Q, M12T	0.9	94 ± 4	82 ± 4
	7	K2Q, M12Y	2.6	98 ± 1	69 ± 2
	8	K2Q, Y13W	3.7	102 ± 4	89 ± 8
	9	M12T, Y13W	0.9	99 ± 6	101 ± 7
	10	M12Y, Y13W	2.0	98 ± 3	66 ± 4
	11	K2Q, M12Y, Y13W	1.9	100 ± 2	78 ± 7
2	1	MVIIC	7.8	20 ± 3	18 ± 3
	12	A6K	3.1	5 ± 3	18 ± 6
	13	P7K	2.0	10 ± 3	−1 ± 4
	14	S17K	5.3	46 ± 6	17 ± 4
	15	S19R	5.7	58 ± 3	18 ± 2
	16	A6K, S17K	0.8	25 ± 4	22 ± 5
	17	A6K, S19R	2.9	73 ± 7	57 ± 5
	18	P7K, S17K	0.9	25 ± 1	6 ± 4
	19	P7K, S19R	3.0	59 ± 1	18 ± 3
	20	S17K, S19R	5.1	18 ± 1	−1 ± 1
3	21	S19H	3.3	58 ± 3	32 ± 3
	22	S19W	0.6	103 ± 7	51 ± 6
	23	S17H, S19H	2.0	54 ± 3	10 ± 4
	24	S17H, S19W	0.2	84 ± 1	28 ± 9
	25	S17K, S19H	0.3	76 ± 3	16 ± 8
	26	S19H, K25R	2.7	89 ± 1	24 ± 0
	27	S19R, K25R	0.9	95 ± 8	15 ± 4
	28	S19W, K25R	1.3	83 ± 4	15 ± 3
	29	S17H, S19W, K25R	0.4	91 ± 6	34 ± 6
	30	S17K, S19W, K25R	0.2	100 ± 5	24 ± 1
4	31	S17K, K25R	8.4	39 ± 5	14 ± 6
	32	K4R, S17K, K25R	3.2	60 ± 5	5 ± 6
	33	D14N, S17K, K25R	0.4	26 ± 9	8 ± 4
	34	S17K, S19R, K25R	3.8	99 ± 2	8 ± 1
	30	S17K, S19W, K25R	4.4	100 ± 5	24 ± 1
	35	S17K, S19Y, K25R	4.5	52 ± 6	25 ± 5
	36	K4R, S17K, S19R, K25R	1.5	103 ± 7	7 ± 0
	37	K4R, S17K, S19W, K25R	1.2	94 ± 7	27 ± 8
	38	K4R, S17K, S19Y, K25R	1.7	56 ± 4	20 ± 0
5	39	G-MVIIC	5.8	68 ± 3	45 ± 1
	40	K-MVIIC	3.2	68 ± 1	26 ± 7
6	41	DG-MVIIC	1.0	100 ± 2	72 ± 2
	42	KG-MVIIC	1.2	61 ± 7	32 ± 5
	43	YG-MVIIC	0.6	64 ± 1	42 ± 2
	44	DK-MVIIC	0.4	100 ± 0	80 ± 9
	45	KK-MVIIC	0.9	89 ± 2	56 ± 4
	46	YK-MVIIC	0.7	90 ± 2	82 ± 4
	47	DN-MVIIC	0.5	97 ± 1	101 ± 2
	48	KN-MVIIC	0.4	91 ± 7	61 ± 0

[a]Customary names are as follows: K2Q is [Gln^2]-MVIIC, and G-MVIIC and KG-MVIIC are glycyl-MVIIC and lysyl-glycyl-MVIIC, respectively.
[b]The value is calculated as the ratio of the weight of purified peptide to the theoretical weight calculated for the assembly on a peptide synthesizer.
[c]Binding displacement of [^{125}I]GVIA or [^{125}I]MVIIC by 10^{-7} M or 10^{-8} M MVIIC and its analogues. The values represent means ± S.D.

filter was washed twice with the ice-cold buffer used for diluting. In both assays, binding was measured in triplicate and non-specific binding was estimated by use of unlabeled GVIA or MVIIC at 1 μM as a competitor.

2.4. NMR analysis

^{1}H NMR spectra were measured at 288 K on a Bruker DRX-500 spectrometer operating at 500 MHz. The samples were prepared at a concentration of 9 mM in 90% H_2O/10% D_2O at pH 4.0.

3. Results

3.1. Synthesis and purification of MVIIC analogues

We selected the residues to be replaced on the basis of previous studies on the structure–activity relationship of MVIIC. The sequence and substitutions are shown in Fig. 1. In run 1, three residues essential for activity, namely Lys2, Met12, and Tyr13, were replaced with residues which were expected to cause significant modulation of activity and selectivity. In run 2 basic residues were substituted for residues which had not been identified as residues important for activity in previous work [22]. Combinations of substitutions in run 3 and run 4 were selected on the basis of the results obtained in the assay of analogues from run 2. Replacements of some charged residues, namely Lys4, Asp14, and Lys25, were also incorporated in run 3 and run 4. Run 5 and run 6 were constructed for examining the effects of elongation of the N-terminus, based on the fact that the N-terminus of MVIIC is exposed to the solvent in the solution structure determined by NMR study.

The mixtures of protected linear precursors were synthesized by usual Fmoc solid-phase methodology, and all the possible precursors in each run were detected by HPLC and mass analysis. The crude mixture of linear precursors was subjected to oxidative disulfide bond formation, affording a mixture of MVIIC analogues. The mixture contained many kinds of impurities, which were also observed in the usual preparation of a single analogue, i.e. polymers, disulfide bond isomers, and so on. The polymers were removed by gel filtration, then the monomers were separated by ion exchange chromatography into several groups and analyzed by reversed-phase HPLC and MALDI-TOF-MS measurement. Although disulfide bond isomers of an analogue are indistinguishable from the analogue by mass analysis, we tentatively assigned prominent peaks on HPLC profiles to the analogues which had the same disulfide bond pairings as MVIIC. A profile of the ion exchange chromatography and HPLC profiles of each fraction obtained in run 1 are shown in Fig. 2. All

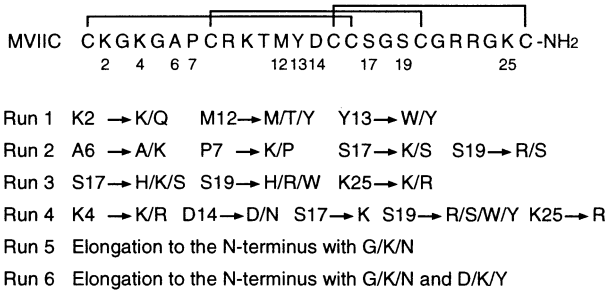

Fig. 1. The primary structure of MVIIC and the combinatorial replacement or elongation sites in MVIIC. The disulfide bond pairings are designated over the sequences.

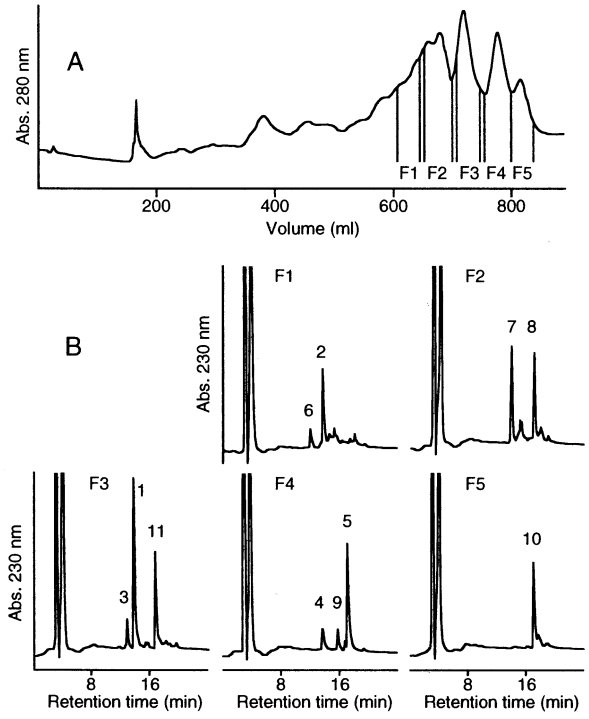

Fig. 2. A: Ion exchange chromatography of the monomer fraction obtained in run 1. The peptides were eluted from CM-cellulose CM-52 (18×200 mm) in a gradient formed from 500 ml of 0.01 M ammonium acetate buffer (pH 4.6) and 500 ml of 0.8 M ammonium acetate buffer (pH 6.5). Fractions were combined into five groups (F1–F5). B: Reversed-phase HPLC profiles of peptides in each combined fraction. The peptides were eluted from an ODS column (4.6×250 mm) in a linear gradient of 5–29% acetonitrile with aqueous 0.1% trifluoroacetic acid for 24 min at a flow rate of 1 ml/min. The numbers above the peaks correspond to the analogue numbers designated in Table 1.

the purified peptides, which are listed in Table 1, competed with radiolabeled MVIIC at 10^{-7} M, so that we regarded them as MVIIC analogues, not isomers, because calcium channels recognize conotoxins in highly rigorous mode and it is unlikely that isomers bind to the channels specifically. Although the disulfide bond pairings should be determined for final confirmation, our purpose was to develop a blocker more specific than MVIIC, so that structural information was obtained only for the most potent analogue.

The ratios of isolated analogues to the theoretical number of analogues were 11/12, 10/16, 10/18, 9/16, 2/3 and 8/9 in runs 1–6 respectively. Among the excluded analogues, some were not formed in sufficient amounts to be isolated and others could not be adequately purified. For example, the replacement of Asp14 with Asn appeared to prevent correct folding and seven analogues which should contain Asn14 were not isolated in run 4. In run 3 all the possible analogues seemed to be formed sufficiently but eight analogues were concluded to be inadequately purified, and were excluded. Finally we could prepare MVIIC and 47 analogues from six runs, taking duplication into account (Table 1).

3.2. Binding activities of MVIIC analogues to N- and P/Q-type calcium channels

Binding activities of MVIIC and the 47 analogues to the N- and P/Q-type calcium channels were estimated at 10^{-8} M for

competition with radiolabeled MVIIC and at 10^{-7} M for competition with radiolabeled GVIA (Table 1). The analogues obtained in run 1 generally showed lower affinities than MVIIC in both assays, while the effect on selectivity varied. For example, replacement of Tyr13 with Trp (in analogue 5) decreased the affinity more in competition with GVIA, whereas replacement of Met12 with Thr (in analogue 3) decreased the affinity only in competition with MVIIC. In the case of the analogues from run 2, affinities were almost fully maintained, especially in competition with MVIIC, and replacement of Ser17 or Ser19 with a basic residue tended to increase selectivity for P/Q-type channels (analogues 14 and 15). The effects of substitutions of Ser17 and Ser19 were further investigated in run 3 and it was found that replacements of Ser19 with basic or bulky residues decreased the affinity for N-type channels. Replacement of Lys25 with Arg was also found to be effective for increasing selectivity for P/Q-type channels when combined with replacements of Ser19. Elongation of the N-terminus in runs 5 and 6 generally decreased affinity for both N- and P/Q type channels, but some analogues showed higher selectivity for P/Q-type channels than MVIIC. We selected analogues 34 in which Ser17, Ser19, and Lys25 were replaced with Lys, Arg, and Arg respectively as a candidate P/Q-type selective blocker because it showed the lowest affinity for N-type channels among the analogues which retained similar affinity to that of MVIIC for P/Q-type channels. The concentration inhibition of [^{125}I]GVIA or [^{125}I]MVIIC binding relationship of analogue 34 is shown in Fig. 3, along with that of analogue 3, which has decreased affinity for P/Q-type and retained affinity for N-type channels.

3.3. NMR spectra

A preliminary NMR structural analysis of analogue 34

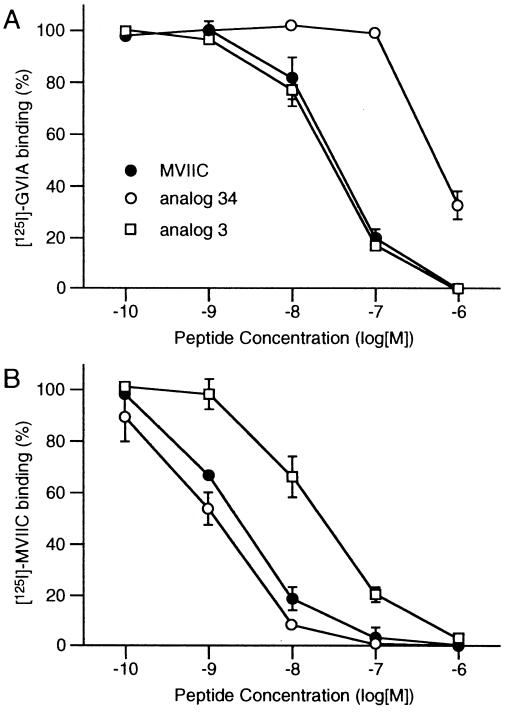

Fig. 3. Concentration–inhibition relationship of representative analogues and MVIIC for [^{125}I]GVIA or [^{125}I]MVIIC binding to rat cerebellar P_2 membranes.

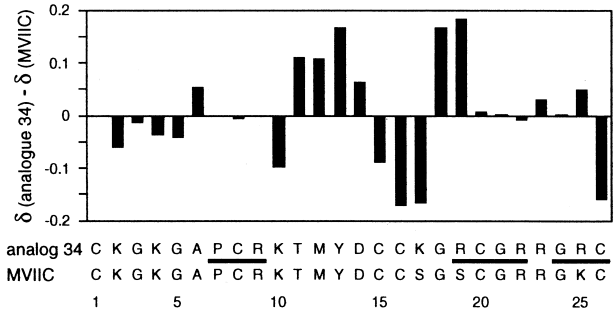

Fig. 4. Deviations of NH chemical shifts from MVIIC native values in analogue 34. The residues which are located in β-strands in MVIIC are designated by the bold lines.

showed dispersion of NH chemical shifts, some of which were preserved, while others deviated somewhat from those of MVIIC (Fig. 4). The residues for which the NH chemical shifts were well preserved are distributed mainly on β-strands, whereas deviations are observed for residues in the other region.

4. Discussion

We wished to find MVIIC analogues with higher selectivity for P/Q-type calcium channels than MVIIC, and obtained some analogues which retained the affinity for P/Q-type channels but exhibited decreased affinity for N-type channels in binding assay. Tyr13 is the most important residue for the activity of GVIA, MVIIA, and MVIIC [14–18] and its replacement with Trp in MVIIC decreased the affinity for both channels, but the decrease was greater for N-type, so that the selectivity for P/Q-type channels was increased. This tendency, which was also noted by Nadasdi et al. [23], implies that Trp13 is acceptable to P/Q-type calcium channels and unfavorable to N-type channels, or that the contribution of Tyr13 to the binding affinity is relatively low for binding with P/Q-type channels. The latter possibility is supported by our previous work, which showed that recognition sites for binding with P/Q-type channels are spread over the whole molecule [19]. The former possibility may be relevant to the effect of the replacement of Ser19 with Arg or Trp, which also increased selectivity for P/Q-type channels. Ser19 is located near Tyr13 in the solution structure so that its replacement with residues possessing bulky side chains may have an effect comparable to that of replacement of Tyr13 with Trp.

The effect of elongation at the N-terminus was studied in runs 5 and 6 and it was revealed that elongation with two residues was acceptable to some extent when Gly was introduced as a spacer (analogues 42 and 43). This implies that the channels may have some latitude around the N-terminus of MVIIC or that the N-terminus may be located at an outer vestibule of the channels, exposed to the solvent.

One of the important observations in this study is that multiple substitutions showed unpredictable effects on the activity and selectivity. For example, Arg25 replacing Lys25 acted as an auxiliary to decrease affinity for N-type channels when combined with Arg19 replacing Ser19, but it had little effect when Ser17 was replaced with Lys. Such a result suggests that further multiple substitutions would be helpful for understanding the orientation of the whole molecule interacting with the channels.

Recent studies on the structure–activity relationships of ω-conotoxins have revealed that some substitutions might cause unexpected local conformational alteration in spite of the rigid molecular scaffold [18,24]. Taking up the question of whether conformational alteration contributes to increased selectivity, we sought structural information for analogue 34. Whereas the circular dichroism spectrum of analogue 34 was similar to that of MVIIC (data not shown), some deviations of NH chemical shifts were observed in NMR analysis (Fig. 4). Although analogue 34 seems to retain the canonical structure of ω-conotoxins, the local conformational changes may influence the activity and selectivity, as well as the substituted side chains.

In this work, we succeeded in preparing MVIIC and 47 analogues in only six runs by use of a combinatorial method, which was developed from the ordinary chemical synthesis method for MVIIC. The amount of each obtained analogue was inevitably smaller than that of an individually synthesized analogue, but was sufficient for identification and estimation of activities, and no tendency for increased polymerization or aggregation was observed in comparison with a single analogue preparation. The most important factor which made this method feasible is that the method for preparing a single MVIIC analogue has been well established in terms of oxidative folding conditions and purification method.

The simultaneous disulfide bond formation method described here is still at a preliminary stage and technical improvements are necessary for its application to the preparation of peptide libraries. However, we believe it has considerable potential for the discovery of ion channel blockers with higher affinity and selectivity. Moreover, it should be applicable for creating functional molecules other than ion channel blockers, for example, molecules which modulate protein–protein interaction. Such a strategy should help us to develop many types of pharmacological tools and lead molecules for drug design.

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