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Discovery of a Potent and Selective α3β4 Nicotinic Acetylcholine Receptor Antagonist from an α-Conotoxin Synthetic Combinatorial Library

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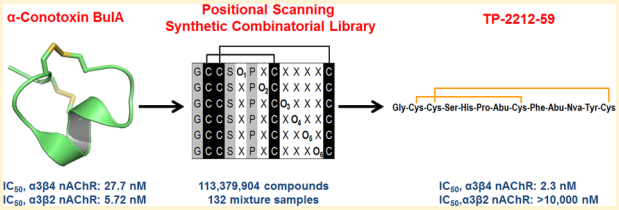
ABSTRACT: α-Conotoxins are disulfide-rich peptide neurotoxins that selectively inhibit neuronal nicotinic acetylcholine receptors (nAChRs). The α3β4 nAChR subtype has been identified as a novel target for managing nicotine addiction. Using a mixture-based positional-scanning synthetic combinatorial library (PS-SCL) with the α4/4-conotoxin BuIA framework, we discovered a highly potent and selective α3β4 nAChR antagonist. The initial PS-SCL consisted of a total of 113 379 904 sequences that were screened for α3β4 nAChR inhibition, which facilitated the design and synthesis of a second generation library of 64 individual α-conotoxin derivatives. Eleven analogues were identified as α3β4 nAChR antagonists, with TP-2212-59 exhibiting the most potent antagonistic activity and selectivity over the α3β2 and α4β2 nAChR subtypes. Final electrophysiological characterization demonstrated that TP-2212-59 inhibited acetylcholine evoked currents in α3β4 nAChRs heterogeneously expressed in *Xenopus laevis* oocytes with a calculated IC_{50} of 2.3 nM and exhibited more than 1000-fold selectivity over the α3β2 and α7 nAChR subtypes. As such, TP-2212-59 is among the most potent α3β4 nAChRs antagonists identified to date and further demonstrates the utility of mixture-based combinatorial libraries in the discovery of novel α-conotoxin derivatives with refined pharmacological activity.



INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) are a family of ligand gated ion channels that are involved in a variety of central and peripheral nervous systems functions including memory, cognition, and reward.[1] Neuronal nAChRs are composed of a pentameric complex of closely related, yet functionally distinct protein subunits arranged around a central cation-conducting pore.[2] In the CNS, nAChRs may consist exclusively of α-subunits (homomeric) or contain combinations of α- and β-subunits (heteromeric). This gives rise to a large number of different receptor subtypes, each of which exhibits distinct pharmacological functions and may be involved in various neuropathological states.[3]

Neuronal nAChRs that are present in mesocorticolimbic reward pathways are important targets for studying nicotine addiction and in the development of smoking cessation therapeutics.[4] For example, the α4β2 nAChR is the most predominant nAChR subtype found in the central nervous system and, as such, is a target for varenicline, a currently approved smoking cessation medication.[5] However, the α3β4 nAChR expressed in the medial habenula has also been found to play a significant role in nicotine addiction.[6−8] For example, 18-methoxycoronaridine (18-MC) and α-conotoxin AuIB were shown to block the α3β4 nAChR in the medial habenula in rats, thus reducing nicotine self-administration.[9,10] More recently, AT-1001, a potent and relatively selective α3β4 nAChR antagonist, was shown to block nicotine self-administration in rats following systemic administration.[11] On the other hand, varenicline also exhibits α3β4 nAChR agonist activity,[12,13] which may account for several observed peripheral and central side effects, including nausea, gastrointestinal symptoms, and suicidal ideation. Therefore, novel antagonists that potently and selectively block the α3β4 nAChR are valuable tools for probing the role that this receptor plays in nicotine addiction and could lead to the development of safer smoking cessation therapeutics.

α-Conotoxins are a family of small, disulfide-rich peptides isolated from the venoms of carnivorous marine cone snails.[14] The remarkable nAChR selectivity of α-conotoxins provides a unique structural template for designing novel nAChR antagonists with increased inhibitory potencies.[15] Typically, α-conotoxins consist of 12−20 amino acids and contain two highly conserved disulfide bonds. In native α-conotoxins, the disulfide bonds are linked via the Cys1-Cys3 and Cys2-Cys4 (globular) connectivity, although two additional misfolded isomers are also possible with connectivity between Cys1-Cys4 and Cys2-Cys3 (ribbon) and between Cys1-Cys2 and Cys3-

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Cys4 (beads). Native α-conotoxins exhibit a well-defined structural framework that projects two loops of intervening amino acid residues between Cys2-Cys3 and Cys3-Cys4 that are denoted as the *m*- and *n*-loops, respectively (Figure 1). The

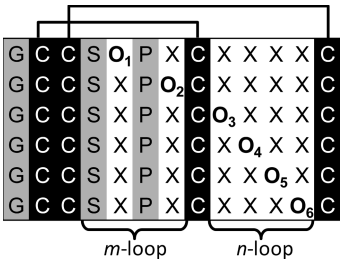

Figure 1. Design of the mixture-based PS-SCL based on the α4/4-conotoxin framework. The conserved cysteine framework and native (globular) disulfide bond connectivity are indicated. Conserved Gly, Ser, and Pro residues are shown in gray. $\mathbf{O}_n$ is a single defined position, and **X** is an equimolar mixture of 22 natural and non-natural L-amino acids.

amino acids contained within these loops are highly variable across the α-conotoxin family, with subtle modifications of these residues often having a profound influence on the potency and selectivity toward different nAChR subtypes.[14] Therefore, the relative ease of chemical synthesis and conserved structural characteristics of α-conotoxins allow further optimization of their nAChR selectivity and antagonist potency.[16]

Several α-conotoxins are known to target the $\alpha3\beta4$ nAChR with varying degrees of potency and selectivity (Table 1). For example, α-conotoxin AuIB has been used as a probe to study the role of the $\alpha3\beta4$ nAChR in the medial habenula, although such studies are limited by its relatively low inhibitory potency.[10,17] Recently, α-conotoxin TxID was characterized as being the most potent and selective $\alpha3\beta4$ nAChR α-conotoxin antagonist, thus making it a valuable probe for studying this receptor.[18] On the other hand, α-conotoxin BuIA is a potent $\alpha3\beta4$ nAChR antagonist, but it also targets a broad range of subtypes, including $\alpha3\beta2$, $\alpha6/\alpha3\beta2\beta3$, and $\alpha6/\alpha3\beta4$ nAChRs.[19] Significantly, BuIA exhibits a unique 4/4 loop framework that is not commonly found in other known α-conotoxins (Figure 1).[20] The inhibitory function and binding kinetics of BuIA have been examined on cloned nAChRs expressed in *Xenopus* oocytes.[19,21] BuIA blocks several $\beta2$- and $\beta4$-containing heteromeric nAChRs, with the highest potency against $\alpha3$- and chimeric $\alpha6$-containing receptors.[19,22] Significantly, nAChR with $\beta4$-containing subunits exhibited slower off-rates when compared to the corresponding $\beta2$-containing nAChR.[19,21] Similar results were found in both human and mouse $\alpha3\beta2$ nAChRs and their $\alpha3\beta4$ counterparts, suggesting that β-subunit selectivity is conserved across species.[19] Notably, Pro6 of BuIA is a critical determinant for distinguishing between $\beta2$ and $\beta4$ subunits, where substitution with 4-hydroxyproline (Hyp) (i.e., BuIA[P6O]) exhibited significant selectivity for $\alpha6\beta4$ over $\alpha6\beta2$ nAChR.[23]

With continued interest in developing novel nAChR ligands as probes for studying the role that these receptors play in nicotine addiction, we have utilized a synthetic combinatorial strategy to develop a potent and highly selective $\alpha3\beta4$ nAChR antagonist using the unique $\alpha4/4$-conotoxin framework exhibited by BuIA. A positional scanning synthetic combinatorial library (PS-SCL) based on the variable positions within the $\alpha4/4$-conotoxin framework was synthesized and screened for $\alpha3\beta4$ nAChR inhibitory activity to identify novel amino acid residues in each variable position within this framework. This led to the design and synthesis of a second generation library of individual BuIA derivatives, of which one analogue was identified as being among the most potent and selective of α-conotoxins targeting the $\alpha3\beta4$ nAChR characterized to date.

RESULTS

Design and Synthesis of the $\alpha4/4$-Conotoxin PS-SCL.

A PS-SCL based on the $\alpha4/4$-conotoxin loop framework was prepared to facilitate the identification of amino acid substitutions at key positions within the *m*- and *n*-loops that give rise to $\alpha3\beta4$ nAChR antagonistic activity (Figure 1). In native α-conotoxins targeting neuronal nAChRs, the four cysteine residues, together with Gly1, Ser4, and Pro6, are generally conserved. However, the remaining six positions are highly variable and were used as diversity positions within the framework. As such, six sublibraries were prepared, where $\mathbf{O}_n$ is a single defined position, and **X** is an equimolar mixture of 22 natural and non-natural L-amino acids (see *x*-axes in Figure 2).

Table 1. α-Conotoxins That Target the $\alpha3\beta4$ nAChR[a]

Name	Loop Spacing	Sequence	nAChR selectivity	IC_{50} ($\alpha3\beta4$)	Reference
TP-2212-59	4/4	GCCSHPBCFBZY---C	α3β4	2.3 nM	This work
BuIA	4/4	GCCSTPPCAVLY---C	α3β2 > α3β4 > α6α3β4	27.7 nM	19
AuIB	4/6	GCCSYPPCFATNPD-C	α3β4	770 nM	17
TxID	4/6	GCCSHPVCSAMSPI-C	α3β4	12.5 nM	18
GIC	4/7	GCCSHPACAGNNQHIC	α3β2 > α4β2 > α3β4	755 nM	48
PeIA	4/7	GCCSHPACSVNHPELC	α9α10 > α3β2 > α3β4	480 nM	49
RegIIA	4/7	GCCSHPACNVNNPHIC	α3β2 > α3β4 ≈ α7	97 nM	50

[a]Conserved cysteine residues are indicated with a box. The native disulfide bond connectivity is between Cys1-Cys3 and Cys2-Cys4. B is 2-aminobutyric acid (Abu). Z is norvaline (Nva).

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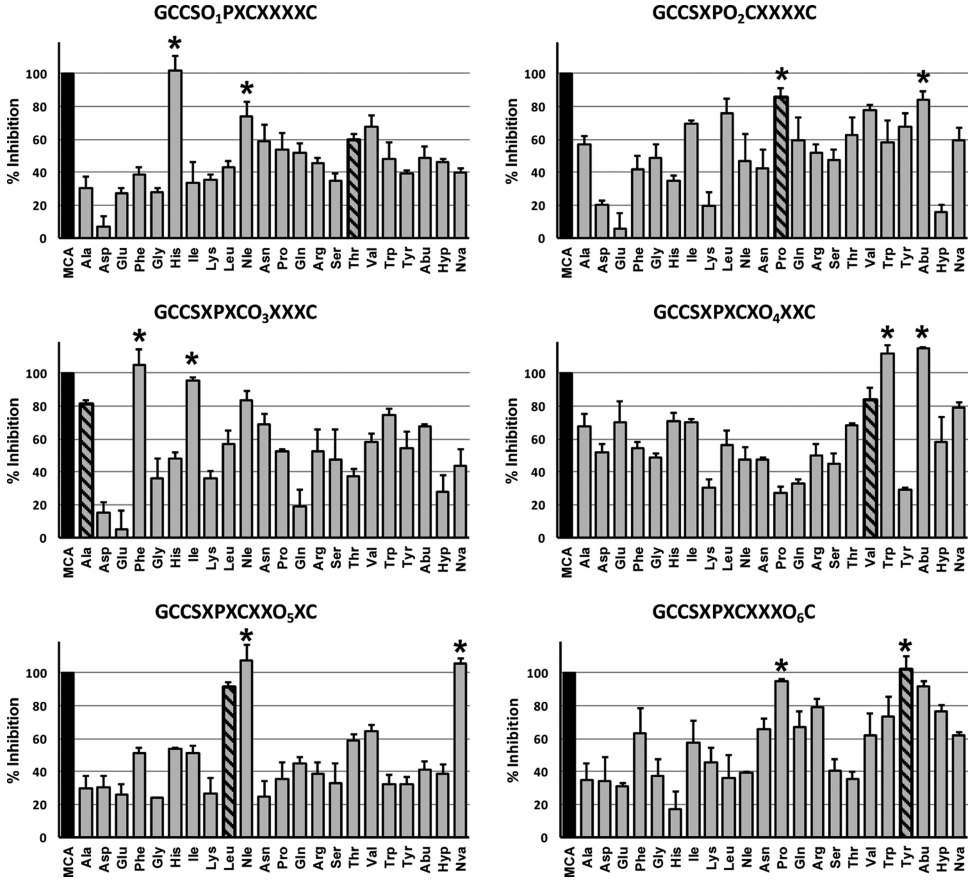

Figure 2. Initial screening of the α4/4-conotoxin BuIA PS-SCL for $\alpha3\beta4$ nAChR inhibition using the fluorescent membrane potential assay. The library was screened in triplicate at 100 μM, and the percentages of inhibition were calculated by comparing the potency of 10 μM mecamylamine (MCA), which was defined as 100% inhibition. Residues that were selected for the synthesis of a second generation library of individual analogues are marked with an asterisk. Amino acids indicated with a cross-hatch pattern correspond to native α-conotoxin BuIA residues.

All proteinogenic amino acids were used, with the exceptions of Cys and Met, which were omitted from the sample mixtures to avoid the formation of oxidation byproducts. As such, 2-aminobutyric acid (Abu) and norleucine (Nle) were included as isosteric replacements of Cys and Met, respectively, in the **X** and $\mathbf{O}_n$ positions. Norvaline (Nva) was also included to complete the series of side chains containing hydrophobic alkyl groups. Moreover, Hyp is a commonly occurring post-translational modification found in several α-conotoxins that was also included in the construction of the library.[24,25]

The PS-SCL was composed of 132 mixture samples, each of which contained 5 153 632 compounds across six sublibraries, with a total of 113 379 904 possible individual amino acid combinations in the entire library. The library was assembled using the "tea bag" method with Boc-SPPS chemistry,[26] followed by a two-step "low–high" HF cleavage procedure.[27] **X**-positions were coupled as a cocktail of amino acids using adjusted predetermined ratios to compensate for the differences in reactivity between different amino acid resides in competitive couplings.[28] The formation of disulfide bonds was achieved by cosolvent assisted oxidative folding (50% isopropanol in aqueous ammonium bicarbonate buffer at pH 8.2), which was previously shown to maximize the accumulation of the native globular isomer of α-conotoxin BuIA.[29] A simplified desalting procedure that employed disposable solid-phase extraction (SPE) columns was used for the rapid and efficient preparation of library samples in parallel prior to initial pharmacological screening.[29] Note that the globular and ribbon isomers of selected second generation individual analogues were later synthesized separately to confirm their identity and were purified by RP-HPLC prior to formal functional characterization.

Screening of the α4/4-Conotoxin PS-SCL for $\alpha3\beta4$ nAChR Inhibition. The 132 PS-SCL mixture samples were each screened for inhibition of rat $\alpha3\beta4$, $\alpha3\beta2$, and $\alpha4\beta2$ nAChRs in the fluorescent membrane potential assay,[30] using HEK293 cell lines stably expressing each nAChR subtype.[31,32] Each sample was screened at 100, 10, and 1 μM, based on the total concentration of α-conotoxin contained within the mixture. Screening of the PS-SCL for $\alpha3\beta4$ nAChR inhibition indicated a dose-dependent inhibitory activity, with a concentration of 100 μM allowing discrete active mixtures corresponding to specific amino acid substitutions to be identified in each position within the α4/4-conotoxin BuIA framework (Figure 2). However, no significant preference for amino acids was apparent at each position for $\alpha3\beta2$ and $\alpha4\beta2$ nAChRs at 100 μM, with a majority of the PS-SCL mixtures exhibiting >80% inhibition. Because no discrete active hits could be identified from $\alpha3\beta2$ and $\alpha4\beta2$ nAChR screening, individual second generation analogues were designed based on the $\alpha3\beta4$ nAChR screen. As such, the two amino acid residues identified in each position as exhibiting the highest antagonistic activity for the $\alpha3\beta4$ nAChR subtype were selected for the design of a second generation library of individual α-conotoxin analogues (Table 2).

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Table 2. Selection of Amino Acid Residues for Second Generation Library Synthesis

position					
O_1	O_2	O_3	O_4	O_5	O_6
Amino Acid					
His	Pro	Phe	Trp	Nle	Pro
Nle	Abu	Ile	Abu	Nva	Tyr

At the O_1 position, His was clearly identified as the most potent, exhibiting 100% inhibition of the $\alpha3\beta4$ nAChR subtype, and was selected for the synthesis of a second generation library. Nle, which exhibited 80% inhibition of the $\alpha3\beta4$ nAChR, was also selected. Thr, which is the native amino acid in BuIA, exhibited 60% inhibition of $\alpha3\beta4$ nAChR and was not selected for the second generation library.

At the O_2 position, the native Pro residue in BuIA exhibited a 90% inhibitory potency for the $\alpha3\beta4$ nAChR subtype, indicating that the structure of two consecutive Pro residues in the *m*-loop is important for sustaining conformational integrity and thus was selected for the synthesis of the second generation library. Abu exhibited a similar inhibitory potency to Pro (90%) and was also selected for the second generation library synthesis. Furthermore, the hydrophobic amino acids Leu and Val each exhibited >80% inhibition of $\alpha3\beta4$ nAChR.

At the O_3 position, the hydrophobic amino acids Phe and Ile were selected for the second generation library. Notably, Ala, which is the native residue at this position in BuIA, together with Nle also exhibited greater than 80% of inhibitory activity for the $\alpha3\beta4$ nAChR subtype.

At the O_4 position, Trp and Abu clearly produced the greatest inhibitory potency for the $\alpha3\beta4$ nAChRs (approximately 100%) and were selected for the synthesis of the second generation library.

At the O_5 position, the unbranched hydrophobic amino acids Nva and Nle were selected for the second generation library. Leu, which is the native residue at this position in BuIA, also produced 90% inhibition.

At the O_6 position, the native Tyr residue in BuIA exhibited a 100% inhibitory potency for the $\alpha3\beta4$ nAChR subtype and was selected for the second generation library. Interestingly, a constrained Pro residue at this position that would be expected to induce a structural distortion also gave rise to high inhibitory activity (90%).

Synthesis and Screening of a Second Generation Library of Individual α-Conotoxin Analogues. A second

Table 3. Sequences of Individual Second Generation $\alpha4/4$-Conotoxin Analogues and Their % Inhibition of $\alpha3\beta4$ nAChR at 10 μM Relative to Mecamylamine in the Fluorescent Membrane Potential Assay[a]

TP-2212	O_1	O_2	O_3	O_4	O_5	O_6	% inhibtion (±SEM)	TP-2212	O_1	O_2	O_3	O_4	O_5	O_6	% inhibition (±SEM)
BuIA	Thr	Pro	Ala	Val	Leu	Tyr	94.4 ± 3.2	**33**	His	Pro	Phe	Trp	Nle	Tyr	44.0 ± 4.9
1	His	Pro	Phe	Trp	Nle	Pro	15.3 ± 4.1	**34**	Nle	Pro	Phe	Trp	Nle	Tyr	60.2 ± 1.9
2	Nle	Pro	Phe	Trp	Nle	Pro	14.2 ± 7.7	**35**	His	Abu	Phe	Trp	Nle	Tyr	101.4 ± 0.7
3	His	Abu	Phe	Trp	Nle	Pro	22.3 ± 4.6	**36**	Nle	Abu	Phe	Trp	Nle	Tyr	76.0 ± 1.3
4	Nle	Abu	Phe	Trp	Nle	Pro	22.1 ± 2.7	**37**	His	Pro	Ile	Trp	Nle	Tyr	43.9 ± 3.8
5	His	Pro	Ile	Trp	Nle	Pro	10.1 ± 2.3	**38**	Nle	Pro	Ile	Trp	Nle	Tyr	51.8 ± 4.1
6	Nle	Pro	Ile	Trp	Nle	Pro	6.3 ± 1.2	**39**	His	Abu	Ile	Trp	Nle	Tyr	55.7 ± 1.7
7	His	Abu	Ile	Trp	Nle	Pro	15.4 ± 2.4	**40**	Nle	Abu	Ile	Trp	Nle	Tyr	68.6 ± 0.9
8	Nle	Abu	Ile	Trp	Nle	Pro	51.4 ± 2.0	**41**	His	Pro	Phe	Abu	Nle	Tyr	106.2 ± 1.1
9	His	Pro	Phe	Abu	Nle	Pro	29.1 ± 4.0	**42**	Nle	Pro	Phe	Abu	Nle	Tyr	106.6 ± 0.7
10	Nle	Pro	Phe	Abu	Nle	Pro	54.6 ± 2.9	**43**	His	Abu	Phe	Abu	Nle	Tyr	110.2 ± 0.6
11	His	Abu	Phe	Abu	Nle	Pro	35.5 ± 5.4	**44**	Nle	Abu	Phe	Abu	Nle	Tyr	106.3 ± 0.9
12	Nle	Abu	Phe	Abu	Nle	Pro	36.6 ± 4.1	**45**	His	Pro	Ile	Abu	Nle	Tyr	67.2 ± 1.0
13	His	Pro	Ile	Abu	Nle	Pro	38.1 ± 3.7	**46**	Nle	Pro	Ile	Abu	Nle	Tyr	63.8 ± 2.1
14	Nle	Pro	Ile	Abu	Nle	Pro	31.3 ± 5.4	**47**	His	Abu	Ile	Abu	Nle	Tyr	110.1 ± 0.6
15	His	Abu	Ile	Abu	Nle	Pro	36.2 ± 2.9	**48**	Nle	Abu	Ile	Abu	Nle	Tyr	72.6 ± 1.1
16	Nle	Abu	Ile	Abu	Nle	Pro	42.9 ± 4.6	**49**	His	Pro	Phe	Trp	Nva	Tyr	47.8 ± 3.2
17	His	Pro	Phe	Trp	Nva	Pro	49.0 ± 3.3	**50**	Nle	Pro	Phe	Trp	Nva	Tyr	61.6 ± 1.0
18	Nle	Pro	Phe	Trp	Nva	Pro	37.1 ± 5.3	**51**	His	Abu	Phe	Trp	Nva	Tyr	72.1 ± 2.0
19	His	Abu	Phe	Trp	Nva	Pro	36.2 ± 6.9	**52**	Nle	Abu	Phe	Trp	Nva	Tyr	67.9 ± 1.2
20	Nle	Abu	Phe	Trp	Nva	Pro	41.1 ± 5.3	**53**	His	Pro	Ile	Trp	Nva	Tyr	56.5 ± 3.0
21	His	Pro	Ile	Trp	Nva	Pro	44.8 ± 5.9	**54**	Nle	Pro	Ile	Trp	Nva	Tyr	58.3 ± 2.8
22	Nle	Pro	Ile	Trp	Nva	Pro	38.9 ± 4.0	**55**	His	Abu	Ile	Trp	Nva	Tyr	47.5 ± 6.2
23	His	Abu	Ile	Trp	Nva	Pro	38.7 ± 1.8	**56**	Nle	Abu	Ile	Trp	Nva	Tyr	61.9 ± 3.1
24	Nle	Abu	Ile	Trp	Nva	Pro	44.8 ± 2.9	**57**	His	Pro	Phe	Abu	Nva	Tyr	92.6 ± 0.5
25	His	Pro	Phe	Abu	Nva	Pro	36.0 ± 3.9	**58**	Nle	Pro	Phe	Abu	Nva	Tyr	96.4 ± 1.1
26	Nle	Pro	Phe	Abu	Nva	Pro	35.7 ± 3.9	**59**	His	Abu	Phe	Abu	Nva	Tyr	109.2 ± 0.6
27	His	Abu	Phe	Abu	Nva	Pro	47.0 ± 1.6	**60**	Nle	Abu	Phe	Abu	Nva	Tyr	104.0 ± 0.5
28	Nle	Abu	Phe	Abu	Nva	Pro	40.3 ± 4.1	**61**	His	Pro	Ile	Abu	Nva	Tyr	62.3 ± 2.4
29	His	Pro	Ile	Abu	Nva	Pro	45.1 ± 3.7	**62**	Nle	Pro	Ile	Abu	Nva	Tyr	50.9 ± 4.0
30	Nle	Pro	Ile	Abu	Nva	Pro	45.7 ± 5.4	**63**	His	Abu	Ile	Abu	Nva	Tyr	84.5 ± 1.6
31	His	Abu	Ile	Abu	Nva	Pro	41.2 ± 2.8	**64**	Nle	Abu	Ile	Abu	Nva	Tyr	59.4 ± 3.8
32	Nle	Abu	Ile	Abu	Nva	Pro	19.2 ± 1.9								

[a]The canonical sequence for each individual compound is Gly-Cys-Cys-Ser-O_1-Pro-O_2-Cys-O_3-O_4-O_5-O_6-Cys.

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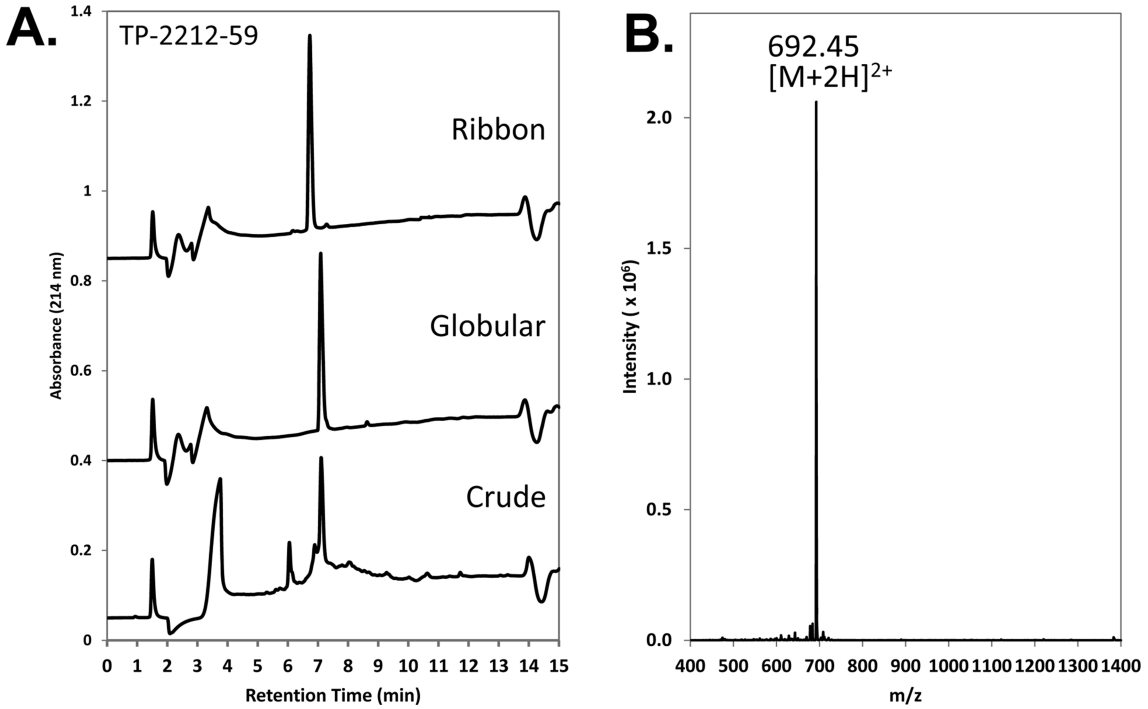

Figure 3. Representative LC–MS analysis of TP-2212-59. (A) LC analysis of TP-2212-59 samples. Samples were analyzed using a C_{18} column (50 mm × 4.6 mm i.d.) with a gradient of 0–60% acetonitrile containing 0.1% formic acid over 12 min at a flow rate of 0.5 mL/min and monitored at 214 nm. Crude samples (bottom) were desalted in parallel using SPE cartridges prior to initial library screening. Globular and ribbon isomers for further pharmacological characterization (center and top, respectively) were synthesized using a two-step regioselective folding approach and purified to >95% homogeneity as described in the Experimental Section. (B) Representative electrospray ionization MS of TP-2212-59. The final mass was calculated from the observed $[M + 2H]^{2+}$ ion: calculated mass, 1382.9; expected mass, 1382.5.

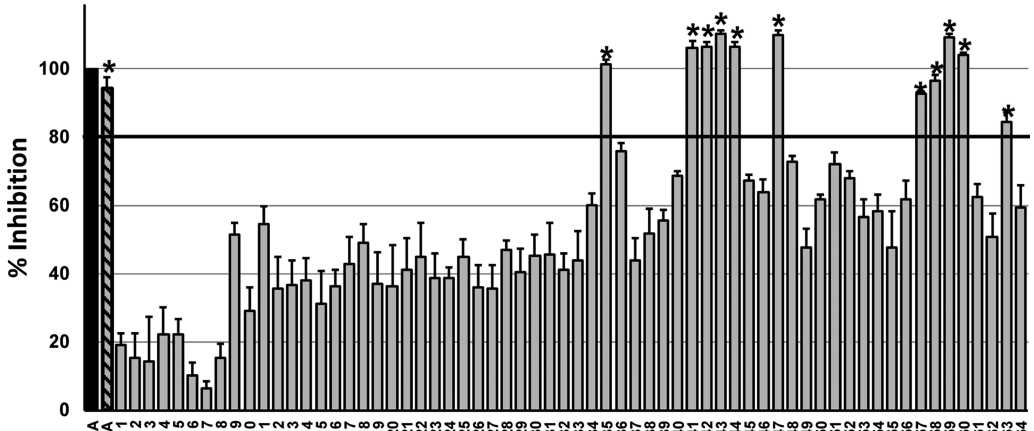

Figure 4. Fluorescent membrane potential assay screening of the second generation individual library at 10 μM. Compounds that exhibited >80% inhibition at 10 μM (indicated with a solid line and asterisk) were selected for further chemical and pharmacological characterization.

generation library (TP-2212) was synthesized and consisted of 64 individual α-conotoxin sequences that were constructed from systematic combinations of selected amino acid residues identified from PS-SCL screening against the $\alpha3\beta4$ nAChR at 100 μM (Table 3). Following assembly and cleavage, each α-conotoxin analogue was oxidized using 50% isopropanol in aqueous ammonium bicarbonate buffer at pH 8.2 as used to prepare the PS-SCL. A majority of samples indicated efficient folding to one predominant isomer as determined by analytical LC–MS (Figure 3). For the initial screen of the second generation library, all samples were desalted in parallel using SPE columns and screened as crude samples (Figure 3), which allowed any side products including misfolded disulfide bond isomers that were present in the preparation of the PS-SCL to be retained during the synthesis of individual analogues. This approach increased the probability of identifying the major active component.

The second generation library compounds were screened for inhibition of the $\alpha3\beta4$ nAChR at 10 μM using the fluorescent membrane potential assay (Figure 4 and Table 3). Of the 64

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Table 4. Inhibitory Potencies (IC_{50} ± SEM (nM)) of 11 Selected Compounds Identified as Active Hits from Initial Screening of the Second Generation Library for $\alpha3\beta4$, $\alpha3\beta2$, and $\alpha4\beta2$ nAChRs Using the Fluorescent Membrane Potential Assay (n = 4)

TP-2212	sequence	$\alpha3\beta4$	Hill slope	$\alpha3\beta2$	Hill Slope	$\alpha4\beta2$	Hill slope
BuIA	Gly-Cys-Cys-Ser-Thr-Pro-Pro-Cys-Ala-Val-Leu-Tyr-Cys	265 ± 17	−1.06	26 ± 1.0	−1.20	>10000	ND
35	Gly-Cys-Cys-Ser-His-Pro-Abu-Cys-Phe-Trp-Nle-Tyr-Cys	1727 ± 151	−1.08	>10000	ND	>10000	ND
41	Gly-Cys-Cys-Ser-His-Pro-Pro-Cys-Phe-Abu-Nle-Tyr-Cys	469 ± 19	−0.94	>10000	ND	>10000	ND
42	Gly-Cys-Cys-Ser-Nle-Pro-Pro-Cys-Phe-Abu-Nle-Tyr-Cys	397 ± 24	−0.96	>10000	ND	>10000	ND
43	Gly-Cys-Cys-Ser-His-Pro-Abu-Cys-Phe-Abu-Nle-Tyr-Cys	307 ± 37	−0.94	>10000	ND	>10000	ND
44	Gly-Cys-Cys-Ser-Nle-Pro-Abu-Cys-Phe-Abu-Nle-Tyr-Cys	310 ± 29	−1.00	>10000	ND	>10000	ND
47	Gly-Cys-Cys-Ser-His-Pro-Abu-Cys-Ile-Abu-Nle-Tyr-Cys	463 ± 9.0	−0.96	>10000	ND	>10000	ND
57	Gly-Cys-Cys-Ser-His-Pro-Pro-Cys-Phe-Abu-Nva-Tyr-Cys	298 ± 22	−0.97	>10000	ND	>10000	ND
58	Gly-Cys-Cys-Ser-Nle-Pro-Pro-Cys-Phe-Abu-Nva-Tyr-Cys	345 ± 31	−1.01	>10000	ND	>10000	ND
59	Gly-Cys-Cys-Ser-His-Pro-Abu-Cys-Phe-Abu-Nva-Tyr-Cys	183 ± 36	−1.05	>10000	ND	>10000	ND
60	Gly-Cys-Cys-Ser-Nle-Pro-Abu-Cys-Phe-Abu-Nva-Tyr-Cys	320 ± 14	−0.99	>10000	ND	>10000	ND
63	Gly-Cys-Cys-Ser-His-Pro-Abu-Cys-Ile-Abu-Nva-Tyr-Cys	1934 ± 77	−0.93	>10000	ND	>10000	ND

individual α-conotoxin samples screened, 11 exhibited greater than 80% inhibition and were defined as active hits. Of these 11 active hits, the substitution of His in the O_1 position (compounds **35**, **41**, **43**, **47**, **57**, **59**, and **63**) and Nle (compounds **42**, **44**, **58**, and **60**) exhibited more than 80% inhibition. The native Pro residue (compounds **41**, **42**, **57**, and **58**) along with Abu (compounds **35**, **43**, **44**, **47**, **59**, **60**, and **63**) were compatible at the O_2 position. For the O_3 position, the substitution of Phe (compounds **35**, **41**, **42**, **43**, **44**, and **57**-**60**) and Ile (compounds **47** and **63**) also produced >80% inhibitory activity. Of the 11 active hits, 10 (compounds **41**−**44**, **47**, **57**−**60**, and **63**) contain Abu at the O_4 position, with compound **35** being the only compound containing Trp at this position. Compounds containing two selected amino acids at the O_5 position (Nle in compounds **35**, **41**, **42**, **43**, **44**, and **47**; Nva in compounds **57**−**60** and **63**) were also identified as active hits. Notably, a distinct trend was observed at the O_6 position between Pro (compounds **1**−**32**) Tyr (compounds **33**−**64**), where the substitution of Pro significantly decreased inhibitory activity, while the native Tyr residue of α-conotoxin BuIA at this position retained its potency for $\alpha3\beta4$ nAChR.

Identification and Characterization of Active Hits. The 11 active hits were selected for further characterization and assessment of nAChR antagonistic activity. First, both the globular and ribbon isomers of each α-conotoxin analogue were synthesized to confirm the identity of the active component from second generation library screening. Each pair of Cys residues was differentially protected using MeBzl and Acm protecting groups followed by a two-step oxidation procedure and purified using RP-HPLC to obtain each isomer in >95% purity (Figure 3). Both isomers were analyzed by LC−MS and co-injected with the major products obtained from the second generation library, which confirmed that the globular isomer was the major product in each of the selected second generation library samples. However, the ribbon isomer was also present as a minor isomer when Nle was substituted in the O_1 position.

To confirm the identity of the active component of each of the 11 active hits from the second generation library, both globular and ribbon isomers were tested for $\alpha3\beta4$ nAChR inhibitory activity using the fluorescent membrane potential assay (Table 4 and Figure 5). For each compound, the globular isomer potently inhibited the $\alpha3\beta4$ nAChR while the ribbon isomer of each compound exhibited no inhibitory activity up to 10 μM, thus confirming that the globular isomer was the active

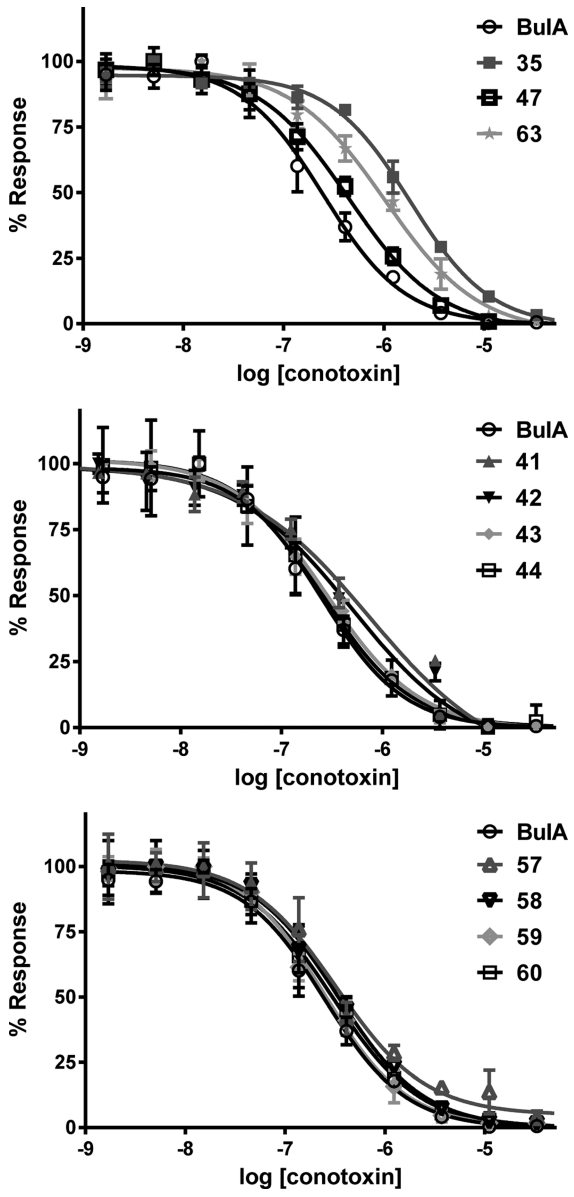

Figure 5. Functional characterization of selected individual α4/4-conotoxins for $\alpha3\beta4$ nAChR inhibition using the fluorescent membrane potential assay.

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component of each of the selected second generation library screening samples.

With the exception of compounds **35** and **63**, all of the selected compounds exhibited inhibitory potencies for the $\alpha3\beta4$ nAChR subtype that were comparable to that of BuIA in the fluorescent membrane potential assay (Table 4 and Figure 5). To further test for selectivity, each of the 11 active hits was also tested for $\alpha3\beta2$ and $\alpha4\beta2$ nAChR inhibitory activity. Significantly, all of compounds exhibited increased selectivity for the $\alpha3\beta4$ nAChR when compared to α-conotoxin BuIA, with no antagonist activity observed for the $\alpha3\beta2$ and $\alpha4\beta2$ nAChR subtypes up to 10 μM (Table 4).

Compounds **57**, **58**, **59**, and **60** were further tested for competitive inhibition of [^{3}H]epibatidine binding to $\alpha3\beta4$ and $\alpha4\beta2$ nAChRs expressed in HEK293 cell membranes (Figure 6

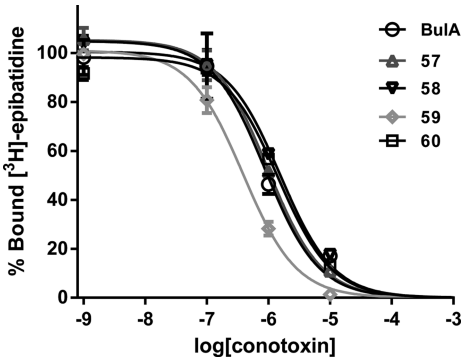

Figure 6. Radioligand binding assays of BuIA and compounds **57**–**60** bound to the $\alpha3\beta4$ nAChR subtype. [^{3}H]Epibatidine was used as a hot ligand in the $\alpha3\beta4$ nAChR ligand–receptor binding assay.

Table 5. Inhibition of [^{3}H]Epibatidine Binding of Selected Compounds to $\alpha3\beta4$ nAChR Expressed in HEK293 Cell Membranes

TP-2212	K_i ± SEM (nM)
BuIA	851 ± 137
57	794 ± 65
58	1107 ± 9
59	270 ± 11
60	980 ± 122

and Table 5). The binding data correlate with results obtained from fluorescent membrane potential assay, with compound **59** exhibiting moderately increased potency toward $\alpha3\beta4$ nAChR when compared to BuIA. Furthermore, no significant binding to the $\alpha4\beta2$ nAChR was observed for any compound up to 10 μM (data not shown). These results provided further evidence that these compounds selectively bind to the endogenous $\alpha3\beta4$ nAChR binding site.

Electrophysiological Characterization. Compounds **57**, **58**, **59**, and **60** were further tested for $\alpha7$ nAChR inhibitory activity by recording ACh-evoked currents in *Xenopus* oocytes. While compound **57** inhibited the response by 49.8% ± 3.2% at 10 μM, no inhibition of the $\alpha7$ nAChR was observed for compounds **58**, **59**, and **60** up to 10 μM. TP-2212-59 (compound **59**) was selected for further functional characterization by recording ACh-evoked currents mediated by $\alpha3\beta4$ and $\alpha3\beta2$ nAChR subtypes heterologously expressed in *Xenopus* oocytes. TP-2212-59 potently blocked the $\alpha3\beta4$ subtype. However, TP-2212-59 exhibited very slow washout kinetics, with 90–120 min required to reach steady-state equilibrium that prohibited the accurate measurement of inhibition at concentrations below 10 nM (Figure 7A). Despite

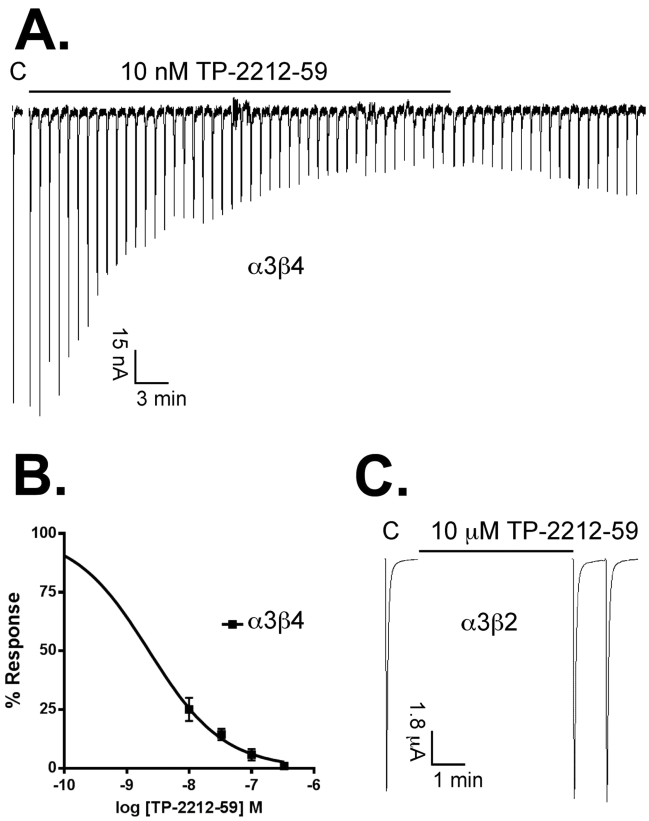

Figure 7. Functional characterization of TP-2212-59 for $\alpha3\beta4$ and $\alpha3\beta2$ nAChR inhibition of acetylcholine evoked currents in *Xenopus* oocytes. ACh (300 μM) was applied as a 1 s pulse once per minute to *Xenopus* oocytes expressing rat nAChRs. (A) TP-2212-59 (10 nM) was perfusion applied to oocytes expressing $\alpha3\beta4$ nAChRs until steady-state block of the ACh current was achieved. TP-2212-59 was then washed out and recovery of block measured. (B) Concentration response of TP-2212-59 on the $\alpha3\beta4$ nAChR. The top and bottom of the curve were constrained to 100 and 0, respectively. The IC_{50} value is calculated as 2.3 nM. n = 3–5 oocytes for each peptide concentration. (C) TP-2212-59 was applied as a 10 μM static bath for 5 min to oocytes expressing rat $\alpha3\beta2$ nAChRs (1000 times higher peptide concentration than that used for $\alpha3\beta4$ nAChRs). Representative traces are shown for each nAChR subtype.

the slow washout kinetics at the $\alpha3\beta4$ nAChR, TP-2212-59 clearly exhibited dose-dependent inhibition to a concentration of 10 nM (Figure 7B). In order to calculate an IC_{50} value for TP-2212-59 at the $\alpha3\beta4$ nAChR, the top and bottom of the curve were constrained to 100% and 0% responses, respectively. This allowed determination of an IC_{50} value for TP-2212-59, which was calculated to be 2.3 nM. In contrast, no inhibition of the $\alpha3\beta2$ nAChR was observed up to 10 μM, thus confirming a >1000-fold selectivity for the $\alpha3\beta4$ subtype (Figure 7C). Together, these results suggest that TP-2212-59 is among the most potent and selective α-conotoxins that target the $\alpha3\beta4$ nAChR subtype to be characterized thus far.

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DISCUSSION

Venomous marine cone snails utilize natural combinatorial libraries of peptide neurotoxins that target an array of receptors in the central and peripheral nervous systems to immobilize and capture their more agile prey.[33] Among these are the α-conotoxins, which exhibit exquisite selectivity against different nAChR subtypes and thus are important research tools for studying a variety of neuropathological conditions, including pain, memory, cognition, and tobacco addiction. Their highly conserved three-dimensional structure and relative ease of synthesis by solid-phase peptide synthesis mean that α-conotoxins represent excellent structural scaffolds for developing large synthetic combinatorial libraries for functional screening.

Genomic and pharmacologic data have suggested the α3β4 nAChR subtype as a novel target for studying tobacco dependence and drug abuse.[7,8] Furthermore, α3-containing nAChRs have been implicated in neuropathic pain.[34,35] However, to determine the precise role of the α3β4 nAChR subtype in nicotine addiction and pain, potent and selective ligands for this receptor are rare. As a result, novel subtype-selective nAChR antagonists are crucial for the fundamental understanding of reward and pain pathways and may pave the way for the development of clinically effective smoking cessation drugs and analgesics that circumvent unwanted side effects.

In this study, we have utilized the unique α4/4-conotoxin framework as a template for constructing a high diversity mixture-based synthetic α-conotoxin combinatorial library for functional screening of the α3β4 nAChR subtype. BuIA is among only a few α-conotoxins identified so far that possesses the 4/4 framework, with a vast majority of neuronally active α-conotoxins possessing the more ubiquitous 4/7 framework.[14] BuIA is a potent antagonist of a broad range of nAChRs, including α3β2 and α3β4 nAChRs. Importantly, it is able to kinetically discriminate between β2- and β4-containing nAChR subtypes.[19,21] Modifications to key amino acid residues within the *m*- and *n*-loop allow its kinetics and selectivity to be fine-tuned. However, the design of functionally active and selective compounds remains a challenging task due to the conserved binding site of different nAChR subtypes that play an important role in the allosteric binding of ligands.

Previously, we reported the use of PS-SCLs based on α-conotoxin and small molecule frameworks for identifying pharmacologically active compounds that target nAChRs.[36−38] Here, we have extended our synthetic combinatorial approach using the α4/4-conotoxin framework as a template for synthesizing and screening a mixture-based PS-SCL for the discovery of potent and selective antagonists of the α3β4 nAChR. The PS-SCL consisted of a total of 113 379 904 α-conotoxin sequences, which expands immensely upon the diversity of conotoxin libraries previously reported by our group.[36,37]

The fluorescent membrane potential assay was used to screen the PS-SCL mixtures for α3β4, α3β2, and α4β2 antagonistic activity.[30] Though previous data suggest that inhibitory potencies obtained by this assay are significantly lower than those obtained from electrophysiological recordings, the assay has the advantage of allowing screening in a high-throughput manner and has proven to be useful for determining relative inhibitory potencies and selectivity of α-conotoxins for various nAChR subtypes and was used for initial screening and characterization of compounds.[37,39−41] Screening of the 132 PS-SCL mixture samples for inhibition of rat α3β4 nAChR expressed in HEK293 cells using the fluorescent membrane potential assay allowed discrete active hits to be rapidly identified for this receptor. However, complementary counter-screening against α3β2 and α4β2 nAChRs could not readily identify active hits for these subtypes, suggesting that the unique α4/4-conotoxin framework may be in an optimized conformation to interact within the α3β4 nAChR binding site.

Significantly, novel amino acid residues were identified at each position, although each of the native residues of BuIA was also found to exhibit a moderate to high inhibitory potency for α3β4 nAChR. Notably, mixtures corresponding to native α-conotoxin BuIA residues were identified as the most active samples in the $\mathbf{O_2}$ and $\mathbf{O_6}$ positions (i.e., Pro and Tyr, respectively). A second generation library of individual analogues was designed based exclusively on the screening of α3β4 nAChR. From the initial screening of the 64 individual second generation α4/4-conotoxin sequences that were synthesized, 11 compounds exhibited >80% inhibition of α3β4 nAChR and were selected for further chemical and pharmacological characterization. Despite extensive mutation of key residues within the α4/4-conotoxin framework, each of these 11 analogues spontaneously formed the correct folded globular isomer upon oxidative folding, although the misfolded ribbon isomer was also present when Nle was substituted in the $\mathbf{O_1}$ position. For all analogues, the globular isomer was confirmed as the active component of the second generation library samples.

His and Nle were identified at the $\mathbf{O_1}$ position, suggesting a possible hydrogen bond acceptor within a hydrophobic pocket of the endogenous binding site. Of significance, His is present in the $\mathbf{O_1}$ position in several conotoxins targeting α3β4 nAChR, including α-conotoxins TxID, GIC, PeIA, and RegIIA (see Table 1). Therefore, it is important that His was clearly defined as an active hit from initial PS-SCL screening among all other residues, which strongly suggests that the $\mathbf{O_1}$ is a major determinant for selectively targeting the α3β4 nAChR.

The substitution of Abu was tolerated at the $\mathbf{O_2}$ position and indicates that conformationally relaxed α-conotoxin BuIA analogues may have a positive effect on the binding kinetics and thus are able to improve both binding affinity and specificity. The result correlates with a NMR structural study where the active globular isomer was found to be structurally relaxed while the inactive ribbon isomer exhibited a well-defined conformation.[42] The substitution of Phe at the $\mathbf{O_3}$ position appears to play a crucial role in the increasing of activity. When compared to α-conotoxin BuIA, it is apparent that bulkier and smaller amino acids are preferred at the $\mathbf{O_4}$ and $\mathbf{O_5}$ positions, respectively. Significantly, all of the active sequences contained tyrosine at the $\mathbf{O_6}$ position, which is the native amino acid residue found in BuIA. Though Pro was selected at the $\mathbf{O_6}$ position based on PS-SCL screening, none of the individual sequences containing Pro at this position were identified as active hits. This was not surprising, since substitution with proline would be expected to introduce detrimental structural distortion into the well-defined α-conotoxin framework. Interestingly, the eight of the canonical α4/4-conotoxin sequences, defined as Gly-Cys-Cys-Ser-(His or Nle)-Pro-(Abu or Pro)-Cys-Phe-Abu-Nle-Tyr-Cys-NH_2 (compounds **41**−**44** and **57**-**60**), exhibited the most potent inhibition of α3β4 nAChR.

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Significantly, of the 11 compounds selected for further functional characterization in the fluorescent membrane potential assay, each was highly selective for $\alpha3\beta4$ nAChR, with no inhibitory activity for the $\alpha3\beta2$ and $\alpha4\beta2$ subtypes observed up to 10 μM. In contrast, α-conotoxin BuIA was shown to be 10-fold more selective for the $\alpha3\beta2$ in the fluorescent membrane potential assay, which directly correlates with previously reported data obtained from electrophysiological recordings.[19]

Of the four analogues selected for further characterization in the radioligand binding assay, each compound displayed potent inhibition of [^{3}H]epibatidine binding to $\alpha3\beta4$ nAChR expressed in HEK293 cell membranes, which indicates competitive binding to the endogenous ligand binding site as exhibited by other α-conotoxins. By contrast, other ligands that potently block the $\alpha3\beta4$ nAChR in functional assays, such as 18-MC and mecamylamine, do not inhibit [^{3}H]epibatidine binding because these compounds bind to the central lumen of the receptor.[43,44] Notably, compound **59** (TP-2212-59) was shown to exhibit moderately increased inhibition of [^{3}H]-epibatidine binding when compared to the other conotoxin analogues tested, including native α-conotoxin BuIA, and was thus selected for further functional characterization.

TP-2212-59 was tested for inhibition of ACh-evoked currents in $\alpha3\beta4$, $\alpha3\beta2$, and $\alpha7$ nAChRs expressed in *Xenopus* oocytes. Though the slow washout kinetics prohibited measurements at concentrations lower than 10 nM, compound **59** inhibited $\alpha3\beta4$ nAChR activity by 75% at this concentration, with a calculated IC_{50} value of 2.3 nM. α-Conotoxin BuIA also exhibits slow off-rates for $\beta4$-containing subtypes, which may contribute to the higher binding affinity and thus increased potency. However, while the off-rates for $\beta4$-containing receptors are in general slower, BuIA exhibits an approximately 5-fold greater selectivity for $\alpha3\beta2$ over $\alpha3\beta4$ nAChR subtypes, with IC_{50} values of 5.72 and 27.7 nM, respectively.[19] In contrast, TP-2212-59 displayed a >1000-fold selectivity for $\alpha3\beta4$ nAChR, with no inhibition of the $\alpha3\beta2$ and $\alpha7$ nAChR observed up to 10 μM. These data confirm that TP-2212-59 exhibits increased potency and selectivity for the $\alpha3\beta4$ nAChR compared to α-conotoxin BuIA and is therefore among the most potent and selective α-conotoxin derivatives that target the $\alpha3\beta4$ nAChR characterized to date.

This work demonstrates that a synthetic mixture-based combinatorial approach using the $\alpha4/4$-conotoxin framework provides an excellent template for the identification of selective $\alpha3\beta4$ nAChR antagonists. Moreover, TP-2212-59 is among the most potent and selective α-conotoxin antagonists that target the $\alpha3\beta4$ nAChR and therefore is a valuable probe to elucidate the function of this receptor in a wide range of experimental paradigms that require $\alpha3\beta4$ nAChR activation. For example, the effects of TP-2212-59 on nicotine self-administration and associated side effects in rodents are currently being examined by our group.[11] Such studies may lead to the development of $\alpha3\beta4$ nAChR antagonists as novel smoking cessation drugs with fewer side effects than presently available options.

EXPERIMENTAL SECTION

Chemical Synthesis of PS-SCL and Individual α-Conotoxin Analogues. The PS-SCL and individual $\alpha4/4$-conotoxin analogues were assembled by solid-phase peptide synthesis using 4 cm × 4 cm polypropylene tea bags, each containing 100 mg of 4-methylbenzhydrylamine resin (ChemImpex, Wood Dale, IL). Couplings were performed using *tert*-butyloxycarbonyl (Boc)/2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) chemistry with in situ neutralization.[45] For the PS-SCL, **X**-positions were coupled as a cocktail of N^{α}-Boc protected amino acids using adjusted concentration ratios to compensate for the relative reaction rates in competitive couplings.[28] The following amino acid side chain protecting groups were used for each respective amino acid: Arg, 4-toluenesulfonyl (Tos); Asn, 1-xanthanyl (Xan); Asp, *O*-cyclohexyl (OcHxl); Cys, 4-methylbenzyl (MeBzl) or *N*-acetomidomethyl (Acm); Glu, OcHxl; His, Tos; Hyp, benzyl (Bzl); Ser, Bzl; Trp, formyl (For); Tyr, 2-bromobenzyloxycarbonyl (BrZ). Following assembly, the tea bags were treated with 30% piperidine to remove the formyl protecting group from Trp; the N-terminal Boc protecting group was removed with trifluoroacetic acid (TFA), neutralized, and then pretreated with low HF (25% HF, 60% dimethylsulfide, 10% *p*-cresol, 5% 1,2-ethanedithiol) for 2 h at 0 °C. Finally, the peptides were cleaved from the resin using HF/*p*-cresol (9:1) for 2 h at 0 °C. The HF was evaporated under a stream of nitrogen. The peptide was precipitated with cold diethyl ether, centrifuged, washed again with additional ether, centrifuged, and lyophilized from 50% acetonitrile/0.1% TFA. The purity and molecular mass of individually synthesized peptides were confirmed using analytical LC−MS (Shimadzu, Kyoto, Japan).

Crude samples were individually oxidized by rigorously agitating each sample in a solution of 0.1 M ammonium bicarbonate, 50% isopropyl alcohol, pH 8.2, for 3 days at room temperature in an open vessel on an orbital shaker platform.[29] Following evaporation of the isopropyl alcohol in vaccuo (Genevac Rocket, Ipswich, U.K.), samples were isolated in parallel with solid-phase extraction (SPE) cartridges using a 24-sample vacuum manifold.[29] For SPE cartridge semi-purification, the sample was loaded by passing through an Oasis; 3 cc, 540 mg SPE cartridge (Waters) pre-equilibrated with buffer A and washed with a further 10 mL of buffer A to remove hydrophilic impurities (i.e., buffer salts). The α-conotoxin product was then eluted with 65% aqueous acetonitrile/0.1% TFA (buffer E, 10 mL), collected in a clean tube, and lyophilized. Selected compounds for further pharmacological characterization were purified to >95% homogeneity by preparative RP-HPLC using a C_{18} Luna column (Phenomenex) using a linear gradient of 0−45% buffer B (95% aqueous acetonitrile/0.1% TFA) over 60 min at a flow rate of 20 mL/min.

Regioselective synthesis of selected compounds for further characterization (i.e., globular and ribbon isomers) was achieved by stirring reduced/*S*-acetomidomethyl-protected peptides in 0.1 M ammonium acetate containing 30% DMSO, pH 5.8, for 2 days, followed by isolation by preparative RP-HPLC. Partially protected purified peptides (~10 mg) were dissolved in 80% methanol (25 mL), and 0.1 M HCl was added (1 mL), followed by 0.1 M I_2 in methanol (2.5 mL). The solution was stirred for 10 min before quenching with 0.1 M sodium thiosulfate. Finally, fully oxidized peptides were purified to >95% homogeneity using preparative RP-HPLC as described above.

Fluorescent Membrane Potential Assay. The fluorescent plate reader membrane potential blue assay was used for initial library screening and to determine inhibition of rat $\alpha3\beta4$, $\alpha3\beta2$, and $\alpha4\beta2$ nAChRs by individual α-conotoxins. Briefly, KX$\alpha3\beta4$R2, KX$\alpha3\beta2$R2, or KX$\alpha4\beta2$R2 HEK293 cells (kindly provided by Drs. Yingxian Xiao and Kenneth Kellar, Georgetown University, Washington, DC)[32] were cultured in a humidified 5% CO_2 incubator and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (100 units/mL), and G-418 (0.5 mg/mL). The cells were split into poly-D-lysine coated clear bottom 96-well plates 24 h prior to the assay and grown to ~80% confluency. The culture medium was aspirated, and the cells were washed twice with Hank's buffered salt solution (HBSS). After washing, 75 μL of HBSS was added to each well, followed by 25 μL of PS-SCL mixtures or α-conotoxin test samples. Finally, membrane potential blue dye (100 μL) was added and the cells were incubated for a further 30 min at 37 °C prior to the assay with the FlexStation 3 microplate reader (Molecular Devices) measuring emission at 530 nm caused by excitation at 565 nm (cutoff 550 nm) before and up to 60 s after addition of epibatidine agonist solution to a concentration of 0.1 μM in assay buffer. The concentration−response for antagonists was

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measured on the basis of the maximal responses at different concentrations of the respective ligands to determine IC_{50} values, which were calculated using GraphPad Prism software (La Jolla, CA).

Nicotinic Acetylcholine Receptor Binding Assay. KXα3β4R2 and KXα4β2R2 HEK293 cells were cultured on 150 mm dishes as described above. Cells were harvested when confluent by scraping the plates with a rubber policeman, suspended in 50 mM Tris buffer, pH 7.4, homogenized using a Polytron homogenizer, and washed twice by centrifugation at 20000*g* (13 500 rpm) for 20 min. For binding, the cell membranes were incubated with the test compounds or mixtures in the presence of 0.31 nM [^{3}H]epibatidine. After 2h of incubation at room temperature, samples were filtered using a Tomtec cell harvester through glass fiber filters presoaked in 0.05% polyethyleneimine. Filters were counted on a betaplate reader (Wallac). Nonspecific binding was determined by using 0.1 μM unlabeled epibatidine. IC_{50} values were determined by using the program GraphPad Prism. K_i values were calculated using the Cheng−Prusoff transformation: $K_i = IC_{50}/(1 + L/K_D)$,[46] where L is radioligand concentration and K_D is the binding affinity of the radioligand, as determined previously by saturation analysis.

Electrophysiological Recordings. Capped cRNA for the various subunits was made using the mMessage mMachine in vitro transcription kit (Ambion, Austin, TX) following plasmid linearization. cRNA of the α3 chimera was combined with cRNA of high expressing β2 and β4 subunits or homomeric α7 subunits (in the pGEMHE vector) to give 200−500 ng/μL of each subunit cRNA. An amount of 50 nL of this mixture was injected into *Xenopus* oocytes, and the sample was incubated at 17 °C. Oocytes were injected within 1 day of harvesting, and recordings were made 2−4 days after injection. Oocytes were voltage-clamped and exposed to a 1 s pulse of 300 M μACh and peptide as described previously.[47] For screening of receptor subtypes and for toxin concentrations of 1 μM and higher, once a stable agonist-response baseline was achieved, either ND-96 alone or ND-96 containing varying BuIA analogue concentrations was manually preapplied for 5 min under static bath conditions prior to agonist addition. For toxin concentrations of <1 μM, toxin was perfusion applied and responses to 1 s pulses of ACh were measured every 1 min. All recordings were performed at room temperature (~22 °C).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

18-MC, 18-methoxycoronaridine; ACh, acetylcholine; Acm, acetomidomethyl; Abu, 2-aminobutyric acid; Boc, *tert*-butyloxycarbonyl; Hyp, 4-hydroxyproline; LC−MS, liquid chromatography−mass spectrometry; MeBzl, 4-methylbenzyl; MBHA, 4-methylbenzylhydrylamine; nAChR, nicotinic acetylcholine receptor; Nle, norleucine; Nva, norvaline; PS-SCL, positional scanning synthetic combinatorial library; TFA, trifluoroacetic acid

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