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Discovery of Novel Antinociceptive α-Conotoxin Analogues from the Direct In Vivo Screening of a Synthetic Mixture-Based Combinatorial Library

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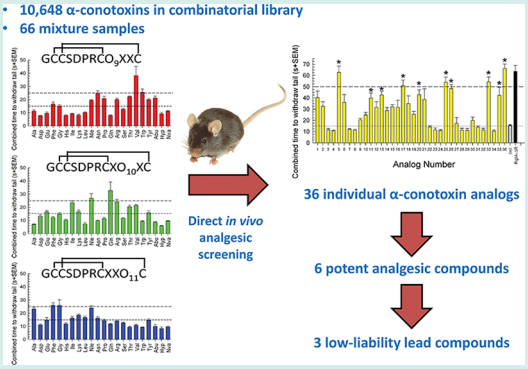
Supporting Information

ABSTRACT: Marine cone snail venoms consist of large, naturally occurring combinatorial libraries of disulfide-constrained peptide neurotoxins known as conotoxins, which have profound potential in the development of analgesics. In this study, we report a synthetic combinatorial strategy that probes the hypervariable regions of conotoxin frameworks to discover novel analgesic agents by utilizing high diversity mixture-based positional-scanning synthetic combinatorial libraries (PS-SCLs). We hypothesized that the direct in vivo testing of these mixture-based combinatorial library samples during the discovery phase would facilitate the identification of novel individual compounds with desirable antinociceptive profiles while simultaneously eliminating many compounds with poor activity or liabilities of locomotion and respiration. A PS-SCL was designed based on the α-conotoxin RgIA-ΔR *n*-loop region and consisted of 10,648 compounds systematically arranged into 66 mixture samples. Mixtures were directly screened in vivo using the mouse 55 °C warm-water tail-withdrawal assay, which allowed deconvolution of amino acid residues at each position that confer antinociceptive activity. A second generation library of 36 individual α-conotoxin analogues was synthesized using systematic combinations of amino acids identified from PS-SCL deconvolution and further screened for antinociceptive activity. Six individual analogues exhibited comparable antinociceptive activity to that of the recognized analgesic α-conotoxin RgIA-ΔR, and were selected for further examination of antinociceptive, respiratory, and locomotor effects. Three lead compounds were identified that produced dose-dependent antinociception without significant respiratory depression or decreased locomotor activity. Our results represent a unique approach for rapidly developing novel lead α-conotoxin analogues as low-liability analgesics with promising therapeutic potential.



KEYWORDS: *α-conotoxin, in vivo screening, mixture-based combinatorial libraries, analgesia*

INTRODUCTION

Marine cone snails have evolved their venoms as gene-encoded combinatorial libraries of disulfide-constrained peptide neurotoxins, collectively known as conotoxins, to swiftly immobilize and capture their fast moving prey.[1−3] Cone snail venoms are highly complex, and each species may express thousands of individual conotoxins. Over the past three decades, conotoxins have provided researchers with a vast repertoire of pharmacologically relevant ligands with profound therapeutic potential.[4−8] As such, the diversity of ion-channels and receptors targeted by the numerous classes of conotoxins has led to the identification of novel compounds with potent analgesic properties, as demonstrated by Ziconotide, an N-type calcium channel inhibitor that is an FDA-approved treatment for chronic neuropathic pain.[9]

α-Conotoxins are selective competitive antagonists of nicotinic acetylcholine receptors (nAChRs).[10] They contain two highly conserved disulfide bonds that give rise to two loops of amino acids denoted *m*- and *n*- respectively (Figure 1).

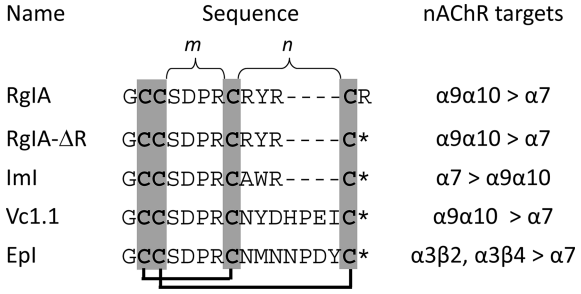


Figure 1. Sequence alignment of α-conotoxins that contain an Asp-Pro-Arg motif in their *m*-loop. Conserved cysteine residues are boxed and disulfide bond connectivity is indicated. Asterisk indicates C-terminal amidation.

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The amino acid composition of these loops varies widely among α-conotoxins, producing significant differences in function and receptor subtype specificity. Of interest, α-conotoxins RgIA and Vc1.1 have proven to be affective models for treating neuropathic pain in animal models[11,12] and in human trials.[13] RgIA and Vc1.1 share an Asp-Pro-Arg motif in their *m*-loop sequence thought to be critical for α9α10 nAChR inhibition (Figure 1).[14] Although the Asp-Pro-Arg motif is also found in EpI and ImI, these α-conotoxins are less selective for α9α10 nAChRs, and do not exhibit significant analgesic activity.[15,16] Deletion of the C-terminal Arg residue on RgIA (yielding RgIA-ΔR) was found to have no effect on activity,[14,17] yet ImI differs from RgIA-ΔR by only two residues in its *n*-loop sequence (Figure 1).[18] As such, the analgesic activity and selectivity for the α9α10 nAChR exhibited by Vc1.1 and RgIA may be attributed to their *n*-loop sequences. This suggests that substitutions in this region would result in effective analgesic compounds, provided that key functional amino acids at each position can be determined.

Synthetic mixture-based combinatorial libraries are systematically arranged, very dense mixtures of compounds that project both defined and mixture (diversity) positions within a chemical scaffold.[19] When systematically arranged in a positional-scanning synthetic combinatorial library (PS-SCL) format, one can gain valuable information regarding active amino acid residues at each position directly from pharmacological screening.[20,21] The screening of mixture-based combinatorial libraries in vivo has facilitated the rapid identification of novel opioid analgesics.[22]

We have previously reported a mixture-based synthetic combinatorial strategy for the discovery of potent α7 nAChR antagonists.[23] This strategy involved the screening of a 66 sample PS-SCL based on the three *n*-loop residues of α-4/3 conotoxins containing the Asp-Pro-Arg motif, which led to the discovery of a series of potent α7 nicotinic acetylcholine receptor antagonists. With the view that ImI differs from RgIA-ΔR by only two residues, but does not possess analgesic activity, we reasoned that direct in vivo screening of our α4/3-conotoxin *n*-loop mixture-based PS-SCL would yield individual α-conotoxin analgesics, while simultaneously allowing the identification of compounds with fewer harmful side effects.

In the present study, we theorized that direct in vivo testing of an α-conotoxin PS-SCL based on the *n*-loop residues of RgIA-ΔR with the mouse 55 °C warm water tail-withdrawal assay would facilitate both the deconvolution of the mixture-based samples to identify key functionalities and the initial characterization of novel individual α-conotoxins with desirable antinociceptive potencies. Selected individual antinociceptive compounds were further examined for effects on respiration rate and locomotor activity, thus eliminating candidates displaying potential liabilities early in preclinical development.

RESULTS

The PS-SCL consisted of three sublibraries, defined as O_9, O_{10}, and O_{11} respectively. An equimolar mixture of 22 natural and non-natural L-amino acids was used in the diversity (or "X") positions. A total of 10,648 possible individual conotoxin sequences were obtained in 66 mixture samples across three sublibraries, with each mixture sample containing 484 compounds. Isosteric aminobutyric acid (Abu) and norleucine (Nle) were included, together with norvaline (Nva) to complete the series of side chains containing hydrophobic alkyl groups. Additionally, hydroxyproline (Hyp), a commonly occurring post-translational modification that is found in several conotoxins, was also incorporated into this library.[24,25]

The library was assembled using manual solid-phase peptide synthesis with Boc chemistry in polypropylene tea bags as described previously.[23,26] Following assembly of the PS-SCL, each sample was cleaved using a two-step low-high HF procedure. Each sample was oxidized as a crude mixture in aqueous buffer consisting of 0.1 M NH_4HCO_3, pH 8.2, containing 50% isopropyl alcohol. These conditions have been reported as optimum for generating the native disulfide bond isomer of α-conotoxin ImI, which shares the same 4/3 *m*- and *n*-loop spacing as RgIA-ΔR.[27,28] Each mixture was subsequently isolated from the oxidation buffer by elution with 62% isocratic acetonitrile with preparative RP-HPLC.

To validate the tail-withdrawal assay as a model for α-conotoxin-induced antinociception, RgIA-ΔR, Vc1.1, and ImI were tested to determine the optimal dose for screening. Nicotine was also tested as a positive control. Nicotine, Vc1.1, and RgIA-ΔR all displayed dose dependent antinociceptive activity, albeit with varying potencies that proved significantly different ($F_{(1,98)}$ = 10.52; P = 0.0016, nonlinear regression modeling; Figure 2). Nicotine and Vc1.1 exhibited statistically

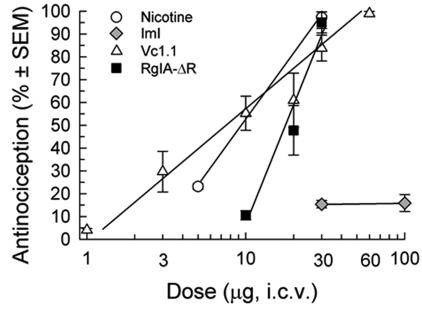

Figure 2. Antinociceptive activity of nicotine and α-conotoxins Vc1.1, RgIA-ΔR, and ImI was assessed in vivo following i.c.v. administration in the 55 °C warm-water tail-withdrawal assay in C57BL/6J mice. All points represent antinociception at peak response, which was 10 min (for nicotine) or 20 min (for Vc1.1, RgIA-ΔR, and ImI). All points represent average % antinociception ± SEM from 8 to 16 mice.

equivalent antinociceptive potencies in this assay with ED_{50} (and 95% confidence interval) values of 9.57 (9.00−10.1) and 7.82 (5.74−10.3) μg i.c.v., respectively, although the duration of Vc1.1-induced antinociception was twice (110 min) that induced by nicotine at the same dose (30 μg, i.c.v.). The ED_{50} (and 95% confidence interval) value for RgIA-ΔR-mediated antinociception was approximately twice that of nicotine (19.4 (11.2−32.1) μg, i.c.v.). In contrast, but consistent with previous reported observations,[15] ImI produced negligible significant antinociception at doses up to 100 μg, i.c.v. (diamonds, Figure 2). As such, synthetic combinatorial libraries based on α-conotoxin frameworks could be screened using the tail-withdrawal assay and would likely lead to the identification of novel analgesic compounds.

The amino acids among the 22 substituted in each position (O_9, O_{10}, and O_{11}) of the α-conotoxin RgIA-ΔR *n*-loop PS-SCL that yield antinociceptive activity were identified with the 55 °C warm-water tail-withdrawal assay (Figure 3). Mice were evaluated for antinociceptive activity every 10 min for 120 min following administration of a 30 μg dose (i.c.v.) of each of the 66 mixture samples in the PS-SCL. The combined latency to tail withdrawal, minus the respective animal's baseline response,

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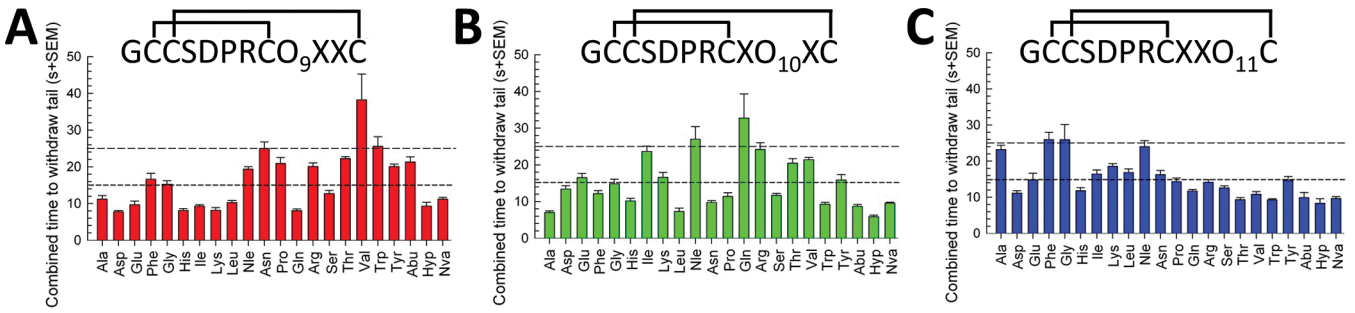

Figure 3. Direct antinociceptive screening in vivo of the α-conotoxin RgIA-ΔR PS-SCL. Mixture samples (O_{9-11} amino acids defined in *x*-axes) were administered at a dose of 30 μg i.c.v prior to testing in the mouse 55 °C warm-water tail-withdrawal assay across a 120 min period. (A) Library defined at Position 9 (O_9; red bars); (B) Library defined at Position 10 (O_{10}; green bars); (C) Library defined at Position 11 (O_{11}; blue bars). Bars represent the average of eight mice (+SEM) calculated from individual animal responses minus baseline latency summed across all time points produced by each mixture sample. (Lower dashed line = designation as inactive/baseline response (<15 s); upper dashed line = designation as moderately antinociceptive (>25 s). X, mixture positions).

was summed over the 12 time points examined for each sample tested and is reported by substitution position (Figure 3A–C). Notably, a number of samples defined at each substitution position increased the combined tail-withdrawal time as compared to the effect of the nonanalgesic α-conotoxin, ImI (30 μg, i.c.v.; lower dashed line on each graph in Figure 3), facilitating deconvolution of the PS-SCL. Specifically, definition of Asn, Val, and Trp in position O_9; Ile, Nle, Gln, and Arg in position O_{10} and Phe, Gly, and Nle in position O_{11} were identified as potentially promoting antinociceptive activity.

On the basis of the initial in vivo PS-SCL screening results, a second generation library of 36 individual α-conotoxin RgIA-ΔR analogues were synthesized which consisted of all possible combinations of amino acids identified in the *n*-loop PS-SCL screening as being significantly antinociceptive (Table 1).

Table 1. List of Individual α-Conotoxin Analogues Synthesized for the Second Generation Library[a]

	GCCSDPRCO$_9$O$_{10}$O$_{11}$C*						
analogue #	O_9	O_{10}	O_{11}	analogue #	O_9	O_{10}	O_{11}
1	Val	Ile	Phe	**19**	Trp	Gln	Gly
2	Val	Nle	Phe	**20**	Trp	Arg	Gly
3	Val	Gln	Phe	**21**	Asn	Ile	Gly
4	Val	Arg	Phe	**22**	Asn	Nle	Gly
5	Trp	Ile	Phe	**23**	Asn	Gln	Gly
6	Trp	Nle	Phe	**24**	Asn	Arg	Gly
7	Trp	Gln	Phe	**25**	Val	Ile	Nle
8	Trp	Arg	Phe	**26**	Val	Nle	Nle
9	Asn	Ile	Phe	**27**	Val	Gln	Nle
10	Asn	Nle	Phe	**28**	Val	Arg	Nle
11	Asn	Gln	Phe	**29**	Trp	Ile	Nle
12	Asn	Arg	Phe	**30**	Trp	Nle	Nle
13	Val	Ile	Gly	**31**	Trp	Gln	Nle
14	Val	Nle	Gly	**32**	Trp	Arg	Nle
15	Val	Gln	Gly	**33**	Asn	Ile	Nle
16	Val	Arg	Gly	**34**	Asn	Nle	Nle
17	Trp	Ile	Gly	**35**	Asn	Gln	Nle
18	Trp	Nle	Gly	**36**	Asn	Arg	Nle

[a]Asterisk denotes C-terminal amidation.

Following synthesis, each analogue was folded using the same oxidation conditions employed for the preparation of the PS-SCL (described above), and the major component of each sample was purified to >95% homogeneity using preparative RP-HPLC (see Supporting Information, Figures S1–S6, for analytical LC-MS data of selected individual analogues).

Each individual second generation analogue was screened for antinociceptive activity in the 55 °C tail-withdrawal assay following administration of a 30 μg, i.c.v. dose (Figure 4). While a

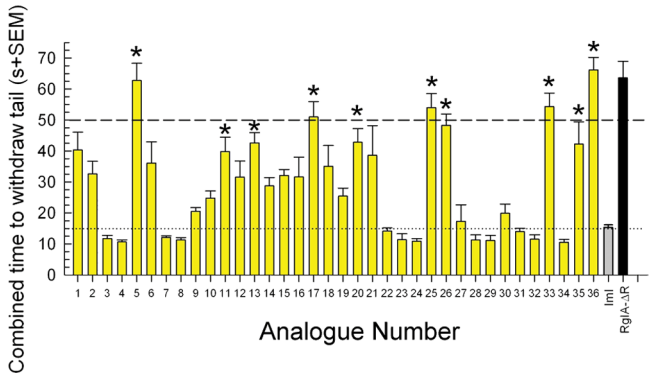

Figure 4. Direct antinociceptive screening in vivo of individual second generation RgIA-ΔR analogues. Bars represent the average (+SEM) response summed across all time points produced by analogues defined at positions O_9, O_{10}, and O_{11} (30 μg i.c.v.) as measured in the mouse 55 °C warm-water tail-withdrawal assay. Functionalities of each analogue are shown in Table 1. ImI (30 μg i.c.v., gray bar) and RgIA-ΔR (30 μg i.c.v., black bar) are included as negative and positive comparison controls, respectively. Dotted lower line represents ImI response; dashed upper line represents minimal response for additional compound characterization. Data calculated from individual animal responses summed across all time points produced by each animal every 10 min over a 120 min period. Bars = 8 mice each. * = significant difference from the ImI treated negative control group (p < 0.05; ANOVA with Tukey HSD post hoc test).

majority of the second generation analogues yielded negligible antinociception, ten individual analogues demonstrated significantly greater summed antinociceptive responses than that produced by ImI ($F_{(36,342)}$ = 18.75, p < 0.0001; one-way ANOVA with Tukey HSD post hoc test).

The six individual analogues that produced the greatest antinociceptive activity (compounds **5**, **17**, **25**, **26**, **33**, and **36**) were selected for more detailed antinociceptive characterization in vivo using the 55 °C warm-water tail-withdrawal assay following i.c.v. administration. Like RgIA-ΔR, each of the

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selected individual analogues exhibited antinociceptive activity in vivo, albeit with varying potencies (Figure 5A). All six analogues produced maximal antinociception 20 to 30 min after i.c.v. administration of each dose tested. Significant differences in potency were demonstrated by comparison of the shift in ED_{50} values using nonlinear regression modeling ($F_{(4,553)}$ = 4.553; P = 0.0016; Figure 5A). Analogues **5**, **17**, **25**, and **36** exhibited similar antinociceptive potencies to RgIA-ΔR, with ED_{50} (and 95% confidence interval) values of 21.3 (16.9−27.5), 20.5 (17.5−23.9), 24.5 (18.3−30.2), and 20.6 (9.64−38.8) μg, i.c.v., respectively. In contrast, analogue **33** was nearly twice as potent as RgIA-ΔR (ED_{50} = 10.8 (5.62−15.6) μg, i.c.v.). Interestingly, analogue **26** did not demonstrate a linear antinociceptive effect in this assay when tested at doses up to 60 μg, i.c.v. (Figure 5A, blue squares). Accordingly, an ED_{50} value for this compound was not calculable.

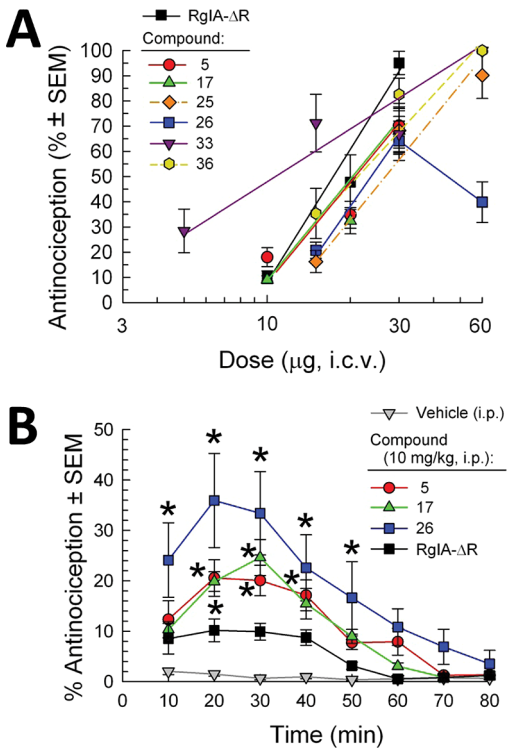

Figure 5. Antinociceptive activity of RgIA-ΔR and the six most active second generation α-conotoxins compounds was assessed in vivo following (A) i.c.v. or (B) i.p. administration in the 55 °C warm-water tail-withdrawal assay in C57BL/6J mice. All points represent antinociception at peak antinociception, either 20 min (RgIA-ΔR) or 30 min post administration. (A) Dose response effect for all seven compounds. (B) Time course of antinociceptive activity induced by vehicle (10% DMSO, gray inverted triangles), RgIA-ΔR (black squares), or compounds **5** (circles), **17** (triangles), and **26** (blue squares) after intraperitoneal administration at a dose of 10 mg/kg. All points represent average % antinociception ± SEM from 8 to 16 mice. * = significant difference from vehicle effect (p < 0.05; two-way ANOVA with Tukey HSD post hoc test).

To determine if any of the α-conotoxin analogues could produce antinociception after peripheral administration, additional mice were administered vehicle (10% DMSO), RgIA-ΔR or analogue **5**, **17**, or **26** through the intraperitoneal (i.p.) route at a dose of 10 mg/kg. While vehicle treatment did not significantly increase tail-withdrawal latency over baseline (1.73 ± 0.28 s) at any point for 80 min ($F(8,71)$ = 2.37, P > 0.05; one-way ANOVA), RgIA-ΔR and each analogue significantly increased antinociception as compared to vehicle ($F(4,245)$ = 21.05, P < 0.0001; two-way ANOVA; Figure 5B). The antinociception produced was time-dependent ($F(7,245)$ = 20.6, P < 0.0001; two-way ANOVA; Figure 5B), and was greatest following treatment with analogue **26** (Figure 5B). Notably, although significant overall in effect, the antinociception induced by Rg1A-ΔR was not found to significantly differ from vehicle effects at any time point tested (P > 0.05, Bonferroni multiple comparison post hoc test).

The effects of RgIA-ΔR and the six characterized α-conotoxin analogues **5**, **17**, **25**, **26**, **33**, and **36** on spontaneous locomotor activity and respiration rate were assessed over 2 h after administration of a 30 μg (i.c.v.) dose using the Comprehensive Laboratory Animal Monitoring System (CLAMS). Treatment with the conotoxin samples generally produced a significant decrease in respiration ($F_{(7,102)}$ = 5.232, P < 0.0001; two-way ANOVA with Tukey's HSD post hoc test; Figure 6A). Analogues **5**, **17**, and **26** displayed no significant change in respiration rate compared to vehicle-treated (10% DMSO, i.c.v.) mice (p > 0.05, Tukey's HSD post hoc test).

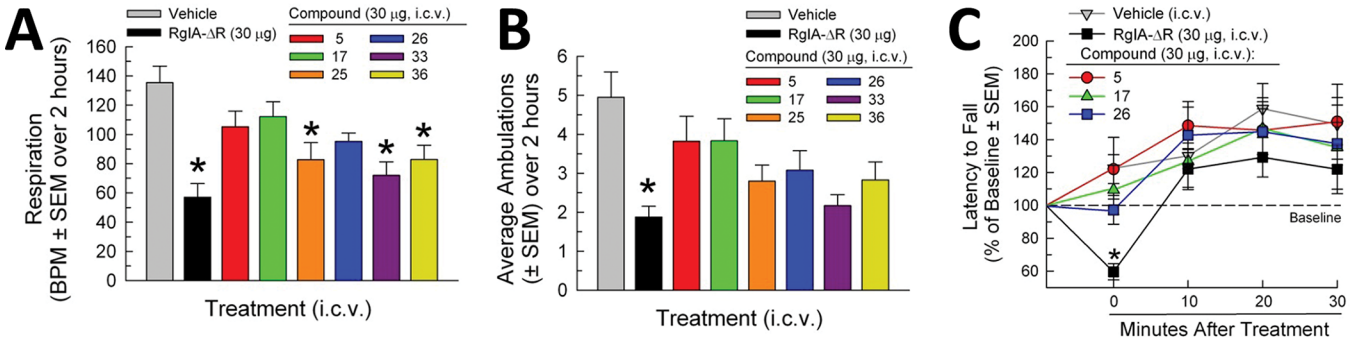

Figure 6. Respiratory and locomotor effects of selected second generation α-conotoxin RgIA-ΔR analogues. Compounds **5**, **17**, and **26** do not induce respiratory or locomotor deficits at therapeutic doses. Mice were administered vehicle (10% DMSO in 0.9% saline; gray bar), RgIA-ΔR (30 μg i.c.v.; black bar), or one of six conotoxin compounds (**5**, **17**, **25**, **26**, **33**, or **36**; 30 μg i.c.v. each) and effect measured for 120 min on (A) respiration rate (as breaths/min (BPM) and (B) spontaneous locomotor effects (ambulations/min) in the Comprehensive Lab Animal Monitoring System (CLAMS). (C) Effects of vehicle (10% DMSO, i.c.v.; gray triangles), RgIA-ΔR (30 μg i.c.v.; black squares) or each of the lead compounds (**5**, **17**, and **26**; 30 μg i.c.v. each) were further assessed in the rotorod assay of evoked locomotor activity (indicated by latency to fall from a rotorod as the percent change from baseline performance/10 min) of mice (n = 8−16/group). * = significant difference from the vehicle treated control group (p < 0.05; one-way (for Figure 6A and B) or two-way (for Figure 6C) ANOVA with Tukey HSD post hoc test).

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In contrast, RgIA-ΔR and compounds **25**, **33**, **36** each significantly decreased respiration rate ($P < 0.0001$, 0.042, 0.006, and 0.025, respectively; Tukey's HSD post hoc test). Although treatment generally produced a significant reduction of spontaneous locomotor activity ($F_{(7,103)} = 2.68$, $P = 0.014$; one-way ANOVA), and some samples showed a trend toward significance (e.g., compound **33**, $P = 0.076$; Tukey's post hoc test), only RgIA-ΔR significantly reduced the ambulatory response from those observed by vehicle-treated mice ($P = 0.033$; Tukey's post hoc test; Figure 6B).

To further test for possible locomotor impairment, analogues **5**, **17**, and **26** were administered (30 μg, i.c.v.) to mice subsequently tested in the rotarod assay (Figure 6C). Additional mice were administered vehicle (10% DMSO, i.c.v.) or RgIA-ΔR (30 μg, i.c.v.) to serve as controls. As mice showed significant improvement in rotorod performance over time ($F_{(3,105)} = 19.7$, $P < 0.0001$; two-way ANOVA), however, analogue-treated mice did not show a significant deficit in coordinated locomotor activity at any time point tested (Figure 6C), as produced RgIA-ΔR when compared to vehicle-treated mice.

DISCUSSION

Conotoxins have attracted intense interest as research tools and drug leads because of their remarkable ability to differentiate between different ion channels and receptors, and for their potential as clinically useful analgesics.[4−8] For example, conotoxin derived drugs such as Ziconotide and Xen2174 have proven to be highly effective analgesics when delivered to neuropathic patients via intrathecal administration.[9,29] With the view that conotoxins are expressed in cone snail venom glands as naturally occurring combinatorial libraries, we theorized that direct in vivo antinociceptive screening of synthetic combinatorial libraries based on conotoxin frameworks would yield novel low-liability analgesic compounds.

α-Conotoxins RgIA and Vc1.1 have been previously shown to be effective analgesics in the chronic constriction injury (CCI) model of neuropathic pain following intramuscular administration.[12,30] More recently, Vc1.1 was shown to reverse mechanical allodynia in CCI rats following intrathecal administration.[31] Although CCI is an effective chronic neuropathic pain model, it is not feasible to screen large numbers of samples in a high-throughput format because of the complex surgical procedures involved. As such, we used the mouse 55 °C warm-water tail-withdrawal test as a high-throughput in vivo screening assay, which was more compatible with our synthetic combinatorial strategy. We have previously reported the use of this assay for identifying potent analgesics using a similar strategy involving synthetic combinatorial libraries.[22] This model for analgesia was validated using α-conotoxins RgIA-ΔR and Vc1.1, which both demonstrated significant antinociceptive activity, and ImI, which did not.

Combinatorial libraries have become widely-used tools for translational drug discovery and development. Two primary approaches are used to prepare and screen large numbers of compounds produced by combinatorial libraries: (1) the massive parallel synthesis and screening of large individual compound arrays; and (2) the generation and screening of extremely large mixture based libraries that focus on a small region of chemical space. While the screening of such a large number of individual conotoxins using in vivo animal assays would be impractical, a mixture-based combinatorial approach has the advantage of rapidly identifying novel compounds with potent biological activity in a relatively short time frame.[19,32] Furthermore, direct in vivo screening allows compounds with unfavorable pharmacokinetic properties to be readily eliminated, since those compounds would not be identified as active hits.

Through direct antinociceptive screening of PS-SCL mixtures in vivo, residues that confer antinociceptive activity in the *n*-loop of the α-conotoxin RgIA-ΔR framework were defined in all positions, although positions 9 and 10 were more clearly defined than position 11. Notably, native RgIA-ΔR residues (Arg9, Tyr10, and Arg11) were identified as being only moderately antinociceptive following PS-SCL screening, and thus were not selected for further consideration in the synthesis of a second generation library. Indeed, given that the six individual lead compounds identified from PS-SCL screening exhibited comparable antinociceptive activity to RgIA-ΔR, it would be expected that the native RgIA-ΔR residues would also be identified in this manner. It should be noted that the inability to identify highly active compounds from PS-SCL screening may occur for several reasons, including the following: the compound not being present in high enough concentration in the mixture-based sample; the response may reflect a combined action of both agonists and antagonists in the mixture tested; or potential synergistic activity from two or more compounds in a sample mixture may produce an additive effect.[33] In this study, 10,648 α-conotoxins were screened across 66 PS-SCL mixtures. Each mixture was administered at a dose of 30 μg, corresponding to 62 ng of each conotoxin within the mixture, which is significantly less than the lowest dose tested for all individual compounds. Therefore, the antinociceptive activity exhibited by the PS-SCL mixtures is likely attributable to the additive effect of several closely related sequences within each mixture. Accordingly, highly active compounds within a mixture may not be readily detected, unless they are extraordinarily active at such a low dose.

Of the 36 individual analogues that were synthesized for the second generation library, 24 analogues displayed antinociceptive activity that was significantly higher than baseline, with ten analogues demonstrating comparable activity to RgIA-ΔR. Such a "hit rate" of active sequences is comparable with other PS-SCL screening and deconvolution studies.[21,22,34,35] Six of the analogues selected for further analysis produced dose-dependent antinociceptive activity, with compound **33** exhibiting the highest antinociceptive activity. Despite the observation that the three compounds predicted to be the most analgesic based directly on PS-SCL screening (i.e., Val-Gln-Phe, Val-Gln-Gly, or Val-Gln-Nle, compounds **3**, **15**, and **27** respectively) were not significantly antinociceptive, other combinations of residues predicted from PS-SCL did produce α-conotoxin analogues with robust antinociceptive activity that was comparable to RgIA-ΔR.

Although some patterns were apparent, no clear structure−activity relationships could be discerned, with all amino acid residues selected for the second generation library at each position exhibiting potent antinociceptive activity in at least one compound. For example, the Trp-Ile-X identities were present in lead analgesic compounds **5** and **17.** Analogue **29** also shares this sequence, yet displayed no significant antinociceptive activity. With the exception of compound **36**, which contained arginine at position 10, hydrophobic or polar uncharged residues were preferred for analgesic activity. Overall, each of the active analogues identified shared no sequence similarities with RgIA-ΔR. However, this finding did demonstrate the

utility of our approach for identifying novel antinociceptive compounds.

Although we successfully demonstrated that the tail-withdrawal assay is a useful method for measuring antinociceptive activity, a significant limitation is that analgesia could possibly be attributed to decreased locomotor activity caused by sedation or paralysis. To this end, six of the most antinociceptive compounds were tested for effects on spontaneous locomotor activity and respiration rate using the CLAM system. Three of the six individual analogues (compounds **5**, **17**, and **26**) did not exhibit reduced locomotor activity or respiratory depression, suggesting their activity could be attributed to analgesic effects. Further testing of lead compounds in the rotarod performance assay confirmed no significant decrease in coordinated locomotor activity. Together, these findings highlight an advantage of our systematic screening for potential liabilities, where lead compounds with potentially deleterious side effects may be eliminated early in the drug discovery process.[36,37]

Interestingly, RgIA-ΔR was shown to exhibit significant sedative effects when compared to the vehicle control in both the CLAMS and rotarod assays, which has not been reported previously. Furthermore, compounds **5**, **17**, and **26** also displayed moderate increases in antinociceptive activity compared to RgIA-ΔR when delivered systemically via intraperitoneal administration. Considering that α-conotoxins are not expected to cross the blood-brain barrier, this result suggests that their molecular target could be found in both central and peripheral nervous systems. Nonetheless, direct antinociceptive screening of mixture-based combinatorial libraries in vivo following systemic administration may lead to the identification of a completely different series of individual second generation analogues with increased peripheral antinociceptive activity as clinically viable analgesics.

Even though the lead compounds identified in this study were significantly antinociceptive, each compound displayed a relatively short duration of action when compared to other nonpeptide-based analgesics. Such a short duration of activity may be attributed to inherently low biological stability associated with peptide-based drugs. While these issues present an ongoing challenge in the development of peptide-based analgesics, cyclization has shown promise as a useful strategy for addressing the issue of α-conotoxin stability.[38] For example, a cyclized analogue of Vc1.1 was previously reported to exhibit significant antinociceptive activity in the CCI model for chronic pain following oral administration.[39] Similarly, a cyclized analogue of RgIA was reported to be more stable than wild type RgIA in human serum, yet retained inhibitory activity against α9α10 nAChRs, and full agonist activity at $GABA_B$ receptors.[17] Therefore, cyclized derivatives of the lead analogues arising from the current study could yield compounds with a longer duration of action.

Though antagonists of α9α10 nAChRs, including Vc1.1 and RgIA are believed to be important targets for antinociceptive drugs that treat chronic neuropathic pain, there has been some debate regarding their true mechanism of action.[40,41] In addition to their potent α9α10 nAChR inhibitory activity, Vc1.1 and RgIA have also been shown to act as G-protein coupled $GABA_B$ receptor agonists, which may modulate N-type calcium channels to produce their analgesic activity.[42] In contrast, AuIB and MII are also effective as analgesics, yet both display very weak activity at α9α10 nicotinic acetylcholine and $GABA_B$ receptors.[30,43] Recent studies have also proposed that their analgesic action may be attributed to inhibition of nAChRs containing combinations of α3, α7 or other subunits.[31] Though we speculate that the mechanism of action of lead compounds arising from this study is related to that of RgIA and Vc1.1, further follow up studies including screening for inhibition of nAChRs and activity at $GABA_B$ are required to confirm a molecular target.

CONCLUSION

Direct in vivo screening of the 66 mixture samples comprising 10,648 individual compounds that made up the α-conotoxin RgIA-ΔR *n*-loop PS-SCL yielded mixtures that produced robust antinociception in the 55 °C warm-water tail-withdrawal assay. Deconvolution of the PS-SCL led to the design and synthesis of 36 individual α-conotoxin analogues. Although a precise molecular mechanism underlying their actions is yet to be determined, systematic testing and elimination of the samples resulted in three potential lead analogues for further evaluation that demonstrated significant antinociceptive activity and did not exhibit respiratory distress or decreased locomotor activity. Together, these data further validates screening of synthetic combinatorial libraries based on α-conotoxin frameworks using the tail-withdrawal assay to identify novel compounds with potent antinociceptive activity. Advanced studies involving more complex animal models for neuropathic pain would be useful to further determine the potential of the lead analogues as clinically viable analgesics. Nonetheless, this work demonstrates combined screening of synthetic combinatorial libraries based on α-conotoxin frameworks using the tail-withdrawal assay together with liability testing to discover novel compounds with potent analgesic activity as a promising approach for developing novel α-conotoxin analogues as clinical analgesics with fewer liabilities of use.

EXPERIMENTAL PROCEDURES

Materials and Methods. N^{α}-*tert*-butyloxycarbonyl (Boc) protected amino acid derivatives, 4-methylbenzylhydrylamine (MBHA) resin, diisopropylethylamine, and trifluoroacetic acid (TFA) were obtained from ChemImpex (Wood Dale, IL). The following amino acid protecting groups were used: Asn and Gln, *N*-xanthan-1-yl; Asp and Glu, *O*-cyclohexyl; Arg and His, 4-toluenesulfonyl; Cys, 4-methylbenzyl (MeBzl); Lys, 2-chlorobenzyloxycarbonyl; Hyp, Ser and Thr, benzyl; Trp, N^{in}-formyl; Tyr, 2-bromobenzyloxycarbonyl. 1*H*-Benzotriazol-1-yl-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Chempep (Wellington, FL). Dichloromethane, *N*,*N*-dimethylformamide, isopropanol, acetonitrile, and *p*-cresol were obtained from Sigma-Aldrich (Milwaukee, WI). Anhydrous HF was obtained from Airgas (Radnor, PA).

Analytical LC-MS was performed using a LC-2010 mass spectrometer equipped with a photodiode array detector (Shimadzu, Columbia, MD). A Jupiter, 50 × 4.6 mm ID reversed phase C_{18} HPLC column (Phenomenex, Torrance, CA) was used for analytical separations. Buffer "A" 0.05% aqueous formic acid; Buffer "B" 95% acetonitrile, 5% water 0.05% formic acid; Linear gradient, 0−60% over 12 min; Flow rate 0.5 mL/min. Detection wavelength 214 nm.

Peptides were purified using a preparative HPLC system (Waters, Milford, MA) using a Luna, 150 × 21.2 mm ID reversed phase C_{18} HPLC column (Phenomenex); Buffer "A" 0.1% aqueous TFA; Buffer "B" 95% acetonitrile, 5% water, 0.1% TFA; Linear gradient, 0−40% buffer "B" over 40 min. Flow rate

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20 mL/min. Detection wavelength 214 nm. The product peak was fractionated and the purity analyzed by LC-MS.

PS-SCL and Individual Conotoxin Synthesis. The RgIA-ΔR *n*-loop PS-SCL and second generation individual α-conotoxin analogues were assembled using solid phase peptide synthesis in 4 cm × 4 cm polypropylene tea-bags containing 100 mg of MBHA resin,[26] by coupling HBTU activated Boc-protected amino acids as previously described.[23] For PS-SCLs, "X" positions were coupled as a cocktail of 22 natural and non-natural amino acids using predetermined concentration ratios to compensate for competitive coupling rates of protected amino acids.[44] Only L-amino acids were used for PS-SCL synthesis. Cysteine and methionine were excluded from "X" positions to avoid complications during oxidative folding. Peptides were cleaved from the resin using a two-step low-high HF procedure as previously described.[28,45] Oxidative folding of α-conotoxins was performed using 0.1 M NH_4HCO_3/50% isopropanol, pH 8.2.[27,28] Complete oxidation was monitored by the qualitative Ellman's test (for PS-SCL mixtures),[46] or liquid-chromatography mass-spectrometry (LC-MS) analysis (for individual analogues, including α-conotoxins Vc1.1, RgIA-ΔR and ImI). Following evaporation of isopropanol in vacuo, samples were desalted using a reversed-phase HPLC column and eluted with 62% acetonitrile (for PS-SCL mixtures) or purified to >95% homogeneity using a linear HPLC gradient of 0 to 40% acetonitrile (for individual analogues).

In Vivo Screening Assays. *Animals.* All in vivo experiments used male C57BL/6J mice (20 to 32 g, Jackson Laboratories, Bar Harbor, ME). The C57BL/6J mouse strain was chosen for this work, as it is a well-established strain for antinociceptive screening and for determining locomotor and respiratory activity.[47,48] Mice were housed four per cage in a temperature-controlled room. Cages were kept in a room with 12-h light-dark cycle, and food and water were available ad libitum. All in vivo experimental procedures were in accordance with the policies and recommendations of the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals edited by International Association for the Study of Pain and of the Guiding Principles in the Care and Use of Animals approved by the Council of The American Physiological Society. All in vivo experimental procedures were approved by the Institutional Animal Care and Use Committee of the Torrey Pines Institute for Molecular Studies.

Administration Techniques. Intraperitoneal (i.p.) injections were made as described previously.[22] All intracerebroventricular (i.c.v.) injections were made directly into the lateral ventricle according to the method of Haley and McCormick.[49] Compounds were delivered via the i.c.v. route to initially measure the inherent pharmacological activity of the analogues in vivo without the complications associated with distribution (i.e., blood-brain barrier penetration) that could affect activity following systemic administration. The mice were lightly anesthetized with isoflurane, an incision made in the scalp, and the injection made 2 mm lateral and 2 mm caudal to bregma at a depth of 3 mm. A volume of 5 μL was injected, using a 10 μL Hamilton microliter syringe with 10% DMSO/90% saline as the vehicle.

Antinociceptive Screening (55 °C Tail Withdrawal Assay). The 55 °C warm-water tail-withdrawal assay was performed in mice as previously described,[22,50] with the latency of tail withdrawal from the water taken as the end point. Mice showing no response within 5 s during the determination of baseline responses were excluded from the experiment. After determining baseline tail-withdrawal latencies, mice were administered conotoxin test samples (either mixtures or individual compounds as described below) in the vehicle (10% DMSO) and tested for antinociceptive every 10 min up to 120 min postinjection, or until latencies returned to baseline values. For positional screening studies, results are presented as the average of individual animal responses summed across all time points. For more detailed analysis across time, antinociception at each time point was calculated according to the formula:

$$\%\text{antinociception} = 100 \times (\text{test latency} - \text{control latency}) / (15 - \text{control latency})$$

A cutoff time of 15 s was used to avoid tissue damage; those mice failing to withdraw their tails within this time were assigned a maximal antinociceptive score of 100%. For the purpose of the initial PS-SCL screening, average summed responses of mixture samples determined a definition as being either inactive/baseline (<15 s; lower dashed lines in Figure 3), or moderately antinociceptive (>25 s; upper dashed lines in Figure 3). For screening of the second generation library, analogues were defined as producing antinociception adequate for additional study if they displayed an average summed tail withdrawal response of >50 s (upper dashed line in Figure 4).

Oxymax/CLAMS Measurement of Respiration Rate and Locomotor Activity. Respiration rates and locomotor activity were recorded using the automated, computer-controlled Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH) as described previously.[22] Mice were placed in closed apparatus cages (23.5 cm × 11.5 cm × 13 cm). Before testing, mice were confined to the locomotor chambers for 30 min to habituate the animals to the apparatus. Mice were administered vehicle (10% DMSO, i.c.v.), or a 30 μg dose of conotoxin sample and returned to chambers for 120 min. Using a pressure transducer built into the sealed CLAMS cage, the respiration rate (breaths/min) of each occupant mouse was counted. Additionally, each cage also contained infrared emitters and receivers spaced every half inch along the longitudinal axis to calculate locomotor activity (as ambulations) from beam breaks.

Rotorod Assay. Possible sedative or hyperlocomotor effects of vehicle, RgIA-ΔR, or compounds **5**, **17**, and **26** were assessed by rotorod performance, as modified from previous protocols.[51] Following seven habituation trials (the last utilized as a baseline measure of rotorod performance), mice were administered intracerebroventricular vehicle (10% DMSO), RgIA-ΔR, or analogues **5**, **17**, or **26** (30 μg/kg, i.c.v., each) 5 min prior to assessment in accelerated speed trials (180 s max. latency at 0−20 rpm) over a 30 min period. Decreased latencies to fall in the rotorod test indicate impaired motor performance. Data are expressed as the percent change from baseline performance.

Statistical Analysis. All dose−response lines were analyzed by regression and ED_{50} (dose producing 50% antinociception) values and 95% confidence limits determined using each individual data point with the Prism 5.0 software package (GraphPad, La Jolla, California, U.S.A.). Data for antinociception experiments were analyzed with Student's *t* tests and analysis of variance (ANOVA) with Bonferroni or Tukey's Honestly Significant Difference (HSD) post hoc tests as appropriate as previously described,[22,50] using Prism 5.0 software.[13] Analyses examined the main effect of baseline and post-treatment tail-withdrawal latencies to determine statistical significance for

all tail-withdrawal data. Statistical significance of ED_{50} values was determined by evaluation of the ED_{50} value shift via nonlinear regression modeling using Prism 5.0. Respiration and spontaneous locomotor activity data collected with CLAMS were analyzed via two-way matching-samples ANOVA, with treatment and time as factors. Rotorod data were analyzed via repeated measures ANOVA, with drug treatment condition as a between-groups factor. For all repeated measures ANOVAs simple main effects and simple main effect contrasts are presented following significant interactions. Where appropriate, Tukey's HSD post hoc tests were used to assess group differences. Effects were considered significant when $p < 0.05$. All effects are expressed as mean ± SEM.

■ ASSOCIATED CONTENT

Supporting Information

Analytical LC-MS data for the six most active lead compounds is provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

C.J.A., R.A.H., and J.P.M. conceived and designed the experiments; C.J.A., J.B., and R.G. synthesized the libraries and individual compounds tested; M.L.G., K.J.R., S.O.E., and E.M. performed the experiments; C.J.A., M.R.H., and J.P.M. analyzed the data, C.J.A. and J.P.M. cowrote the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Abu, aminobutyric acid; ANOVA, analysis of variance; Boc, *tert*-butyloxycarbonyl; CCI, chronic constriction injury; CLAMS, comprehensive laboratory animal monitoring system; ED_{50}, effective dose to produce 50% antinociception; HSD, honestly significant difference; Hyp, hydroxyproline; LC-MS, liquid-chromatography mass-spectrometry; MBHA, 4-methylbenzylhydrylamine; nAChR, nicotinic acetylcholine receptor; Nle, norleucine; Nva, norvaline; PS-SCL, positional scanning synthetic combinatorial library; RP-HPLC, reversed-phase high-performance liquid chromatography

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