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Discovery of non-peptide, small molecule antagonists of $\alpha 9\alpha 10$ nicotinic acetylcholine receptors as novel analgesics for the treatment of neuropathic and tonic inflammatory pain

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Abstract

A series of azaaromatic quaternary ammonium analogs has been discovered as potent and selective $\alpha 9\alpha 10$ nicotinic acetylcholine receptor (nAChR) antagonists. The preliminary structure-activity relationships of these analogs suggest that increased rigidity in the linker units results in higher potency in inhibition of $\alpha 9\alpha 10$ nAChRs and greater selectivity over $\alpha 7$ nAChRs. These analogs represent a new class of analgesic for the treatment of neuropathic and tonic inflammatory pain.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channel complexes, composed of five (α and/or β) subunits, each spanning the membrane four times and aligning around the central ion channel.^{1–3} Nine α ($\alpha 1$ – $\alpha 7$, $\alpha 9$, $\alpha 10$) and four β ($\beta 1$ – $\beta 4$) subunits in mammals have been identified.⁴ Homomeric or heteromeric combinations of the α and β subunits produce a variety of nAChR subtypes with distinct pharmacological properties.⁵

The $\alpha 7$ and $\alpha 9$ subunits are the only known nAChR subunits that can assemble into a functional homomeric subtype.⁶ $\alpha 9$ nAChRs exist as both homomeric $\alpha 9$ and heteromeric $\alpha 9\alpha 10$ receptors; the latter is suggested to be the major subtype.^{7–8} Mutagenesis studies indicate that the stoichiometry of the major form is $\alpha 9(2)\alpha 10(3)$.⁹ The $\alpha 10$ subunit has only been co-expressed functionally with an $\alpha 9$ subunit.⁷ In *Xenopus* oocytes, the co-injection of $\alpha 9$ and $\alpha 10$ subunits boosts functional nAChR expression 100-fold or more compared to

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injection of $\alpha 9$ subunits alone.⁷⁻⁸ The pharmacological profiles of the homomeric and heteromeric $\alpha 9$ nAChRs are essentially indistinguishable, and closely resemble those reported for endogenous cholinergic receptors found in vertebrate hair cells.⁷ A number of pharmacologically unique features of $\alpha 9\alpha 10$ nAChRs have been observed:¹⁰ i) $\alpha 9\alpha 10$ nAChRs can be activated not only by acetylcholine (ACh), but also by the classical muscarinic agonist oxotremorine, indicating a mixed nicotinic/muscarinic pharmacology; ii) $\alpha 9\alpha 10$ nAChRs are antagonized by most classical nAChR agonists, such as nicotine, cytosine, and epibatidine; iii) the classical muscarinic agonist, muscarine, and the classical antagonist, atropine, both block $\alpha 9\alpha 10$ nAChRs; iv) α -bungarotoxin (α -BTX) and methyllycaconitine (MLA) are antagonists of $\alpha 9\alpha 10$ nAChRs; and v) a number of non-cholinergic antagonists, including strychnine (glycine receptor antagonist), bicuculline (GABA-antagonist) and ICS-205,930 (5HT₃ receptor antagonist), also potently block $\alpha 9\alpha 10$ nAChRs. Thus, $\alpha 9\alpha 10$ nAChRs have a pharmacological profile different from any other nicotinic or muscarinic cholinergic receptor subtype.

The $\alpha 9$ nAChR has been identified in a rich variety, yet restricted set of critical tissues, including lymphocytes and developing thymocytes, bronchial epithelium and airway fibroblasts, sensory organs, nervous tissue (dorsal root ganglia), and sperm, but are not present in brain.¹¹ To date, with the exception of innervation of the sensory hair cells in the inner ear,⁷ the function of $\alpha 9\alpha 10$ receptors is still obscure, and research has been hampered by the paucity of selective ligands for probing receptor function. In this regard, chronic constriction of the sciatic nerve increases the number of nAChR-producing lymphocytes at the site of nerve injury,¹² and data suggests that locally released ACh from the lymphocytes stimulates $\alpha 9\alpha 10$ nAChRs to maintain the immune response and behavioral hypersensitivity.¹² Thus, the lymphocytic cholinergic system may be regulating local immune response associated with nerve injury.

Chronic pain afflicts approximately 20% of the adult population in developed nations.¹³ Although there are numerous marketed analgesic drugs, they appear to act through a limited number of molecular mechanisms. Even when these medications are used in combination, substantial pain often persists. Therefore, it is highly desirable to develop new treatments that act through alternative mechanisms of action.¹⁴

A growing body of evidence indicates that $\alpha 9\alpha 10$ nAChR antagonism may represent a novel mechanism to produce analgesia.^{10, 15-16} α -Conotoxins Vc1.1 and RgIA are antagonists of $\alpha 9\alpha 10$ nAChRs.¹² The subcutaneous or intramuscular administration of Vc1.1 and RgIA acutely alleviates pain resulting from traumatic, inflammatory, and metabolic neuronal injury.¹⁵ Intriguingly, analgesia appears to continue for days or weeks after administration of the conotoxin is discontinued, indicating that blockade of $\alpha 9\alpha 10$ nAChRs with these peptides may have disease-modifying effects.¹⁵ Thus, non-peptide, small molecule antagonists of $\alpha 9\alpha 10$ nAChRs on immune cell membranes may have the ability to mediate restorative effects rather than just symptomatic management of nerve injury, and thus, represent novel, potential therapeutics for modulating neuropathic and tonic inflammatory chronic pain.^{10, 12, 15-16}

During the course of our search for potent and selective small molecule nAChR antagonists, we generated a diverse library of novel quaternary ammonium analogs.¹⁷⁻²⁵ Recently, a number of these analogs were found to be highly potent $\alpha 7$ nAChR antagonists.²⁶ Since the classic $\alpha 7$ antagonists α -BTX and MLA are also potent $\alpha 9\alpha 10$ antagonists,⁷ we hypothesized that these quaternary ammonium analogs might also be antagonists at $\alpha 9\alpha 10$ nAChRs. Thus, in the present work, we evaluated a family of *bis*-, *tris*-, and *tetakis*-azaaromatic quaternary ammonium analogs (general structures **1-8**, Fig. 1; **9** and **10**, Fig. 2; and **11** and **12**, Fig. 2, respectively) for their ability to inhibit $\alpha 9\alpha 10$ nAChR-mediated

responses in *Xenopus* oocytes. *bis*-Quaternary ammonium analogs of general structures **1–8** (Fig. 1 and Table 1) were prepared by coupling of the linker precursors **13–20** with the appropriate azaaromatic head-group precursors (Table 1, Fig. 1). Dibromide **13–18**, linker precursors for the synthesis of analogs **1–6**, have been synthesized previously.²³ Dibromide **19** and **20**, linker precursors for the synthesis of analogs **7** and **8**, were prepared utilizing similar procedures as those used for the synthesis of **13–18**, via Sonogashira coupling of 4,4'-dibromobiphenyl with propargyl alcohol. The synthesis of the *tris*- and *tetrakis*-quaternary ammonium analogs **9–12** has been described previously.^{24–25}

The analogs were tested initially at a probe concentration of 100 nM on cloned $\alpha 9\alpha 10$ nAChRs heterologously expressed in *Xenopus* oocytes for their ability to block ACh-gated currents. Data represent percentile inhibitory responses during the co-application of ACh and analog, and are normalized to the responses to ACh alone (Table 1).²⁷

The preliminary screen (Table 1) identified a number of analogs that potently inhibited $\alpha 9\alpha 10$ ACh-gated currents, from which *tetrakis*-analogs **11b**, **11e**, and **12b** exhibited nearly complete inhibition at the probe concentration. In addition, analogs containing three or four quaternary ammonium head-groups were more effective in inhibiting $\alpha 9\alpha 10$ receptors at the probe concentration when compared with analogs within the *bis*-, *tris*-, and *tetrakis*-series. Furthermore, analogs bearing bulky, more hydrophobic cationic head-groups generally exhibited greater inhibition of $\alpha 9\alpha 10$ nAChRs, with the exception of the *tetrakis*-analog series, in which the 3,4-lutidinium analogs **11b** and **12b** were more potent than the isoquinolinium analogs **11d** and **12d**.

Selected compounds, including **7a**, **8a**, **9e**, **10c**, **10d**, **10e**, **11e**, and **12e**,²⁸ were evaluated further in full concentration-response studies for inhibition of ACh-gated currents (Table 2). Analog **11e**, a *tetrakis*-quaternary ammonium compound with four 3-phenylpyridium head-groups, exhibited the highest potency in this series, with an IC_{50} value of 0.56 nM. Notably, analogs containing the more rigid triple bond linker units, i.e. **7a** (IC_{50} =16 nM), **9e** (IC_{50} =1.7 nM), and **11e** (IC_{50} =0.56 nM), were more potent than their corresponding alkyl linked analogs **8a** (IC_{50} =670 nM), **10e** (IC_{50} =3.7 nM), and **12e** (IC_{50} =5.4 nM), respectively, indicating that a conformationally rigid central core scaffold appears to be critical for optimal inhibitory activity at $\alpha 9\alpha 10$ nAChRs.

The above quaternary ammonium analogs are expected to have very limited brain bioavailability due to the highly polar nature of their cationic head-groups, which makes such compounds more attractive as antagonists for $\alpha 9\alpha 10$ nAChRs, due to the absence of this receptor in brain. On the other hand, in the design of antagonists of $\alpha 9\alpha 10$ nAChRs for chronic pain, their selectivity over peripheral $\alpha 7$ nAChRs is critical, due to the fact that antagonism of these two peripheral nAChRs produces diametrically opposite pharmacodynamic effects.¹⁵ Thus, the lead analogs in Table 2 also were evaluated for their ability to block ACh-gated currents in $\alpha 7$ nAChRs heterologously expressed in *Xenopus* oocytes (Table 2). Interestingly, the presence of a rigid central core scaffold appears to be important in affording selectivity for the $\alpha 9\alpha 10$ subtype over the $\alpha 7$ subtype. *bis*-Analog **7a** and **9e**, which incorporate conformationally restricted linker units in their structures, were 75-fold and 109-fold more selective, respectively, for the $\alpha 9\alpha 10$ subtype vs. the $\alpha 7$ subtype. Their corresponding alkyl linker counterparts, **8a** and **10e**, were only 9- and 25-fold more selective, respectively, for the $\alpha 9\alpha 10$ vs. $\alpha 7$ subtypes. However, conformational restriction of the linker units in the *tetrakis*-series of analogs appears to have no significant effect on selectivity, since analogs **11e** and **12e** were equipotent, at $\alpha 9\alpha 10$ and $\alpha 7$ nAChRs. Interestingly, the nature of the head-group appears to have greater influence on selectivity than potency. For example, IC_{50} values of the *tris*-analogs **10c**, **10d**, and **10e** at $\alpha 9\alpha 10$ are similar, whereas selectivity for the $\alpha 9\alpha 10$ subtype vs. the $\alpha 7$ subtype is 360-, 35-, and 25-

fold, respectively, for these three analogs. Thus, analog **11e** was the most potent $\alpha 9\alpha 10$ antagonist ($IC_{50}=560$ pM), but was less selective vs $\alpha 7$ when compared to the slightly less potent analog **10c**.

In preliminary proof-of-concept studies, the most potent $\alpha 9\alpha 10$ antagonist, compounds **7a**, **10c**, and **11e** have recently been evaluated in *in vivo* rat models of pain.^{16, 29} These three analogs were found to be ineffective as an analgesic in the rat tail-flick test, but demonstrated an analgesic effect in the rat formalin model of tonic inflammatory pain (phase 2) and reversed mechanical hyperalgesia in a dose-related manner in the rat chronic constriction nerve injury (CCI) model of neuropathic pain. Furthermore, tolerance to the antihyperalgesic effect was not observed after repeated dosing (7 days) of drug treatment. In addition, efficacy was achieved at doses well below those that produced toxicity in the motor function rotarod test. The above analogs were 10 to 5000-fold less potent as antagonists at other nAChRs, including $\alpha 1\beta 1\delta\epsilon$, $\alpha 2\beta 2$, $\alpha 2\beta 4$, $\alpha 3\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2$, $\alpha 4\beta 4$, $\alpha 6/\alpha 3\beta 2\beta 3$, and $\alpha 6/\alpha 3\beta 4$ subtypes; The most potent analog **11e** is a selective inhibitor of $\alpha 9\alpha 10$ nAChRs, having relatively poor inhibitory potency against $\alpha 4\beta 2$ ($IC_{50}=3.8$ μ M), $\alpha 3\beta 4$ ($IC_{50}=0.8$ μ M), and muscle ($IC_{50}=0.12$ μ M) nAChRs.¹⁶ We have also shown that analog **11e** does not interact with GABA_A or GABA_B receptors, and has several orders of magnitude lower affinity for 5HT₃ and κ -opioid receptors compared to $\alpha 9\alpha 10$ nAChRs (unpublished data). More recently, the *bis*-analog **7a**, was shown to be effective in alleviating vincristin-induced neuropathic pain in the rat CCI pain model.³⁰ These collective results provide support for the contention that $\alpha 9\alpha 10$ nAChRs are a new target for studying pain modulation, and suggests that further development of small, non-peptide $\alpha 9\alpha 10$ antagonists as treatments for chronic pain may lead to novel, clinically useful, non-opioid analgesics.

In conclusion, a series of azaaromatic quaternary ammonium analogs have been discovered as potent and selective antagonists of $\alpha 9\alpha 10$ nAChRs. These first-in-class compounds represent excellent leads for the subsequent development of highly potent and subtype-selective small molecule antagonists at $\alpha 9\alpha 10$ nAChRs as treatments for chronic pain.

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27. In the voltage clamp electrophysiology assays (Tables 1 and 2), *Xenopus* oocytes were voltage-clamped and exposed to ACh and test compounds as previously described.¹¹ Briefly, the oocyte chamber consisting of a cylindrical well (~30 μ L in volume) was gravity perfused at a rate of ~2 mL/min with ND96 containing 0.01% (wt/vol) BSA and 1 μ M atropine to block potential contaminating signal from endogenous muscarinic receptors. For experiments involving $\alpha 7$ and $\alpha 9\alpha 10$, atropine was excluded from the perfusion solution because it has been shown to block these receptor subtypes. Oocytes were exposed once a minute to 1 sec pulses of ACh. ACh concentrations used were 200 μ M for $\alpha 7$, 10 μ M for $\alpha 1\beta 18e$ and $\alpha 9\alpha 10$ and 100 μ M for all other subtypes. Compounds were applied at the beginning of a five min static bath incubation. The % block was calculated as a % of ND96 control (no compound) response. Concentration-response data were fit to the equation $Y = 100/(1 + 10^{-(\text{LogIC}_{50} - \text{Log}[\text{Toxin}]) \cdot \text{Hill Slope}})$ by nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA). Data points in the concentration-response represent the mean $\pm$ SEM from at least 3 oocytes.
28. Spectra data of selective compounds: **7a**, ¹H NMR (300MHz, CDCl₃) δ 9.01 (s, 2H), 8.97 (d, J=6.3 Hz, 2H), 7.99 (d, J=6.3 Hz, 2H), 7.76 (dd, J=6.6, 2.1 Hz, 4H), 7.61 (dd, J=6.6, 2.1 Hz, 4H), 5.81 (s, 4H), 2.52 (s, 6H), 2.40 (s, 6H) ppm; ¹³C NMR (75 MHz, CDCl₃) δ 159.6, 143.1, 142.0, 140.4, 138.7, 133.1, 128.9, 127.7, 120.9, 88.8, 83.1, 50.4, 20.7, 17.2 ppm; **8a**, ¹H NMR (300MHz, CDCl₃) δ 8.82 (s, 2H), 8.73 (d, J=6.3 Hz, 2H), 7.78 (d, J=6.3 Hz, 2H), 7.46 (d, J=8.4 Hz, 4H), 7.29 (d, J=8.4 Hz, 4H), 4.64 (t, J=7.2 Hz, 4H), 2.79 (t, J=7.2 Hz, 4H), 2.47 (s, 6H), 2.39 (s, 6H), 2.32–2.42 (m, 4H) ppm; ¹³C NMR (75 MHz, CDCl₃) δ 158.4, 143.0, 141.4, 139.6, 138.5, 138.3, 129.0, 128.3, 126.6, 60.6, 32.6, 32.0, 19.4, 16.1 ppm; **9e**, ¹H NMR (300 MHz, CD₃OD) δ 9.50 (s, 3H), 9.09 (d, J=6.0 Hz, 3H), 8.78 (d, J=8.1 Hz, 3H), 8.16 (dd, J=8.1, 6.0 Hz, 3H), 7.75–7.87 (m,

6H), 7.42–7.65 (m, 9H), 7.17 (s, 3H), 4.96 (t, J=6.9 Hz, 6H), 2.69 (t, J=6.3 Hz, 6H), 2.42 (m, 6H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 144.4, 144.3, 144.2, 142.6, 134.9, 134.5, 131.5, 130.8, 129.5, 128.7, 125.1, 90.0, 81.5, 62.6, 30.8, 17.4 ppm; **10c**, ^1H NMR (300 MHz, CD_3OD) δ 9.46 (dd, J=6.0, 1.5 Hz, 3H), 9.22 (d, J=8.4 Hz, 3H), 8.57 (d, J=9.0 Hz, 3H), 8.45 (dd, J=8.4, 1.5 Hz, 3H), 8.30 (m, 3H), 8.02–8.14 (m, 6H), 6.80 (s, 3H), 5.11 (t, J=7.5 Hz, 6H), 2.56 (t, J=7.5 Hz, 6H), 2.14 (m, 6H), 1.69 (m, 6H), 1.52 (m, 6H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 150.3, 148.9, 143.4, 139.4, 137.3, 132.2, 131.8, 131.4, 127.2, 123.1, 119.9, 59.4, 36.7, 32.3, 31.1, 27.3 ppm; **10d**, ^1H NMR (300 MHz, CD_3OD) δ 9.99 (s, 3H), 8.69 (dd, J=6.9, 1.5 Hz, 3H), 8.47–8.54 (m, 6H), 8.22–8.36 (m, 6H), 8.07 (m, 3H), 6.78 (s, 3H), 4.78 (t, J=7.5 Hz, 6H), 2.53 (t, J=7.5 Hz, 6H), 2.15 (m, 6H), 1.67 (m, 6H), 1.44 (m, 6H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 150.8, 143.2, 138.8, 138.2, 135.8, 132.5, 131.5, 129.0, 128.5, 127.5, 127.1, 62.8, 36.5, 32.3, 32.0, 26.8 ppm; **10e**, ^1H NMR (300 MHz, CD_3OD) δ 9.39 (s, 3H), 8.98 (d, J=6.0 Hz, 3H), 8.85 (ddd, J=6.0, 1.8, 1.2 Hz, 3H), 8.15 (dd, J=8.1, 6.0 Hz, 3H), 7.78–7.90 (m, 6H), 7.50–7.65 (m, 9H), 6.82 (s, 3H), 4.74 (t, J=7.8 Hz, 6H), 2.55 (t, J=7.5 Hz, 6H), 2.11 (m, 6H), 1.69 (m, 6H), 1.45 (m, 6H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 144.2, 143.9, 143.4, 142.8, 134.6, 131.5, 130.8, 129.4, 128.7, 127.2, 63.3, 36.7, 32.7, 32.2, 27.0 ppm; **11e**, ^1H NMR (300 MHz, CD_3OD) δ 9.50 (s, 4H), 9.09 (d, J=6.0 Hz, 4H), 8.74 (dd, J=8.4, 1.2 Hz, 4H), 8.14 (dd, J=8.1, 6.0 Hz, 4H), 7.70–7.85 (m, 8H), 7.45–7.62 (m, 12H), 7.23 (s, 2H), 4.95 (t, J=6.9 Hz, 8H), 2.71 (t, J=6.6 Hz, 8H), 2.30–2.47 (m, 8H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 144.2, 142.6, 136.2, 134.5, 131.5, 130.8, 129.6, 128.7, 126.1, 94.8, 81.0, 62.6, 31.1, 17.7 ppm; **12e**, ^1H NMR (300 MHz, CD_3OD) δ 9.48 (s, 4H), 9.05 (d, J=6.0 Hz, 4H), 8.83 (d, J=8.4 Hz, 4H), 8.14 (dd, J=7.8, 6.3 Hz, 4H), 7.89 (dd, J=7.8, 1.2 Hz, 8H), 7.40–7.65 (m, 12H), 6.85 (s, 2H), 4.80 (t, J=7.5 Hz, 8H), 2.52 (t, J=6.9 Hz, 8H), 2.11 (t, J=6.6 Hz, 8H), 1.37–1.69 (m, 16H) ppm; ^{13}C NMR (75 MHz, CD_3OD) δ 142.9, 142.8, 142.6, 141.2, 137.3, 133.3, 130.3, 130.1, 129.6, 128.4, 127.6, 62.1, 32.0, 31.7, 31.0, 26.2 ppm.

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Figure 1.
General synthesis and structures of the *bis*-azaaromatic quaternary ammonium analogs.

Figure 2.
General structures of the *tris*- and *tetrakis*-azaaromatic quaternary ammonium analogs.

Table 1

ACh-gated current responses at $\alpha 9\alpha 10$ nAChRs after coapplication of 100 nM of azaaromatic quaternary ammonium analogs.

Compound ^a	$\alpha 9\alpha 10$ nAChRs (% response) ^b	Compound ^a	$\alpha 9\alpha 10$ nAChRs (% response) ^b
1a	31.3±5.8	4a	72.0±6.9
1b	67.6±16.9	4b	124.4±10.2
		4c	10.9±1.4
1d	28.03±3.87	4d	31.15±8.65
2a	76.9±11.9	5a	62.7±7.7
2c	41.6±6.7	5c	42.6±9.5
2d	39.7±11.4	5d	37.5±6.6
2e	23.2±3.8		
3a	10.6±4.6	6a	36.7±11.3
3b	76.2±8.3	6b	97.1±9.9
7a	8.1±6.1	8a	89.3±10.8
		8b	113.8±4.7
9a	61.12±6.95	10a	62.74±7.85
9b	66±12.61	10b	79.77±9.71
9c	10.46±3.48	10c	15.9±3.5
		10d	6.9±4.71
9e	5.5±2.6	10e	3.84±1.95
9f	36.15±7.23	10f	16.1±9.3
11a	47.4±8.2	12a	11.0±3.1
11b	0.3±0.07	12b	0.6±0.4
11d	11.6±3.1	12d	17.9±3.3
11e	0.3±0.09	12e	6.5±2.9

^aThe number in the compound designation corresponds to the type of linker as shown in Figure 1. The letter corresponds to the head groups.

^bInhibition of $\alpha 9\alpha 10$ -mediated responses to 10 μ M ACh in the presence of 100 nM of analog, the data represent % of response; Each value represents data from 3–8 independent experiments.

Table 2

IC₅₀ values for the azaaromatic quaternary ammonium analog inhibition of $\alpha 9\alpha 10$ and $\alpha 7$ nAChRs.

Compounds	$\alpha 9\alpha 10$ nAChR (IC ₅₀ , nM)	$\alpha 7$ nAChR (IC ₅₀ , nM)
7a	16	1190
8a	670	6010
9e	1.7	185
10c	4.2	1510
10d	4.8	170
10e	3.7	91
11e	0.56	8.8
12e	5.4	99