Chem. Biodiversity

A small library of 1,2,3-triazole analogues of CAP-55: synthesis and binding affinity at nicotinic acetylcholine receptors

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Alpha7 nicotinic acetylcholine receptor is emerging as a central regulator in inflammatory processes, as documented by increasing studies reported in the literature. For instance, the activation of this nicotinic receptor subtype in resident macrophages inhibits the production of pro-inflammatory cytokines, thereby attenuating local inflammatory responses, and may open a new window in the treatment of chronic inflammatory disease, such as Crohn's disease, rheumatoid arthritis, psoriasis and asthma. In continuation of our ongoing research for the development of new cholinergic drug candidates, we selected the nicotine derivative CAP55, which was previously shown to exert anti-inflammatory effects via nicotinic stimulation, as a suitable compound for lead optimization. Through the isosteric replacement of its 3,5-disubstituted 4,5-dihydroisoxazole core with a 1,4-disubstituted 1,2,3-triazole ring, we could rapidly generate a small library of CAP55-related analogues via a one-pot copper(I)-catalyzed azide-alkyne cycloaddition. Receptor binding assays at nAChRs led to the identification of two promising derivatives, compounds **4** and **10**, worthy of further pharmacological studies.

Keywords: click chemistry • nicotine • alpha7 • cholinergic • inflammation

Introduction

Convergence from many unrelated research areas has identified a primary role in health and disease for one of the most abundant subtype of the nicotinic acetylcholine receptor (nAChR), the α7 nAChR. The α7 nAChR belongs to the family of acetylcholine-gated ion channels and exhibits distinct biophysical and pharmacological profiles relative to other nAChR subtypes. This receptor, expressed in the CNS, in the autonomous nervous system, and in the vascular system, is emerging as a central regulator in processes ranging from cognitive mechanisms through modulation of specific neurotransmitters, neuroprotection following various insults (e.g., chemical toxicity and β-amyloid-induced cell death), normalization of sensory gating in schizophrenic patients, and inflammatory processes. Resident macrophages express the α7 nAChR, and activation of this receptor inhibits the production of pro-inflammatory cytokines (e.g., TNF, IL-1, IL-6, HMGB-1), but does not inhibit anti-inflammatory cytokine production (e.g., IL-10 and TGF-β), thereby attenuating local inflammatory responses.[1,2,3] Previous studies have demonstrated that targeting pro-inflammatory cytokines, such as TNF, IL-1 and IL-6, represents a promising approach in the treatment of inflammatory disorders, such as Crohn's disease, psoriasis, rheumatoid arthritis and asthma.[4,5,6] Therefore, α7 nAChRs may represent a relevant target in view of new therapeutics for the treatment of such inflammatory diseases.

Several studies supporting a role for α7 nAChR in inflammatory reflexes can be found in the literature. For instance, GTS-21, an α7 nAChR partial agonist, was shown to inhibit levels of serum TNF-α, which was associated with improved survival in a murine endotoxemia and severe sepsis model,[7] and decreased severity of experimental pancreatitis.[8] Moreover, GTS-21 strongly inhibited LPS-induced TNF-α release into the peritoneal cavity and the circulation.[9] In 2011, Li and co-workers described the α7-mediated anti-inflammatory effects of A-833834, a new-generation α7 nAChR agonist endowed with exceptionally high binding affinity for this receptor subtype.[10] A-833834 inhibited LPS-induced TNF-α release in a methyllycaconitine (MLA)-sensitive manner in macrophages. These effects were also confirmed using human whole blood assay where A-833834 was active. The role for α7 nAChR in regulating TNF-α release was further confirmed in two different *in vivo* mouse models, the i.v. LPS-induced TNF-α release from whole blood and the i.p. zymosan-induced peritonitis models. Li and coll. also showed that A-833834 reduced LPS-induced release of IL-1β and IL-6 in human blood. This is consistent with recent reports that acetylcholine and other α7 nAChR agonists including PNU-282987 and AR-R17779 reduced the release of TNF-α and IL-6 in human fibroblast-like synoviocytes via α7 nAChR,[11,12] even though the precise mechanisms underlying α7 nAChR-mediated cytokine reduction remain to be elucidated.[10]

In 2005, Saeed and coll. developed a new synthetic derivative of the major tobacco alkaloid nicotine (from *Nicotiana tabacum*), named CAP55, and investigated its anti-inflammatory effects, which were shown to be mediated by selective activation of the α7 nAChR subtype (**Figure 1**).[5,13] CAP55 significantly inhibited endothelial cell inflammatory responses *in vitro* and *in vivo*. Using the localized Shwartzman reaction model, characterized by

sustained E-selectin and VCAM-1 expression,[14] CAP55 was found to be very effective in inhibiting endothelial cell activation *in vivo*. Moreover, CAP55 significantly blocked TNF-induced adhesion molecule expression and chemokine expression by human microvascular endothelial cells (HuMVECs) in a dose-dependent manner. The inhibitory effects of CAP55 on endothelial cell activation were reverted both *in vitro* and *in vivo* by mecamylamine, a noncompetitive nAChR blocker, suggesting that the observed anti-inflammatory effects were mediated by nAChRs. In the carrageenan air pouch model, CAP55 was also found to suppress leukocyte migration during inflammation *in vivo*, and again this effect was blocked by administration of mecamylamine.[5]

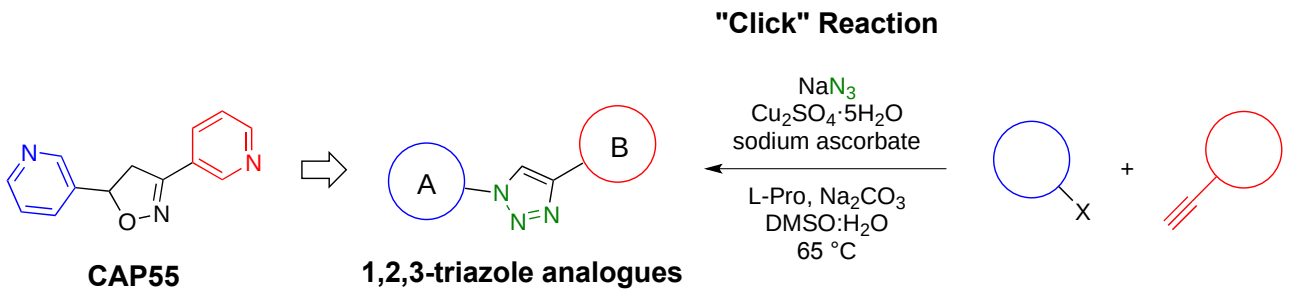


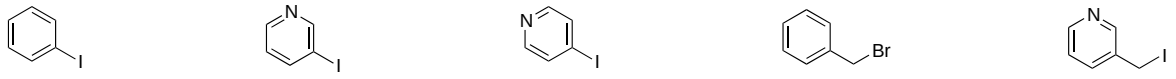
Figure 1. Chemical structure of the cholinergic agonist CAP55, design strategies of the 1,2,3-triazole analogues reported in this study and general scheme of the "click" reaction applied. Blue rings represent aryl, pyridyl, benzyl and pyridylmethyl halides; red rings represent aryl and pyridyl alkynes.

The above discussed results demonstrate the suppressive effects of α7 nAChR agonists on inflammation-related events and highlight the potential therapeutic usefulness of such agents in the treatment of a variety of inflammatory disorders. In continuation of our ongoing research for the development of selective nicotinic agents,[15,16,17,18,19,20] we identified CAP55 as a suitable candidate for lead optimization. In order to rapidly generating a small library of CAP55 analogues, we aimed at applying a "click chemistry" approach.[21] For its molecular structure, CAP55 is well-suited to this purpose: in fact, we observed that the replacement of its 3,5-disubstituted 4,5-dihydroisoxazole core with a 1,4-disubstituted 1,2,3-triazole ring would have allowed us to prepare a set of new derivatives via one-pot copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) (**Figure 1**).[22] Herein, we report the synthesis of a "click chemistry"-based small library of 1,2,3-triazole analogues of CAP55 and the assessment of their binding affinity at α7 and α4β2 nAChR subtypes.

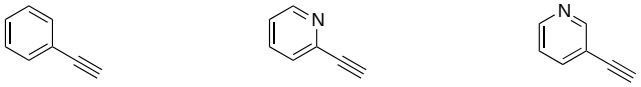
Results and Discussion

The desired 1,4-disubstituted 1,2,3-triazole derivatives **1-10** (**Figure 2**) were all prepared via a copper(I)-promoted 1,3-dipolar cycloaddition between an *in situ* generated azide and a terminal alkyne, following the one-pot two-step procedure described by Fokin and co-workers.[23] The starting halide and alkyne building blocks were all selected in such a way that the new derivatives differed for the presence and/or the position of the two pyridine nitrogen atoms, which normally play an important role in the orthosteric binding at nAChRs (**Figure 2**).[24,25] All the new derivatives were obtained by simply mixing and stirring the needed reagents in a sealed vial at 65 °C and isolated in a pure form by filtration (see Experimental section for details). Through this experimental protocol, we did not observe the formation of any undesired N–H triazole byproduct, which may result from the competing reaction between inorganic azide and an alkyne. Moreover, the expected regioselectivity was maintained and yields ranged from fair to excellent. Finally, we tested the binding affinity of CAP55 (so far not reported in the literature) and the new analogues **1-10** towards α7 and α4β2 nAChR subtypes. K_i values obtained from the competition assays are reported in **Table 1**.

a) aryl, pyridyl, benzyl and pyridylmethyl halides used in this work



b) aryl and pyridyl alkynes used in this work



c) 1,2,3-triazole analogues of CAP55 synthesized in this work

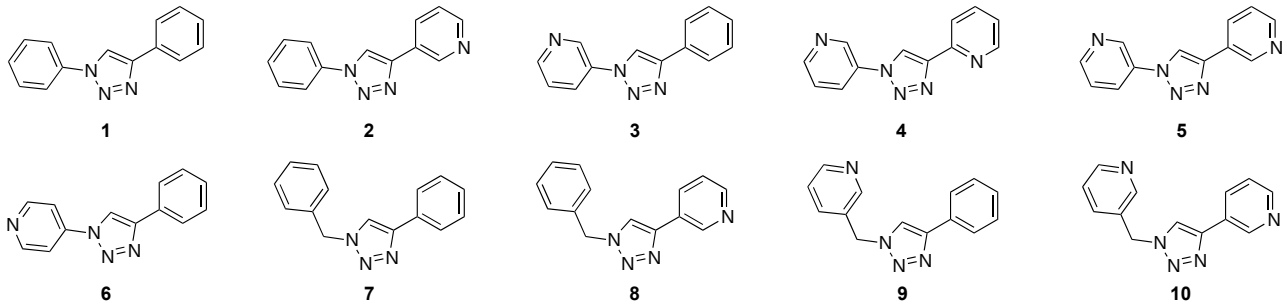
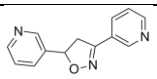
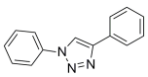
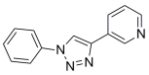
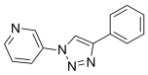
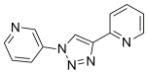
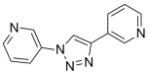
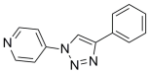
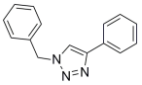
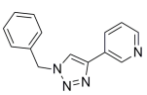
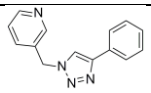
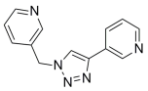


Figure 2. Chemical structure of the CAP55 1,2,3-triazole analogues synthesized and their building blocks for the one-pot copper(I)-catalyzed azide-alkyne cycloaddition.

Table 1. CAP55 and compounds **1–10**: binding affinity for native receptor subtypes present in rat cortex and labelled by [^{125}I]αBungarotoxin (α7 nAChRs) or [^{3}H]epibatidine (α4β2 nAChRs). Numbers in brackets represent the % coefficient of variation (CV).

Compound	Structure	α7 [^{125}I]α-Bungarotoxin (K_i, μM)	α4β2 [^{3}H]Epibatidine (K_i, μM)
CAP55		407 (83)	70 (28)
1		686 (88)	76 (12)
2		100 (65)	48 (46)
3		> 100	> 100
4		6.5 (48)	20 (75)
5		> 100	50 (53)
6		346 (125)	178 (14)
7		189 (94)	150 (15)
8		91 (85)	47 (55)

9		73 (64)	34 (58)
10		24 (63)	> 100

The parent compound CAP55 showed a moderate affinity for both receptor subtypes, with a certain preference for α4β2. Based on the effects reported for this compound as an α7 activator in *in vitro* and *in vivo* models of inflammation,[5] its binding profile may seem quite surprising. However, similar nicotinic ligands, such as GTS-21, have been proven to selectively stimulate α7 nAChRs but also inhibit some other nicotinic receptors, including those containing β2 subunits, and, in our opinion, it is reasonable to hypothesize a similar scenario for CAP55.[26,27] Most of the new derivatives showed a similar binding profile, with moderate or poor affinity for both receptor subtypes. Indeed, compounds **4**, **8**, **9** and **10** displayed an increased affinity for α7, with compounds **4** (60-fold increase) and **10** (17-fold increase) —both characterized by the presence of a pyridine ring on the two substituted positions of the triazole core— emerging as the most interesting analogues. In addition, compound **10** also showed a moderate gain in selectivity versus the α4β2 subtype. Overall, the replacement of the 4,5-dihydroisoxazole core of CAP55 with a triazole system turned out to be a well-tolerated and in some cases a beneficial substitution. Interestingly, increasing the distance between the two pyridine nitrogen atoms produced an inversion of the selectivity profile in compound **10** compared to its lower homologue **5**.

Conclusions

In conclusion, we generated a small library of CAP55 analogues via a click chemistry approach and demonstrated that the 1,4-disubstituted 1,2,3-triazole system may serve as an effective bioisosteric replacement of the 3,5-disubstituted 4,5-dihydroisoxazole nucleus. We have also reported for the first time the binding affinity profile of the cholinergic agonist CAP55 at the two main nAChR subtypes. Compounds **4** and **10**, which both showed a relevant increase in affinity for the α7 subtype, emerged as the most promising derivatives. Further studies are ongoing to elucidate, on one hand, the functional and anti-inflammatory properties of these two nicotinic ligands and, on the other, to deepen the structure-activity relationships in this set of compounds through the rational design of novel analogues.

Experimental Section

Chemistry

Materials and methods. All chemicals were purchased from Sigma-Aldrich and used without any further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (^{1}H, 300.063; ^{13}C, 75.451 MHz) spectrometer at 20 °C. ESI Mass spectra were obtained on a Varian 320 LC-MS-MS instrument; data are reported as mass-to-charge ratio (m/z). TLC analyses were performed on commercial Silica Gel 60 F254 aluminium sheets; the spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution or a phosphomolybdic acid solution (10% in ethanol). Melting points were determined on a model B 540 Büchi apparatus and are uncorrected.

Typical experimental procedure. The aryl- or arylmethyl halide (1 equiv) was mixed with ethynylbenzene or ethynylpyridine (1 equiv) in a sealable round bottom vial. To the mixture were added L-proline (0.2 equiv), Na_2CO_3 (0.2 equiv), NaN_3 (1.2 equiv), sodium ascorbate (0.1 equiv), 9:1 DMSO/H_2O and $CuSO_4 \cdot 5H_2O$ (0.05 equiv). The vial was sealed and the mixture was stirred overnight at 65 °C. Upon completion (monitored by TLC), the crude mixture was poured into ice-cold water. The off-white precipitate was isolated by filtration and washed with dilute NH_4OH to remove any traces of explosive copper azides before the final drying step.

1,4-Diphenyl-1*H*-1,2,3-triazole [1]. 71% yield, white powder, mp: 183-185 °C; R_f = 0.79 (DCM/MeOH = 99:1); ^{1}H NMR (DMSO-d_6): δ (ppm) 9.29 (s, 1H), 7.95 (d, J = 8.1 Hz, 4H), 7.62 (t, J = 7.5 Hz, 2H), 7.52-7.46 (m, 3H), 7.37 (t, J = 7.4 Hz, 1H); ^{13}C NMR (DMSO-d_6): δ (ppm) 148.0, 137.3, 131.0, 130.6, 129.7, 129.4, 128.9, 126.0, 120.7, 120.3; MS (m/z) calcd. for $C_{14}H_{12}N_3^+$ [M+H$^+$] 222.10, found 222.1.

3-(1-Phenyl-1*H*-1,2,3-triazol-4-yl)pyridine [2]. 59% yield, light yellow powder, mp: 170-173 °C; R_f = 0.20 (cyclohexane/AcOEt = 1:1); ^{1}H NMR ($CDCl_3$): δ (ppm) 9.09 (s, 1H), 8.62 (d, *J* = 4.7 Hz, 1H), 8.31 (t, *J* = 1.9 Hz, 1H), 8.29 (s, 1H), 7.83-7.79 (m, 2H), 7.58 (t, *J* = 7.2 Hz, 2H), 7.50 (d, *J* = 7.4 Hz, 1H), 7.42 (dd, *J* = 8.0 and 5.0 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ (ppm) 149.7, 147.3, 145.5, 133.4, 130.1, 129.8, 129.3, 126.7, 124.1, 120.9, 118.2; MS (m/z) calcd. for $C_{13}H_{11}N_4^+$ [M+H$^+$] 223.10, found 223.1.

3-(4-Phenyl-1*H*-1,2,3-triazol-1-yl)pyridine [3]. 52% yield, yellow powder, mp: 176-178 °C; R_f = 0.22 (cyclohexane/AcOEt = 1:1); ^{1}H NMR ($CDCl_3$): δ (ppm) 9.07 (s, 1H), 8.73 (d, *J* = 4.4 Hz, 1H), 8.25 (s, 1H), 8.23-8.19 (m, 1H), 7.92 (d, *J* = 8.3 Hz, 2H), 7.53 (dd, *J* = 8.3 and 5.0 Hz, 1H), 7.48 (t, *J* = 7.4 Hz, 2H), 7.41 (d, *J* = 7.7 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ (ppm) 150.2, 149.2, 141.8, 134.0, 130.0, 129.2, 129.0, 128.3, 126.2, 124.5, 117.6; MS (m/z) calcd. for $C_{13}H_{11}N_4^+$ [M+H$^+$] 223.10, found 223.1.

2-(1-(Pyridin-3-yl)-1*H*-1,2,3-triazol-4-yl)pyridine [4]. 63% yield, light brown powder, mp: 203-206 °C; R_f = 0.50 (DCM/MeOH = 95:5); ^{1}H NMR ($CDCl_3$): δ (ppm) 9.20 (s, 1H), 8.81 (m, 1H), 8.69-8.60 (m, 2H), 8.24 (dd, *J* = 11.8 and 8.0 Hz, 2H), 7.84 (t, *J* = 7.7 Hz, 1H), 7.64-7-52 (m, 1H), 7.30 (dd, *J* = 7.2 and 5.0 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ (ppm) 150.1, 149.5, 149.0, 137.5, 137.3, 134.0, 130.1, 129.2, 128.3, 126.2, 123.4, 117.6; MS (m/z) calcd. for $C_{12}H_{10}N_5^+$ [M+H$^+$] 224.09, found 224.0.

3,3'-(1*H*-1,2,3-Triazole-1,4-diyl)dipyridine [5]. 87% yield, white powder, mp: 218-220 °C; R_f = 0.45 (DCM/MeOH = 96:4); ^{1}H NMR ($CDCl_3$): δ (ppm) 9.10 (s, 1H), 9.08 (d, *J* = 2.7 Hz, 1H), 8.75 (dd, *J* = 4.7 and 1.1 Hz, 1H), 8.65-8.60 (m, 1H), 8.35 (s, 1H), 8.29 (dt, *J* = 8.0 and 1.7 Hz, 1H), 8.21 (dq, *J* = 8.3 and 1.7 Hz, 1H), 7.55 (dd, *J* = 8.3 and 4.7 Hz, 1H), 7.43 (dd, *J* = 8.0 and 5.0 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ (ppm) 150.5, 150.1, 147.4, 146.2, 141.9, 133.7, 133.5, 128.4, 126.3, 124.6, 124.1, 118.1; MS (m/z) calcd. for $C_{12}H_{10}N_5^+$ [M+H$^+$] 224.09, found 224.1.

4-(4-Phenyl-1*H*-1,2,3-triazol-1-yl)pyridine [6]. 65% yield, light brown powder, mp: 170-172 °C; R_f = 0.30 (cyclohexane/AcOEt = 6:4); ^{1}H NMR ($CDCl_3$): δ (ppm) 8.83 (m, 2H), 8.30 (s, 1H), 7.92 (d, *J* = 7.2 Hz, 2H), 7.80 (d, *J* = 5.0 Hz, 2H), 7.48 (t, *J* = 7.2 Hz, 2H), 7.41 (d, *J* = 7.2 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ (ppm) 152.0, 149.4, 143.2, 129.8, 129.3, 129.1, 126.2, 116.8, 113.9; MS (m/z) calcd. for $C_{13}H_{11}N_4^+$ [M+H$^+$] 223.10, found 223.1.

1-Benzyl-4-phenyl-1*H*-1,2,3-triazole [7]. 82% yield, white powder, mp: 128-130 °C; R_f = 0.38 (cyclohexane/AcOEt = 7:3); ^{1}H NMR (DMSO-d_6): δ (ppm) 8.63 (s, 1H), 7.83 (d, *J* = 7.4 Hz, 2H), 7.45-7.30 (m, 8H), 5.64 (s, 2H); ^{13}C NMR (DMSO-d_6): δ (ppm) 136.7, 131.4, 129.6, 129.5, 128.9, 128.6, 128.1, 125.8, 122.3, 120.7, 53.7; MS (m/z) calcd. for $C_{15}H_{14}N_3^+$ [M+H$^+$] 236.12, found 236.1.

3-(1-Benzyl-1*H*-1,2,3-triazol-4-yl)pyridine [8]. 70% yield, light brown powder, mp: 100-102 °C; R_f = 0.25 (cyclohexane/AcOEt = 3:7); ^{1}H NMR ($CDCl_3$): δ (ppm) 8.95 (bs, 1H), 8.56 (d, *J* = 3.4 Hz, 1H), 8.18 (ddd, *J* = 7.7, 3.9 and 2.2 Hz, 1H), 7.74 (s, 1H), 7.43-7.30 (m, 6H), 5.60 (s, 2H); ^{13}C NMR ($CDCl_3$): δ (ppm) 149.4, 147.2, 145.4, 134.5, 133.1, 129.4, 129.1, 128.3, 126.9, 123.9, 120.0, 54.6; MS (m/z) calcd. for $C_{14}H_{13}N_4^+$ [M+H$^+$] 237.11, found 237.1.

3-((4-Phenyl-1*H*-1,2,3-triazol-1-yl)methyl)pyridine [9]. 67% yield, white powder, mp: 112-115 °C; R_f = 0.40 (AcOEt); ^{1}H NMR ($CDCl_3$): δ (ppm) 8.67-8.62 (m, 2H), 7.79 (d, *J* = 6.9 Hz, 2H), 7.71 (s, 1H), 7.62 (d, *J* = 7.7 Hz, 1H), 7.41 (t, *J* = 6.9 Hz, 2H), 7.32 (t, *J* = 7.2 Hz, 2H), 5.60 (s, 2H); ^{13}C NMR ($CDCl_3$): δ (ppm) 150.6, 149.4, 148.8, 135.9, 130.8, 130.5, 129.1, 128.6, 126.0, 124.2, 119.6, 51.8; MS (m/z) calcd. for $C_{14}H_{13}N_4^+$ [M+H$^+$] 237.11, found 237.1.

3-((4-(Pyridin-3-yl)-1*H*-1,2,3-triazol-1-yl)methyl)pyridine [10]. 72% yield, white powder, mp: 132-134 °C; R_f = 0.33 (AcOEt); ^{1}H NMR ($CDCl_3$): δ (ppm) 8.97 (d, *J* = 1.9 Hz, 1H), 8.68 (d, *J* = 2.2 Hz, 1H), 8.65 (dd, *J* = 5.0 and 1.7 Hz, 1H), 8.58 (dd, *J* = 4.7 and 1.4 Hz, 1H), 8.19 (dt, *J* = 8.0 and 1.9 Hz, 1H), 7.80 (s, 1H), 7.66 (dt, *J* = 8.0 and 2.2 Hz, 1H), 7.39-7.33 (m, 2H), 5.64 (s, 2H); ^{13}C NMR ($CDCl_3$): δ (ppm) 150.7, 149.5, 148.9, 147.7, 147.4, 136.0, 134.6, 130.8, 130.6, 126.1, 124.2, 120.1, 51.8; MS (m/z) calcd. for $C_{13}H_{12}N_5^+$ [M+H$^+$] 238.11, found 238.1.

Receptor binding assays

Animal tissues. Adult male pathogen-free Sprague-Dawley rats (Harlan-Nossan, Milan, Italy) (at least 4-month-old) were used. Rats were reared on a 12 h light/dark cycle (animals were kept in a 12 h light/12 h dark cycle) with free access to food and water. All experiments were conducted according to Ministry of Health (the regulatory authority for controlling the use of laboratory animals and ethics on animal experiments in Italy) guidelines (Legislative Decree n. 116/92) and in accordance with the European Community guidelines European Directive 86/609/EEC). All the experiments were performed according to protocols (n.2/2010) approved by the University of Milan Animal Care and Use Committees and by the Ministry of Health.

Membranes binding of [^{125}I]α-Bungarotoxin and [^{3}H]Epibatidine. The cortex tissues were dissected, immediately frozen on dry ice and stored at 80 °C for later use. In each experiment, the cortex tissues from two rats were homogenized in 10 mL of a buffer solution (50 mM Na_3PO_4, 1 M NaCl, 2 mM ethylenediaminetetraacetic acid [EDTA], 2 mM ethylene glycol tetraacetic acid [EGTA] and 2 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4) using a potter homogenizer; the homogenates were then diluted and centrifuged at 60,000 *g* for 1.5 h. The total membrane homogenization, dilution and centrifugation procedures were performed twice, then the pellets were collected, rapidly rinsed with a buffer solution (50 mM Tris-HCl, 120 mM NaCl, 5

mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 2 mM PMSF, pH 7), and resuspended in the same buffer containing a mixture of 20 μg/mL of each of the following protease inhibitors: leupeptin, bestatin, pepstatin A, and aprotinin. K_i values were calculated from the competition curves with the LIGAND software.[28]

[^{125}I]α-Bungarotoxin binding. The saturation binding experiments were performed using aliquots of cortex membrane homogenates incubated overnight with 0.1-10 nM concentrations of [^{125}I]α-bungarotoxin (specific activity 200–213 Ci/mmol, GE Healthcare) at room temperature. Nonspecific binding was determined in parallel by means of incubation in the presence of 1 μM unlabelled α-bungarotoxin. After incubation, the samples were filtered as described above and the bound radioactivity was directly counted in a γ counter.

[^{3}H]Epibatidine binding. (±)-[^{3}H]Epibatidine with a specific activity of 56–60 Ci/mmol was purchased from Perkin–Elmer (Boston MA); the non radioactive α-bungarotoxin and epibatidine were purchased from Sigma. It has been previously reported that [^{3}H]epibatidine also binds to α-bungarotoxin binding receptors with nM affinity.[29] To prevent the binding of [^{3}H]epibatidine to the α-bungarotoxin binding receptors, the membrane homogenates were pre-incubated with 2 μM α-bungarotoxin and then with [^{3}H]epibatidine. The saturation experiments were performed by incubating aliquots of cortex membrane homogenates with 0.01-2.5 nM concentrations of (±)-[^{3}H]epibatidine overnight at 4 °C. Nonspecific binding was determined in parallel by means of incubation in the presence of 100 nM unlabelled epibatidine. At the end of the incubation, the samples were filtered on a GFC filter soaked in 0.5% polyethylenimine and washed with 15 mL of a buffer solution (10 mM Na_3PO_4, 50 mM NaCl, pH 7.4), and the filters were counted in a β counter.

Author Contribution Statement

LR and CM designed and synthesized the new compounds; CG performed receptor binding assays; MDA and CD supervised the project; CM and MDA wrote the manuscript with contributions from all authors.

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