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# Pharmacological profile of zacopride and new quaternarized fluorobenzamide analogues on mammalian $\alpha 7$ nicotinic acetylcholine receptor

Céline M. Bourdin <sup>a,b</sup>, Jacques Lebreton <sup>c</sup>, Monique Mathé-Allainmat <sup>c</sup>, Steeve H. Thany <sup>a,\*</sup>

<sup>a</sup> Université d'Orléans, Laboratoire de Biologie des Ligneux et des Grandes Cultures (LBLGC) UPRES EA 1207, Rue de Chartres, BP 6759, 45067 Orléans, France

<sup>b</sup> Université d'Angers, RCIM UPRES EA 2647 US INRA 1330, 2 rue Lavoisier, 49045 Angers, France

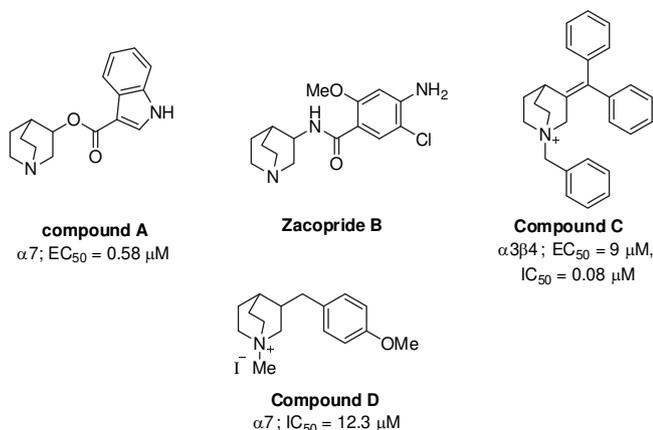
<sup>c</sup> Université de Nantes, CEISAM UMR CNRS 6230, UFR des Sciences et des Techniques, 2 rue de la Houssinière, BP 92208, Nantes 44322, France

From quaternarization of quinuclidine enantiomers of 2-fluoro benzamide LMA10203 in dichloro-methane, the corresponding *N*-chloromethyl derivatives LMA10227 and LMA10228 were obtained. Here, we compared the agonist action of known zacopride and its 2-fluoro benzamide analogues, LMA10203, LMA10227 and LMA10228 against mammalian homomeric  $\alpha 7$  nicotinic acetylcholine receptor expressed in *Xenopus* oocytes. We found that LMA10203 was a partial agonist of  $\alpha 7$  receptor with a  $pEC_{50}$  value of  $4.25 \pm 0.06 \mu M$  whereas LMA10227 and LMA10228 were poorly active on  $\alpha 7$  homomeric nicotinic receptor. LMA10227 and LMA10228 were identified as antagonists of acetylcholine-induced currents with  $IC_{50}$  values of  $28.4 \mu M$  and  $39.3 \mu M$  whereas LMA10203 and zacopride possessed  $IC_{50}$  values of  $8.07 \mu M$  and  $7.04 \mu M$ , respectively. Moreover, despite their  $IC_{50}$  values, LMA10227 was the most potent inhibitor of nicotine-induced current amplitudes ( $65.7 \pm 2.1\%$  inhibition). LMA10203 and LMA10228 had the same inhibitory effects ( $26.5 \pm 7.5\%$  and  $33.2 \pm 4.1\%$ , respectively), whereas zacopride had no significant inhibitory effect ( $4.37 \pm 4\%$ ) on nicotine-induced responses. Our results revealed different pharmacological properties between the four compounds on acetylcholine and nicotine currents. The mode of action of benzamide compounds may need to be reinterpreted with respect to the potential role of  $\alpha 7$  receptor.

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate fast excitatory synaptic transmission in central and peripheral nervous system of mammals.<sup>1</sup> They can be homomeric receptors composed of one subunit like the homomeric  $\alpha 7$  receptor<sup>2</sup> which was proposed as a therapeutic targets for several pathologies such as Alzheimer's and Parkinson's diseases, neuroinflammation and schizophrenia.<sup>3-8</sup> Several studies demonstrated that quinuclidine compounds could show dual affinity at both serotonergic (5-HT) and nicotinic receptors, due to significant sequence homology between both receptor types which is up to 30%.<sup>9</sup> Because of their simple synthetic access, families of ester or amide derivatives of 3-substituted quinuclidine were reported in the literature as potent ligands of ion channel receptors. Some of them were identified as  $\alpha 7$  nAChR ligands with nanomolar affinity or activity.<sup>10-12</sup> As an example, the quinuclidine

ester analogue **A** (Fig. 1) of the well known 5-HT<sub>3</sub> antagonist tropisetron, was identified as a potent and selective partial agonist of  $\alpha 7$  nAChR (Tropisetron:  $EC_{50} = 1.3 \mu M$  and compound **A**:  $EC_{50} = 0.58 \mu M$ ).<sup>13</sup> To our knowledge, no similar data were published in the literature with the structurally close zacopride **B** also known as a potent 5-HT<sub>3</sub> antagonist.<sup>14</sup> Even, if they are not members of these ester or amide families, we could also notice that quaternarized quinuclidine derivatives such as arylidene compound **C** was revealed as  $\alpha 3\beta 4$  nAChR antagonist<sup>15</sup> and that the methiodide of 2-benzyl quinuclidine compound **D** (Fig. 1) also displayed antagonist profile on  $\alpha 7$  nAChR.<sup>16</sup> Furthermore, simple quinuclidine benzamide such as the enantiomerically pure 4-fluoro benzamide **E** (Fig. 2) was characterized as a potent  $\alpha 7$  nAChR agonist ( $EC_{50} = 0.56 \mu M$ ) and then selected as a suitable [<sup>18</sup>F]PET tracer for in vivo  $\alpha 7$  nAChR imaging.<sup>17</sup> We also recently demonstrated that the 2-fluoro analogue compound LMA10203 (Fig. 2) acted as a partial agonist of insect nicotinic acetylcholine receptors with a specific affinity for  $\alpha$ -bungarotoxin-insensitive nAChR2 expressed in the insect neurosecretory cells.<sup>18</sup>

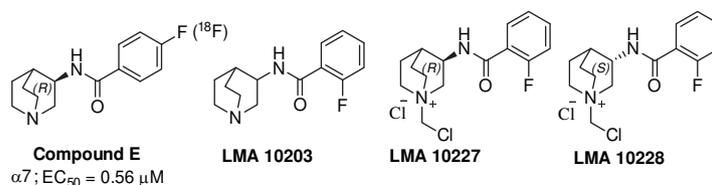
\* Corresponding author. Tel.: +33 (0) 238417058; fax: +33 (0) 238494089.  
E-mail address: steeve.thany@univ-orleans.fr (S.H. Thany).



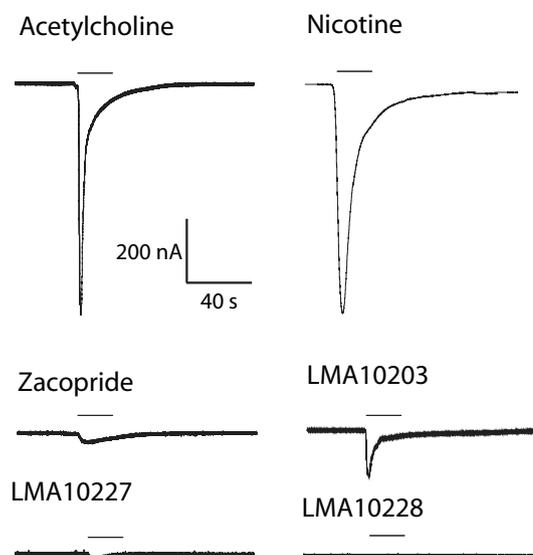
**Figure 1.** Some quinuclidine derivatives targeting nicotinic acetylcholine receptors.

In this work, we synthesized two analogues of LMA10203, the (*R*)-*N*-(chloromethyl) quinuclidinium compound LMA10227 and the (*S*)-enantiomer LMA10228 (Fig. 2) and studied their pharmacological profiles on the mammalian homomeric  $\alpha_7$  nicotinic acetylcholine receptor. The novel enantiomerically pure compounds LMA10227 and LMA10228 were prepared, respectively, from commercial (*R*)- and (*S*)-3-aminoquinuclidine using a two steps reaction sequence. The first step was acylation of the corresponding 3-aminoquinuclidine with 2-fluorobenzoyl chloride and the second step was the simple quaternarization of the purified benzamide in dichloromethane used as the solvent (Fig. 1).<sup>19</sup> There effects were compared to zacopride<sup>20</sup> and LMA10203<sup>18</sup> earlier prepared, and using oocyte two-electrode voltage-clamp electrophysiology.<sup>21</sup>

As illustrated in Figure 3, compared to ACh- and nicotine-induced current amplitudes, zacopride has a very weak effect whereas LMA10203 is a partial agonist of  $\alpha_7$  receptors which was in accordance with the previous studies showing that it was a partial agonist of insect nAChR subtypes.<sup>18</sup> No currents were detected in all control oocytes tested (oocytes injected with saline solution). The maximum currents of LMA10203, zacopride and LMA10227 responses at 1 mM were  $0.19 \pm 0.01 \mu\text{A}$  ( $n = 8$ ),  $0.038 \pm 0.002 \mu\text{A}$  ( $n = 7$ ) and  $0.021 \pm 0.004 \mu\text{A}$  ( $n = 8$ ) whereas LMA10227 and LMA10228 ( $n = 8$ ) were ineffective in activating the  $\alpha_7$  nicotinic acetylcholine receptor (Table 1). The concentration-response curves allowed us to determine the pEC<sub>50</sub> and the Hill's coefficient values for each compound (Fig. 4). For ACh and nicotine, the pEC<sub>50</sub> were  $3.68 \pm 0.06$  and  $3.96 \pm 0.07$ , corresponding to the EC<sub>50</sub> of 205.3  $\mu$ M and 108.7  $\mu$ M, respectively (Table 1). The pEC<sub>50</sub> value obtained for LMA10203 ( $4.25 \pm 0.06$  corresponding to an EC<sub>50</sub> at 56.5  $\mu$ M, Table 1) was significantly different to those determined for ACh (*t* test,  $p < 0.0001$ ) and nicotine (*t* test,  $p = 0.01$ ). Unfortunately, very small currents were recorded following the application of zacopride and LMA10227 and it was not possible



**Figure 2.** Fluorinated quinuclidine benzamides and quaternarized analogues LMA10227 and LMA10228.



**Figure 3.** Comparisons of the current responses to acetylcholine and nicotine with those of LMA10203, LMA10227, LMA10228 and zacopride on  $\alpha_7$  nAChRs expressed on *Xenopus* oocytes, at concentration inducing the maximum response (1 mM). Each bar indicates when the compound is added.

**Table 1**

$I_{\text{max}}$ , pEC<sub>50</sub> and Hill coefficient values<sup>a</sup> for acetylcholine, nicotine, LMA10203, LMA10227, LMA10228 and zacopride on  $\alpha_7$  nicotinic acetylcholine receptor

	$I_{\text{max}}$ <sup>b</sup> ( $\mu\text{A}$ )	pEC <sub>50</sub>	Hill
Acetylcholine	$0.97 \pm 0.05$	$3.68 \pm 0.06$	$1.75 \pm 0.29$
Nicotine	$0.93 \pm 0.06$	$3.96 \pm 0.07$	$1.99 \pm 0.95$
LMA10203	$0.19 \pm 0.01$	$4.25 \pm 0.06$	$2.77 \pm 1.14$
LMA10227	$0.021 \pm 0.004$	ND <sup>c</sup>	ND <sup>c</sup>
LMA10228	ND <sup>c</sup>	ND <sup>c</sup>	ND <sup>c</sup>
Zacopride	$0.038 \pm 0.002$	ND <sup>c</sup>	ND <sup>c</sup>

Each line represents a mean  $\pm$  SEM of at least eight oocytes.

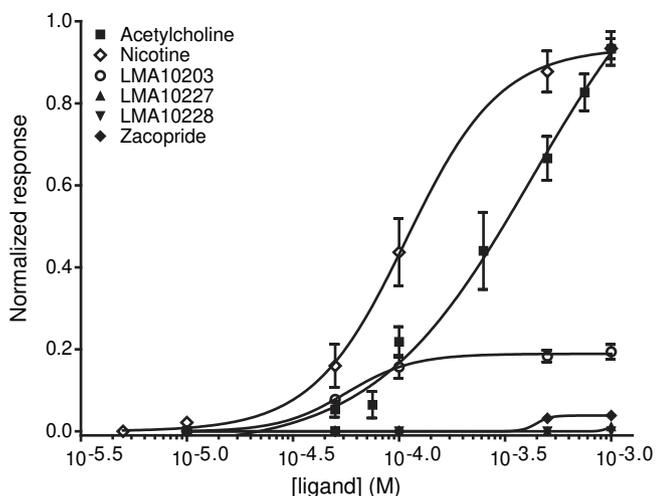
<sup>a</sup> The values shown were determined using the concentration-response curves illustrated in Figure 4.

<sup>b</sup> The maximum currents ( $I_{\text{max}}$ ) were shown as the ratio of the maximum response to each compound to that induced by 1 mM ACh.

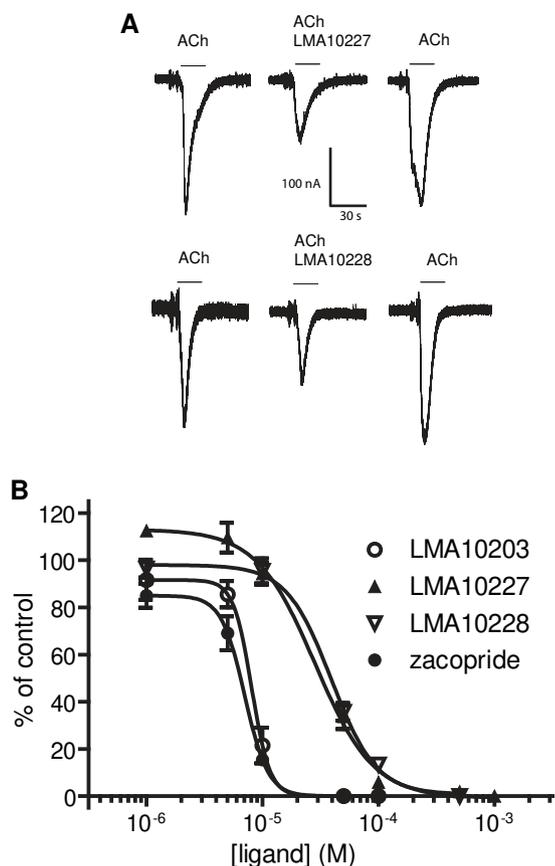
<sup>c</sup> ND: not determined.

to determine their pEC<sub>50</sub> as for LMA10228. Nevertheless, we proposed that LMA10203 was a partial agonist of  $\alpha_7$  receptors.

The current studies showed that exposure to LMA10227 and LMA10228 resulted in no activation of  $\alpha_7$  receptors, which may suggest that they can have a potential effect as inhibitors and/or in the desensitization of ACh currents. We determined the inhibitory curves by co-applying ACh (EC<sub>50</sub>: 205  $\mu$ M) with each compound. Each oocyte received two initial applications of ACh to establish an internal control, then after 10 min, a co-application of ACh and zacopride, LMA10203, LMA10227 or LMA10228. As shown, the amplitude of control ACh responses was comparable both before and after the co-application (Fig. 5A) but significantly



**Figure 4.** Concentration-response relationships for acetylcholine (ACh), nicotine, LMA10203, LMA10227, LMA10228 and zacopride. Data are peak current responses calculated relative to control ACh responses obtained from the same cells and subsequently normalized to the maximum ACh responses. Control ACh concentration was 500  $\mu$ M for all experiments. Each point plotted in the concentration-response curves represents mean  $\pm$  SEM of at least seven oocytes.



**Figure 5.** Effects of zacopride, LMA10203, LMA10227 and LMA10228 on the amplitude of ACh responses. An example of oocyte responses expressing rat  $\alpha$ 7 nAChR to the application of 205  $\mu$ M ( $EC_{50}$ ) ACh before and after co-application with 50  $\mu$ M LMA10227 and 50  $\mu$ M LMA10228. Each bar indicates when the compound is added. (B) Inhibitory curves illustrating the effects of zacopride, LMA10203, LMA10227 and LMA10228 on ACh responses. Responses for  $\alpha$ 7 nAChR are based on peak current amplitudes, calculated relative to ACh control responses obtained from the same cells. Data are plotted as mean  $\pm$  SEM from at least six oocytes.

decreased during co-application with zacopride, LMA10203, LMA10227 and LMA10228 (Fig. 5B). We first observed that LMA10203 and zacopride were the most effective inhibitors with the following  $pIC_{50}$  values,  $5.09 \pm 0.04$  ( $n = 5$ ) and  $5.15 \pm 0.02$  ( $n = 5$ ), corresponding to  $IC_{50}$  of 8.07  $\mu$ M and 7.04  $\mu$ M, respectively, whereas LMA10227 and LMA10228 were less effective ( $pIC_{50}$  values:  $4.55 \pm 0.04$  corresponding to an  $IC_{50}$  of 28.4  $\mu$ M ( $n = 5$ ) and  $4.41 \pm 0.04$  corresponding to an  $IC_{50}$  of 39.3  $\mu$ M ( $n = 5$ ), respectively (Table 2)). Interestingly, when we co-applied nicotine ( $EC_{50}$ : 108  $\mu$ M) and each compound at their  $IC_{50}$  values obtained with ACh (Table 2), we observed that they differently acted on nicotine-induced responses (Fig. 6). LMA10227 had a better and significant inhibitory effect on nicotine-induced responses ( $65.7 \pm 2.1\%$ ,  $t$  test,  $p < 0.001$ ,  $n = 6$ , Fig. 6A and B) whereas LMA10203 and LMA10228 showed a lower effect ( $26.5 \pm 7.5\%$  and  $33.2 \pm 4.1\%$ ,  $p < 0.05$ ,  $n = 6$ , respectively) compared to zacopride which was not effective ( $4.37 \pm 4\%$ ,  $t$  test,  $p > 0.05$ ,  $n = 6$ , Fig. 6B). Thus, our results suggested that the (*R*)-benzamide LMA10227 was more potent than its (*S*)-enantiomer on nicotine responses ( $t$  test,  $p < 0.05$ ,  $n = 6$ , Fig. 6B).

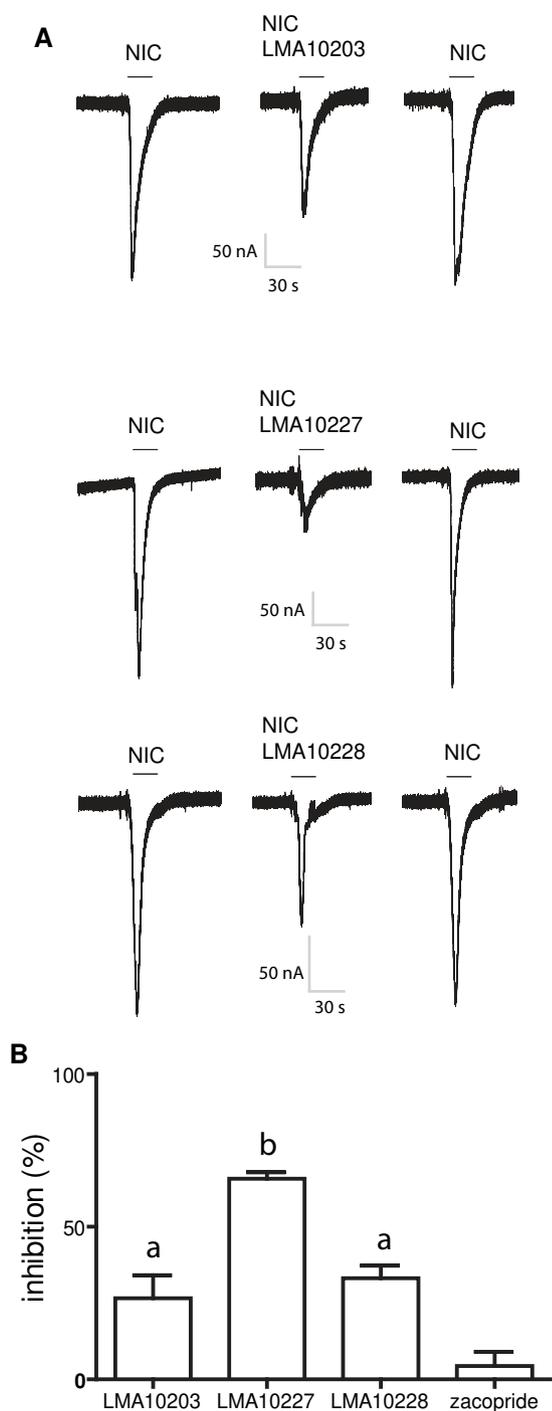
In conclusion, our data suggested that LMA10227 and LMA10228 cannot activate  $\alpha$ 7 nAChR compared to LMA10203. However, they had all an inhibitory effect on ACh-induced responses. Interestingly, these results were modified with nicotine and the (*R*)-enantiomer LMA10227 showed the best inhibitory effect on nicotine responses, compared to LMA10203 and LMA10228. Similar results were earlier published on using non quaternized quinuclidine benzamide compounds, toward  $\alpha$ 7-5-HT<sub>3</sub> chimera.<sup>11</sup> They were found to have agonist activities and the (*R*)-enantiomers were more active than the corresponding (*S*)-enantiomers.<sup>11</sup> It was suggested that on the benzamide portion, small substituents in the para position gave the most active analogues.<sup>11</sup> In the present studies, we demonstrated that the quaternized fluorobenzamide analogues acted as  $\alpha$ 7 nAChRs antagonists. Despite that several data are needed to identify their binding sites on  $\alpha$ 7 nAChRs and also their mode of action on other nAChRs such as  $\alpha$ 4 $\beta$ 2, we hypothesize that (1) nicotine is a poor competitor against LMA10227. Thus, the affinity of LMA10228 for  $\alpha$ 7 nAChR sites is lower than for LMA10227. (2) LMA10227 and LMA10228 could act differently as allosteric inhibitors, a property allowing them to modulate the  $\alpha$ 7 receptors at various concentrations of ACh or nicotine. In addition, the discrepancy between LMA10203, LMA10227 and LMA10228 on  $\alpha$ 7 nAChRs suggested that they have different *in vivo* effects. We propose that LMA10227 could be used as an available tool to pharmacologically characterized nAChRs activated with exogenous drugs such as nicotine. Our future work will address this issue and how these quaternized analogues will activate different mammalian nAChR subtypes. In final, we also demonstrate for the first time that zacopride, known as a gastrointestinal prokinetic drug<sup>22</sup> and a specific antagonist of 5-HT<sub>3</sub> receptors,<sup>23</sup> can induce an inhibitory effect on ACh-induced currents.

**Table 2**  
Inhibitory effect of LMA10203, LMA10227, LMA10228 and zacopride on ACh-induced currents

	$pIC_{50}$	Hill
LMA10203	$5.09 \pm 0.04$	$5.53 \pm 2.01$
LMA10227	$4.55 \pm 0.05$	$1.72 \pm 0.22$
LMA10228	$4.41 \pm 0.04$	$2.24 \pm 0.53$
Zacopride	$5.15 \pm 0.02$	$4.30 \pm 0.58$

Each line represents a mean  $\pm$  SEM of at least eight oocytes.

The values shown were determined using the inhibitory curves presented in Figure 5.



**Figure 6.** The effects of LMA10203, LMA10227, LMA10228 and zacopride on nicotine-induced current amplitudes. (A) An example of nicotine currents before and after co-application with LMA10203, LMA10227 and LMA10228. Nicotine was tested at  $EC_{50}$  (108.7  $\mu$ M). Each bar indicates when the compound is added. (B) Histogram illustrating the nicotine inhibition. Responses for  $\alpha 7$  nAChR are based on peak current amplitudes, calculated relative to nicotine control responses obtained from the same cells. Data are plotted as mean  $\pm$  SEM from at least six oocytes. 'a' and 'b' indicate significant inhibition ( $p < 0.05$  and  $p < 0.001$ , respectively) of nicotine current amplitudes when co-applied with LMA10203, LMA10227 and LMA10228 and zacopride.

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Grant. Rat  $\alpha 7$  nAChR and RIC-3 clones used for expression in *Xenopus* oocytes are kindly provided by Pr. Roger L. Papke and Dr. Clare Stokes (Department of Pharmacology and Therapeutics, College of Medicine, University of Florida, Gainesville, Florida, USA) and Prof. Millet Treinin (School of Medicine-IMRIC-Medical Neurobiology, The Hebrew University of Jerusalem, Israel).

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- General procedure for quinuclidine derivatives quaternization:** The dihydrochloride salt of (R)- or (S)-3-aminoquinuclidine (0.25 g, 1.25 mmol) was dissolved in dry chloroform (5 mL) under argon in the presence of  $Et_3N$  (0.4 mL, 3 mmol) and pyridine (1 mL) during 30 min. The 2-fluorobenzoyl chloride (0.15 mL, 1.25 mmol) was then added dropwise at 0 °C under argon. The reaction mixture was stirred at room temperature during 18 h and quenched with 2.5 N solution of NaOH. The aqueous phase was extracted twice with chloroform and the organic layer was dried over anhydrous  $MgSO_4$ , filtered and evaporated under reduced pressure. The residue was purified by flash chromatography on neutral alumina column (1–5% MeOH in  $CHCl_3$ ) to give 0.15 g (51%) of the expected neutral quinuclidine benzamide as a white powder:  $[\alpha]_D^{20} +24$  (c 0.25 g/100 mL, MeOH);  $^1H$  NMR (300 MHz,  $CDCl_3$ ):  $\delta$  = 8.08 (td, 1H,  $J$  = 7.92 and 1.87 Hz,  $H_{ar}$ ), 7.47 (m, 1H,  $H_{ar}$ ), 7.26 (td, 1H,  $J$  = 8.77 and 1.06 Hz,  $H_{ar}$ ), 7.12 (ddd, 1H,  $J$  = 8.27 and 0.9 Hz,  $H_{ar}$ ), 6.98 (bm, 1H, NH), 4.21 (m, 1H, CH), 3.46 (dd, 1H,  $J$  = 14.2 and 9.9,  $CHH'$ ), 2.88 (m, 4H,  $2 \times CH_2$ ), 2.69 (m, 1H,  $CHH'$ ), 2.08 (m, 1H, CH), 1.74 (m, 3H,  $CH_2$  and  $CHH'$ ), 1.56 (m, 1H,  $CHH'$ ) ppm. HRMS (MALDI-DHB-PEG200):  $C_{14}H_{18}FN_2O$   $[M+H]^+$   $m/z$ , calcd 249.1398, found 249.1398.  
The (R)-benzamide (0.05 g, 0.2 mmol) was dissolved in dry dichloromethane and standing 48 h at room temperature, a white precipitate appeared which was filtered to give 0.053 g (80%) of the expected chloromethylated benzamide LMA10227, as a white powder.  $^1H$  NMR (300 MHz,  $DMSO-d_6$ ):  $\delta$  = 9.01 (d, 1H,  $J$  = 5.7 Hz, NH), 7.63 (td, 1H,  $J$  = 7.40 and 1.67 Hz,  $H_{ar}$ ), 7.55 (m, 1H,  $H_{ar}$ ), 7.30 (m, 2H,  $2 \times H_{ar}$ ), 5.42 (s, 2H,  $CH_2Cl$ ), 4.42 (m, 1H, CH), 4.00 (ddd, 1H,  $J$  = 9.6 and 2.56 Hz, CH), 3.56 (m, 5H,  $2 \times CH_2$  and  $CHH'$ ), 2.27 (m, 1H, CH), 2.2 (m, 1H,  $CHH'$ ), 2.05 (m, 2H,  $CH_2$ ), 1.90 (m, 1H,  $CHH'$ ) ppm;  $^{13}C$  NMR (75 MHz,  $CD_3OD$ ):  $\delta$  = 167.68 (CO), 161 (d,  $J_{CF}$  = 248 Hz,  $C_{ivar}$ ), 134.44 (d,  $J_{CF}$  = 8 Hz,  $C_{ar}$ ), 131.32 ( $C_{ar}$ ), 125.74 ( $C_{ar}$ ), 124.20 (d,  $J_{CF}$  = 14.25 Hz,  $C_{ivar}$ ), 117.3 (d,  $J_{CF}$  = 22.5 Hz,  $C_{ar}$ ), 69.42 ( $CH_2Cl$ ), 60.17 ( $CH_2$ ), 54.92 ( $CH_2$ ), 54.66 ( $CH_2$ ), 47.23 (CH), 26.08 (CH), 23.37 ( $CH_2$ ), 19.61 ( $CH_2$ ) ppm. HRMS (MALDI, DHB-PEG200):  $C_{15}H_{19}FN_2O$   $[M]^+$   $m/z$ , calcd 297.1164, found 297.1169.  
The (S)-benzamide (0.05 g, 0.2 mmol) was dissolved in dry dichloromethane and after 48 h at room temperature, a white precipitate appeared which was filtered to give 0.053 g (80%) of the expected chloromethylated benzamide LMA10228, as a white powder.  $^1H$  NMR (300 MHz,  $CD_3OD$ ):  $\delta$  = 7.69 (td, 1H,  $J$  = 7.46 and 1.76 Hz,  $H_{ar}$ ), 7.55 (m, 1H,  $H_{ar}$ ), 7.29 (td, 1H,  $J$  = 7.57 and 1.02 Hz,  $H_{ar}$ ), 7.24 (ddd, 1H,  $J$  = 8.4 and 0.87 Hz,  $H_{ar}$ ), 5.23 (s, 2H,  $CH_2Cl$ ), 4.56 (m, 1H, CH), 4.10 (ddd, 1H,  $J$  = 9.6 and 2.66 Hz,  $CHH'$ ), 3.66 (m, 4H,  $2 \times CH_2$ ), 3.52 (m, 1H,  $CHH'$ ), 2.45 (m, 1H, CH), 2.33 (m, 1H,  $CHH'$ ), 2.21 (m, 2H,  $CH_2$ ), 2.08 (m, 1H,

CHH') ppm;  $^{13}\text{C}$  NMR (75 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  = 167.69 (CO), 161.13 (d,  $J_{\text{CF}}$  = 249 Hz,  $\text{C}_{\text{IVar}}$ ), 134.4 (d,  $J_{\text{CF}}$  = 9 Hz,  $\text{C}_{\text{ar}}$ ), 131.32 ( $\text{C}_{\text{ar}}$ ), 125.73 ( $\text{C}_{\text{ar}}$ ), 124.22 (d,  $J_{\text{CF}}$  = 13.5 Hz,  $\text{C}_{\text{IVar}}$ ), 117.31 (d,  $J_{\text{CF}}$  = 22.5 Hz,  $\text{C}_{\text{ar}}$ ), 69.42 ( $\text{CH}_2\text{Cl}$ ), 60.20 ( $\text{CH}_2$ ), 54.92 ( $\text{CH}_2$ ), 54.66 ( $\text{CH}_2$ ), 47.24 (CH), 26.08 (CH), 23.37 ( $\text{CH}_2$ ), 19.61 ( $\text{CH}_2$ ) ppm. HRMS (MALDI, DHB-PEG200):  $\text{C}_{15}\text{H}_{19}\text{ClF}_2\text{O}$  [ $\text{M}^+$ ]  $m/z$ , calcd 297.1164, found 297.1158.

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21. Recombinant plasmid containing rat  $\alpha 7$  nAChR and RIC-3 were linearized with *Sma*I (New England Biolabs, Ipswich, USA) or *Nhe*I (Promega, Madison, USA), respectively, and capped RNA were transcribed in vitro using the T7 mMESSAGE mMACHINE kit (Ambion, Austin, USA). Ovarian lobes were surgically harvested from adult *Xenopus laevis*. Briefly, *X. laevis* female was anesthetized in tricaine (0.15 M, Sigma-Aldrich) and washed in a standard oocyte saline (SOS) solution composed of (in mM): NaCl 100, KCl 2,  $\text{CaCl}_2$  1.8, Hepes 5, pH 7.5. Defolliculated oocytes were obtained after 1 h treatment at room temperature with 2 mg/ml collagenase (type 1A, Sigma-Aldrich, Saint Quentin Fallavier, France) and 0.8 mg/ml trypsin inhibitor (Sigma-Aldrich) in calcium-free SOS. *Xenopus* oocytes were injected with 27 nl of 6 ng nAChR  $\alpha 7$  subunit RNA and 3 ng RIC-3 RNA. Injected oocytes were incubated in sterile medium composed of SOS supplemented with gentamycin (50  $\mu\text{g}/\text{ml}$ ),

penicillin (100 U/ml), streptomycin (100  $\mu\text{g}/\text{ml}$ ) and sodium pyruvate (2.5 mM) at 21 °C. Recordings were performed 4-7 days after injection. Injected oocytes with 27 nl of water were used as control. Experiments were carried out using the two electrode voltage-clamp (TEVC) techniques. Oocytes were tested using 1 M-KCl/2 M-Acetate-filled borosilicate glass electrodes connected to a TEVC amplifier (GeneClamp 500, Axon Instruments, Foster City, USA). Digidata 1320A interface (Axon Instruments) and pCLAMP 8 software (Axon Instruments) were used for acquisition (sampling rate: 100 kHz; band pass-filter: 500 Hz). Oocytes were voltage-clamped at -60 mV. The oocytes were bath-perfused with SOS solution at 2 ml/min. Baseline conditions were evaluated by two initial applications of ACh performed before application of putative partial agonists. For concentration-response experiments, ACh, nicotine and others drugs were applied during 3-4 s followed by 4 min washout. For inhibition experiments, a 10 min pre-incubation of drug was performed before application of both ACh or nicotine combined with other drugs. In all cases, significant results between experiments were determined using the unpaired Student's *t*-test.

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