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*N*3,*N*7-diaminophenothiazinium derivatives as antagonists of α7-nicotinic acetylcholine receptors expressed in *Xenopus* oocytes☆

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ABSTRACT

Derivatization of phenothiazine (PTZ, **1**) has been a commonly used method to develop drugs with various pharmacological properties. In the present study, a series of PTZ derivatives **1**–**11** were investigated on the inhibition of the cloned α7 subunit of the human nicotinic acetylcholine receptor (α7-nAChR) expressed in *Xenopus* oocytes by using the two-electrode voltage-clamp technique. In the first series of experiments, the effect of unsubstituted phenothiazine **1** on α7-*n*AChRs was compared with that of the *N*3,*N*7-diaminophenothiazin-5-ium derivative **2**, and of sequentially methylated derivatives **3**–**6**. In the second set of experiments, the effects of *N*3,*N*7-tetra-ethyl- to n-hexylphenothiazin-5-ium derivatives **7**–**11** were tested. Despite the lack of activity found for **1**, a reversible inhibition of α7-*n*AChRs, ranging from moderate to potent, was observed as a result of a sequential amine- and methylamine substitution of **1**. The inhibition of ACh (100 μM)-induced currents was concentration-dependent with IC_{50} values ranging from 0.4 to 16.8 μM. However, an optimal inhibitory activity was achieved by prolongation of alkyl chains up to propyl size, as found in PTZ derivative **8**, whereas further lengthening of alkyl chains to *n*-butyl-, *n*-pentyl-, or *n*-hexyl groups resulted in inactive derivatives **9**–**11**. The results evidently suggest the presence of a lipophilic binding pocket of narrow tolerability on the receptor protein. These results emphasize the importance of amine and/or alkylamine moieties for the inhibitory effect of PTZ derivatives and provide further insights for the development of novel antagonists targeting α7-*n*AChRs.

1. Introduction

Since their introduction into medicine during the late 19th century, phenothiazines (PTZs) have been widely used for various clinical applications, ranging from the treatment of nausea and vomiting to the management of anxiety and psychosis [1,2]. The PTZ ring constitutes the building block for many clinically used drugs, such as antipsychotics, antiemetics, antibiotics and antihistamines [1,3,4]. PTZs have been used primarily for the treatment of psychotic disorders and are, in fact, among the most widely prescribed antipsychotic drugs [1,2]. Recently, a PTZ-class dye, methylene blue, has attracted much attention due to its possible therapeutic actions in Alzheimer disease (AD) and psychosis [5,6]. Several lines of evidence indicate that PTZs interact with mostly uncharacterized site(s) located at ligand-gated ion channels, and it seems likely that the modulation of these receptors is, at least partly, responsible for their therapeutic and/or side effect profiles [7–9]. The effects of PTZs on the functional properties of muscle type and neuronal nicotinic acetylcholine receptors (*n*AChRs) have been reported in several earlier studies [8–10]. Recent studies have suggested that the α7-*n*AChR, a major neuronal nicotinic receptor subtype, plays an important role in cognitive dysfunction, neurodegenerative diseases, epilepsy, and smoking cessation [11,12]. Moreover, α7-*n*AChR-selective antagonists have been explored as potential treatment for non-small cell lung cancer, organophosphorus nerve agent intoxication and smoking cessation [11,13,14]. Evidently, there is a need to further elucidate the pharmacological functions of α7-*n*AChR antagonists on this receptor in the brain and explore potential clinical uses. In our earlier studies, we have demonstrated for the first time that PTZ-class antipsychotic drugs inhibit the function of human α7-*n*AChRs [10]. In the present study, effects of major PTZ-class dyes **2**–**6** (Table 1) and their alkyl-amino

Abbreviations: ACh, acetylcholine; BAPTA, 1,2-bis(*o*-aminophenoxy) ethane-*N*,*N*,*N*′,*N*v-tetraacetic acid; HEPES, 4-(2-hydroxyethyl) piperazineethanesulfonic acid; LGIC, ligand-gated ion channel; *n*AChR, nicotinic acetylcholine receptor.

☆ Perspective articles contain the personal views of the authors who, as experts, reflect on the direction of future research in their field.

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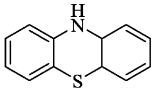
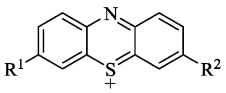
Table 1
Observed inhibitory potencies of PTZ derivatives **1**–**11**.



1
10*H*-Phenothiazine



2-11
N3-N7-Diaminophenothiazin-5-ium derivatives

Compound	R^1	R^2	$IC_{50} \pm SEM$ [μM]
1 10H-phenothiazine	–	–	NE[a]
2 3,7-diaminophenothiazin-5-ium (Thionine)	NH_2	NH_2	16.6 ± 1.8
3 *N3*-methyl-3,7-diaminophenothiazin-5-ium (Azure C)	$NHCH_3$	NH_2	11.7 ± 1.9
4 *N3*,*N3*-dimethyl-3,7-diaminophenothiazin-5-ium (Azure A)	$N(CH_3)_2$	NH_2	6.7 ± 1.2
5 *N3*,*N3*,*N7*-trimethyl-3,7-diaminophenothiazin-5-ium (Azure B)	$N(CH_3)_2$	$NHCH_3$	4.8 ± 1.4
6 *N3*,*N3*,*N7*,*N7*-tetramethyl-3,7-diaminophenothiazin-5-ium (Methylene blue)	$N(CH_3)_2$	$N(CH_3)_2$	3.3 ± 1.2
7 *N3*,*N3*,*N7*,*N7*-tetraethyl-3,7-diaminophenothiazin-5-ium	$N(C_2H_5)_2$	$N(C_2H_5)_2$	0.7 ± 0.1
8 *N3*,*N3*,*N7*,*N7*-tetra-*n*-propyl-3,7-diaminophenothiazin-5-ium	$N(C_3H_7)_2$	$N(C_3H_7)_2$	0.4 ± 0.1
9 *N3*,*N3*,*N7*,*N7*-tetra-*n*-butyl-3,7-diaminophenothiazin-5-ium	$N(C_4H_9)_2$	$N(C_4H_9)_2$	NE[b]
10 *N3*,*N3*,*N7*,*N7*-tetra-*n*-pentyl-3,7-diaminophenothiazin-5-ium	$N(C_5H_{11})_2$	$N(C_5H_{11})_2$	NE[b]
11 *N3*,*N3*,*N7*,*N7*-tetra-*n*-hexyl-3,7-diaminophenothiazin-5-ium	$N(C_6H_{13})_2$	$N(C_6H_{13})_2$	NE[b]

[a] NE, no inhibitory effect up to 30 μM concentration of the compound tested.
[b] No inhibitory effect up to 3 μM concentration; above 3 μM was toxic to cells.

group-substituted derivatives **7**–**11** were investigated on the function of human α7-*n*AChRs expressed in *Xenopus* oocytes.

2. Materials and methods

2.1. Chemistry

All chemicals used in preparing the solutions were purchased from Sigma–Aldrich (St. Louis, MO). Phenothiazine derivatives **1**–**6**, ACh and α-bungarotoxin were obtained from Sigma (St. Louis, MO). Compounds **7**–**11** were synthesized in the laboratory of Dr. M. Wainwright according to previously published methods [15–17]. Procedures for the injections of BAPTA (50–100 nl, 100 mM) were performed as described previously [18]. BAPTA was prepared in Cs_4-BAPTA and injections were performed 1 h prior to recordings using an oil-driven ultra microsyringe pump (Micro4, WPI, Inc., Sarasota, FL). Stock solutions of compounds used in this study were prepared in distilled water at a concentration of 10 mM.

2.2. Pharmacology

The procedures followed in this study were approved by the Institutional Review Board (FMHS Animal Research Ethics Committee) of the UAEU. Mature female *Xenopus laevis* frogs were supplied by Xenopus Express (Haute-Loire, France) and were housed in a water tank at 19–21 °C with a 12/12-h light/dark cycle and fed with food pellets. Oocytes were removed surgically under local anesthesia (Tricaine, Sigma, St. Louis, MO; 0.15%, w/v), and dissected manually in a solution containing (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $MgSO_4$, 0.8; HEPES, 10 (pH 7.5). Oocytes were stored two to seven days in modified Barth's solution (MBS) containing (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $Ca(NO_3)_2$, 0.3; $CaCl_2$, 0.9; $MgSO_4$, 0.8; HEPES, 10 (pH 7.5), supplemented with sodium pyruvate, 2 mM, penicillin 10,000 IU/L, streptomycin, 10 mg/L, gentamicin, 50 mg/L, and theophylline, 0.5 mM. Briefly, oocytes were placed in a 0.2 ml recording chamber and superfused at a rate of 2–3 ml/min. The bathing solution consisted of (in mM): NaCl, 95; KCl, 2; $CaCl_2$, 2; and HEPES 5 (pH 7.5). Using two glass microelectrodes filled with 3 M KCl (1–10 MΩ), cells were voltage clamped at a holding potential of −70 mV. Currents were recorded using GeneClamp-500 amplifier (Axon Instruments Inc., Burlingame, CA) and data acquisition software, Clampex 8.1 (Axon Instruments Inc.). Test compounds were applied by gravity flow via a micropipette positioned about 2 mm from the oocyte. During continuing applications, compounds were applied by addition to the superfusate.