Toxicology Letters 206 (2011) 100–104

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

# Interaction of bispyridinium compounds with the orthosteric binding site of human α7 and *Torpedo californica* nicotinic acetylcholine receptors (nAChRs)

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ARTICLE INFO

*Article history:*
Available online 16 June 2011

*Keywords:*
Nicotinic acetylcholine receptor
Human α7
*Torpedo californica*
Bispyridinium compound
Radioligand binding assay

ABSTRACT

Standard treatment of poisoning by organophosphorus (OP) nerve agents with atropine and oximes lacks efficacy with different nerve agents. A direct pharmacologic intervention at the nicotinic acetylcholine receptor (nAChR) was proposed as an alternative therapeutic approach and promising in vitro and in vivo results were obtained with the bispyridinium compound SAD-128. In addition, a number of SAD-128 analogues improved neuromuscular transmission of soman-poisoned diaphragms in vitro. We investigated the interaction of six of these SAD-128 analogues with the orthosteric binding site of the human α7 nAChR and *Torpedo californica* nAChR with a high-throughput assay using radioactive ligands. The determined affinity constants indicate a weak interaction of three test compounds ($K_i$ in the micromolar range) with both receptors, but no interaction could be recorded with the other three test compounds. The six SAD-128 analogues showed a low intrinsic inhibitory potency with human acetylcholinesterase ($IC_{50}$ > 400 μM). In conclusion, the results of the present study do not indicate a correlation between the affinity to the orthosteric binding site and the functional improvement of neuromuscular transmission and it is assumed that other mechanisms contribute to the therapeutic effect of the tested compounds.


## 1. Introduction

Organophosphorus (OP) nerve agents inhibit the pivotal enzyme acetylcholinesterase (AChE) irreversibly. Inhibition of AChE in the synaptic cleft leads to accumulation of acetylcholine (ACh) and subsequent overstimulation of central and peripheral muscarinic (mAChR) and nicotinic (nAChR) receptors. The resultant cholinergic syndrome is characterized by disturbance of numerous body functions and may lead finally to central and peripheral respiratory failure and death.

Standard treatment of nerve agent poisoning includes the administration of atropine as an anticholinergic agent and oximes, e.g., obidoxime or pralidoxime, as AChE reactivators (Eyer and Worek, 2007).

At therapeutic concentrations atropine acts exclusively as a reversible antagonist at mAChRs while oximes may provide a causal treatment by reactivating OP-inhibited AChE which is of utmost importance to restore neuromuscular transmission at respiratory muscles (Thiermann et al., 2010). Clinically used and experimental oximes were shown to be potent reactivators of AChE inhibited by different nerve agents and pesticides but lack efficacy with tabun- and soman-inhibited AChE (Worek et al., 2004).

In order to overcome the limited therapeutic efficacy of oximes in cases of poisoning by different nerve agents a direct, pharmacologic intervention at nAChRs was proposed as a new therapeutic approach to improve nerve agent-impaired neuromuscular transmission (Sheridan et al., 2005).

Previous studies with the bispyridinium non-oxime SAD-128 demonstrated its therapeutic effect against soman in vitro and in vivo which was partly attributed to its interaction with nAChRs (Schoene and Oldiges, 1973; Harris et al., 1977; Štalc and Šentjurc, 1990; Alkondon and Albuquerque, 1989; Grubič and Tomažič, 1989).

In vertebrates, nAChRs mediate synaptic transmission at the skeletal neuromuscular junction. The muscle-type ($\alpha1\beta1\delta\varepsilon$) nAChRs show a high degree of homology with *Torpedo* nAChRs ($\alpha\beta\delta\gamma$) (Millar, 2003) and the α7 nAChR subtype, which was originally classified as a neuronal receptor, appears to be widespread in the human body (Fagerlund and Eriksson, 2009).

In the present study, six novel SAD-128 analogues (Fig. 1) (Timperley et al., 2005), which showed promising effects in improving soman-impaired neuromuscular transmission (Turner, 2007),

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0378-4274/$ – see front matter. 
doi:10.1016/j.toxlet.2011.06.009

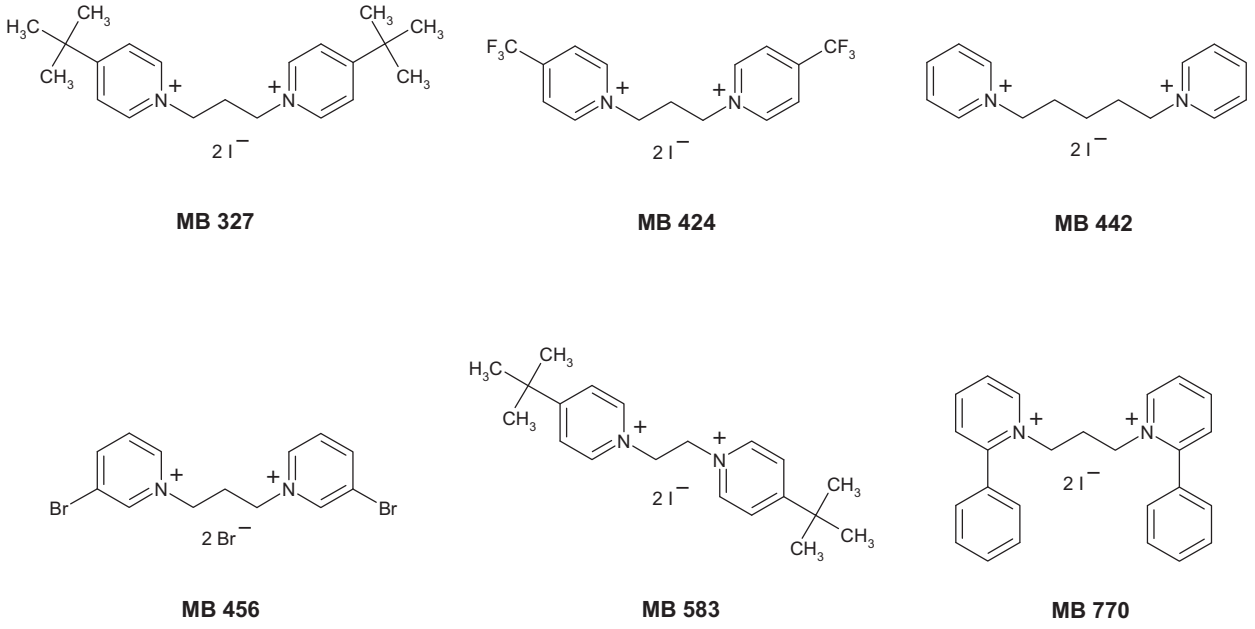


**Fig. 1.** Bispyridinium compounds with different alkyl chains and pyridinium-substituents.

were investigated to determine their interaction with the orthosteric binding site of nAChRs. The experiments were performed with two nAChR subtypes, the human homomeric α7 nAChR and the heteromeric αβδγ-nAChR isolated from *Torpedo californica* electric tissue applying a newly developed high-throughput binding assay.

## 2. Materials and methods

### 2.1. Materials

The $GH_4C_1$/hα7 nAChR cell line was obtained from Genionics, Schlieren, Switzerland. *T. californica* electroplaque tissue was purchased from Aquatic Research Consultants, San Pedro, CA, USA. Disposables and cell culture flasks were from Becton & Dickinson, Heidelberg, Germany, and Nunc, Thermo Scientific, Langenselbold, Germany. 500 $cm^2$ cell-culture plates were supplied by Corning, Amsterdam, The Netherlands, and the complete cell culture media and supplements were purchased from Gibco, distributed by Invitrogen, Darmstadt, Germany. Epibatidine, [5,6-cycloheptyl-$^3$H] with a specific activity of approx. 2 TBq/mol was from Perkin Elmer, Jügesheim, Germany. PNU 282987, α-bungarotoxin, methyllycaconitine and (±)-epibatidine were obtained from Tocris, Bristol, UK, and pancuronium, carbamoylcholine, acetylthiocholine iodide (ATCh) and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB, Ellman's reagent) from Sigma–Aldrich, Taufkirchen, Germany.

The bispyridinium compounds MB442, MB583, MB327, MB770, MB456 and MB424 (Fig. 1) were synthesized according to Turner (2007) at Dstl Porton Down, UK.

Stock solutions of MB compounds, methyllycaconitine, pancuronium and carbamoylcholine were prepared in distilled water (10 mM), (±)-epibatidine and PNU 282987 in ethanol (10 mM), and α-bungarotoxin in 10% aqueous DMSO (1 mM).

### 2.2. Cell culture

$GH_4C_1$ cells, derived from a rat pituitary tumor cell line, were stably transfected with the human α7 nAChR (Genionics, Schlieren, Switzerland) and were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4500 mg/l glucose and GlutaMAX supplemented with 10% heat-inactivated fetal bovine serum and 100 μl/ml of the selective antibiotic, geneticin (G418) (Steiner et al., 2010). Cells were maintained at 37 °C and 5% $CO_2$/humidified air up to 70–80% confluence and split twice a week, using enzyme-free dissociation buffer for detachment.

### 2.3. Cell membrane preparation

$GH_4C_1$ cells, stably expressing the human α7-nAChR ($GH_4C_1$/hα7-nAChR cells), were grown to 70–80% confluence (~400 × $10^6$ cells) in 500 $cm^2$ cell-culture plates. After removal of the cell-culture media, the cell monolayer was washed with ice-cold phosphate-buffered saline (4 mM phosphate and 150 mM NaCl), supplemented with 1 mM EDTA (PBS-EDTA) and 25 ml of PBS-EDTA buffer was added. Then, the cell-culture plates were incubated for 60 min at 4 °C to detach the cells which were then transferred into polypropylene tubes and subsequently centrifuged at 700 × *g* for 10 min and 4 °C to gain a pellet. The pellet was shock frozen in liquid nitrogen and stored at −80 °C until use.

All subsequent steps were conducted at 4 °C to avoid receptor degradation. The frozen pellets were thawed in a 10-fold volume of lysis buffer (1 mM $NaH_2PO_4/Na_2HPO_4$, 0.5 mM EDTA, pH 7.4), suspended by Dounce homogenisation (50 up and down strokes), and centrifuged at 1000 × *g* for 10 min. The supernatant was combined, mixed with a 5-fold volume of pellet buffer (10 mM $NaH_2PO_4/Na_2HPO_4$, 120 mM NaCl, 5 mM KCl, pH 7.4), and centrifuged at 100,000 × *g* for 30 min (Beckman ultracentrifuge). The supernatant was discarded and the pellet homogenised in storage buffer (10 mM $NaH_2PO_4/Na_2HPO_4$, 120 mM NaCl, 5 mM KCl, 300 mM sucrose, 0.5 mM EDTA, pH 7.4), corresponding to about 5 mg protein/ml. Total protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985), using bovine serum albumin as standard. Aliquots were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

### 2.4. Preparation of (αβδγ) nAChR enriched membrane fragments

Membranes were prepared from frozen electric organ of *T. californica* (Aquatic Consultants, San Pedro, USA) as described before (Elliot et al., 1980) with minor modifications. All subsequent steps were consequently performed at 4 °C.

A threefold volume of extraction buffer (20 mM $Na_2HPO_4/NaH_2PO_4$, 400 mM NaCl, 2 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride (freshly added), pH 7.4) was added to frozen tissue. After thawing, the mixture was dispersed with an Ultra-Turrax at 13,500 rpm. The homogenate was centrifuged for 90 min at 27,000 × *g* (Beckman ultracentrifuge). The pellet was resuspended in wash buffer (extraction buffer without NaCl) and centrifuged for 60 min at 37,000 × *g*. After three washes, the suspension was centrifuged for 10 min and 1000 × *g*. The supernatant was mixed with ≈5-fold volume of pellet buffer (10 mM $Na_2HPO_4/NaH_2PO_4$, 120 mM NaCl, 5 mM KCl, pH 7.4) and centrifuged for 30 min at 100,000 × *g*. The residue was suspended with a double volume of storage buffer (10 mM $Na_2HPO_4/NaH_2PO_4$, 120 mM NaCl, 5 mM KCl, 300 mM sucrose, 0.5 mM EDTA, pH 7.4), aliquots were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

### 2.5. Radioligand binding

Radioligand binding experiments were performed according to described [$^3$H] epibatidine assay methods (Quik et al., 1996) with few modifications.

Pipetting and incubation were carried out with a modular pipetting platform (EVO 150 workstation, Tecan, Crailsheim, Germany). All radioligand experiments were conducted in 96-deep well plates, in assay binding buffer (10 mM $NaH_2PO_4/Na_2HPO_4$, 120 mM NaCl, 5 mM KCl, pH 7,4). An aliquot of the $GH_4C_1$/hα7 nAChR membrane fraction was rapidly thawed and diluted in a fivefold volume of cold binding buffer. *Torpedo* membranes were 200-fold diluted with binding buffer, containing additional 0.2% bovine serum albumin. In the case of *Torpedo* nAChRs, total protein amount was 10 μg per well and with hα7 nAChRs 50 μg per well. Total volume in each well was 250 μl. The membrane suspension was stirred (200 rpm) at +4 °C. After the incubation period of 120 min at 25 °C, bound and free [$^3$H] epibatidine were separated by rapid vacuum filtration using a cell harvester (Perkin Elmer, Jügesheim, Germany) onto GF/B filter plates, previously pre-soaked in 0.1%