The effects of novel 7-MEOTA-donepezil like hybrids and N-alkylated tacrine analogues in the treatment of quinuclidinyl benzilate-induced behavioural deficits in rats performing the multiple T-maze test

Jan Misik, Jan Korabecny, Eugenie Nepovimova, Pavla Cabelova, Jiri Kassa

Aims. The number of approved drugs for the clinical treatment of Alzheimer’s disease remains limited. For this reason, there is extensive search for novel therapies. Of these, cholinesterase inhibitors have some proven benefit in slowing the disease progression and still remain the first-line therapeutic approach. In this study, the pro-cognitive effect of four novel tacrine-related inhibitors was evaluated and compared with the standards, tacrine and donepezil.

Methods. Wistar rats trained to perform the multiple T-maze were treated intra-peritoneally with the anticholinergic agent 3-quinuclidinyl benzilate (QNB, 2.0 mg/kg), followed 30 min later by another injection containing a therapeutic dose of standard or novel cholinesterase inhibitor. The rats were repeatedly subjected to the multiple T-maze task at several time points following QNB administration (1, 24, 48 and 72 h). The passage time and number of errors were recorded. The inhibitory potential of selected therapeutic doses was assessed in a separate in vivo experiment using a spectrophotometric method.

Results. QNB significantly impaired the performance of the rats within 48 h. The four novel cholinesterase inhibitors attenuated the effect of QNB at 1 h, 24 h and 48 h test intervals. The novel compounds resulted in brain cholinesterase inhibition ranging from 5.4 to 11.3 %, and their effect on the QNB-induced deficit recorded in the T-maze performance was comparable to that of the standards or higher at some time points. The best result was achieved with derivative 4, followed by derivatives 2 and 3, suggesting that these compounds could be candidates for the treatment of Alzheimer’s disease.

Key words: acetylcholine, acetylcholinesterase, Alzheimer’s disease, spatial orientation, donepezil, tacrine

INTRODUCTION

Alzheimer’s disease (AD) is a multifactorial neurodegenerative disorder for which the aetiology and pathogenesis are still not well-understood. Globally, there is a rising incidence in the aging population and lack of appropriate treatment, leading to an estimated 115 million patients suffering from AD in 2050 (ref.1,2). One of the leading disease hypotheses over the several past decades is the “cholinergic” hypothesis; concerning the loss of cholinergic connections in the CNS, mediated by degeneration of the basal forebrain nuclei3. Thus, original treatment strategies addressed the decreased levels of the neurotransmitter acetylcholine (ACh). Efforts to elevate cholinergic neurotransmission have included supplementation of ACh precursors and the use of cholinergic receptor agonists or cholinesterase inhibitors. Most of these approaches have not proven effective, with the exception of cholinesterase inhibitors4,5.

Cholinesterase inhibitors (ChEIs) are a varied group of compounds that are able to inhibit the enzymatic cleavage of the neurotransmitter ACh and thus indirectly strengthen cholinergic neurotransmission. This is beneficial particularly in the early stages of AD, by reducing disease progression and the severity of symptoms. The beneficial effects of ChEIs on cognition have been demonstrated several times in laboratory animals, using either lesion- or drug-induced behavioural impairment models6-8, as well as in human clinical trials4.

Currently, there is extensive research into variable treatment approaches for AD, including anti-tau strategies, beta-secretase inhibitors, or vaccination4,9. However, the number of approved drugs is still limited.

Besides memantine which is a N-methyl-D-aspartate (NMDA) receptor antagonist, several ChEIs, including rivastigmine, donepezil and galantamine, are used in clinical practice8. Although treatment with ChEIs is rather symptomatic with only temporary beneficial effect4,10,11, ChEIs remain the first-line therapeutic approach12,13. The extensive search for new ChEIs has focused on innovative alternatives to existing drugs. Novel approaches include combinations (dual inhibitors) and/or modifications of existing compounds, as well as multi-target hybrid compounds as potential candidates for the treatment of AD (ref.14,21). The potential of these novel analogues lies in their favourable inhibitory properties, as well as other biological actions including inhibition of beta-amyloid aggregation, antioxidant and neuro-protective effects22.
The present study is focused on novel tacrine-related reversible ChEIs 1, 2, 3 and 4 (Fig. 1) synthesized originally at the Faculty of Military Health Sciences\textsuperscript{23,24}. ChEIs 1 and 2 are dual binding site ChEIs representing 7-MEOTA-donepezil like hybrids. These novel compounds combine the 7-MEOTA moiety as a peripheral anionic site ligand, with differently substituted benzyl fragments of donepezil acting as catalytic anionic site (CAS) ligands. In contrast to donepezil, 1 and 2 exhibit non-selective cholinesterase inhibition and less inhibitory activity, whereas their inhibition profile is better than that of the parent compound 7-MEOTA (ref.\textsuperscript{24}).

ChEIs 3 and 4 are \textit{N}-alkylated tacrine analogues\textsuperscript{23,25,26} representing a group of compounds bearing 9-amino-1,2,3,4-tetrahydroacridine moiety as the CAS ligand and a short \textit{N}-alkyl chain, which improves binding of the inhibitor to the enzyme active site. Their \textit{in vitro} inhibitory potential was demonstrated; compound 3 revealed even higher inhibitory potential than the parent compound tacrine.

As all four novel reversible ChEIs were shown to be potent inhibitors of cholinesterase \textit{in vitro}\textsuperscript{23,24} their potential as cognitive enhancers in the treatment of AD was considered using a pharmacological animal model of cholinergic depletion\textsuperscript{27}. The effect of these novel ChEIs was compared to that of standard tacrine (the basis of 3, 4 and 7-MEOTA - the parent compound of 1 and 2), although tacrine is no longer utilized in clinical practice - it was withdrawn due to dose-dependent hepatotoxicity and severe side effects including nausea, vomiting, diarrhoea and weight loss\textsuperscript{28}. From this point of view, novel 7-MEOTA-donepezil like hybrids (1 and 2) are promising due to decreased acute toxicity, especially 2, which is considered to be a low toxic compound\textsuperscript{29}. As another standard, donepezil (the parent compound of 1 and 2), approved for the treatment of mild and moderate stages of AD in 1996, was used. To evaluate the pro-cognitive effects of these novel compounds, a single dose of each tested ChEI (standard or novel) was administered therapeutically, 30 min after injecting a behaviour-impairing agent, to male Wistar rats performing the multiple T-maze - a standard behavioural test of spatial learning and memory.

**MATERIAL AND METHODS**

**Animals**
Male Wistar rats (8 to 10-week-old, 150-200 g b.w.) were obtained from Velaz Ltd. (Czech Republic). The rats were housed in groups of 4, in temperature- and light-controlled breeding units (temperature 21±1 °C, 12/12 h light/dark cycle) at an approved animal facility. The animals received a standard rodent diet (Cerea corp.) and drinking water \textit{ad libitum}. The food supply was limited to approximately 75% of the free feeding rate during the T-maze experiment. The acclimatization period was a minimum of 10 days prior to the initiation of training sessions, without any food restrictions. The use of animals in this study was formally approved by the Ethics Committee of the Faculty of Military Health Sciences, Czech Republic. All procedures involving animals were performed in accordance with current legislation.

**Chemicals**
The cognition-impairing agent 3-quinuclidinyl benzilate hydrochloride (QNB) and novel ChEIs were synthesized \textit{de novo} at the University of Defence (Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy). All compounds were of 90-95% purity (HPLC determination). Standards tacrine (9-amino-1,2,3,4-tetrahydroacridine) hydrochloride, donepezil hydrochloride (Bayer) and 7-MEOTA (a gift of Czech Chemical Society) were used for the characterization of the novel ChEIs.

**Fig. 1.** Novel 7-MEOTA-donepezil like hybrids (1 and 2) and \textit{N}-alkylated tacrine analogues (3 and 4).
A novel series of cholinesterase inhibitors (ChEIs) 1,2,3,4-tetrahydroacridine hydrochloride and donepezil (1-benzyl-4-[5,6-dimethox-1-indanon]-2-yl) methylpiperidine hydrochloride) were purchased from Sigma Aldrich Ltd. (Czech Republic) as well as other chemicals for assessment of brain cholinesterase inhibition [Tris-HCl buffer, acetylthiocholine, 5,5‘-dithio-bis-(2-nitrobenzoic acid)]. QNB, tacrine and donepezil were administered to experimental animals in a standardised volume of 1 mL/kg; diluted in saline (0.9% natrium chloride, B. Braun Medical Ltc., Czech Republic) immediately before administration. Novel ChEIs were administered with the ChEIs at the same dose and were considered significant at 2α=0.05.

**Novel ChEIs**

Novel ChEIs 1 (7-methoxy-N-(2-[4-[(3-methylphenyl) methyl]piperazin-1-yl]ethyl)-1,2,3,4-tetrahydroacridine-9-amine trihydrochloride), 2 (N-[2-[4-[(2-bromophenyl) methyl]piperazin-1-yl]ethyl]-7-methoxy-1,2,3,4-tetrahydroacridine-9-amine trihydrochloride), 3 (N-ethyl-1,2,3,4-tetrahydroacridine-9-amine hydrochloride) and 4 (7-methoxy-N-hexyl-1,2,3,4-tetrahydroacridine-9-amine hydrochloride) were synthesized de novo at the University of Defence (Faculty of Military Health Sciences, Department of Toxicology and Military Pharmacy) as hybrids consisting of a 7-MEOTA unit connected with analogues of N-benzylpiperazine moieties (1, 2) or as N-alkylated tacrine analogues (3, 4). Therapeutic doses of novel ChEIs were arbitrarily determined to correlate with 20% of the median lethal dose (LD50), except for compound 1, where 40% of LD50 was chosen, due to negligible brain cholinesterase inhibition at 20% of LD50 (unpublished data).

**Assessment of brain cholinesterase inhibition in vivo**

The inhibitory potential of selected therapeutic doses was evaluated in a separate in vivo experiment. The rats were administered with the ChEIs at the same dose and via the same route as rats in the T-maze test and euthanized by CO2, 30 min after administration of the ChEI. The brains were removed immediately after euthanasia and cholinesterase activity was assessed, using a standard spectrophotometric method. Brains were homogenized (homogenizator Ultra-Turrax T25 Basic, IKA*- WERKE, Germany) in Tris-HCl buffer (0.02 mol/L, pH 7.6, 1:10). Acetylthiocholine was used as a substrate (Tris-HCl buffer, N = 0.1 mol/L, pH 7.6). The Helios Alpha spectrophotometer was used for determination of absorbance at 436 nm and the cholinesterase activity was expressed as μkat/kg (μmol substrate hydrolyzed/kg wet tissue within 1 second). Untreated control values for brain cholinesterase activity were obtained from rats who received saline instead of ChEI (saline control).

**T-maze test**

The T-maze apparatus consisted of segments measuring 12 × 20 × 11 cm (L×W×H) with several different choice points, enabling possible left or right exits. The distance between the starting position and the goal compartment was 185 cm (ref.31). The rats were food-deprived, receiving approximately 75% of common daily food intake during training and test sessions. The training period took 30 consecutive days (excluding weekends). Once a day, the rats were released from the starting position and the time taken to reach the goal compartment (containing a reward of several food pellets) was measured, as well as the number of wrong entries. To avoid odour cues, the maze was properly cleaned with 70% ethanol between trials. Well trained rats were able to pass through the maze in less than 3 seconds without entering a wrong arm. 5 out of 68 rats taken into training were excluded after the training period due to lack of performance.

On the day of the experiment, and 24 h after the last training session, the rats were randomly divided into 8 groups containing 8 individuals each (except the tacrine-treated group - n=7). QNB was administered to the animals via an i.p. injection at a dose of 2.0 mg/kg 1 h before the test. Treated groups received a dose of either 1 (25.6 mg/kg), 2 (12.3 mg/kg), 3 (2.85 mg/kg), 4 (5.2 mg/kg), donepezil (2.65 mg/kg) or tacrine (5.2 mg/kg) 30 min after administration of the QNB. A positive control group received QNB followed by saline in place of a ChEI. The saline control group received saline (1 mL/kg) 30 min and 1 h before the test. The performance of rats running the maze was evaluated at several time points - 1, 24, 48 and 72 h after the QNB injection. The passage time and the number of entries into the wrong arms of the maze were recorded.

**Statistical analysis**

Statistical analysis was performed using Statistica software98 Edition. Non-parametric tests were used for analysis – the Kruskal-Wallis test with post hoc tests or the Mann-Whitney U test. All values were presented as means±S.E.M. (Standard Error of the Mean). Differences were considered significant at 2α=0.05.

**RESULTS**

The in vivo inhibitory potential of selected doses of ChEIs is shown in table 1. Tested doses of novel ChEIs caused brain cholinesterase inhibition ranging from approx. 5 to 11%. The lowest inhibitory potential was observed for compound 4 (5.4%) and highest for compound 1 (25.6%). There was a significant difference in T-maze performance between groups in the 1 h test (H(7, 63)=31.8, P<0.001; Fig. 2) as well as in the 24 h test (H(7, 63)=26.0, P<0.001; Fig. 2) and 48 h (H(7, 63)=25.5, P<0.001; Fig. 2) test, whereas no difference was found at the latest time point 72 h (H(7, 63)=12.5, P<0.09; Fig. 2). The cholinergic antagonist QNB significantly increased the passage time of rats compared to saline controls (P<0.001; 1-48 h) and this effect was long-lasting, persisting until 48 h. QNB-
treated rats (positive control) also showed increased incidence of wrong entries and the largest number of aberrant animals at all time points except the latest (table 2).

When treated with ChEIs, the passage time of the QNB treated rats was obviously improved, although none of the treated groups achieved the high performance of saline controls (Fig. 2). Statistically, there was no significant difference between the saline control group and groups treated with 2, 3 and 4 in the 1 h test (all \( P \geq 0.1 \), Fig. 2), whereas at the 24 and 48 h time points, the performance of rats improved significantly for all groups and there was no difference between saline controls and ChEI-treated rats (Fig. 2). No significant difference in T-maze performance was found between rats treated with novel compounds compared to standards at all time points (all \( P \geq 0.9 \)). In contrast to the positive QNB control, no error entries occurred in the 24 and 48 h test in groups treated with all tested ChEIs, except for the compound-3-treated group, with one aberrant individual observed. Generally, the best result was achieved with analogue 4 in regard to the shortest passage time and least error entry at all time points of testing.

**DISCUSSION**

The potential of novel 7-MEOTA-donepezil like hybrids and \( N \)-alkylated tacrine analogues was evaluated in the current study according to the assumption that restoring cholinergic activity indirectly, via inhibition of cholinesterase, could improve cognition. As a standard amnesic drug, scopolamine is frequently used in similar

---

**Table 1.** In vivo brain cholinesterase inhibition (%) in male wistar rats, 30 min after i.p. injection of cholinesterase inhibitors.

<table>
<thead>
<tr>
<th>Treatment/(dose)</th>
<th>Cholinesterase activity (µkat.kg(^{-1}))</th>
<th>Inhibition (%)</th>
<th>% of LD(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>saline (1 mL/kg)</td>
<td>170.6 ± 7.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 (25.6 mg/kg)</td>
<td>161.0 ± 9.6</td>
<td>5.6</td>
<td>40</td>
</tr>
<tr>
<td>2 (12.3 mg/kg)</td>
<td>151.3 ± 9.1</td>
<td>11.3</td>
<td>20</td>
</tr>
<tr>
<td>3 (2.85 mg/kg)</td>
<td>137.8 ± 7.4</td>
<td>6.9</td>
<td>20</td>
</tr>
<tr>
<td>4 (5.2 mg/kg)</td>
<td>152.7 ± 6.0</td>
<td>5.4</td>
<td>20</td>
</tr>
<tr>
<td>donepezil (2.65 mg/kg)*</td>
<td>99.3 ± 11.8</td>
<td>41.8</td>
<td>-</td>
</tr>
<tr>
<td>tacrine (5.2 mg/kg)</td>
<td>136.8 ± 5.2</td>
<td>19.8</td>
<td>20</td>
</tr>
</tbody>
</table>

Mean ± S.E.M. Percentage of median lethal dose corresponding to selected dose of novel cholinesterase inhibitors and tacrine is shown. *LD\(_{50}\) was not established, the therapeutic dose was approximated from a previous study\(^3\).
central activity and selectivity to acetylcholinesterase
advantage in favour of donepezil against tacrine is better
passage time in the 1 h test and no error entries at all
with tacrine analogue 4 as evidenced by the shortest
dards in the 1 h test. Excellent results were achieved
in vivo evaluation29. Nevertheless, there is an expectation
potential can be only estimated on the basis of current
investigated and not yet known. The central inhibitory
ity might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
therapy might be a consideration. Unfortunately, the detailed
potential is equivalent to that of standards.
in vivo effect has been found to date\textsuperscript{51}. Only a few compounds showed behavioural effects in preliminary tests, including tacrine-6-ferulic acid, which effectively reversed scopolamine-induced behavioural impairment in mice\textsuperscript{41}. In this study, the effect of novel compounds on a pharmacologically-induced deficit of spatial navigation in rats proved to be comparable to standard drugs with minor inter-individual differences. The possible therapeutic application and variable effectiveness of different ChEIs relates to such factors as central/peripheral inhibitory potential, selectivity for individual brain areas (targeted mainly at the hippocampus and cortex), cholinesterase selectivity, and overall biological and chemical properties, including toxicity and side effects. From this point of view, the potential of novel ChEIs lies in their dual binding properties, lower toxicity of 7-MEOTA-donepezil like hybrids and other potential benefits, including inhibition of beta-amyloid aggregation. Such novel compounds should be further investigated as promising anti-Alzheimer’s agents; however, whether these compounds will ultimately prove more efficacious to known standard drugs depends on future detailed evaluation in pharmacology, physiology and biochemistry.

ACKNOWLEDGEMENT

This work was supported by the Grant Agency of the Czech Republic (No. P303/12/0611). The authors would like to thank Mrs E. Reslova and Mrs J. Hatlapatkova for their skilful technical assistance and Dr Daren Hanshaw for providing language help.

Authorship contributions: JM: manuscript writing, conception and experimental design, data collection, analysis and interpretation; JK: conception and design; EN: in vitro synthesis, evaluation, manuscript revision; PC: in vitro synthesis, evaluation, data collection; JK: data interpretation, manuscript revision.

Conflicts of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

REFERENCES


27. Misik J, Vanek J, Musilek K, Kassa J. Cholinergic antagonist 3-quinu-


34. Falsafi SK, Deli A, Höger H, Pollak A, Lubec G. Scopolamine admin-

35. Gacar N, Mutfu O, Utkan T, Komsuoglu Celikyurt I, Gocmez SS, Ulak G. Beneficial effects of resveratrool on scopolamine but not meca-
mamylione induced memory impairment in the passive avoidance and Morris water maze tests in rats. Pharmacol Biochem Behav 2011;99:316-23.


38. Spowart-Manning L, van der Staay FJ. The T-maze continuous alter-

39. Deiana S, Platt B, Riedel G. The cholinergic system and spatial learn-
ing. Behav Brain Res 2011;221:389-411.

40. Kirkby DL, Jones DN, Barnes JC, Higgins GA. Effects of anticholin-

41. Kirkby DL, Jones DN, Barnes JC, Higgins GA. Effects of anticholin-

42. Spowart-Manning L, van der Staay FJ. The T-maze continuous alter-

43. Misik J, Vanek J, Musilek K, Kassa J. Cholinergic antagonist 3-quinu-


48. Deiana S, Platt B, Riedel G. The cholinergic system and spatial learn-
ing. Behav Brain Res 2011;221:389-411.


50. Fleck C, Appenroth D, Fang L, Schott Y, Lehmann J, DeckerM. Investigation into the in vivo effects of five novel tacrine/fe-
rulic acid and beta-carboline derivatives on scopolamine-in-