

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

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Aims. The number of approved drugs for the clinical treatment of Alzheimer 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.

Conclusion: 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 disease.

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

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INTRODUCTION

Alzheimer 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 inhibitors^{4,5}.

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 vaccination^{4,9}. 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 practice⁴. Although treatment with ChEIs is rather symptomatic with only temporary beneficial effect^{4,10,11}, ChEIs remain the first-line therapeutic approach^{12,13}. 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^{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.²⁴).

ChEIs 3 and 4 are *N*-alkylated tacrine analogues^{23,25,26} representing a group of compounds bearing 9-amino-1,2,3,4-tetrahydroacridine moiety as the CAS ligand and a short *N*-alkyl chain, which improves binding of the inhibitor to the enzyme active site. Their *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 *in vitro*^{23,24} their potential as cognitive enhancers in the treatment of AD was considered using a pharmacological animal model of cholinergic depletion²⁷. 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 loss²⁸. 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 compound²⁹. 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 *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 *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-

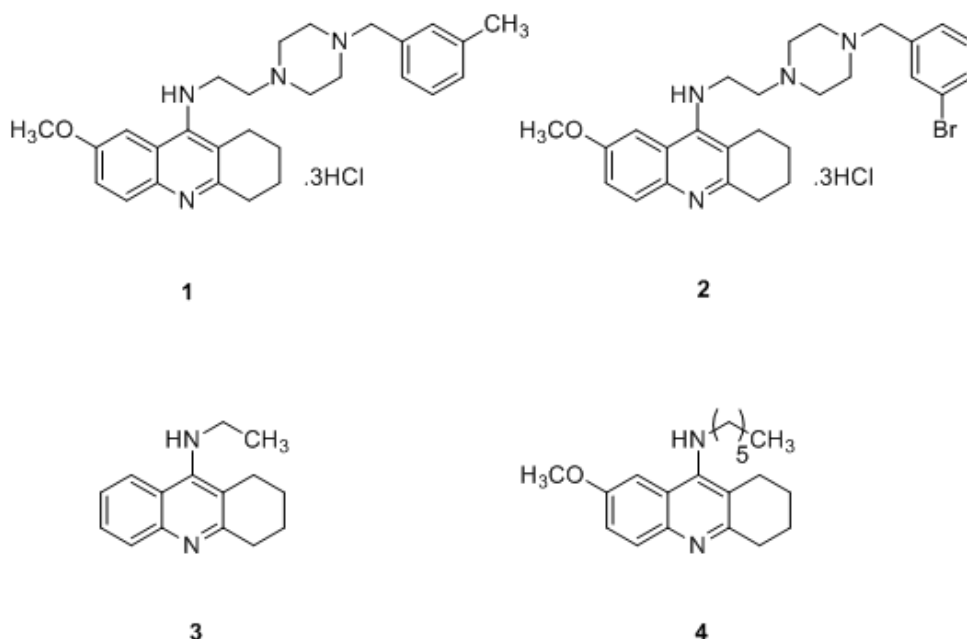


Fig. 1. Novel 7-MEOTA-donepezil like hybrids (1 and 2) and *N*-alkylated tacrine analogues (3 and 4).

1,2,3,4-tetrahydroacridine hydrochloride) and donepezil (1-benzyl-4-[(5,6-dimethoxy-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'-dithiobis-(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% sodium chloride, B. Braun Medical Ltd., Czech Republic) immediately before administration. Novel ChEIs were diluted in dimethylsulfoxide/saline solution (1/10), due to poorer solubility in saline.

Novel ChEIs

Novel ChEIs 1 (7-methoxy-*N*-(2-[4-[(3-methylphenyl) methyl]piperazin-1-yl]ethyl)-1,2,3,4-tetrahydroacridin-9-amine trihydrochloride), 2 (*N*-(2-[4-[(2-bromophenyl) methyl]piperazin-1-yl]ethyl)-7-methoxy-1,2,3,4-tetrahydroacridin-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 (LD_{50}), except for compound 1, where 40% of LD_{50} was chosen, due to negligible brain cholinesterase inhibition at 20% of LD_{50} (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 CO_2 30 min after administration of the ChEI. The brains were removed immediately after euthanasia and cholinesterase activity was assessed, using a standard spectrophotometric method³⁰. 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 μ kat/kg (μ mol 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 $12 \times 20 \times 11$ 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 software'98 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 $2\alpha=0.05$.

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 2 (11.3%), which was below the inhibition caused by standards donepezil (41.8%) and tacrine (19.8%). Selected doses were well tolerated and no signs of cholinergic overstimulation, such as mastication, salivation or tremor, occurred. Mild and transient hyperactivity was observed following QNB injection, rising approx. 3 min post administration and diminishing spontaneously after 15 min.

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-

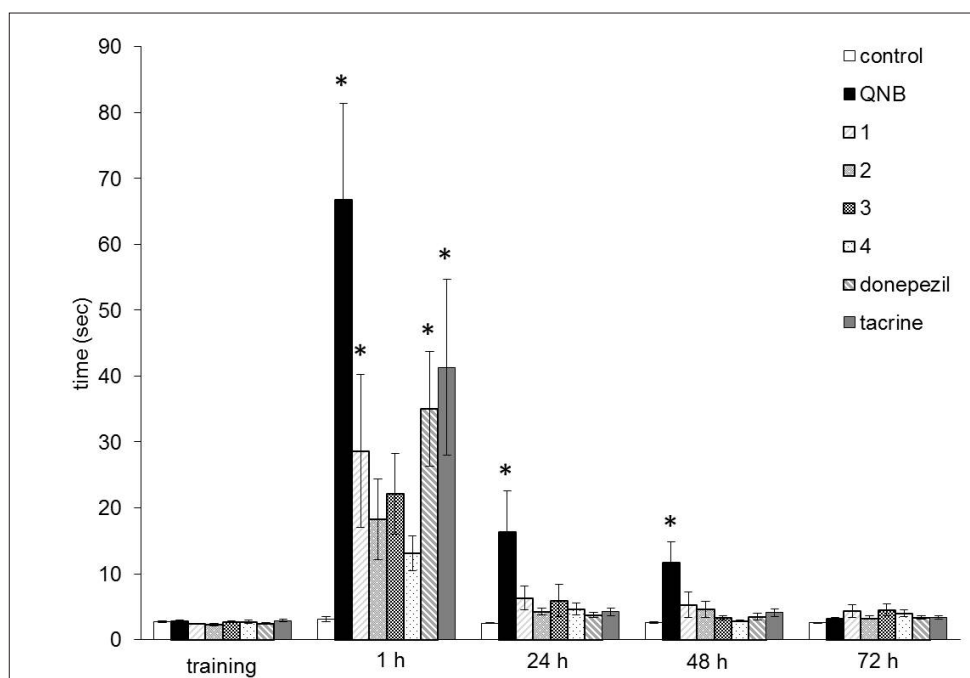


Fig. 2. Passage times (sec) of rats performing the multiple T-maze test at training trial, 1 h, 24 h, 48 h and 72 h. Treatments provided 1 h (saline/QNB) and 30 min (saline/ChEI) before 1 h trial. Experimental groups - control (saline+saline; saline control), QNB (QNB+saline; positive control), 1 (QNB+compound 1), 2 (QNB+compound 2), 3 (QNB+compound 3), 4 (QNB+compound 4), donepezil (QNB+donepezil), tacrine (QNB+tacrine). Mean±SEM. *Significantly different from the saline control.

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

Treatment/(dose)	Cholinesterase activity ($\mu\text{kat.kg}^{-1}$)	Inhibition (%)	% of LD ₅₀
saline (1 mL/kg)	170.6 ± 7.5	-	-
1 (25.6 mg/kg)	161.0 ± 9.6	5.6	40
2 (12.3 mg/kg)	151.3 ± 9.1	11.3	20
3 (2.85 mg/kg)	137.8 ± 7.4	6.9	20
4 (5.2 mg/kg)	152.7 ± 6.0	5.4	20
donepezil (2.65 mg/kg)*	99.3 ± 11.8	41.8	-
tacrine (5.2 mg/kg)	136.8 ± 5.2	19.8	20

Mean ± S.E.M. Percentage of median lethal dose corresponding to selected dose of novel cholinesterase inhibitors and tacrine is shown. *LD₅₀ was not established, the therapeutic dose was approximated from a previous study³⁶.

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 2. Mean number of error entries and number of aberrant individuals in rats performing the multiple T-maze during training, 1 h, 24 h, 48 h and 72 h test (mean±S.E.M.).

Treatment	Mean no. of error entries/no. of aberrant individuals				
	Training	1 h	24 h	48 h	72 h
saline + saline	0	0	0	0	0
QNB + saline	0	2.13±1.15/6	0.63±0.49/2	1.13±0.30/6	0
QNB + 1	0	0.13±0.13/1	0	0	0
QNB + 2	0	0.25±0.25/1	0	0	0
QNB + 3	0	0.13±0.13/1	0.38±0.38/1	0.13±0.13/1	0
QNB + 4	0	0	0	0	0
QNB + donepezil	0	0.38±0.26/2	0	0	0
QNB + tacrine	0	0.72±0.36/3	0	0	0

Error entries in the group treated with compound 3 occurred in the same individual throughout different time points.

studies³²⁻³⁵. In this study, scopolamine was successfully substituted by another non-selective muscarinic receptor antagonist – QNB. This approach enabled long-term observation of anti-cholinergic effects due to the prolonged action of QNB compared to scopolamine^{27,36}.

To evaluate the pro-cognitive effects of novel compounds, the multiple T-maze test was selected^{26,34,37,38}. This behavioural task reflects spatial learning and memory, requiring recollection of spatial information and the construction of a cognitive map, which is considered to be analogous to human episodic memory in animals^{39,40}. Thus, the multiple T-maze seems to be ideal for investigating the pro-cognitive effects of potential anti-Alzheimer drugs.

The experimental amnesia was induced by systemic injection of QNB 1 h before testing, followed by a therapeutic dose of ChEI after 30 min, which was considered an appropriate time to allow for systemic distribution of the drug and restoration of cholinergic activity via cholinesterase inhibition. Indeed, there was a general significant reduction of passage time in rats treated with ChEIs at the 1 h test against non-treated controls regardless whether novel or standard ChEIs were used. Also the number of error entries was substantially reduced. Selected standards donepezil and tacrine have been confirmed as cognitive enhancers in several different animal models of neurodegenerative disorders^{7,8,41,42}. Donepezil was usually more effective than tacrine, but the difference could be attributed to variable therapeutic doses³⁶. In this study, the effects of both standards were equivalent at all time points. An advantage in favour of donepezil against tacrine is better central activity and selectivity to acetylcholinesterase⁴³⁻⁴⁵ and also reduced incidence of peripheral side effects due to lower toxic potential at therapeutic doses. The narrow therapeutic index of tacrine explains for the failure of tacrine (10 mg/kg) in the multiple T-maze test due to cholinergic overstimulation³⁶.

Novel ChEIs were at least equally effective to standards in the 1 h test. Excellent results were achieved with tacrine analogue 4 as evidenced by the shortest passage time in the 1 h test and no error entries at all time points. An obvious improvement was observed in all ChEI-treated groups in the 24 and 48 h tests. There was

no significant difference between the novel compounds and standards at all time points, indicating that the action of novel ChEIs is equivalent to that of standards. Moreover, novel compounds were subjectively better than standards in the 1 h test, but this effect might be related to the dose-dependent cholinesterase inhibition. The therapeutic doses of ChEI were mainly selected according to 20% of LD₅₀ and, as anticipated, responsible for variable brain cholinesterase inhibition ranging from 5 to 11% in the novel compounds. Good bioavailability, blood-brain barrier penetration ability and related brain cholinesterase inhibition are crucial requirements for evaluation of novel treatments. The main disadvantage of the novel compounds against standards was poorer solubility in aqueous solutions, and thus reduced bioavailability might be a consideration. Unfortunately, the detailed pharmacokinetics of these novel ChEIs is currently being investigated and not yet known. The central inhibitory potential can be only estimated on the basis of current *in vivo* evaluation²⁹. Nevertheless, there is an expectation that novel compounds, at least partly, penetrate the blood-brain barrier, and this is supported by *in vitro* observation in similar tacrine-donepezil hybrids⁴⁶.

The greatest brain cholinesterase inhibition was found for standard donepezil, touching the limit of 40 %, which is considered a threshold. The therapeutic efficacy of ChEIs is usually reduced at the expense of cholinergic overstimulation, if this threshold is exceeded^{6,47}. Nevertheless no cholinergic signs were observed in the donepezil group, even if the brain inhibition was slightly higher. In general, ChEIs seem to be effective at an inhibition level range of 10-40% (ref.⁶). In this study, compounds 1, 3 and 4 showed inhibition levels of less than 10%. Nevertheless, the doses of ChEIs might be responsible for *in vivo* brain inhibition at a higher level than was observed via the spectrophotometric method due to a confounding dilution effect⁴⁸.

Recently, a number of novel tacrine-related analogues including mono-tacrine derivatives, homodimers, and most promisingly, heterodimers have been introduced^{22,49}. Unfortunately, few have met the requirements for an optimal anti-Alzheimer drug and hence not evaluated further. Some have shown promising results *in vitro* but no *in vivo*

effect has been found to date⁵⁰. Only a few compounds showed behavioural effects in preliminary tests, including tacrine-6-ferulic acid, which effectively reversed scopolamine-induced behavioural impairment in mice⁵¹.

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 agents; however, whether these compounds will ultimately prove more efficacious to known standard drugs depends on future detailed evaluation in pharmacology, physiology and biochemistry.

CONCLUSION

The newly synthesized 7-MEOTA-donepezil like hybrids and N-alkylated tacrine analogues were evaluated as possible candidates for the treatment of Alzheimer disease in this study. The effect of all novel compounds was comparable, or even better at selected doses, to parent compounds tacrine and donepezil. In summary, the potential of novel ChEIs (especially compound 4) was proved and further detailed evaluation of these compounds, including investigation of toxicological and biochemical properties, is proposed.

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