In vitro effects of acetylcholinesterase inhibitors and reactivators on Complex I of electron transport chain

Jana Hroudová [1], Zdeněk Fišar [1], Jan Korábečný [2], Kamil Kuča [3,4]

1 Department of Psychiatry, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic
2 Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Hradec Králové, Czech Republic
3 Center of Advanced Studies, Faculty of Military Health Sciences, University of Defence, Hradec Králové, Czech Republic
4 University Hospital Hradec Králové, Hradec Králové, Czech Republic

Correspondence to: Assoc. Prof. Zdeněk Fišar, PhD.
Department of Psychiatry, First Faculty of Medicine,
Charles University in Prague and General University Hospital in Prague
Ke Karlovu 11, 120 00 Prague 2, Czech Republic.
TEL/FAX: +420 224 965 313; E-MAIL: zfisar@lf1.cuni.cz

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Abstract

OBJECTIVES: Inhibition of the enzyme acetylcholinesterase (AChE) is the main mechanism both of therapeutic action of drugs for the treatment of Alzheimer's disease and toxic action of organophosphorus compounds. Various types of oximes reactivate AChE and are commonly used as antidotes against organophosphates (pesticides, nerve agents).

METHODS: Effects both of AChE inhibitors (tacrine, 7-methoxytacrine) and oximes (pralidoxime, trimedoxime, obidoxime, methoxime, HI-6) on Complex I of electron transport chain (ETC) were examined. The enzyme activity was measured spectrophotometrically in crude mitochondrial fraction isolated from pig brain.

RESULTS: Our results showed statistically significant Complex I inhibition by tacrine, other drugs did not affect the enzyme activity significantly.

CONCLUSIONS: These observations suggest the possibility of tacrine-induced side effects related to disturbance in ETC. On the contrary, it seems that oximes do not affect cellular energetic metabolism.

Abbreviations:

AChE	- acetylcholinesterase
AD	- Alzheimer's disease
COX	- cytochrome c oxidase
ETC	- electron transport chain
7-MEOTA	- 9-amino-7-methoxy-1,2,3,4-tetrahydroacridine
NADH	- reduced nicotinamide adenine dinucleotide

ORIGINAL ARTICLE

INTRODUCTION

Cognitive impairment in Alzheimer's disease (AD) is treated with cholinesterase inhibitors, especially with reversible acetylcholinesterase (AChE, EC 3.1.1.7) inhibitors. The first AChE inhibitor licensed for AD therapy was tacrine, which was withdrawn from the market due to its toxicity. Current treatment of AD is predominantly based on donepezil, rivastigmine, and galantamine. These drugs are related to cholinergic central transmission, decrease the enzymatic degradation of acetylcholine by the inhibition of AChE, and increase both the concentration and persistence of acetylcholine in synaptic cleft (Nieoullon 2010). However, derivative of tacrine called 7-MEOTA (9-amino-7-methoxy-1,2,3,4-tetrahydroacridine) has been developed and further tested as AD modifying drug with better toxicity profile in comparison to tacrine (Filip *et al.* 1991; Korabecny *et al.* 2010a,b,c).

During recent years, AChE inhibitors were tested for their capacity to protect AChE from inhibition by organophosphorus compounds (Lorke *et al.* 2010). The current standard antidotal treatment includes oximes and atropine. Oximes effect as cholinesterase reactivators are applied in antidote therapy against organophosphates – nerve agents (e.g. sarin, tabun) and pesticides (e.g. paraoxon, chlorpyrifos) (Lorke *et al.* 2008; Oh *et al.* 2006). Currently, only five AChE reactivators (pralidoxime, trimedoxime, obidoxime, methoxime, HI-6) are worldwide used together with combined treatment with atropine and diazepam (Kuca *et al.* 2007). The effectiveness of oximes treatment is limited by different factors: reactivating properties and pharmacokinetics as well as sedation, artificial ventilation and other individual characteristics of oximes (Antonijevic & Stojiljkovic 2007).

Complex I of electron transport chain (ETC) plays a major role in controlling of oxidative phosphorylation and its abnormal activity can lead to defects in energy metabolism and thereby to changes in neuronal activity (Maurer *et al.* 2000; Pathak & Davey 2008). Furthermore, induction of oxidative stress is one of the causative factors in AD and Complexes I and III are the most responsible for production of reactive oxygen species (ROS) (Ezoulin *et al.* 2006; Reddy & Beal 2005). Complex I is the most pharmacologically affected from all ETC complexes. Haloperidol, chlorpromazine and fluphenazine inhibited Complex I activity, similarly antidepressants decrease its activity. Contrary, clozapine did not cause changes (Prince *et al.* 1997; Hroudova & Fisar 2010).

We investigated *in vitro* activities of tacrine, 7-MEOTA and five oximes on the NADH dehydrogenase (Complex I of ETC) activity. We suppose that *in vitro* effects of these drugs on Complex I activity provide us information about potential contribution of changes in cellular energetics to their therapeutic and/or adverse effects.

MATERIAL & METHODS

Pig brain mitochondria isolation

The mitochondria were isolated from pig brain cortex as described previously (Fišar *et al.* 2010; Fišar 2010). Briefly, the homogenate was centrifuged at 1000 g for 10 min to remove unbroken cells, nuclei and cell debris. The supernatant was carefully decanted; the pellet was resuspended in buffered sucrose and centrifuged again under the same conditions. Supernatants were collected and recentrifuged at 10000 g for 15 min. The final pellet containing mitochondria was washed twice with buffered sucrose (10 000 g, 15 min), resuspended to a protein concentration of 20–40 mg/ml, and stored at –70°C until the assay. Protein concentration was determined by the method of Lowry (Lowry *et al.* 1951), with bovine serum albumin as the standard.

Effect of drugs on Complex I (NADH dehydrogenase (ubiquinone), EC 1.6.5.3) activity

Crude mitochondrial extract was resuspended with hypotonic buffer (25 mmol/l potassium phosphate, 5 mmol/l $MgCl_2$, pH 7.2); further, they were repeatedly frozen and thaw two times to achieve the maximum of enzyme activity.

Complex I activity was determined as rotenone sensitive rate of NADH oxidation at 340 nm. Previously published method was used with a slight modification (Ragan *et al.* 1987; Folbergrová *et al.* 2007; Hroudova & Fisar 2010). The reaction mixture contained 25 mmol/l potassium phosphate (pH 7.2), 5 mmol/l $MgCl_2$, 2.5 mg/ml bovine serum albumin (BSA), 2 mmol/l KCN, 0.3 mmol/l NADH, 33 µmol/l decylubiquinone, and 150 µg/ml of sample proteins. Samples were incubated with selected oxime for 30 minutes at 30 °C, final drug concentration was 50 µmol/l for all drugs tested. Samples were measured in a total volume of 3 ml. The reaction was started by the addition of NADH and measured for 1 min, afterward rotenone was added in final concentration of 50 µmol/l, and the inhibited rate was measured for further 2 min.

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), drugs tested (pralidoxime, trimedoxime, obidoxime, methoxime, HI-6, tacrine and 7-MEOTA) were obtained from Department of Toxicology (Faculty of Military Health Sciences, University of Defence). Uvicon XL spectrophotometer (SECOMAM, Alès, France) was used for all assays.

Data analysis and statistics

Enzyme activities were evaluated as a slope of time dependence of absorbance of samples using LabPower Junior software (SECOMAM). Each independent measurement had a control, i.e. sample containing all components except for the drug. Relative changes of enzyme activities evoked by drugs were determined assuming that the activity of the control sample is equal to 100%. All data presented are expressed as the mean

± standard deviation. Results were analyzed by STATISTICA (data analysis software system, version 9.0, StatSoft, Inc., Tulsa, OK, USA). The Wilcoxon matched pairs test was used to calculate test statistics in order to compare the enzyme activities in samples with and without the drug.

RESULTS

Potency of tested drug in affecting of Complex I activity is summarized in the Figure 1. All drugs showed inhibitory effect on the Complex I activity; however, only tacrine induced statistically significant inhibition.

DISCUSSION

We evaluated Complex I activity of ETC after the interaction with AChE inhibitors (tacrine, 7-MEOTA) and reactivators (pralidoxime, trimedoxime, obidoxime, methoxime, HI-6). The study on the interaction of drugs with components of ETC is important for prediction of their potential influence on cellular energetics both in periphery and in the brain, i.e. on processes related to some therapeutic and/or adverse effects of the pharmacotherapy. The aim of the present study was to examine whether the interaction between selected inhibitors and reactivators of AChE and Complex I of ETC leads to changes in the NADH dehydrogenase activity. Mitochondria isolated from pig brain were used to study *in vitro* effects of these drugs.

It can be concluded that although our study indicates the interaction of oximes with Complex I of ETC, the extent of inhibition is relatively small compared to known Complex I inhibitors such as conventional antipsychotics, and the oxime interaction with Complex I seems not to be clinically significant. Drug concentration used in our experiment was near to maximal expected brain concentration of oximes. Results obtained with AChE inhibitors confirm that 7-MEOTA do not significantly inhibit NADH dehydrogenase activity. Tacrine was the only significant inhibitor of Complex I in our study (Figure 1).

Tacrine shows various side effects. In study with human hepatoma cell line Hep G2, tacrine caused increase of the citric acid cycle, which could be a signature of uncoupling of the oxidative phosphorylation (Niklas *et al.* 2009). Another study compared parameters leading to oxidative stress – differences were found between newly developed AChE inhibitor PMS777 and tacrine; PMS777 was able to fight inflammatory event whereas tacrine was able to minimize them (Ezoulin *et al.* 2007). It was shown that tacrine induces cytotoxicity both via inhibition of mitochondrial energization and by destabilization of membrane phospholipids associated with oxidative stress (Ezoulin *et al.* 2006). In our study, drug concentrations were higher (50 μmol/l); in spite of this fact, other drugs tested than tacrine have not influenced significantly Complex I activity.

According to cumulative evidences, mitochondrial insufficiencies contribute to pathology of AD: mitochondrial abnormalities, alterations in mitochondrial enzymes, and deficiency of cytochrome c oxidase (COX) have been observed (Bosetti *et al.* 2002; Cardoso *et al.* 2004; Gibson *et al.* 1998). Down-regulation of mitochondrial genes in Complex I was found in early as well as in definite AD brain specimens (Reddy & Beal 2005). Studies reported decreased Complex I activity in AD brains (Chandrasekharan *et al.* 1996; Parker *et al.*

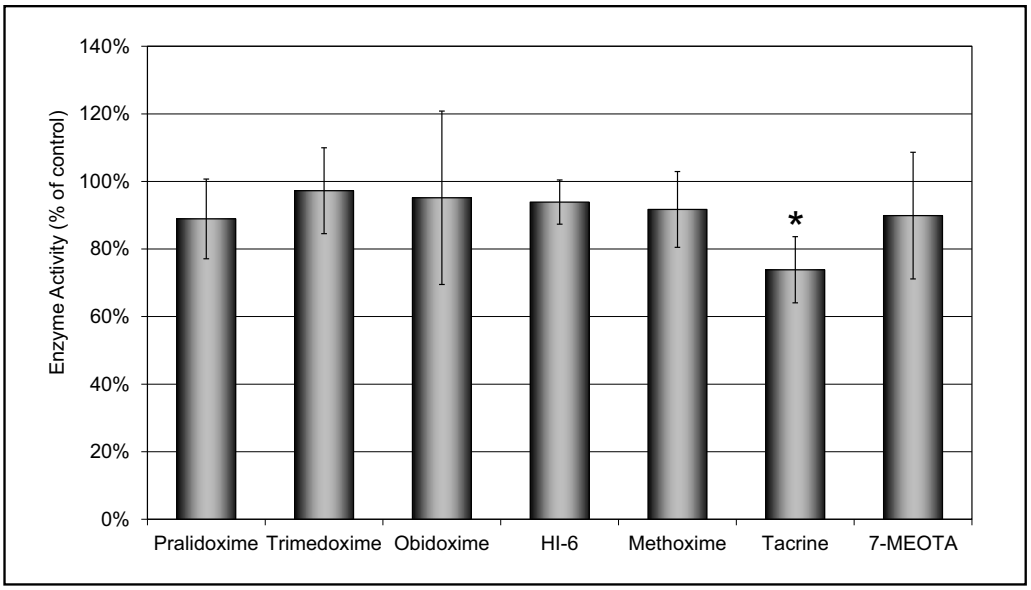

Fig. 1. Effects of acetylcholinesterase inhibitors (tacrine and 7-MEOTA) and reactivators (pralidoxime, trimedoxime, obidoxime, HI-6, and methoxime) on activity of the Complex I of electron transport chain. Values are means as ± standard deviation of at least 5 independent measurements; comparison between controls and drugs tested was determined using Wilcoxon matched paired test (*p<0.05).

1994), gene expression of ND4 – subunit of Complex I was found decreased in temporal cortex of AD patients (Fukuyama *et al.* 1996). Changes of the expression of mitochondrial and nuclear genes, encoding parts of COX and NADH dehydrogenase enzyme complexes, may contribute to alterations of oxidative metabolism in AD (Aksenov *et al.* 1999). Therefore, inhibition of this complex may be concerned with influence of energetic metabolism and was connected with possible extrapyramidal side effects caused often by haloperidol and chlorpromazine (Maurer & Möller 1997; Maurer *et al.* 2000).

CONCLUSIONS

Present results indicate that AChE reactivators pralidoxime, obidoxime, trimedoxime, methoxime and HI-6 can be most probably taken as relatively safe compound regarding to drug-induced changes in Complex I activity and related changes in cellular energetics. It corresponds to study that observed influence of oximes on mitochondrial COX activity and showed only slightly inhibited activity by 2-PAM (Sakurada *et al.* 2009). Contrary, the interactions with AChE inhibitor tacrine significantly affect the Complex I activity and we suppose that this effect can contribute to its adverse effects. Because of these data, more experiments will be done in order to get information about *in vivo* therapeutic and adverse effects of treatment with AChE inhibitors.

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REFERENCES

1 Aksenov MY, Tucker HM, Nair P, Aksenova MV, Butterfield DA, Estus S *et al.* (1999) The expression of several mitochondrial and nuclear genes encoding the subunits of electron transport chain enzyme complexes, cytochrome c oxidase, and NADH dehydrogenase, in different brain regions in Alzheimer's disease. Neurochemical Res. **24**: 767–774.
2 Antonijevic B, Stojiljkovic MP (2007) Unequal efficacy of pyridinium oximes in acute organophosphate poisoning. Clin Med Res. **5**: 71–82.
3 Bosetti F, Brizzi F, Barogi S, Mancuso M, Siciliano G, Tendi EA *et al.* (2002) Cytochrome c oxidase and mitochondrial F_1F_0-ATPase (ATP synthase) activities in platelets and brain from patients with Alzheimer's disease. Neurobiol Aging. **23**: 371–376.
4 Cardoso SM, Proença MT, Santos S, Santana I, Oliveira CR (2004) Cytochrome c oxidase is decreased in Alzheimer's disease platelets. Neurobiol Aging. **25**: 105–110.
5 Chandrasekharan K, Hatanppa K, Brady DR, Rapoport SI (1996) Evidence for physiological down-regulation of brain oxidative phosphorylation in Alzheimer's disease. Exp Neurol. **142**: 80–88.
6 Ezoulin MJ, Dong CZ, Liu Z, Li J, Chen HZ, Heymans F *et al.* (2006) Study of PMS777, a new type of acetylcholinesterase inhibitor, in human HepG2 cells. Comparison with tacrine and galanthamine on oxidative stress and mitochondrial impairment. Toxicol In Vitro. **20**: 824–831.
7 Ezoulin MJ, Liu Z, Dutertre-Catella H, Wu G, Dong CZ, Heymans F *et al.* (2007) A new acetylcholinesterase inhibitor with anti-PAF activity modulates oxidative stress and pro-inflammatory mediators release in stimulated RAW 264.7 macrophage cells. Comparison with tacrine. Int Immunopharmacol. **7**: 1685–1694.
8 Filip V, Vachek J, Albrecht V, Dvorak I, Dvorakova J, Fusek J *et al.* (1991) Pharmacokinetics and tolerance of 7-methoxytacrine following the single dose administration in healthy-volunteers. Int J Clin Pharmacol Ther Toxicol. **29**: 431–436.
9 Fišar Z (2010) Inhibition of monoamine oxidase activity by cannabinoids. Naunyn-Schmied Arch Pharmacol. **381**: 563–572.
10 Fišar Z, Hroudová J, Raboch J (2010) Inhibition of monoamine oxidase activity by antidepressants and mood stabilizers. Neuroendocrinol Lett. **31**: 645–656.
11 Folbergrová J, Ješina P, Drahota Z, Lisý V, Haugvicová R, Vojtíšková A *et al.* (2007) Mitochondrial complex I inhibition in cerebral cortex of immature rats following homocysteic acid-induced seizures. Exp Neurol. **204**: 597–609.
12 Fukuyama R, Hatanpää K, Rapoport SI, Chandrasekaran K (1996) Gene expression of ND4, a subunit of complex I of oxidative phosphorylation in mitochondria, is decreased in temporal cortex of brains of Alzheimer's disease patients. Brain Res. **713**: 290–293.
13 Gibson GE, Sheu KF, Blass JP (1998) Abnormalities of mitochondrial enzymes in Alzheimer disease. J Neural Transm. **105**: 855–870.
14 Hroudova J, Fisar Z (2010) Activities of respiratory chain complexes and citrate synthase influenced by pharmacologically different antidepressants and mood stabilizers. Neuroendocrinol Lett. **31**: 336–342.
15 Korabecny J, Holas O, Musilek K, Pohanka M, Opletalova V, Dohnal V *et al.* (2010a) Synthesis and in vitro evaluation of new tacrine derivates-bis-alkylene linked 7-MEOTA. Lett Org Chem. **7**: 327–331.
16 Korabecny J, Musilek K, Holas O, Binder J, Zemek F, Marek J *et al.* (2010b) Synthesis and *in vitro* evaluation of *N*-alkyl-7-methoxytacrine hydrochlorides as potential cholinesterase inhibitors in Alzheimer disease. Bioorg Med Chem Lett. **20**: 6093–6095.
17 Korabecny J, Musilek K, Holas O, Nepovimova E, Jun D, Zemek F *et al.* (2010c) Synthesis and *in vitro* evaluation of *N*-(brombut-3-en-2-yl)-7-methoxy-1,2,3,4-tetrahydroacridin-9-amine as a cholinesterase inhibitor with regard to Alzheimer's disease treatment. Molecules. **15**: 8804–8812.
18 Kuca K, Jun D, Bajgar J (2007) Currently used cholinesterase reactivators against nerve agent intoxication: comparison of their effectivity in vitro. Drug Chem Toxicol. **30**: 31–40.
19 Lorke DE, Nurulain SM, Hasan MY, Kuča K, Musilek K, Petroianu GA (2008) Eight new bispyridinium oximes in comparison with the conventional oximes pralidoxime and obidoxime: *in vivo* efficacy to protect from diisopropylfluorophosphate toxicity. J Appl Toxicol. **28**: 920–928.
20 Lorke DE, Hasan MY, Nurulain SM, Shafiullah M, Kuča K, Petroianu GA (2011) Pretreatment for acute exposure to diisopropylfluorophosphate: *in vivo* efficacy of various acetylcholinesterase inhibitors. J Appl Toxicol. doi: 10.1002/jat.1589.
21 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem. **193**: 265–275.
22 Maurer I, Möller H-J (1997) Inhibition of complex I by neuroleptics in normal human brain cortex parallels the extrapyramidal toxicity of neuroleptics. Mol Cell Biochem. **174**: 255–259.
23 Maurer I, Zierz S, Möller H-J (2000) A selective defect of cytochrome c oxidase is present in brain of Alzheimer disease patients. Neurobiol Aging. **21**: 455–462.
24 Nieoullon A (2010) Acetylcholinesterase inhibitors in Alzheimer's disease: further comments on their mechanisms of action and therapeutic consequences. Psychol Neuropsychiatr Vieil. **8**: 123–131.

25 Niklas J, Noor F, Heinzle E (2009) Effects of drugs in subtoxic concentrations on the metabolic fluxes in human hepatoma cell line Hep G2. Toxicol Appl Pharmacol. **240**: 327–336.
26 Oh KA, Yang GY, Jun D, Kuca K, Jung Y-S (2006) Bis-pyridiumaldoxime reactivators connected with $CH_2O(CH_2)_nOCH_2$ linkers between pyridinium rings and their reactivity against VX. Bioorg Med Chem Lett. **16**: 4852–4855.
27 Parker WD, Parks J, Filley CM, Kleinschmidt-DeMasters BK (1994) Electron transport chain defects in Alzheimer's disease brain. Neurology. **44**: 1090–1096.
28 Pathak RU, Davey GP (2008) Complex I and energy thresholds in the brain. Biochim Biophys Acta. **1777**: 777–782.
29 Prince JA, Yassin MS, Oreland L (1997) Neuroleptic-induced mitochondrial enzyme alterations in the rat brain. J Pharmacol Exp Ther. **280**: 261–267.
30 Ragan CI, Wilson MT, Darley-Usmar VM, Lowe PN (1987) Subfractionation of mitochondria and isolation of the proteins of oxidative phosphorylation. In: Darley-Usmar VM, Rickwood D, Wilson MT, editors. Mitochondria. A Practical Approach. London: IRL Press. p. 79–112.
31 Reddy PH, Beal MF (2005) Are mitochondria critical in the pathogenesis of Alzheimer's disease? Brain Research Reviews. **49**: 618–632.
32 Sakurada K, Ikegaya H, Ohta H, Fukushima H, Akutsu T, Watanabe K (2009) Effects of oximes on mitochondrial oxidase activity. Toxicol Lett. **189**: 110–114.