New performance of biosensor technology for Alzheimer´s disease drugs: *in vitro* comparison of tacrine and 7-methoxytacrine

Miroslav Pohanka [1,2], Kamil Kuca [1,2], Jiri Kassa [2]

1. Center of Advanced Studies, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic
2. Department of Toxicology, Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic

Correspondence to: Miroslav Pohanka, PhD.
Centre of Advanced Studies and Department of Toxicology,
Faculty of Military Health Science, University of Defense
Trebesska 1575, 500 01 Hradec Kralove, Czech Republic
TEL.: +420973251519
E-MAIL: rau@atlas.cz

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Abstract

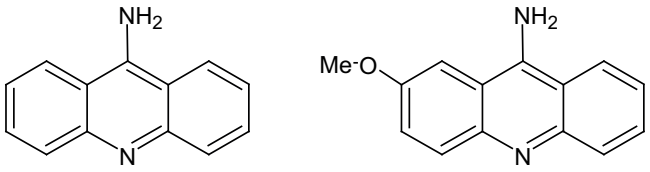
Two drugs were tested using electrochemical biosensor with immobilized acetylcholinesterase (AChE). The first was commercialized drug tacrine (known also as Cognex) used for treatment of cognitive manifestation of Alzheimer´s disease (AD). The second one was its 7-methoxy derivate (7-MEOTA) that has not been marketed. We determined the IC_{50} $(6.67\pm0.92)\times10^{-7}$ M for tacrine and $(1.66\pm1.43)\times10^{-9}$ M for 7-MEOTA. In this *in vitro* study, 7-MEOTA acts as stronger inhibitor of AChE and in this way could be more favorable for treatment of cognitive manifestation of AD. Our study shows that biosensor technology could be used as a quick and cheap tool for testing of promising AChE inhibitors (AD drug candidates).

INTRODUCTION

Tacrine (1,2,3,4-tetrahydroacridine-9-amine) is an reversible inhibitor of cholinesterases. It is also well known under brand name Cognex. When administered as drug, it penetrates into central nervous system and affects nerve synapses. It was found as an effective drug for treatment of cognitive manifestation of Alzheimer´s disease (AD; Dejmek, 1990; Tumiattit *et al*., 2008). Tacrine induced partial modulation of oxidative stress was described, too (Ezoulin *et al*., 2007). The depletion of nicotinic acetylcholine receptors (nAChRs) is one of proved biochemical mechanisms of AD (Whitehouse *et al*., 1982). Tacrine is able to act as effective molecular protector of nAChRs (Newhouse and Kelton, 2000; Takatory *et al*., 2006). However, mechanism of AD seems to be more complicated (Stuerenburg *et al*., 2006; Baranowska-Bik *et al*., 2008).

There is another similar drug to tacrine: 7-methoxytacrine (7-MEOTA). The chemical structures of tacrine and 7-MEOTA are presented in Figure 1; the only slight difference in one methoxy group is clearly visible. This drug was found effective when administered in the same way like

ORIGINAL ARTICLE



tacrine 7-MEOTA

Figure1. Chemical structures of tacrine and methoxy derivate 7-MEOTA

tacrine (Bajgar *et al*., 1979; 1994). Although the intensively testing of 7-MEOTA appointed at promising usability (Bajgar *et al*., 1995), this derivate has been neither commercialized nor advanced in extensive preclinical testing. Since the last studies were performed on the obsolete technologies using brain homogenates, we decided to compare tacrine and 7-MEOTA using an artificial system: electrochemical biosensor with immobilized pure acetylcholinesterase (AChE). This type of biosensors was found suitable for detection of organophosphates (Pohanka *et al*., 2008a,b), detection of aflatoxins (Pohanka *et al*., 2008c), and characterization of AChE reactivator drugs such as HI-6 (Pohanka *et al*., 2007a). Interaction of chosen biorecognition component and analyte could be evaluated just by biosensor. E.g. kinetics of association of monoclonal antibody and immobilized lipopolysaccharide antigen was obtained just in this way (Pohanka *et al*., 2007b).

In this work, electrochemical biosensor with AChE immobilized on a clean surface of platinum working electrode and acetylthiocholine chloride as substrate is considered for *in vitro* testing and comparing tacrine and 7-MEOTA inhibitory effect towards AChE.

MATERIAL AND METHODS

Chemicals and sensors

Lyophilized human recombinant AChE (2,000 IU/mg of protein), acetylthiocholine chloride (ATChCl), glutaraldehyde and bovine albumine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tacrine and 7-MEOTA were previously synthesized at the Department of Toxicology. The purity was estimated by HPLC prior to use. Phosphate buffered saline (PBS) was prepared using the standard procedures. All others reagents were obtained in the standard analytical purity. Three electrode screen printed sensors were obtained from BVT (Brno, Czech Republic). It contained platinum working (circle shaped with diameter 1 mm), Ag/AgCl reference, and platinum auxiliary electrodes.

Biosensor and biorecognition component immobilization

AChE was suspended into PBS up final activity 0.1 IU/μl. On the working electrode previously polished with ethanol, there were mixed 1 μl of AChE suspension, 1 μl of 1% glutaraldehyde, and 1 μl of BSA 1 mg/ml. Suspension and the electrode were replaced into a closed chamber, let to interact and slowly dry. After washing of surface with PBS and drying, the immobilization was repeated again. Prepared biosensor was used immediately or stored in a fridge for not more than two weeks.

Measuring protocol

The chosen principle of enzyme activity evaluation was based on two steps reaction. In the first step, ATChCl was enzymatically digested into acetic acid and thiocholine chloride. The second step was carried out by applied voltage (+450 mV). Thiocholine chloride concentration was evaluated chronoamperometrically. Thiol functional group was oxidized on working electrode. The dithiolcholine chloride was created in this reaction. The realization of measurement was following:

- 20 μl of 1 mM ATChCl was spread over the electrode.
- Signal (current in nA) was let to stabilize (approx. 10 s).
- 20 μl of tacrine or 7-MEOTA solution in given concentration was spread over electrode.
- Outputting signal was read 1 minute after tacrine (or 7-MEOTA) application.

The achieved data were processed using mathematical software Origin 6.1 (Northampton, MA, USA).

RESULTS AND DISCUSSION

Tacrine and 7-MEOTA were successfully assayed in the chosen concentrations (10^{-3}–10^{-14} M). Inhibition curves expressed as achieved current (nA) vs. drug concentration are presented as Figure 2. The Boltzmann

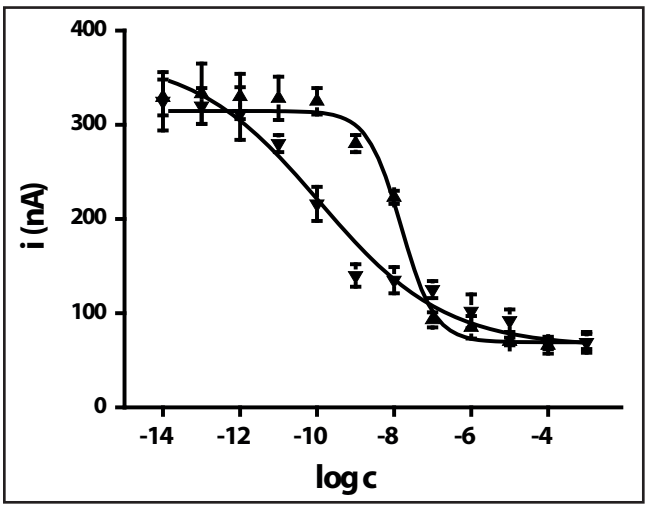


Figure 2. Inhibition of AChE immobilized onto screen printed electrodes by 7-MEOTA (▼) and tacrine (▲). Error bars indicate standard deviation (n=4). The experimental points were interpolated with Boltzmann function. Meaning of function is discussed in text.

function was found suitable for data fitting. It could be mathematically expressed as follows:

$$i = \frac{(i_1 - i_2)}{(1 + e^{(c - IC_{50})/dc})} + i_2 \qquad (1)$$

Where i_1 is the upper and i_2 is the bottom curve limit on y-axis (current). Concentration of tacrine or 7-MEOTA responds to *c*. IC_{50} responds to 50% inhibition of AChE. The last parameter *dc* correlates steepness of tangent in IC_{50} point. Unfortunately, we have not found relevant comparison using human AChE. Bajgar *et al*., (1995) used rat brain AChE. However, it seems to be either less sensitive to inhibition than the human brain or brain artifacts could scavenged 7-MEOTA. Own results carried out IC_{50} $(6.67\pm0.92)\times10^{-7}$ M for tacrine and $(1.66\pm1.43)\times10^{-9}$ M for 7-MEOTA.

The achieved values of IC_{50} appoint at fact that 7-MEOTA seems to be approximately 400 times stronger inhibitor. The effect of 7-MEOTA was quite gradual when the steepness of tacrine inhibition curve is considered. The first positively detected inhibition of AChE was evaluated as the point on the curve responding to triple noise of blank sample. For tacrine, it was approximately 10^{-8} M respectively 10^{-10} M for 7-MEOTA. These data appoint at very strong inhibition when other known inhibitors such as pesticides and aflatoxins are considered (Pohanka *et al*., 2008 b,c). These obtained data favor 7-MEOTA. Pertinent treatment of AD could be realized with the approximately 400 times lower dose of 7-MEOTA leading to significantly lower toxicological stress for the treated person and reduction of costs. In view of tacrine hepatotoxicity, 7-MEOTA could be considered as non-toxic replacement; however, with lower concentration needed for the same effect. The differences of *IC50* values confirm the older data achieved by Bajgar *et al*., (1994 – 1995) provided by *in vivo* models.

The steepness of inhibition curves around IC_{50} value is another important parameter. The slope in IC_{50} should be evident from the second equation:

$$i = \frac{i_1 - i_2}{4dc} c \qquad (2)$$

Meaning of symbols is the same as used in Eequation 1. The found slope was 143 nA.l.mol^{-1} for tacrine and 48 nA.l.mol^{-1} for 7-MEOTA. The higher steepness of curve for tacrine when compared with 7-MEOTA was expected. Vesela *et al*., (2006) achieved similar ratio of slopes for an *in vivo* model represented by *Daphnia magna* organisms.

Another experimental value was bottom limit of inhibition (i_2) and its comparison with the upper one (i_1). We found unambiguous agreement for the upper and bottom limits for the both compounds. Calculation of maximal inhibition provided value around 80% for both tacrine and 7-MEOTA. This fact was quite surprising when organophosphates inhibition effect was considered. E.g. paraoxon could inhibit immobilized AChE with the IC_{50} approximately equal to 7-MEOTA (Pohanka *et al*., 2007c). However, only about 50% of AChE was inhibited by paraoxon in concentration highly above IC_{50} (Pohanka *et al*., 2007c, 2008b). We could explain the higher maximal inhibition of AChE by tacrine and 7-MEOTA by interaction enzyme – inhibitor that is non-binding interaction and it represents a fast process. On the other hand, paraoxon is covalently bound in the active site of AChE. Kinetics of this interaction is completely different. The creation of covalent bond is slower and only short preincubation was used in the mentioned studies. Longer preincubation of AChE with paraoxon could cause higher inhibition.

Another object of this work was performance of in vitro method for fast, cheap but precision characterization of new drugs. The *in vitro* approach is an intriguing alternative for drug testing when typical *in vivo* drug testing considered (Palenicek *et al*., 2007; Kopecek *et al*., 2007). Further improvement of proposed method is expected. Especially, interaction kinetics is developed upgrade.

CONCLUSIONS

Two drugs applicable for treatments of AD cognitive manifestation have been investigated using biosensor system. The first drug was commonly available tacrine, the second one was 7-MEOTA that is not commercially available. Our preliminary results achieved by biosensor technology performance appointed at more than two orders higher inhibition of AChE by 7-MEOTA when *in vitro* compared with tacrine. According our achieved data, we could emphasize that 7-MEOTA provided better data for performance rather than tacrine; however, the *in vitro* study is not able to describe all parameters and extensive *in vivo* testing should provide final comparison. In another point of view, biosensor technology could be used for very quick and cheap testing of drug candidates for AD treatment in the future.

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