Journal of
APPLIED
BIOMEDICINE

J. Appl. Biomed.
4: 105–110, 2006
ISSN 1214-021X

ORIGINAL ARTICLE

Freshwater microcrustacean *Daphnia magna* Straus as an early screen model to compare toxicity of acetylcholinesterase inhibitors

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Received 1st November 2005.
Revised 3rd January 2006.
Published online 20th February 2006.

Summary
Daphnia magna is a freshwater microcrustacean which is often used for acute and chronic toxicity testing in aquatic ecotoxicology. Recently, tests with daphnids have been used to prescreen the toxicity of newly synthesized acetylcholinesterase reactivators (oximes), which appear as weak inhibitors of acetylcholinesterase (AChE). In our study we investigated and compared the toxicity of five reversible acetylcholinesterase inhibitors, two of them non ionic (tacrine and 7-MEOTA) and three ionic (berberine chloride, 1-methyltacridium iodide and ethidium bromide). Tests were carried out according to the methodology described in the standard EN ISO 6341. EC_{50} values as well as dose-response curves were calculated for all tested inhibitors. In general, non ionic substances were found to be less toxic than ionic substances. Estimated EC_{50} values in tests taking respectively 24 h and 48 h were as follows: tacrine 3000 resp. 1515 $\mu g.l^{-1}$, 7-MEOTA 3606 resp. 707.2 $\mu g.l^{-1}$, berberine chloride 903.6 resp. 822.4 $\mu g.l^{-1}$, 1-methyl-acridinium iodide 714.3 resp. 233.5 $\mu g.l^{-1}$ and ethidium bromide 644.3 resp. 291.1 $\mu g.l^{-1}$. The higher toxicity of 7-MEOTA in time compared to tacrine and the similar toxicity of berberine chloride in both tests are explained as a consequence of their metabolism in the daphnid's body.

Keywords: antidotes – cholinesterase – crustacean – inhibition – tacrine – toxicity

INTRODUCTION

Daphnids, commonly called water fleas, are freshwater microcrustaceans belonging to the class *Crustacea*, order *Cladocera* (Benzie 2005). The largest of all *Daphnia* species, *Daphnia magna*, is often used as an experimental animal for acute and chronic toxicity testing in aquatic ecotoxicology (Adema 1978, Cooney 2003). The main objectives of these tests are to determine the critical amount of toxicants or their mixtures for water ecosystems and to predict their influence and their fate. However, those tests and daphnids may be of use also in other areas of research, for example in veterinary medicine (De Bosschere et al. 2001) or experimental pharmacology (Postmes et al. 1989, Villegas-Navarro et al. 2003).

Recently, it was demonstrated that it is possible to use acute toxicity tests with daphnids to

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prescreen the toxicity of newly synthesized acetylcholinesterase reactivators (oximes) (Veselá et al. 2006). Oximes used in that study bind to the active surface of enzyme acetylcholinesterase (AChE) and appear like its weak inhibitors (Skrinjaric-Spoljar et al. 1999). As a further step in establishing tests with daphnids as an alternative method to be used to prescreen the toxicity of substances of known mechanism of action, we decided to investigate the toxicity of reversible inhibitors of AChE. To perform this, five substances – two of them non-ionic (tacrine and 7-MEOTA) and three of them ionic (berberine chloride, 1-methylacridinium iodide and ethidium bromide) – were chosen for investigation.

Of the chosen substances, tacrine is used as a therapeutic for the treatment of Alzheimer´s disease (Passmore and Patterson 2001); berberine is an alkaloid present in a number of clinically-important medicinal plants, including *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (coptis or goldenthread), *Berberis aquifolium* (oregon grape), *Berberis vulgaris* (barberry) and *Berberis aristata* (tree turmeric) [Berberine has been shown to exhibit multiple pharmacological effects such as antimicrobial, antidiarrheic and antiarrhytmic (Tsai and Tsai 2004)], and ethidium bromide is commonly used as a non-radioactive marker for identifying and visualizing nucleic acid bands in electrophoresis and in other methods of gel-based nucleic acid separation. The aim of the present study was twofold: to test and to compare the toxicities of non-ionic and ionic AChE inhibitors to juveniles of *Daphnia magna* and to evaluate the method to be used for further research in military toxicology.

MATERIAL AND METHODS

Chemicals and reagents

Tacrine (9-amino-1,2,3,4-tetrahydroacridine) and ethidium bromide were purchased from Sigma-Aldrich; berberine chloride was purchased from Fluka; 7-MEOTA (9-amino-7-methoxy-1,2,3,4-tetrahydroacridine) and 1-methylacridinium iodide were synthesized in the laboratory of the Department of Toxicology, Faculty of Military Health Sciences, University of Defense, Czech Republic (Dejmek 1990, Kuča et al. 2004). Stock solutions of all substances were prepared immediately before the start of tests by adding them to ultra pure wate (conductivity less than 5μS.cm^{-1}). The ISO medium was prepared according to EN ISO 6341 (1998). Chemicals used for its preparation ($CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$, KCl) were purchased from Lachner–Lachema (Czech Republic) in analytical grade. The water was ultra pure.

Culture of maternal animals

Neonates of *Daphnia magna*, clone HK (clone *a* sensu Baird et al. 1989) that originated from third to sixth brood females were used as mothers to produce test neonates. Mothers were kept in groups of 10 animals per 1000 ml of medium, at a room temperature 20±1 °C, with a light regime 16 h light/8 h dark and a food level of c. 2 mg C.l^{-1} of *Scenedesmus acutus* MEYEN.

Experiment methodology

In all tests, female juveniles from third to sixth brood no older than 24 hours were used. Test concentrations were subsequently prepared by adding an appropriate aliquot volume of the tested substance from the stock solution to the ISO medium. Concentration ranges consisting of enough treatments to get a good description of the whole dose-response curve were used. Per each treatment, three replicates of 7 animals were used. Experiments were run with reference to fittings of circadian biorythms (Berger 2003) at a light/dark regime of 16/8 h and temperature of 20±1 °C for 24 and 48 hours; no food was added. For more details of methodology and experimental conditions see EN ISO 6341 (EN ISO 1998).

The aim of the tests is to determine the median effective concentration, EC_{50}. This is defined as the concentration at which 50% of the exposed organisms are affected by the measured effect (Newman 1995). In our tests, the incapacitation of daphnids due to the toxic action was chosen as the adverse effect. Such animals are so immobilized that they cannot start swimming within 15 seconds after gentle shaking of the medium in the test beaker (EN ISO 1998).

Data analysis

EC_{50} values and dose-response curves were calculated by nonlinear regression with four parameter logistic equations (Motulsky and Christopoulos 2003) using the computer program GraphPad PRISM, version 4.0.

RESULTS

The relationship between percentage inhibition in daphnids, and substance concentration at the beginning of the test are shown in Fig. 2. This figure is divided into five graphs; each showing results for one substance. In each graph, the values of percentage inhibition in daphnids and fitted dose-response curves for both tests, one lasting 24 and one 48 h, are plotted. The largest increase between the slopes of the 24 and 48 h curves was observed for ethidium bromide, followed by 1-methylacridinium iodide and tacrine. In contrast, for

berberine chloride and less for 7-MEOTA, a decrease in the slopes was found.

Tests results are summarized in Table 1. The highest EC_{50} value (which means the lowest toxicity) from all tests lasting 24 h was found for 7-MEOTA followed by tacrine, the lowest for ethidium bromide. EC_{50} values of berberine chloride and 1-methylacridinium iodide were slightly higher than those of ethidium bromide.

Table 1. **EC_{50} values for reversible AChE inhibitors using *Daphnia magna* as test species in tests lasting 24 and 48 hours**

AChE inhibitor	EC_{50} after 24 h ($\mu g.l^{-1}$)			EC_{50} after 48 h ($\mu g.l^{-1}$)		
	EC_{50}	95% CL	Slope	EC_{50}	95% CL	Slope
Tacrine	3000.0	2753–3269	4.29	1515.0	1425–1612	6.97
7-MEOTA	3606.0	3411–3813	4.60	707.2	640.6–780.7	3.57
Berberine chloride	903.6	843.3–968.2	6.64	822.4	732.9–922.9	4.42
1-methylacridinium iodide	714.3	589.1–866.1	1.42	233.5	215.7–252.7	5.50
Ethidium bromide	644.3	566.5–732.9	2.17	291.1	274.7–308.5	8.28

The highest EC_{50} value in all tests lasting 48 h was found for tacrine, the lowest for 1-methylacridinium iodide. The EC_{50} value of ethidium bromide was close to that of 1-methylacridinium iodide, 95% CL overlapped. The EC_{50} values of berberine chloride and 7-MEOTA, 95% CL of those also overlapped, and were approximately at the middle of the EC_{50} values range found in all 48 hours tests.

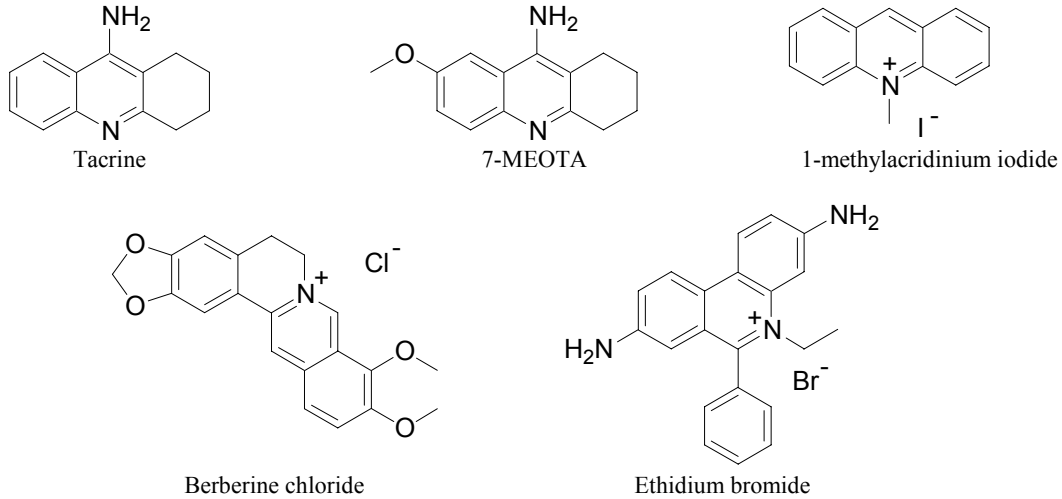

Fig. 1. **Chemical structures of five tested reversible AChE inhibitors**

The calculated 95% CL for ethidium bromide and 1-methylacridinium iodide overlapped, as well as those of 1-methylacridinium iodide and berberine chloride.

The EC_{50} values of all tested inhibitors decreased with time (Table 1, Fig. 2); the highest decrease was observed for 7-MEOTA and the lowest for berberine chloride.

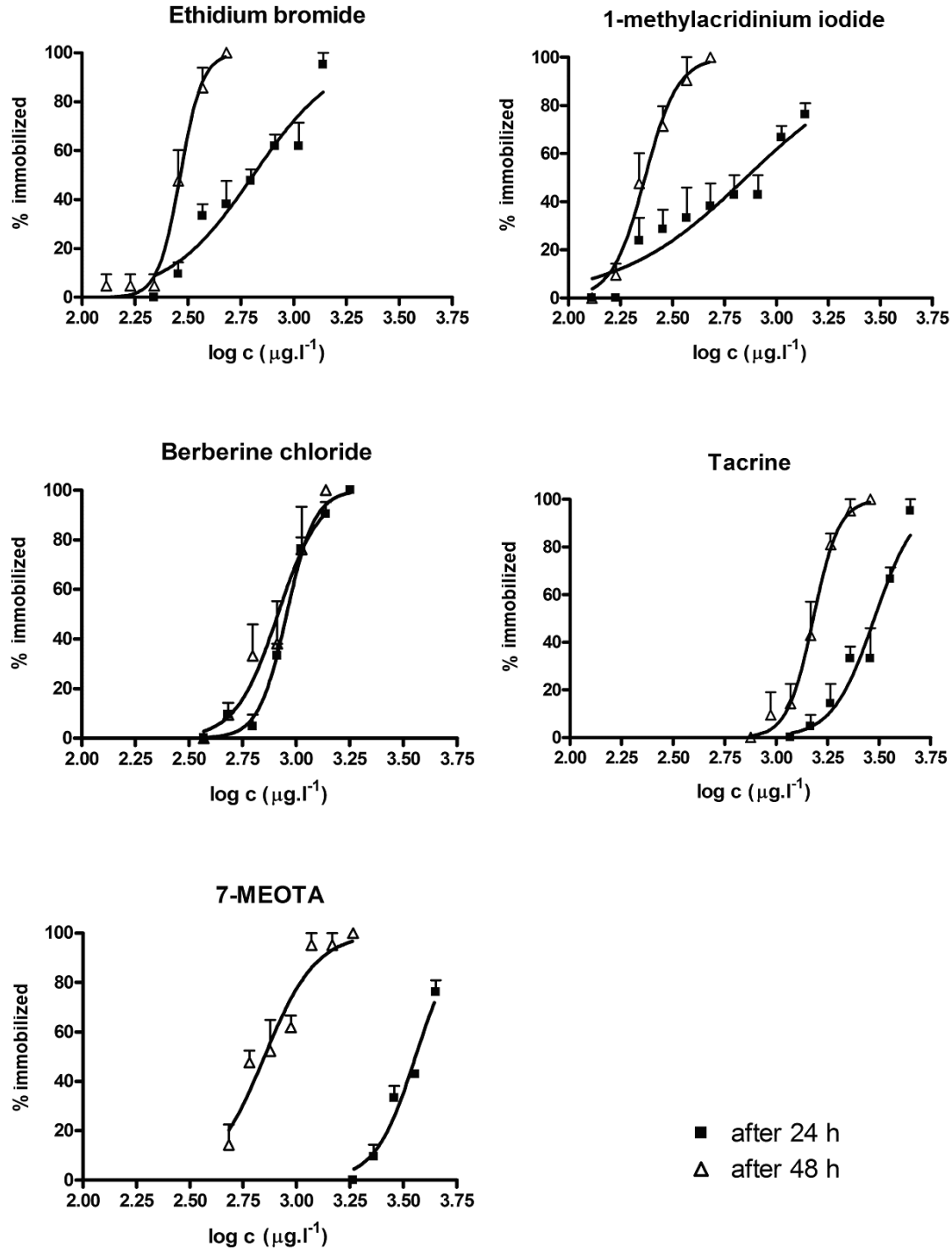

Fig. 2. **Relationship between percentage inhibition in daphnids and concentrations of tested AChE inhibitors at the beginning of tests taking 24 and 48 h.** Error bars represent + 1SD

DISCUSSION

Daphnids have a cholinergic nervous system. Their cholinesterase shows the characteristics of a pseudocholinesterase, since it prefers propionylthiocholine to acetylthiocholine. However, like mammalian AChE, it is inhibited by high concentrations of substrate (Guilhermino et al. 1996, Diamantino et al. 2003). Daphnids, therefore may be used as a model for the preliminary investigation of the toxicity of AChE inhibitors. It is also known that the value EC_{50} (median effective value for *Daphnia* immobility) after exposure to organophosphate and carbamate pesticides could correlate with IC_{50} of AChE inhibition (Printes and Callaghan 2004). This means that the inhibition potency of tested compounds could be expressed by EC_{50}.

Reversible inhibitors of AChE, depending on their concentration at the place of toxic effect, block molecules of enzyme AChE. This induces an increase of the actual concentration of acetylcholine on postsynaptic receptors followed by an increase in their stimulation (Pope et al. 2005). $EC_{50}(24)$ values of non ionic substances (tacrine and 7-MEOTA) which penetrate membranes and barriers, were found to be 3 – 4 times higher (which means those substances are less toxic) than EC_{50} values of non ionic substances. In 48 h tests, the EC_{50} value of tacrine was approximately 2 times higher than the values of 7-MEOTA and berberine chloride, and approximately 5 times higher than the EC_{50} values of 1-methylacridinium iodide and ethidium bromide. The difference in toxicity between ionic and non ionic substances can be explained by the penetratation of non ionic substances into cells where they bind to intracellular enzymes and other molecules and structures. As a consequence, there is a decrease in their concentration at the places of their toxic effect. Ionic substances are not expected to penetrate into the cells in significant amounts. The established results are in a good agreement with findings that the lipid solubility of a cholinesterase inhibitor and the metabolic fate influence the clinical picture of poisonings (Eddleston et al. 2005).

In experiments with mice and rats, the acute toxicity of 7-MEOTA was lower than the acute toxicity of tacrine (Dejmek 1990). The results of the 24 h experiment are in agreement with this finding, but, in the experiment lasting 48 h, the toxicity of tacrine was about half of that found for 7-MEOTA. This may indicate that the higher toxicity of 7-MEOTA in time may be caused by its slower or less effective elimination. In human and rats, the majority of 7-MEOTA is eliminated by urine and later by feces in unchanged form; the minority is metabolized (Dejmek 1990). In contrast, tacrine is rapidly eliminated through hepatic transformation mainly to the hydroxyl metabolite velnacrine (Passmore and Patterson 2001). In daphnids, the organ with functions similar to the mammalian liver is gut diverticula located at the anterior midgut region (Peters 1987).

Interesting results were found for berberine chloride. Its toxicity in tests running for 24 and 48 h was found to be similar, calculated 95% CL of EC_{50} values overlapped. In the 48 h test, the decrease in a slope of the fitted dose-response curve was found. This can be explained by the ability of juveniles of *Daphnia magna* to effectively eliminate or metabolize this substance. This also explains why the $EC_{50}(48)$ value found for the substance is higher than would be expected if compared to values of other ionic inhibitors. In rats, berberine chloride is intensively metabolized in the liver and excreted hepatobiliarly; only very small amounts are excreted in an unchanged form (Tsai and Tsai 2004).

The results obtained indicate that tests with daphnids can provide valuable information about tested substances and can be used satisfactorily as an alternative method to prescreen toxicity of substances of known mechanism of action. The need for the relative stability of the tested substance in water in order to exclude its breakdown during the test is the only limitation of the described methodology. This paper is the second of a series of papers describing experiments targeted to investigate daphnids as new animals to be used in prescreening tests in military toxicology. Because of this, we have focused only on estimating the EC_{50} values of tested substances and interpretation of results. Comparison of results found for daphnids with IC_{50} values of human AChE as well as other data will be the content of a following paper.

In summary, it was found that non ionic reversible AChE inhibitors are less toxic to *Daphnia magna* than ionic ones. The higher toxicity of 7-MEOTA in time if compared to tacrine and similar toxicity of berberine chloride in both tests is explained as a consequence of their metabolism in the daphnid's body. Tests with daphnids can provide valuable information and can be used to prescreen and compare the toxicity of the tested substances.

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