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Comparative *in vitro* study of the inhibition of human and hen esterases by methamidophos enantiomers

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ABSTRACT

The current Organisation for Economic Co-operation and Development (OECD) guidelines for evaluating organophosphorus-induced delayed neuropathy (OPIDN) require the observation of dosed animals over several days and the sacrifice of 48 hens. Adhering to these protocols in tests with enantiomers is difficult because large quantities of the compound are needed and many animals must be utilized. Thus, developing an *in vitro* screening protocol to evaluate chiral organophosphorus pesticides (OPs) that can induce delayed neuropathy is important. This work aimed to evaluate, in blood and brain samples from hens, human blood, and human cell culture samples, the potential of the enantiomeric forms of methamidophos to induce acetylcholinesterase (AChE) inhibition and/or delayed neurotoxicity. Calpain activation was also evaluated in the hen brain and SH-SY5Y human neuroblastoma cells. The ratio between the inhibition of neuropathy target esterase (NTE) and AChE activities by the methamidophos enantiomers was evaluated as a possible indicator of the enantiomers' abilities to induce OPIDN. The (−)-methamidophos exhibited an IC_{50} value approximately 6 times greater than that of the (+)-methamidophos for the lymphocyte NTE (LNTE) of hens, and (+)-methamidophos exhibited an IC_{50} value approximately 7 times larger than that of the (−)-methamidophos for the hen brain AChE. The IC_{50} values were 7 times higher for the human erythrocyte AChE and 5 times higher for AChE in the SH-SY5Y human neuroblastoma cells. Considering the esterases inhibition and calpain results, (+)-methamidophos would be expected to have a greater ability to induce OPIDN than the (−)-methamidophos in humans and in hens.

1. Introduction

Although the organophosphorus compounds (OPs), employed as insecticides exhibit preferential toxicity to insects, they are also toxic to humans and other animals due to the inhibition of AChE and the subsequent accumulation of acetylcholine at the neuron synapses (Johnson et al., 2000). In addition, some OPs can inhibit and age another esterase, known as the neuropathy target esterase (NTE) (Johnson, 1988), and cause a delayed effect that is known as organophosphorus-induced delayed neuropathy (OPIDN). OPIDN is characterized by a central–peripheral distal axonopathy and Wallerian-type degeneration that develops 8–14 days after poisoning by a neuropathic OP (Jortner et al., 2005). The OPs that cause OPIDN include phosphates, phosphonates and phosphoramidates. Some examples of compounds that have been reported to cause OPIDN include tri-o-cresyl phosphate (TOCP), methamidophos, mipafox, dichlorvos and leptophos (Johnson, 1975, 1981; Lotti, 1992).

However, the simple inhibition of NTE by OPs is not sufficient to cause OPIDN, which occurs along with the acute effects observed after AChE inhibition. Generating a negative charge on the terminal portion of the phosphate group bonded to the enzyme is also necessary and occurs as a result of a second reaction, known as "aging." In this step, the cleavage of one bond in the R—O—P chain and the loss of R lead to the formation of a charged mono-substituted phosphoric acid residue that is still attached to the protein. The "aging" reaction is possible when the OP has its radical R attached to the central phosphorus atom through a connection P—O—R or P—S—R. This reaction is called "aging" because it is a progressive process and the product is no longer responsive to nucleophilic reactivating agents (Glynn, 2000).

Abbreviations: OP, organophosphorus pesticide; OPIDN, organophosphorus-induced delayed neuropathy; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; NTE, neuropathy target esterase; LNTE, lymphocyte neuropathy target esterase; TOCP, tri-ortho-cresyl phosphate; CNS, central nervous system; IC_{50}, inhibitory concentration of 50% of enzyme activity; *ki*, bimolecular rate constant of inhibition; LD_{50}, median lethal dose; OECD, Organisation for Economic Co-operation and Development; ATCC, American Type Culture Collection; SD, standard deviation.

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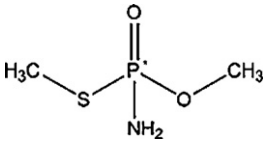

Fig. 1. Chemical structure of methamidophos. *Chiral center.

Current OECD guidelines (OECD 418, 1995; OECD 419, 1995) mandate the clinical observation of dosed animals for 21 or 48 days and the sacrifice of 48 hens as the experimental model for evaluating OPIDN. Following these protocols in tests with enantiomers is difficult because to obtain large quantities of these isomers is very exhaustive and expensive. Several *in vitro* methods using cultured neuroblastoma cells or tissue homogenates (blood and brain) are employed before the *in vivo* methods to avoid unnecessary expenses and excessive animal sacrifices (Fedalei and Nardone, 1983; Ehrich et al., 1997).

Methamidophos (O,S-dimethyl phosphoramidothioate), which contains an asymmetric center at the phosphorus atom and one radical attached to the central phosphorus through a connection P—O—R and the other through a connection P—S—R (Fig. 1), is an insecticide widely used in agriculture, both in developed and developing countries (Lin et al., 2006). Several previous studies have investigated the ability of methamidophos or its analogues to cause delayed neuropathy in hens (Vilanova et al., 1987; Johnson et al., 1989, 1991; Bertolazzi et al., 1991; Lotti et al., 1995). McConnell et al. (1999) provided a case report suggesting that lymphocyte NTE (LNTE) inhibition would predict OPIDN in patients who ingested methamidophos. They suggested that reference values of this esterase in lymphocytes could be used as a bioindicator of OPIDN in humans. However, the potential of the racemate methamidophos in inducing neuropathy could be greater in human than in hens. This was suggested by a study in which the racemate methamidophos was administered to hens without the development of neuropathy because the cholinergic crisis was so severe (Lotti et al., 1995). One possible explanation for the differential effects observed between humans and hens is the fact that this compound has a chiral center in its chemical structure and, thus, the compound exists as two enantiomers. When the racemic mixture reaches the bloodstream, the enantiomers exhibit different affinities for NTE and AChE (Bertolazzi et al., 1991). Furthermore, metabolic differences between these two species could favor a lower metabolism of the enantiomer with apparently much greater affinity for NTE in humans, and the opposite could be true in hens (Battershill et al., 2004).

Thus, the aim of this study was to evaluate, in the blood and brain of hens, in the blood of humans, and in SH-SY5Y human neuroblastoma cells the potential of the methamidophos enantiomers to induce delayed neurotoxicity using the ratio between NTE inhibition and AChE inhibition as a possible indicator. Mipafox was also used as a positive control because it is known as a compound that induces OPIDN. In addition, reference values for LNTE and AChE in erythrocytes are presented in a sample of donors not exposed to pesticides. Calpain activation was also evaluated because it has been suggested as contributor to OPIDN (El-Fawall et al., 1990; Glynn, 2000; Choudhary and Gill, 2001; Emerick et al., 2010).

2. Materials and methods

2.1. Chemicals

Sodium dodecyl sulfate (SDS), paraoxon, bovine serum albumin (BSA), Coomassie Brilliant Blue G-250, Histopaque-1077, tris(hydroxymethyl) aminomethane, ethylenediaminetetraacetic acid (EDTA), phosphoric acid 85%, acetylthiocholine (ACTh) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma, St. Louis, MO, USA; mipafox and phenyl valerate were obtained from Oryza Laboratories, Inc., Chelmsford, MA, USA; sodium citrate and triton X-100 were purchased from Rhiedel-de Haën, Hannover, Germany; 4-aminoantipyrine, potassium ferricyanide, and dimethylformamide were purchased from Merck, Darmstadt, Germany; heparin 25,000 IU/5 ml was obtained from Roche, Rio de Janeiro, Brazil; Deltametrin (K-otrine®) was obtained from Bayer Cropscience Ltd., Rio de Janeiro, RJ, Brazil; and piperazine citrate (Proverme®) was purchased from Tortuga Agrarian Zootechnical Company, São Paulo, Brazil. The analytical standard (±)-methamidophos was obtained from Sigma, St. Louis, MO, USA, and the enantiomeric separation was conducted according to the method described by Emerick et al. (2011). The enantiomers of methamidophos were obtained with 99.5% of optical purity for the (+)-methamidophos and 98.3% of optical purity for the (−)-methamidophos. Initially, mipafox was prepared at 0.1 mM concentration level, (+)-methamidophos was prepared at 1000 mM concentration level and (−)-methamidophos was prepared at 10,000 mM concentration level. All these solutions were prepared in absolute ethanol. These concentrates were then diluted at least 100× for incubation with neuroblastoma cells and other tissues to obtain a final concentration of 1% for ethanol. This solvent was chosen based on methamidophos solubility and on previous work that employed SH-SY5Y cells (Ehrich et al., 1997). All other chemicals employed in this study were of analytical grade.

2.2. Animals

Twelve *isabrown leghorn* hens (aged 70–90 weeks, weighing 1.5–2.0 kg) were obtained from the Hayashi farm cooperative of Guatapará, SP, Brazil. Before the experiments were initiated, the hens were treated to eliminate ecto-parasites and endo-parasites, as described elsewhere (DeOliveira et al., 2002; Emerick et al., 2010). After this treatment (1 month), the hens were housed at a density of 3 per cage in a temperature- and humidity-controlled room (24 ± 2 °C and 55% ± 10 RH) on an automatic 12:12 light–dark photocycle with lights activated at 8 a.m. Purina® feed and filtered tap water were provided *ad libitum*. All experimental procedures were conducted with the approval of the Research Ethics Committee of the School of Pharmaceutical Sciences of Araraquara, SP, Brazil in accordance with their guidelines for the care and use of laboratory animals (Resolution 24/2009).

2.3. Human volunteers

Blood was collected from 80 volunteers at the hemocenter of the School of Pharmaceutical Sciences of Araraquara – UNESP, SP, Brazil. Donors were invited to participate in this study after undergoing the standard screening required of all blood donors, and, after this first step, the purpose of this study was explained to them. After declaring that they accepted the terms of participation in the study, volunteers were invited to sign the Form of Consent and Statement of Grant for Biological Material that are requirements of 196/1996 Resolution of the Brazilian National Health Council. In addition to the various requirements that a blood donor must satisfy, we applied a questionnaire prior to screening to investigate the volunteers' habits. We asked the following key questions: Do you smoke? Are you taking any medicine? Did you drink any alcoholic beverages in the last two days? Did you have some contact with pesticides in the last 30 days? These questions were applied to reduce confounding factors. Next, an employee of the hemocenter collected approximately 5 ml of blood in heparinized tubes for vacuum collection. All of these procedures were conducted with the approval of the Research Ethics Committee of the School of Pharmaceutical Sciences of Araraquara, SP, Brazil in accordance with their guidelines for the care and use of humans in research (Resolution 09/2009).

2.4. Cell culture

SH-SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Passages 10–22 were used for these experiments. The human cells were grown in 15–20 ml F12 nutrient mixture (F12 HAM; Sigma Cell Culture, St. Louis, MO) containing 15% fetal bovine serum (FBS; Summit Biotechnology, FL Collins, CO) and 1% of an antibiotic–antimycotic solution (10,000 IU/ml penicillin, 10,000 μg/ml streptomycin, 25 μg/ml amphotericin B, Mediatech Inc., Manassas, VA) in 225-cm^2 flasks (Coming Costar Corporation, Cambridge, MA). Previous studies determined that these media provided optimal esterase activities for these cell lines (Ehrich et al., 1995). Cells were observed daily. To induce differentiation and maximize basal AChE activity, SH-SY5Y human neuroblastoma cells were treated with 10 μM retinoic acid when reaching 60–80% confluency. The SH-SY5Y cells remained in the retinoic acid-containing medium for 4 days before being harvested. To harvest SH-SY5Y cells, the medium was removed and the cells incubated in 3.0 ml of trypsin 0.5% (diluted in medium) for 5 min before being removed from the flask by pipetting. After harvesting, viability was determined by trypan blue exclusion to be >80%. Following centrifugation, the cells were resuspended in PBS at a concentration of 1×10^7 cells/ml and kept with the inhibitors for one hour before assays.

2.5. Sample collection

For determination of LNTE activity, 2.5 ml of blood were collected from the axillary veins of the hens in 3-ml syringes already containing 0.1 ml of heparin per ml