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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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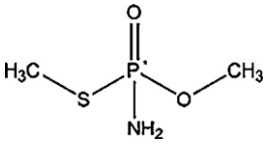

Fig. 1. Chemical structure of methamidophos. *Chiral center.

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

of blood (5000 IU/ml diluted 1/5 with 0.9% saline solution). For the determination of AChE and NTE activity in the brain of the hens, they were sacrificed by cervical torsion followed by decapitation. Next, a small amount (about 0.4 g) of tissue was extracted from the frontal part of the brain. This tissue was homogenized in the sodium phosphate buffer (0.1 M, pH 8.0) for the AChE assay and in the Tris buffer (50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0, 25 °C) for the NTE assay at a concentration of 1 g tissue to 20 ml of buffer. To measure the activity of AChE in human erythrocytes, 0.5 ml of whole blood was extracted, and erythrocytes were separated from the plasma by centrifugation (500 × *g*, 10 min). These erythrocytes were subsequently washed twice with 1.5 ml (3 times the volume of blood) of isotonic saline solution using the same spin cycle for plasma separation to avoid interference from other plasma esterases. After this step, the erythrocytes were diluted 1/600 in water for further analysis. For the determination of the LNTE activity of humans, 2.5 ml of blood was collected, as described above for the hens. Fifty microliters of 1×10^7/ml of cells were used as sample for the determinations of AChE and NTE in the human neuroblastoma cells.

2.6. Enzyme assays

To assay the LNTE activity, the lymphocytes were separated from the blood using Histopaque-1077® according to the Sigma diagnostic procedure. The lymphocytes and brains were diluted in a buffer (50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0, 25 °C) and their protein concentrations were determined by the method of Bradford (1976). The NTE and LNTE activity were assayed, as described by Correll and Ehrich (1991) using phenyl valerate as substrate. In addition, in the same volume of the sample (50 μL), 6 different concentrations of the OPs (ranging from 0.01 to 100 mM, see Section 2.1) were employed. The incubations were done for 1 h, at 37 °C.

The activity of cholinesterases was determined using the method described by Ellman et al. (1961), with 6 different concentrations of the OPs as inhibitors (ranging from 0.0001 to 10 mM in ethanol, see Section 2.1). The incubations proceeded for 1 h, at 37 °C. Four readings of each concentration were recorded at intervals of 60 s at 37 °C and 450 nm with constant stirring at 600 rpm in a UV/visible HP 8453 spectrophotometer. The absorbance used to calculate the enzyme activity was the average per min of these 4 readings. The concentrations of protein samples were evaluated using the Bradford method (1976) before the enzyme evaluations because all of the enzyme activities were reported in terms of μmol/min/g of protein.

Calpain activity in the chicken brain and neuroblastoma cells was analyzed as described elsewhere (Emerick et al., 2010), but before the assay, tissue homogenate was incubated with mipafox (0.01 mM) or (+)-methamidophos (10 mM) or (−)-methamidophos (100 mM) for one hour, at 37 °C. $CaCl_2$ in a concentration of 4 mM was added in the follow proportion: 1 g of tissue or 1 ml of cells (1×10^7/ml)/0.01 ml of OP in ethanol/1 ml of $CaCl_2$. The concentrations of OPs used were based on the NTE inhibition with concentration for each compound causing at least 80% NTE inhibition.

2.7. Statistical analyses

Inhibitor concentrations capable of inhibiting 50% of enzyme activity (IC_{50}) were determined using the equation of the line graph of the log of % activity *versus* the concentration of inhibitor (semilog plots). The semilog plots are not shown to avoid repetitions of results. The regression coefficients of these lines were calculated using the method of least squares. Differences in biochemical analyses were examined for statistical significance by one way ANOVA (Analysis Of Variance) followed by Tukey's test for multiple comparisons. These tests were performed in Microsoft Office Excel 2007 for Windows. The definition of significance was $p < 0.05$ for all statistical analyses. All biochemical data are presented as the averages of three samples done in triplicate ($n = 3$). All biochemical data are expressed as means ± the standard deviation (SD).

3. Results

3.1. Neuropathy target esterase

Control values for NTE and AChE activities in hens and humans are presented in Table 1. All of the coefficients of variation remained below 20%. AChE activity was not evaluated in the hens' erythrocytes because a previous study showed that this activity could not be detected (Wilson and Henderson, 1992).

The potencies of the isomers of methamidophos against NTE and LNTE differed. The inhibition curves of NTE in hens and humans are depicted in Fig. 2A, C, E and G and IC_{50} values are reported in Table 2. These data indicate that the (+)-methamidophos form was a more potent inhibitor of NTE than the (−)-methamidophos form. The (−)-methamidophos isomer exhibited an IC_{50} value approximately 5.6 times greater than did the (+)-methamidophos isomer for the LNTE activity of hen and approximately 4 times that observed for the inhibition of human LNTE activity. The percentage activity *versus* inhibitor concentrations exhibited high inverse regression coefficients for all NTE activities (Table 2). For the NTE in the hen brain and in the neuroblastoma cells the (−)-methamidophos isomer exhibited IC_{50} values approximately 41 and 160 times greater than did the (+)-methamidophos isomer (Table 2). Mipafox had a lower IC_{50} value for the hen brain and for the SH-SY5Y cells when compared to the isoforms of methamidophos (Fig. 2H and Table 2). Comparing the results of IC_{50} values for both species, it was possible to see that human cells (SH-SY5Y and lymphocytes) are more sensitive to the methamidophos enantiomers compared to tissues from hens. This was not true, however, for mipafox, as hen brain was more sensitive than SH-SY5Y cells (Fig. 2H).

3.2. Acetylcholinesterase

The curves of inhibition for AChE in the brain of hens are depicted in Fig. 2D and indicate that the isoform (−)-methamidophos was a more potent enzyme inhibitor than the (+)-methamidophos form. Human AChE in SH-SY5Y cells and erythrocytes (Fig. 2B and F) presented similar behavior to that of AChE in hen brains with the (−)-methamidophos form a more potent inhibitor than the (+)-methamidophos. The (+)-methamidophos isomer exhibited an IC_{50} value approximately 7 times greater than that of the (−)-methamidophos isomer for the inhibition of AChE in hen brain (Table 2). The lines of the log of percentage activity *versus* inhibitor concentration demonstrated strong inverse regression coefficients in all tissue tested (Table 2). Mipafox was used as a known inducer of OPIDN and presented a lower IC_{50} value for the chicken brain and an intermediate IC_{50} value for the SH-SY5Y cells compared to the isoforms of methamidophos (Fig. 2H and Table 2). Comparing the results of IC_{50} values for both species, it was noted that human cells (SH-SY5Y and erythrocytes) are more sensitive to the compounds tested in relation to hen tissues. These results are summarized in Table 2. The ratios of enzyme IC_{50} values presented in Table 2 show that the isoforms of methamidophos are stronger inhibitors for AChE than NTE. On the other hand, mipafox is a stronger inhibitor of NTE.

3.3. Calpain

Calpain activation was evaluated in hen brain and in the SH-SY5Y neuroblastoma cells. Although (+)-methamidophos exposure resulted in a small calpain activation, neither enantiomer of methamidophos was able to produce activation of calpain statistically different from control. In contrast, mipafox was able to induce a 5% increase in the calpain activity in hen brain and a 15% increase in the human cells (Fig. 3).

4. Discussion

The results of the present study demonstrated differences between the enantiomers of methamidophos in their ability to inhibit both NTE and AChE. This study also demonstrated that these differences could be determined *in vitro*. Enantioseparation has become an important tool in the discernment of the actual toxic agent responsible for a particular purpose. However, when neurotoxicity studies in animals require large quantities of the compounds in question, an initial *in vitro* screening is useful.

Although the sensitivities of NTE and AChE may be similar in hens and humans when a racemic mixture is tested, our results with the enantiomers of the methamidophos demonstrated that they may not be predicted or extrapolated from one species to another. One explanation may relate to metabolic differences between species. Methamidophos can cause a cholinergic crisis in hens so strong that it will be lethal before the onset of clinical signs of

Table 1
Summary of the normal activity of the enzymes NTE and AChE in human and hen tissues.

Assessed enzyme	Mean (μmol/min/g of protein)	Standard deviation	Coefficient of variation
LNTE in humans	7.0	1.2	17.1
NTE in SH-SY5Y cells	19.9	3.7	18.6
LNTE in hens	9.4	1.8	19.1
NTE in hen brains	29.3	4.4	15.1
AChE in human erythrocytes	6.9	1.3	18.8
AChE in SH-SY5Y cells	82.9	12.8	15.4
AChE in hen brains	978.2	101.4	10.4

$n = 12$ for hens and SH-SY5Y cells and 80 for humans. Each sample was assayed in triplicate.
AChE, acetylcholinesterase; LNTE, lymphocyte neuropathy target esterase.

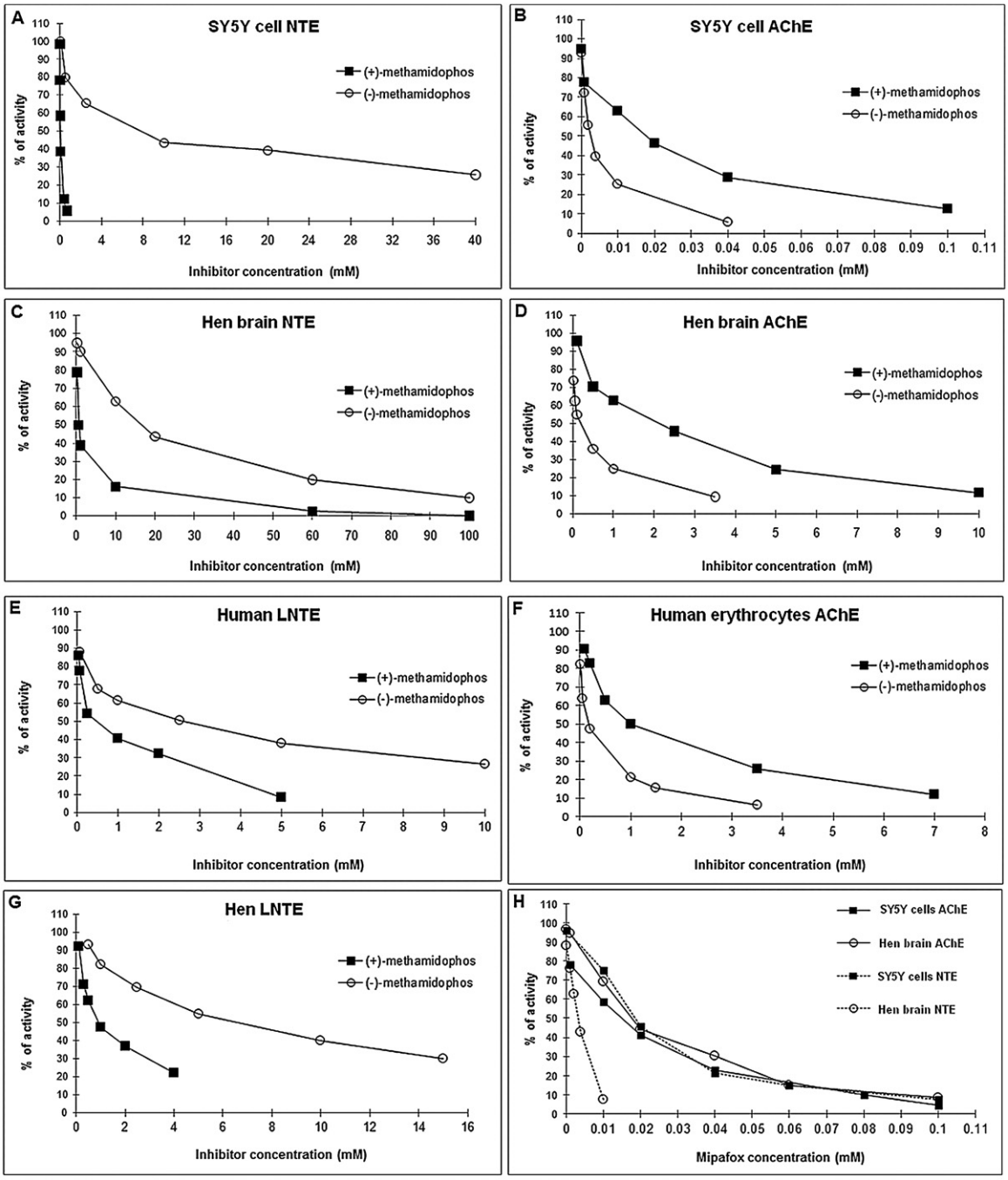

Figure 2

Fig. 2. Comparison among the curves of % of activity *versus* the concentrations of mipafox, (+)-methamidophos and (−)-methamidophos for NTE and AChE in hens and humans. Results for methamidophos are in (A)–(G); results for mipafox are in (H). Each point was obtained by averaging three samples. Each sample was analyzed in triplicate ($n = 3$). IC_{50} values and their SD are given in Table 2.

Table 2
Summary of the IC_{50} values and *R-squared* of NTE and AChE in human and hen tissues.

Assessed enzyme	Inhibitor	IC_{50} (mM)	*R*-squared	Ratio IC_{50} NTE/IC_{50} AChE
LNTE of humans	(+)-Methamidophos	0.862 ± 0.0710*	0.9725	0.55
	(−)-Methamidophos	3.43 ± 0.252	0.9278	**14.8**
LNTE of hens	(+)-Methamidophos	1.32 ± 0.202*	0.9385	
	(−)-Methamidophos	7.42 ± 0.285	0.9744	
NTE in the brain of hens	Mipafox	0.0027 ± 0.0008	0.9899	0.13
	(+)-Methamidophos	0.550 ± 0.0926*	0.9731	0.23
	(−)-Methamidophos	22.8 ± 3.24	0.9767	**66.1**
NTE in SH-SY5Y cells	Mipafox	0.0193 ± 0.0015	0.9538	1.27
	(+)-Methamidophos	0.0892 ± 0.0221*	0.9536	3.96
	(−)-Methamidophos	14.3 ± 2.32	0.9401	**3325**
AChE in the brain of hens	Mipafox	0.0206 ± 0.0025	0.9720	
	(+)-Methamidophos	2.42 ± 0.0091*	0.9814	
	(−)-Methamidophos	0.345 ± 0.0112	0.9661	
AChE in SH-SY5Y cells	Mipafox	0.0152 ± 0.0017	0.9926	
	(+)-Methamidophos	0.0225 ± 0.0031*	0.9543	
	(−)-Methamidophos	0.0043 ± 0.0010	0.9364	
AChE in human erythrocytes	(+)-Methamidophos	1.58 ± 0.061*	0.9683	
	(−)-Methamidophos	0.231 ± 0.0121	0.9246	

IC_{50} values are represented as means ± SD. These results were obtained with the means of three concentration-response curves.
AChE, acetylcholinesterase; LNTE, lymphocyte neuropathy target esterase; IC_{50}, inhibitory concentration for 50% of enzymatic activity.
* A statistically significant difference compared with the other isomer of methamidophos ($p < 0.05$, according to one way ANOVA followed by Tukey's test for multiple comparisons).

OPIDN. Therefore, in hens, the enantiomer with a higher affinity for AChE may be less metabolized than in other species, and the enantiomer that exhibits greater affinity for NTE may be less metabolized in humans. Studies done only with tissue from hens could lead to the erroneous conclusion that methamidophos does not induce OPIDN in humans. Therefore, the combination of *in vitro* studies on human and hen enzymes and studies of metabolism in hens could predict whether the OP is capable of generating OPIDN in both species (Battershill et al., 2004).

There are several research studies that describe calpain activation in hens after intoxication by a neuropathic OP (El-Fawall et al., 1990; Choudhary and Gill, 2001; Emerick et al., 2010). In Wallerian-type degeneration an excessive intake of calcium by the cell can activate calpain. This enzyme promotes digestion of the terminal portion of axons, preventing the transmission of nerve impulses to the post-synaptic cells (Moser et al., 2007). In the present work, an *in vitro* calpain assay demonstrates that only mipafox was able to promote calpain activation. This effect was greater with human neuroblastoma cells, probably because they are relatively pure compared to the multiple cell types found in a brain homogenate.

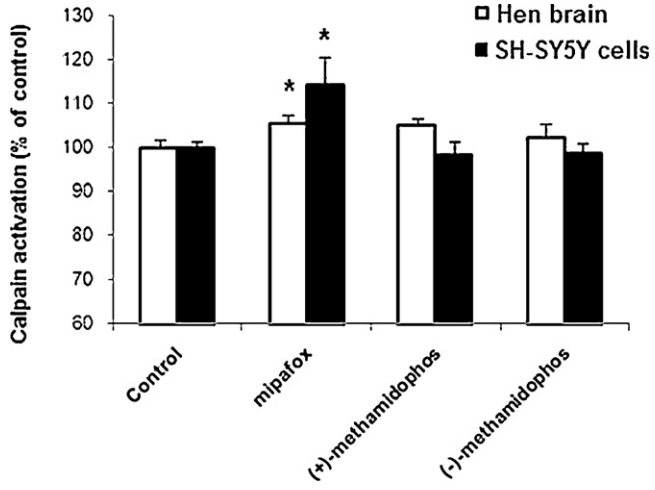

Fig. 3. Calpain activation caused by mipafox (0.01 mM), (+)-methamidophos (10 mM) and (−)-methamidophos (100 mM) in the hen brain and SH-SY5Y human neuroblastoma cells after 1 h of incubation at 37 °C. Each point was obtained by averaging three samples. Each sample was analyzed in triplicate ($n = 3$). *Values statistically different from controls ($p < 0.05$).

An early study by Ehrich et al. (1997) showed that capability to cause or not cause OPIDN could be predicted by ratios of the IC_{50} values in human and mouse neuroblastoma cells. Later, Sogorb et al. (2010) proposed an alternative methodology to predict whether an OP is able to induce OPIDN. This method is based on the comparison of the *in vitro* inhibition (and aging of NTE) of both enzymes (NTE and AChE) in human and hen cells. The authors tested 10 OPs (6 neuropathic and 4 non-neuropathic), and stated that if the IC_{50}NTE/IC_{50}AChE ratio is greater than five, then the compounds would not be able to induce the neuropathy. This was because the concentrations necessary for inhibition and aging of greater than 70% of NTE would not be compatible with the survival of individuals due to strong cholinergic crisis before the onset of delayed effects. However, if the IC_{50}NTE/IC_{50}AChE ratio is less than five, the OP may be a neuropathic compound if it has the ability to induce the "aging" reaction. Applying this hypothesis to the results of this *in vitro* study, we conclude that the (−)-methamidophos form would not be able to generate OPIDN in humans and hens, even if the aging reaction of NTE was to occur. However, other variables exist *in vivo*, such as differences in metabolism. Aging studies were not performed because prior works demonstrate that both enantiomers of methamidophos are weak inducers of NTE aging and that greater than 90% inhibition of NTE is required to induce OPIDN *in vivo* (Vilanova et al., 1987; Johnson et al., 1989; Sogorb et al., 1997; Kellner et al., 2000). However, the aging protocol is essential to make conclusions based on *in vitro* tests in an unknown chiral organophosphate.

Previous experiments using different species have demonstrated toxicological differences between the stereoisomers of methamidophos, noting differences in the potential to induce OPIDN (Senanayake and Johnson, 1982; Lotti et al., 1995; McConnell et al., 1999; Battershill et al., 2004). Using brain from human and hen Bertolazzi et al. (1991) examined the ratio between the inhibition constant of AChE and the inhibition constant of NTE.

The authors observed, as did the present study with IC_{50} values, that the *ki* AChE/*ki* NTE ratio of (−)-methamidophos was much higher than that observed for the other isomer. Thus, the most probable hypothesis is that the (+)-methamidophos form can induce OPIDN in humans and hens. However, further studies are necessary to determine if differences between the two species in their ability to induce OPIDN is related to metabolism or to the enantioselectivity of these compound for inhibiting and aging NTE and inhibiting AChE activities.

In conclusion, significant differences were observed between the IC_{50} values of the three isoforms of methamidophos regarding their *in vitro* inhibition of the activities of the NTE and AChE enzymes. The (−)-methamidophos form exhibited an IC_{50} value approximately 6 times greater than did the (+)-methamidophos form in inhibiting LNTE activity in chickens, and the (+)-methamidophos form demonstrated a IC_{50} value approximately 7 times greater than that of the (−)-methamidophos form in inhibiting hen AChE activity. Differences between species were noted, as human esterases showed more sensitivity than hen esterases to both enantiomers. The model of SH-SY5Y human cells showed the higher difference between the NTE inhibition of methamidophos enantiomers and the hen brain showed the higher difference between the AChE inhibition of methamidophos enantiomers. Finally, considering only the *in vitro* results (NTE and AChE inhibition), the (+)-methamidophos form exhibited a greater potential to induce OPIDN than did the (−)-methamidophos form both for humans and for hens. However, this potential in inducing OPIDN was lower than the potential observed with mipafox considering NTE and AChE inhibition and calpain activation as indicators.

Conflict of interest

There are no conflicts of interest.

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