SHORT COMMUNICATION

Effects of Physostigmine on the Pharmacokinetics of Intravenous Parathion in Rats

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ABSTRACT: It was reported that the area under the plasma concentration-time curve from time zero to time infinity (AUC) of parathion was significantly smaller, and the time-averaged total body clearance (Cl) of parathion was significantly faster after intravenous administration of parathion to rats pretreated with dexamethasone than those in control rats. This was supported by significantly faster intrinsic clearance of parathion to form paraoxon in hepatic microsomal fraction of rats pretreated with dexamethasone. The above data suggested that parathion was metabolized to paraoxon by dexamethasone-inducible hepatic cytochrome P450 (CYP) 3A in rats. The purpose of this study is to explain the protective effects of physostigmine against paraoxon toxicity by suppressing CYP3A, and hence, decreasing formation of a toxic metabolite, paraoxon. The pharmacokinetic changes of parathion and paraoxon were investigated after intravenous administration of parathion, 3 mg/kg, to control Sprague–Dawley rats, and the rats pretreated with physostigmine (100 µg/kg, intraperitoneal injection 30 min before parathion administration). After a 1-min intravenous infusion of parathion to rats pretreated with physostigmine, the AUC of parathion (60.4 compared with 73.7 µg min/mL) was significantly greater, Cl of parathion (49.7 compared with 40.7 mL/min/kg) was significantly slower, and amount of paraoxon recovered from liver, mesentery and large intestine at 5 min was smaller than those in control rats. Based on in vitro rat hepatic microsomal studies, physostigmine inhibited significantly the erythromycin N-demethylase activity (1.03 compared with 0.924 nmol/mg protein/min), mainly mediated by hepatic cytochrome P450 3A in rats. The above data suggested that the formation of paraoxon was inhibited in rats pretreated with physostigmine by inhibiting CYP3A. Copyright © 2000 John Wiley & Sons, Ltd.

Key words: CYP3A; paraoxon; parathion; pharmacokinetics; physostigmine; rats

Introduction

Parathion was one of the toxic organophosphorus insecticides usually used for agricultural purposes. In animals, biotransformation of parathion involves a deactivating pathway to form diethylphosphorothioate and p-nitrophenol, and an activating pathway to form paraoxon through hepatic cytochrome P450 (CYP)-dependent oxidative desulphuration followed by a deactivating pathway to form diethylphosphate and p-nitrophenol by hydrolysis [1,2]. The acute mammalian toxicity of parathion results from paraoxon, a potent and irreversible inhibitor of acetylcholinesterase.

It was reported [3] that physostigmine (100 µg/kg, intraperitoneal injection) inhibited acetylcholinesterase and cholinesterase activities in rat blood, brain and lung when the enzyme activities were measured 30 min after pretreatment with physostigmine and they were reactivated rapidly. Pretreatment with physostigmine
(100 μg/kg, intraperitoneal injection) decreased the mortality of rats challenged with a lethal dose of parathion (4 mg/kg, intraperitoneal injection), either when treated alone or in combination with atropine (10 mg/kg, intraperitoneal injection) [3]. As protection against parathion toxicity was evident, even when this organophosphate was injected into rats of which acetylcholinesterase had been depressed by the carbamate, it was suggested that simple competition between paraoxon, an active form of parathion, and the carbamate may not be the sole underlying mechanism of protection provided by physostigmine [3].

It was reported from our laboratories [4] that the area under the plasma concentration–time curve from time zero to time infinity (AUC) of parathion was significantly smaller and the time-averaged total body clearance (Cl) of parathion was significantly faster after intravenous administration of parathion to rats pretreated with dexamethasone than those in control rats. This was supported by in vitro intrinsic clearance (Clint) of parathion to form paraoxon in hepatic microsomal fraction; the Clint in rats pretreated with dexamethasone was significantly faster than that in control rats [4]. The above data suggested that parathion was metabolized to paraoxon by dexamethasone-inducible hepatic CYP3A23, the induction of which was confirmed by Western blot analysis [4]. It was also reported [5] that the major dexamethasone-inducible form of CYP3A in rats was CYP RL33/cDEX. The purpose of this study is to explain the protective effects of physostigmine against parathion toxicity in terms of pharmacokinetics, by suppressing CYP3A and hence decreasing formation of a toxic metabolite, paraoxon, by physostigmine using rats as an animal model.

### Materials and Methods

#### Chemicals

Parathion (diethyl-p-nitrophenyl phosphorothioate, purity of 99.2%) was purchased from Chem Service (West Chester, PA, USA). Paraoxon (diethyl-p-nitrophenyl phosphate), physostigmine sulphate, β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH) and erythromycin were purchased from Sigma Chemical Company (St. Louis, MO, USA). YH439 [isopropyl 2-(1,3-dithioetane-2-ylidene)-2-(N-4-methylthiazol-2-yl)carbamoylacetate], an internal standard of high-performance liquid chromatographic (HPLC) assay, was donated by Yuhan Research Center of Yuhan Corporation (Kunpo, Republic of Korea). Other chemicals were of reagent grade or HPLC grade and, therefore, were used without further purification.

#### Animal Pretreatment

The protocol of animal study was approved by Animal Care and Use Committee of College of Pharmacy, Seoul National University (Seoul, Republic of Korea). Male Sprague–Dawley rats (weighing 250–360 g) purchased from Charles River Company (Atsugi, Japan) were randomly divided into control rats and rats pretreated with physostigmine. The rats were housed in a light-controlled room (light 07:00–19:00 h, dark 19:00–07:00 h) kept at a temperature of 22 ± 2°C and a humidity of 55 ± 5°C (Animal Centre for Pharmaceutical Research, College of Pharmacy, Seoul National University, Seoul, Republic of Korea). The rats were fed food (Agribrands Purina Korea, Seoul, Republic of Korea) and tap water ad libitum. The pretreatment procedures and cannulation methods of the carotid artery and the jugular vein for each rat were similar to those reported previously [6].

#### Pretreatment of Physostigmine

Thirty minutes prior to parathion administration, physostigmine sulphate was dissolved in water for injection and was injected (100 μg/kg as free physostigmine) once intraperitoneally in rats [3]. Total injection volume was 2 mL/kg. The same volume of water for injection was also injected intraperitoneally to control rats.

#### Intravenous Study

Parathion (1.2 μL) was dissolved in 1 mL of polyethylene glycol 400, and 3 mg/kg was infused for 1 min via the jugular vein of control rats (n = 7) and rats pretreated with physostigmine (n = 9). The total injection volume was
approximately 0.6 mL. Blood samples (0.22 mL) were collected via the carotid artery at 0 (to serve as a control), 1 (at the end of infusion), 5, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420 and 480 min following parathion administration. Approximately 0.25 mL of heparinized 0.9% NaCl injectable solution (20 units/mL) was used to flush the cannula after each blood sampling to prevent blood clotting. Blood samples were centrifuged immediately, and a 100-μL aliquot of each plasma sample was mixed with pre-mixed extraction solvent (1 mL of ethyl acetate, 150 μL of distilled water and 50 μL of internal standard, YH439) to minimize degradation of paraoxon. The mixture was stored in a −70°C freezer (model DF8517, Ilshin Lab Company, Seoul, Republic of Korea) until HPLC analysis of parathion and paraoxon [4]. At the end of 8 h (after intravenous administration of parathion), each rat was sacrificed by cervical dislocation, and the abdomen was opened. The entire gastrointestinal tract (including its contents and faeces) was removed, transferred to a beaker containing 30 mL of methanol (to facilitate the extraction of parathion and paraoxon), and cut into small pieces using scissors. After stirring with a glass rod, two 100-μL aliquots of the extract were collected from each beaker and stored in a −70°C freezer until HPLC analysis of parathion and paraoxon [4]. At the same time, the metabolic cage was rinsed with 15 mL of distilled water, and the rinsings were combined with urine sample. After measuring the exact volume of the combined urine sample, two 100-μL aliquots of the combined urine sample were stored in a −70°C freezer until HPLC analysis of parathion and paraoxon [4]. Small portions of liver, lung and brain were fixed in 10% neutral phosphate-buffered formalin, and were processed for routine histological examination by haematoxylin-eosin staining. The 8-h bile juice was collected after intravenous administration of parathion, 3 mg/kg, to eight control rats with bile duct cannulation.

Thirty minutes after intraperitoneal injection of physostigmine (n = 4) or water for injection (n = 4), plasma was collected and plasma protein binding of parathion was measured at parathion plasma concentration of 5 μg/mL using an equilibrium dialysis technique [7].

**Measurement of Hepatic Microsomal CYP3A Activity**

Rats were sacrificed 30 min after intraperitoneal injection (total injection volume was 2 mL/kg) of physostigmine (the physostigmine sulphate powder was dissolved in water for injection), 100 μg/kg as free physostigmine (n = 8) or water for injection (n = 8). For the measurement of microsomal CYP3A enzyme activities, liver was homogenized in an ice-cold buffer of 0.154 M KCl/50 mM Tris–HCl in 1 mM ethylenediamine tetracetate (EDTA), pH 7.4. The homogenate was centrifuged at 12 000 × g for 20 min, and the supernatant fraction was further centrifuged at 104 000 × g for 60 min. The microsomal pellet was resuspended in buffer of 0.154 M KCl/50 mM Tris–HCl in 1 mM EDTA, pH 7.4 and recentrifuged at 104 000 × g for 60 min. The concentration of CYP was estimated from carbon monooxide difference spectrum [8]. Protein content was measured using the reported method [9]. The activity of erythromycin N-demethylase, which is primarily catalysed by CYP3A in rats [10], was determined at 37°C for 10 min in an incubation mixture containing 0.5 mM substrate, 1.0 mg of microsomal protein and 1 mM of NADPH in a final volume of 1 mL of 0.1 M Tris–HCl, pH 7.4. The reaction was terminated by addition of 0.3 mL of ice-cold 20% trichloroacetic acid. The formaldehyde produced was determined spectrophotometrically at 412 nm [11].

**Tissue Distribution Study**

Parathion (the same solution as used in the intravenous study), 3 mg/kg, was administered intravenously to control rats and rats pretreated with physostigmine. At 5 (n = 6, each), 30 (n = 5, each) and 120 (n = 5, each) min after administration of parathion, as much blood as possible was collected via the carotid artery and each rat was sacrificed by cervical dislocation. Approximately 1 g each of liver, lung, brain, kidney, heart, muscle, fat, mesentery, stomach, small intestine and large intestine was excised, rinsed with cold 0.9% NaCl injectable solution to eliminate blood remaining in the tissues, blotted dry with paper tissue and homogenized with 2 vol of methanol (to facilitate the extraction of parathion and
paraoxon) using tissue homogenizer (Ultra-Turrax T25, Janke and Kunkel, IKA-Labortechnik, Staufen, Germany). After centrifugation, a 50-μL aliquot of plasma or the 9000 × g supernatant fraction of each tissue was stored in a −70°C freezer until HPLC analysis of parathion and paraoxon [4]. All procedures were conducted at 4°C on an ice-bath.

Pharmacokinetic Analysis
The AUC was calculated by the trapezoidal rule-extrapolation method; this method employed the logarithmic trapezoidal rule for the calculation of the area during the declining plasma-level phase [12] and the linear trapezoidal rule for the rising plasma-level phase. The area from the last data point to time infinity was estimated by dividing the last measured plasma concentration by the terminal rate constant.

Standard methods [13] were used to calculate the following pharmacokinetic parameters; the Cl, area under the first moment of the plasma concentration-time curve (AUMC), mean residence time (MRT) and apparent volume of distribution at steady state (Vss) [6].

The mean values of Cl [14], Vss [15] and terminal half-life [16] were calculated by the harmonic mean method.

Statistical Analysis
Levels of statistical significance were assessed using the t-test between two means for unpaired data. Significant differences were judged as a p value of less than 0.05. All data were expressed as mean ± standard deviation.

Results
After intravenous administration, the mean arterial plasma concentrations of parathion declined in a polyexponential fashion for both groups of rats (Figure 1) with mean terminal half-lives of 210 and 277 min for control rats and rats pretreated with physostigmine, respectively (Table 1); this difference was significant (p < 0.05). The plasma concentrations of parathion declined fast during the first hour and declined slowly thereafter for both groups of rats (Figure 1). The fast declining of plasma concentrations could be a result of the rapid distribution of parathion into tissues (the tissue-to-plasma (T/P) ratios of parathion were greater-than-unity at 5 min in all rat tissues studied, as will be discussed in the tissue distribution study) and rapid elimination of parathion from the plasma (Cl of 49.7 and 40.7 mL/min/kg for control rats and rats pretreated with physostigmine, respectively, Table 1). Similar results were also obtained from other rat studies [17].

Some pharmacokinetic parameters of parathion were changed by pretreatment with physostigmine. For example, in rats pretreated with physostigmine, the AUC was greater (22% increase, p < 0.05) than that in control rats, and this was a result of the slower Cl of parathion (18% decrease, p < 0.05) in the rats (Table 1). In rats pretreated with physostigmine, the Vss was larger (40% increase, p < 0.05) than that in control rats (Table 1), and the reason is not clear. However, this could not be mainly a result of the increase in the unbound fraction of parathion in plasma; the unbound fraction of parathion in plasma was comparable between control rats (3.56 ± 0.565%) and rats pretreated with physostigmine (3.20 ± 0.854%). In rats pretreated with physostigmine, the larger Vss and slower Cl of parathion resulted in longer terminal half-life (32% increase, p < 0.05) and MRT.
demethylase activity, which is primarily water-bath shaker kept at 37°C up to 8-h incubation. More than 98.0% of the spiked amount of parathion were recovered for 50 oscillations per min; more than 98.0% of the juice at parathion concentration of 10 mg/L were excreted in 8-h bile. Parathion was fairly stable for up to 8-h incubation with fresh rat bile juice at parathion concentration of 10 μg/mL in a water-bath shaker kept at 37°C, and at a rate of 50 oscillations per min; more than 98.0% of the spiked amount of parathion were recovered for up to 8-h incubation.

Physostigmine inhibited erythromycin N-demethylation activity, which is primarily catalysed by CYP3A in rats [10]; the activities were 1.03 ± 0.0642 and 0.924 ± 0.0865 nmol/mg protein/min for control rats and rats pretreated with physostigmine, respectively (p < 0.05). There were no significant differences in cytochrome P450 contents and p-nitrophenol hydroxylase activity (which represents the level of CYP2E1) between two groups of rats.

Parathion had a good affinity to all rat tissues studied; the T/P ratios were greater-than-unity in all rat tissues studied (Figure 2) and this could be supported by considerably large values of V_{ss} of 4550 and 6360 mL/kg for control rats and rats pretreated with physostigmine, respectively (p < 0.05). Note that, in control rats, the amounts of parathion in brain tissues were 22.2 and 45.6 times, respectively, greater than those in liver (Figure 2), and similar explanation could also be applied, as mentioned above concerning the brain. Tissue distribution of parathion was not significantly different between control rats and rats pretreated with physostigmine in all rat tissues studied (Figure 2). After intravenous administration of parathion, paraoxon was detected in liver, lung, fat, mesentery and large intestine at 5 min, and only in fat and mesentery at 30 and 120 min (Table 2). High

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control rats (n = 7)</th>
<th>Rats pretreated with physostigmine (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>324 ± 19.5</td>
<td>303 ± 21.1</td>
</tr>
<tr>
<td>AUC (μg min/mg)</td>
<td>60.4 ± 6.91</td>
<td>73.7 ± 10.2*</td>
</tr>
<tr>
<td>Terminal half-life (min)</td>
<td>210 ± 47.8</td>
<td>277 ± 56.1*</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>95.8 ± 27.1</td>
<td>170 ± 52.5**</td>
</tr>
<tr>
<td>V_{ss} (mL/kg)</td>
<td>4550 ± 1140</td>
<td>6360 ± 1890*</td>
</tr>
<tr>
<td>Cl (mL/min/kg)</td>
<td>49.7 ± 5.53</td>
<td>40.7 ± 5.54*</td>
</tr>
<tr>
<td>Amount excreted in 8-h urine (% of intravenous dose)</td>
<td>0.0626 ± 0.0534</td>
<td>0.239 ± 0.145***</td>
</tr>
<tr>
<td>Recovery from gastrointestinal tract at 8-h (% of intravenous dose)</td>
<td>0.34 ± 0.31</td>
<td>0.140 ± 0.0721</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01 and *** p < 0.001.

(77% increase, p < 0.01) in the rats (Table 1), indicating that parathion resides longer in the rats. In rats pretreated with physostigmine, the total amount of unchanged parathion excreted in 8-h urine (expressed in terms of percentages of intravenous dose of parathion) was greater (282% increase, p < 0.001) than that in control rats (Table 1). The Cl of parathion based on plasma data, 49.7 mL/min/kg for control rats and 40.7 mL/min/kg for rats pretreated with physostigmine (Table 1) was faster than 55.2 mL/min/kg of the reported hepatic blood flow based on blood data in rats [18], suggesting that parathion is metabolized in other tissues in addition to liver. It was reported [19,20] that lung, brain, kidney and intestine activate parathion with a much lesser extent than liver. Paraoxon was below detection limit in plasma, urine and gastrointestinal tract, and this could be due to our HPLC assay sensitivity, rapid conversion of paraoxon to form diethylphosphate and p-nitrophenol, and/or rapid elimination of paraoxon in rats, terminal half-life of paraoxon of 3.3 min [17].

In control rats, the biliary excretion of unchanged parathion was almost negligible; less than 0.0546% of intravenous dose of parathion were excreted in 8-h bile. Parathion was fairly stable for up to 8-h incubation with fresh rat bile juice at parathion concentration of 10 μg/mL in a water-bath shaker kept at 37°C, and at a rate of 50 oscillations per min; more than 98.0% of the spiked amount of parathion were recovered for up to 8-h incubation.
amount of paraoxon was detected in liver, mesentery and large intestine at 5 min suggesting that paraoxon was rapidly generated in rats. However, paraoxon was below detection limit in liver at 30 and 120 min; the lack of accumulation of paraoxon in liver is owing to the breakdown of paraoxon by liver esterases, and not owing to the lack of formation of paraoxon by liver microsomes [21]. Although the tissue distribution of paraoxon was not significantly different between the two groups of rats, the amount of paraoxon at 5 min in the liver, mesentery and large intestine tended to be smaller in rats pretreated with physostigmine than those in control rat (Table 2), suggesting that formation of paraoxon seemed to be inhibited by physostigmine.

Based on the liver microscopy, hepatocellular degeneration and acute inflammation were observed in control rats, however, inflammation was not observed in rats pretreated with physostigmine. Based on lung microscopy, minimal edema and reactive changes (margination of neutrophils) were observed for both groups of rats, suggesting that lung toxicity induced by paraoxon could not be entirely protected by physostigmine. Based on brain microscopy, no significant findings were observed for both groups of rats suggesting that brain toxicity seemed not to be observed at the parathion dose employed in the present study.

Discussion

It was reported that the metabolism of parathion to paraoxon increased by CYP3A23 in rats pretreated with dexamethasone [4] and the major dexamethasone-inducible form of CYP3A in rats

![Figure 2. Mean T/P ratios of parathion collected at 5 (n = 6, each), 30 (n = 5, each) and 120 (n = 5, each) min after intravenous administration of parathion, 3 mg/kg, to control rats (white) and rats pretreated with physostigmine (grey). The plasma concentrations of parathion (µg/mL) are shown in the last figure. Vertical bars represent S.D. No significant difference (p < 0.05) was observed at each tissue.](image-url)
Table 2. Mean (± S.D.) amount (μg/g tissue) of paraoxon recovered from each tissue collected at 5, 30 and 120 min after intravenous administration of parathion, 3 mg/kg, to control rats and rats pretreated with physostigmine, 100 μg/kg

<table>
<thead>
<tr>
<th>Tissue</th>
<th>5 min</th>
<th>30 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control rats (n = 6)</td>
<td>Rats pretreated with</td>
<td>Control rats (n = 5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>physostigmine (n = 6)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.763 ± 0.194</td>
<td>0.666 ± 0.0992</td>
<td>BD</td>
</tr>
<tr>
<td>Lung</td>
<td>0.227 ± 0.0483</td>
<td>0.237 ± 0.104</td>
<td>BD</td>
</tr>
<tr>
<td>Fat</td>
<td>0.235 ± 0.09993</td>
<td>0.211 ± 0.0745</td>
<td>0.377 ± 0.376</td>
</tr>
<tr>
<td>Mesentery</td>
<td>0.559 ± 0.415</td>
<td>0.232 ± 0.0745</td>
<td>0.208 ± 0.227</td>
</tr>
<tr>
<td>Large intestine</td>
<td>0.891 ± 0.844</td>
<td>0.489 ± 0.345</td>
<td>BD</td>
</tr>
</tbody>
</table>

BD, below detection limit.

was CYP RL33/cDEX [5]. In rats pretreated with physostigmine, the greater AUC of parathion (Table 1) and the smaller amount of paraoxon in tissues (Table 2) suggested that physostigmine could, at least partly, inhibit the metabolism of parathion to paraoxon, possibly by inhibiting hepatic cytochrome P450 3A, and this could be supported by the following data. Erythromycin N-demethylase activity, which is primarily catalysed by CYP3A in rats [10], was inhibited in rats pretreated with physostigmine.

It was reported [19] that at least 4.4% of administered parathion (in the range of LD₅₀) were metabolized to paraoxon in 1 h by rat liver, gut, lung, kidney and suprarenal gland based on in vitro tissue slices studies; from these amounts, approximately 64% are converted in liver, 21% in gut, 13% in lung and 1.5% in kidney. Hence, the biliary excretion of unchanged parathion could play a considerable role in the Cl of parathion if the biliary excretion of unchanged parathion is considerable, as parathion is metabolized to paraoxon in rat gastrointestinal tract [19]. However, this could be almost negligible; the total amount of 8-h biliary excretion of parathion after intravenous administration of parathion, 3 mg/kg, to eight rats was less than 0.0546% of the intravenous dose of parathion. Parathion was fairly stable for up to 8-h incubation in fresh rat bile juice at a parathion concentration of 10 μg/mL. Based on the above data and the negligible urinary excretion of unchanged parathion (Table 1), it could be concluded that the Cl values of parathion listed in Table 1 could represent the metabolic clearances of parathion mainly metabolized in rat tissues (or organs).

Parathion is a high hepatic extraction ratio compound; the hepatic extraction ratio was approximately 80% [23] for rat isolated perfused liver. Therefore, the Cl of parathion is affected by hepatic blood flow and/or hepatic intrinsic clearance. It was reported that pretreatment with phenobarbital increased hepatic blood flow by 26.8–175% ([24] and references therein) in rats. However, the AUC and Cl of parathion were not significantly different between control rats and rats pretreated with phenobarbital [4], indicating that the hepatic blood flow changes by physostigmine, if any, could not contribute considerably to slower Cl of parathion in rats pretreated with physostigmine. Therefore, the slower Cl of parathion in rats pretreated with physostigmine may be due to slower hepatic intrinsic clearance of parathion to form paraoxon in the rats.

Although the Cls of parathion (Table 1) and erythromycin N-demethylase activities were significantly different between two groups of rats, the differences were not considerable and the amounts of paraoxon in liver, mesentery and large intestine at 5 min were slightly smaller (Table 2) in rats pretreated with physostigmine. These factors could not be a sole mechanism for the protection against parathion toxicity by physostigmine. More studies are required to find any possible mechanisms for the protection against parathion toxicity by physostigmine in
addition to the pharmacokinetic differences and the depressed activities of acetylcholinesterase by the carbamate.

Acknowledgements

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References