Use of OpdA, an Organophosphorus (OP) Hydrolase, Prevents Lethality in an African Green Monkey Model of Acute OP Poisoning

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Abstract

Organophosphorus (OP) pesticides are a diverse class of acetylcholinesterase (AChE) inhibitors that are responsible for tremendous morbidity and mortality worldwide, killing approximately 300,000 people annually. Enzymatic hydrolysis of OPs is a potential therapy for acute poisoning. OpdA, an OP hydrolase isolated from *Agrobacterium radiobacter,* has been shown to decrease lethality in rodent models of OP poisoning. This study investigated the effects of OpdA on AChE activity, plasma concentrations of OP, and signs of toxicity after administration of dichlorvos to nonhuman primates.

A dose of 75 mg/kg dichlorvos given orally caused apnea within 10 minutes with a progressive decrease in heart rate. Blood AChE activity decreased to zero within ten minutes. Respirations and AChE activity did not recover. The mean dichlorvos concentration rose to a peak of 0.66 μg/ml. Treated monkeys received 1.2 mg/kg OpdA iv immediately after poisoning with dichlorvos. In Opda-treated animals, heart and respiratory rates were unchanged from baseline over a 240-minute

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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observation period. AChE activity slowly declined, but remained above 25% of baseline for the entire duration. Dichlorvos concentrations reached a mean peak of 0.19 μg/ml at 40 minutes after poisoning and decreased to a mean of 0.05 μg/ml at 240 minutes.

These results show that OpdA hydrolyzes dichlorvos in an African Green Monkey model of lethal poisoning, delays AChE inhibition, and prevents lethality.

Keywords

organophosphorus; pesticide; dichlorvos; hydrolysis; monkey

Introduction

Organophosphorus (OP) pesticide poisoning is a major global health problem. An estimated 3,000,000 people are acutely poisoned each year, with a case fatality rate in excess of 9% in Sri Lanka (Eddleston *et al.*, 2012). In nearly all countries the deliberate oral ingestion of OP pesticides is the most common method of self-harm (Chang *et al.*, 2012). In the developed world, however, intentional self-harm with OP pesticides is an uncommon event. The main concern is the risk of terrorist attack through the release of OP gases in public spaces or introduction of highly toxic OP pesticides into water supplies.

The acute toxicity of OPs is primarily due to inhibition of acetylcholinesterase (AChE) (Sidell, 1994; Thiermann *et al.*, 2005). Current therapy for OP poisoning requires the use of atropine to antagonize the muscarinic effects of AChE inhibition, followed by administration of oximes to reactivate AChE, plus benzodiazepines to prevent neurocognitive sequelae (Bird *et al.*, 2003; Eddleston *et al.*, 2005b). However, the effectiveness of these antidotes is limited. Despite intensive care support in the best of Western hospitals, up to 40% of patients die. (Eyer *et al.*, 2003). Although OP pesticides have been a clinical problem for 50 years, no new therapies have been introduced since the 1960s.

One promising and novel approach to treating OP pesticide poisoning is the use of therapeutic enzymes to degrade pesticides *in vivo*. These enzymes, called OP hydrolases, are found endogenously in bacteria and humans. The use of OP hydrolases has the potential to decrease morbidity and mortality after acute OP poisoning, and to decrease limited resource utilization in the developing world. One particularly attractive feature of OP hydrolases is that they are amendable to genetic engineering, allowing the construction of mutant enzymes capable of hydrolyzing a diverse group of OPs (Vilanova *et al.*, 1999; Bird *et al.*, 2010). Recombinant enzymes with enhanced catalytic activities against many OPs have been developed (Sutherland *et al.*, 2004; Jackson *et al.*, 2009). However, few of these enzymes have undergone efficacy testing in animals, and no catalytic enzymes have ever been tested in non-human primates (NHP).

One metal-dependent OP hydrolase called OpdA (accession number EU002557), from *Agrobacterium radiobacter,* that shows high activity against many OPs and military G-series nerve agents, has been characterized (Horne *et al.*, 2002; Yang *et al.*, 2003) (figure 1).

The combination of its high catalytic efficiency, broad substrate range, and stability make it an excellent therapeutic OP hydrolase candidate.

The persistent shortcomings in currently available treatment options have led us to investigate OpdA as a possible therapeutic agent for acute OP pesticide poisoning. The pharmacokinetics and efficacy of OpdA against dichlorvos, ethyl-parathion, and methyl-parathion in rats (Herkert *et al.*, 2009; Gresham *et al.*, 2010) have been published. Furthermore, the pharmacokinetics of OpdA nonhuman primates (Bird *et al.*, 2008) have been published and are favorable for the further development of OpdA as a countermeasure to acute OP poisoning. This current work is an extension of previously published studies in rodents, and the purpose was to determine whether the results with OpdA in rodents can be extrapolated to NHP. The goals of this study were to: 1) determine if OpdA preserves AChE activity in an African Green Monkey model of severe dichlorvos (2,2-dichlorovinyl dimethyl phosphate) poisoning; 2) determine if OpdA hydrolyzes dichlorvos *in vivo*, and; 3) determine if OpdA prevents dichlorvos associated lethality in a novel nonhuman primate model.

Methods

OpdA preparation

The wild-type *opdA* gene was inserted between the NdeI and EcoR1 restrction sites of the pETMCSI plasmid (Neylon *et al.*, 2000). BL21(DE3)$^{RecA-}$ (Invitrogen, Carlsbad, California, U.S.A) cells were transformed with pETMCSI-*opdA* vector heat-shock as per manufacturers instructions. Cells were grown on a Luria-Bertani broth-agar plate (containing 100 μg/mL ampicillin) at 37°C overnight. A single colony was inoculated into 50 mL Terrific broth (TB) medium supplemented with 1 mM $CoCl_2$ (Sigma-Aldrich, St. Louis, Missouri, USA) and 100 μg/mL ampicillin (Sigma-Aldrich, St. Louis, Missouri, USA) and incubated at 37°C until mid-log phase. This start-culture was then diluted 1:50 in 2 L of the same medium and grown at 30 °C for 40 h. Cells were harvested by centrifugation at 6000 x *g* for 20 min at 4°C and resuspended in 50 mL buffer containing 50 mM HEPES (Sigma-Aldrich, St. Louis, Missouri, USA), pH 8.0, with 1 mM $CoCl_2$ and 1 x Bugbuster cell lysis reagent and 1 U/mL benzonase (Novagen, EMD Chemicals, Gibbstown, New Jersey, U.S.A.). Lysis occurred at 20 °C for 30 minutes before centrifugation at 30,000 x *g* for 40 min at 4°C to sediment the cell debris. The supernatant was loaded onto a 60 mL DEAE Fractogel column (Merck, Frankfurt, Germany) and the unbound fraction containing OpdA was collected and dialysed against buffer containing 50 mM HEPES (Sigma-Aldrich, St. Louis, Missouri, USA), pH 7.0, overnight. This fraction was then twice loaded onto a 5 mL HiTrap SPFF column (GE Healthcare, Piscataway, New Jersey, U.S.A.) equilibrated with 50 mM HEPES, pH 7.0. Bound OpdA was eluted over a linear gradient of 0 to 0.5 M NaCl (Sigma-Aldrich, St. Louis, Missouri, USA). SDS-PAGE indicated that OpdA was >95% pure and the overall yield was in excess of 50 mg OpdA per L of growth medium. For storage, the protein was dialysed against 50 mM HEPES, 1 mM $CoCl_2$, 150 mM NaCl, pH 7.5. For storage, the enzyme was dialyzed against 50 mM HEPES, 1 mM $CoCl_2$, 150 mM NaCl, pH 7.5. In previous studies, there was negligible loss of activity after 8 months of storage at 4°C.(Bird *et al.*, 2008)

Endotoxin removal and assays

After preparation, the amount of endotoxin present in the OpdA as determined by the Pyrotell *Limulus* Amebocyte Lysate gel-clot assay (Associates of Cape Cod, Inc., East Falmouth, Massachusetts, USA) was 51.4 EU/mg OpdA. Endotoxin was removed by running the enzyme through an endotoxin removal column (Detoxi-GeL Endotoxon Removing Column, Pierce Protein Research Products, Rockford, Illinois, USA). After one passage through the column, endotoxin concentration decreased to 2.1 EU/mg OpdA. This level of endotoxin concentration corresponds to less than 0.5 ng/ml (Petsch *et al.*, 2000) is safe when injected iv (Xiao *et al.*, 1998) and meets the standards of the FDA for therapeutics (FDA, 2012). Prior to administration, the OpdA was dialyzed overnight at 4°C against a solution of 150mM NaCl and 20 mM HEPES buffer to remove Co^{2+} metal ions present in the storage buffer.

Dichlorvos poisoning model

NHP studies were performed in 5–7 kg male African green monkeys (*Chlorocebus sabaeus*) that were naïve to OpdA. All African green monkeys were purchased from the New Iberia Research Center, New Iberia, Louisiana, and housed at the New England Primate Research Center (NEPRC) in accordance with the standards of the American Association for Accreditation of Laboratory Animal Care and Harvard Medical School's Animal Care and Use Committee. The animal quarters were under a 12-hour: 12-hour light-dark cycle, with fresh water freely available in the home cage. Animals were fed a diet *ad libitum* of commercial primate food supplemented with fresh fruit daily. On arrival at NEPRC the animals were quarantined for 45 days during which time they underwent physical examinations, tuberculosis testing, fecal analysis for bacterial and parasitic pathogens, complete blood counts, serum chemistries and virological screening.

Animals were fasted overnight and weighed immediately before experimentation. Monkeys were sedated with intramuscular ketamine (10–15 mg/kg, Fort Dodge, IA, USA) and intubated endotracheally. The entire experiment was performed under isoflurane anesthesia with continuous end-tidal carbon dioxide monitoring. A saphenous or cephalic vein catheter was placed for serial blood sampling. A continuous three-lead electrocardiogram monitor was used to accurately measure changes in cardiac activity.

After vital signs were stabilized, dichlorvos (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 75 mg/kg (approximating 3 x the rat oral LD_{50}) suspended in 0.5 ml/kg of peanut oil was administered through an orogastric tube, followed by a flush of 2 ml of peanut oil, after which the orogastric tube was removed. This dose of dichlorvos was based upon previous rodent models of dichlorvos poisoning (Bird *et al.*, 2008). Development of the dichlorvos poisoning model required that dichlorvos alone be administered to 2 animals. Timing of physiologic changes was recorded and was used to guide our treatment regimen.

Due to the rapid and severe physiological changes seen after dichlorvos administration, the animals enrolled in the OpdA-treatment study were given 1.2 mg/kg intravenous OpdA immediately following oral administration of dichlorvos. This dose of OpdA was based upon known pharmacokinetics of OpdA in the African green monkey. Approximately 1 ml

blood samples were taken every 5 minutes for 20 minutes, then every 10 minutes for 20 minutes, then every 20 minutes until 240 minutes post-poisoning. The blood was placed immediately on ice, then centrifuged to separate the serum, and frozen at −20 °C until analyzed.

The primary endpoint was survival to 240 minutes after poisoning. Secondary endpoints were area under the curves (AUC) of AChE activity and dichlorvos concentration. Animals were humanely euthanized with >50 mg/kg pentobarbital intravenously (Sleepaway, Fort Dodge, IA) at 240 minutes post poisoning or after 20 minutes of apnea, whichever occurred first.

All personnel working with dichlorvos or animal carcasses wore nitrile gloves and disposable gowns, according to standard procedures. Carcasses were disposed of in an incinerator equipped with afterburner and scrubber per current institutional protocol.

AChE Determination

Measurement of RBC AChE activity was performed using the point-of-care Test-mate ChE Cholinesterase Test System (EQM Research, Cincinnati, OH, USA). This device determines AChE activity using a modified Ellman method(Ellman *et al.*, 1961) from just 10 μL of blood. The Test-mate device has been validated and used in both research and clinical applications in humans and NHP (Taylor *et al.*, 2003; Rajapakse *et al.*, 2011).

Dichlorvos Quantification

Quantification of serial dichlorvos concentrations was performed in triplicate on the serum samples according to published methods (Inoue *et al.*, 2007). In summary, stock solutions of dichlorvos were prepared in methanol. The working solution for the internal standard (IS), dichlorvos-D6, (Santa Cruz Biotechnology, Dallas, Texas) was prepared by diluting an aliquot of stock solution with methanol.

Monkey serum calibration standards of dichlorvos solutions (0.001, 0.005, 0.01, and 0.05 mcg/mL) were prepared by spiking the working standard solution and internal standard (2 mcg/mL) into a sample of dichlorvos-free NHP serum (25 μL).

Serum aliquots of 50 μL were added to each 5 μL of the IS solutions prepared in methanol. Acetonitrile (50 μL) was added to the mixture. The resulting mixture was vortexed for 1 minute and then centrifuged at $3000 \times g$ for 5 minutes. The supernatant was filtered through a C18 spin filter (Pierce) and 20 μL of the filtrate was injected and separated using an XTerra C18 3.5μ 1.0×50 mm (Waters, Inc) using a Surveyor (Thermo) HPLC with mobile phase solvents consisting of 10 mM ammonium formate in water (solvent A) and methanol (solvent B). The elution gradient was 15% B to 95% B (0–3 minutes), 95% B (3–9.5 minutes), 95% B to 15% B (9.5–10 minutes), and 15% B (10–20 minutes) at a flow rate of 0.3 mL/minute. The data were acquired using a LTQ (Thermo) and by monitoring the tandem mass fragment ion *m/z* 109 from *m/z* 221 (dichlorvos) and fragment ion *m/z* 115 from *m/z* 227 (D6-dichlorvos). A linear calibration curve (1/x weighting, excluding origin) was created from the ratio of the area of the standard vs. the area of the internal standard and the sample concentrations were determined against the curve.

The method was validated by establishing linearity, intra- and interassay accuracy and precision, limits of detection (LODs) and limits of quantitation (LOQs). Batches consisting of triplicate calibration standards for each concentration were analyzed two different times. Graph of the dichlorvos-to-internal standard peak-area ratios versus the theoretical concentration was constructed using linear least- squares regression analysis.

Statistical analyses

Area under the curves for AChE activity and dichlorvos concentration were calculated using GraphPad Software's Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). In order to normalize the AUC for dichlorvos for time, the total AUC for serum dichlorvos concentration was divided by the time in minutes for both groups of animals (treated vs. un-treated).

Results

Oral Dichlorvos administration model

Dichlorvos was administered orally to two monkeys. Both monkeys experienced a progressive decrease in heart rate (figure 2). Both monkeys also experienced apnea within 10 minutes (figure 2). Neither respirations nor AChE activity recovered after 20 minutes, at which time the monkeys were euthanized.

OpdA treatment model

Due to the rapidity with which dichlorvos exerted it toxic effects on the monkeys, OpdA at a dose of 1.2 mg/kg/IV (0.5 ml/kg) was given immediately after dichlorvos administration to a group of 3 monkeys. No other antidote was administered. All three monkeys maintained normal heart rates (figure 2) and normal respiratory rates (figure 3) for the entire 240 minutes.

AChE activity

Blood AChE activity rapidly decreased to zero by ten minutes in both dichlorvos-only animals. In the OpdA-treated animals, AChE activity slowly declined over the 240 minutes, but remained above 25% of baseline (figure 4).

Dichlorvos concentrations

In the dichlorvos-alone monkeys, the mean dichlorvos serum concentration steadily rose to a peak of 0.66 µg/ml at the time of euthanasia (figure 5). In the OpdA-treated monkeys, the mean serum dichlorvos concentration reached a peak of 0.19 mcg/ml 40 min post-poisoning, and decreased to a mean value of 0.05 mcg/ml by 240 minutes. The AUCs for dichlorvos concentrations were 9.69 and 22.93 for the control and OpdA-treated animals, respectively (p<0.01). However, because the treated animals survived much longer than the control animals, the AUCs were normalized to time of survival (by minutes). Doing so yielded a value of 0.484 for the control animals and 0.095 for the OpdA-treated animals (p<0.01)

Discussion

We found that OpdA, when used alone without atropine or an oxime, prevented lethality in a NHP model of lethal OP pesticide poisoning. Furthermore, we have shown that OpdA prevents changes in respiratory rate and heart rate after OP administration, protects AChE from inhibition for up to 4 hours, and effectively hydrolyzes an *OP in vivo*. This is the first study demonstrating the proof-of-principal of a catalytic enzyme treating acute OP poisoning in a NHP.

Organophosphorus (OP) pesticides are commonly used in the developing world and are the most common class of chemicals used for deliberate self-harm (Van der Hoek *et al.*, 1998; Eddleston *et al.*, 2004b)). In the United States, the situation is quite different. Only a few thousand cases of exposure to OP pesticides were reported to the U.S. Association of Poison Control Centers in 2011, with just 2 deaths (Bronstein *et al.*, 2012). The greatest concern from the perspective of U.S. authorities and the National Institutes of Health is the risk of terrorist attacks with OP compounds, including nerve agents. These concerns has led the NIH to develop the Countermeasures Against Chemical Threats (CounterACT) program, whose mission is the development of new and improved medical countermeasures designed to prevent, diagnose, and treat the conditions caused by potential and existing chemical agents of terrorism.

Prophylactic therapy is not feasible for either pesticide self-poisoning or for a terrorist attack on civilians. Therefore, novel and highly effective post-exposure therapy is needed. Overall mortality after OP poisoning in the developing world is unknown, but is believed to be between 4% and 30% (Karalliedde, 1999). Mortality for patients admitted to the hospital is as high as 50% (Munidasa *et al.*, 2004). Patients exhibiting severe clinical signs associated with OP exposure are a challenge to treat, even in the most sophisticated hospitals: mortality for parathion poisoning in the ICUs of Munich, Germany, where toxicologist specialist services were available, was 40% (Eyer *et al.*, 2003). It could be expected that in the event of a mass poisoning due to terrorist attack that the mortality would be even higher.

Dichlorvos is a commonly used OP in laboratory and animal studies (Sinha *et al.*, 2003; Bird *et al.*, 2008), and is the most common lethal OP poisoning in China (Phillips *et al.*, 2002). In order to more closely mimic these clinical poisoning scenarios, we elected to administer dichlorvos via gavage. At a dose of 75 mg/kg dichlorvos very rapidly and completely inhibited AChE. Furthermore, apnea and bradycardia soon developed, necessitating the administration of OpdA immediately following dichlorvos administration in the treatment group. As the goal of this investigation was to determine the efficacy of of OpdA treatment alone, we did not treat with either atropine or an oxime.

Despite treatment only with OpdA, monkeys maintained normal heart and respiratory rates throughout the entire 4-hour experiment. In addition, while there was a steady decrease in AChE activity, the mean AChE activity 4 hours after poisoning remained above a clinically relevant 25%. Although OpdA was effective in hydrolyzing dichlorvos *in vivo* to a low concentration, this hydrolysis took some time (peak dichlorvos concentration occurred at 40 minutes after dosing) and was not entirely complete.

The reasons for discrepancy between the inhibitory constant of human AChE by dichlorvos (1×10^{-7} M to 1×10^{-5} M *in vitro*) (Nakagawa *et al.*, 1977) and our measured serum concentration of dichlorvos and AChE are unclear, but likely multi-factorial. For instance, we were able to measure free dichlorvos in serum, but methods to quantify OpdA-, AChE-, or butyrylcholinesterase-bound dichlorvos do not exist. Perhaps even more importantly, human AChE has been shown to undergo significant spontaneous reactivation after inhibition by dichlorvos (Askar *et al.*, 2011).

Due to IACUC requirements, animals were euthanized while under general anesthesia 4 hours after poisoning. It is important to keep in mind that this severe poisoning model was successful despite including no other antidotal therapies. Longer term survival and behavioral studies using OpdA plus conventional therapies (atropine and an oxime) in NHP will require significantly more animal subjects (as the survival rate in would be expected to be high). The current study is one of the first steps in demonstrating the efficacy of OpdA for severe acute OP poisoning.

This study demonstrates that when used as a monotherapy, OpdA protects AChE from inhibition, hydrolyzes dichlorvos *in vivo*, and prevents lethality in an African green monkey model of severe dichlorvos poisoning. This is the first study of a catalytic enzyme for treating OP poisoning in a non-human primate, and supports the contention that further research into expanding the catalytic properties of the enzyme and the development of OpdA as a post-exposure therapy for acute OP pesticide poisoning are warranted.

Acknowledgments

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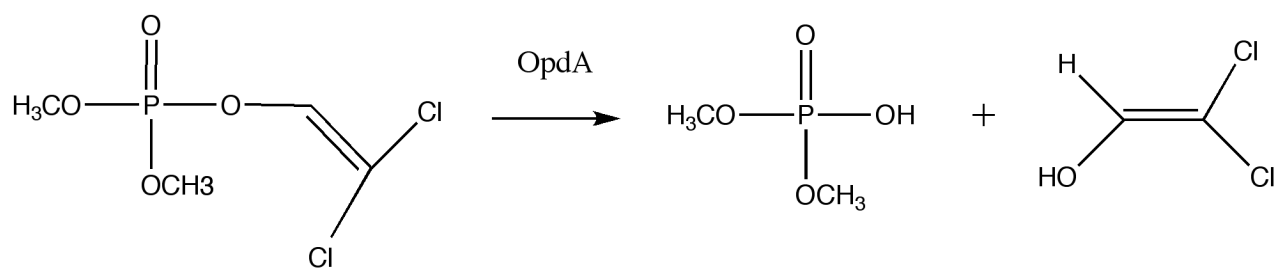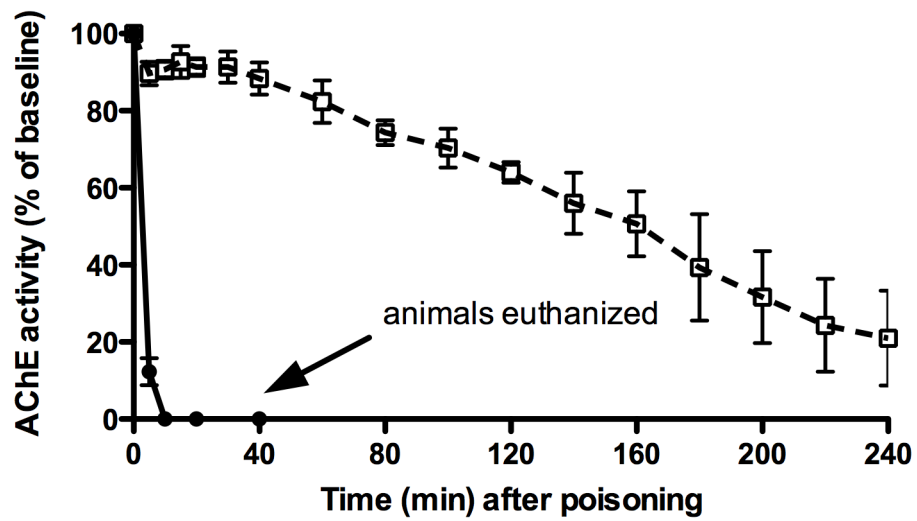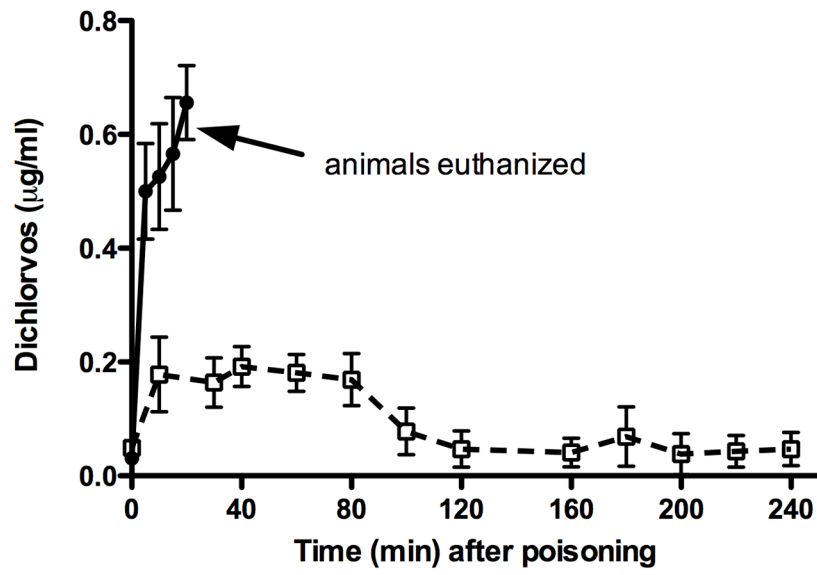
Figure 1.
Hydrolysis of dichlorvos by OpdA
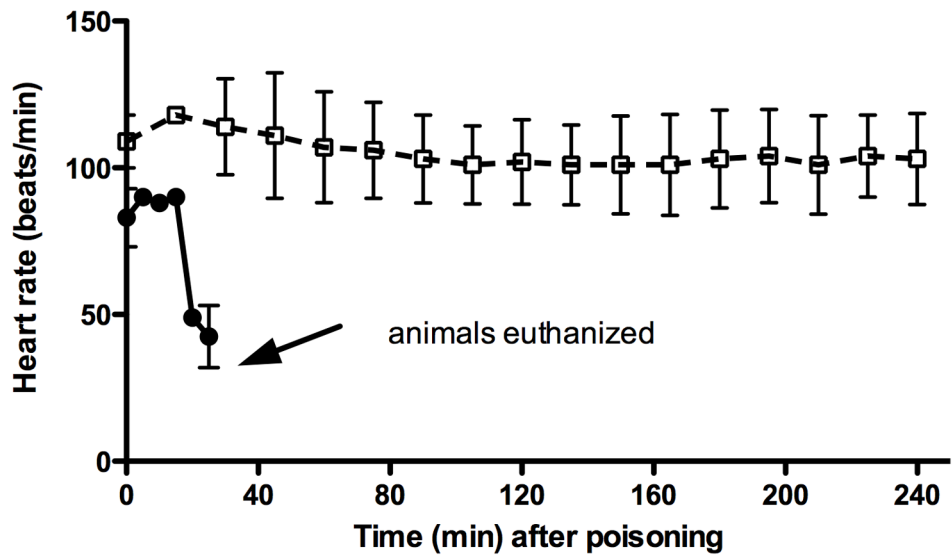
Figure 2.
Heart rate (●) in animals given dichlorvos alone (n=2) and heart rate (□) in those treated with OpdA immediately after dichlorvos (dashed line, n=3). Error bars represent SD.
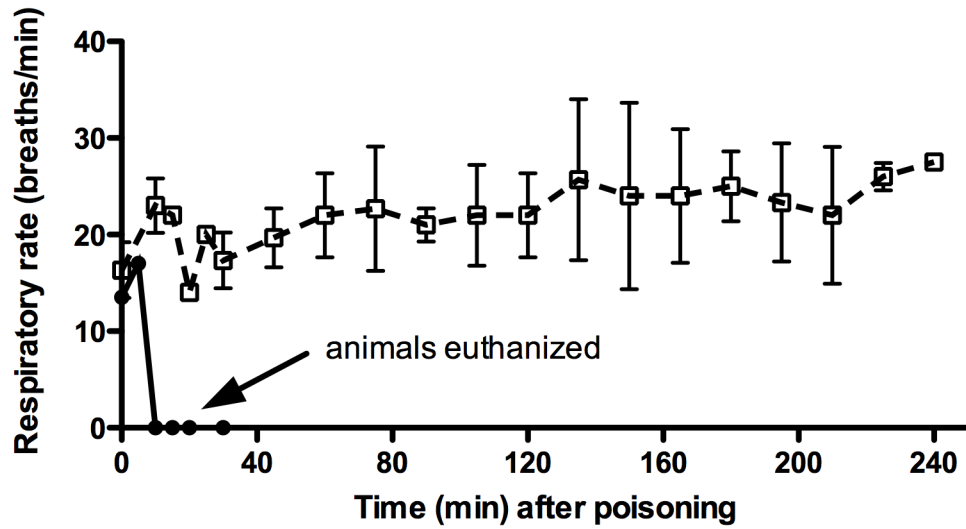
Figure 3.
Respiratory rate (●) in animals given dichlorvos alone (n=2) and respiratory rate (□) in those treated with OpdA immediately after dichlorvos (dashed line, n=3). Error bars represent SD.

Figure 4.
Acetylcholinesterase activity as a percentage of baseline in animals given dichlorvos alone (●) and those treated with OpdA immediately after dichlorvos (□, dashed line). Error bars represent SD. Mean initial baseline AChE activity was 0.25 U/gm hemoglobin.

Figure 5.
Serum dichlorvos concentrations in animals given dichlorvos alone (●) and those treated with OpdA (□, dashed line). Error bars represent SD.