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CHOLINESTERASE ACTIVITY IN GUINEA PIGS FOLLOWING INTRAVENOUS EXPOSURE TO THE OPTICALLY PURE ENANTIOMERS OF VX

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The center phosphorus atom of VX (*O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate) rotates linearly polarized light both clockwise (P(+)-isomer) and anticlockwise (P(–)-isomer) so that its synthesis results in equal amounts of two enantiomers with the same chemical and physical properties. The toxicological properties of the two enantiomers, however, are anticipated to be different because they exert their effects in a chiral, biologic environment. We recently estimated median lethal doses (24 h LD_{50}) for intravenous exposure to the optically pure enantiomers of VX in adult, male guinea pigs; the P(+)-isomer of VX was 60× less toxic than the P(–)-isomer. Serial blood samples were collected from a subset of these guinea pigs to characterize the toxicodynamic profile of each enantiomer. Acetylcholinesterase (AChE) activity was inhibited more slowly with the P(+)-isomer of VX than with the P(–)-isomer. In addition, the P(+)-isomer of VX inhibited nearly 95% of the butyrylcholinesterase (BuChE) activity compared to only 40% with the P(–)-isomer. The stereoselectivity differences between AChE and BuChE inhibition may explain why the P(+)-isomer of VX was less toxic than the P(–)-isomer in the guinea pig model.

15. SUBJECT TERMS
O-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX)
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PREFACE

The work described in this report was authorized under project no. CB3281 (Dr. Robert Kristovich, Principal Investigator). The work was started in January 2016 and completed in September 2016, as recorded in ECBC notebook 15-0101. At the time this work was performed, the U.S. Army Combat Capabilities Development Command Chemical Biological Center (CCDC CBC; Aberdeen Proving Ground, MD) was known as the U.S. Army Edgewood Chemical Biological Center (ECBC).

In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* (National Academy Press: Washington, DC, 2016). These investigations were also performed in accordance with the requirements of AR 40-33, *The Care and Use of Laboratory Animals in DoD Programs* (Headquarters: Washington, DC, 2005); and the Institutional Animal Care and Use Committee (IACUC), which oversees the use of laboratory animals by reviewing for approval all CCDC CBC research protocols requiring laboratory animals. This project, assigned IACUC Protocol No. 16-472, was approved in December 2015.

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This report has been approved for public release.

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- Mr. Jeffry Forster and Mses. Ruth Moretz and Julie Renner (CCDC CBC) for their efforts with the exposures and biosample collections,
- Drs. Mark Winemiller and Sue Bae (CCDC CBC) for synthesizing the optically pure enantiomers of VX and ensuring their purity before each exposure, and
- Drs. Christopher Whalley and Robert Kristovich (CCDC CBC) for their support.

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CHOLINESTERASE ACTIVITY IN GUINEA PIGS FOLLOWING INTRAVENOUS EXPOSURE TO THE OPTICALLY PURE ENANTIOMERS OF VX

1. INTRODUCTION

Chemical warfare nerve agents (CWNAs) exert their toxicological effects by inhibiting the activity of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of acetylcholine in the central and peripheral nervous systems. The accumulation of acetylcholine within the synaptic cleft prolongs the stimulation of muscarinic and nicotinic receptors, leading to an acute cholinergic crisis characterized by hypersecretions, involuntary movements, respiratory distress, seizures, and ultimately death. In vitro studies have demonstrated that the P(+)-isomers of CWNA are several orders of magnitude less potent inhibitors of AChE than the P(–)-isomers (Nordgren et al., 1984; Benschop and de Jong, 1988; Ordentlich et al., 2004). However, only a few in vivo studies have been conducted with optically pure stereoisomers (reviewed in Benschop and de Jong, 1988; 2001). By exposing mice to each of the stereoisomers of soman, tabun, and VX, it was determined that the median lethal dose (LD_{50}) values of the P(+)-isomers of these agents were higher than the LD_{50} values of the P(–)-isomers. However, mice are not the ideal animal model for studying the toxicity associated with CWNA exposure as they have relatively high levels of carboxylesterase activity compared to humans (Bahar et al., 2012).

Guinea pigs have lower levels of carboxylesterase activity than mice (Maxwell et al., 1987; Bahar et al., 2012); thus, our laboratory recently estimated LD_{50} values for adult, male guinea pigs intravenously (IV) exposed to the optically pure enantiomers and the racemic mixture of VX (Wright et al., 2017). The 24 h LD_{50} value for the P(+)-isomer of VX was estimated to be 261.3 μg/kg, which is 1-2 orders of magnitude higher than the values estimated for the P(–)-isomer and the racemic mixture (4.4 and 5.4 μg/kg, respectively). In this concurrent study, we collected blood and tissue samples from VX-exposed guinea pigs to characterize the toxicodynamic profile of each enantiomer, as well as the racemic mixture, in terms of AchE and butyrylcholinesterase (BuChE) inhibition.

2. METHODS

2.1 Animals

Young adult, male guinea pigs (350–400 g) with surgically implanted double jugular vein catheters were purchased from Charles River Laboratories International, Inc. (Kingston, NY). Guinea pigs were single-housed in temperature- and humidity-controlled rooms (21 ± 1 °C and 50 ± 20%, respectively) with the lights on from 0600 to 1800. Food and water were provided ad libitum, and the animals had access to enrichment items such as tunnels and chew toys. Guinea pigs weighed 434 ± 26 g at the time of the VX exposures, which occurred no less than 5 days after their arrival.

2.2 Agent

Optically pure enantiomers were separated from the racemic (50:50) VX mixture by members of the Agent Chemistry Branch (U.S. Army Edgewood Chemical Biological Center (ECBC); Aberdeen Proving Ground (APG), MD) using procedures described by Bae and Winemiller (2016). An aliquot of neat (undiluted) agent was transferred from the Agent Chemistry Branch to the Operational Toxicology Branch on the morning of each exposure (approximately 30 min before the first guinea pig was exposed to agent). The purity of the agent in each aliquot was verified by nuclear magnetic resonance spectroscopy no more than 24 h before it was transferred between branches.

2.3 Exposures

Guinea pigs (n = 3-4 animals/dose) were IV exposed via the catheters on their left-hand sides to one of the optically pure enantiomers or the racemic mixture of VX. The solvent used was saline, and the injection volume was 0.5 mL/kg. The guinea pigs were exposed to doses identical to those used to determine the LD_{50} for each agent (Wright et al., 2017), which ranged from 175 to 280 µg/kg for the P(+)-isomer, 3.0 to 5.0 µg/kg for the P(–)-isomer, and 3.7 to 7.0 µg/kg for the racemic mixture of VX. Blood samples (300 µL/time point) were collected from each guinea pig via the catheter on its right-hand side at 0, 1, 10, 20, 30, 40, 50, 60, 180, 360, and 1440 min post-exposure. If the catheters were no longer patent, blood was collected from the saphenous vein on one of the guinea pigs' hind legs. Guinea pigs were IV administered a barbiturate euthanasia solution (390 mg/mL sodium pentobarbital) at 24 h post-exposure, and biosamples (brain, heart, liver, lung, and kidneys) were collected once death was confirmed. Blood was stored at -20 °C, whereas the other biosamples were stored at -80 °C until they could be analyzed.

Guinea pigs with non-patent catheters that could not be exposed to agent (n = 13) were intraperitoneally administered a barbiturate euthanasia solution. Biosamples were collected from these naïve guinea pigs and stored as described above.

2.4 Cholinesterase Assays

Cholinesterase activities were determined using the Model 700 Cholinesterase Chemistry Set from EQM Research, Inc. (Cincinnati, OH), which is a modified version of the Ellman assay described by Worek and colleagues (1999). First, a CryoPrep system (Covaris, Inc.; Woburn, MA) was used to pulverize 0.5 to 1.0 g of each tissue sample, which was then homogenized in 1.0 mL of deionized water. Using a microcap from Drummond Scientific Company (Broomall, PA), 20 µL of whole blood or supernatant from the homogenized tissue was added to 4 mL of distilled water in a cryogenic vial (Corning, Inc.; Corning, NY). The vial was then shaken vigorously to remove all of the blood or supernatant from the microcap before adding 0.4 mL of 0.69 mM phosphate buffer, pH 7.4 to it and vortexing. Next, 200 µL aliquots of the vortexed solution were transferred to four wells on a 96-well plate. Twenty-five microliters of 30 mM 5,5-dithiobis-2-nitrobenzoic acid was added to each well before covering the plate and incubating it at 37 °C for 10 min. Twenty-five microliters of a solution containing 10 mM acetylcholine and 200 M 10-(α-diethylaminopropionyl)-phenothiazine was added to two

of the four wells to determine AChE activity, whereas 25 µL of a solution containing 20 mM butyrylthiocholine was added to the other two wells to determine BuChE activity. The plate was then read at 450 nm and 37 °C on a SpectraMax Plus^{384} microplate spectrophotometer (Molecular Devices, LLC; Sunnyvale, CA) for 10 min, and cholinesterase activities were determined using SoftMax Pro LS 5.1. To determine the amount of hemoglobin in each whole blood sample, 225 µL aliquots of the vortexed solution were transferred to two wells on a 96-well plate. Twenty-five microliters of a solution containing 25 mM sodium dodecyl sulfate (SDS) was then added to each well before incubating the plate at room temperature for 10 min. The plate was read at 536 nm on the spectrophotometer. To determine the amount of total protein in each whole blood sample, 50 µL of blood was collected in a microhematocrit capillary tube from Fisher Scientific (Pittsburgh, PA). The amount of protein was then measured using a temperature-compensated refractometer (AO Spencer; Buffalo, NY).

All readings from the spectrophotometer were adjusted to a 1 cm light path and corrected for background. Cholinesterase activities were calculated using the molar absorption coefficient for 5-thio-2-nitrobenzoic acid, which is 8.26 $cm^{-1}M^{-1}$ at 450 nm and 37 °C (Eyer et al., 2003). The amount of hemoglobin was calculated using the molar absorption coefficient of hemoglobin-SDS, which is 0.19 $cm^{-1}M^{-1}$ at 536 nm (Prahl, 1999). AChE activity in each of the whole blood samples was normalized to the amount of hemoglobin, whereas BuChE activity in each of the whole blood samples was normalized to the amount of total protein. Cholinesterase activity in each of the tissue samples was normalized to the weight of the sample.

2.5 Data Analysis

Cholinesterase activities in the whole blood samples were expressed as a percentage of the activities measured at baseline (0 min post-exposure), whereas cholinesterase activities in the tissue samples were expressed as a percentage of the activities measured in the tissues of the naïve guinea pigs. Data are presented as means ± standard deviations, and SigmaPlot 13.0 (Systat Software, Inc.; San Jose, CA) was used to create the graphs. Data from guinea pigs that did not survive until euthanasia (24 h post-exposure) were excluded from the graphs of cholinesterase activity in the tissue samples. However, data from all of the guinea pigs may be found in the appendices.

3. RESULTS

AChE and BuChE activities in whole blood collected from guinea pigs prior to exposure measured 8.6 ± 1.9 units of activity per gram of hemoglobin and 54.4 ± 11.9 units of activity per gram of total protein, respectively. Table 1 shows AChE and BuChE activities in tissue samples collected from naïve guinea pigs. The highest amounts of AChE activity were measured in the brains of these guinea pigs, whereas the highest amounts of BuChE activity were measured in their livers.

Table 1. Cholinesterase Activities in Tissue Samples Collected from Naïve Guinea Pigs

Tissue	AChE (U/g)	BuChE (U/g)
Brain	499 ± 162	215 ± 67
Heart	111 ± 19	475 ± 140
Liver	170 ± 68	8,226 ± 6,038
Lung	132 ± 12	1,244 ± 249
Kidney	88 ± 22	690 ± 168

U/g, units of activity per gram of tissue

Figure 1 shows AChE activities in whole blood samples collected from guinea pigs IV exposed to the P(+)-isomer (left), the P(–)-isomer (middle), or the racemic mixture (right) of VX. Four of the five groups of guinea pigs exposed to the P(+)-isomer had AChE activities greater than 20% of their baseline values at 1 min post-exposure. The 210 µg/kg group dropped below 20% at 10 min, whereas the 175, 245, and 260 µg/kg groups dropped below 20% at 20 min. Only two groups of guinea pigs exposed to this isomer had AChE activities less than 10%: 210 µg/kg from 40 to 180 min and 280 µg/kg from 40 to 50 min. In contrast, all of the guinea pigs exposed to the P(–)-isomer had AChE activities less than 20% at 1 min post-exposure. The 3.0 µg/kg group had AChE activities less than 10% from 1 to 60 min, whereas the 4.0 and 5.0 µg/kg groups had AChE activities less than 10% from 1 to 180 min. The 4.2 µg/kg group had AChE activities less than 10% at 10, 50, and 60 min. Similarly, all of the guinea pigs exposed to the racemic mixture had AChE activities less than 20% at 1 min post-exposure. The 4.8 µg/kg group had AChE activities less than 10% from 1 to 10 min; the 5.0 µg/kg group had AChE activities less than 10% from 1 to 40 min; and the 6.0 and 7.0 µg/kg groups had AChE activities less than 10% from 1 to 60 min. Regardless of agent, all of the guinea pigs had AChE activities greater than 20% at 1440 min post-exposure.

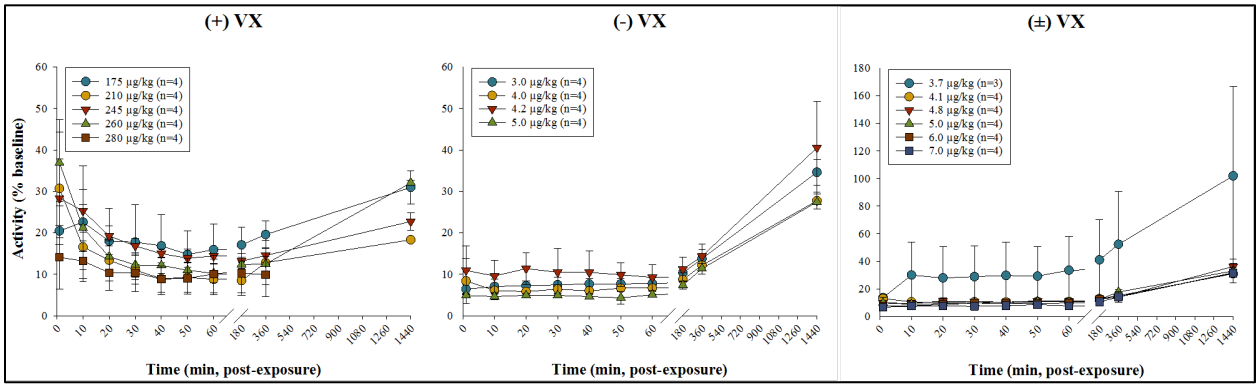

Figure 1. AChE activities in whole blood samples collected from guinea pigs IV exposed to one of the optically pure enantiomers or the racemic mixture of VX.

Figure 2 shows BuChE activities in whole blood samples collected from guinea pigs IV exposed to the P(+)-isomer (left), the P(–)-isomer (middle), or the racemic mixture (right) of VX. All of the guinea pigs exposed to the P(+)-isomer had BuChE activities less than

10% of their baseline values from 1 to 360 min post-exposure. BuChE activities were still less than 20% at 1440 min for all of these guinea pigs except for those exposed to 260 µg/kg. In contrast, all of the guinea pigs exposed to the P(–)-isomer had BuChE activities greater than 50% at 1 min post-exposure. The only groups to have BuChE activities less than 50% were 4.0 µg/kg from 20 to 180 min (except for 30 min), 4.2 µg/kg at 1440 min, and 5.0 µg/kg from 10 to 1440 min; however, none of these groups had BuChE activities less than 30% at any time. Similarly, all of the guinea pigs exposed to the racemic mixture had BuChE activities greater than 50% at 1 min post-exposure. The only groups to have BuChE activities less than 50% were 4.8 µg/kg from 20 to 180 min, 5.0 µg/kg at 50 min, 6.0 µg/kg at 10 and 1440 min, and 7.0 µg/kg from 20 to 1440 min; however, none of these groups had BuChE activities less than 30% at any time.

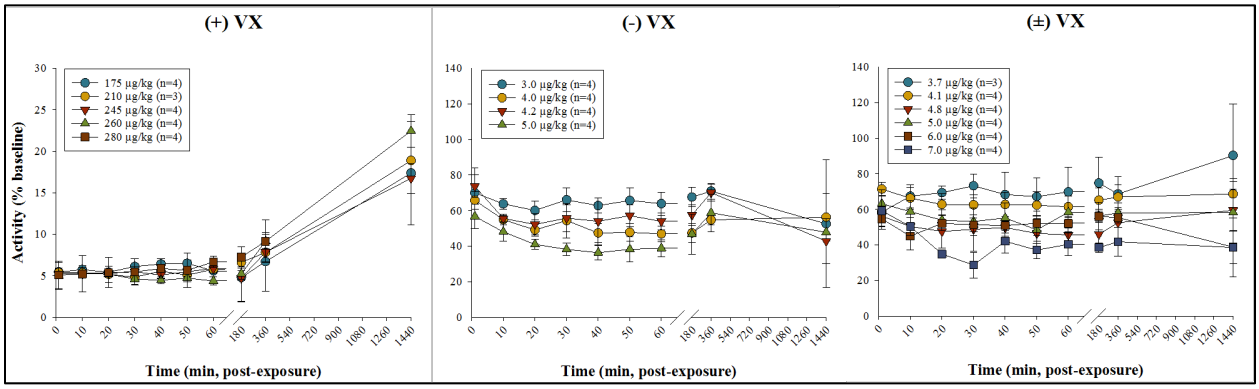

Figure 2. BuChE activities in whole blood samples collected from guinea pigs IV exposed to one of the optically pure enantiomers or the racemic mixture of VX.

Figure 3 shows AChE activities in tissue samples collected from guinea pigs IV exposed to the P(+)-isomer (left), the P(–)-isomer (middle), or the racemic mixture (right) of VX. At 24 h post-exposure, heart, lung, and kidney samples collected from guinea pigs exposed to P(+)-isomer had AChE activities 80% greater than samples collected from naïve guinea pigs. AChE activities were less than 50% in brain samples taken from all guinea pigs exposed to this isomer except for the lone survivor in the 210 µg/kg group. AChE activities were less than 80% in liver samples taken from all of the guinea pigs except for those exposed to 245 µg/kg of the P(+)-isomer ; however, none of these guinea pigs' liver samples had AChE activities less than 50%. Lung and kidney samples collected from all guinea pigs exposed to the P(–)-isomer had AChE activities greater than 80% at 24 h post-exposure. AChE activities were greater than 80% in all of the heart samples except for those collected from the 4.2 µg/kg group. AChE activities were less than 80% in all of the brain and liver samples except for those collected from the 4.2 µg/kg group. Brain samples collected from guinea pigs exposed to 4.0 µg/kg of the P(–)-isomer were the only ones to have AChE activities less than 50%. Brain, heart, and lung samples collected from guinea pigs exposed to the racemic mixture had AChE activities greater than 80% at 24 h post-exposure. AChE activities in liver samples collected from guinea pigs exposed to 3.7, 4.1, or 4.8 µg/kg were greater than 80%; whereas AChE activities in liver samples collected from guinea pigs exposed to 5.0, 6.0, or 7.0 µg/kg were between 50 and 80%. AChE activities were greater than 80% in all of the kidney samples except for those collected from the 5.0 µg/kg group; AChE activities in those samples were between 50 and 80%.

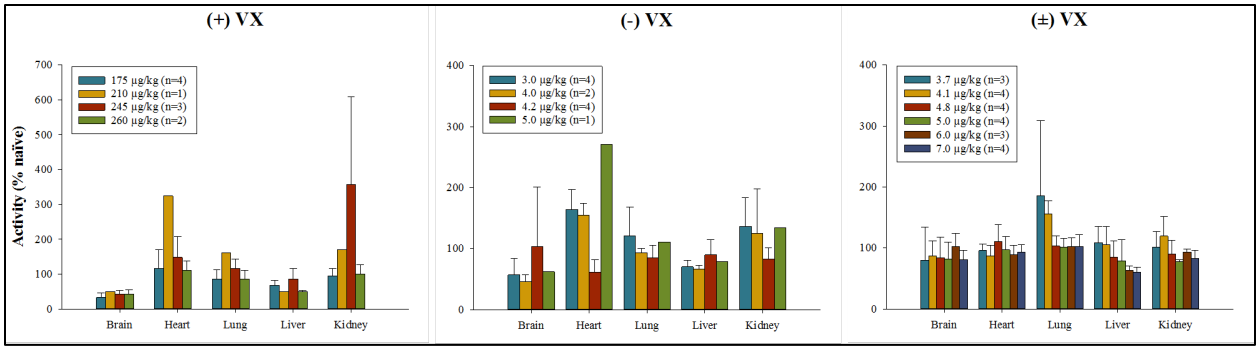

Figure 3. AChE activities in tissue samples collected from guinea pigs IV exposed to one of the optically pure enantiomers or the racemic mixture of VX.

Figure 4 shows BuChE activities in tissue samples collected from guinea pigs IV exposed to the P(+)-isomer (left), the P(–)-isomer (middle), or the racemic mixture (right) of VX. At 24 h post-exposure, all of the tissue samples collected from guinea pigs exposed to the P(+)-isomer had BuChE activities 80% less than samples collected from naïve guinea pigs with four exceptions: liver samples from the 175 µg/kg group, heart and kidney samples from the 210 µg/kg group, and kidney samples from the 245 µg/kg group. All of the lung samples collected from guinea pigs exposed to this isomer had BuChE activities less than 50%. BuChE activities less than 50% were also measured in heart and brain samples from the 175 µg/kg group, liver samples from the 210 µg/kg group, liver samples from the 245 µg/kg group, and heart samples from the 260 µg/kg group. All of the tissue samples collected from guinea pigs exposed to the P(–)-isomer had BuChE activities greater than 80% with five exceptions: lung samples from the 3.0 µg/kg group, brain samples from the 4.0 µg/kg group, heart samples from the 4.2 µg/kg group, and liver and lung samples from the 5.0 µg/kg group. However, none of these tissue samples had BuChE activities less than 50%. All of the tissue samples collected from guinea pigs exposed to the racemic mixture had BuChE activities greater than 80% with three exceptions: brain samples from the 5.0 µg/kg group and brain and kidney samples from the 7.0 µg/kg group. However, none of these tissue samples had BuChE activities less than 50%.

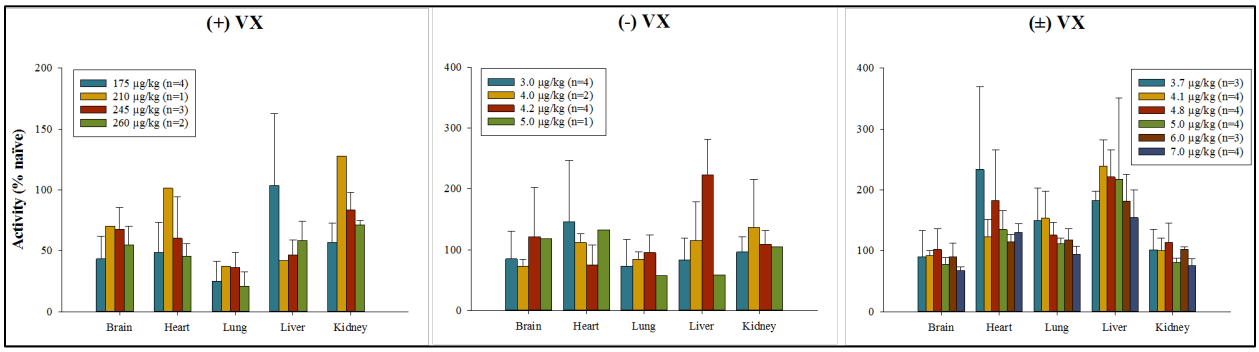

Figure 4. BuChE activities in tissue samples collected from guinea pigs IV exposed to one of the optically pure enantiomers or the racemic mixture of VX.

4. DISCUSSION

AChE activity in the blood of young adult, male guinea pigs was inhibited more slowly with the P(+)-isomer of VX than with the P(–)-isomer or the racemic mixture. Guinea pigs exposed to the P(+)-isomer of VX did not reach 80% AChE inhibition until 10–20 min post-exposure, whereas guinea pigs exposed to the P(–)-isomer or racemic mixture reached that level of inhibition almost immediately. This was in keeping with in vitro studies reporting the rate constants for the inhibition of AChE activity by the enantiomers of VX. In bovine erythrocyte ghosts, the rate constant for the inhibition of AChE activity by the P(+)-isomer of VX was 200× lower than that by the P(–)-isomer (Benshop and De Jong, 1988; 2001). In human and swine erythrocyte ghosts, the rate constants for the inhibition of AChE activity by the P(+)-isomer of VX were 350× and 380×, respectively, lower than that by the P(–)-isomer (Reiter et al., 2015). The rate constants for the racemic mixture of VX were comparable to those for the P(–)-isomer in both human and swine erythrocytes. Using X-ray crystallography, Bester et al. (2018) showed that recombinant human AChE reacted preferentially with the P(–)-isomer of VX in the presence of the racemic mixture.

The P(+)-isomer of VX inhibited nearly 95% of BuChE activity in the blood of young adult, male guinea pigs, while the P(-)-isomer and the racemic mixture inhibited only 50-60% of BuChE activity. This was at odds with the study by Reiter et al. (2015) that showed the rate constants for the inhibition of BuChE activity by the P(+)-isomer of VX were 2× and 13× lower than that by the P(–)-isomer in human and swine erythrocyte ghosts, respectively. Using X-ray crystallography, Wanderhammer et al. (2011) showed that recombinant human BuChE reacted preferentially with the P(+)-isomer of VX in the presence of the racemic mixture. The active site of BuChE had large binding pockets, allowing the P(+)-isomer of VX to fit better than the P(–)-isomer and form the P_S adduct, which did not age. Crystals of recombinant human BuChE inhibited with the P(+)-isomer or the P(–)-isomer of VX formed the P_S adduct and the P_R adduct, respectively. With the P_R adduct, the ethoxy substituent of the P(–)-isomer of VX was positioned next to two catalytic residues known to promote aging. The stereoselectivity differences between AChE and BuChE inhibition most likely explain why the P(+)-isomer of VX was 60× less toxic than the P(–)-isomer in the guinea pig model.

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ACRONYMS AND ABBREVIATIONS

AChE	acetylcholinesterase
BuChE	butyrylcholinesterase
CWNA	chemical warfare nerve agent
IV	intravenously
LD_{50}	median lethal dose
N/A	not applicable
SDS	sodium dodecyl sulfate
(+) VX	P(+)-isomer of VX
(–) VX	P(–)-isomer of VX
(±) VX	racemic mixture of VX

Blank

APPENDIX A
CHOLINESTERASE ACTIVITIES IN BLOOD SAMPLES

Table A-1. AChE Activities in Whole Blood Samples Collected from Guinea Pigs IV Exposed to the P(+)-Isomer of VX

Animal ID	Dose (µg/kg)	Time Point (% baseline)									
		1 min	10 min	20 min	30 min	40 min	50 min	60 min	180 min	360 min	1440 min
207	175	N/A	N/A	N/A	N/A	N/A	N/A	N/A	14.1	17.3	35.2
252	175	27.6	32.2	23.6	24.2	22.1	18.8	20.3	20.1	21.9	30.3
254	175	13.4	13.0	12.4	11.3	11.6	10.8	11.6	N/A	N/A	N/A
312	175	20.4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	27.4
262	210	20.0	12.1	8.5	9.1	8.2	7.3	7.0	5.7	N/A	N/A
266	210	26.2	15.1	13.1	11.0	9.6	10.2	8.7	7.8	9.0	18.3
269	210	N/A	14.7	N/A	8.2	4.4	5.6	5.5	6.8	N/A	N/A
307	210	46.0	24.4	18.5	16.0	13.6	13.8	14.0	13.6	16.6	N/A
311	245	N/A	32.5	22.2	19.0	14.3	15.1	14.9	14.3	16.6	23.3
303	245	25.6	22.9	17.0	15.8	14.8	11.1	15.0	12.2	13.2	20.3
304	245	20.5	20.5	17.5	14.8	14.3	13.3	13.9	12.4	13.1	N/A
305	245	38.9	25.3	20.4	17.7	16.5	16.1	13.7	13.6	15.3	24.6
306	260	N/A	13.4	9.3	8.2	N/A	6.9	6.8	N/A	11.3	N/A
308	260	45.9	25.7	13.5	13.0	12.9	13.2	11.9	12.6	14.6	32.5
309	260	25.5	20.4	15.9	12.9	10.0	10.9	10.5	11.2	11.5	N/A
310	260	39.3	25.1	18.3	15.0	13.6	12.9	11.6	13.1	N/A	31.6
268	280	7.1	12.6	7.1	7.5	N/A	6.5	N/A	N/A	6.1	N/A
270	280	13.5	12.8	11.4	8.1	8.1	7.2	6.9	6.9	7.7	N/A
271	280	10.8	7.8	7.0	N/A	6.1	N/A	N/A	N/A	N/A	N/A
272	280	25.0	19.7	16.1	15.5	12.4	13.5	13.3	13.7	15.9	N/A

N/A, not applicable

Table A-2. AChE Activities in Whole Blood Samples Collected from Guinea Pigs IV Exposed to the P(–)-Isomer of VX

Animal ID	Dose (µg/kg)	Time Point (% baseline)									
		1 min	10 min	20 min	30 min	40 min	50 min	60 min	180 min	360 min	1440 min
253	3.0	7.7	8.7	8.4	9.7	9.2	9.4	9.7	12.0	16.1	34.0
255	3.0	N/A	N/A	6.6	6.8	7.2	6.9	6.9	9.1	11.7	32.6
256	3.0	6.4	7.4	8.0	8.0	8.3	8.1	7.9	10.8	14.9	32.8
265	3.0	5.3	5.1	6.1	5.5	6.0	6.3	6.5	9.4	12.3	39.1
209	4.0	16.6	5.6	5.1	5.0	5.0	6.1	5.6	6.3	9.4	26.3
257	4.0	6.4	6.2	6.1	6.6	6.2	7.3	7.5	9.9	14.6	N/A
258	4.0	5.6	5.8	6.2	7.6	6.3	6.0	6.5	9.0	12.0	N/A
259	4.0	5.0	6.8	5.9	6.5	6.4	7.2	7.0	9.7	12.6	29.2
188	4.2	N/A	7.9	10.4	8.3	6.9	8.3	8.3	9.0	13.3	32.2
189	4.2	15.1	14.0	8.9	17.0	9.4	8.0	9.0	12.2	14.8	41.1
192	4.2	6.8	6.9	9.1	6.2	7.5	9.2	6.2	8.8	11.2	32.8
194	4.2	N/A	N/A	17.1	N/A	18.0	14.1	13.5	14.9	18.1	56.1
260	5.0	5.2	5.4	5.5	4.4	4.1	5.4	5.2	7.8	10.9	27.5
261	5.0	5.4	3.8	4.4	5.0	4.7	2.1	5.0	7.7	12.8	N/A
264	5.0	4.7	5.1	4.9	5.3	5.2	5.0	5.3	8.0	10.4	N/A
267	5.0	4.2	4.3	5.0	5.0	4.6	4.8	4.8	6.0	N/A	N/A

N/A, not applicable

Table A-3. AChE Activities in Whole Blood Samples Collected from Guinea Pigs IV Exposed to the Racemic Mixture of VX

Animal ID	Dose (µg/kg)	Time Point (% baseline)									
		1 min	10 min	20 min	30 min	40 min	50 min	60 min	180 min	360 min	1440 min
204	3.7	13.6	20.6	17.2	18.1	16.9	22.4	25.2	30.2	49.0	119.3
206	3.7	N/A	57.2	54.0	54.5	57.6	53.1	61.0	74.2	92.3	156.3
208	3.7	13.8	12.2	12.5	14.0	14.6	12.3	14.3	18.7	15.7	30.3
200	4.1	14.3	11.8	11.4	10.5	10.3	10.3	10.5	13.6	15.8	28.6
201	4.1	13.3	10.2	9.6	11.8	10.7	12.3	11.4	13.2	17.0	31.7
202	4.1	10.8	10.0	9.6	7.9	9.7	9.3	10.3	12.4	14.0	32.0
203	4.1	13.7	10.4	10.5	12.5	11.4	11.5	11.8	12.6	13.6	31.7
180	4.8	10.1	10.5	11.7	10.8	12.0	11.5	12.5	11.4	16.1	37.4
182	4.8	12.3	11.2	14.4	12.7	12.4	13.9	11.7	14.3	11.6	43.0
183	4.8	9.0	9.1	9.3	9.7	7.5	9.4	9.7	12.0	13.0	31.3
185	4.8	7.5	7.5	9.0	9.4	9.4	10.6	11.0	12.7	15.7	33.3
196	5.0	7.4	6.6	7.5	8.0	7.8	9.1	8.8	11.9	18.6	34.0
197	5.0	8.8	8.9	10.6	11.1	10.1	12.0	10.6	14.3	N/A	31.7
198	5.0	7.4	7.5	8.4	8.7	9.0	10.9	11.6	10.4	15.7	32.4
199	5.0	15.8	12.4	11.1	11.1	10.7	11.2	11.9	13.0	18.8	34.0
190	6.0	7.3	6.4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
191	6.0	7.2	8.8	9.9	9.5	10.4	10.0	10.4	12.4	11.2	35.6
193	6.0	9.0	9.1	8.4	10.4	10.0	9.3	10.4	14.7	18.4	30.0
195	6.0	7.3	6.4	7.7	9.3	8.3	8.6	8.8	10.3	13.1	29.5
181	7.0	6.6	7.7	N/A	N/A	8.0	N/A	N/A	N/A	15.8	41.3
184	7.0	N/A	N/A	N/A	6.7	N/A	N/A	N/A	N/A	14.7	27.2
186	7.0	7.0	7.5	7.5	8.1	7.6	9.4	8.8	11.4	11.7	30.8
187	7.0	6.5	7.4	N/A	N/A	8.0	7.7	6.6	9.7	14.2	26.5

N/A, not applicable

Table A-4. BuChE Activities in Whole Blood Samples Collected from Guinea Pigs IV Exposed to the P(+)-Isomer of VX

Animal ID	Dose (µg/kg)	Time Point (% baseline)									
		1 min	10 min	20 min	30 min	40 min	50 min	60 min	180 min	360 min	1440 min
207	175	N/A	N/A	N/A	N/A	N/A	N/A	N/A	2.7	4.2	12.3
252	175	5.1	6.1	5.5	6.1	6.7	6.2	5.6	6.9	9.2	24.3
254	175	5.8	5.4	5.4	6.2	6.2	6.8	N/A	N/A	N/A	N/A
312	175	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	15.5
262	210	5.5	4.7	5.2	3.9	N/A	4.5	N/A	5.8	N/A	N/A
266	210	5.3	5.9	5.2	5.3	5.5	6.1	6.0	6.3	7.8	18.9
269	210	N/A	5.6	N/A	5.4	5.6	4.7	5.5	7.8	N/A	N/A
307*	210	27.1	25.1	26.8	25.9	28.1	26.8	27.5	28.8	43.0	N/A
311	245	N/A	4.8	5.3	4.8	4.6	4.9	5.1	0.6	7.6	16.6
303	245	5.1	5.2	5.4	6.2	5.1	6.0	5.9	5.9	7.5	18.6
304	245	5.4	5.8	5.6	5.5	5.7	6.2	6.4	6.4	9.6	N/A
305	245	5.2	5.2	5.0	5.4	5.0	5.0	6.1	6.1	6.8	15.0
306	260	N/A	N/A	N/A	4.7	N/A	4.9	4.5	N/A	12.1	N/A
308	260	6.8	4.9	5.8	5.6	4.9	4.9	5.0	5.9	8.3	21.1
309	260	3.6	4.1	4.1	4.4	4.0	4.4	3.8	4.5	7.2	N/A
310	260	4.6	4.6	5.7	3.8	4.5	4.7	4.2	5.3	N/A	23.8
268	280	2.7	2.4	3.1	3.7	N/A	3.3	N/A	N/A	8.3	N/A
270	280	6.2	6.4	6.8	5.9	6.3	6.4	6.2	6.4	9.1	N/A
271	280	5.2	4.7	4.8	N/A	4.6	N/A	N/A	N/A	N/A	N/A
272	280	6.3	7.4	6.9	6.8	6.8	7.2	7.1	8.1	10.0	N/A

*Outlier (Grubbs' test) removed from the analyses
N/A, not applicable

Table A-5. BuChE Activities in Whole Blood Samples Collected from Guinea Pigs IV Exposed to the P(–)-Isomer of VX

Animal ID	Dose (µg/kg)	Time Point (% baseline)									
		1 min	10 min	20 min	30 min	40 min	50 min	60 min	180 min	360 min	1440 min
253	3.0	76.4	N/A	67.4	74.3	65.9	75.7	70.1	72.9	70.6	18.9
255	3.0	N/A	N/A	56.2	64.0	63.3	61.0	56.1	71.9	74.8	85.2
256	3.0	54.2	61.7	56.2	58.3	56.6	60.1	61.1	61.6	64.9	24.2
265	3.0	79.6	66.0	60.5	67.6	65.6	66.1	68.6	64.3	73.6	82.0
209	4.0	70.5	59.0	51.6	68.1	51.1	51.3	48.2	45.4	50.8	46.7
257	4.0	61.9	53.1	49.7	51.9	51.0	49.3	47.5	54.3	55.6	N/A
258	4.0	61.3	52.1	44.2	45.2	40.1	40.5	40.8	41.6	56.3	N/A
259	4.0	69.7	55.5	51.0	52.4	47.5	49.9	51.1	48.4	56.5	65.8
188	4.2	N/A	57.3	55.0	55.8	51.6	56.6	55.1	59.3	74.6	23.9
189	4.2	69.2	55.8	51.0	48.2	49.6	53.5	55.3	53.1	63.7	47.4
192	4.2	78.2	53.7	58.4	63.3	72.9	66.2	58.3	65.0	72.3	50.8
194	4.2	N/A	N/A	44.6	N/A	42.0	52.3	47.2	52.6	69.9	49.3
260	5.0	55.9	46.6	39.3	38.7	37.4	N/A	41.3	39.6	47.4	47.8
261	5.0	64.8	54.1	44.8	43.1	41.2	45.7	43.3	45.9	60.7	N/A
264	5.0	57.9	49.9	41.1	35.8	34.8	37.0	37.9	63.0	68.1	N/A
267	5.0	48.2	41.6	38.8	35.3	32.0	32.1	32.6	38.8	N/A	N/A

N/A, not applicable

Table A-6. BuChE Activities in Whole Blood Samples Collected from Guinea Pigs IV Exposed to the Racemic Mixture of VX

Animal ID	Dose (µg/kg)	Time Point (% baseline)									
		1 min	10 min	20 min	30 min	40 min	50 min	60 min	180 min	360 min	1440 min
204	3.7	57.8	66.5	72.5	72.4	74.8	67.5	79.0	85.5	70.7	118.0
206	3.7	N/A	74.3	70.3	80.4	76.4	77.7	76.8	80.9	77.1	92.8
208	3.7	59.1	61.4	65.5	67.1	53.8	56.4	53.8	58.1	58.3	60.2
200	4.1	67.1	57.6	54.4	55.3	54.8	51.9	53.2	55.8	57.3	61.7
201	4.1	69.5	66.5	65.7	62.2	66.2	63.0	67.0	71.3	69.2	74.8
202	4.1	72.7	70.7	68.6	69.1	59.4	64.5	61.1	65.0	73.0	60.7
203	4.1	76.3	70.5	62.4	63.5	70.6	70.1	64.5	68.8	68.4	77.6
180	4.8	60.7	60.7	57.0	64.8	54.5	54.5	53.7	52.5	61.6	75.0
182	4.8	61.6	49.4		44.2	48.7	47.1	46.3	49.3	55.4	46.7
183	4.8	50.2	44.9	38.7	36.0	52.2	36.1	36.2	37.1	41.1	56.7
185	4.8	52.2	46.7	47.1	50.2	43.7	48.1	46.5	45.3	51.7	59.6
196	5.0	61.1	57.0	52.8	50.3	57.1	41.7	59.6	51.9	60.3	75.7
197	5.0	69.5	66.2	59.6	63.8	63.0	49.6	69.5	67.2	N/A	63.1
198	5.0	53.1	51.0	46.2	45.5	43.3	43.7	47.5	49.5	51.5	34.1
199	5.0	69.0	60.8	57.1	53.2	57.4	59.0	57.0	56.4	62.2	60.1
190	6.0	46.4	37.1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
191	6.0	54.1	54.4	54.3	50.5	53.1	60.1	55.7	58.7	55.2	36.1
193	6.0	58.8	47.7	52.1	47.9	48.0	49.4	46.8	56.5	56.7	49.3
195	6.0	59.4	40.6	49.9	55.6	51.6	46.9	53.9	52.9	54.2	31.1
181	7.0	49.8	50.8	N/A	N/A	46.7	N/A	N/A	N/A	47.2	51.7
184	7.0	N/A	N/A	N/A	23.5	N/A	N/A	N/A	N/A	32.5	16.9
186	7.0	61.6	45.2	34.9	33.8	34.3	33.6	35.9	36.6	37.7	51.5
187	7.0	66.3	55.2	N/A	N/A	45.2	40.6	44.8	40.4	49.8	34.1

N/A, not applicable

APPENDIX B
CHOLINESTERASE ACTIVITIES IN TISSUE SAMPLES

Table B-1. AChE Activities in Tissue Samples Collected from Guinea Pigs IV Exposed to the P(+)-Isomer of VX

Animal ID	Dose (µg/kg)	Latency to Death (min)	% naïve				
			Brain	Heart	Liver	Lung	Kidney
207	175	N/A	47.1	51.2	54.0	58.2	78.4
252	175	N/A	23.6	115.8	67.4	70.7	85.2
254	175	N/A	18.8	113.1	61.6	90.4	127.3
312	175	N/A	41.5	183.2	88.0	120.1	85.2
262	210	153	23.4	187.6	76.2	83.6	163.6
266	210	N/A	50.5	324.1	50.4	160.3	169.3
269	210	239	46.7	368.0	105.6	202.9	113.6
307	210	<1440	N/A	N/A	N/A	N/A	N/A
311	245	N/A	33.7	196.6	54.0	135.3	626.1
303	245	N/A	42.7	80.8	113.2	83.6	318.2
304	245	<1440	N/A	N/A	N/A	N/A	N/A
305	245	N/A	52.3	167.0	89.7	127.7	126.1
306	260	335	31.5	135.5	41.6	101.1	142.0
308	260	N/A	51.3	92.5	48.7	104.1	119.3
309	260	<1440	N/A	N/A	N/A	N/A	N/A
310	260	N/A	33.9	130.2	52.2	68.4	81.8
268	280	203	46.3	300.7	78.0	148.2	112.5
270	280	<1400	N/A	N/A	N/A	N/A	N/A
271	280	63	21.6	232.5	62.8	221.1	111.4
272	280	<1440	N/A	N/A	N/A	N/A	N/A

<1440 min as a latency to death indicates that the guinea pig died outside of business hours.
N/A, not applicable

Table B-2. AChE Activities in Tissue Samples Collected from Guinea Pigs IV Exposed to the P(–)-Isomer of VX

Animal ID	Dose (µg/kg)	Latency to Death (min)	% naïve				
			Brain	Heart	Liver	Lung	Kidney
253	3.0	N/A	23.6	115.8	67.4	70.7	85.2
255	3.0	N/A	51.5	180.4	85.6	183.9	196.6
256	3.0	N/A	64.7	180.4	61.6	107.1	142.0
265	3.0	N/A	87.4	180.4	65.7	122.3	122.7
209	4.0	N/A	37.6	168.8	62.2	88.4	72.7
257	4.0	<1400	N/A	N/A	N/A	N/A	N/A
258	4.0	<1440	N/A	N/A	N/A	N/A	N/A
259	4.0	N/A	54.1	140.0	70.4	98.0	176.1
188	4.2	N/A	96.3	47.6	79.8	109.7	102.3
189	4.2	N/A	243.0	84.4	125.5	69.2	83.0
192	4.2	N/A	27.7	41.3	85.0	92.8	86.4
194	4.2	N/A	45.6	71.8	69.2	67.0	58.0
260	5.0	N/A	61.7	271.1	79.2	110.9	134.1
261	5.0	<1440	N/A	N/A	N/A	N/A	N/A
264	5.0	<1440	N/A	N/A	N/A	N/A	N/A
267	5.0	167	93.0	134.6	88.6	155.0	123.9

<1440 min as a latency to death indicates that the guinea pig died outside of business hours.
N/A, not applicable

Table B-3. AChE Activities in Tissue Samples Collected from Guinea Pigs IV Exposed to the Racemic Mixture of VX

Animal ID	Dose (µg/kg)	Latency to Death (min)	% naïve				
			Brain	Heart	Liver	Lung	Kidney
204	3.7	N/A	20.2	86.2	97.4	77.3	71.6
206	3.7	N/A	124.2	106.8	89.7	157.6	108.0
208	3.7	N/A	96.1	94.3	138.4	320.3	122.7
200	4.1	N/A	116.7	108.6	140.2	164.9	136.4
201	4.1	N/A	61.4	64.6	103.8	181.9	94.3
202	4.1	N/A	71.4	89.8	67.4	133.3	156.8
203	4.1	N/A	97.0	82.6	111.4	141.4	89.8
180	4.8	N/A	126.5	149.0	110.3	125.2	123.9
182	4.8	N/A	93.3	105.9	100.3	106.8	77.3
183	4.8	N/A	50.7	79.9	80.9	89.1	78.4
185	4.8	N/A	65.1	105.0	49.9	90.6	79.5
196	5.0	N/A	53.7	96.9	55.7	98.7	81.8
197	5.0	N/A	110.5	126.6	51.0	84.7	78.4
198	5.0	N/A	63.6	88.0	128.4	117.8	75.0
199	5.0	N/A	99.1	75.4	79.2	104.6	73.9
190	6.0	21	20.4	38.6	45.2	77.3	55.7
191	6.0	N/A	93.0	81.7	66.9	114.9	90.9
193	6.0	N/A	86.4	77.2	54.5	106.0	98.9
195	6.0	N/A	127.0	106.8	67.4	86.2	89.8
181	7.0	N/A	87.4	104.1	62.2	131.8	77.3
184	7.0	N/A	87.8	78.1	59.2	89.1	76.1
186	7.0	N/A	57.9	98.7	51.0	96.5	75.0
187	7.0	N/A	91.0	93.4	69.8	92.0	102.3

N/A, not applicable

Table B-4. BuChE Activities in Tissue Samples Collected from Guinea Pigs IV Exposed to the P(+)-Isomer of VX

Animal ID	Dose (µg/kg)	Latency to Death (min)	% naïve				
			Brain	Heart	Liver	Lung	Kidney
207	175	N/A	54.5	59.6	182.5	33.8	51.0
252	175	N/A	19.6	26.1	109.5	9.7	80.1
254	175	N/A	38.2	29.7	78.0	12.4	50.9
312	175	N/A	61.0	78.3	44.3	43.7	44.9
262	210	153	35.4	54.5	96.0	12.9	61.3
266	210	N/A	70.3	101.3	41.9	37.5	127.8
269	210	239	65.7	108.5	168.0	27.9	28.1
307	210	<1440	N/A	N/A	N/A	N/A	N/A
311	245	N/A	55.0	97.3	36.9	50.2	82.9
303	245	N/A	58.7	29.7	42.4	30.2	98.4
304	245	<1440	N/A	N/A	N/A	N/A	N/A
305	245	N/A	88.5	53.9	60.2	28.0	69.0
306	260	335	50.8	52.2	55.0	22.7	40.6
308	260	N/A	65.7	52.7	47.3	29.3	68.5
309	260	<1440	N/A	N/A	N/A	N/A	N/A
310	260	N/A	44.2	38.5	69.5	12.3	73.9
268	280	203	58.7	142.4	68.1	46.8	46.5
270	280	<1400	N/A	N/A	N/A	N/A	N/A
271	280	63	41.0	49.9	52.0	27.8	41.9
272	280	<1440	N/A	N/A	N/A	N/A	N/A

<1440 min as a latency to death indicates that the guinea pig died outside of business hours.
N/A, not applicable

Table B-5. BuChE Activities in Tissue Samples Collected from Guinea Pigs IV Exposed to the P(–)-Isomer of VX

Animal ID	Dose (µg/kg)	Latency to Death (min)	% naïve				
			Brain	Heart	Liver	Lung	Kidney
253	3.0	N/A	19.6	26.1	109.5	9.7	80.1
255	3.0	N/A	88.5	133.7	114.3	112.2	133.1
256	3.0	N/A	110.9	152.3	69.3	84.6	84.0
265	3.0	N/A	120.2	271.9	38.8	84.9	86.9
209	4.0	N/A	64.3	101.1	160.3	74.8	80.0
257	4.0	<1400	N/A	N/A	N/A	N/A	N/A
258	4.0	<1440	N/A	N/A	N/A	N/A	N/A
259	4.0	N/A	81.0	122.2	70.0	92.8	192.6
188	4.2	N/A	149.0	46.8	170.2	133.6	117.9
189	4.2	N/A	224.0	92.0	255.9	66.2	136.2
192	4.2	N/A	47.0	48.9	175.2	97.0	96.3
194	4.2	N/A	66.1	112.7	289.2	83.6	84.6
260	5.0	N/A	118.3	132.5	58.2	57.8	105.0
261	5.0	<1440	N/A	N/A	N/A	N/A	N/A
264	5.0	<1440	N/A	N/A	N/A	N/A	N/A
267	5.0	167	323.7	103.6	103.4	88.1	80.3

<1440 min as a latency to death indicates that the guinea pig died outside of business hours.
N/A, not applicable

Table B-6. BuChE Activities in Tissue Samples Collected from Guinea Pigs IV Exposed to the Racemic Mixture of VX

Animal ID	Dose (µg/kg)	Latency to Death (min)	% naïve				
			Brain	Heart	Liver	Lung	Kidney
204	3.7	N/A	43.3	157.3	178.8	97.1	65.3
206	3.7	N/A	126.7	390.9	169.9	146.3	130.5
208	3.7	N/A	100.1	152.9	199.6	204.2	109.2
200	4.1	N/A	102.9	159.6	222.0	117.3	106.9
201	4.1	N/A	87.6	94.4	197.6	217.7	78.4
202	4.1	N/A	87.8	128.9	236.1	131.9	126.1
203	4.1	N/A	94.6	108.5	299.4	146.8	87.4
180	4.8	N/A	145.3	298.4	270.6	121.7	126.2
182	4.8	N/A	113.2	158.2	220.9	153.2	85.9
183	4.8	N/A	74.5	103.0	229.9	103.7	88.1
185	4.8	N/A	75.5	172.1	163.8	123.2	152.1
196	5.0	N/A	77.8	127.2	161.8	104.4	76.4
197	5.0	N/A	86.6	180.5	113.2	107.2	71.9
198	5.0	N/A	63.3	110.6	411.8	125.3	86.8
199	5.0	N/A	84.3	121.5	184.0	110.9	86.8
190	6.0	21	45.6	72.0	136.4	42.3	63.3
191	6.0	N/A	64.7	128.7	149.9	138.7	99.1
193	6.0	N/A	107.1	108.7	162.3	108.9	101.9
195	6.0	N/A	97.8	105.5	232.3	106.6	106.6
181	7.0	N/A	61.9	143.2	109.3	86.4	62.0
184	7.0	N/A	65.2	113.9	134.7	112.7	72.7
186	7.0	N/A	67.1	122.8	159.0	92.5	88.8
187	7.0	N/A	76.4	141.3	215.3	83.1	78.2

N/A, not applicable

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