In vitro Characterization and Rescue of VX Metabolism in Human Liver Microsomes

Thomas R. Lane¹, David Koebel², Eric Lucas², Robert Moyer² and Sean Ekins¹.

¹Collaborations Pharmaceuticals, Inc. 840 Main Campus Drive, Lab 3510, Raleigh, NC 27606.

²Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201.
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VX Metabolism in Human Liver Microsomes

To whom correspondence should be addressed:

Sean Ekins Ph.D., D.Sc.

Collaborations Pharmaceuticals, Inc. 840 Main Campus Drive, Lab 3510

Raleigh, NC 27606.

E-mail: sean@collaborationspharma.com

Phone: 215-687-1320.

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Abbreviations
Acetylcholinesterase (AChE); Army Materiel Command (AMC); Battelle Biomedical Research Center (BBRC); Butyrylcholinesterase (BChE); central nervous system (CNS); chemical warfare nerve agents (CWNAs); diisopropylaminoethyl methyl thiolophosphonate (EA-2192); Ethylenediaminetetraacetic acid (EDTA); ethyl methylphosphonic acid (EMPA); human liver microsomes (HLM); liquid chromatography with tandem mass spectrometry (LC-MS/MS); methylphosphonic acid (MPA); organophosphate (OP); multiple-reaction monitoring (MRM); nicotinamide adenine dinucleotide phosphate (NADPH); organophosphorus hydrolase (OPH); Paraoxonase-1 (PON1), phosphotriesterase (PTE)
Abstract

VX is an organophosphate acetylcholinesterase (AChE) inhibitor and while it is one of the most toxic AChE inhibitors known the extent of metabolism in humans is not currently well understood. The known metabolism in humans is limited to the metabolite identification from a single victim of the Osaka poisoning in 1994, which allowed for the identification of several metabolic products. VX has been reported to be metabolized in vitro by paraoxonase-1 and phosphotriesterase, although their binding constants are many orders of magnitude above the LD$_{50}$, suggesting limited physiological relevance.

Using incubation with human liver microsomes (HLM) we have now characterized the metabolism of VX and the formation of multiple metabolites, as well as identifying an FDA-approved drug (EDTA) that enhances the metabolic rate. HLM incubation alone shows a pronounced increase in the metabolism of VX as compared to buffer, suggesting that CYP-mediated metabolism of VX is occurring. We identified a biphasic decay with two distinct rates of metabolism. The enhancement of VX metabolism in multiple buffers was assessed to attempt to mitigate the effect of hydrolysis rates. The formation of VX metabolites was shown to be shifted with HLMs, suggesting a pathway enhancement over simple hydrolysis. Additionally, our investigation of hydrolysis rates in various common buffers used in biological assays discovered dramatic differences in VX stability. The new human in vitro VX metabolic data reported points to a potential in vivo treatment strategy for rescue in individuals that are poisoned though enhancement of metabolism alongside existing treatments.
Significance Statement

VX is a potent acetylcholinesterase inhibitor and chemical weapon. To date we do not possess a clear understanding of its metabolism in humans that would assist us in treating those exposed to it. We now describe the human liver microsomal metabolism of VX and identify EDTA which appears to enhance the rate of metabolism. This may provide a potential treatment option for human VX poisoning.
Introduction

The threat of exposure to organophosphate (OP) chemical warfare nerve agents (CWNAs) such as VX, tabun, sarin, soman, cyclosarin, and others is a continued concern (Singh et al., 2016). This was highlighted by the assassination of Kim Jong-Nam (Nakagawa and Tu, 2018), an assassination attempt in the UK using Novichok (Haslam et al., 2022), and the use of chemical weapons in the ongoing war in Syria (Worek et al., 2016). In addition, OP pesticide accidental exposure and intentional self-poisoning kills hundreds of thousands of people every year (Zayed et al., 2015). OPs can induce prolonged and uncontrolled excitation of the cholinergic system through inhibition of acetylcholinesterase (AChE), which may ultimately lead to death (Costanzi et al., 2018). Depending on the route of exposure and amount absorbed, the peripheral and/or central nervous system (CNS) can be affected. The result is the prolonged stimulation of muscarinic and/or nicotinic receptors.

While reviews have focused on the chemistry, physiology of cholinergic transmission, biochemistry of cholinesterase of nerve agent inhibition and treatment options, (Costanzi et al., 2018; Pope and Brimijoin, 2018) there has been limited assessment of the metabolism of these molecules in humans. Evaluation of OP and CWNA metabolism in humans would clearly be unethical, but it is possible to learn from in vitro systems and studies which have assessed genetic polymorphisms in acute toxicity patients that have pointed to the role of paraoxonase 1 (PON1) and CYP2D6 (Zayed et al., 2015). There has been little published work on the metabolism of CWNAs using human hepatocytes, or the assessment of the drug metabolizing enzymes involved
(Cao et al., 1999; Bruinink et al., 2002). There has been limited study of CWNA metabolism in other species, except for soman metabolism in mice (Tuovinen, 2004) and VX in swine (Reiter et al., 2015). While we understand the primary targets of the OPs, knowledge of their metabolism in humans and inter-individual differences in metabolic enzymes is limited. There is some data on OP pesticide metabolism in humans (Hodgson and Rose, 2007a; Hodgson and Rose, 2007b; Foxenberg et al., 2011) and animals, which may serve as a starting point to help bridge in vitro CWNA metabolism to in vivo.

VX is a lethal, OP AChE inhibitor (LD$_{50}$ = 0.022 - ~0.1 mg/kg in mice) (Pike, 1992) (Reiter et al., 2008) the metabolism of which in humans is poorly understood. Reports of VX metabolism in humans are limited to metabolite identification (e.g. from specific poisoning incidents like in Osaka in 1994) which provide part of the metabolic pathway, while other relevant parameters (e.g., rates and proportions of metabolite formation) remain unknown (Tsuchihashi et al., 1998). VX has been shown to be metabolized in vitro by PON1 (Kirby et al., 2013), phosphotriesterase (PTE) (Tsai et al., 2012) and organophosphorus hydrolase (OPH) (Rastogi et al., 1997; Reeves et al., 2008) although with binding constants orders of magnitude above peak level ranges found following toxic exposure (100 nM to 10 µM) (Benschop and De Jong, 1991; Reiter et al., 2015), suggesting limited physiological relevance. Touvrey and coauthors (2019) recently reported the interaction of human bile salt-activated lipase, (pancreatic lipase), with paraoxon and sarin-, tabun-, VX-surrogates (Touvrey et al., 2019) with binding constants within achievable physiological concentrations. They suggested this enzyme
may act as a stoichiometric biological scavenger for VX but noted that both the VX and sarin surrogates showed a 50-fold lower affinity for pancreatic lipase as compared to AChE. Human butyrylcholinesterase (BChE) is also an effective VX bioscavenger, with in vivo studies in guinea pigs showing BChE enabled both post-exposure and prophylactic protection with up to 8x LD$_{50}$ of VX (Lenz et al., 2010; Saxena et al., 2011).

A few of the known metabolic products of VX retain some AChE inhibitory activity with the potent AChE inhibitor diisopropylaminoethyl methyl thiolophosphonate (EA-2192) (Munro et al., 1999), but hydrolysis of one or more alkyl ester bonds of organophosphonic acids generally result in the final formation of nontoxic alkyl methylphosphonic acids such as ethyl methylphosphonic acid (EMPA) and methylphosphonic acid (MPA). Preliminary studies in rat liver microsomes suggested a role for CYP450s and showed that aniline (a CYP inhibitor) competitively inhibited the inactivation of VX in this system (Fu and Sun, 1990). The metabolism of VX is therefore complex (Figure 1) and the role of specific enzymes is currently unclear. To rectify this lack of knowledge we have used human liver microsomes (HLM) to characterize the metabolism of VX and in the process have identified an FDA-approved drug that significantly enhances the in vitro metabolic rate.

**Materials and Methods**

**Materials:**

Target analytes were either provided by U.S. DEVCOM Chemical Biological Center (Aberdeen Proving Ground, MD, USA), synthesized at Battelle Memorial Institute...
(Columbus, OH, USA), or purchased from Sigma-Aldrich (St. Louis, MO, USA) or equivalent. Mixed-gender human liver microsomes (HLMs, 150-donor pool, lot number ZZQ) were purchased from BioIVT (Hicksville, NY, USA). Methanol, acetonitrile, and formic acid (Fisher Scientific, Florence, KY, USA) were all high-performance liquid chromatography (HPLC) grade. All other chemicals and reagents were purchased from Sigma-Aldrich or equivalent.

Methods:

Metabolism of VX in HLMs

HLM experiments were performed as described previously for organophosphate pesticides (Agarwal et al., 2023) with final concentrations of VX and HLMs at 200 ng/ml (~740 nM) and 0.5 mg/ml, respectively. Final buffer concentrations were 10 mM for both TRIS and MOPS (pH 7.4) and when included, a 5 mM final concentration of EDTA was used. At minimum, samples were taken at timepoints 0, 1, 2, 3, 4, 8, 24, 32, 48, 75, 96, 120, 144, 168, 192, 216, and 240 hr. A calibration curve of VX and VX metabolites/degradation products was prepared in a diluent to matrix match the calibration standards to the samples.

Each sample was prepared at 2 mL final volume in a 3 mL vial with a Teflon lined cap. Samples were prepared by combining 1.51 mL of 13.25 mM buffer, 10 µL of 40 µg/mL VX and or 400 µL of 12.5 mM nicotinamide adenine dinucleotide phosphate (NADPH), 50 µL of 20 mg/mL HLM, 20 µL of 0.5 M EDTA, 10 µL of 20 mM atipamezole, with water added to obtain a final volume of 2 mL (Table S1). Once samples were prepared, they were incubated on a heating block at 37°C placed on an orbital shaker table (100 rpm)
for the duration of the experiment (typically 240 hrs). To inhibit CYP activity, 100 µM of the pan-CYP inhibitor atipamezole was pre-incubated for 1 hour prior to the addition of VX in HLMs (Li et al., 2019).

At the appropriate sampling time point, vials were carefully inverted to mix and 50 µL of each sample was removed and placed into a vial with a conical insert. 100 µL of internal standard solution (EMPA-d4 at 150 ng/mL in acetonitrile) was aliquoted into each conical insert. Each analysis vial was shaken vigorously and vortexed for approximately 10 seconds. The sample vial was returned to the heating block for the remainder of each experiment. Samples were then analyzed using a Waters TQ-XS quadrupole mass spectrometer coupled with a Waters I-Class UPLC (Waters Corporation, Milford, MA USA) to obtain liquid chromatography with tandem mass spectrometry (LC-MS/MS) data. The LC-MS/MS software was Water’s MassLynx version 4.2 (Table S2).

A calibration curve of VX and VX metabolites/degradation products was prepared in a diluent to matrix match the calibration standards to the samples. The diluent was 10 mM buffer, depending on the buffer used in the experiment, and 2.5 mM NADPH. These calibration standards were prepared fresh each day of analysis. At the time of analysis, 50 µL of calibration standard was combined with 100 µL of internal standard solution (EMPA-d4 at 150 ng/mL in acetonitrile) in a vial with a conical insert. Each vial was shaken vigorously and vortexed for approximately 10 seconds.

All calibration standards, blanks, quality controls standards and time point samples were analyzed on a reversed-phase HPLC column (Prodigy ODS (3) column, 3 µm particle
size, 2 mm X 100 mm, made by Phenomenex (Torrance, CA, USA). All compounds were detected by electrospray ionization in positive ion mode using multiple-reaction monitoring (MRM). Prior to sample analysis the MRM method was optimized by infusing individual 10 µg/mL solutions of each target analyte into the electrospray ionization source while scanning for the appropriate precursor ion (typically protonated ions). Optimal cone energies were obtained, and product ions were generated utilizing the optimal collision energy for each.

The preparation of stock solutions of various chemicals is described in detail in the Supplemental Materials and Methods. Quantitation of each targeted analyte was performed using calibration standard concentrations prepared at 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 200 ng/mL. Calibration curves were either linear or quadratic, depending on the best fit, with a weighting factor of $1/x^2$.

**Buffer stability experiments**

VX stability studies were performed in 10 mM MOPS, ADP, HEPES, MES, PIPES, TRIS, or sodium phosphate (~pH 7.2). A calibration curve of VX and VX degradation products was prepared in a diluent to matrix match the calibration standards to the samples. pH was confirmed to have minimal drift during the experiment (Table S3).

**Statistics:**

Statistical analysis was performed in GraphPad Prism 10.1.1 (Boston, MA).

**Ethical**
VX was provided by the U.S. DEVCOM Chemical Biological Center (Aberdeen Proving Ground, MD). All tests involving VX were performed at the Battelle Biomedical Research Center (BBRC) located in West Jefferson, Ohio or Battelle’s Dilute Chemical Agent laboratories located in Columbus, Ohio. Battelle is certified to work with chemical surety material under a Provisioning Agreement with oversight by the U.S. Army Materiel Command (AMC; Provisioning Agreement Battelle-1). Wherever applicable and required, the reporting requirements for this agreement were followed. VX originated from the same synthesis lot, and its identity and purity were confirmed by gas chromatography. Neat/non-exempt VX was stored in accordance with BBRC security and chemical warfare agent storage policies until needed for testing. Dilute/exempt stocks of VX were transferred to Battelle’s Dilute Chemical Agent laboratories in Columbus, OH for the experiments described herein.

Results

HLM incubation with VX (200 ng/ml (~0.75µM)) showed a pronounced increase in the breakdown of VX compared to buffer, suggesting a strong enzyme-mediated metabolism of VX was occurring (Figure 2). The relative proportion of the key metabolites were also significantly shifted in HLMs, indicating a pathway enhancement over simple hydrolysis (Figure 2C, F). A significant shift in metabolism also occurred between the time points 8 h and 24 h (Figure 2A), which was corroborated using a non-linear regression fit (Prism 10.1.1), showing a 2nd rather than 1st order reaction based on the extra sum-of-squares F test (P < 0.0001). Initially this reaction appears to be dominated by the fast rate (t_{1/2} (fast) = 5.2 h), which represents ~30% of the total VX
breakdown, but after this initial stage the reaction vastly slows down ($t_{1/2}$ (slow) = 191.6 h). Starting at 24 h, VX appears to be stabilized well beyond the rate of hydrolysis in HLMs (Figure 2B). To further demonstrate that the large shift in metabolism is between the 8h and 24h time points these data were analyzed with the natural log of concentration. As a first-order exponential decay, the ln[VX] of VX controls (buffer without HLM, Figure 2E) can be fit to a straight line, but with addition of HLMs there are clearly two distinct regions of metabolism (Figure 2B).

Surprisingly, the pan-CYP inhibitor atipamezole (Li et al., 2019) only partially inhibited VX metabolism, suggesting that non-CYP dependent enzymatic activity is also partially responsible for VX metabolism in HLMs (Figure 3A). To determine if PON1, an enzyme known to be important in the metabolism of other CWNA agents, had a role in HLM VX metabolism EDTA (Gonzalvo et al., 1997) was used as an inhibitor. When EDTA (5 mM) was added initially to the HLM reaction the $t_{1/2}$ (fast) rates are similar, but the reaction with EDTA added shows a drastic increase in the overall metabolism (Figure 3B). The $t_{1/2}$ (slow) is much faster (approximately 10-fold lower) with the addition of EDTA, demonstrating that EDTA drastically affects this rate. There was no significant change in VX stability when EDTA was added to the VX control (extra sum-of-squares test $p = 0.1964$), suggesting this is an effect on the enzymes in the HLM. Interestingly, atipamezole also had similar effects on the metabolic rate of VX with and without EDTA (Figure 3C).
To determine if this was caused by a buffer effect, we repeated these experiments in another frequently used biological buffer, MOPS, which has a similar pKa but is less prone to shifts in pH based on temperature variations. We found a very similar EDTA-mediated rescue effect (Figure S1), but the stabilization effect of VX in HLM (no EDTA) was dramatically increased over the hydrolysis rate (Figure S1A,E), to the point that there was almost no appreciable hydrolysis past 24 hr. Interestingly, the $t_{1/2}$ (slow) was much longer in the VX + HLM + EDTA in MOPS as compared to TRIS (Figure S2A). A direct comparison of VX-HLM stability in these two buffers shows a significant difference in metabolism, with and without EDTA, although the stability in buffer alone is unchanged (Figure S2C). Statistically, the hydrolysis of VX-only controls in MOPS were not different from TRIS using an extra sum-of-square F test ($p = 0.1814$) using Prism 10.1.1.

To determine if the chelating agent EDTA was able to rescue the metabolism of VX in HLMs later in the reaction, a time course was explored. Ultimately, EDTA added at all points (0, 4, 24, 72 hr) eliminated the stabilization effect found in HLM with no EDTA (Figure 4). When added at 4 hr, the metabolic rate appears to have increased between 24-48 hrs to a similar level when EDTA was added at time zero, but from 48 hrs on this increase appears to be diminished.

Further investigation into VX buffer-dependent stability found a drastic difference in the VX decay rates in commonly used buffers (Figure 5). An approximate 5-fold difference in $t_{1/2}$ was found, with sodium phosphate (35.4 hr) and PIPES (197.3 hr) buffers
demonstrating the lowest and highest stability, respectively. As VX stability is known to be drastically effected by pH, this was measured before and after with either no or minimal shifts found (Table S3). With the exception of TRIS, the relative % contribution of each metabolite was the same for each of the buffers, suggesting that the rate of decay was altered rather than the hydrolysis pathway (Figure S3).

**Discussion**

While much is known about AChE inhibition by VX its human metabolism is poorly characterized and limited to partial reports (Tsuchihashi et al., 1998). There has been the suggestion of the involvement of PON1 (Kirby et al., 2013), PTE (Tsai et al., 2012), OPH (Rastogi et al., 1997; Reeves et al., 2008), human bile salt-activated lipase (Touvrey et al., 2019), BChE (Lenz et al., 2010; Saxena et al., 2011) and P450 (Fu and Sun, 1990). In comparison there has been some discussion of the role of CYP2B6 in the metabolism of OP pesticides like chlorpyrifos (Hodgson and Rose, 2007b), however, this is largely in regard to the oxidation of the inactive thion/thioate to the active oxon (D'Agostino et al., 2015) which is not relevant for CWNAs or many pesticides. Nonetheless, efforts to inhibit the CYPs responsible for this bioactivation of pesticides using molecules like menadione has been proposed as one way to counteract them (Jan et al., 2015; Jan et al., 2016).

All HLM experiments were performed with a final VX concentration of 200 ng/ml (~0.75 μM), which is over an order of magnitude above the maximum plasma concentration found with a 3x-LD$_{50}$ percutaneous dosing in swine (30nM) (Reiter et al., 2015). The *in vitro* assessment of VX metabolism in HLM demonstrated that it is enzymatically...
metabolized in this system (Figure 3). The relative proportion of the key metabolites were significantly shifted in HLMs, suggesting a pathway enhancement over spontaneous hydrolysis (Figure 2). We also identified a biphasic metabolism in HLM which may point to two or more enzymes involved in VX metabolism. In HLMs, there is significant increase in the abundance of metabolites that are formed by hydrolysis (Figure 1), suggesting that hydrolases may have an important in the metabolism of VX. As the pan-CYP inhibitor does not completely eliminate metabolism in HLMs (Figure 3A) this provides further evidence that there are multiple enzymes involved in the metabolism of VX. It is also possible that the product of the first enzyme may create a metabolite that could interfere with other reactions or even a direct enzymatic poisoning by VX.

Microsomes contain a wide variety of xenobiotic-metabolizing proteins in addition to CYPs, with over 300 hydrolases identified in HLMs with 36 of these being associated with drug metabolism (Wang et al., 2020). The most abundance hydrolase found in human microsomes is carboxylesterase (CES) 1, which has previously been shown to hydrolyze the G-Agent sarin (Hemmert et al., 2010) and is potently inhibited by the activated organophosphate pesticides chlorpyrifos oxon, paraoxon, and methyl paraoxon (Crow et al., 2008). The role of carboxylesterases in VX metabolism is unknown. In the proteomics study by Wang et al., a comparison of HLMs and the S9 fraction of liver homogenate, which contains a more significant representation of cytosolic proteins, showed major overlap in the proteins present, although with variable
concentrations. This suggests that the role of cytosolic proteins should not be dismissed when assessing metabolism in HLMs.

We have also now shown additional effects of hydrolysis rates of VX in common biological buffers (Figure 5). The reason for this is unknown, but analysis of the metabolite formation shows a difference with TRIS as compared to the other buffers tested. The slight shift in VX metabolite formation with TRIS is likely due to it being nucleophilic (Brasch et al., 1994), which would provide a plausible explanation why there is an increase in the relative abundance of EA-2192 and EMPTA in TRIS over other buffers.

In the process of assessing VX metabolism we identified that the addition of EDTA to HLM showed a drastic increase in the overall metabolism and that this could be partially inhibited with a pan CYP inhibitor (Li et al., 2019) (Figure 3,4). Of the tracked metabolite, BDAED is likely the only one formed by VX that is CYP-mediated (Figure 1). As BDAED does not appear to be statistically significantly different (either a paired t-test or one-way ANOVA with follow-up multiple comparisons test) with the addition of EDTA, this suggests that other as yet unknown enzymes may also be responsible for the increase in VX metabolism upon the addition of EDTA. EDTA is a widely used chelating agent that binds Iron (Fe^{2+}/Fe^{3+}) and calcium ions (Ca^{2+}) that is also FDA approved for lead poisoning and to our knowledge has not been previously suggested as a treatment for CWNA or for that matter VX poisoning. EDTA has been long known as an inhibitor of lipid peroxidation in rat microsomes (Kamataki and Kitagawa, 1974). 5mM EDTA has
previously been used as a lipid peroxidation inhibitor in liver S9 assays (Paolini et al., 1988). There may also be precedent for the enhanced metabolism observed after EDTA treatment in humans in which antipyrine was used as a probe for metabolism and which was inhibited by lead (Meredith et al., 1977). EDTA is widely used in liver microsome isolation and microsomal incubations such that it is possible that in some cases it may also be influencing the metabolism of molecules being tested in vitro.

Future work could involve in vivo assessment of EDTA on VX metabolism in mouse or rats to assess whether it could be used as an adjunct to the use of AChE reactivators for treating poisoning. An understanding of the time window for treatment may also be important to see if it agrees with our in vitro data. Limitations of this work are that we have only focused on one CWNA (VX) and that other nerve agents may have very different metabolism such that using EDTA to increase metabolism may not be ideal as increased metabolism could exacerbate toxicity of some CWNAs. For example, PON1 has been suggested to be important for the metabolism of some CWNAs (Cheng et al., 1997; Melzer et al., 2009; Gupta et al., 2011; Kirby et al., 2013). Also, we have assessed a single chelating agent when in fact the testing of several may be warranted to identify the optimal agent. EDTA has been widely used in in vitro experiments with liver microsomes for decades and it may need further assessment as to whether it could also be influencing the experimental outcome in a compound dependent manner. The involvement of CYPs in the metabolism of VX has yet to be confirmed but is hypothesized as the pan-CYP inhibitor reduced the overall metabolism of VX in our assays. As the majority of the tracked metabolites are the product of hydrolysis, there
are likely CYP-mediated metabolites that have yet not been identified. We have not identified the specific enzymes involved in VX metabolism and therefore do not know which one (or more) is being affected by EDTA specifically. Metabolic studies could also be performed in either S9 fractions, hepatocytes and or liver organoids to further determine the potential role of cytosolic proteins in the metabolism of VX.

In conclusion, this study now updates our knowledge on human metabolism of VX and may suggest a new avenue to develop treatments for poisoning by the targeted increase in the CYP mediated metabolism using an already FDA approved drug (EDTA).

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Data Availability Statement
All data are provided in this publication.

Authorship Contributions
Participated in research design: Lane, Koebel, Lucas, Moyer, and Ekins
Conducted experiments: Koebel
Contributed new reagents or analytic tools: Koebel and Moyer
Performed data analysis: Lane, Koebel, Lucas and Moyer
Wrote or contributed to the writing of the manuscript: Lane and Ekins

References


Footnotes

a) Financial support for this research was provided by DTRA WHDTRA1-19-1-0020.

b) Statement on dual use: The in vitro data described in this study has potential dual-use capabilities and may be equally used to design more potent analogs with decreased metabolism.

c) Financial disclosure: SE is owner and TRL is employee of Collaborations Pharmaceuticals. DK, EL and RM are employees of Battelle Memorial Institute.

d) Pursuant to Grant # HDTRA11910020, DTRA authorizes the public release of the VX + EDTA poster, manuscript and related documentation, as authored by Collaborations Pharmaceuticals, Inc. and Battelle Memorial Institute, in support of the scope of the program.

e) Reprint requests Sean Ekins Ph.D., D.Sc., Collaborations Pharmaceuticals, Inc. 840 Main Campus Drive, Lab 3510, Raleigh, NC 27606., E-mail: sean@collaborationspharma.com, Phone: 215-687-1320.
Figure Legends

Figure 1. VX metabolism overview. Compounds highlighted in red were quantified in our studies using targeted LC/MS. HLM and EDTA were not present.

Figure 2. *In vitro* clearance rates of VX (200 ng/ml or ~0.75 µM) in pooled HLM versus control (n=2) over a 10-day period. (A, B) The breakdown of VX in HLM versus (D, E) the breakdown of VX in HLM-free buffer (Table S1; C). The metabolism and or the hydrolysis of VX was visualized and analyzed using a standard (A, D) or natural log scale (B, E). The % metabolite contribution (C, F) was calculated on the fraction of the total molarity of all tracked metabolites (BDAED, BDAES, DIPAE, EA-2192, EMPA, EMPTA, MPA). Both linear and non-linear regression (NLR) fits were calculated using Prism 9.3.1. For NLR, 1st or 2nd order decay was based on the outcome of the extra sum-of-squares F test. The $t_{1/2}$ calculated from the ln assumed a first-order decay and used the following equation: $(\ln(2))/(-\text{slope})$. The transition regions were chosen based on what maximized the $R^2$ values of the line fit. Dashed lines represent the 95% confidence intervals and error bars (where applicable) are SDs.

Figure 3. Effects of (A) CYP inhibition, (B) EDTA or (C) a combination of both on VX metabolism in HLMs (Table S1; G). For clarity, only the first 96 hrs of metabolism are shown.
**Figure 4.** Time-dependent rescue of VX metabolism using EDTA in HLMs. The effects of EDTA addition on VX metabolism at times 0, 4, 24 or 72 hr in HLMs (Table S1; D, E, G).

**Figure 5.** Comparison of VX metabolite formation half-life in different buffers (pH 7.2) (Table S1; F).
Figure 1
Figure 2
Figure 3

A. Effect of CYP Inhibition on VX Metabolism
- VX Control
- VX + HLM + Alpamezole
- VX + HLM

B. Effect of EDTA on VX Metabolism
- VX + HLM
- VX + HLM + EDTA
- $t_{1/2}^{(fast)} = 3.1$ hr
- $t_{1/2}^{(slow)} = 266.1$ hr
- $t_{1/2}^{(fast)} = 3.24$ hr
- $t_{1/2}^{(slow)} = 29.77$ hr

C. Effects of CYP Inhibition on VX Metabolism
- VX (+HLM, +Alpamezole, -EDTA)
- VX (+HLM, -Alpamezole, -EDTA)
- VX (+HLM, +Alpamezole, -EDTA)
- VX (+HLM, -Alpamezole, -EDTA)
Effects of EDTA on VX Metabolism (Full)

- VX (HLM + EDTA @ 0hr)
- VX (HLM + EDTA @ 4hr)
- VX (HLM + EDTA @ 24hr)
- VX (HLM + EDTA @ 72hr)
- VX (HLM - EDTA)

Figure 4
Figure 5

Buffer Stability of VX

- VX (NaPO₄)  $t_{1/2} = 35.4$ hr
- VX (ADP)   $t_{1/2} = 66.48$ hr
- VX (HEPES) $t_{1/2} = 83.7$ hr
- VX (TRIS)   $t_{1/2} = 90.41$ hr
- VX (MOPS)   $t_{1/2} = 107.3$ hr
- VX (MES)    $t_{1/2} = 133.1$ hr
- VX (PIPS)   $t_{1/2} = 197.8$ hr
Supplemental Data

*In vitro* Characterization and Rescue of VX Metabolism in Human Liver Microsomes

Thomas R. Lane¹, David Koebel², Eric Lucas², Robert Moyer² and Sean Ekins¹.

¹Collaborations Pharmaceuticals, Inc. 840 Main Campus Drive, Lab 3510, Raleigh, NC 27606.

²Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201.
Supplemental Materials and Methods

Stock solution preparation:

Stock solution of VX was prepared in Acetone at 40 µg/mL and stored at ≤ -20°C when not in use. Stock solutions of VX metabolites/degradation products (BDAED, BDAES, DIPAE, EA-2192 (VX Acid), EMPA, EMPTA, and MPA) were prepared in one solution, each at 20 µg/mL and stored at ≤ -20°C when not in use. Stock solutions of NADPH (12.5 mM) and MOPS, ADP, HEPES, MES, PIPES, TRIS, and Sodium Phosphate (13.25 mM) were prepared in purified water and stored at 1-7°C when not in use. A stock solution of internal standard (EMPA-d4) was prepared at 150 ng/mL in acetonitrile and stored at ≤ -20°C when not in use.
Table S1. Experimental details summary:

(A) Experiment 1 (EDTA added at T=0, MOPS buffer):

(A1) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM MOPS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(A2) 200 ng/mL VX + 10 mM MOPS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(A3) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM MOPS @ pH 7.4 + 2.5 mM NADPH

(A4) 200 ng/mL VX + 10 mM MOPS @ pH 7.4 + 2.5 mM NADPH

Timepoints: 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 24, 28, 32, 49, 52, 56, 72, and 96 hr.

(B) Experiment 2 (EDTA added at T=0, MOPS buffer):

(B1) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM MOPS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(B2) 200 ng/mL VX + 10 mM MOPS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(B3) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM MOPS @ pH 7.4 + 2.5 mM NADPH

(B4) 200 ng/mL VX + 10 mM MOPS @ pH 7.4 + 2.5 mM NADPH

Timepoints: 0, 1, 2, 3, 4, 5, 6, 7, 8, 21, 24, 28, 32, 48, 52, 56, 72, 96, 120, 144, 168, 192, 216, and 240 hr.

(C) Experiment 3 (EDTA added at T=0, TRIS buffer):
(C1) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(C2) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(C3) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH

(C4) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH

Timepoints: 0, 1, 2, 3, 4, 8, 24, 32, 48, 75, 96, 120, 144, 168, 192, 216, and 240 hr.

(D) Experiment 4 (EDTA added at T=0 and T=72hr, TRIS buffer):

(D1) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(D2) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(D3) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH

(D4) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH

(D5) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=72hr

(D6) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=72hr

Timepoints: 0, 1, 2, 3, 4, 5, 6, 7, 8, 22, 24, 25, 26, 27, 29, 30, 31, 32, 46, 48, 49, 50, 51, 52, 53, 55, 56, 70, 72, 73, 74, 75, 77, 78, 79, 80, 94, 96, 97, 98, 99, 101, 102, 103, 104, 124, 144, 168, 192, 216, and 240 hr.

(E) Experiment 5 (EDTA added at T=0 and T=24hr, TRIS buffer):
(E1) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(E2) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=0hr

(E3) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH

(E4) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH

(E5) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=24hr

(E6) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ T=24hr

Timepoints: 0, 1, 2, 3, 4, 5, 6, 7, 8, 23, 24, 25, 26, 28, 29, 30, 31, 32, 47, 48, 49, 50, 51, 52, 54, 55, 56, 72, 76, 80, 96, 99, 102, 119, 143, 167, 191, 216, and 238 hr.

(F) Experiment 6 (VX stability in MOPS, ADP, HEPES, MES, PIPES, TRIS, and Sodium Phosphate:

(F1) 200 ng/mL VX + 10 mM MOPS @ pH 7.4 + 2.5 mM NADPH

(F2) 200 ng/mL VX + 10 mM ADP @ pH 7.4 + 2.5 mM NADPH

(F3) 200 ng/mL VX + 10 mM HEPES @ pH 7.4 + 2.5 mM NADPH

(F4) 200 ng/mL VX + 10 mM MES @ pH 7.4 + 2.5 mM NADPH

(F5) 200 ng/mL VX + 10 mM PIPES @ pH 7.4 + 2.5 mM NADPH

(F6) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH

(F7) 200 ng/mL VX + 10 mM Sodium Phosphate @ pH 7.4 + 2.5 mM NADPH

Timepoints: 0, 5, 8, 24, 28, 32, 48, 53, 56, 72, 76, 96, 122, 144, 168, 192, 216, 240 and 288 hr.
**G) Experiment 7** (EDTA added at $T=0$ and $T=4$ hr, TRIS buffer):

(G1) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ $T=0$hr

(G2) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ $T=4$hr

(G3) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ $T=0$hr

(G4) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + 5 mM EDTA @ $T=4$hr

(G5) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH

(G6) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + Atipamezole @ 100 µM + 5 mM EDTA @ $T=0$hr

(G7) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + Atipamezole @ 100 µM + 5 mM EDTA @ $T=0$hr

(G8) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + Atipamezole @ 100 µM + 5 mM EDTA @ $T=4$hr

(G9) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + Atipamezole @ 100 µM + 5 mM EDTA @ $T=4$hr

(G10) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + Atipamezole @ 100 µM

(G11) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH + Atipamezole @ 100 µM

(G12) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4 + 5 mM EDTA @ $T=0$hr
(G13) 200 ng/mL VX + 0.5 mg/mL HLM + 10 mM TRIS @ pH 7.4

(G14) 200 ng/mL VX + 10 mM TRIS @ pH 7.4 + 2.5 mM NADPH

Timepoints: 0, 4, 6, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240 hr.
Table S2. LC-MS/MS Instrument Conditions

| Instrument          | Waters Acquity I-Class Ultra Performance Liquid Chromatograph  
|                    | Waters Xevo TQ-XS Quadrupole Mass Spectrometer |
| MS Software        | Waters MassLynx version 4.2 |
| MS Ionization Mode | Electrospray Positive Ionization |
| LC Column          | Phenomenex Prodigy ODS(3), 100A, 3µm, 100x2.0mm |
| LC Column Temperature | 50°C |
| Mobile Phase Components | A=0.1% (v:v) formic acid in water |
|                     | B=0.1% (v:v) formic acid in acetonitrile |
| Gradient Profile   | Time, min | %A | %B | Flow rate, mL/min |
|                    |           |    |   |                 |
|                    | 0         | 98 | 2 | 0.2             |
|                    | 1         | 98 | 2 | 0.2             |
|                    | 5         | 0  | 100 | 0.2         |
|                    | 5.01      | 0  | 100 | 0.3         |
|                    | 6.5       | 0  | 100 | 0.3         |
|                    | 6.6       | 98 | 2  | 0.3         |
|                    | 8         | 98 | 2  | 0.3         |

All changes are linear with respect to time.

<p>| Injection Volume | 2 µL |
| MS Capillary Voltage | 0.5 kV |
| MS Source Temperature | 110°C |
| MS Desolvation | 400°C |</p>
<table>
<thead>
<tr>
<th>Temperature</th>
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<td><strong>MS Desolvation Gas</strong></td>
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<td><strong>Precursor&gt;Product Ions</strong></td>
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<td><strong>Calibration Curve</strong></td>
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**Table S3.** pH Measurements for VX buffer stability experiments.

<table>
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<tr>
<th>Buffer (10 mM) + 2.5 mM NADPH</th>
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<th>t=384 hr</th>
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<td>Sodium Phosphate</td>
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<tr>
<td>TRIS</td>
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</tr>
</tbody>
</table>
**Figure S1.** The metabolism and/or the hydrolysis of VX (200 ng/ml) over a 10-day period. All experiments were done with 10 mM MOPS pH 7.4, and 2.5mM NADPH (Table S1; A,B). (A) The breakdown of VX in HLM (0.5 mg/mL HLM), (B) VX alone, (C) VX + HLM + EDTA, or VX + EDTA. (E-H) This is also visualized on a natural log scale to help pinpoint where a single decay rate dominates the reaction. For these the $t_{1/2}$ was calculated assuming a first-order decay using the following equation: $\frac{\ln(2)}{-\text{slope}}$. The transition regions were chosen based on what maximized the $R^2$ values of the line.
Regression fits and $t_{1/2}$s were calculated using Prism 10.1.1. Dashed lines represent the 95% confidence intervals and error bars (where applicable) are SDs.

**Figure S2.** Comparison of the hydrolysis/metabolism of VX in TRIS versus MOPS buffers (pH 7.4). (A) VX + HLM + 5 mM EDTA, (B) VX + HLM – EDTA, (C) VX Control (Table S1; B,C). Non-linear regression lines represent either a 1$^{st}$ (A, B) or 2$^{nd}$ order decay (C) and were determined as the better fit using an extra sum-of-squares F Test (simpler model if $P \geq 0.05$). For the control (C), parameters (plateau and half-life) are not significantly different from each other (extra sum-of-squares F test, $P = 0.1814$) therefore the shared half-life is given (95% CI).
Figure S3. Comparison of VX metabolism in different buffers (pH7.4) (Table S1; F).