**STUDY PROTOCOL**

**1.0 STUDY TITLE**

Inhibition kinetics of six organophosphate compounds on human and rat erythrocyte acetylcholinesterase

**2.0 OBJECTIVE**

The objective of this study is to determine experimentally the inhibition kinetics constants (*KI, KA*, and *kp*) for the oxons of six organophosphate (OP) test compounds plus paraoxon (oxon analog of ethyl parathion for comparison) on acetylcholinesterase (AChE) assayed in “ghost” preparations (i.e., erythrocyte cell membranes separated from hemoglobin and other cytoplasmic constituents) obtained from human and rat erythrocytes. Because the AChE of erythrocytes is the same gene product as neural AChE, the information obtained from the erythrocytes will be relevant to the neural target AChE. The comparison of the kinetic parameters from human and rat will be useful in addressing the interspecies uncertainty factor in risk assessment. The range of values obtained for individual humans across ages will be useful in addressing the intraspecies uncertainty factor in risk assessment.

**3.0 STUDY SPONSORS**

AMVAC Chemical Corporation

Glenn A. Wintemute Research Center

2110 Davie Avenue

Commerce, CA 90040

Company Representative: Ann Jonynas

Gowan Company, LLC

370 South Main Street

Yuma, Arizona 85364

Company Representative: Elizabeth Codrea

FMC Corporation

2929 Walnut Street

Philadelphia, PA 19104

Company Representative: Terri Spanogle

Study Monitor: Dr. Richard Reiss, Exponent, [rreiss@exponent.com](mailto:rreiss@exponent.com); 571-227-7228

**4.0 TESTING FACILITY AND STUDY DIRECTOR ADDRESS**

Mississippi State University, Center for Environmental Health Sciences

240 Wise Center Road

College of Veterinary Medicine

Mississippi State, MS 39762-6100

Study Director: Janice Chambers, PhD, DABT, ATS

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**5.0 PROPOSED SCHEDULE**

Proposed experimental start date: September 1, 2016

Proposed experimental termination date: March 31, 2017

**6.0 TEST SUBSTANCE INFORMATION**

**6.1 Test Substances**

Six test substances will be: malaoxon, bensulide oxon, omethoate, tebupirimphos oxon, phorate oxon sulfone, and phorate oxon sulfoxide, all of which will be supplied by the study sponsors of the chemical. The following sample information and chemical/physical properties should be provided with each test substance: batch/lot number, sample expiration date, physical description, empirical formula, stability, suggested storage conditions, a Material Safety Data Sheet (MSDS/SDS) or equivalent information, and handling precautions. In the event where the active metabolite is not available from the producer/owner, the test substance will be purchased from a commercial vendor, which will supply similar information.

The positive control for optimization of the kinetics assay procedures (and also to be used for comparative purposes to the test substances) will be paraoxon, which will be synthesized at Mississippi State University (by Dr. Howard Chambers, Professor, Dept of Biochemistry, Molecular Biology, Entomology and Plant Pathology) using established methods. Dr. H. Chambers has frequently synthesized high purity paraoxon. The purity and identify of the paraoxon will be verified through NMR conducted in the Mississippi State Chemical Laboratory. If the test compound is obtained from a commercial vendor, its purity and identity might also be verified through NMR conducted in the Mississippi State Chemical Laboratory.

**6.2 AChE sources**

The human AChE will be from blood samples from individual healthy youth and adult human subjects (age range 8-65 years of age) of either sex and any race or ethnic group. These blood samples will be purchased from either Innovative Research (adult samples) or BioreclamationIVT (youth samples), and shipped refrigerated overnight to Mississippi State University. Additionally, cord blood samples will be purchased from Zenbio and shipped refrigerated overnight to Mississippi State University. The blood samples will be tested for common known pathogens and cleared by the commercial supplier before the MSU laboratory will prepare the ghosts. The ghost preparation will occur two days after the blood is withdrawn from the human subjects.

The rat AChE measurements will be from blood samples from individual healthy adult rats using both sexes. Blood will be obtained from rats purchased from Envigo (Harlan Laboratories) and will be stored at 4°C. The ghost preparation will occur 2 days after blood was obtained from the rats to be consistent with the time until ghost preparation required for the human samples.

There will be 9 replications for the human with the 9 replications representing individuals: 4 will be adult samples, 3 will be youth samples (8-18 years of age) and 2 will be cord blood samples. There will be 6 replications for rats with the 6 replications representing individuals: 3 will be females and 3 will be males.

**7.0 TEST PROCEDURE**

**7.1 Materials**

Blood samples will be obtained as described in Section 6.2. All chemicals and reagents will be purchased from Sigma Aldrich and will be the highest purity available. Buffers will be made bi-weekly and the pH will be verified before use. Acetylthiocholine iodide (ATCh), 5,5’-dithio(bis-nitrobenzoic acid) (DTNB) and eserine sulfate solutions will be stored frozen at -20°C and used within one month.

The following solutions will be prepared:

1. 0.05M Tris-HCl buffer, pH 7.4 at 37°C.
2. Acetylthiocholine plus 5,5’-dithio(bis-nitrobenzoic acid (substrate + chromogen): 5 mM ATCh and 25 mM DTNB in 0.05M Tris HCl buffer, pH 7.4 at 37°C, stored frozen at -20°C.
3. Eserine sulfate (carbamate AChE inhibitor used to assess non-AChE substrate hydrolysis in the blanks): 10-3M in 0.05M Tris-HCl buffer, pH 7.4 at 37°C.
4. The 6 test OPs plus paraoxon, yielding 7 individual OP solutions, in ethanol, stored at -20°C. Concentrations to be determined by initial range finding studies.

**7.2 Methods**

Methods of erythrocyte ghost preparation from human blood were established in a pilot study already conducted. Methods are already in place for preparation of rat erythrocyte ghosts. The test laboratory has already conducted OP inhibition kinetics studies on similar OP anticholinesterases (Carr and Chambers,1996; Coban et al., 2016).

The ghost preparation will involve centrifugation of whole blood anticoagulated with K2EDTA (3000 g for 10 min), plasma will be removed and packed erythrocytes will be washed 3 times (500 g centrifugation) using 2 volumes of 100mM phosphate buffer (pH 7.4). Erythrocytes will be lysed using 20 volumes of hypotonic phosphate buffer (6.7mM, pH 7.4). Cells will lyse for 10 min on ice followed by centrifugation at 50,000 g for 30 min. The pellet of each centrifugation will be washed 3 times with 2 volumes of 100mM phosphate buffer and resuspended to original volume using 100mM phosphate buffer. The final ghost preparation will show minimal evidence of hemoglobin. The ghost preparation will be aliquoted into individual cryogenic tubes and will be stored frozen at -70°C until assayed within 2 months of preparation. The pilot project already conducted provided evidence that the human ghost preparation did not lose AChE activity by freezing at -70°C for two weeks. Protein concentration of the ghost preparations for standardization will be determined using the Folin phenol reagent (Lowry et al., 1951).

A continuous spectrophotometric assay will be used to determine AChE activities (modification of Ellman et al., 1961) with acetylthiocholine (ATCh) as the substrate and 5,5’-dithiobis(nitrobenzoic acid) (DTNB) as the chromogen (Chambers et al., 1988).

The bimolecular rate constant (*ki*), phosphorylation constant (*kp*), and association constant (*KA*) for each inhibitor in brain homogenates and human erythrocyte AChE will be determined for each inhibitor according to the method of Johnson and Wallace (1987) and Carr and Chambers (1996) based on the work of Kitz and Wilson (1962) and Segel (1975). The assays will use a SpectraMax M5 microplate reader with SoftMax Pro Software (Molecular Devices Corporation, Sunnyvale, CA). The software will measure each well's absorbance and calculate the velocity of each reaction by determining the slope of the line from a plot of product formed as a function of time.

Optimization studies will be done to determine the dilution of the uninhibited rat and human ghost preparations that yield an absorbance in the range of 0.7-1.2 Absorbance Units (AU) in a 5 min period. Additionally range finding studies will be conducted to determine the concentration range of each OP that yields 10-90% inhibition.

Each plate with ghost preparation will be warmed to 37°C before starting the assay. 158µl of each preparation will be added into each well of a 96-well plate. No additional proteins will be added to the assays for enzyme stabilization to avoid any additional potential binding sites for the test compounds. To correct for non-enzymatic hydrolysis 60µM eserine sulfate will be incubated with additional wells to serve as blank samples to correct for non-AChE hydrolysis of substrate. Non-enzymatic hydrolysis of ATCh is expected to be <10%.

The inhibition reaction will be initiated by pre-incubation of the ghost preparation with ethanol vehicle or eight concentrations of each test item which were predetermined to give a range of approximately 10-90% AChE inhibition. Six pre-incubation periods will be used (0-5 mins at 1 min intervals). OP’s will be dissolved in ethanol at concentrations 100-fold higher than the desired final concentration and will be added in a volume of 2 µl per well. Each solvent control, OP concentration or eserine blank will be repeated in 2-3 wells on the same plate to account for any variation due to pipetting differences among the wells.

Following the pre-incubation with vehicle or OP, the inhibition reaction will be terminated by addition of the substrate ATCh which, being at least 3 orders of magnitude greater in concentration than the inhibitors, will out-compete the OP for binding to the active site. The AChE reaction will be initiated by the addition of a 40µl mixture of 5mM substrate ATCh and 25mM DTNB (to yield final concentrations of 1mM ATCh and 5mM DTNB). For the 0-min time point, the substrate/chromogen mixture will be added at the same time with the OP. Each reaction will be monitored by recording the absorbance at 412nm for 3 min at 30 second intervals (6 readings) and the velocity of each pre-incubation time will be obtained for each concentration and control.

Note: the above procedures have been followed in our laboratories with brain and purified AChE.

**7.3 Glassware, Pipets, and Other Containers**

All microtiter plates in which the AChE reactions will be conducted will be new. Microliter pipettes are calibrated twice yearly in the College of Veterinary Medicine by a pipette calibration firm. All pipet tips, cryovials and other disposables will be new. Any reusable glassware will be washed thoroughly with Alconox laboratory glassware detergent and rinsed thoroughly with tap water, followed by a deionized water rinse before air drying. Any reusable glassware that has had an OP compound in it will be rinsed twice with acetone after the tap water rinse and prior to the deionized water rinse.

**8.0 CALCULATIONS**

For each OP pre-incubation time, the AChE velocity following inhibition (*[E]t*) will be divided by the original uninhibited velocity (*[E]o*) to obtain the fraction of AChE velocity remaining (*[E]t*/*[E]o*). The apparent rate of AChE phosphorylation (slope = *-kapp*) for each concentration will be calculated by linear regression of the natural log (ln) of the *[E]t*/*[E]o* as a function of time. Finally, a double reciprocal plot of *kapp* as a function of the molar inhibitor concentration of AChE will be used to calculate each inhibitor’s *ki* (slope = 1/*ki*), *kp* (y intercept = 1/*kp*), and *KA* (x intercept= *-KA*). The reaction describing the inhibition process and definition of kinetics parameters are based on the following scheme:



where [*E*] is the concentration of free enzyme, [*I*] is the concentration of free inhibitor, [*EI*]R is the reversible enzyme-inhibitor complex, and [*EI*]I is the irreversibly phosphorylated enzyme-inhibitor complex (Johnson and Wallace, 1987).

For each species and each inhibitor, all linear regressions and calculations will be performed using Microsoft Excel 2010 to obtain the AChE velocity remaining (*[E]t*/*[E]o*) and apparent rate of AChE phosphorylation (*kapp*) for determination of the bimolecular rate constants (*ki*), association constants (*KA*), phosphorylation constants (*kp*) for each OP.

The assistance of Dr. Kenneth Willeford (enzymologist and collaborator, Professor, Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University) in calculation of the kinetic parameters will be obtained.

**9.0 REPORT**

A separate report for each of the OP compounds will be generated that describes the procedures conducted and shows the compiled data and the kinetic constants for rats and for humans. The three kinetic constants for each OP for each species will be reported as mean ± SEM for the 9 replications for humans or 6 replications for rats. The units for the constants will be: *ki* ,μM-1 min-1; *KA*, μM-1; and *kp*, min-1. The Study Monitor will format this report.

**10.0 PROTOCOL AMENDMENTS AND DEVIATIONS**

While it is not anticipated that amendments or deviations from the approved protocol will occur once optimization procedures and range finding on the seven OP’s (including paraoxon) have been conducted, any necessary adjustments will be made to insure that the data can be obtained reliably and that samples are not wasted. (An example might be adjustment of ghost preparation concentration or of OP concentrations if an individual had unusually low or high activity or low or high sensitivity to the OP.) If an overall change of protocol appears to be needed to obtain better quality data, then this will be discussed with Exponent (and with the study sponsors), as needed, before such adjustments are made.

**11.0 QUALITY ASSURANCE/GLP**

Mississippi State University is not a GLP facility. However, all assays will be conducted in the spirit of GLP with as much care, compliance with the approved protocol, adherence to laboratory standard operating procedures, and attention to detail as possible.

**12.0 RECORDS**

All information will be recorded in bound notebooks and print-outs from the plate reader spectrophotometer will be taped into the notebooks. Each OP will have a separate notebook. All records of the samples, procedures, and any deviations or exceptions to the protocols will be noted in the notebook. Each data entry page will have a date and the name of the individual performing the experiment will be recorded. The print-outs of the calculations and statistical analyses will also be taped into the notebooks. In addition the spread sheets containing the data will be copied into a separate jump drive for each OP. If desired, the Sponsor will be supplied with the original notebooks and the jump drives containing the data and data analysis; Mississippi State University will retain a copy of the data and data analysis. The data (either originals or copies) will be retained by Mississippi State University for 7 years, or as specified by the Sponsor.

**13.0 REFERENCES**

Carr, R.L. and J.E. Chambers. 1996. Kinetic analysis of the in vitro inhibition, aging, and reactivation of brain acetylcholinesterase from rat and channel catfish by paraoxon and chlorpyrifos-oxon. *Toxicol. Appl. Pharmacol*. 139:365-373. PMID: 8806854

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Coban, A., R.L. Carr, H.W. Chambers, K.O. Willeford and J.E. Chambers. 2016. Comparison of inhibition kinetics of several organophosphates, including some nerve agent surrogates, using human erythrocyte and rat and mouse brain acetylcholinesterase. *Toxicol*. *Lett.* 248:39-45. PMID: 26965078.

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Johnson, J.A., Wallace, K.B., 1987. Species-related differences in the inhibition of brain acetylcholinesterase by paraoxon and malaoxon. Toxicol. Appl. Pharmacol., 88, 234-241.

Kitz, R., Wilson, I.B., 1962. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. J. Biol. Chem., 237, 3245-3249.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. 193:265-275.

Segel, I. H.,1975. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. Wiley, New York.