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In Vivo Evaluation of the Protective Efficacy of RS194B as a Centrally Acting Reactivator of Nerve Agent-Inhibited Acetylcholinesterase

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14. ABSTRACT

RS194B is an oxime that was developed by a group at the University of California at San Diego and is purported to penetrate the central nervous system (CNS) and reactivate brain acetylcholinesterase (AChE) inhibited by nerve agents. The first study evaluated the ability of RS194B to reactivate nerve agent-inhibited human or mouse AChE enzyme *in vitro*. In the second study, RS194B was administered to mice 15 min after they had been challenged with a toxic dose (1xLD_{50}) of the nerve agent sarin (GB), VX or cyclosarin (GF), and AChE activity was determined using a modified Ellman method. In the third study, mice were challenged with 5xLD_{50} of GB or VX or 3.5xLD_{50} of GF and then treated 1 min later with atropine sulfate, midazolam and either RS194B or 2-PAM. Survival at 24 hr was measured. In summary, RS194B produced occasional AChE reactivation in some brain structures inhibited by GB or VX, but not GF, but it did consistently produce reactivation in peripheral tissues and blood. RS194B was no more effective than 2-PAM in providing protection against a 5xLD_{50} challenge of GB or VX and provided no protection against a 3.5xLD_{50} GF challenge. RS194B appeared to provide minimal to modest reactivation of brain AChE at best and was no more effective than 2-PAM in protecting against the lethal effects of these nerve agents.

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ABSTRACT

RS194B is an oxime that was developed by a group at the University of California at San Diego and is purported to penetrate the central nervous system (CNS) and reactivate brain acetylcholinesterase (AChE) inhibited by nerve agents. This ability to penetrate and reactivate brain AChE inhibited by nerve agents is considered by its inventors/developers as one of the prime therapeutic features of this novel compound. In the present report three experiments were performed. The first study evaluated the ability of RS194B to reactivate nerve agent-inhibited human or mouse AChE enzyme *in vitro*. In the second study, RS194B was administered to mice 15 min after they had been challenged with a toxic dose (1xLD_{50}) of the nerve agent sarin (GB), VX or cyclosarin (GF). Forty-five min later the mice were euthanized, and both peripheral (heart, skeletal muscle, diaphragm, blood) and brain (cortex, hippocampus, midbrain, cerebellum, brainstem) tissues were dissected and processed to determine AChE activity using a modified Ellman method. In the third study, mice were challenged with 5xLD_{50} of GB or VX or 3.5xLD_{50} of GF and then treated 1 min later with atropine sulfate, midazolam and either RS194B or 2-PAM. Survival at 24 hr was measured. In the *in vivo* reactivation experiment, RS194B at 45 mg/kg produced significant reactivation of GB-inhibited AChE in brainstem and cerebellum; at a dose of 63 mg/kg, reactivation was seen in midbrain but in no other brain structures. The 45 mg/kg dose of RS194B produced significant AChE reactivation of heart tissue, and 63 mg/kg RS194B produced significant reactivation in diaphragm, skeletal muscle and heart tissue. All three dose of RS194B (25, 45 and 63 mg/kg) produced a dose-dependent reactivation of whole blood and red blood cell (RBC) AChE. In VX-challenged animals, reactivation was seen only in midbrain at the 45 mg/kg RS194B dose; RS194B produced significant reactivation in diaphragm (63 mg/kg), heart (45 and 63 mg/kg), skeletal muscle (63 mg/kg) and both RBC and whole blood (45 and 63 mg/kg doses). In GF-challenged animals, RS194B (63 mg/kg) produced no reactivation in any brain structure, but did reactivate diaphragm, heart, RBC and whole blood AChE. In the survival study, RS194B produced comparable survival to 2-PAM against 5xLD_{50} challenges of sarin (RS194B 75%; 2-PAM 75%) and VX (RS194B 75%; 2-PAM 38%), and no survival against 3.5xLD_{50} of GF (RS194B 0%; 2-PAM 25%). In summary, RS194B produced occasional AChE reactivation in some brain structures inhibited by GB or VX, but not GF, but it did consistently produce reactivation in peripheral tissues and blood. RS194B was no more effective than 2-PAM in providing protection against a 5xLD_{50} challenge of GB or VX and provided no protection against a 3.5xLD_{50} GF challenge. RS194B appeared to provide minimal to modest reactivation of brain AChE at best and was no more effective than 2-PAM in protecting against the lethal effects of these nerve agents.

INTRODUCTION

A research group out of the University of California at San Diego (UCSD) under the leadership of Dr. Palmer Taylor and Dr. Zoran Radic has been working for a number of years to develop improved oxime reactivators of organophosphate-inhibited acetylcholinesterase (AChE). Specifically, they have tried to develop compounds that can enter into the brain past the blood-brain barrier (BBB) and reactivate brain AChE inhibited by organophosphate compounds such as the nerve agents and some pesticides (Sit et al., 2011; Radic et al., 2012). Current clinically used oximes such as 2-pyridine aldoxime (2-PAM) or obidoxime are charged molecules and as such do not penetrate the BBB to any extent and do not reactivate brain AChE inhibited by nerve agents. Reactivation of brain AChE that has been inhibited by nerve agents has been shown to be very beneficial in reversing the central nervous system (CNS) signs of intoxication and minimizing post-exposure neurobehavioral debilitation (Shih et al., 2010; Skovira et al., 2010). Different research groups have taken a number of different experimental approaches to this problem (Chambers et al., 2013, 2016a,b). The approach taken by the UCSD group was to develop a library of uncharged oximes from which, after initial *in vitro* screening, they chose a lead structure (RS41A). They then systematically varied the structure of RS41A and evaluated the reactivating properties of the different analogs and eventually identified RS194B (Figure 1) as having substantially improved *in vitro* reactivation kinetics (Radic et al., 2012). RS194B is a zwitterion, which is a molecule with two or more functional groups of which at least one has a positive and one has a negative electrical charge and the net charge of the entire molecule is zero. It is this feature, the molecule being uncharged, that should allow penetration of this compound through the BBB. Subsequent to this work, RS194B had been tested *in vivo* for the ability to protect against the lethal effects of various nerve agents (sarin, VX, tabun) and pesticide compounds (paraoxon) in mice (Radic et al., 2012; Sit et al., 2018) and rapidly reversed toxic signs in macaques inhaling sarin or paraoxon (Rosenberg et al., 2017, 2018).

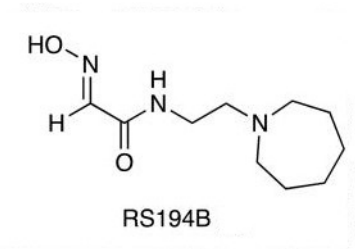


Figure 1. Structure of RS194B.

The current report describes the results of three studies to examine the effectiveness of RS194B to reactivate AChE inhibited by different nerve agents. In the first study, the ability of RS194B to reactivate AChE inhibited with a number of nerve agents (tabun, GA; sarin, GB; soman, GD; cyclosarin, GF; VX; Russian V, VR) was assessed in several *in vitro* assays. In the second study, mice were administered a median lethal dose (1xLD_{50}) of one of three nerve agents (GB, VX, GF), and then after a period of time when brain and peripheral tissues were maximally inhibited the mice were administered test doses of RS194B; after a subsequent period of time the animals were euthanized, and brain and peripheral tissues were assayed for AChE activity levels. The assumption of this study was that if a dose of RS194B were effective in reactivating nerve agent-inhibited tissue in either the brain or periphery, then that tissue would show higher levels of AChE activity than tissue from animals exposed to the nerve agent and treated with saline. The third study assessed if RS194B provided protection against the lethal effects of these same nerve agents when combined with standard medical countermeasures, atropine and midazolam.

METHODS

In Vitro Reactivation Studies

Reactivation assays were conducted using recombinant human acetylcholinesterase (huAChE, AT1002, Allotropic Tech, LLC, Halethorpe, MD) or mouse acetylcholinesterase (mAChE, AA874, Chesapeake-PERL, Inc, Savage, MD). In short, enzyme was inhibited with a molar excess of nerve agent, and excess free nerve agent was removed using small scale size-exclusion columns. The resulting inhibited samples and an uninhibited positive control sample were diluted to an appropriate working concentration. Enzyme samples were mixed with reactivator compound at several concentrations to initiate reactivation, and aliquots were removed at various time points, diluted to stop reactivation and measured for residual AChE activity. Percent reactivation was calculated by comparing each sample to the uninhibited positive control. Nonlinear regression assuming maximal reactivation of 100% was used to determine the half-time of reactivation ($t_{1/2}$) at each reactivator concentration (Sit et al., 2011; Worek et al., 2010).

In Vivo Studies

Subjects:

Adult male carboxylesterase knock-out (Es-1KO) mice from the USAMRICD breeding colony served as subjects in both experiments. Animals were allowed food and water ad lib. On the day of study they were weighed and an identification number was written on their tail in indelible ink. In the *in vivo* AChE study the animals were group housed; in the agent challenge survival study the animals were single housed.

In Vivo Reactivation Study

Procedure: Mice (N=6-8/group) were injected subcutaneously (SC) with saline or doses of nerve agent near the LD_{50} for the Es-1KO mice. These doses were GB = 27 ug/kg; GF = 69 ug/kg; VX = 17 ug/kg. Fifteen (15) min after agent injection, the animals received either saline (no reactivator control) or RS194B intraperitoneally (IP). RS194B was prepared in sterile water, and small amounts of 17% hydrochloric acid were added while vortexing to dissolve the drug in the solution, with the final pH = 6.0-6.4. RS194B was a generous gift of Dr. Palmer Taylor, University of California San Diego. The mice received 25, 45 or 63 mg/kg RS194B in the GB experiments; 45 and 63 mg/kg RS194B in the VX experiments; and 63 mg/kg RS194B in the GF experiment. Forty-five (45) min after saline or RS194B administration (i.e., 60 min after nerve agent administration) mice were euthanized by decapitation while under deep isoflurane anesthesia. Trunk blood was collected in microfuge tubes containing 50 µl heparin sodium (15 units/ml). The brain was dissected into the following parts: cerebral cortex, hippocampus, midbrain, cerebellum, and brain stem. Three peripheral tissues, heart, diaphragm and gastrocnemius muscle, were dissected. Brain regions were diluted (1:30) and peripheral tissues were diluted (1:10) in 1% Triton-X100 solution (in water) and homogenized. Homogenized samples were centrifuged at 31,000 x g. Brain regions were centrifuged for 20 minutes, while peripheral tissues were centrifuged for 30 minutes. The supernatant from each sample was then frozen at -80^{o}C until assayed. For a whole blood (WB) sample, 20 µl of collected blood was diluted (1:25) in 1% Triton–X100 (in water) solution. The original blood sample was then centrifuged (5 min at 16,000 × g), and 10 µl of the packed red blood cells (RBC) were then diluted (1:50) in 1% Triton–X100 solution. WB and RBC samples were immediately flash frozen and stored at -80^{o}C until assayed.

AChE Analysis: Tissue samples were assayed for AChE activity using a modification of the Ellman method (Ellman et al., 1961) adapted to a 96-well plate format (Shih et al., 2010). On the day of AChE

analysis, the brain and peripheral tissue samples were thawed, and three 7 µl replicates of each were pipetted into a 96-well microplate (UV Star, Greiner, Longwood, FL). Three 10 µl replicates of the WB and RBC samples were pipetted into the microplates. Standard curves were established by adding 7 µl (for brain and peripheral tissue samples) or 10 µl (for WB and RBC samples) AChE from electric eel at 3.75, 7.5, and 15 U/ml. Twenty microliters of deionized water was added to each well containing brain and peripheral tissue samples, and 17 µl of deionized water was added to each WB and RBC sample. Following the addition of water, 200 µl of DTNB (0.424 M, pH 8.2) was added as the chromatophore to each sample well. Each microplate was then incubated for 10 min at 37°C before being placed in the Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA) where it was allowed to shake for 2 min. Immediately after, 30 µl of the substrate acetylthiocholine iodide (51.4 mM) was added to each well. The samples were read at 412 nm (at 20 s intervals) for 3.5 min, and the activity (µmole/ml/min) was determined using Softmax plus 4.3 LS software (Molecular Devices).

Protein Analysis: Protein levels in the tissue samples were determined by a bovine serum albumin BCA protein assay method (Pierce Biotechnology, Inc.). The standard curve was created using bovine serum albumin at the following concentrations: 0.5, 0.75, 1.0, 1.5, and 2.0 mg/ml. Three replicates of 10 µl for each brain tissue sample were added to individual microplate wells. To each well of brain tissue samples 200 µl of working reagent was then added. Three replicates of 5 µl for each peripheral tissue sample were added to individual microplate wells. The peripheral tissue samples were further diluted by adding 5 µl of deionized water before adding 200 µl of BCA working reagent. The microplates were shaken for 30 s and then incubated at 37°C for 30 min. The microplates were allowed to cool to room temperature before being read using the Spectramax Plus microplate reader and Softmax Plus 4.3 LS software as described above. After obtaining the protein contents of each tissue sample, the AChE activity was then expressed as µmol substrate hydrolyzed/g protein/min for brain and peripheral tissues.

Data Analysis: AChE activity was initially expressed as µmol substrate hydrolyzed/ml/min for RBC and WB and then converted to percentage of the control animals (saline/saline) baseline AChE values. In peripheral tissues and brain regions the AChE activity was initially expressed as µmol substrate hydrolyzed/g protein/min) and then expressed as percentage of the saline-treated control AChE value The enzymatic activities of the treatment groups were then expressed as percentage of the saline/saline control group (mean ± SEM % of control value) within a nerve agent. Statistical analysis of sarin- and VX-exposed groups was performed using a one-way ANOVA to compare AChE activity for all tissues across treatment groups for each nerve agent. A Tukey's test was used for multiple comparisons. Statistical analysis of GF-exposed groups was performed using an independent-samples t-test. Statistical significance was defined as $p<0.05$.

Survival Study

Procedures: Adult male Es-1KO mice were injected SC with ~5xLD_{50} of GB (105 ug/kg) or VX (104 ug/kg) or 3.5xLD_{50} of GF (268 ug/kg); one min later they received (IP) atropine sulfate (1.06 mg/kg) admixed with midazolam (3.5 mg/kg) and RS194B (25 mg/kg) or 2-PAM (25 mg/kg). The doses of atropine and oxime were meant to be human equivalent doses of drug contained in three Antidote Treatment Nerve Agent Autoinjector (ATNAA) and human equivalent dose of midazolam in two Anticonvulsant Autoinjector Systems (AAS). Animals were observed for survival at 1, 4 and 24 hr after nerve agent administration. Differences in survival fractions between 2-PAM- and RS194B-treated groups were evaluated with Fisher's exact test.

RESULTS

In Vitro Reactivation Assessment

Initial Screening Results: An initial assay measuring the reactivation potential of RS194B against frozen samples of inhibited huAChE was conducted and revealed significant reactivation potential of the compound against GB-, GF-, VX-, and VR-inhibited enzyme. Included in Table 1 are the calculated half-times of reactivation for these agents with RS194B at the highest concentration tested (500 µM). No significant reactivation was noted for GA- or GD-inhibited enzyme (data not shown).

Table 1. Half-times of RS194B-induced reactivation (500 µM) of huAChE inhibited by different nerve agents.

Agent:	**GB**	**GF**	**VX**	**VR**
Half-time of Reactivation ($t_{1/2}$ in minutes)	80 (64.8 – 104.3)	900 (759 – 1105)	175 (134 – 251)	266 (220 – 337)

(95% CI intervals indicated below values.)

In conjunction with the *in vivo* reactivation assays described below in this report, RS194B was evaluated for reactivation potential using recombinant forms of mouse and human AChE. In these assays, all enzyme was inhibited by the indicated agents and used immediately in assays. This procedural change resulted in slightly different half-times of reactivation when compared to the earlier screening efforts using frozen enzyme. No significant differences, with the exception of reactivation against GB-inhibited enzyme at 100µM reactivator, were noted between the reactivation of mouse and human AChE, indicating that mice should be a suitable *in vivo* model for this compound. These results are displayed below in Table 2 and Figure 2.

Table 2. Half-times of reactivation of RS194B in human and mouse recombinant AChE

Agent	[RS194B] (μM)	Mouse AChE ($t_{1/2}$ in minutes)	Human AChE ($t_{1/2}$ in minutes)
GB	500	33.6 (31.1 – 36.4)	25.6 (22.3 – 29.7)
	100	142* (121 - 171)	70.6 (57.3 – 90.9)
	10	820 (687 – 1015)	502 (388 – 708)
VX	500	29.6 (26.2 – 33.8)	24.5 (21.2 – 28.8)
	100	103 (85.3 – 130)	72.8 (60.6 – 90.6)
	10	439 (387 – 507)	422 (345 – 541)

(95% CI intervals indicated below values, * indicates a significant difference compared to human AChE [p≤0.05])

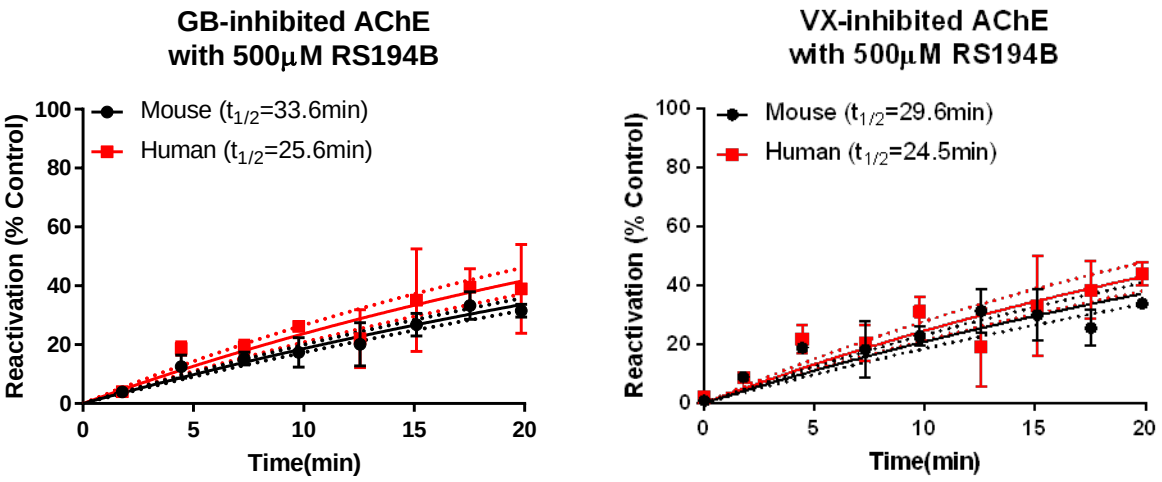


Figure 2. Reactivation of human and mouse recombinant AChE by RS194B after inhibition by GB (left) or VX (right).

In Vivo Reactivation Study

GB: The ability of RS194B to reactivate GB-inhibited brain tissue was sporadic and did not appear to be dose-dependent. RS194B at 45 mg/kg produced significantly higher levels of AChE activity in brain stem and cerebellum when compared to saline-treated animals. However, the next higher dose, 63 mg/kg RS194B, produced no significant increase in AChE activity in these tissues, but this dose did result in increased AChE activity in the midbrain tissue. In peripheral tissue, the 45 and 63 mg/kg doses of RS194B resulted in dose-dependent increases in AChE activity in heart, while only the 63 mg/kg RS194B dose produced increased AChE activity in diaphragm and skeletal muscle. RS194B produced dose-dependent increases in AChE activity in both WB and the RBC blood fraction at the three doses tested (25, 45 and 63 mg/kg). These data are summarized in more detail in Table 3 and graphically in Figure 3.

Table 3. AChE activity in the brain, peripheral tissues, and blood in control, GB-intoxicated/saline-treated, and GB-intoxicated/RS194B-treated ES1KO mice.

(A) Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain)

			µmol substrate hydrolyzed/minute/g protein				
Agent	Treatment (mg/kg)	N	Brainstem	Cerebellum	Cortex	Hippocampus	Midbrain
Control AChE activity, #mean ± SEM							
Saline	Saline	10	234.98 ± 5.49	61.93 ± 2.39	173.33 ± 11.87	131.62 ± 3.35	266.78 ± 6.11
% of control AChE activity at 60 min, #mean ± SEM							
GB	Saline	8	40.65 ± 2.06	29.80 ± 2.17	24.04 ± 1.41	31.46 ± 2.72	42.50 ± 2.86
GB	RS194B (25.0)	7	41.57 ± 1.97	38.14 ± 1.89	21.69 ± 2.32	30.61 ± 1.60	37.83 ± 1.25
	RS194B (45.0)	8	50.68 ± 1.89*	44.02 ± 2.84*	30.22 ± 2.82	39.35 ± 2.53	49.62 ± 3.13
	RS194B (63.0)	8	47.05 ± 1.31	38.59 ± 3.64	25.54 ± 1.52	34.39 ± 1.47	59.42 ± 3.97*

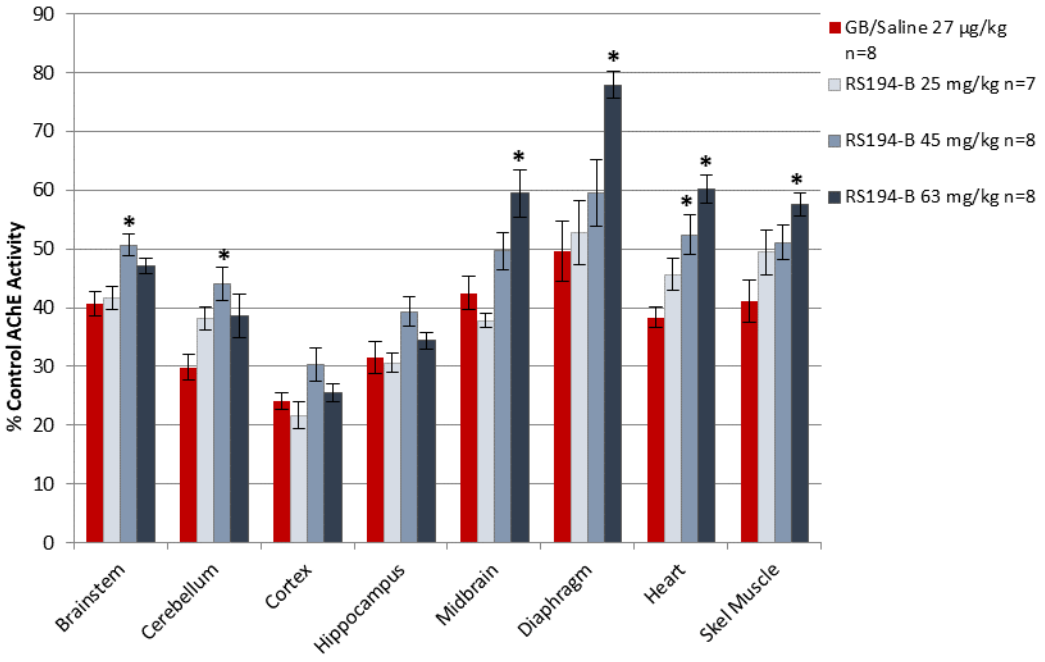
***p ≤ 0.05 vs. GB/Saline 60-minute Control**

(B) Peripheral tissues (diaphragm, heart, skeletal muscle) and blood (red blood cells and whole blood)

			µmol substrate hydrolyzed/minute/g protein			µmol substrate hydrolyzed/ml/min	
Agent	Treatment (mg/kg)	N	Diaphragm	Heart	Skeletal Muscle	Red Blood Cells	Whole Blood
Control AChE activity, #mean ± SEM							
Saline	Saline	10	21.97 ± 0.54	14.32 ± 0.68	20.18 ± 0.85	0.70 ± 0.06	1.78 ± 0.04
% of control AChE activity at 60 min, #mean ± SEM							
GB	Saline	8	49.70 ± 5.12	38.32 ± 1.72	41.09 ± 3.54	34.15 ± 2.68	13.25 ± 0.36
GB	RS194B (25.0)	7	52.71 ± 5.47	45.64 ± 2.72	49.42 ± 3.83	54.53 ± 4.28*	24.01 ± 0.86*
	RS194B (45.0)	8	59.50 ± 5.69	52.43 ± 3.42*	51.12 ± 3.03	63.61 ± 3.43*	34.31 ± 1.49*
	RS194B (63.0)	8	77.91 ± 2.30*	60.14 ± 2.40*	57.56 ± 1.99*	72.28 ± 1.81*	39.16 ± 1.97*

***p ≤ 0.05 vs. GB/Saline 60-minute Control**

A.



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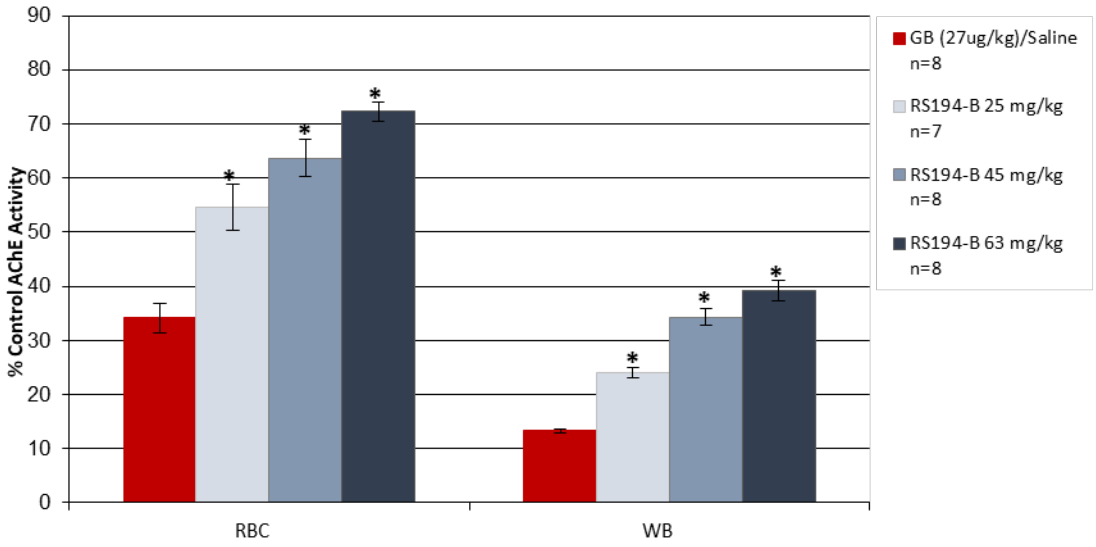


Figure 3. Effects of GB and RS194B treatment on brain and peripheral tissues (**A**) and blood (**B**) AChE activity in ES1KO mice. (*p<0.05 vs. GB/Saline 60-minute control group).

VX: The ability of RS194B to reactivate VX-inhibited brain tissue was seen in only one tissue (midbrain) and did not appear to be dose-dependent. AChE activity was significantly higher in the midbrain area of animals challenged with VX and treated with 45 mg/kg of RS194B than the VX-saline-treated controls. However, this significant increase in AChE activity was not seen in animals tested with the higher (63 mg/kg) dose of RS194B. In peripheral tissue, the 45 and 63 mg/kg doses of RS-194B resulted in dose-dependent increases in AChE activity in heart, while the 63 mg/kg RS-194B dose produced increased AChE activity in diaphragm and skeletal muscle in VX-challenged animals. The 45 and 63 mg/kg doses of RS194B produced significantly higher AChE activity in both WB and RBC blood fractions of the VX-challenged animals. This pattern of results in peripheral tissue and blood is virtually identical to the results produced by these same two doses of RS194B seen following GB challenge. These data with VX are summarized in more detail in Table 4 and graphically in Figure 4.

GF: In GF-challenged animals, there was no indication that 63 mg/kg RS194B could reactivate any of the brain tissues sampled. In fact, in the brainstem and hippocampus, AChE activities were significantly lower in RS194B-treated animals than in those that just received GF alone. The 63 mg/kg dose of RS194B produced significantly increased AChE activity in diaphragm and heart tissue, but not in skeletal muscle. Likewise, this dose of RS194B significantly increased AChE activities in both WB and RBC blood fractions much like with the GB- and VX-challenged animals. These data with GF are summarized in more detail in Table 5 and graphically in Figure 5.

Survival Study: RS194B (25 mg/kg) or 2-PAM (25 mg/kg) was combined with human equivalent doses of atropine (1.06 mg/kg) contained in three (3) ATNAAs and midazolam (3.5 mg/kg) contained in two (2) AAS autoinjectors and given 1 min after challenge with 5xLD_{50}s of GB or VX or 3.5xLD_{50} of GF. The 24 hr survival fractions were statistically equivalent for RS194B- and 2-PAM-treated animals challenged with GB, VX or GF. RS194B was unable, under these circumstances, to provide any protection against GF challenge, while 2-PAM provided 25% protection, but this difference was not large enough to reach statistical significance between these two oxime conditions. These data are displayed in Table 6.

Table 4. AChE activity in the brain, peripheral tissues, and blood in control, VX-intoxicated/saline-treated, and VX-intoxicated/RS194B-treated ES1KO mice.

(A) Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain)

			µmol substrate hydrolyzed/minute/g protein				
Agent	Treatment (mg/kg)	N	Brainstem	Cerebellum	Cortex	Hippocampus	Midbrain
Control AChE activity, #mean ± SEM							
Saline	Saline	10	234.98 ± 5.49	61.93 ± 2.39	173.33 ± 11.87	131.62 ± 3.35	266.78 ± 6.11
% of control AChE activity at 60 min, #mean ± SEM							
VX	Saline	8	26.52 ± 4.01	16.59 ± 2.14	18.41 ± 5.56	17.00 ± 3.21	29.35 ± 4.54
VX	RS194B (45.0)	8	36.62 ± 6.19	21.62 ± 2.87	14.64 ± 1.42	18.61 ± 2.78	45.72 ± 5.73*
	RS194B (63.0)	8	32.72 ± 2.15	21.87 ± 2.21	17.82 ± 3.31	20.91 ± 2.55	38.77 ± 2.67

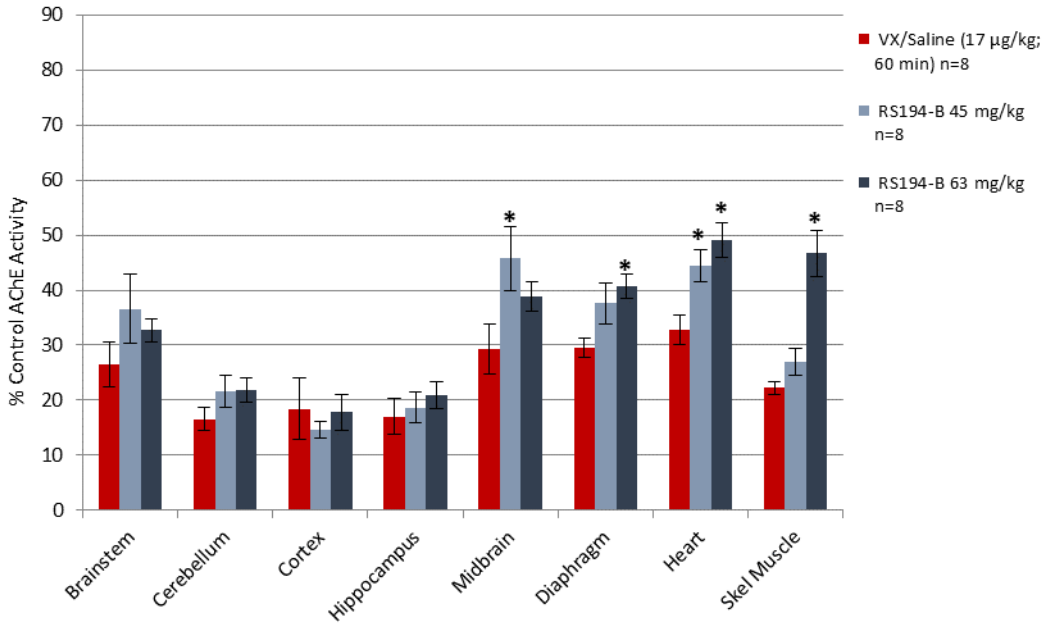
***p ≤ 0.05 vs. VX/Saline 60-minute Control**

(B) Peripheral tissues (diaphragm, heart, skeletal muscle) and blood (red blood cells and whole blood)

			µmol substrate hydrolyzed/minute/g protein			µmol substrate hydrolyzed/ml/min	
Agent	Treatment (mg/kg)	N	Diaphragm	Heart	Skeletal Muscle	Red Blood Cells	Whole Blood
Control AChE activity, #mean ± SEM							
Saline	Saline	10	21.97 ± 0.54	14.32 ± 0.68	20.18 ± 0.85	0.70 ± 0.06	1.78 ± 0.04
% of control AChE activity at 60 min, #mean ± SEM							
VX	Saline	8	29.62 ± 1.73	32.77 ± 2.57	22.22 ± 1.09	31.79 ± 2.65	12.02 ± 1.10
VX	RS194B (45.0)	8	37.63 ± 3.75	44.41 ± 2.86*	26.96 ± 2.39	54.72 ± 6.29*	26.49 ± 2.02*
	RS194B (63.0)	8	40.61 ± 2.21*	49.11 ± 3.13*	46.66 ± 4.15*	53.98 ± 2.66*	29.22 ± 1.95*

***p ≤ 0.05 vs. VX/Saline 60-minute Control**

A.



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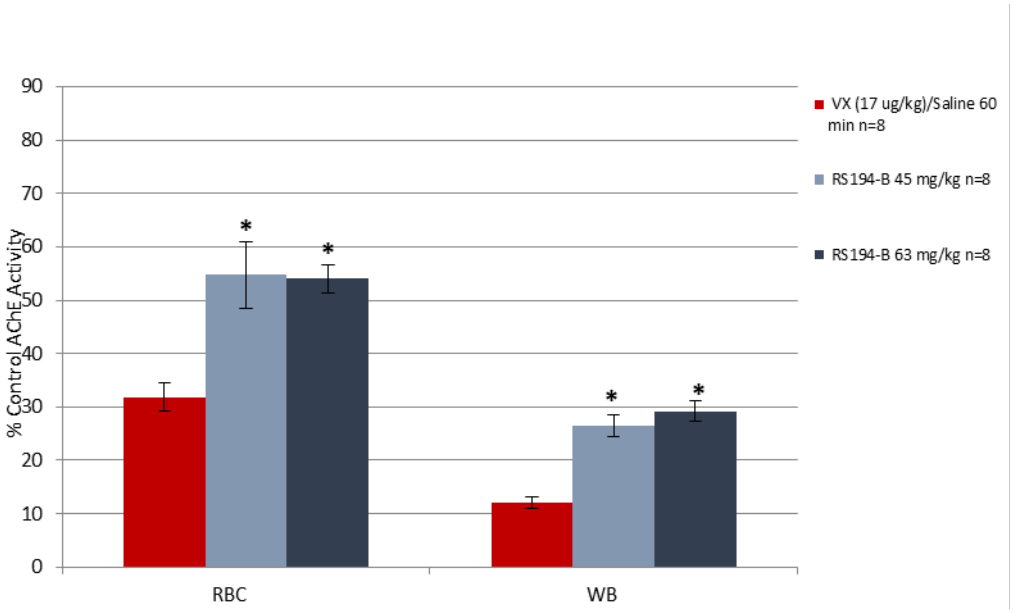


Figure 4. Effects of VX and RS194B treatment on brain and peripheral tissues (**A**) and blood (**B**) AChE activity in ES1KO mice. (*p<0.05 vs. VX/Saline 60-minute control group).

Table 5. AChE activity in the brain, peripheral tissues, and blood in control, GF-intoxicated/saline-treated, and GF-intoxicated/RS194B-treated ES1KO mice.

(A) Brain regions (brainstem, cerebellum, cortex, hippocampus, midbrain)

			µmol substrate hydrolyzed/minute/g protein				
Agent	Treatment (mg/kg)	N	Brainstem	Cerebellum	Cortex	Hippocampus	Midbrain
Control AChE activity, #mean ± SEM							
Saline	Saline	10	234.98 ± 5.49	61.93 ± 2.39	173.33 ± 11.87	131.62 ± 3.35	266.78 ± 6.11
% of control AChE activity at 60 min, #mean ± SEM							
GF	Saline	8	14.62 ± 0.69	13.71 ± 1.66	12.63 ± 3.31	17.86 ± 1.46	13.98 ± 1.11
GF	RS194B (63.0)	8	9.59 ± 1.85*	13.81 ± 1.93	8.35 ± 1.23	11.68 ± 1.77*	18.88 ± 4.61

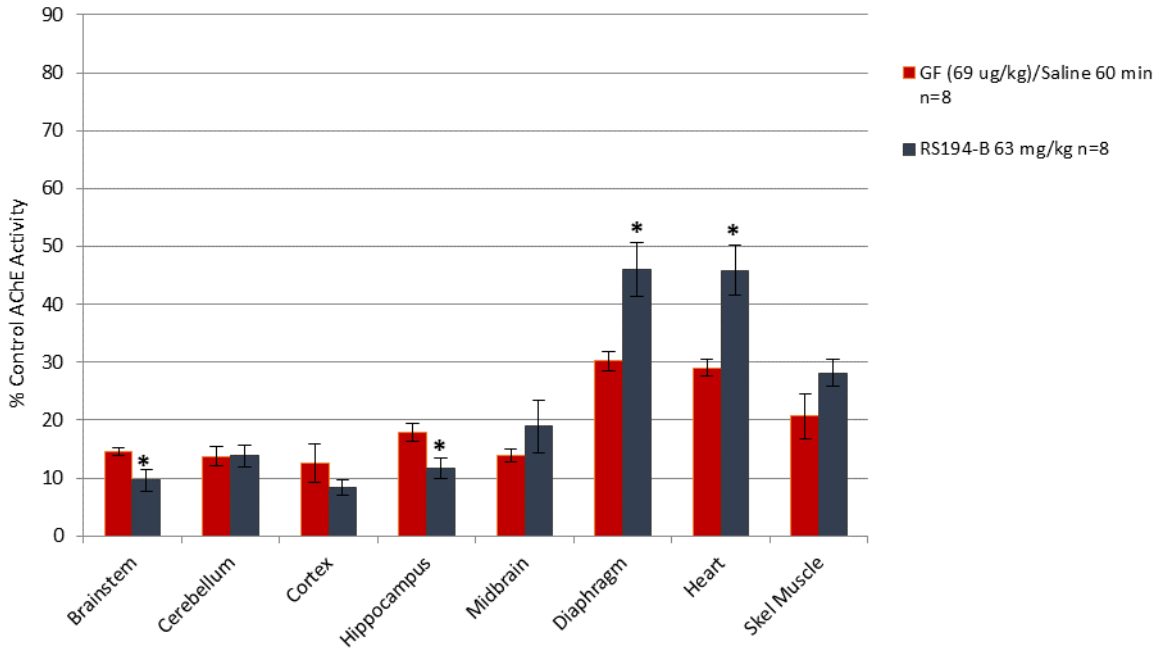
***p ≤ 0.05 vs. GF/Saline 60-minute Control**

(B) Peripheral tissues (diaphragm, heart, skeletal muscle) and blood (red blood cells and whole blood)

			µmol substrate hydrolyzed/minute/g protein			µmol substrate hydrolyzed/ml/min	
Agent	Treatment (mg/kg)	N	Diaphragm	Heart	Skeletal Muscle	Red Blood Cells	Whole Blood
Control AChE activity, #mean ± SEM							
Saline	Saline	10	21.97 ± 0.54	14.32 ± 0.68	20.18 ± 0.85	0.70 ± 0.06	1.78 ± 0.04
% of control AChE activity at 60 min, #mean ± SEM							
GF	Saline	8	30.21 ± 1.71	29.04 ± 1.36	20.68 ± 3.85	19.94 ± 3.90	7.82 ± 0.67
GF	RS194B (63)	8	45.95 ± 4.62*	45.85 ± 4.37*	28.14 ± 2.28	37.99 ± 1.78*	19.21 ± 2.05*

***p ≤ 0.05 vs. GF/Saline 60-minute Control**

A.



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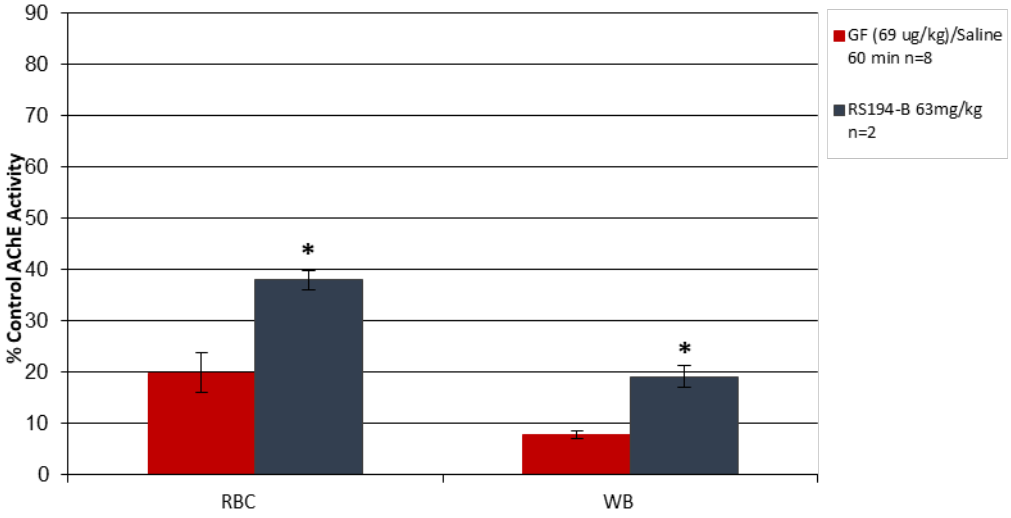

Figure 5. Effects of GF and RS194B treatment on brain and peripheral tissues (**A**) and blood (**B**) AChE activity in ES1KO mice. (*p<0.05 vs. GF/Saline 60-minute control group).

Table 6. Twenty-four-hour survival fractions and percentages for ES-1 KO mice given human equivalent doses of atropine (1.06 mg/kg in three ATNAAs) and midazolam (3.5 mg/kg in two AAS autoinjectors) along with RS194B (25 mg/kg) or 2-PAM (25 mg/kg) 1 min after a 5xLD_{50} challenge of GB or VX or 3.5xLD_{50} of GF

	GB	VX	GF
2-PAM	6 of 8; 75%	3 of 8; 37%	2 of 8; 25%
RS194B	8 of 12; 75%	6 of 8; 75%	0 of 13; 0%

DISCUSSION

RS194B showed a reactivation profile very similar to 2-PAM. *In vitro* it reactivates GB- and VX-inhibited human and mouse AChE relatively quickly and at concentrations that would be clinically relevant. Reactivation against VR-inhibited enzyme was less robust, and RS194B was least active against GF-inhibited enzyme. These *in vitro* data are in line with the results obtained in the *in vivo* studies where there was relatively good dose-dependent *in vivo* reactivation of peripheral tissue inhibited by GB and VX, with significantly less reactivation seen in the GF-exposed animals. Utilizing known pharmacokinetic parameters for RS194B determined in guinea pigs (Malfatti et al., 2017) to estimate the maximum plasma concentration of the compound for each dose administered, the estimated reactivation level for each dose 45 minutes after administration was calculated using a rate of reactivation extrapolated from *in vitro* data. When these calculated values (corrected for the maximum inhibition achieved *in vivo*) were compared to the measured reactivation in red blood cells and whole blood of GB-exposed animals (which displayed the most noticeable dose-dependent response to RS194B), nearly all of the values fall within the predicted range (Figure 6). These results help to support the correlation noted between *in vitro* comparisons of human and mouse AChE reactivation overall while also pointing to the possibilities of developing *in vitro*/*in vivo* correlation models in the future. The correlation between *in vitro* and *in vivo* reactivation data appeared to carry over to the survival study, where RS194B was able to provide protection comparable to 2-PAM against the lethal effects of GB and VX, but provided no protection against GF challenge. However, RS194B was promoted as being superior to oximes like 2-PAM because of its purported ability to enter the brain and reactivate CNS AChE that had been inhibited by the nerve agents. The data from the *in vivo* reactivation study show that RS194B provided only sporadic and non-dose dependent reactivation of some brain areas. For example, in the GB exposed animals, the 45 mg/kg RS194B dose produced higher AChE activity levels in brain stem and cerebellum, while the 63 mg/kg dose produced higher levels only in the midbrain. One would expect that if 45 mg/kg RS194B dose was effective in reactivating AChE in brain stem and cerebellum, then the 63 mg/kg dose would also be effective in these tissues (it was not) and not just midbrain. In contrast, RS194B did produce orderly, dose-dependent reactivation of peripheral tissue (diaphragm, heart, skeletal muscle, blood) AChE inhibited by the three nerve agents. On the basis of these data, RS194B does not seem to provide significant widespread reactivation of CNS AChE.

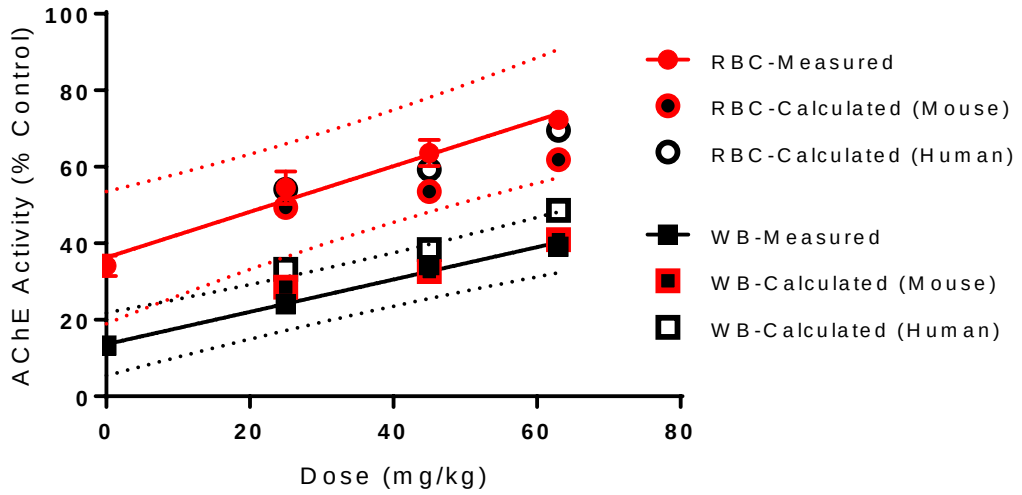

Figure 6. AChE reactivation in red blood cells (RBC, solid red circle) and whole blood (WB, solid black square) was measured in mice exposed to GB 45 minutes after administration of RS194B at 0, 25, 45, or 63 mg/kg dose. Using the rate of reactivation determined *in vitro* with human or mouse AChE and the estimated plasma levels of RS194B, the level of reactivation in the tissue was estimated (human values used for black-lined, unfilled circle or square; mouse values used for red-lined, black-filled circle or square). The prediction intervals (95% CI) for a linear relationship of the measured RBC (red dashes) or WB (black dashes) reactivation are indicated.

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