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ECBC-TN-074

SYNTHESIS AND ACETYLCHOLINESTERASE ASSAY REPORT FOR RS182A AND RS150D

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January 2018

U.S. ARMY RDECOM

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
XX-01-2018	Final	Mar 2017 – Jul 2017

4. TITLE AND SUBTITLE
Synthesis and Acetylcholinesterase Assay Report for RS182A and RS150D

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Myslinski, James*; Walz, Andrew J.; and Height, Jude (ECBC)

5d. PROJECT NUMBER
DTRA 3889-ECBC

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Excet, Inc.; 6225 Brandon Ave., Suite 360, Springfield, VA 22150-2519
Director, ECBC, ATTN: RDCB-DRC-C, APG, MD 21010-5424

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TN-074

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Defense Threat Reduction Agency, 8725 John J. Kingman Road, MSC 6201, Fort Belvoir, VA 22060-6201

10. SPONSOR/MONITOR'S ACRONYM(S)
DTRA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release: distribution unlimited.

13. SUPPLEMENTARY NOTES
*During the period this research was performed, James Myslinski was employed by Excet, Inc.

14. ABSTRACT-LIMIT 200 WORDS

The synthesis and the reactivation kinetics of two cholinergic reactivators, RS150D and RS182A, are reported, and the results are compared to the kinetics of the standard reactivator, 2-pyridine aldoxime methyl chloride (2-PAM). In a 2011 article, Sit and his coworkers highlighted RS150D as a promising reactivator of ethyl *N*,*N*-dimethyl phosphoramidocyanidate (GA)-inhibited acetylcholinesterase (AChE), although it was not found to perform better than the standard 2-PAM reactivator. The reaction schemes for RS150D and RS182A are described, and the kinetic assays with several agents are reported. The synthesis produced acceptable overall yields.

15. SUBJECT TERMS
Nerve agent Acetylcholinesterase (AChE) Reactivator

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Renu B. Rastogi
U	U	U	UU	26	**19b. TELEPHONE NUMBER** *(include area code)* (410) 436-7545

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PREFACE

The work described in this report was authorized under project no. DTRA 3889-ECBC. The work was started in March 2017 and completed in July 2017.

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Acknowledgments

The authors thank Dr. Sue Bae and Dr. Mark Winemiller for the generous gift of pure samples for testing.

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SYNTHESIS AND ACETYLCHOLINESTERASE ASSAY REPORT FOR RS182A AND RS150D

1. INTRODUCTION

Chemical nerve agents covalently bind to acetylcholinesterase (AChE) causing a buildup of the neurotransmitter acetylcholine, which results in toxic effects including seizures and death. Standard medical treatment for organophosphate (OP) poisoning includes atropine to treat muscarinic effects, 2-pyridine aldoxime methyl chloride (2-PAM) as the cholinergic reactivator, and diazepam for seizure control. RS182A and RS150D (Figure 1) are potential oxime-based reactivators of agent-inhibited AChE. These compounds were developed by multiple researchers (Kovarik et al., 2013; Radić et al., 2012; Sit et al., 2011). The two compounds were synthesized and assayed to estimate their potential as reactivators and to compare their performance to the current Army standard reactivator 2-PAM (Figure 1). RS150D was noted as the most promising new candidate for the reactivation of GA–AChE conjugate (GA is the code for ethyl *N*,*N*-dimethyl phosphoramidocyanidate), which has unique reactivation requirements due to the dimethylamino group characteristic of GA-inhibited AChE. However, RS150D was still not as effective for the reactivation of GA–AChE as were the bis-pyridinium aldoximes (e.g., asoxime chloride [HI-6] and 1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2,4-bis [(hydroxyimino)methyl]pyridinium dimethanesulfonate [HLo-7]) (Kovarik et al., 2013). In addition, RS150D and RS182A do not have a permanently charged nitrogen, and they may cross the blood–brain barrier to reactivate the central nervous system AChE.

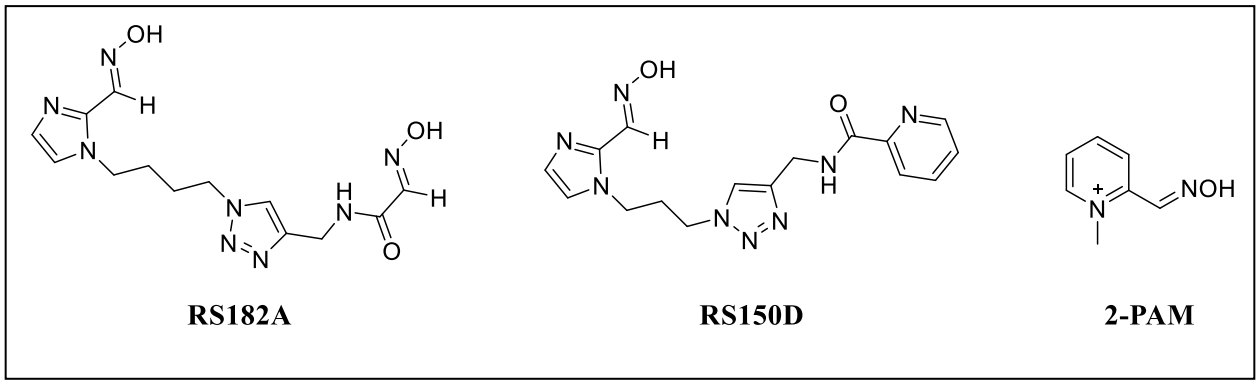


Figure 1. Structures of RS182A, RS150D, and 2-PAM.

The inhibition of AChE can be represented as shown in Figure 2, wherein the catalytically active serine (Ser_{203}) is covalently bound to the OP via a nucleophilic attack at the phosphorus atom of the agent. The Ser_{203}–OP conjugate effectively blocks the catalytic activity of AChE. Subsequent reactivation with an appropriate nucleophile can occur only prior to an aging process.

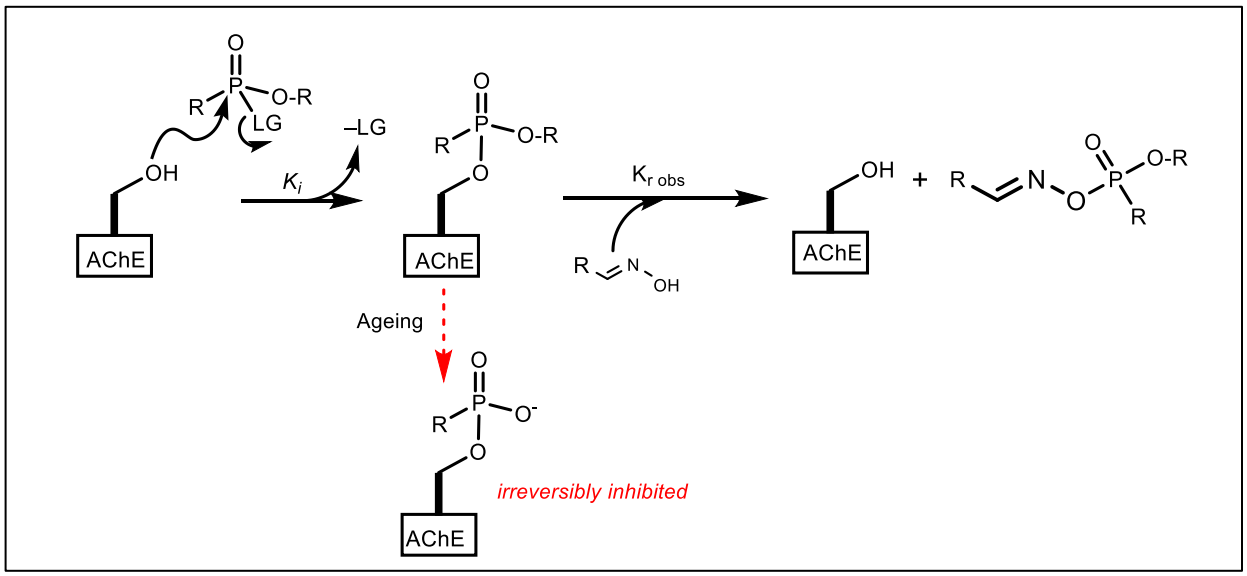


Figure 2. Scheme representing the proposed mechanism for inhibition of AChE and subsequent reactivation using an oxime.

The inhibition and reactivation process may also be represented as shown in Figure 3, wherein K_i is the rate constant of inhibition, K_D is the rate constant of the formation of the inhibited AChE-reactivator complex, k_r is the second-order reactivation rate constant, and $k_{r\ obs}$ is the observed rate of reactivation.

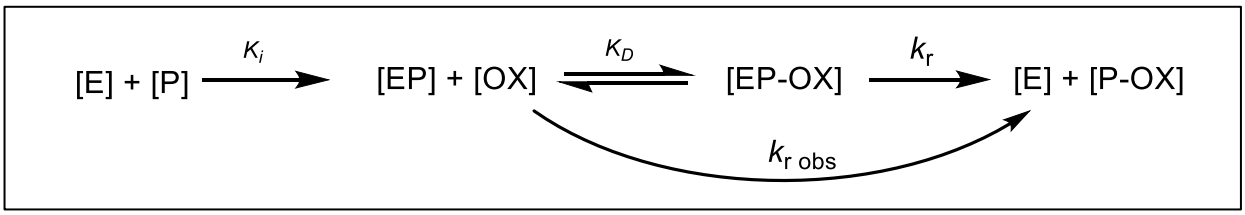
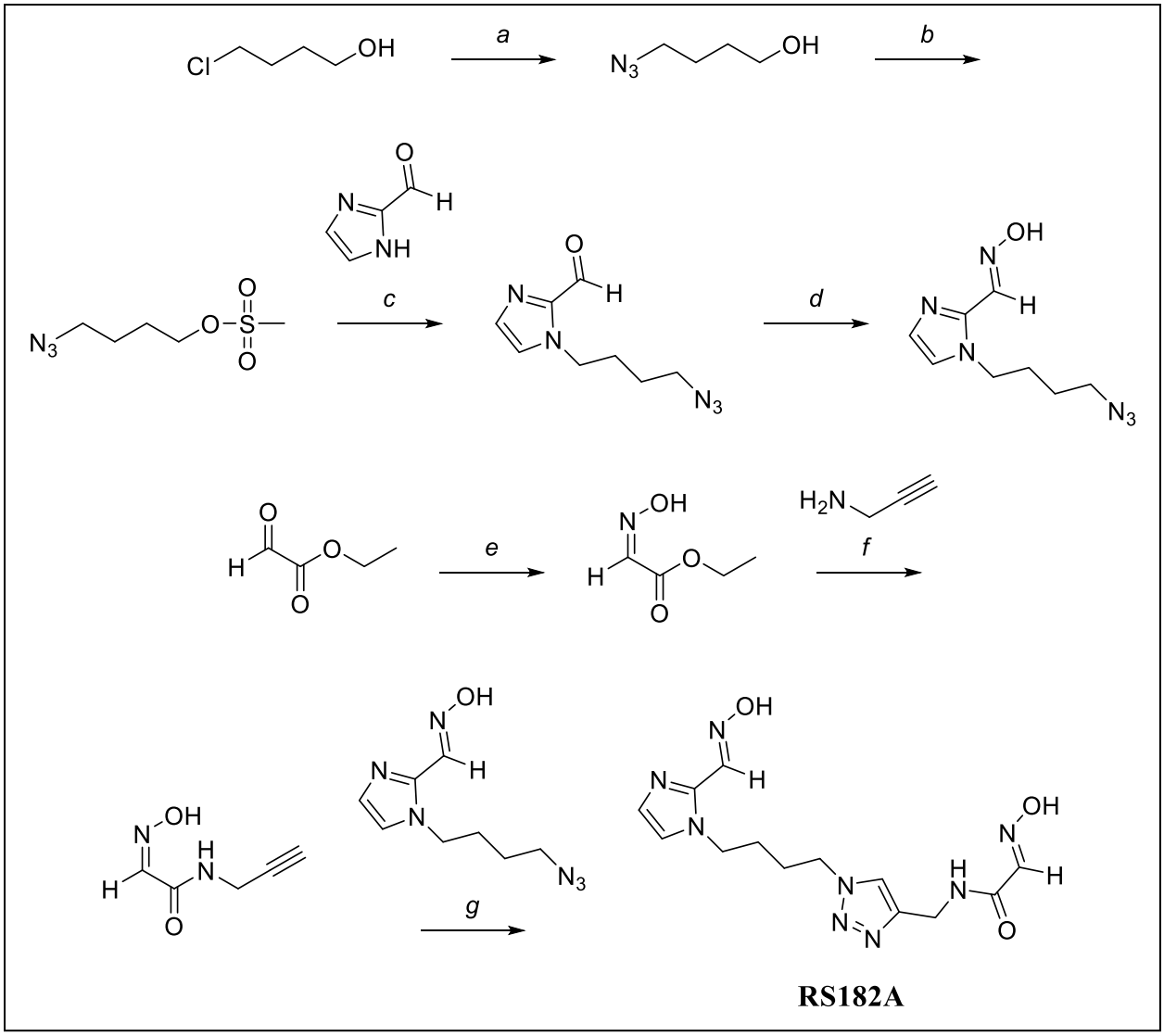


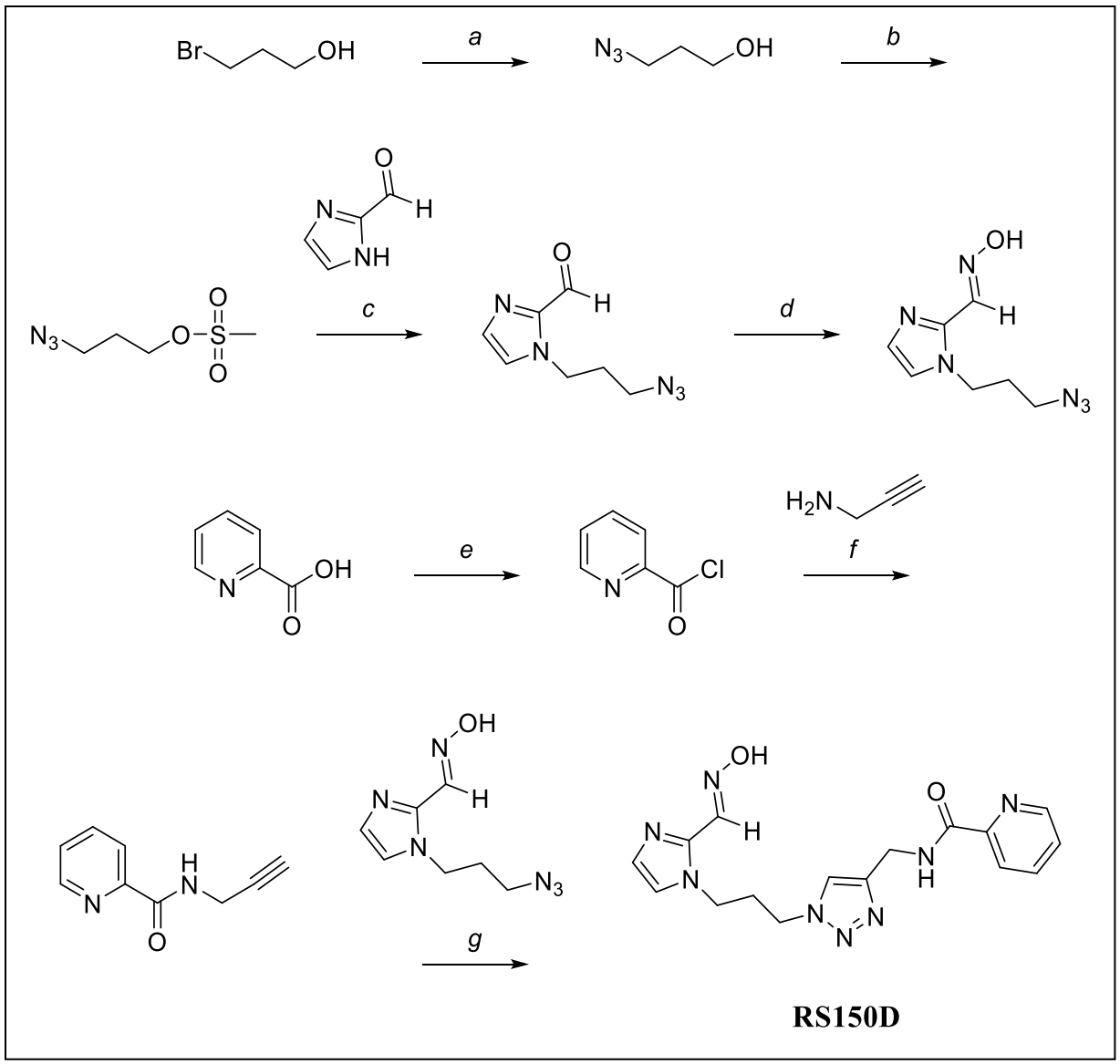
Figure 3. The kinetic parameters governing the inhibition and reactivation of AChE. Where [E] is enzyme concentration, [P] is inhibitor concentration, [EP] is the inhibited enzyme concentration, [OX] is oxime concentration, [EP–OX] is the inhibited enzyme–oxime complex concentration, and [P–OX] is the organophosphate–oxime product concentration

2. METHODS

The compounds RS182A and RS150D were synthesized as shown in reaction Schemes 1 and 2, respectively. The chemistry followed the methods of Radić et al., 2012; Reiter et al., 2014; and Sit et al., 2011.



Scheme 1. Synthesis of RS182A. (a) NaN_3, NaI, ACN, H_2O; (b) MsCl, TEA, CH_2Cl_2; (c) K_2CO_3, DMF; (d) $NH_2OH(HCl)$, K_2CO_3, H_2O, MeOH; (e) $NH_2OH(HCl)$, TEA, ACN, H_2O; (f) EtOH; and (g) sodium ascorbate, $CuSO_4$, *t*-BuOH, H_2O. ACN is acetonitrile, MsCl is methanesulfonyl chloride, TEA is triethylamine, DMF is dimethylformamide, EtOH is ethanol, and *t*-BuOH is tert-butanol.



Scheme 2. Synthesis of RS150D. (a) NaN_3, H_2O; (b) MsCl, TEA, CH_2Cl; (c) K_2CO_3, DMF; (d) $NH_2OH(HCl)$, K_2CO_3, H_2O, MeOH; (e) $SOCl_2$, DMF, CH_2Cl_2; (f) DIEA, CH_2Cl_2; and (g) sodium ascorbate, $CuSO_4$, *t*-BuOH, H_2O. DIEA is diisopropylethylamine.

Plots of the kinetic analysis of RS182A and RS150D reactivation of human acetylcholinesterase (*h*AChE) inhibited by chemical agents GA ((*RS*)-ethyl *N*,*N*-dimethylphosphoramidocyanidate), GB (isopropyl methylphosphonofluoridate), and the chiral isomers of VX, including ethyl ({2-[bis(propan-2-yl)amino]ethyl}sulfanyl)(methyl)phosphinate, (P_{RS})-VX, (P_R)-VX, and (P_S)-VX, (where P_R and P_S represent the stereochemical environment at the phosphorus atom as given by the Cahn–Ingold–Prelog sequence rules, and P_{RS} represents a racemic mixture of both enantiomers), and experimental compounds S2 and S3 are shown. Agent-inhibited dimeric *h*AChE was treated with an oxime, then the rate of hydrolysis of acetylthiocholine was determined and compared to uninhibited *h*AChE as a function of time with

the reactivator. The time courses of reactivation using RS150D and RS182A are shown in Figures 4 and 5, respectively.

*h*AChE was inhibited with an excess of the inhibitor for 30 min at 0 °C, and then the unreacted inhibitor was removed by centrifugation through a CentriSep spin column (Thermo Fisher Scientific, Inc.; Waltham, MA) (Cadieux et al., 2016). The resulting *h*AChE samples were diluted in buffer (0.100 M PO_4^-, pH 8.0, 5% bovine serum albumin [BSA]) and stored at –80 °C. Samples were thawed on ice immediately before use. Rates of hydrolysis of acetylthiocholine (v_t) were determined by a modified Ellman assay (Ellman et al., 1961) at 0.5, 1, 2, 3, 4, 5, and 30 min after the addition of a reactivator at 37 °C. The rate of hydrolysis in the absence of OP (v_o) was determined by assaying *h*AChE that was treated with buffer only. The oxime-promoted breakdown of 5, 5′-dithio-bis-(2-nitrobenzoic acid) (DTNB; v_{ox}) was determined for each reactivator at each concentration and was subtracted from both v_o and v_t in the determination of k_{obvs}. The kinetic parameters k_r and K_D were determined by the nonlinear fit of eqs 1 and 2. The results are summarized in Tables 1 and 2 for each reactivator (Reiter et al., 2014).

$$k_{\mathrm{obvs}} = \frac{k_2[\mathrm{OX}]}{K_{\mathrm{OX}}+[\mathrm{OX}]} \tag{1}$$

$$\%(E_{\mathrm{react}})_t = \frac{v_t - v_{\mathrm{OX}}}{v_o - v_{\mathrm{OX}}} = A(1 - e^{-k_{\mathrm{obvs}}t}) \tag{2}$$

where k_{obvs} is the first-order rate constant of reactivation at a given oxime concentration, k_2 is the maximum first-order rate constant, [OX] is oxime concentration, K_{OX} is approximately the dissociation constant of the [EP–OX] complex, $\%E_{react}$ is the specific activity of uninhibited AChE that was recovered, t is time in seconds, and A is the maximum activity recovered.

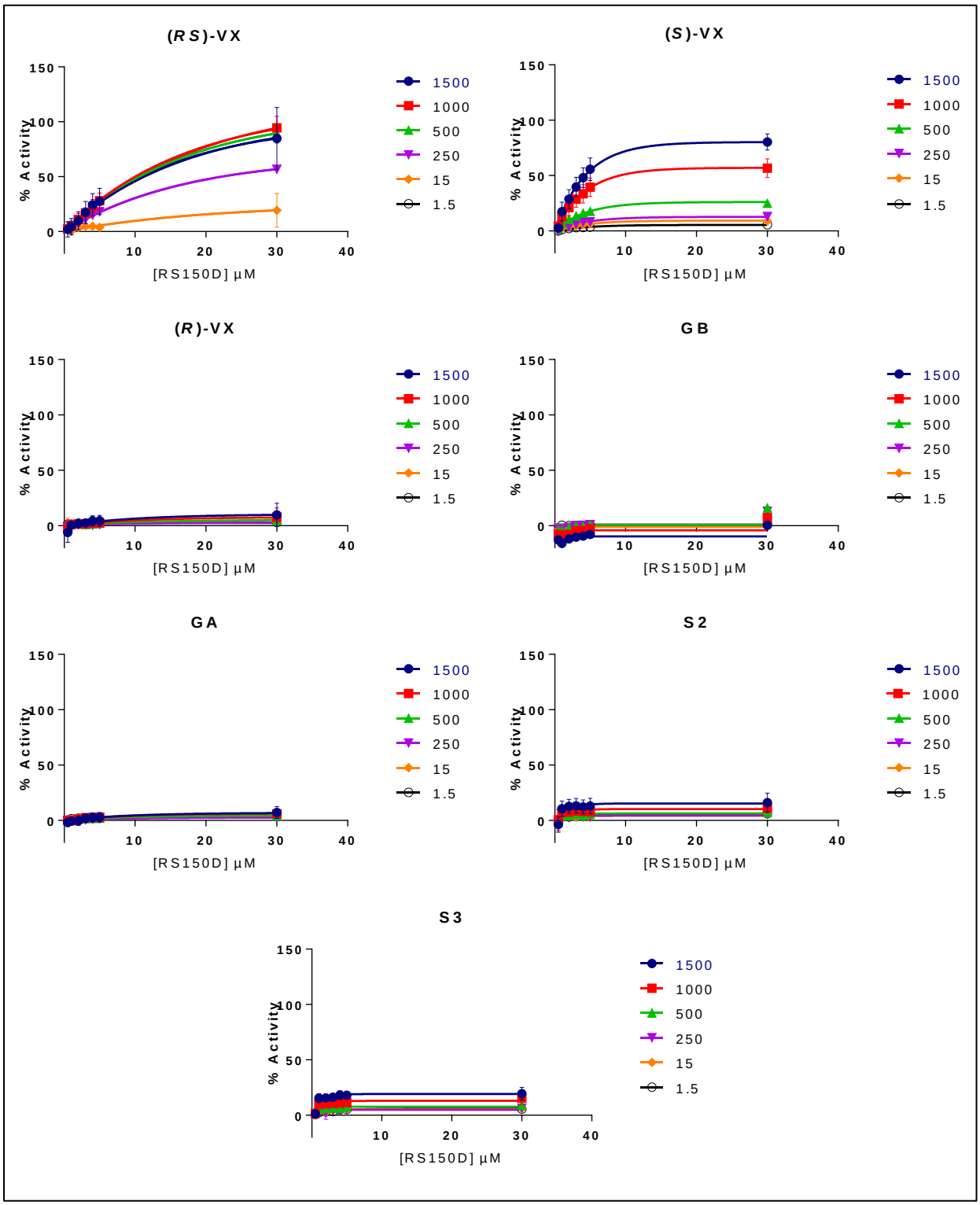


Figure 4. AChE activity recovered (%) relative to uninhibited AChE with RS150D as a function of time. Agents are shown in micromolar concentration.

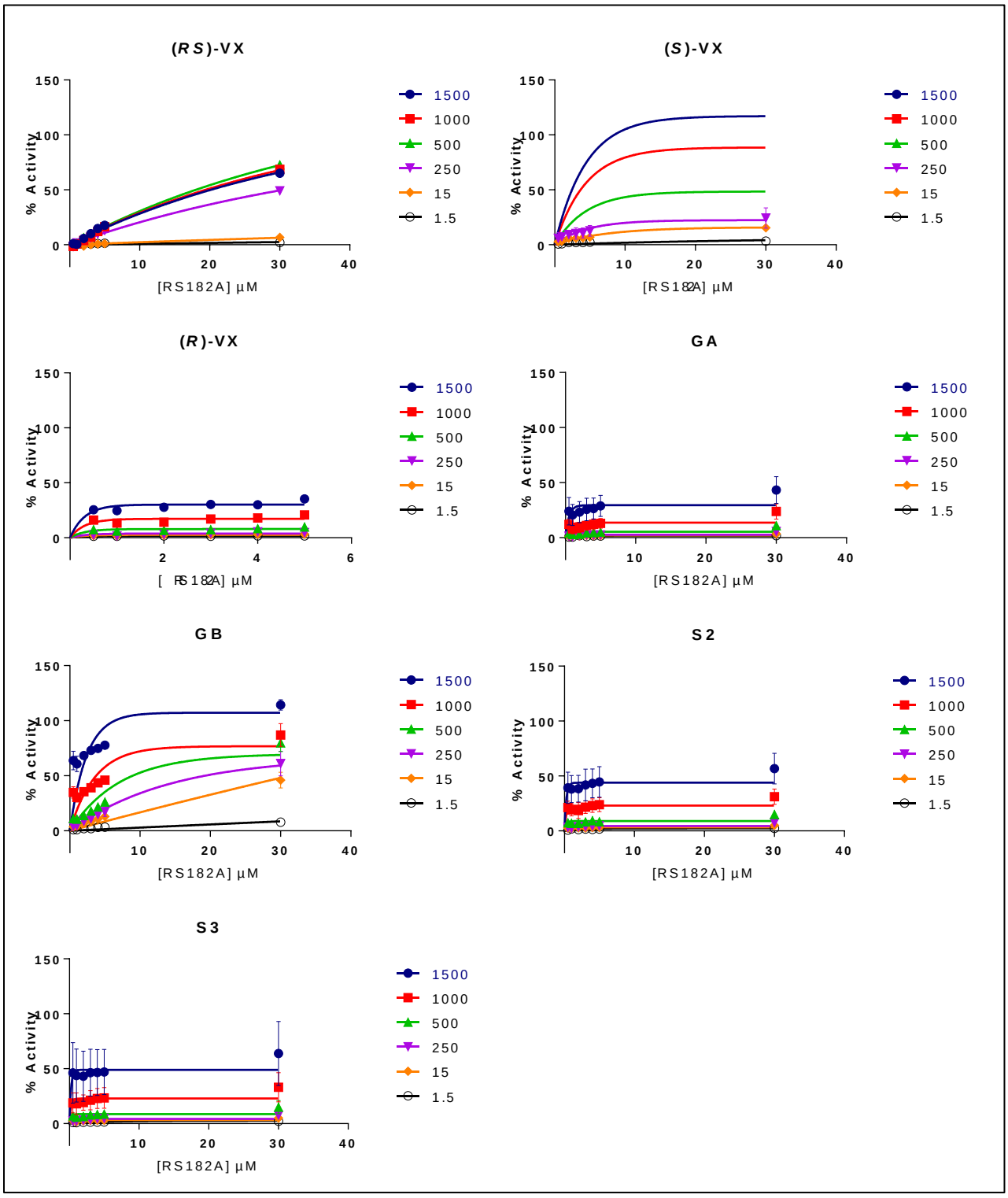


Figure 5. AChE activity recovered (%) relative to uninhibited AChE with RS182A as a function of time. Agents are shown in micromolar concentration.

Table 1. Reactivation Kinetics for RS150D with the Indicated Inhibitor

Agent	k_2 **(min^{-1})**	K_{OX} **(μM)**	K_r **(μM^{-1} min^{-1})**	**Reactivation at 15 μM (%)**
GA	0.10 ± 0.03	–1.12 ± 0.30	–0.09 ± 0.04	3
GB	–	–	–	0
(P_{RS})-VX	0.058 ± 0.007	3.0 ± 19	0.02 ± 0.13	25
(P_S)-VX	0.228 ± 0.008	0.0340 ± 1.08	6.73 ± 214	9
(P_R)-VX	0.094 ± 0.031	–1.34 ± 0.24	–0.07 ± 0.03	3
S2	0.561 ± 0.103	0.736 ± 1.38	0.76 ± 1.39	4
S3	0.786 ± 0.091	1.85 ± 1.64	0.425 ± 0.367	5

– Indicates that the data could not be fit to eqs 1 and 2.

Table 2. Reactivation Kinetics for RS182A with the Indicated Inhibitor

Agent	k_2 **(min^{-1})**	K_{OX} **(μM)**	K_r **(μM^{-1} min^{-1})**	**Reactivation at 15 μM (%)**
GA	2.00 ± 0.39	5.48 ± 22	0.365 ± 1.45	2
GB	–	–	–	84
(P_{RS})-VX	0.029 ± 0.005	62.4 ± 116.2	0.00047 ± 0.00079	41
(P_S)-VX	0.229 ± 0.016	10.4 ± 19	0.022 ± 0.04	16
(P_R)-VX	0.234 ± 0.019	–0.05 ± 3	–4.7 ± 282	6
S2	3.97 ± 0.79	14.0 ± 35.3	0.281 ± 0.705	3
S3	4.59 ± 1.76	37.6 ± 117	0.122 ± 365	3

– Indicates that the data could not be fit to eqs 1 and 2.

To compare the data from this study (Figure 6 and Table 3) with that of Sit et al. (2011), the k_{obvs} of reactivation at 0.50 mM (Table 4) was compared to the k_{obvs} of reactivation at 0.67 mM oxime (Table 5), which was determined in the cited reference. k_{obvs} was determined using eq 1 at the noted concentration of oxime. Unfortunately, the cited paper only discusses k_{obvs} relative to 2-PAM but not in absolute terms. Therefore, the rate constants relative to 2-PAM are reported in Figure 7 and Table 6 for comparison to the data determined in this study.

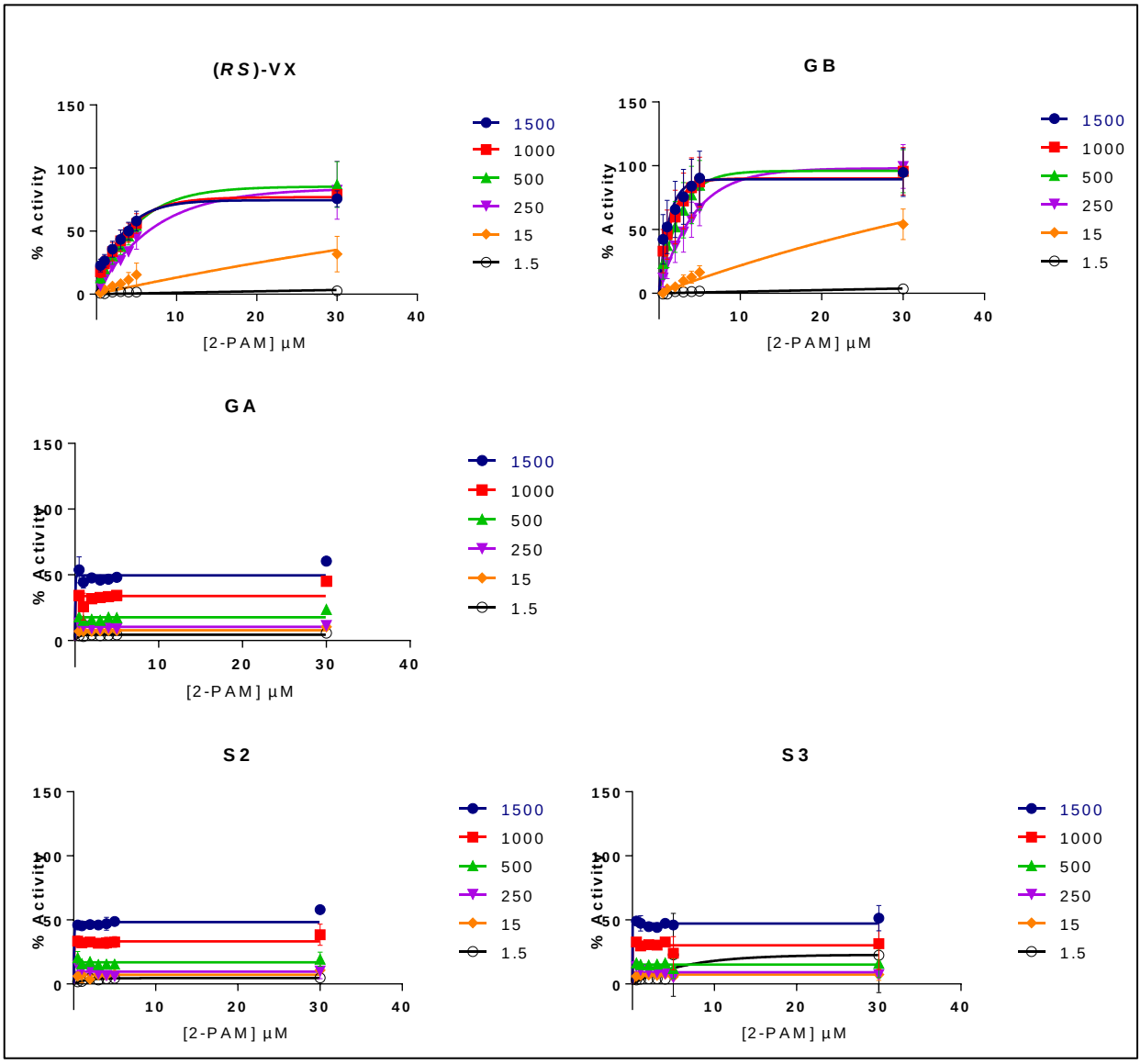


Figure 6. AChE activity recovered (%) relative to uninhibited AChE with 2-PAM as a function of time. Agents are shown in micromolar concentration.

Table 3. Reactivation Kinetics for 2-PAM with the Indicated Inhibitor

Agent	k_2 (min^{-1})	K_{OX} (μM)	K_r (μM^{-1} min^{-1})	Reactivation at 15 μM (%)
GA	24 ± 1677	12.6 ± 988	1.9 ± 16.3	8
GB	1.67 ± 0.81	1538 ± 1043	0.0011 ± 0.0002	>100
(P_R and P_S)-VX	0.424 ± 0.072	506.7 ± 181.9	0.00084 ± 0.00017	>100
S2	14.8 ± 66.7	30.8 ± 161	0.48 ± 0.58	7
S3	–	–	–	7

– Indicates that the data could not be fit to eqs 1 and 2.

Table 4. K_{obvs} (min^{-1}) (0.50 mM) for Reactivation of Inhibited AChE with Selected Agents

Oxime	(P_{RS})-VX	(P_S)-VX	(P_R)-VX	GB	GA	S2	S3
RS150D	0.06809 ± 0.003010	0.03242 ± 0.001905	0.001321 ± 0.004291	0.01152 ± 0.001728	N/A	0.004344 ± 0.002000	0.002327 ± 0.002377
RS182A	0.03571 ± 0.001199	0.05536 ± 0.01092	0.004888 ± 0.01070	0.04236 ± 0.002613	0.003826 ± 0.004214	0.005123 ± 0.002667	0.006148 ± 0.003392
2-PAM	0.2150 ± 0.01573	0.1148 ± 0.003573	N/A	0.2497 ± 0.008106	0.0009271 ± 0.002760	N/A	N/A

N/A, not applicable.

Table 5. Relative Rate Constants for Reactivation Using 0.67 mM Reactivator Relative to 2-PAM (Reproduced from Cadieux et al., 2016.)

Oxime	GA	GB	(P_{RS})-VX
RS150D	0.58	0.20	1.0
RS182A	0.33	0.25	1.7

Table 6. Rate Constant k_{obvs} Expressed as a Ratio Relative to 2-PAM, Determined at 0.5 mM

Oxime	(P_{RS})-VX	(P_S)-VX	(P_R)-VX	GB	GA
RS150D	0.32	0.28	N/A	0.05	N/A
RS182A	0.17	0.48	N/A	0.17	4.13

N/A, not applicable.

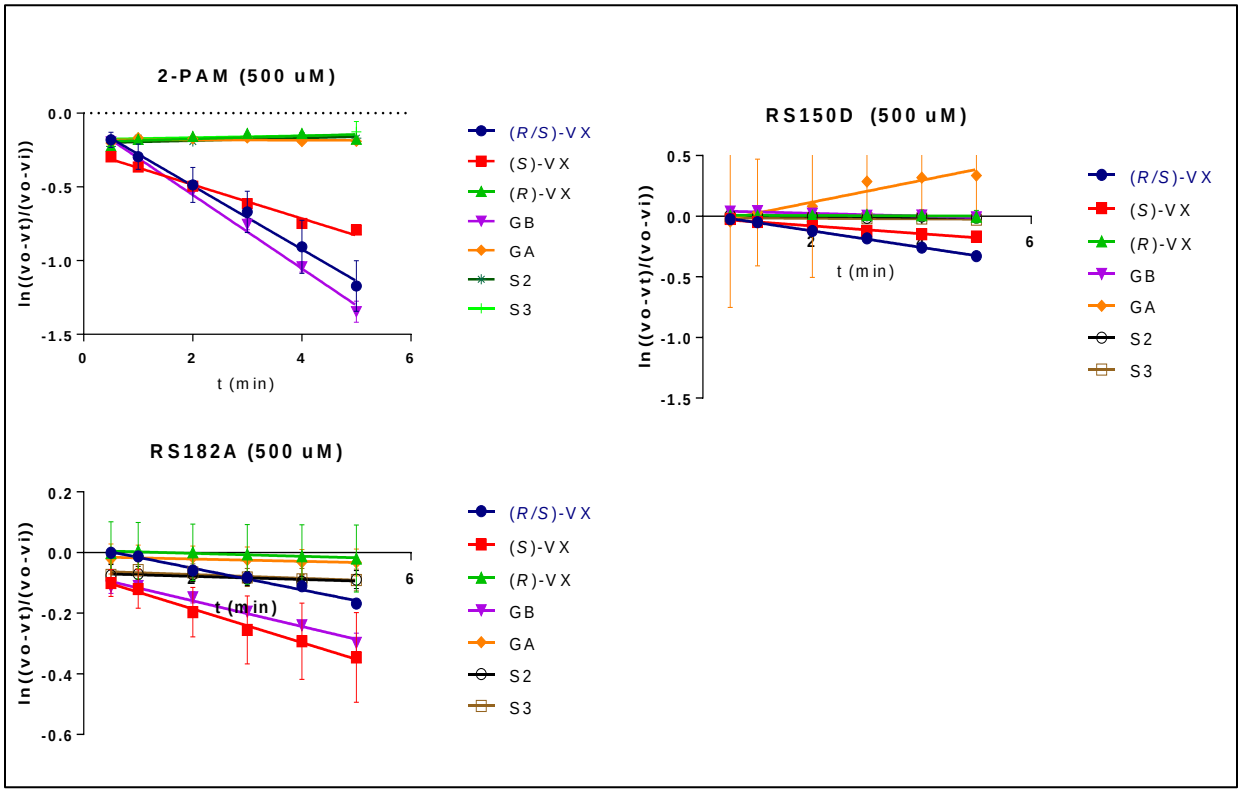


Figure 7. Determination of k_{obvs} for 2-PAM, RS150D, and RS182A at 500 µM. k_{obvs} is given by the slope of the best-fit line.

3. SUMMARY

The synthesis of RS182A and RS150D proceeded with acceptable overall yields and produced solid material that was amenable for biological study. Solubility issues were addressed with aqueous HCl-mediated pH adjustments.

A comparison of Tables 5 and 6 shows that the RS182A and RS150D were outperformed by 2-PAM in both our work and the work of Sit et al. (2011) against GA-inhibited *h*AChE (Figure 8). However, neither compound was more successful than the other at reactivating VX-inhibited *h*AChE, as had been reported by Sit et al. Although it appears that RS182A was a better reactivator of GA-inhibited *h*AChE than was 2-PAM, when the relative k_{obvs} values were compared, our analysis revealed that RS182A was a very poor reactivator of GA-inhibited *h*AChE.

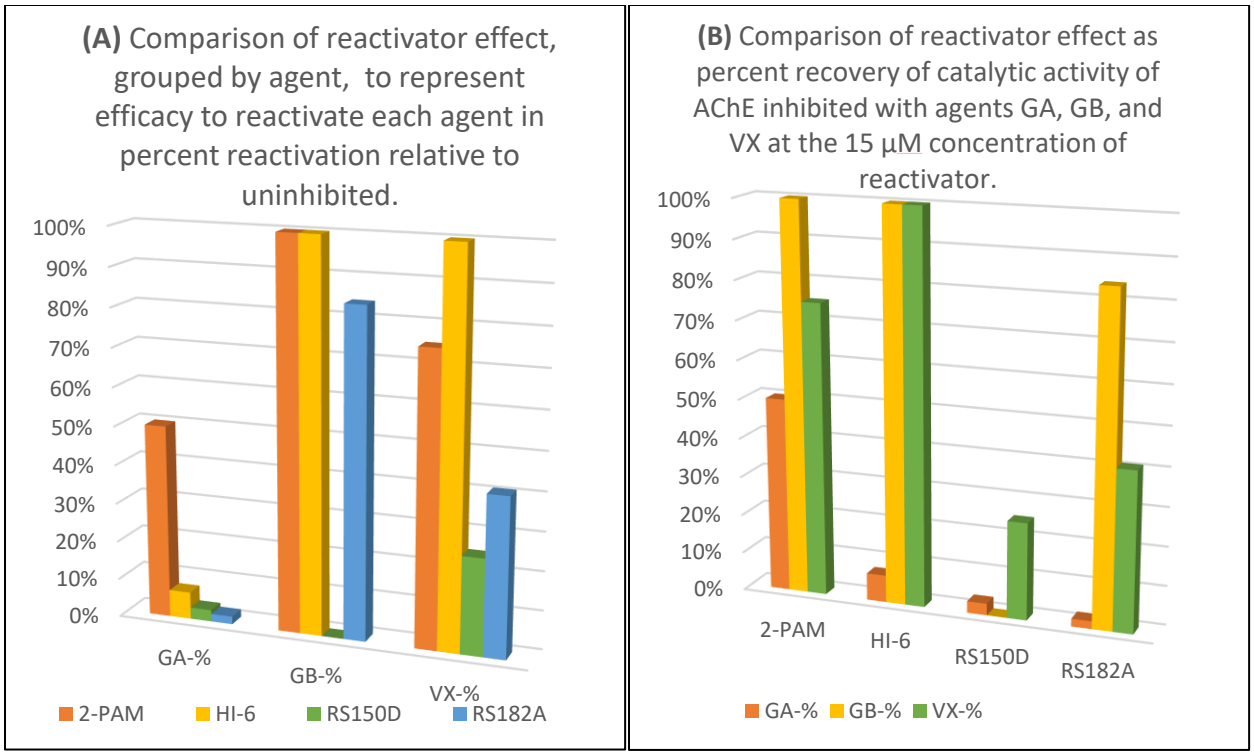


Figure 8. (A) Shows a comparison of the effectiveness of each color-coded reactivator on GA, GB, and VX agents. (B) Shows a comparison of the efficacy of each reactivator by each color-coded chemical agent.

Some notable differences between the experiments might lead to variations in the reactivation. However, the use of a k_{obvs}, relative to 2-PAM at 0.67 mM, rather than at 0.50 mM, might result in some differences as well. Therefore, comparison of the differences between the reactivation potential is complicated without disclosure of the full characterization of the kinetics. The largest differences between the observed reactivation potential and the reported reactivation trends could be attributed to differences between the *h*AChE itself. In the work performed by Sit et al., a monomeric mutant of AChE was used, whereas in this study, the wild-type sequence was used, and in our hands it has been shown to produce a dimeric form of *h*AChE.

REFERENCES

Cadieux, C.L.; Wang, H.; Zhang, Y.; Koenig, J.A.; Shih, T.M.; McDonough, J.; Cerasoli, D. Probing the Activity of a Non-Oxime Reactivator for Acetylcholinesterase Inhibited by Organophosphorous Nerve Agents. *Chem. Biol. Interact.* **2016,** *259*, 133–141.

Ellman, G.L.; Courtney, K.D.; Andres, V.; Featherstone, R.M. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochem. Pharm.* **1961,** *7*, 88–95.

Kovarik, Z.; Maćek, R.K.; Sit, R.K.; Radić, Z.; Fokin, V.V.; Sharpless, K.B.; Taylor, P. Centrally Acting Oximes in Reactivation of Tabun-Phosphoramidated AChE. *Chem. Biol. Interact.* **2013,** *203* (1), 77–80.

Radić, Z.; Sit, R.K.; Kovarik, Z.; Berend, S.; Garcia, E.; Zhang, L.; Amitai, G.; Green, C.; Radić, B.; Fokin, W.; Sharpless, K.B.; Taylor, P. Refinement of Structural Leads for Centrally Acting Oxime Reactivators of Phosphylated Cholinesterases. *J. Biol. Chem.* **2012,** *287*, 11798–11809.

Reiter, G.; Müller, S.; Hill, I.; Weatherby, K.; Thiermann, H.; Worek, F.; Mikler, J. In Vitro and in Vivo Toxicological Studies of V Nerve Agents: Molecular and Stereoselective Aspects. *Toxicol. Lett.* **2014,** *232* (2), 438–448.

Sit, R.K.; Radić, Z.; Gerardi, V.; Zhang, L.; Garcia, E.; Katalinic, M.; Amitai, G.; Kovarik, Z.; Fokin, V.V.; Sharpless, K.B.; Taylor, P. New Structural Scaffolds for Centrally Acting Oxime Reactivators of Phosphylated Cholinesterases. *J. Biol. Chem.* **2011,** *286* (22), 19422–19430.

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ACRONYMS AND ABBREVIATIONS

2-PAM	2-pyridine aldoxime methyl chloride
AChE	acetylcholinesterase
ACN	acetonitrile
BSA	bovine serum albumin
DIEA	diisopropylethylamine
DMF	dimethylformamide
DTNB	5, 5′-dithio-bis-(2-nitrobenzoic acid)
DTRA	Defense Threat Reduction Agency
EtOH	ethanol
GA	ethyl *N*,*N*-dimethyl phosphoramidocyanidate; tabun
GB	isopropyl methylphosphonofluoridate; sarin
*h*AChE	human acetylcholinesterase
HI-6	asoxime chloride; (*E*)-1-(((4-carbamoylpyridin-1-ium-1-yl)methoxy)methyl)-2-((hydroxyimino)methyl)pyridin-1-ium
HLo-7	1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2,4-bis [(hydroxyimino)methyl]pyridinium dimethanesulfonate
MsCl	methanesulfonyl chloride
OP	organophosphate
OX	oxime
P_R and P_S	represent the stereochemical environment at the phosphorus atom as given by the Cahn–Ingold–Prelog sequence rules
P_{RS}	represents a racemic mixture of two enantiomers
Ser_{203}	catalytically active serine
t-BuOH	*tert*-butanol
TEA	triethylamine
VX	*O*-ethyl-*S*-(2-diisopropylaminoethyl) methyl phosphonothiolate

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