JPET Fast Forward. Published on September 6, 2018 as DOI: 10.1124/jpet.118.249383
This article has not been copyedited and formatted. The final version may differ from this version.

`**Title Page**

Title:

Pharmacology, pharmacokinetics and tissue disposition of zwitterionic hydroxyiminoacetamido alkylamines as reactivating antidotes for organophosphate exposure

Authors:

Rakesh K. Sit, Zrinka Kovarik, Nikolina Maček Hrvat, Suzana Žunec, Carol Green, Valery V. Fokin, K. Barry Sharpless, Zoran Radić and Palmer Taylor

Primary laboratory of origin: Department of Pharmacology, Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California San Diego

Affiliations:

RKS, VVF, BS: Skaggs Institute for Chemical Biology, The Scripps Research Institute, San Diego, CA 92037

ZK, NMH, SŽ: Institute for Medical Research and Occupational Health, HR−10001, Zagreb, Croatia

CG: SRI International, Menlo Park, CA 94025−3493

ZR, PT: Department of Pharmacology, Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093−0650

Running Title Page

Running title:

Pharmacokinetics of Zwitterionic Reactivators of AChE

Corresponding Author:

Palmer Taylor, Ph.D.

Department of Pharmacology; Skaggs School of Pharmacy & Pharmaceutical Sciences 2256 Pharmaceutical Sciences Bldg. 0650

University of California, San Diego, 9500 Gilman Dr. La Jolla, CA 92093

Telephone (858) 534-1366; Fax: (858) 822-5591

e-mail pwtaylor@ucsd.edu

Number of Pages: 33

Number of pages including Title and Running title pages, Abstract, main text, and Footnote page: 22

Number of Tables: 4

Number of Figures: 4

Number of References: 43

Number of words in the Abstract: 245

Number of words in the Introduction: 686

Number of words in the Discussion: 1056

Non-standard Abbreviations: AChE, acetylcholinesterase; ACh, acetylcholine; ATCh, acetylthiocholine; OP, organophosphate

Recommended section assignment: Drug Discovery and Translational Medicine

ABSTRACT

In the development of antidotal therapy for treatment of organophosphate exposure from pesticides used in agriculture and nerve agents insidiously employed in terrorism, the alkylpyridinium aldoximes have received primary attention since their early development by I.B. Wilson in the 1950's. Yet these agents, by virtue of their quaternary structure, are limited in rates of crossing the blood-brain barrier, and require administration parenterally to achieve full distribution in the body. Oximes lacking cationic charges or presenting a tertiary amine have been considered as alternatives. Herein, we examine the pharmacokinetic properties in mice of a lead ionizable, zwitterionic hydroxyiminoacetamido alkylamine to develop a framework for studying these agents *in vivo* and generate sufficient data for their consideration as appropriate antidotes for humans. Consequently, *in vitro* and *in vivo* efficacies of immediate structural congeners were explored as leads or backups for animal studies. We have compared oral and parenteral dosing, and developed an intramuscular loading and oral maintenance dosing scheme in mice. Steady-state plasma and brain levels of the antidote are achieved with sequential administrations out to 10 hours, with brain exceeding plasma levels shortly after administration. Moreover, the zwitterionic oxime shows substantial protection after gavage, whereas the classic methylpyridinium aldoxime, 2-PAM, is without evident protection. While further studies in other animal species are necessary, ionizing zwitterionic aldoximes present viable alternatives to existing antidotes for prophylaxis and treatment of large numbers of individuals in terrorist-led events with nerve agent organophosphates, such as sarin, and in organophosphate pesticide exposure.

1. INTRODUCTION

Treatment of pesticide and nerve agent poisoning (Dolgin, 2013) has relied on a combination of agents: antagonists to minimize excessive muscarinic stimulation affecting cardiovascular and respiratory parameters, anticonvulsant agents to avert seizures presumably initiated from central acetylcholine accumulation, and acetylcholinesterase (AChE) reactivating agents as antidotes to reduce the excessive acetylcholine levels through cholinesterase reactivation (Somani et al., 1992; Taylor, 2018). The classic reactivating agents are derivatives of a lead compound 2-PAM, a *N*-methylpyridinium 2-aldoxime, that was shown over 60 years ago to be site-directed to the then presumed active site of AChE (Wilson, 1951, 1959; Wilson and Ginsburg, 1955). These agents, while intrinsically active in reactivating the organophosphate-conjugated AChE, are limited to their peripheral activity in being unable to cross the blood-brain barrier in appreciable concentrations to reactivate central AChE (Bodor et al., 1975; Shih et al., 2012; Worek and Thiermann 2013; Harvilchuck et al., 2013). Since the organophosphates (OPs) are typically neutral molecules, and several have high vapor pressures, they easily cross the blood-brain barrier and therefore inhibit central as well as peripheral AChE. Similar to other quaternary amine congeners interacting at cholinergic target sites, quaternary antidotes are poorly and very slowly absorbed when administered orally (Somani et al., 1992; Taylor, 2018).

To circumvent these intrinsic limitations, investigators have explored other alternatives. These have included protopam, a tertiary prodrug that can cross the blood-brain barrier and through reduction *in situ* form the desired agent 2-PAM (Bodor et al., 1975), attaching 2-PAM or its analogues to various transportable sugars in which the oxime becomes the cargo (Bhonsle et al., 2013; Garcia et al., 2010), attaching extended hydrophobic moieties to classic pyridinium aldoximes (Chambers et al., 2016), small neutral oximes and other tertiary oximes or amidines that may have multiple ionization states (Rajapurkar et al., 1958; Kalisiak et al., 2011; de Koning et al., 2011; Mercey et al., 2011; Kliachya et al., 2014; Renou et al., 2013). Recognizing the importance of the limited angle of access of the nucleophile within the narrow and tortuous active center gorge, further impacted with the conjugated OP, we have developed a series of oximes, the hydroximinoacetamido alkylamines (Sit et al., 2011; Radić et al., 2012). Here the aldoxime is attached to a carbamoyl residue of small steric dimensions compared to the quaternary methylpyridinium moiety, thereby enhancing the angle of attack at the electrophilic, conjugated phosphorus. This minimizes orientation constraints on a SN_2 nucleophilic attack by the oxime or oximate in formation of a phospho-oxime and/or in cleaving the phosphonyl or phosphoryl serine bond.

The ionization equilibria of such zwitterionic molecules offer the possibility of achieving sufficient neutral species at physiological pH to cross the blood-brain barrier, as well as presenting a cationic species upon entry to the AChE active center gorge. Oximes, among the candidate nucleophiles, also present an intrinsic capacity to form an oximate anion for nucleophilic attack at the conjugated phosphorus atom. This process may be facilitated by proton abstraction in the active center gorge.

Scheme 1 shows the four species of the hydroxyiminoacetamido alkyl amines that exist in various fractional concentrations at physiological pH resulting from the protonation state of the amine and loss of a proton from the oxime.

Scheme 1. *Place HERE*

We examine here the ionization equilibria, pharmacokinetic properties, and oral bioavailability of RS194B as a lead compound in the hydroxyiminoacetamido alkylamine series. Furthermore, we designed and synthesized eleven immediate structural congeners of RS194B to rank order the respective reactivation rates. Given its favorable Antidote Index, defined by the oxime-elicited reactivation rate of OP-conjugated human AChE (hAChE) *in vitro*, relative to the LD_{50} toxicity in mice (Radić et al., 2012), we developed a profile of pharmacokinetic studies directed to RS194B effectiveness as an oral and parenteral antidote. In addition, we examine the capacity of a single dose of RS194B to reactivate blood and brain AChE and BChE in mice exposed to sub-lethal doses of sarin and VX.

2. Materials and methods

2.1. Chemicals and enzyme

Low toxicity, nonvolatile fluorescent methylphosphonates were used in all *in vitro* experiments as analogues of the methylphosphonate nerve agents: sarin, cyclosarin, and VX. These surrogate analogues differ from actual nerve agent OPs, only by the structure of their respective leaving groups (Sit et al., 2011; Radić et al., 2012). Inhibition of hAChE by these fluorescent analogues results in OP-hAChE covalent conjugates identical to the ones formed upon inhibition with the corresponding volatile, nerve agent OPs. Their lack of volatility insures greater safety in laboratory use, and the reaction can also be monitored by release of the fluorophore. Paraoxon was purchased from Sigma. Nerve agent OPs used in *in vivo* experiments were purchased from NC Laboratory (Spiez, Switzerland). 2-Pyridinealdoxime methiodide (2-PAM) was purchased from Sigma.

Our previous studies indicated that the tertiary amine containing region of the zwitterionic oxime molecule is a critical modulator of activity (Sit et al., 2011; Radić et al., 2012; Sit et al., 2014). Herein, we explored the effect of the tertiary amine ring flexibility and dimensions on the rate of reactivation of OP- inhibited AChE. Building on our lead compound, RS194B, we investigate here 11 additional derivatives (**Table 1).**

Synthetic schemes and characterizations of each derivative are detailed in the Supplement. Highly purified monomeric hAChE was prepared as described earlier (Radić et al., 2012; Sit et al., 2011).

2.2. *In vitro* oxime reactivation assays

In *in vitro* assays we employed fluorescent analogs of the toxic methylphosphonates that are non-volatile to minimize laboratory risk. They form conjugates identical to the nerve agents of congeneric structure (Radic' et al., 2012) and with similar enantiomeric ratios of the racemic nerve agent (Kovarik et al., 2004). hAChE activities were measured using a spectrophotometric assay (Ellman et al., 1961) at 37 °C in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.01 % BSA and substrate acetylthiocholine (ATCh). OP-hAChE conjugates were prepared, and oxime reactivation was performed and analyzed as described earlier (Radić et al., 2012). The dependence of reactivation rates, k_{obs}, on oxime concentrations and determination of maximal reactivation rate constant k_2, an apparent Michaelis-Menten type constant K_{ox}, and the overall second order reactivation rate constant k_r, were previously described (Kovarik et al., 2004). k_{obs} , obtained from *in vitro* reactivation of human AChE was then related to the LD_{50} in mice to obtain an Antidote Index, analogous to the Therapeutic Index, employed in efficacy rankings of pharmacologic agents.

2.3. Acute oxime toxicity and oxime treatment of OP exposed mice

Male CD-1 mice (25−30 g body weight) were purchased from Rudjer Bošković Institute, Zagreb, Croatia and maintained as previously described (Radić et al., 2012). For the experimental sequences, mice were randomly divided into groups of four. Mice were treated in accord with the approval of the Ethics Committee of the Institute for Medical Research and Occupational Health in Zagreb, Croatia.

Acute toxicity (LD_{50}) was based upon 24 h mortality rates calculated according to Thompson (1947) and Weil (1952). Antidotal activity against OP exposure was tested by intramuscularly (*i.m.*) administering oximes (at the specified dose) to male CD-1 mice, together with atropine sulfate (10 mg/kg) 1 min after subcutaneous (*s.c.*) OP administration. Alternatively, mice were pretreated orally (*p.o.*) with oximes by gavage (at the specified dose, but without atropine) 15 min before *s.c.* OP administration. Concentrated stock solutions of nerve agents were prepared in isopropyl alcohol. Further dilutions were made in saline immediately before use.

Antidotal efficacy of oximes in the intact mouse was expressed as a Protective Index (PI) with 95 % confidence limits and the maximal dose of OP (MDP). The Protective Index (PI) is the ratio of LD_{50} values between OP with RS194B with atropine and OP given alone. The Maximal Dose of OP Poison (MDP) is the highest multiple dose of the OP LD_{50} that was fully counteracted by the antidotal treatment.

2.4. Blood and brain AChE and blood BChE activity measurements

Mice were divided into five groups and administered OP and antidotes as follows: i) Saline Control: *i.m.* with saline only (2 ml/kg); ii) a & b) Separate OP Controls: *s.c.* administration with 0.75 LD_{50} dose of VX (LD_{50} = 28.3 μg/kg body weight) or 0.75 LD_{50} dose of sarin (LD_{50} = 864 μg/kg body weight); iii) a and b) *i.m.* with RS194B (125 mg/kg) plus atropine (10 mg/kg) one minute after the above s.c. OP exposure (Thierman et al, 2011). All animals were humanely euthanized at 0.5, 1, 3, or 6 h after the antidotal treatments.

Blood was drawn directly from the heart into heparinized vacutainers (Becton Dickenson, Plymouth, UK), diluted 25 times in 0.1 M Na phosphate buffer, pH 7.4, and stored over night at –20 °C. Prior to activity measurements, samples were centrifuged for 10 min at 4 °C and 2,100 x g. Brain samples were rinsed with saline, frozen in liquid nitrogen and stored at –20 °C. To prepare 40 mg/ml homogenates, brains were weighed and diluted in corresponding volume of 1% Triton X-100 in 0.1 M Na phosphate buffer, pH 7.4. After homogenization, samples were sonicated for 10 min in an ultrasonic bath filled with ice. The supernatants from 21,255 x g centrifugation for 30 min were assayed for activity promptly after preparation (Ellman at al., 1961). Final sample dilutions were 200 times for blood and 2,000

times for brain. AChE and BChE activity were determined using selective inhibitors 10 μM BW284C51 (Sigma, SAD) and 20 μM ethopropazine (Sigma, SAD), respectively as described previously (Katalinić et al., 2015).

2.5. Oxime pharmacokinetics in mice

Female CD-1 mice 4−8 weeks old (22−34 g of body weight) were purchased from Harlan (Livermore, CA). Mice were fed Purina Certified Rodent Chow #5002. Food and purified water were provided *ad libitum*. Mice were kept in hanging polycarbonate cages at 21−23 °C, exchanging light and dark cycles every 12 h. General procedures for animal care and housing were in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (1996) and the Animal Welfare Standards Incorporated in 9 CFR Part 3, 1991.

For the pharmacokinetic single dose and multiple loading and maintenance doses, mice were divided into groups of three for parenteral dosing and oral dosing by gavage. In the single dose pharmacokinetic studies, RS194B was administered by various routes in the absence of OP: *i.v.* 20mg/kg, *i.m.* 80 mg/kg, *p.o*, gavage, 50 and 200mg/kg. Three animals were injected for every time point analyzed and blood (~300 μl) was collected at each time point from the retro-orbital sinus of mice under isoflurane anesthesia into tubes containing EDTA, processed to plasma within 30 min of collection, and then stored frozen at ≤ −80 °C (±10 °C).

In the sequential loading and maintenance dose studies, mouse brains and plasma were collected and analyzed individually at each time point. Brain weight was documented for each animal before storage on dry ice. Brains were stored at ≤ –80 °C (±10 °C) until analysis. Concentration of the oxime in body compartments was determined by LC-MS using multiple reaction monitoring electrospray ionization detection in positive ion mode (Radić et al., 2012).

3. Results

3.1. *In vitro* reactivation kinetics by hydroxyimino acetamido alkyl cyclic amines.

In search for efficient reactivators with the RS194B structural scaffold, we tested *in vitro* reactivity of eleven new congeners **(Table 1)**, in addition to analogs studied previously (Sit et al., 2011; Radić et al., 2012; Kovarik et al., 2013). Several reactivators of moderate potency were identified, where the azepine ring of RS194B was replaced by bridged azatricyclo undecane (RS2-138B, RS2-150C) or azabicyclo octane (RS3-43D) rings. Substitution by aza adamantane (RS3-36D), azabicyclo octane (RS2-237D) and heptane (RS2-234D) that create larger steric bulk in the immediate vicinity of the heteroatom resulted in reduced reactivation potential due to a diminished reversible binding component (an increase in K_{ox}). Substitutions of the azepine ring (RS2-140B) or within its linker region (RS2-245C and RS2-90C) reduced reactivation parameters by compromising both, the reversible interaction (K_{ox}) and optimal approach to the reactivation target, the conjugated phosphate (reduction in k_2). The most significant compromise of reactivation resulted from steric/electronic distortion of aldoxime (RS2-292A) and alkyl (RS2-90C) substitutions. It is also evident that reactivation parameters for the oxime antidotes vary substantially with the conjugated organophosphate. Through this and prior analyses, we created four nearly equipotent, uncharged acetamido RS194B congeners as potential leads.

3.2. Reactivation capacity measured as an Antidote Index

Our previous studies comparing RS194B and RS41A with 2-PAM in terms of reactivation of human AChE had shown comparable properties in terms of reactivation rates and its two deconstructed catalytic parameters, K_{ox} and k_2, obtained from an analysis of reactivator concentration dependence against human AChE (Sit et al., 2011; Radić et al., 2012). Superiority against MINA and DAM, two other neutral oximes, was also demonstrated (**Fig. 1**).

If we relate these kinetic parameters with hAChE to the toxicity of the antidote in the mouse, the superiority of RS194B, as well as RS2-138B, becomes manifest as an Antidote Index that relates the reactivation rate constant, k_{obs}, for human AChE measured *in vitro* in relation to the LD_{50} of the antidote in the mouse (**Fig. 1**).

These parameters provide a justification for a more detailed pharmacokinetic analysis in the mouse to ascertain the rate of crossing the blood-brain barrier of the oxime, its tissue disposition, plasma lifetime and clearance within the body.

3.3 Single dose comparisons of plasma and brain levels in the mouse

In this sequence, we examine the plasma and tissue concentrations after single administrations of RS194B following *i.v., i.m.* and *p.o.* dosing (**Fig. 2**). Following *i.v.* and *i.m.* dosing, plasma levels show maxima within the first 5 min and decline thereafter with a half-life of approximately one hour. Upon oral dosing by gavage, systemic absorption also appears quite rapid, where a maximal plasma level is seen within 30 min with a similar rate of decline of plasma concentrations, as oral absorption diminishes. A previous comparison of brain and plasma levels of RS194B after *i.m.* administration shows rapid penetration into the central nervous system, where CNS levels exceed plasma levels after 30 min (Radić et al., 2012). However, the cross-over of brain and plasma values arises largely from the rapid decline of plasma concentrations with more retention in the brain. Accordingly, a comparison of plasma levels in **Figs 2** and **3**, suggests that oral administration as a maintenance dose following a parenteral loading dose affords a means of sustaining sufficiently high levels of antidote in the brain.

3.4. Loading and maintenance dose profiles

To examine antidote levels following an *i.m.* loading dose and oral maintenance dose sequence, we administered RS194B by gavage at sequential 2 hour intervals following an initial loading dose given intramuscularly (**Fig. 3**). The study required separate groups of three mice that were sacrificed immediately or 2 hours after the loading dose, but before the first oral maintenance dose. Others in the group received the loading dose followed by maintenance doses at each two hour interval. Each group of mice was sacrificed prior to the time of the projected next loading dose, while the remaining mice received the next dose in the sequence. Thus, at the final 10 hour time point, mice in this group received the loading dose, and subsequent maintenance doses at 2, 4, 6 and 8 hours and were sacrificed at 10 hours post the initial antidote exposure.

The sequential dosing study shows that within the two hour time period brain concentrations exceed the plasma concentrations, as to be expected from the previous single dose kinetic studies. Hence, if elimination approximates a first order decline after a short distribution period, then an apparent steady-state in plasma concentrations is obtained with the two hour intervals. If larger numbers of mice were used and plasma and brain samples were taken at various times after each administration, then we might anticipate a "saw tooth" appearing profile within each injection interval. Importantly, our data shown here reveal no evidence for antidote accumulation when administered successively over the 10 hour interval.

Over this interval mice were monitored for toxicity symptoms of RS194B administration, and at this dose level, we noted no gross evidence of toxicity in terms of compromised motor activity and overt symptoms. Hence, these findings warrant higher dose studies with samples to be taken from other tissues containing cholinesterase targets or presumed to be involved in the disposition of the antidote.

3.5. Antidotal efficacy of RS194B and RS2-138B as therapy or pretreatment in OP exposed mice

In order to compare oral efficacies of RS194B and 2-PAM, we administered RS194B and 2-PAM by gavage 15 min prior to VX exposure of mice and determined their Protection Indices with increasing doses of VX (**Table 2**). What is plainly evident is that protection by gavage of RS194B is substantial, whereas no protection is afforded by 2-PAM, when given by the same route in mice. We also note in this study that both 2-PAM and RS194B confer comparable protection when administered intramuscularly 1 minute post-exposure to VX, when scaled to 25% of the LD_{50} of the two oximes.

The oral efficacy of RS194B in treatment of VX exposed mice was further examined with higher dose and repetitive dosing of RS194B (**Table 3**). However, the Protective Index is only slightly higher when increasing the oxime dose to 400 mg/kg with only minimal improvement in terms of survival and lessened toxicity symptoms, when compared to dose of 200 mg/kg.

Repetitive *p.o.* dosing of RS194B (a total of 800/mg/kg) at 6, 4 and 2 hrs pre-exposure afforded less protection of mice than was the case of pre-exposure treatment with 200 mg/kg 15 min before the OP. This result is consistent with the short half-life and rapid clearance of RS194B. Oral administration of 200 mg/kg of RS194B in paraoxon, tabun and soman exposure was not as efficient as intramuscular RS194B administration, reported previously for RS194B (Radić et al., 2012).

Since *in vitro* experiments indicate RS2-138B is a potentially effective VX and sarin reactivator, we tested its antidotal efficacy in the case of different OP exposures: VX, sarin, tabun, paraoxon and soman. The acute toxicity of RS2-138B administered *i.m.* to mice was found to be relatively low (283 mg/kg), even though it appeared more toxic than RS194B (LD_{50}>500 mg/kg; Radić et al., 2012). Nevertheless, we tested the efficacy of oxime RS2-138B as therapy or pretreatment in the case of OP exposed mice with a dose roughly equivalent to 25 % of RS2-138 LD_{50}. It was evident that mice recovered better if pretreated with oxime RS2-138B (*i.m.*) 15 min before OP (*s.c.*) and received therapy (RS2-138B plus atropine, *i.m.*) 1 min after OP exposure (**Table 4**). However, the lower acute toxicity and

higher Protective Indices determined for *i.m.* administered RS194B (Radić et al., 2012) justify its choice for further *in vivo* studies on mice. RS2-138B can be considered as a back-up, zwitterionic compound.

3.6. Blood and brain cholinesterase activity in sarin and VX exposed mice upon administration of RS194B

Mice in separate groups of four were exposed to: i) sub-lethal doses of sarin or VX (0.75 LD_{50}, *s.c.*); ii) to sub-lethal doses of sarin or VX (0.75 LD_{50}, *s.c.*) and treated with RS194B (125 mg/kg in 10 mg/kg atropine) administered *i.m.* 1 min after *s.c.* OP exposure, and iii) control group was treated with saline only (2 mL/kg, *i.m.*). The AChE and BChE activity was monitored in whole blood and brain 0.5, 1, 3 and 6 h after sarin or VX exposure. Although all mice survived the poisoning, symptoms and their intensity differed in regards to OP. Mice exposed to VX showed only mild symptoms of poisoning (mice were calm), while mice exposed to sarin exhibited immediate moderate to severe tremors that last up to 2 h after exposure. Later the mice were very calm, but exhibited continued tachycardia. Symptoms were attenuated if mice were treated with oxime RS194B. This difference in symptoms between VX and sarin can be assigned to different physical and chemical properties of sarin and VX (John et al., 2015) and their tissue dispositions, as reported by Shih et al. (2005, 2010). Indeed, blood AChE and BChE activity were significantly inhibited by both OPs, while AChE in brain was more inhibited by sarin than by VX.

The effect of the RS194B oxime in terms of the AChE/BChE activity in whole blood and brain is shown in **Fig 4**. Despite large variability, it seems that recovery of brain activity by RS194B in mice exposed to sarin or even VX was evidence for its fast distribution to tissues and efficient blood-brain barrier penetration. The efficacy of antidotal therapy was most evident in the brain of mice exposed to sarin with the peak in the 1 h group. The obtained results are in accordance with RS194B half-life of 1 h of less in plasma, and brain levels exceeding those in plasma after 40 min (Radić et al., 2012), staying elevated for longer time periods. RS194B reactivation was not evident in other peripheral tissues studied (data not shown). A similar phenomenon was reported in a study on tabun exposed rats where significant AChE reactivation with a pyridinium oxime was observed only in whole blood (Katalinić et al., 2015).

4. Discussion

These studies with RS194B and related hydroxyiminoacetamido alkylamines provide justification for further investigations of zwitterionic oxime antidotes in the treatment of OP exposure. The superior efficacy of these hydroxyiminoacetamido alkyl amines may arise from multiple pharmacodynamic and pharmacokinetic considerations that are applicable to antidotes, the principles of which have been shown for candidate therapeutic agents. First, we note that a comparison of Antidote Indices, **Fig. 1**, shows that when reactivation rates are scaled to toxicity of the respective antidotes, the values for RS194B exceed that for the 2-PAM standard in mice. The compounds have roughly comparable reactivation rates for human AChE with the various organophosphate conjugates, but the toxicity of 2-PAM in the mouse is 104 mg/kg, whereas for RS194B, it is greater than 500 mg/kg (Radić et al., 2012).

When antidote efficacy is analyzed in terms of intrinsic activity of the antidote at the target site, several factors are likely to come into play. First, the quaternary ligands, such as 2-PAM, may be favored in terms of their affinity for the active center over a tertiary amine that exists in association with H_2O as a hydronium ion or in hydrogen bonding. Hence, binding or association within the active center gorge is likely reflected in the parameter K_{ox}, an apparent dissociation constant. However, in the case of a nucleophilic reaction at the phosphorus atom in the conjugated alkyl phosphonate or phosphorate, orientation of the bound ligand positioning the oximate nucleophile may become the critical factor. Amongst the aldoximes, the attached methylpyridinium ring is far larger than the proximal carbamoyl group in our zwitterionic oximes. Hence, within the spatially impacted active center gorge, a smaller molecular moiety proximal to the aldoxime that has electron withdrawing characteristics may be preferred. An extensive analysis of various oximes and in particular those of the hydroxyiminoacetamido alkylamine series, shows a wide variation in the deconstructed reactivation parameters, K_{ox} and k_2, when examined for alkylphosphonates and phosphorates of different structure (Radić et al., 2012). Among the enantiomeric R_p and S_p methylphosphonates, substantial differences exist between enantiomers for reactivation (Wong et al., 2000; Kovarik et al., 2003, 2004). Thus, the more reactive organophosphate enantiomer is also the one with the highest propensity for reactivation, implying a similarity in transition states associated with organophosphate conjugation and oxime or general base catalyzed hydrolysis of the cholinesterase conjugate.

A second parameter of efficacy that arises relates to achieving pharmacokinetics and tissue disposition that favor the zwitterionic oximes, such as RS194B and RS2-138B, over the quaternary oximes, 2-PAM and its congeners such as MMB4 and HI-6. Here several factors, both known or predictable and unknown, come into play. OP compromise of

respiratory and cardiovascular parameters arises from *both* central and peripheral inhibition of AChE activity and enhanced acetylcholine concentrations at synapses (Buccafusco, 1996; Edery et al., 1986). Moreover, oximes can reverse the actions of locally applied OP's in the ventral (rostral) medulla oblongata (Edery et al., 1986). OP's are hydrophobic compounds; some, such as sarin, have high vapor pressure and would be expected to cross the blood-brain barrier rapidly, exhibiting acute toxicity. They may also become sequestered at or partition into hydrophobic sites, where over time, they may redistribute from these storage sites back into the circulation. Accordingly, an antidote with the potential to cross the blood-brain barrier rapidly would not only reactivate the immediately exposed and inhibited OP-sensitive sites in the CNS, but should also prevent secondary inhibition. Hence, there may be a justification for longer term reactivation potential of an oxime antidote that is retained in brain. Multiple ionization states, a capacity to be retained in tissue, a larger Volume of Distribution and prolonged oral absorption may favor less frequent repeat dosing of the antidote.

Several pharmacokinetic studies have been reported for 2-PAM and the more extended pyridinium structures, such as MMB4 and HI-6 (Sidell and Groff, 1971; Baggot et al., 1993; Hong et al., 2013). Typically small Volumes of Distribution (0.3 to 0.5 L/kg) and short half-lives (~30 min) are evident. This likely arises from appreciable glomerular filtration and renal tubular secretion of the quaternary amine (Swartz and Sidell, 1974). Thus, antidotes retained within tissues with larger Volumes of Distribution may carry a pharmacokinetic preference.

A final question relates to the fractional distribution of species at physiological pH. In the case of RS194B, its p*K*a values for the protonation of the amine and dissociation of a proton from the oxime forming the oximate are nearly equivalent at 8.8 and 8.9 (Radić et al., 2012). Approximately equal fractions of neutral species and zwitterion exist at physiological pH values, and one can ask if the non-ionized species population at pH 7.4 is sufficient to meet the pharmacokinetic and pharmacodynamic criteria. In terms of transfer across the blood-brain barrier, the enlarged potential surface area of the blood-brain barrier should allow crossing of the neutral species at a rate sufficient to account for the kinetics of CNS accumulation seen in **Fig. 2**. RS194B shows transfer into and retention in the brain that is superior to RS41A despite its difference only in ring size of the pyrrole and azepine rings. Although it has yet to be measured, RS2-138B with its additional methylene groups, may even show greater brain retention. Further analysis should also determine whether carrier mediated transport processes, efflux from the brain and/or biliary or renal transport are critical factors governing the plasma pharmacokinetics and tissue disposition.

Identifying efficacious antidotes capable of reactivating OP-inhibited AChE in the central and peripheral nervous systems has proven elusive, despite many years of intense effort. RS194B, when compared to several pyridinium aldoximes oximes, did not show high antidote activity, when organophosphates were administered subcutaneously in the guinea pig (Wilheim et al. 2014). Recent studies have demonstrated that a lethal dose of inhaled sarin vapor and paraoxon aerosol in macaques, resulted in a sequelae of symptoms very similar to insecticide poisoned humans exposed, either by inhalation or ingestion, and that these symptoms could be rapidly reversed by intramuscular administration of a centrally acting oxime RS194B (Rosenberg et al. 2017a,b). Herein, our studies in mice and macaques, show that RS194B has the pharmacokinetic and reactivating capabilities, enabling potentially effective antidotal actions for acute exposure to a volatile organophosphate. Subsequent oral dosing offers the potential of sustained antidotal action for resdistributed OP or other thioate OPs pesticides that are slowly converted to the active oxons.

Authorship Contributions:

Participated in research design: Sit, Kovarik, Green, Fokin, Radić, Sharpless, Taylor

Conducted experiments: Sit, Kovarik, Maček-Hrvat, Žunec, Green, Fokin, Radić

Performed data analysis: all authors

Contributed to writing of the manuscript: Sit, Kovarik, Maček-Hrvat, Radić, Sharpless, Taylor

References

Baggot JD, Buckpitt A, Johnson D, Brennan P, and Chung H (1993) Bioavailability and disposition kinetics of HI-6 in Beagle dogs. *Biopharm Drug Dispos* **14**(2): 93-105.

Bhonsle JR, Causey R, Oyler B, Bartolucci C, Lambda D, Pesaresi A, Bhamare NK, Soojhawon I, and Garcia GE (2013) Evaluation and computational characterization of the facilitated transport of Glc carbon C−1 oxime reactivators across a blood brain barrier. *Chem Biol Interact* **203**: 129-134.

Bodor N, Shek E, and Higuchi T (1975) Delivery of a quaternary pyridinium salt across the blood-brain barrier by its dihydropyridine derivative. *Science* **190**: 155-156.

Buccafusco JJ (1996) The role of central cholinergic neurons in the regulation of blood pressure and in exerperimental hypertension. *Pharmacol Rev* **48**: 179-211.

Chambers JE, Chambers HW, Funck KE, Meek EC, Pringle RB, and Ross, MK (2016) Efficacy of novel phenoxyalkyl pyridinium oximes as brain-penetrating reactivators of cholinesterase inhibited by surrogates of sarin and VX. *Chem Biol Interact* **259**: 154-159.

de Koning MC, van Grol M, and Noort D (2011) Peripheral site ligand conjugation to a non -quaternary oxime enhances reactivation of nerve agent-inhibited human acetylcholinesterase. *Toxicol Lett* **206**: 54-59.

Dolgin E (2013) Syrian gas attack reinforces need for better anti-sarin drugs. *Nat Med* **19**:1194-1195.

Edery H, Geyer M, Taylor P, and Berman HA (1986) Target sites for anti-acetylcholinesterase on the ventral surface of the medulla oblongata: Hypotension elicited by organophosphate agents. *J Autonom Pharmacol* **6**: 195-205.

Ellman GL, Courtney KD, Andres Jr. V, and Featherstone RM (1961) New and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7**: 88-95.

Garcia GE, Campbell AJ, Olson J, Moorad-Doctor D, and Morthole VI (2010) Novel oximes as blood −brain barrier penetrating cholinesterase reactivators. *Chem Biol Interact* **187**: 199-206.

Harvilchuck JA, Hong SP, Richey JS, Osheroff MR, and Johnson JD (2013) In vivo acetylcholinesterase reactivation in male guinea pigs and Rhesus macaques following cyclosarin exposure and treatment with 1,1’ −methylenebis{4 −(hydroxylimino)methyl] pyridinium} dimethanesulfonate. *Int J Toxicol* **32**: 99S-107S.

Hong SP, Gibbs ST, Kobs, DJ, Osheroff, MR, Johnson JD, and Burback BL (2013) Pharmacokinetics of MMB4 DMS in rats, rabbits and dogs following a single IV administration. *Int J Toxicol* **32**: 30S−37S.

John H, Balszuweit F, Kehe K, Worek F, and Thiermann H (2015) Toxicokinetic Aspects of Nerve Agents and Vesicants, 2nd ed, in Handbook of Toxicology of Chemical Warfare Agents (Gupta RC ed) pp 817-856, Academic Press, Boston.

Kalisiak J, Ralph EC, Zhang J, and Cashman J (2011) Amidine−oximes: reactivators for organophosphate exposure. *J Med Chem* **54**: 3319-3330.

Katalinić M, Maček Hrvat N, Zdarova Karasová J, Misik J, and Kovarik Z (2015) Translation of in vitro to in vivo pyridinium oxime potential in tabun poisoning. *Arh Hig Rada Toksikol* **6**: 291-298.

Kliachya M, Santoni G, Nussbaum V, Renou J, Sanson B, Colletier JP, Arboléas M, Loiodice M, Weik M, Jean L, Renard PY, Nachon F, and Baati R (2014) Design, synthesis and biological evaluation of novel tetrahydro pyridine −aldoxime and amidooxime hybrids as efficient uncharged reactivators of nerve agent inhibited human acetycholinesterase. *Eur J Med Chem* **78**: 455-467.

Kovarik Z, Radić Z, Berman HA, Simeon−Rudolf V, Reiner E, and Taylor P (2003) Acetylcholinesterase active centre and gorge conformations analysed by combinatorial mutations and enantiomeric phosphonates. *Biochem J* **373**: 33-40.

Kovarik Z, Radić Z, Berman HA, Simeon-Rudolf V, Reiner E, and Taylor, P (2004) Mutant cholinesterases possessing enhanced capacity for reactivation of their phosphonylated conjugates. *Biochemistry* **43**: 3222-3229.

Kovarik Z, Maček N, Sit R K, Radić Z, Fokin VV, Sharpless, KB, and Taylor P (2013) Centrally acting oximes in reactivation of tabun-phosphoramidated AChE. *Chem Biol Interact* **203**: 77-80.

Mercey G, Verdelet T, Saint −André G, Gillon E, Wagner A, Baati R, Jean L, Nachon F, and Renard PY (2011) First efficient uncharged reactivators for the dephosphylation of poisoned human acetylcholinesterase. *Chem Commun* **47**: 5295-5297.

Radić Z, Sit RK, Kovarik Z, Berend S, Garcia E, Zhang L, Amitai G, Green C, Radić B, Fokin VV, Sharpless KB, and Taylor P (2012) Refinement of structural leads for centrally acting oxime reactivators of phosphylated cholinesterases. *J Biol Chem* **287**: 11798-11809.

Rajapukar MV, Koelle GB, and Smart P (1958) Reactivation of DFP-inactivated acetylcholinesterase by mononitrosoacetone and dacetylmonoxome in vivo. *J Pharmacol Exp Ther* **123**: 247-253.

Renou J, Mercey G, Verdelet T, Puanescu E, Gillon E, Arboléas M, Loiodice M, Kliachyna M, Baati R, Nachon F, Jean L, and Renard PY (2013) Synthesis and in vitro evaluations of uncharged reactivators for human acetylcholinesterase inhibited by organophosphorus agents. *Chem Biol Interact* **203**: 81-84.

Rosenberg YJ, Mao L, Jiang X, Lees J, Zhang L, Radić Z, and Taylor P (2017a) Post-exposure treatment with the oxime RS194B rapidly reverses early and advanced symptoms in macaques exposed to sarin vapor. *Chem Biol Interact* **274**: 50-57.

Rosenberg YJ, Wang J, Ooms T, Rajendran N, Mao L, Jiang X, Lees J, Urban L, Momper JD, Sepulveda Y, Shyong, YJ, and Taylor P (2017b) Post-exposure treatment with the oxime RS194B rapidly reactivates and reverses advanced symptoms of lethal inhaled paraoxon in macaques. *Toxicol Lett* (in press) doi: 10.1016/j.toxlet.2017.10.025.

Shih T-M, Kan, RK, and McDonough JH (2005) In vivo cholinesterase inhibitory specificity of organophosphorus nerve agents. *Chem Biol Interact* **157-158**: 293-303.

Shih T-M, Koplovitz I, Kan RK, and McDonough JH (2012) In search of an effective in vivo reactivator for organophosphorus nerve agent −inhibited acetylcholinesterase in the central nervous system. *Adv Stud Biol* **10**: 451-478.

Shih T-M, Skovira JW, O'Donnell JC, and McDonough JH (2010) In vivo reactivation by oximes of inhibited blood, brain and peripheral tissue cholinesterase activity following exposure to nerve agents in guinea pigs. *Chem Bio Interact* **187**: 207-214.

Sidell FR and Groff WA (1971) Intramuscular and intravenous administration of small doses of 2-pyridinium aldoxime methiodide to man. *J Pharm Sci* **60**: 1224-1228.

Sit RK, Fokin VV, Amitai G, Sharpless KB, Taylor P, and Radić Z (2014) Imidazole aldoximes effective in assisting catalysis of organophosphate detoxification. *J Med Chem* **57**: 1378-1389.

Sit RK, Radić Z, Gerardi V, Zhang L, Garcia E, Katalinić M, Amitai G, Kovarik Z, Fokin VV, Sharpless KB, and Taylor P (2011) New structural scaffolds for centrally acting oxime reactivators of phosphylated cholinesterases. *J Biol Chem* **286**: 19422-19430.

Somani SM, Solana RP, and Dube SN (1992) Toxicodynamics of nerve agents, in Chemical warfare agents pp 67-123, Academic Press, New York.

Swartz RD and Sidell, FR (1974) Renal tubular secretion of pralidoxime in man. *Proc Soc Exp Biol Med* **146**: 419-424.

Taylor P (2018) Anticholinesterase agents, in Goodman & Gilman's The Pharmacological Basis of Therapeutics, 12th ed. (Brunton LL, ed) pp. 239-254, McGraw−Hill, New York.

Thiermann H, Steinritz D, Worek F, Radtke M, Eyer P, Eyer F, Felgenhauer N, and Zilker T (2011) Atropine maintenance dosage in patients with severe organophosphate pesticide poisoning. *Toxicol Lett* **206**: 77-83.

Thompson WR (1947) Use of moving averages and interpolation to estimate median-effective dose. *Bacteriol Rev* **11**: 115-145.

Weil CS (1952) Tables for convenient calculation of median-effective dose (LD50 or ED50) and instruction in their use. *Biometrics* **8**:249-263.

Wilheim CM, Snider TH, Babin MC, Jett DA, Platoff GE, and Yeung DT (2014) A comprehensive evaluation of the efficacy of leading oxime therapies in guinea pigs exposed to organophosphorus chemical warfare agents or pesticides. *Toxicol Appl Pharmacol* **281**: 254-265.

Wilson IB (1951) Acetylcholinesterase XI: Reversibility of tetraethylpyrophosphate inhibition. *J Biol Chem* **190**: 110-117.

Wilson IB (1959) Molecular complementarity and antidotes for alkylphosphate poisoning. *Fed Proc* **18**: 752-758.

Wilson IB and Ginsburg S (1955) A powerful reactivator of alkyl phosphate inhibited acetylcholinesterase. *Biochim Biophys Acta* **18**: 168-170.

Wong L, Radić Z, Brüggemann RJM, Hosea N, Berman HA, and Taylor P (2000) Mechanism of oxime reactivation of acetylcholinesterase analyzed by chirality and mutagenesis. *Biochemistry* **39**: 5750-5757.

Worek F and Thiermann H (2013) The value of novel oximes for treatment of poisoning by organophosphosphorus compounds. *Pharmacol Ther* **139**: 249-259.

Conflict of Interest

None.

Footnotes:

This work was supported by CounterACT Program, National Institutes of Health Office of the Director and National Institutes of Health [grants U01 NS 5058048 (to PT) and 1R21 NS084904] (to ZR) from the NINDS, and by Croatian Science Foundation [grant 4307] (to ZK).

Valery V Fokin - Present Address: The Bridge@USC, University of Southern California, Los Angeles, CA 90089, USA

Figure Legends:

Fig 1: *(Top Panels)* Rates of reactivation of (from left to right) paraoxon, sarin, cyclosarin, and VX inhibited human AChE measured as a function of the respective oxime concentration: 2-PAM (blue), RS194B (Red), RS2-138B, (Black), RS 41A (Green); MINA monoisonitrosoacetone (light grey), DAM-diacetylmonoxime (light grey) (Rajapurkar et al., 1958). *(Bottom Panels*) Calculation of an Antidote Indices comparing the *in vitro* rates of reactivation of recombinant human AChE conjugated with the specified organophosphates with the toxic doses (LD_{50}) of the respective oximes.

Fig. 2: Plasma levels after single dose administration of RS194B to mice by intravenous, intramuscular and oral (gavage) routes: *i.v.* 20 mg/kg (inverted triangles); *i.m.* 80 mg/kg (open squares); *p.o.* 50 mg/kg (filled circles); *p.o.* 200 mg/kg (open circles). Measurements were made at 5, 10, 15, 30, 60, 90, 120,180 and 240 min thereafter in separate groups of three mice.

Fig. 3: Plasma (open circles) and whole brain (solid circles) pharmacokinetics of RS194B in mice following an *i.m.* loading dose of 220 mg/kg and 2 hour subsequent oral maintenance doses of 110 mg/kg by gavage. Concentrations (μM) are measured with respect to plasma volume and brain weight. An apparent steady-state is reached with repetitive dosing out to 10 hours.

Fig. 4. The effects of oxime RS194B (*i.m.*) on AChE/BChE activities in whole blood and brain of sarin- or VX-exposed mice at 0.5, 1, 3 and 6 h after treatment. Activities are expressed as median and interquartile range of four values obtained for each mouse and sample, and compared to the control group (mean 100% value). Administration in mice was conducted as follows: The Control Group received 2 ml/kg saline (*i.m.*); Open squares, mice were exposed to 0.75 LD_{50} of sarin or VX (*s.c.*); Solid circles, mice were exposed to the same dose of sarin or VX and one min after exposure to OP received RS194B (125 mg/kg with atropine 10 mg/kg, *i.m.*). Statistical significance between mice that received RS194B and atropine therapy was determined using t-tests (two-sided, with 95% confidence interval). Differences were considered significant when $p<0.05$ (*), $p<0.005$ (**), and $p<0.001$ (***).

Table 1. Kinetic parameters for reactivation of various OP conjugates with human AChE. Rates of reactivation were studied as a function of oxime concentration and kinetic parameters, K_{OX} and k_2 were calculated from the concentration dependencies; k_r is the ratio of k_2 and K_{OX}. [1]Data from Radić *et al*., 2012.

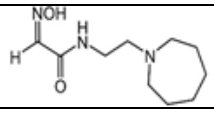
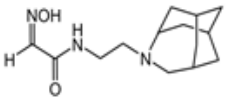
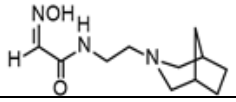
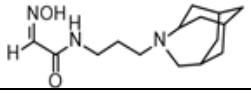
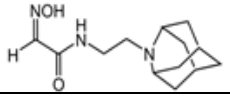
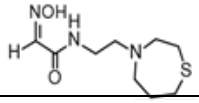
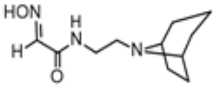
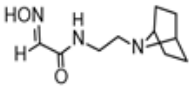
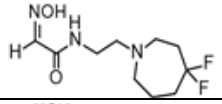
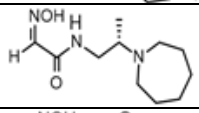
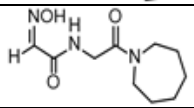
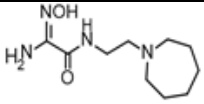
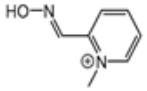
Oxime		OP	k_2 (min^{-1})	K_{OX} (mM)	k_r ($M^{-1}min^{-1}$)
RS194B[1]		VX	3.1	2.1	1600
		Sarin	2.5	1.9	1300
		Cyclosarin	0.88	3.9	230
		Paraoxon	0.38	7.4	51
RS138B		VX	2.6	1.6	1600
		Sarin	2.4	1.2	2000
		Cyclosarin	0.83	6.8	120
		Paraoxon	0.23	2.2	100
RS3-43D		VX	1.6	1.3	1200
		Sarin	1.1	0.8	1400
		Cyclosarin	0.63	3.3	190
		Paraoxon	0.22	11	19
RS2-150C		VX	-	-	≤ 1600
		Sarin	-	-	≤ 2000
		Cyclosarin	-	-	≤ 30
		Paraoxon	-	-	≤ 70
RS3-36D		VX	6.7	7.7	870
		Sarin	>10	>10	820
		Cyclosarin	0.69	5.9	120
		Paraoxon	0.71	29	25
RS2-148B		VX	-	-	≤ 360
		Sarin	-	-	≤ 2000
		Cyclosarin	-	-	≤ 30
		Paraoxon	-	-	≤ 8
RS2-237D		VX	2.1	3.6	570
		Sarin	2.9	4.9	590
		Cyclosarin	-	-	89
		Paraoxon	0.15	5.2	29
RS2-234D		VX	3.2	6.2	520
		Sarin	1.7	1.8	940
		Cyclosarin	0.79	15	51
		Paraoxon	0.14	4.6	30
RS2-140B		VX	-	-	≤ 300
		Sarin	-	-	≤ 600
		Cyclosarin	-	-	≤ 30
		Paraoxon	-	-	≤ 8
RS2-245C		VX	0.5	4.7	110
		Sarin	0.41	4.1	100
		Cyclosarin	0.47	9.5	49
		Paraoxon	0.04	5.9	7
RS2-90C		VX	-	-	≤ 1
		Sarin	-	-	≤ 1
		Cyclosarin	-	-	≤ 1
		Paraoxon	-	-	≤ 1
RS2-292A		VX	-	-	≤ 1
		Sarin	-	-	≤ 1
		Cyclosarin	-	-	≤ 1
		Paraoxon	-	-	≤ 1
2PAM[1]		VX	0.73	0.3	2400
		Sarin	1.1	0.34	3200
		Cyclosarin	0.73	6.6	110
		Paraoxon	0.27	1.8	150

Table 2. Protective Index determined in mice for RS194B and 2-PAM after oral (*p.o.*) administration by gavage in comparison to intramuscular (*i.m.*) administration. Atropine (10 mg/kg) was administered *i.m.* 1 min after VX. Increasing doses of VX were administered in groups of four mice and fractional survival was measured at each OP dose.

Oxime	Dose	Protective Index (confidence limits)	
		1 min after VX	15 min before VX
RS194B	125 mg/kg (*i.m.*)	18 (12.4–25.7)	–
	200 mg/kg (*p.o.*)	–	40 (17.2–93)
2-PAM	26 mg/kg (*i.m.*)	9.3 (7.3–13)	–
	42 mg/kg (*p.o.*)	–	1.1 (–)

Table 3. Protection of OP exposed mice by orally administered RS194B either before or after OP (*s.c.*). The RS194B oxime was administered either as single or multiple dose at indicated times. Atropine (10 mg/kg) was always administered 1 min after OP. Increasing doses of OP were administered in groups of four mice and fractional survival was determined at each OP dose. Protective Index is the ratio of LD_{50} for treated OP-exposed animals and LD_{50} for animals given OP alone. Maximal dose of poison (MDP), a highest multiple dose of OP LD_{50} fully counteracted by the treatment, is given in parentheses.

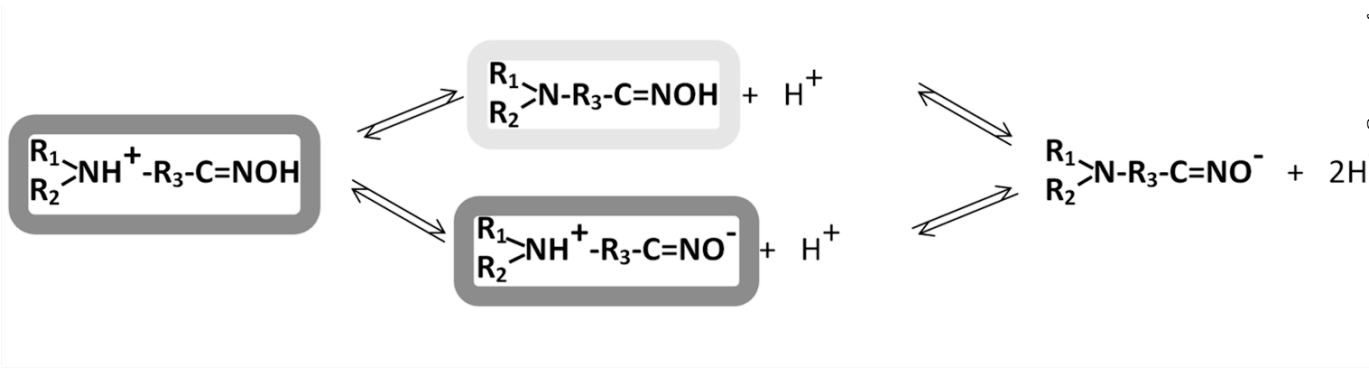
OP	RS194B (mg/kg)	Pretreatment before OP	Therapy 1 min after OP	Protective Index (MDP)
VX	400 + 200 + 200	@ 6h, 4h, 2h	*i.m.* atropine	7.9 (<6.3)
	200	@ 15 min	*i.m.* atropine	40.0 (20.0)
	400	@ 15 min	*i.m.* atropine	43.3 (31.8)
	200	none	*p.o.* RS194B + *p.o.* atropine	3.2 (<2.5)
	200	none	*p.o.* RS194B + *i.m.* atropine	3.2 (2.5)
Paraoxon	200	@ 15 min	*i.m.* atropine	7.3 (5.0)
Tabun	200	@ 15 min	*i.m.* atropine	3.4 (1.6)
Soman	200	@ 15 min	*i.m.* atropine	n.d.[1]

[1]not determined – the survival was 1/4, 0/4, 0/4 and 0/4 on 1, 1.26, 1.59 and 2.0 x LD_{50} of soman.

Table 4. Protection of OP exposed mice by RS2-138B administered *i.m.* either before or after, or both before and after OP (*s.c.*). Atropine (10 mg/kg) was administered *i.m.* 1 min after OP. Increasing doses of OP were administered in groups of four mice and fractional survival was determined at each OP dose. LD_{50} *i.m.* of RS138B was 238.3 mg/kg. The Protective Index is the ratio of LD_{50} for treated OP exposed animals and LD_{50} for animals given OP alone. Maximal Dose of Poisoning (MDP), a highest multiple dose of OP LD_{50} fully counteracted by the treatment, is given in parentheses.

OP	RS2-138B (mg/kg)	Pretreatment 15 min before OP	Therapy 1 min after OP	Protective index (MDP)
VX	60	none	RS2-138B + atropine	10.8 (7.9)
	60 + 60	RS2-138B	RS2-138B + atropine	31.8 (25.2)
	60	RS2-138B	None	3.4 (2.5)
Sarin	60	none	RS2-138B + atropine	1.6 (1.3)
	60 + 60	RS2-138B	RS2-138B + atropine	4.0 (3.2)
Tabun	60	none	RS2-138B + atropine	2.0 (1.6)
	60 + 60	RS2-138B	RS2-138B + atropine	2.7 (2.0)
Paraoxon	60	none	RS2-138B + atropine	5.4 (4.0)
	60 + 60	RS2-138B	RS2-138B + atropine	12.6 (<7.9)
Soman	60	none	RS2-138B + atropine	n.d.[1] (n.d.)
	60 + 60	RS2-138B	RS2-138B + atropine	n.d.[2] (n.d.)

[1]not determined – the survival was 1/4, 0/4, 0/4 and 0/4 on 1, 1.26, 1.59 and 2.0 x LD_{50} of soman.
[2]not determined – the survival was 0/4, 0/4, 0/4 and 0/4 on 1, 1.26, 1.59 and 2.0 x LD_{50} of soman.



Scheme 1. Ionization equilibria of amino oximes with two ionizing groups proceed through formation of a neutral or zwitterionic species. CNS penetrant species is highlighted in light grey and dominant OP-AChE binding and catalytic species are highlighted in grey.

Figure 1

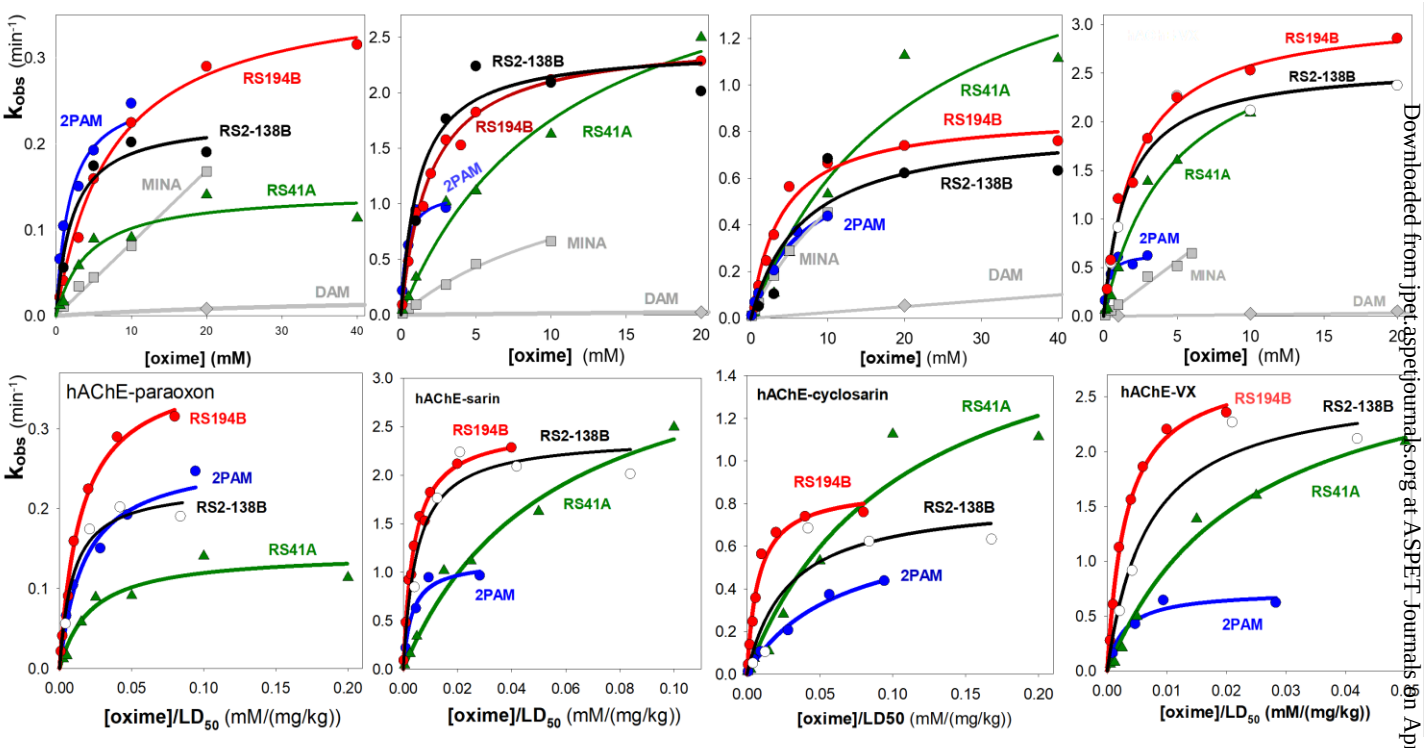


Figure 2

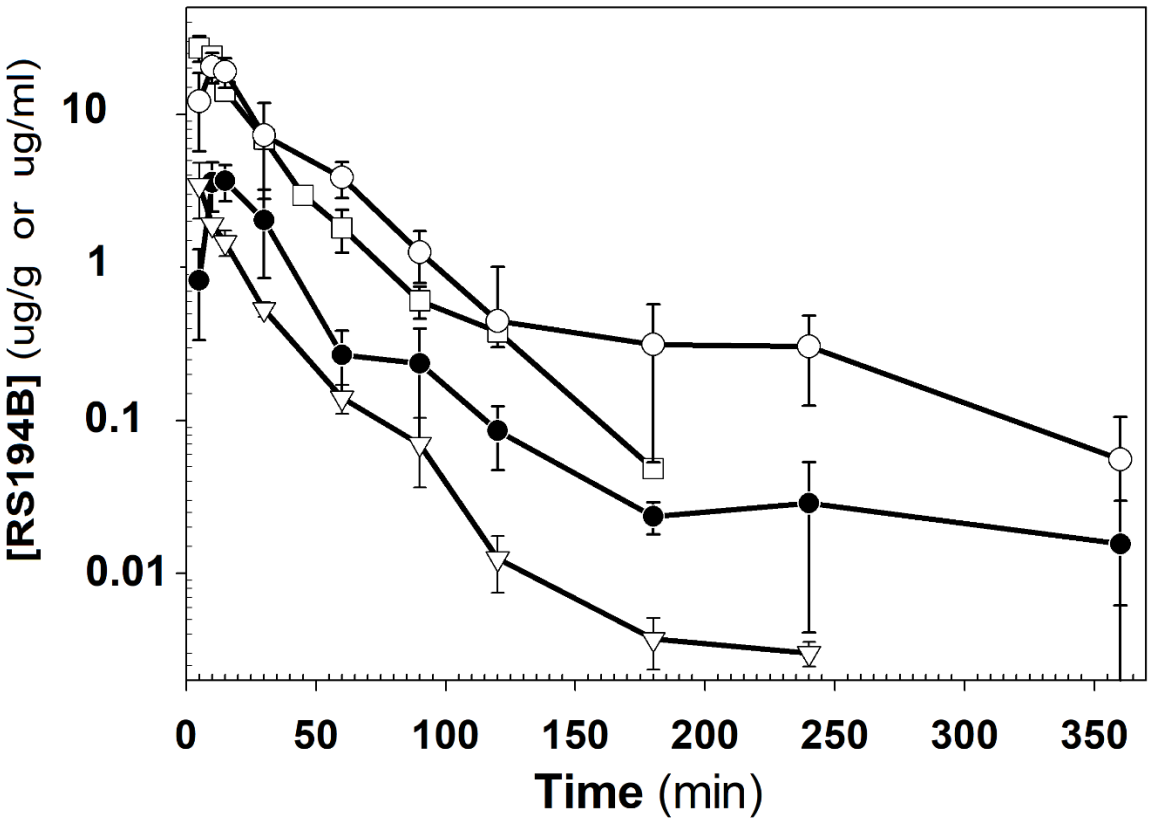


Figure 3

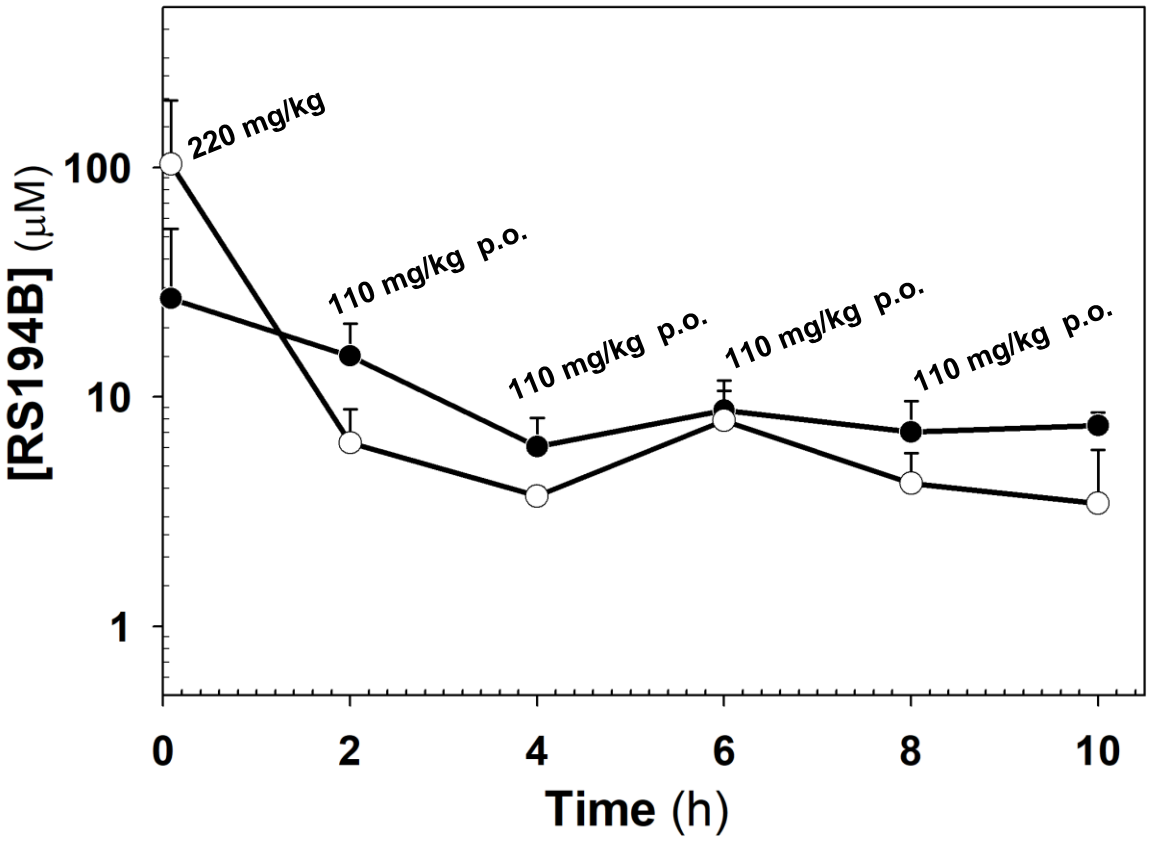
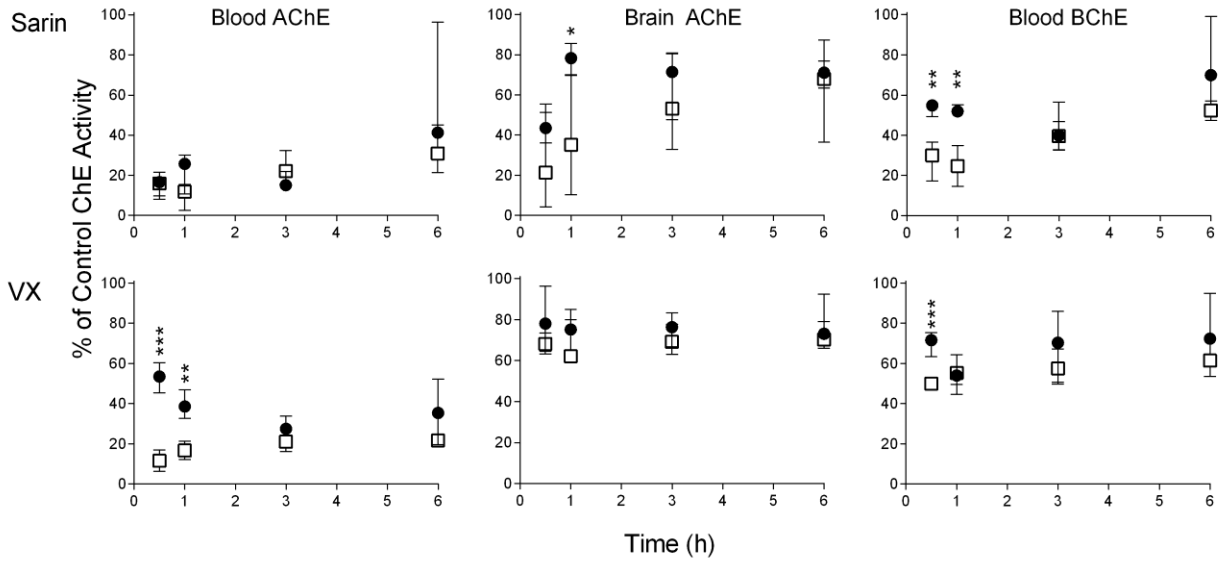


Figure 4



The Journal of Pharmacology and Experimental Therapeutics

SUPPLEMENTAL DATA

Pharmacology pharmacokinetics and tissue disposition of zwitterionic hydroxyiminoacetamido alkylamines as reactivating antidotes for organophosphate exposure

Rakesh K. Sit, Zrinka Kovarik, Nikolina Maček Hrvat, Suzana Žunec, Carol Green, Valery V. Fokin, K. Barry Sharpless, Zoran Radić and Palmer Taylor

Affiliations:

Skaggs Institute for Chemical Biology, The Scripps Research Institute, San Diego, CA 92037: RKS, VVF, KBS

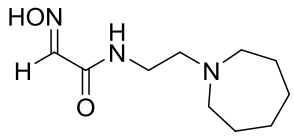
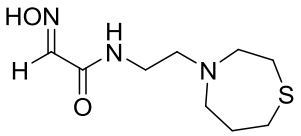
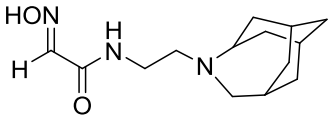
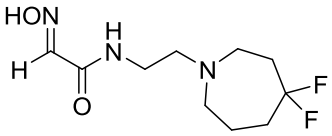
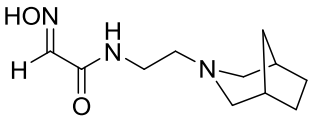
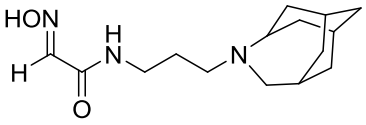
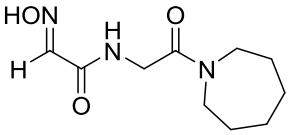
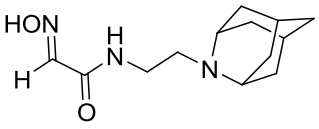
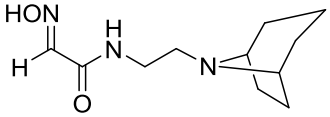
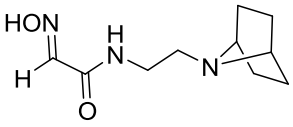
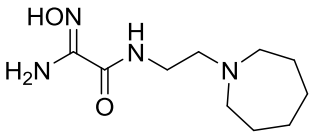
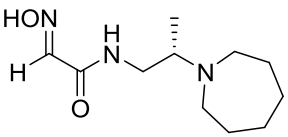
Institute for Medical Research and Occupational Health, HR−10001, Zagreb, Croatia: ZK, NMH, SŽ

SRI International, Menlo Park, CA 94025−3493: CG

Department of Pharmacology, Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093−0650: ZR, PT

Synthetic Chemistry Supplement

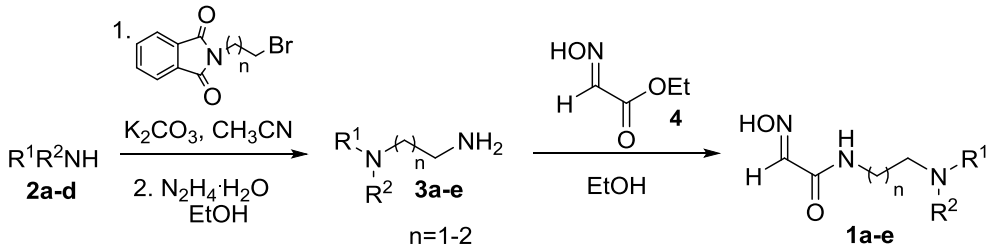
Our previous studies have indicated that the tertiary amine containing region of the oxime molecule is a critical modulator of activity. Herein we explored the effect of the tertiary amine size on the rate of reactivation towards organophosphate inhibited acetylcholinesterase. Uncharged reactivators demonstrate the ability to cross the blood brain barrier and are a logical step towards improving existing treatments for organophosphate poisoning. Building on our lead compound, **RS194B**, we demonstrate here 11 additional derivatives (**1a-k**).

RS194B	RS2-148B (1a)	RS2-138B (1b)
		
RS2-140B (1c)	**RS3-43D (1d)**	**RS2-150C (1e)**
		
RS2-90C (1f)	**RS3-36D (1g)**	**RS2-237D (1h)**
		
RS2-234D (1i)	**RS2-292A (1j)**	**RS2-245C (1k)**
		

By use of previous protocol, *N*-substituted 2-hydroxyiminoacetamides **1a-e** were synthesized, as shown in scheme 1. The synthesis begins by reacting the pthalimide protected amino alkyl bromide with the desired secondary amine (**2a-d**) to yield the protected

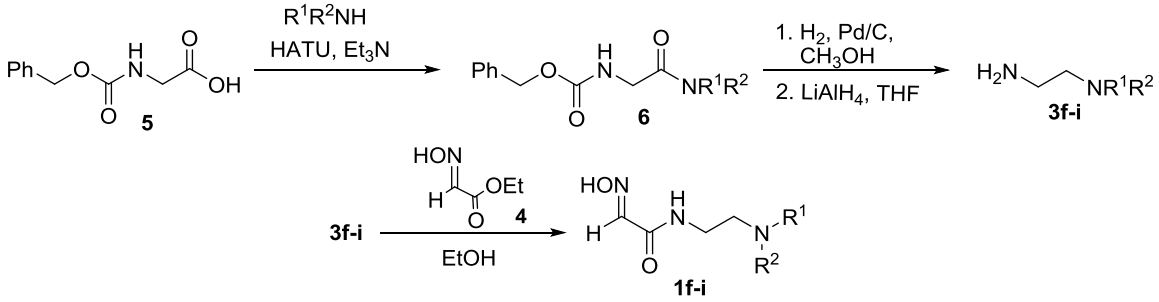
intermediates. In the next step, deprotection of the intermediate using hydrazine reveals the desired amine (**3a-e**) in moderate yield, and followed by subsequent amidation with ethyl glyoxylate oxime **4** delivers oxime derivatives **1a-e**.

Scheme 1



Furthermore, to obtain oxime derivatives **1f-i** the corresponding primary amines **3f-i** were first synthesized following a different route, shown in scheme 2. In the first step the acid chloride reacts with glycine under Schotten–Baumann conditions to form N-protected glycine **5**. Next, carboxamidation of **5** with desired secondary amines (**2e-h**) was done to obtain carboxamide **6**. Removal of Cbz group of **6**, and followed by reduction with $LiAlH_4$ in THF gave primary amines **3f-i**. Amidation of ethyl glyoxylate oxime **4** with corresponding primary amines provided acetamide oxime derivatives **1f-i**.

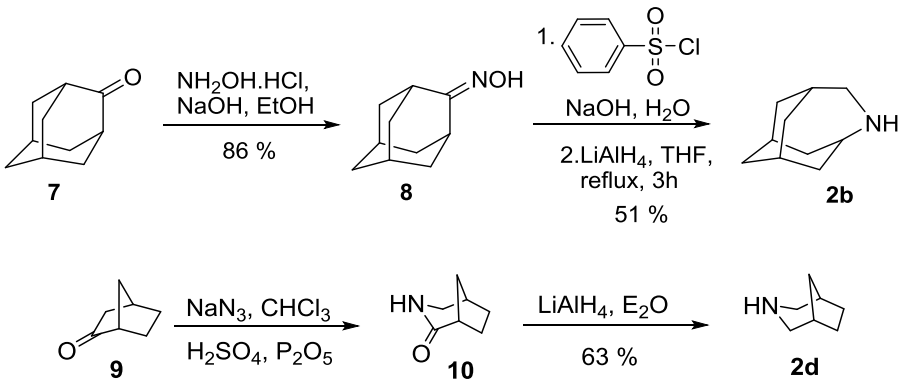
Scheme 2



The synthesis of secondary amine 4-azatricyclo[4.3.1. $1.^{3,8}$]undecan **2b** was straightforward and involved an initial condensation of 2-adamantanone **7** with hydroxylamine to afford

adamantan-2-oxime, followed by Beckmann rearrangement to form the 4-azatricyclo[4.3.1. 1.^{3,8}]-undecan-5-one (**8**) (scheme 3). Reduction of 4-azatricyclo amide derivative **8** furnished 4-azatricyclo [4.3.1. 1.^{3,8}]undecane **2b**. Similarly, the secondary amine 1,4-thiazepane **2a** was also prepared following the same synthetic route. Further, the bicylic amine **2d** was made from starting material norcamphor **9** in two steps. 3-Azabicyclo[3.2.1]octan-2-one (**10**) was obtained by carrying out curtius rearrangement on **9**. Finally, reduction with $LiAlH_4$ in Et_2O delivered 3-azabicyclo[3.2.1]octane in moderate yield **2d**.

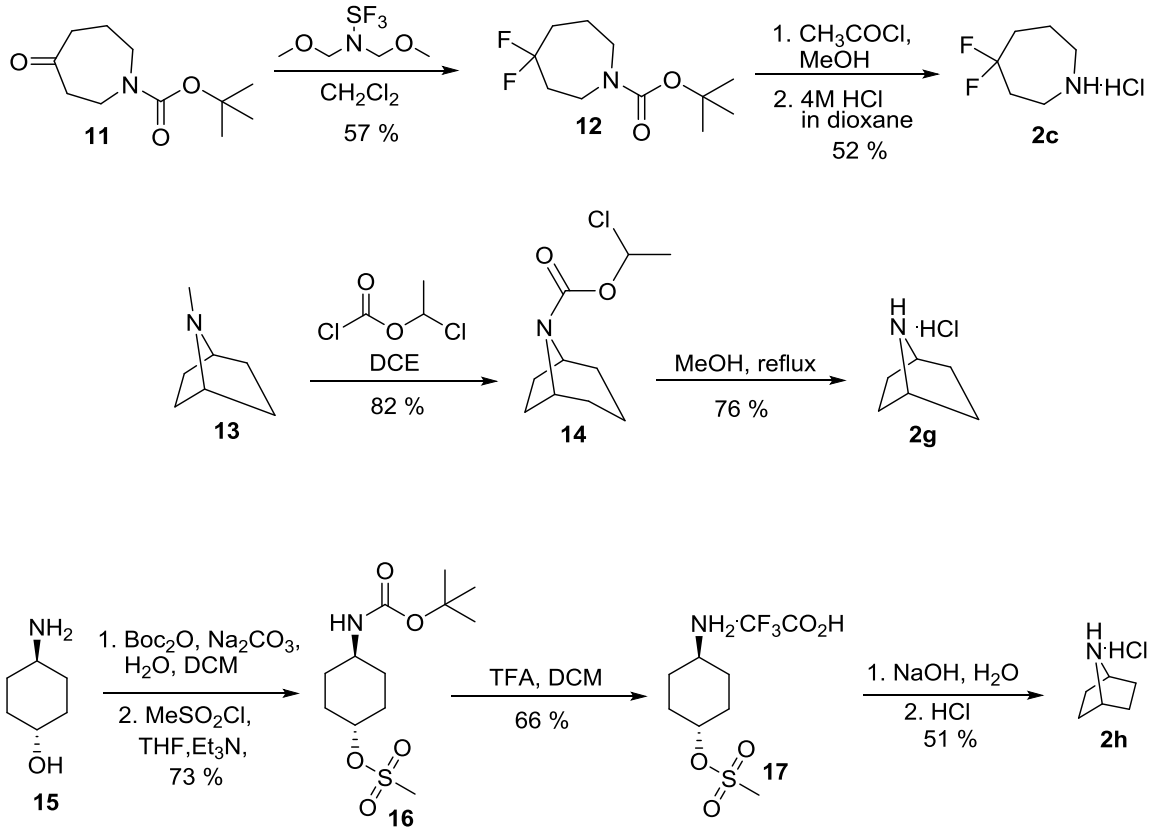
Scheme 3



It was decided to incorporate fluorine in the azepane ring at position **4** in **RS194B**, scheme 4, to achieve a modest p*K*a reduction of the cycylic amine and thus N-(2-(4,4-difluoroazepan-1-yl)ethyl)-2-(hydroxyimino)acetamide **1c** was synthesized. Difluoride **2c** was obtained from **11** following treatment with aminosulfurane and subsequent Boc deprotection. To synthesize oxime derivative **1h**, bicyclic amine 8-azabicyclo[3.2.1]octane hydrochloride **2g** was obtained via chloroethyl chloroformate mediated dealkylation of the tertiary amine **13**. Further, trans-4-aminocyclohexanol **15** was protected using Boc-anhydride and then mesylation was done to obtain Boc-protected cyclohexyl methanesulfonate **16**. In the next step, Boc group was removed

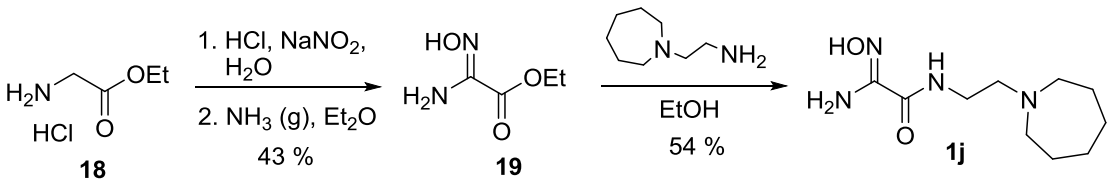
under acidic condition and the TFA salt of cyclohexyl methanesulfonate was cyclized under basic condition to deliver 7-azabicyclo[2.2.1] heptanes **2h** in moderate yield.

Scheme 4



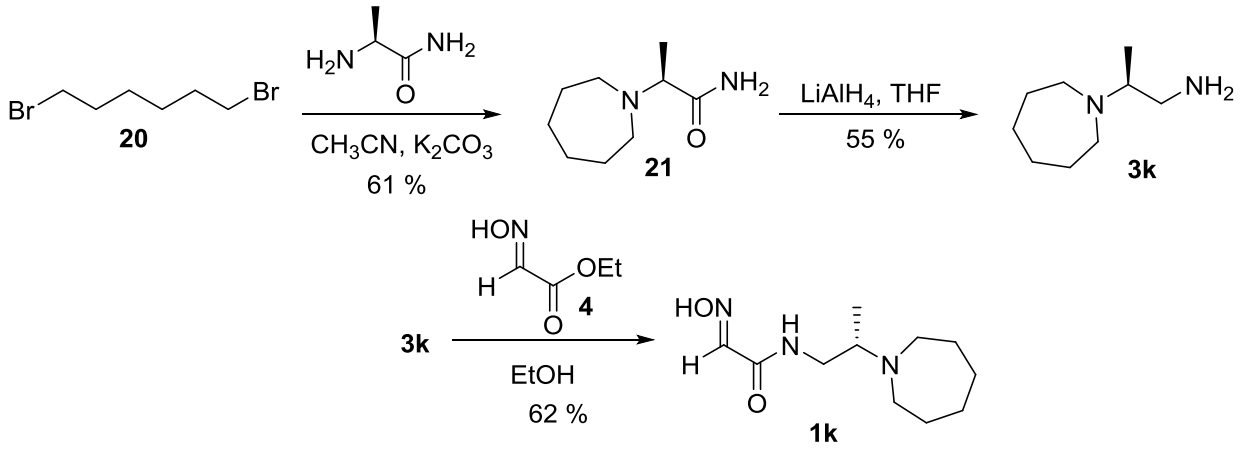
After successful synthesis of oximes **1a−i**, modification of vicinity of the nucleophilic oxime was made and the oxime derivative **1j** was prepared. Glycine ester hydrochloride **18** was converted into 2-chloro-2 (hydroxyimino)acetate, followed by treatment with NH_3 (g) afforded 2-amino-2-(hydroxyimino)acetate **19**. Amidation of **19** with 2-(azepan-1-yl)ethan-1-amine delivered acetamide oxime **1j**.

Scheme 5



To investigate the effect of introducing methyl group in the linker, the oxime derivative **1k** was prepared. The primary amine **3k** was first synthesized in two steps. The appropriate tertiary amine carboxamide **21** was prepared from the readily available L-alaninamide by reaction with 1,6-dibromohexane under basic conditions to construct the azepane ring. Reduction of carboxamide **21** with $LiAlH_4$ in THF afforded **3k** and subsequent amidation with ethyl glyoxylate oxime **4** delivered acetamide oxime **1k**.

Scheme 6



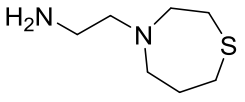
General

All reactions were performed with commercially available ACS grade reagents and solvents. Anhydrous *N*,*N*-dimethylfomamide (DMF), acetonitrile and chloroform, dichloromethane were used as received without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a

Varian 400 MHz spectrometer. All chemical shifts were reported in ppm relative to solvent resonances, as indicated (DMSO-d_6 δ 2.50, ^{1}H; δ 39.50, ^{13}C), ($CDCl_3$ δ 7.26, ^{1}H; δ 77.16, ^{13}C). ^{1}H NMR coupling constants (J) are given in Hz.

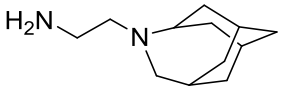
A. General method for the preparation of primary amines (3a-e). A 15 mmol solution of secondary amine (**2a**−**e**) in 80 mL of acetonitrile was treated with 18.75 mmol of *N*-bromopthalimide and K_2CO_3 (45.0 mmol). The resulting mixture was refluxed overnight. After the reaction was completed, 90 mL of saturated $NaHCO_3$ was added followed by extraction with ethyl acetate. The combined organic layers were acidified with 2 N HCl and washed with water. The pH of the aqueous layer was adjusted to pH 12 using 4 N NaOH and then extracted with methylene chloride. The organic solution was dried over Na_2SO_4 and evaporated to yield the product, which is used in the next reaction without further purification.

The *N*-alkylated pthalimide obtained above was dissolved in 80 mL of ethanol and hydrazine hydrate (3 equiv) was added. The reaction was refluxed for 3 h and cooled to room temperature, and the resulting precipitate was removed by filtration. The filtrate was concentrated and the residue was diluted with 30 mL of EtOAc. The resulting precipitate was removed by filtration, the filtrate was concentrated to dryness and kugelrohr distillation was done to obtain pure amine.

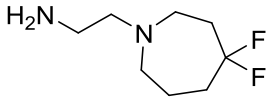


2-(1,4-thiazepan-4-yl)ethanamine (3a). Prepared according to the general method A. Clear oil, yield 1.1 g (46 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.92 (t, J = 5.6 Hz, 2H), 2.85 (t, J = 5.6 Hz,

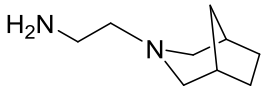
2H), 2.75-2.64 (m, 6H), 2.60 (t, J = 5.7 Hz, 2H), 1.87 (pent, J = 5.8 Hz, 2H), 1.59 (s, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 59.6, 58.8, 53.7, 39.9, 34.4, 32.1, 31.1.



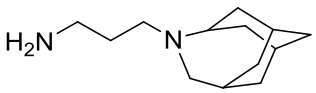
2-(4-Azatricyclo[4.3.1.1^{3,8}]undecan-4-yl)ethanamine (3b). Prepared according to the general method A. Clear oil, yield 1.7 g (58 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.92 (t, J = 5.4 Hz, 1H), 2.76 (d, J = 3.8 Hz, 2H), 2.64 (dd, J = 9.1, 4.5 Hz, 4H), 2.04 (s, 2H), 1.86-1.76 (m, 4H), 1.75-1.40 (m, 9H). ^{13}C NMR (400 MHz, DMSO-d6) δ 60.0, 55.8, 54.8, 40.9, 32.8, 31.3, 29.0, 26.4, 17.3, 16.8.



2-(4,4-Difluoroazepan-1-yl)ethanamine (3c). Prepared according to the general method A. Light yellow oil, yield 1.3 g (48 %).

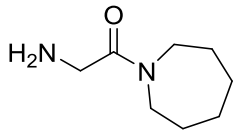


2-(3-Azabicyclo[3.2.1]octan-3-yl)ethanamine (3d). Prepared according to the general method A. Clear oil, yield 1.4 g (59 %)



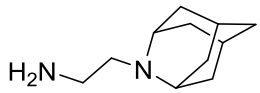
3-(4-Azatricyclo[4.3.1.13,8]undecan-4-yl)propan-1-amine (3e). Prepared according to the general method A. Clear oil, yield 1.6 g (51 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.99 (s, 1H), 2.77

(d, *J* = 3.8 Hz, 2H), 2.72 (t, *J* = 6.7 Hz, 2H), 2.61 (t, *J* = 7.1 Hz, 2H), 2.05 (s, 1H), 1.90-1.75 (m, 6H), 1.69-1.63 (m, 3H), 1.62-1.52 (m, 5H), 1.49-1.45 (m, 2H).



2-Amino-1-(azepan-1-yl)ethanone (3f). To a stirred solution of ((benzyloxy)carbonyl)glycine (1 g, 4.78 mmol) in 35 mL of dichloromethane at rt, HATU (2 g, 5.3 mmol) and DIPEA (1.8 g, 14.3 mmol) were added. The reaction mixture was stirred for 10 min, and then azepane (1 g, 10.5 mmol) was added, and stirred for another 3 h. The resulting reaction mixture was filtered and the filtrate was concentrated. The obtained residue was purified by flash column chromatography (EtOAc/hexane, 8:2) to obtain 1.1 g (79 %) of benzyl(2-(azepan-1-yl)-2-oxoethyl)carbamate as a clear oil.

Benzyl(2-(azepan-1-yl)-2-oxoethyl)carbamate (1 g, 3.4 mmol) was dissolved in 40 mL of MeOH, degassed and placed under argon. 10% Pd/C (0.25 g) was added, and the contents were thoroughly degassed, placed under hydrogen via balloon and stirred overnight. The resulting reaction mixture was filtered through Celite, and the Celite pad was subsequently washed with DCM and MeOH. The resulting filtrate was concentrated in vacuo and kugelrohr distillation was done to afford 0.50 g (94 %) of the title compound as clear oil. ^{1}H NMR (400 MHz, CDCl3) δ 3.50 (t, *J* = 6.0 Hz 2H), 3.41 (s, 2H), 3.30 (t, *J* = 6.0 Hz, 2H), 1.78 (s, 2H), 1.72-1.63 (m, 4H), 1.51 (dd, *J* = 5.9, 3.0 Hz, 4H). ^{13}C NMR (400 MHz, DMSO-d6) δ 172.2, 46.4, 46.2, 43.1, 28.9, 27.6, 27.2, 26.9.

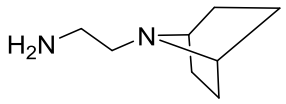


2-(Azaadamantan-2-yl)ethanamine (3g). To a stirred solution of ((benzyloxy)carbonyl)glycine (0.70, 3.3 mmol) in 25 mL of dichloromethane at rt, HATU (1.40 g, 3.6 mmol) and DIPEA (0.90 g, 6.9 mmol) were added. The reaction mixture was stirred for 10 min, and then 2-azaadamantane (0.54 g, 4 mmol) was added, and stirred for another 3 h. The resulting reaction mixture was filtered and the filtrate was concentrated. The obtained residue was purified by flash column chromatography (EtOAc/hexane, 7:3) to obtain 0.90 g (83 %) of benzyl(2-(2-azaadamantane-2-yl)-2-oxoethyl)carbamate as a clear oil.

Benzyl(2-(2-azaadamantane-2-yl)-2-oxoethyl)carbamate (0.85 g, 2.6 mmol) was dissolved in 25 mL of MeOH, degassed and placed under argon. 10% Pd/C (0.25 g) was added, and the contents were thoroughly degassed, placed under hydrogen via balloon and stirred overnight. The resulting reaction mixture was filtered through Celite, and the Celite pad was subsequently washed with DCM and MeOH. The resulting filtrate was concentrated in vacuo to deliver 1-(2-azaadamantane-2-yl)-2-aminoethan-1-one (0.43 g, 85 %).

To a stirred suspension of LAH (0.24 g, 9.7 mmol) in THF (20 mL) at 0 °C was added dropwise to a solution of 1-(2-azaadamantane-2-yl)-2-aminoethan-1-one (0.40 g, 2.1 mmol) in 20 mL of THF. The reaction mixture was stirred overnight at rt. The resulting reaction mixture was quenched with careful addition of H_2O (0.30 mL), followed by 15% aqeous NaOH (0.60 mL), and H_2O (0.90 mL). This crude mixture was filtered through a small pad of Celite, and the filter cake was washed with ether (10 mL). The filtrate was concentrated and kugelrohr distillation was done to afford 0.29 g (76 %) of the title compound as a clear oil. ^{1}H NMR (400 MHz, cdcl$_3$) δ 2.75 (s, 2H), 2.71-2.63 (m, 4H), 2.03-1.95 (m, 6H), 1.78 (s, 2H), 1.72 (s, 2H), 1.53

(s, 2H), 1.50 (s, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 55.9, 51.8, 40.2, 37.3, 33.5, 27.5.

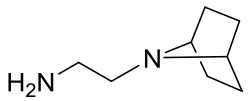


2-(8-Azabicyclo[3.2.1]octan-8-yl)ethanamine (3h). To a stirred solution of ((benzyloxy)carbonyl)glycine (0.80, 3.8 mmol) in 25 mL of dichloromethane at rt, HATU (1.60 g, 4.2 mmol) and DIPEA (1.5 g, 11.6 mmol) were added. The reaction mixture was stirred for 10 min, and then 8-azabicyclo[3.2.1]octane hydrochloride (1.1 g, 7.4 mmol) was added, and stirred for another 3 h. The resulting reaction mixture was filtered and the filtrate was concentrated. The obtained residue was purified by flash column chromatography (EtOAc/hexane, 8:2) to obtain 1.1 g (95 %) of benzyl(2-(8-azabicyclo[3.2.1]heptan-7-yl)-2-oxoethyl)carbamate as a clear oil.

Benzyl(2-(8-azabicyclo[3.2.1]heptan-7-yl)-2-oxoethyl)carbamate (1.1 g, 3.6 mmol) was dissolved in 40 mL of MeOH, degassed and placed under argon. 10% Pd/C (0.28 g) was added, and the contents were thoroughly degassed, placed under hydrogen via balloon and stirred overnight. The resulting reaction mixture was filtered through Celite, and the Celite pad was subsequently washed with DCM and MeOH. The resulting filtrate was concentrated in vacuo to deliver 2-amino-1-(8-azabicyclo[3.2.1]octan-8-yl)ethan-1-one (0.59 g, 97 %).

To a stirred suspension of LAH (0.40 g, 10.5 mmol) in THF (20 mL) at 0 °C was added dropwise to a solution of 2-amino-1-(8-azabicyclo[3.2.1]octan-8-yl)ethan-1-one (0.59 g, 3.5 mmol) in 20 mL of THF. The reaction mixture was stirred overnight at rt. The resulting reaction mixture was quenched with careful addition of H_2O (0.40 mL), followed by 15% aqeous NaOH (0.80 mL), and H_2O (1.3 mL). This crude mixture was filtered through a small pad of Celite, and

the filter cake was washed with ether (10 mL). The filtrate was concentrated and kugelrohr distillation was done to afford 0.35 g (65 %) of the title compound as a clear oil. ^{1}H NMR (400 MHz, cdcl$_3$) δ 3.09 (s, 2H), 2.69 (t, J = 6.3 Hz, 2H), 2.34 (t, J = 6.3 Hz, 2H), 1.91-1.82 (m, 2H), 1.78 (s, 2H), 1.72-1.57 (m, 2H), 1.54-1.47 (m, 2H), 1.45-1.35 (m, 2H), 1.29 (d, J = 12.9 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 60.0, 55.8, 54.8, 40.9, 32.8, 31.3, 29.0, 26.4, 17.3, 16.8.

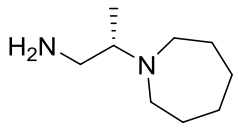


2-(7-Azabicyclo[2.2.1]heptan-7-yl)ethanamine (3i). To a stirred solution of ((benzyloxy)carbonyl)glycine (0.80, 3.8 mmol) in 25 mL of dichloromethane at rt, HATU (1.60 g, 4.2 mmol) and DIPEA (1.5 g, 11.6 mmol) were added. The reaction mixture was stirred for 10 min, and then 7-azabicyclo[2.2.1]heptane hydrochloride (0.76 g, 5.7 mmol) was added, and stirred for another 3 h. The resulting reaction mixture was filtered and the filtrate was concentrated. The obtained residue was purified by flash column chromatography (EtOAc/hexane, 8:2) to obtain 1 g (91 %) of benzyl(2-(7-azabicyclo[2.2.1]heptan-7-yl)-2-oxoethyl)carbamate as a clear oil.

Benzyl(2-(7-azabicyclo[2.2.1]heptan-7-yl)-2-oxoethyl)carbamate (1 g, 3.5 mmol) was dissolved in 40 mL of MeOH, degassed and placed under argon. 10% Pd/C (0.26 g) was added, and the contents were thoroughly degassed, placed under hydrogen via balloon and stirred overnight. The resulting reaction mixture was filtered through Celite, and the Celite pad was subsequently washed with DCM and MeOH. The resulting filtrate was concentrated in vacuo to deliver 2-amino-1-(7-azabicyclo[2.2.1]heptan-7-yl)ethan-1-one (0.50 g, 93 %).

To a stirred suspension of LAH (0.37 g, 9.7 mmol) in THF (20 mL) at 0 °C was added

dropwise to a solution of 2-amino-1-(7-azabicyclo[2.2.1]heptan-7-yl)ethan-1-one (0.50 g, 3.2 mmol) in 20 mL of THF. The reaction mixture was stirred overnight at rt. The resulting reaction mixture was quenched with careful addition of H_2O (0.40 mL), followed by 15% aqeous NaOH (0.80 mL), and H_2O (1.3 mL). This crude mixture was filtered through a small pad of Celite, and the filter cake was washed with ether (10 mL). The filtrate was concentrated and kugelrohr distillation was done to afford 0.31 g (69 %) of the title compound as clear oil. ^{13}C NMR (400 MHz, DMSO-d6) δ 61.6, 56.4, 56.1, 40.9, 37.6, 36.1, 35.7, 32.2, 31.7, 26.7.



(*S*)-2-(Azepan-1-yl)propan-1-amine (3k). 1,5-Dibromopentane (3.0 g, 12.3 mmol mmol) was added to a suspension of L-alaninamide (1.5 g, 12 mmol), potassium carbonate (5 g, 36 mmol), and potassium iodide (6 mg) in CH_3CN (36 mL). The mixture was refluxed overnight and then cooled to rt. The mixture was treated with 75 mL of 1N HCl and extracted with dichloromethane. The organic layer was discarded. The aqueous layer was basified with 4N NaOH and extracted with dichloromethane (3x30 mL). The solvent was evaporated to dryness to obtain 1.50 g (73%) of (S)-2-(azepan-1-yl)propanamide.

To a stirred suspension of LAH (1.5 g, 41.1 mmol) in THF (80 mL) at 0 °C was added dropwise a solution of (S)-2-(azepan-1-yl)propanamide (1.4 g, 8.2 mmol) in 40 mL of THF. The reaction mixture was stirred overnight at 50 °C and cooled to rt. The resulting reaction mixture was quenched with careful addition of H_2O (1.5 mL), followed by 15% aqeous NaOH (3 mL), and H_2O (5 mL). A thick gel-like precipitate was formed. The reaction mixture was filtered

through a small pad of Celite, and the filter cake was washed with ether (50 mL). The filtrate was concentrated and kugelrohr distillation was done to afford 0.90 g (66 %) of the title compound as a clear oil.

^{1}H NMR (400 MHz, cdcl$_3$) δ 2.68-2.56 (m, 3H), 2.56-2.47 (m, 1H), 2.44-2.35 (m, 3H), 1.66-1.43 (m, 10H), 0.81 (d, J = 6.3 Hz, 3H).^{13}C NMR (400 MHz, DMSO-d6) δ 62.8, 50.7, 45.5, 29.9, 26.9, 11.4.

B. General method for the preparation of acetamide oximes 1a-k. The desired primary amine (3a-j) was added to a solution of ethyl glyoxylate oxime 4 in ethanol with stirring. The solution was stirred overnight at room temperature. The resulting precipitate was filtered, washed with cold ethanol and dried under vacuum.

***N*-(2-(4-Azatricyclo[4.3.1.1^{3,8}]undecan-4-yl)ethyl)-2-(hydroxyimino)acetamide (RS2-138B)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.75 g, 6.41 mmol), 2-(4-azatricyclo[4.3.1.1^{3,8}]undecan-4-yl)ethanamine (1.4 g, 7.0 mmol), ethanol (10 mL). White solid, yield (1 g, 60 %). ^{1}H NMR (400 MHz, dmso) δ 11.91 (s, 1H), 7.87 (s, 1H), 7.42 (s, 1H), 3.19 (dd, J = 12.8, 6.4 Hz, 2H), 2.95 (d, J = 5.1 Hz, 1H), 2.77 (d, J = 3.8 Hz, 2H), 2.66 (t, J = 6.8 Hz, 2H), 2.04 (s, 1H), 1.82 (d, J = 10.7 Hz, 4H), 1.69 (d, J = 13.6 Hz, 2H), 1.59-1.40 (m, 6H).^{13}C NMR (400 MHz, DMSO-d6) δ 161.6, 143.7, 60.5, 56.8, 55.9, 37.3, 37.0, 35.7, 35.4.

***N*-(2-(2-Azaadamantan-2-yl)ethyl)-2-(hydroxyimino)acetamide (RS3-36D)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.15 g, 1.3 mmol), 2-(2-azaadamantan-2-yl)ethanamine (0.25 g, 1.4 mmol), ethanol (4 mL). White solid, yield (0.26 g, 79 %). ^{1}H NMR (400 MHz, dmso) δ 11.92 (s, 1H), 7.86 (s, 1H), 7.42 (s, 1H), 3.15 (dd, J = 12.8,

6.5 Hz, 2H), 2.75 (s, 2H), 2.68 (t, *J* = 6.9 Hz, 2H), 1.93 (d, *J* = 6.9 Hz, 6H), 1.75 (s, 2H), 1.50 (d, *J* = 10.8 Hz, 4H).^{13}C NMR (400 MHz, DMSO-d6) δ 161.6, 143.7, 51.7, 50.9, 37.3, 36.6, 32.9, 26.8.

(*S*)-*N*-(2-(Azepan-1-yl)propyl)-2-(hydroxyimino)acetamide (RS2-245C) Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.50 g, 4.27 mmol), (*S*)-2-(azepan-1-yl)propan-1-amine (0.77 g, 4.91 mmol), ethanol (7 mL). White solid, yield (0.60 g, 62 %). ^{1}H NMR (400 MHz, dmso) δ 11.95 (s, 1H), 7.77 (s, 1H), 7.42 (s, 1H), 3.20-3.00(m, 2H), 2.83 (sext, *J* = 7.1 Hz, 1H), 2.67-2.61 (m, 2H), 2.52 (s, 2H), 1.52 (s, 8H), 0.88 (s, 3H). ^{13}C NMR (400 MHz, DMSO-d6) δ 161.5, 143.7, 58.7, 50.2, 41.5, 39.5, 29.2, 26.4, 12.3.

***N*-(2-(Azepan-1-yl)-2-oxoethyl)-2-(hydroxyimino)acetamide (RS2-90C)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.30 g, 2.56 mmol), 2-amino-1-(azepan-1-yl)ethanone (0.46 g, 2.94 mmol), ethanol (6 mL). White solid, yield (0.34 g, 58 %). ^{1}H NMR (400 MHz, DMSO-d6) ^{1}H NMR (400 MHz, dmso) δ 12.04 (s, 1H), 8.02 (s, 1H), 7.50 (s, 1H), 4.02 (d, *J* = 5.2 Hz, 1H), 3.41 (dd, *J* = 11.7, 5.7 Hz, 2H), 1.74-1.63 (m, 1H), 1.63-1.54 (m, 1H), 1.54-1.35 (m, 2H).^{13}C NMR (400 MHz, DMSO-d6) δ 167.2, 161.7, 143.3, 46.1, 45.4, 28.2, 27.1, 26.7, 26.2.

***N*-(2-(8-Azabicyclo[3.2.1]octan-8-yl)ethyl)-2-(hydroxyimino)acetamide (RS2-237D)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.17 g, 1.5 mmol), 2-(8-azabicyclo[3.2.1]octan-8-yl)ethanamine (0.25 g, 1.6 mmol), ethanol (5 mL). White solid, yield (0.24 g, 71 %). ^{1}H NMR (400 MHz, DMSO) δ 11.92 (s, 1H), 7.92 (s, 1H), 7.42 (s, 1H), 3.19 (q, *J* = 6.5 Hz, 2H), 3.11 (s, 2H), 2.36 (t, *J* = 6.9 Hz, 2H), 1.93 -1.73 (m, 2H), 1.65-1.50 (m, 3H), 1.49 (d, *J* = 7.5 Hz, 2H), 1.39-1.19 (m, 3H).

^{13}C NMR (400 MHz, DMSO-d6) δ 161.6, 143.7, 59.2, 51.2, 37.8, 30.6, 26.0, 16.3.

***N*-(2-(7-Azabicyclo[2.2.1]heptan-7-yl)ethyl)-2-(hydroxyimino)acetamide (RS2-234D)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.23 g, 1.94 mmol), 2-(7-azabicyclo[2.2.1]heptan-7-yl)ethanamine (0.30 g, 2.14 mmol), ethanol (5 mL). White solid, yield (0.28 g, 68 %).

***N*-(2-(3-Azabicyclo[3.2.1]octan-3-yl)ethyl)-2-(hydroxyimino)acetamide (RS3-43D)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.35 g, 3.0 mmol), 2-(3-azabicyclo[3.2.1]octan-3-yl)ethanamine (0.53 g, 3.44 mmol), ethanol (7 mL). White solid, yield (0.41 g, 60 %). %). ^{1}H NMR (400 MHz, DMSO) δ 11.92 (s, 1H), 7.82 (s, 1H), 7.41 (s, 1H), 3.21 (d, J = 5.6 Hz, 2H), 2.61 (d, J = 8.5 Hz, 2H), 2.36 (d, J = 6.1 Hz, 2H), 2.07 (s, 2H), 2.00 (d, J = 9.9 Hz, 2H), 1.65-1.35 (m, 5H), 1.29 (d, J = 10.6 Hz, 1H).^{13}C NMR (400 MHz, DMSO-d6) δ 161.6, 143.7, 60.8, 59.5, 56.1, 37.2, 35.9, 34.6, 28.3.

***N*-(3-(4-Azatricyclo[4.3.1.1^{3,8}]undecan-4-yl)propyl)-2-(hydroxyimino)acetamide (RS2-150C)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.50 g, 4.27 mmol), 3-(4-azatricyclo[4.3.1.1^{3,8}]undecan-4-yl)propan-1-amine (1.0 g, 4.91 mmol), ethanol (9 mL). White solid, yield (0.70 g, 59 %). ^{1}H NMR (400 MHz, dmso) δ 11.84 (s, 1H), 8.18 (s, 1H), 7.41 (s, 1H), 3.17 (q, J = 6.6 Hz, 2H), 2.95 (s, 1H), 2.72 (d, J = 3.8 Hz, 2H), 2.55 (t, J = 6.8 Hz, 2H), 2.04 (s, 1H), 1.81 (s, 4H), 1.73-1.62 (m, 2H), 1.60-1.42 (m, 8H).^{13}C NMR (400 MHz, DMSO-d6) δ 161.6, 143.9, 60.8, 55.8, 55.6, 37.2, 37.1, 35.5, 30.9, 27.8, 26.1.

***N*-(2-(Azepan-1-yl)ethyl)-2-(hydroxyamino)-2-iminoacetamide (RS292A)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.30 g, 2.56 mmol), 2-(azepan-1-yl)ethanamine (0.42 g, 2.94 mmol), ethanol (6 mL). White solid, yield (0.32 g, 54 %). ^{1}H NMR (400 MHz, dmso) δ 9.85 (s, 1H), 7.65 (s, 1H), 5.62 (s, 2H), 3.19 (q, J = 6.3 Hz, 2H), 2.61-2.57

(m, 2H), 2.53 (t, J = 6.6 Hz, 2H), 1.53 (s, 8H). ^{13}C NMR (400 MHz, DMSO-d6) δ 161.6, 143.7, 56.0, 54.6, 36.7, 28.0, 26.6.

***N*-(2-(4,4-Difluoroazepan-1-yl)ethyl)-2-(hydroxyimino)acetamide (RS2-140B)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.30 g, 2.56 mmol), 2-(4,4-difluoroazepan-1-yl)ethanamine (0.52 g, 2.94 mmol), ethanol (6 mL). White solid, yield (0.37 g, 58 %).

***N*-(2-(1,4-Thiazepan-4-yl)ethyl)-2-(hydroxyimino)acetamide (RS2-148B)** Prepared according to the general method B. Ethyl glyoxylate oxime 3 (0.35 g, 3 mmol), 2-(1,4-thiazepan-4-yl)ethanamine (0.55 g, 3.45 mmol), ethanol (7 mL). White solid, yield (0.44 g, 61 %). ^{1}H NMR (400 MHz, dmso) δ 11.88 (s, 1H), 7.93 (s, 1H), 7.42 (s, 1H), 3.20 (q, J = 6.3 Hz, 2H), 2.90 (t, J = 5.1 Hz, 2H), 2.85 (t, J = 5.9 Hz, 2H), 2.70 - 2.59 (m, 6H), 1.80 (pent, J = 5.9 Hz, 2H). ^{13}C NMR (400 MHz, DMSO-d6) δ 161.7, 143.7, 58.1, 54.6, 52.9, 36.8, 33.5, 31.0, 30.6.