Published in final edited form as:

Neurotoxicology. 2018 September ; 68: 142–148. doi:10.1016/j.neuro.2018.07.014.

Evaluating the broad-spectrum efficacy of the acetylcholinesterase oximes reactivators MMB4 DMS, HLö-7 DMS, and 2-PAM Cl against phorate oxon, sarin, and VX in the Hartley guinea pig

Christina M. Wilhelm^{a,*}, Thomas H. Snider^a, Michael C. Babin^a, Gennady E. Platoff Jr.^b, David A. Jett^c, and David T. Yeung^b

^aBattelle, 505 King Avenue, JM-7, Columbus, OH 43201-2693, USA

^bNational Institutes of Health/National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892, USA

^cNational Institutes of Health/National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892, USA

Abstract

Organophosphorus (OP) compounds, including pesticides and chemical warfare nerve agents (CWNA), are threats to the general population as possible weapons of terrorism or by accidental exposure whether through inadvertent release from manufacturing facilities or during transport. To mitigate the toxicities posed by these threats, a therapeutic regimen that is quick-acting and efficacious against a broad spectrum of OPs is highly desired. The work described herein sought to assess the protective ratio (PR), median effective doses (ED₅₀), and therapeutic index (TI = oxime 24-h LD₅₀/oxime ED₅₀) of MMB4 DMS, HLö-7 DMS, and 2-PAM Cl against the OPs sarin (GB), VX, and phorate-oxon (PHO). All OPs are representative of the broader classes of G and V chemical warfare nerve agents and persistent pesticides. MMB4 DMS and HLö-7 DMS were previously identified as comparative efficacy leads warranting further evaluations. 2-PAM Cl is the U.S. FDA-approved standard-of-care oxime therapy for OP intoxication. Briefly, PRs were determined in male guinea pigs by varying the subcutaneously (SC) delivered OP dose followed then by therapy with fixed levels of the oxime and atropine (0.4 mg/kg; administered intramuscularly [IM]). ED₅₀s were determined using a similar approach except the OP dose was held constant at twice the median lethal dose (2 × LD₅₀) while the oxime treatment levels were varied. The ED₅₀ information was then used to calculate the TI for each OP/oxime combination. Both MMB4 DMS and HLö-7 DMS provided significant protection, i.e., higher PR against GB, VX, and PHO when compared to atropine controls, but significance was not readily demonstrated across the board when compared against 2-PAM Cl. The ED₅₀ values of MMB4 DMS was

*Corresponding author: wilhelmc@battelle.org (C.M. Wilhelm), babinm@battelle.org (M.C. Babin), platoffg@niaid.nih.gov (G.E. Platoff), jettdd@ninds.nih.gov (D.A. Jett), dy70v@nih.gov (D.T. Yeung).

Conflict of interest

The authors have no known conflicts of interest.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

consistently lower than that of the other oximes against all three OPs. Furthermore, based on those ED₅₀s, the TI trend of the various oximes against both GB and VX was MMB4 DMS > HLö-7 DMS > 2-PAM Cl, while against PHO, MMB4 DMS > 2-PAM Cl > HLö-7 DMS.

Keywords

HED; ED₅₀; Protective ratio; MMB4 DMS; HLö-7 DMS; Phorate-oxon

1. Introduction

Organophosphorus(OP) compounds are highly toxic chemicals that present a serious exposure threat to the general population, OPs include many commonly available agricultural pesticides as well as chemical warfare nerve agents (CWNA). Acute exposure to either OP class, whether deliberate or accidental, is of increasing concern for both military personnel and civilians (Okumura et al., 2005; Zurer, 1998; Baker, 2013; Dolgin, 2013). The primary mode of OP-induced toxicosis is the irreversible inhibition of the enzyme acetylcholinesterase (AChE) leading to the hyperstimulation of post-synaptic parasympathetic nerves terminals (Taylor, 2017). Clinical symptoms of intoxication such as miosis, fasciculations, respiratory distress, seizures, convulsions, increased secretions, and death can quickly occur if not effectively controlled (Bajgar, 2004). The only U.S. FDA-approved and fielded therapeutic regimen to treat OP-induced toxicity entails the rapid administration of an antimuscarinic, i.e., atropine, and an AChE oxime reactivator, i.e., pralidoxime chloride (2-PAM Cl) followed by an anticonvulsant such as diazepam, if convulsion is observed (Eddleston et al., 2008). While 2-PAM Cl is currently fielded to the U.S. military (ATNAA; <http://www.meridianmeds.com/products/atnaa>) and is also available as a civilian therapeutic (DuoDote®; <http://www.meridianmeds.com/products/duodote>), it does not offer broad efficacy across the different OPs (Thiermann et al., 2013; Worek and Thiermann, 2013). As such, identifying a more effective AChE reactivator drug, e.g., one with broad-spectrum efficacy, would greatly reduce the toxicity from both CWNA and OP pesticide exposures and thus likely to be beneficial in incidents of civilian and military exposures.

Attempts to improve the rescue of OP-inhibited AChE have been ongoing in the field for years. These efforts include developing: 1) chemically novel oxime AChE-reactivators, 2) combinations/cocktails of various experimental oximes and oximes licensed in other countries, and 3) non-oxime based reactivators (Winter et al., 2016; Radi et al., 2013; Worek et al., 2016; Bierwisch et al., 2016; Wei et al., 2016). To date, no marked improvement in efficacy of these approaches has been demonstrated against a broad spectrum of OPs. In a previous study comparing the efficacy of various leading oxime therapies under standardized and rigorous conditions in the guinea pig model, the oximes MMB4 DMS and HLö-7 DMS demonstrated the best overall efficacy against a spectrum of eight different OPs by enhancing survival, quality of life, and reactivation of ChE levels (Wilhelm et al., 2014).

The goal of this current work is to further characterize those two previously identified leading oximes by assessing additional measures of efficacy, specifically protective ratios (PRs), 24-h median effective doses (ED₅₀), and therapeutic indices (TIs). PRs were assessed against three OPs: sarin (GB), VX, and phorate oxon (PHO), followed by determination of the 24-h ED₅₀s against each. These three specific OP compounds were chosen as representatives of the G- and V-class of CWNAs and PHO for OP pesticides. All three chosen OPs have also been previously identified by the U.S. Department of Homeland Security (DHS) and the Chemical Countermeasures Research Program (CCRP) at the National Institute of Allergy and Infectious Diseases (NIAID/NIH) as chemical compounds of interest under the DHS Chemical Terrorism Risk Assessment (CTRA) program.

PRs were determined by subcutaneous (SC) administration of each OP in guinea pigs with atropine (0.4 mg/kg) administered as standard therapy post-exposure. In this work, the median lethal dose (LD₅₀) for each OP was determined in guinea pigs administered atropine postexposure that were also administered the oxime therapy (which was fixed at an equimolar dose to ensure standardization across the different oximes (Wilhelm et al., 2014)). This data, when divided by the LD₅₀ for the same OP in guinea pigs administered atropine post-exposure absent oxime therapy, provided the baseline PR for each oxime. To provide additional efficacy insight, ED₅₀ assessments were also performed in guinea pigs administered atropine post-exposure to identify a potential dose of each oxime that could be administered to protect against all three OPs. The dose of the OP was fixed at 2 × 24LD₅₀ while the level of the administered oxime was varied. ED₅₀ values were then determined via probit analysis of the resultant survival data.

Lastly, in addition to the two lead oximes of interest, the baseline PR and ED₅₀ values for 2-PAM Cl (at the currently approved pre-hospital human equivalent dose (HED)) were also identified. These data were then utilized as a comparator to quantitatively measure any differences in efficacy between MMB4 DMS and HLö-7DMS against the current standard of care oxime.

2. Materials and methods

Solutions of GB (O-isopropyl methylphosphonofluoridate), and VX (O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothiolate) were prepared in normal (0.9%, w/v) saline. Phorate oxon (PHO) was synthesized in-house following Snider et al. (2016b) and prepared in Multisol (48.5% water, 40% propylene glycol, 10% ethanol, and 1.5% benzyl alcohol, all v/v). Multisol is a biocompatible solution with no adverse clinical signs when administered alone via the SC route. Gas chromatographic with flame photometric detection (GC-FPD) analyses confirmed nerve agent and pesticide solution concentrations prior to challenge and after administration.

1,1-methylene bis[4(hydroxyimino- methyl)pyridinium] dimethanesulfonate (**MMB4 DMS**, Medical Countermeasure Systems Joint Project Management Office, Frederick, MD) and pyridinium,1-(((4(aminocarbonyl) pyridinio)methoxy)methyl)-2,4- bis((hydroxyimino) methyl), dimethanesulfonate (**HLö-7 DMS**, CAS RN 120103–35-7, procured from Southwest Research Institute (SwRI), San Antonio, TX), and 2-hydroxyiminomethyl-1-

methylpyridinium chloride (**pralidoxime chloride**, 2-PAM Cl, (25.7 mg/kg)) were formulated in normal (0.9%, w/v) saline. Treatment concentrations were 5–259 mg/mL for MMB4 DMS, 20–302 mg/mL for HLö-7 DMS and 20–102 mg/mL for 2-PAM Cl. Oxime concentration was confirmed by high performance liquid chromatography (HPLC) for each oxime solution. Atropine was prepared in normal (0.9%, w/v) saline supplied as a 1.64 mg/mL solution (free base) by King Pharmaceuticals, St. Louis, MO. An atropine free base level of 0.4 mg/kg in the guinea pig was selected for this study based on the body surface area-corrected equivalent dose given to a human victim of OP poisoning in a first responder setting after administration of three DuoDote® autoinjectors (USD HHS, FDA, CDER, 2005).

Male Hartley guinea pigs were purchased from Charles River (Raleigh, NC and Saint-Constant, QC, Canada) and utilized in accordance with protocol specifications approved by the Institutional Animal Care and Use Committee (IACUC). The guinea pig was selected based on low plasma carboxylesterase levels similar to humans (Bahar et al., 2012), homology of AChE protein sequence to humans (Cadieux et al., 2010), affordability, and historical use. Animals randomized by weight were placed into dose appropriate groups. Clinical observations were performed throughout a 24-h period and assessed for quality of life.

2.1. Protective ratio

The PRs were determined based on the LD₅₀ for each OP against fixed doses of atropine and the selected oxime. Varying doses of OP were administered SC (n = 8) based on body weight (mean body weight = 347 g). The atropine plus oxime therapy (2-PAM Cl, MMB4 DMS, or HLö-7 DMS) was administered IM at 1-minute post-challenge for all animals. The oxime therapy dose was fixed at 146 µmol/kg (equimolar to the FDA-approved maximum pre-hospital HED of 2-PAM Cl; Wilhelm et al., 2014). Control group animals, with the exception of VX challenged animals, were challenged with a 1 × 24-h LD₅₀ OP dose followed by atropine followed by the oxime vehicle. For VX, the 0.0092 mg/kg (Maxwell et al., 2006) dose administered was based on historical data from United States Army Medical Research Institute of Chemical Defense (USAMRICD).

2.2. Median effective dose

Challenge dose levels were set at 2 × 24-h LD₅₀ for each OP (GB = 0.096 mg/kg, VX = 0.016 mg/kg and PHO = 3.84 mg/kg). At the 2 × 24-h LD₅₀ for each OP, the challenge dose was higher than the established LD₉₉, ensuring no survival in the non-treated groups. OPs were administered by the SC route at time zero, while atropine therapy was administered IM (0.4 mg/kg; HED of atropine in three ATNAA or DuoDote® injections as recommended by the U.S. FDA for pre-hospital dosing in the absence of definitive/supportive care) at 1-min postchallenge as a standard therapy for all animals. The oxime therapy was initiated immediately following atropine administration. Varying doses of oxime therapy were administered IM to groups (n = 5) based on body weight (mean weight = 332 g). Vehicle control animals were administered agent vehicle only + atropine and positive control animals were administered agent + atropine with no oxime treatment.

2.3. Statistical analysis

Power analyses were conducted to ensure group size for both the PR and ED₅₀ was acceptable for use in probit analysis. The PR for the 2PAM Cl, MMB4 DMS, or HLö7DMS groups was the ratio of its LD₅₀ to that for the no-treatment control group as previously described. The delta method was used to calculate a 95% confidence interval with significance at the 0.05 level for the PR. A comparison was performed for the following groups:

- MMB4 DMS, HLö7 DMS and 2-PAM Cl versus control group to assess oxime protection relative to each OP, i.e., Baseline PR.
- MMB4 DMS and HLö7 DMS versus 2-PAM Cl to assess protection relative to 2-PAM Cl, i.e., Comparative PR.

Separate probit dose-response models were fitted to the survival data versus log₁₀ (oxime dose) for each of the three agent/oxime datasets. The ED₅₀ and 95 percent confidence intervals (CI) were estimated using the Fieller's method (Finney, 1971) for each treatment group. All results are reported at the 0.05 level of significance.

3. Results

3.1. Clinical manifestation

Administration of OPs to animals designated as agent only + atropine exhibited severe cholinergic signs with complete lethality by 24 h. For all GB only + atropine animals, lethality was marked by 30 min post challenge. Animals administered PHO only + atropine were observed with mild signs by 30 min but quickly deteriorated to severe signs by 1 h post challenge. All lethality for animals administered PHO only + atropine were observed between 2–4 h post challenge. The latent period for animals administered VX only + atropine was indicated by moderate signs of cholinesterase inhibition by 30 min post challenge and lethality extended to 2–24 h post challenge.

The progression of cholinergic signs in those animals administered oxime (2-PAM Cl, MMB4 DMS, or HLö7 DMS) were similar to agent only + atropine animals. However, the administration of oxime delayed the severity of signs indicated by higher quality of life. In general, severity of cholinergic signs was similar across all three oximes within a given OP class.

3.2. Protective ratio determination

Baseline PRs were determined using the observed LD₅₀ value of the OP in animals receiving atropine + oxime treatment divided by the LD₅₀ value of the OP in animals receiving atropine alone (control groups). For each OP studied, the percent lethality of the atropine only control groups were at or above 50% with the exception of VX. Co-administration of MMB4 DMS, HLö-7DMS, or 2-PAM Cl with atropine increased the LD₅₀ values of GB, VX, and PHO relative to atropine only animals, i.e., the observed percent lethality decreased in the presence of the oximes. All three atropine + oxime combinations outperformed their counterpart atropine-only control groups in the study. Notably, overall survivability after VX exposure increased to 100% when any one of the three oximes was co-administered with

atropine compared to atropine alone treatments (data not shown). Moreover, against VX, MMB4 DMS and HLö-7 DMS exhibited the two highest statistically significant baseline PRs attained in the study (Table 1).

Comparative PRs, used here to evaluate the potential differences in efficacy of MMB4 DMS and HLö-7 DMS versus 2-PAM Cl, were also determined. Potential difference was. A comparative PR > 1 would indicate the oxime is superior to 2-PAM Cl for that particular OP. Of MMB4 DMS and HLö-7 DMS, the comparative PRs of the former exceeded 1 for all three OPs evaluated (Table 2). Baseline and comparative PRs of MMB4 DMS and HLö-7 DMS are plotted in Figs. 1 and 2.

All therapies against GB provided significant protection, with lethality being less than 20% as compared to the atropine control group at the LD₅₀ challenge dose. There was no statistical significance when MMB4 DMS and HLö-7 DMS were compared to 2-PAM Cl; however, MMB4 DMS did have an approximate 2-fold improvement in protection.

A significant increase in the LD₅₀ occurred when any of the oximes was administered compared to atropine-only control animals against PHO. Therefore, all three oximes outperformed atropine alone with statistical significance at approximately baseline PR 2. Neither of the DMS oximes significantly outperformed 2-PAM Cl against PHO.

3.3. Median effective dose determination

Each positive control animal challenged and treated only with 0.4 mg/kg atropine died by 2-h post challenge. Treatment dose ranges for each oxime are as shown in Table 3.

3.4. MMB4 DMS

A summary of the proportion of survival when treated with MMB4 DMS is provided in Fig. 3. Treatment doses with MMB4 DMS below 1.0 mg/kg resulted in no survival when challenged with GB. Survival was observed at doses above 3.1 mg/kg and complete survival was observed at 65.5 mg/kg (equimolar to the maximum FDA-recommended pre-hospital dose of three autoinjectors of 2-PAM Cl). The ED₅₀ calculated for MMB4 DMS against 2 × 24-h LD₅₀ GB was estimated to be 6.8 mg/kg (95% CI = 2.5, 19) (Table 4).

For VX challenged groups treated with MMB4 DMS, there was a threshold in terms of survival through 24 h. Therapy levels up to 6.0 mg/kg promoted a cumulative survival rate of 15% with clinical observations that were indicative of severe cholinergic impairments. When treated with MMB4 DMS doses of 7.0, 11.0, 22.0, and 65.5 mg/kg, a 95% survival rate was observed against VX with clinical observations ranging from normal to severe. The ED₅₀ calculated for MMB4 DMS against 2 × 24-h LD₅₀ VX was estimated to be 5.3 mg/kg (95% CI = 1.8, 15).

MMB4 DMS dose level ranges were higher for PHO challenges as compared to those against GB and VX. Approximately 27% survival was observed against PHO, with MMB4 DMS doses from 11.0 to 32.7 mg/kg. The groups treated with 50.0, 65.5, and 89.7 mg/kg exhibited 80% survival. The two highest dose groups, 135 and 200 mg/kg, had complete

survival. The ED₅₀ calculated for MMB4 DMS against 2 × 24-h LD₅₀ PHO was estimated to be 29 mg/kg (95% CI = 17, 47).

3.5. HLö-7 DMS

A summary of the proportion of survival when treated with HLö-7 DMS is provided in Fig. 4. Treatment with doses of HLö-7 DMS was inconsistent across all OPs. Survival ranged from 20%–60% with HLö-7 DMS doses of 3.1–30 mg/kg. The treatment dose level of 39.1 mg/kg (80% survival) demonstrated the highest survival. Treatment levels of 49.5, 76.1 (equimolar to three autoinjectors of 2-PAM Cl), 90 and 104 mg/kg demonstrated the same survival rate of 60%. When treated with a dose of 150 mg/kg, survival was only 40%. Deaths in this treated group occurred in a shorter period of time than any other group except the control group animals. The highest delivered dose of 180 mg/kg, resulted in 0% survival (data removed from ED₅₀ calculation). The ED₅₀ calculated for HLö-7 DMS against 2 × 24-h LD₅₀ GB was estimated to be 38 mg/kg (95% CI = 18, 86).

For all HLö-7 DMS treatment levels from 1.0 to 150 mg/kg against VX except for the group treated with 65.0 mg/kg, no difference was seen in terms of lethality or time to death. At 65.0 mg/kg, 100% survival was accomplished with a marked improvement in cholinergic signs throughout the study. The ED₅₀ calculated for HLö-7 DMS against 2 × 24-h LD₅₀ VX was estimated to be 66 mg/kg (95% CI = 29, 183).

No survival was seen in the HLö-7 DMS lowest treatment group against PHO. The treatment dose groups of 38.1 mg/kg and 76.1 mg/kg both had only 20% survival. Improvement in survival (80%) was observed in the 104.3 mg/kg treatment group even though quality of life for the survivors was poor. Complete survival was observed with the 135 mg/kg treated group. In the highest HLö-7 DMS treated group, 265 mg/kg, survival decreased to 20%. The ED₅₀ calculated for HLö-7 DMS against 2 × 24-h LD₅₀ PHO was estimated to be 96 mg/kg (95% CI = 61, 150). However, assuming that the highest dose group was in the toxicity range of the oxime, performing statistical analysis without this dose group decreased the ED₅₀ to 67 mg/kg (95% CI = 44, 101).

3.6. 2-PAM Cl

A summary of the proportion of survival when treated with 2-PAM Cl is provided in Fig. 5. For animals challenged with GB and treated with 2-PAM Cl, survival increased in increments from 20% to 60% in doses of 3.0–25.8 mg/kg. A decline in survival (40%) was observed at 70.6 mg/kg, with clinical signs ranging from normal to severe. At 100.7 mg/kg, 100% survival was provided without indications of 2PAM Cl toxicity. The ED₅₀ for 2-PAM Cl against 2 × 24-h LD₅₀ GB was estimated to be 26 mg/kg (95% CI = 11, 70).

Treatment groups against VX demonstrated a fluctuation in survival from the lowest to the highest treatment level. All animals treated with 20 mg/kg, survived. The calculated ED₅₀ was 62 mg/kg (95% CI = 28,

184).

Similar to the CWNAs, therapy with 2-PAM Cl against PHO varied. 60% survival with normal clinical signs was seen at a dose of 35.3 mg/ kg. Survival increased and quality of life improved with the three highest 2-PAM Cl treatment groups of 60.0, 86.0 and 100 mg/kg. Survivors in these treatment groups were observed as normal at termination. The ED₅₀ calculated for 2-PAM Cl against 2 × 24-h LD₅₀ PHO was estimated to be 31 mg/kg (95% CI = 19, 50).

4. Discussion

considered 'leads' and compare this information against the current U.S.-deployed oxime option, namely 2-PAM Cl. Although promising, it is important to bear in mind that the guinea pig efficacy data presented here can only serve as an illustrative prediction of potential effectiveness in humans. This is especially true as recent studies have demonstrated that just a few amino acid differences within the active site gorge of the guinea pig and human AChE can result in different rates of reactivation to the same oximes (Cadieux et al., 2010). The observed difference in the various oxime-mediated reactivation profiles suggests that the human enzyme may react more favorably (and thus requiring a lower dose) than that of the guinea pig. Accordingly, it has been argued that the guinea pig may not be the most relevant animal model for humans (Cadieux et al., 2010). However, due to its low level of serum carboxylesterase (CaE), the guinea pig is still the most physiologically relevant natural small mammal to humans (Lenz et al., 2017). It is also important to bear in mind that pyridostigmine bromide (a reversible AChE-inhibitor) was approved by the U.S. FDA as a pretreatment for the CWNA soman based on efficacy data derived from the guinea pig model, in addition to non-human primates (FDA, <https://www.fda.gov/newsevents/newsroom/pressannouncements/ucm130342>). Similarly, more recent advanced development efforts within the Department of Defense have utilized the guinea pig (and non-human primates) model to formally replace the current FDA-approved treatment for CWNA-induced seizures (i.e., diazepam) with an improved benzodiazepine anticonvulsant (i.e., midazolam) (<http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.895.6880&rep=rep1&type=pdf>). Based on its lack of serum CaE, historical use and familiarity to the FDA, the guinea pig may still be considered a viable animal model over other lower order mammals.

Efficacy, using the standardized equimolar (146 µmol/kg) approach as described by Wilhelm et al. (2014), was first demonstrated by determining the PRs after treatments with the dimethanesulfonate salts of MMB4 and HLö-7 and the chloride salt of 2-PAM in OP-challenged guinea pigs. ED₅₀ values were then determined to identify a potential dose of each oxime that can offer protection across all three OPs and to calculate the TI (i.e., ratio of the LD₅₀ to the ED₅₀ for a given drug). A small ED₅₀ value is desirable as it indicates less drug is needed to protect half of the population challenged. Conversely, a large TI value is desired since it indicates a wider margin/window of safety.

The identified baseline PRs, ED₅₀s, and TIs of MMB4 DMS, HLö-7 DMS, and 2-PAM Cl against GB, VX, and PHO are as reported in Tables 1 and 4. Based on the baseline PR data presented here, 2-PAM Cl was shown to provide ~2 to 7 times more protection against the three OPs evaluated as compared to animals administered only HED of atropine (i.e., non-oxime treated). This result reinforces the notion that atropine and 2-PAM Cl offer protection

in a complementary manner (Sidell and Borak, 1992). The ED₅₀ values of 2-PAM Cl against both GB and PHO are 26 and 31 mg/kg, respectively. These levels closely correspond to the calculated HED of three DuoDotes® of 2-PAM Cl (25.7 mg/kg as described in Wilhelm et al. (2014) and Brittain et al. (2016)) for guinea pigs. Conversely, the ED₅₀ of 2-PAM Cl against VX was 62 mg/kg, slightly more than twice as high as what was observed for the other two OPs tested. Together, the data suggest that at the currently recommended pre-hospital dose of 2-PAM Cl, protection against up to a 2 × 24 h LD₅₀ OP exposure would only be expected if the OP was either PHO or GB. If the OP was VX, the recommended dose would likely not be sufficient thus necessitating higher levels of the oxime. Unfortunately, pre-hospital administration of additional 2-PAM Cl may not be possible (especially in the absence of adequate supportive care) due to its relatively low TI that is only between 3 and 6.

No HLö-7 DMS dose above 80 mg/kg demonstrated greater than 60% survival against either GB or VX. The ED₅₀ values of HLö-7 DMS for VX and PHO are 66 and 96 mg/kg, respectively. These values are approximately 1.7 and 2.5 times higher than the ED₅₀ against GB (38 mg/kg) suggesting that this oxime would be more efficacious as a therapy for GB than either VX or PHO. Interestingly, this result is not consistent with the results of the baseline PR studies, where HLö-7 DMS had a significantly higher baseline PR against VX than for both GB and PHO. Additionally, as shown in Fig. 4, the 180 mg/kg HLö-7 DMS dose utilized for GB produced complete group mortality within minutes of treatment. A similar increase in mortality was also observed after VX challenge at the 150 mg/kg HLö-7 DMS dose. Based on these observations, it might be postulated that doses of 150 mg/kg and up were approaching the LD₅₀ of the oxime itself. However, this may not be the case as the LD₅₀ of HLö-7 DMS was previously reported to be 314 mg/kg (Snider et al., 2016a), which is approximately twice the level administered here.

Although the doses administered were about half of the reported LD₅₀, the observed increase in mortality rates may still be due, in part, to the inherent toxicity of the oxime, HLö-7 DMS. For example, oxime-induced AChE inhibition can occur which may then lead to transient neuromuscular blockade resulting in systemic muscle weakness (Bartosova et al., 2006; Gupta, 2015; Taylor, 2001). This toxic effect could potentially then be magnified as the dose of the oxime continues to increase. The resultant oxime-induced toxicity when combined with the already toxic anti-ChE effect of the CWNAs could conceivably exacerbate the overall toxicity and lethality observed in the studies thus serving to potentially confound the efficacy results.

In contrast to the GB and VX study results, where protection never exceeded 60% after 80+ mg/kg HLö-7 DMS, the PHO-challenged groups produced increased survival up to 100% at 135 mg/kg before group lethality began to occur. This discrepancy may be attributable to differences in toxicokinetics of PHO versus CWNAs, where, when the level of exposure is controlled, the latter have been found to be substantially more lethal with faster onset of toxicity than the former (Wilhelm et al., 2014; Snider et al., 2016a,b). This delay in onset of toxicity for PHO may explain why a higher level of HLö-7 DMS is necessary to obtain the same 50% protection of the study population (ED₅₀ value of 96 mg/kg), i.e., to ensure enough of the oxime is still in circulation when PHO begins to exert its toxic effects. It

should be noted that similar to 2-PAM Cl, the TI of HLö-7 DMS ranged between 3 and 8. This suggests that the safety window of HLö-7 DMS is just as narrow as 2-PAM Cl, thus additional administration of this oxime is likely to also be detrimental in the absence of supportive care.

Similar to 2-PAM Cl and HLö-7 DMS, the inclusion of MMB4 DMS with atropine consistently offered better protection than atropine alone against all OPs. Relative to 2-PAM Cl, MMB4 DMS consistently demonstrated higher comparative PRs (Table 2), indicating potential superiority when administered at the equimolar level. Additionally, unlike the other two oximes, increasing doses of MMB4 DMS did not increase overall group mortality against any of the three OPs tested in the ED₅₀ determination studies. In fact, all ED₅₀ values determined for MMB4 DMS were far below the previously tested dose level of 65.3 mg/kg used in the PR studies. A dose of 65.3 mg/kg MMB4 DMS was chosen since it represents the molar equivalent of the approved pre-hospital HED of 2-PAM Cl.

For VX, the ED₅₀ value parallels that for GB at 5.3 and 6.8 mg/kg, respectively. Although the ED₅₀ of MMB4 DMS for PHO was 29 mg/kg (or approximately five times higher than for the CWNAs), it was still substantially lower than the 2-PAM Cl equimolar dose of 65.3 mg/kg. With a LD₅₀ of 679 mg/kg (Snider et al., 2016a), the TI for MMB4 DMS ranged from 23 to 128. The substantially higher TI displayed by MMB4 DMS indicates that higher doses of this oxime could still be administered before concerns regarding oxime-induced toxicity arise. This is further confirmed in Fig. 3, where MMB4 DMS doses up to 200 mg/kg was still extremely efficacious. Together these results suggest that an increased dose of MMB4 could prove protective against higher levels of OP exposure.

Overall, both MMB4 DMS and HLö-7 DMS provided significant protection against GB, VX, and PHO as compared to atropine controls. All three oxime therapies offered complete protection against VX when delivered at the equimolar level to the FDA-approved 2-PAM Cl prehospital dose. Of the three OPs tested, PHO generated the lowest baseline PR values for all three oximes (ranging between 2–2.63), this suggests that it may be the ‘toughest’ OP to protect against under this paradigm. Nevertheless, 100% survivability would still be expected against an approximate LD₉₀ of PHO if either MMB4 DMS or HLö-7 DMS was administered at a treatment dose equimolar to 2-PAM Cl.

5. Conclusion

Overall, the guinea pig-derived efficacy data presented here demonstrate a lower quantity of MMB4 DMS is likely to afford the same or better level of protection against GB, VX, and PHO, as afforded by much higher levels of either HLö-7 DMS or 2-PAM Cl. Most importantly, the TI of MMB4 DMS was at least ~4 to ~43-fold higher across the three OPs tested than the other two oximes. Considering the potential for oxime-induced toxicity, it is safe to presume the lower the oxime dose necessary for protection, the better. As such, based on the results described here, an alternative oxime option with MMB4 DMS is likely the more superior candidate than HLö-7 DMS to potentially replace 2-PAM Cl as the AChE reactivator of choice.

Acknowledgments

The authors wish to recognize the excellent technical assistance of Jennifer Webb, Ashley Robertson, Erin Abele, Beth Reed, Ernest Johnson, Nancy Niemuth and Claire Matthews. Additionally, we thank the Medical Countermeasure Systems Joint Project Management Office, Department of Defense, for providing the oxime MMB-4 DMS through an agency to agency material transfer.

The authors also wish to thank Countermeasures Against Chemical Threats (CounterACT) Program Steering Committee (CPSC) members, Drs. John H. McDonough and Irvin Koplovitz (U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland, U.S.A.) and Judith Laney (HHS Biomedical Advanced Research & Development Authority) for their expertise and guidance in the design of this study.

Funding

This work was supported by the National Institutes of Health (NIH) Office of the Director through an interagency agreement (OD#: Y1- OD0387-01) between the National Institute of Allergy and Infectious Diseases (NIAID) and Department of Defense (DoD) and prepared under the auspices of the NIH, NIAID, NINDS, and the DoD Defense Technical Information Center (DTIC) under the Chemical, Biological, Radiological & Nuclear Defense Information Analysis Center (CBRNIAC) program, Contract No. SP0700-00-D-3180, Delivery Order Number 0687, CBRNIAC Task 832/CB-IO-OOI2.

The views expressed in this article are those of the authors and do not reflect the official policy of the NIH, Department of Health and Human Services, or the U.S. Government. No official support or endorsement of this article by the NIAID, NINDS, NIH, or DoD is intended or should be inferred. The experimental protocol was approved by the Institutional Animal Care and Use Committee at Battelle. All procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89544), as amended. The NIH sponsor developed the concept of the study and contributed to its design and the interpretation of the data as well as the preparation of the manuscript and the decision to submit it for publication. The sponsor also made similar contributions to other studies occurring at Battelle during the same time frame.

References

- Bahar FG, Ohura K, Ogihara TI, Imai T, 2012 Species differences of esterase expression and hydrolase activity in plasma. *J. Pharm. Sci* 101 (10), 3979–3988. [PubMed: 22833171]
- Bajgar J, 2004 Organophosphates/nerve agent poisoning: mechanism of action, diagnosis, prophylaxis, and treatment. *Adv. Clin. Chem* 38, 151–216. [PubMed: 15521192]
- Baker A, 2013 Syrian Opposition Says Deadly Chemical Attack Kills Hundreds in Damascus. *Time*. 21-Aug.
- Bartosova L, Kuca K, Kunesova G, Jun D, 2006 The acute toxicity of acetylcholinesterase reactivators in mice in relation to their structure. *Neurotox. Res* 9, 291–296. [PubMed: 16782588]
- Bierwisch A, Wille T, Thiermann H, Worek F, 2016 Kinetic analysis of interactions of amodiaquine with human cholinesterases and organophosphorous compounds. *Tox. Lett* 246, 49–56.
- Brittain MK, McGarry KG, Moyer RA, Babin MC, Jett DA, Platoff GE, Jr., Yeung DT, 2016 Efficacy of recommended prehospital human equivalent doses of atropine and pralidoxime against the toxic effects of carbamate poisoning in the Hartley guinea pig. *Int. J. Toxicol* 35 (3), 344–357. [PubMed: 27102179]
- Cadieux CL, Broomfield CA, Kirkpatrick MG, Kazanski ME, Lenz DE, Cerasoli DM, 2010 Comparison of human and guinea pig acetylcholinesterase sequences and rates of oxime-assisted reactivation. *Chem. Biol. Interact* 187 (1–3), 229–233. [PubMed: 20433814]
- Dolgin E, 2013 Syrian gas attack reinforces need for better anti-sarin drugs. *Nat. Med* 19 (10), 1194–1195. [PubMed: 24100968]
- Eddleston M, Buckley NA, Eyer P, Dawson AH, 2008 Management of acute organophosphorus pesticide poisoning. *Lancet* 371, 597–607. [PubMed: 17706760]
- Finney DJ, 1971 Probit Analysis, Third edition. Cambridge University Press, Cambridge, England.
- Gupta RC, 2015 Handbook of Toxicology of Chemical Warfare Agents, Second edition. pp. 1057–1070 Chapter 71.

- Lenz DE, Cerasoli DM, Maxwell DM, 2017 Radiolabeled Soman binding to Sera from rats, guinea pigs and monkeys. *Toxicol. Lett.* 283.
- Maxwell DM, Brecht KM, Koplovitz I, Sweeney RE, 2006 Acetylcholinesterase inhibition: does it explain the toxicity of organophosphorus compounds? *Arch. Toxicol* 80 (11), 756–760. [PubMed: 16770629]
- Okumura T, Hisaoka T, Naito T, Isonuma H, Okumura S, Miura K, Maekawa H, Ishimatsu S, Takasu N, Suzuki K, 2005 Acute and chronic effects of sarin exposure from the Tokyo subway incident. *Environ. Toxicol. Pharmacol* 3, 447–450.
- Radi Z, Dale T, Kovarik Z, Berend S, Garcia E, Zhang L, Amitai G, Green C, Radi B, Duggan BM, Ajami D, Rebek J, Taylor P, 2013 Catalytic detoxification of nerve agent and pesticide organophosphates by butyrylcholinesterase assisted with non-pyridinium oximes. *Biochem. J* 450 (1), 231–242. [PubMed: 23216060]
- Sidell FR, Borak J, 1992 Chemical warfare agents: II. Nerve agents. *Ann. Emerg. Med.* 21 (July (7)), 865–871. [PubMed: 1610046]
- Snider TH, Babin MC, Jett DA, Platoff GE, Jr., Yeung DT, 2016a Toxicity and median effective doses of oxime therapies against percutaneous organophosphorous pesticide and nerve agent challenges in the Hartley guinea pig. *J. Toxicol. Sci* 41 (4), 511–521. [PubMed: 27432237]
- Snider TH, McGarry KG, Babin MC, Jett DA, Platoff GE, Jr., Yeung DT, 2016b Acute toxicity of phorate oxon by oral gavage in the Sprague-Dawley rat. *Fundam. Toxicol. Sci* 3 (5), 195–204. [PubMed: 28856079]
- Taylor PW, 2001 Anticholinesterase agents In: Hardman G, Limbird LE, Gilman AG (Eds.), Goodman & Gilman's The Pharmacological Basis of Therapeutics, 10th ed. McGraw-Hill, New York, pp. 175–192.
- Taylor PW, 2017 In: Brunton LL (Ed.), Anticholinesterase Agents. Goodman & Gilman's The Pharmacological Basis of Therapeutics, 13th ed. McGraw-Hill, New York, pp. 239–254.
- Thiermann H, Worek F, Kehe K, 2013 Limitations and challenges in treatment of acute chemical warfare agent poisoning. *Chem. Biol. Interact* 206 (3), 435–443. [PubMed: 24091052]
- US Department of Health and Human Services, Food and Drug Administration, 2005 Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. *Pharmacology and Toxicology*. 7..
- Wei Z, Liu Y, Wang Y, Li W, Zhou X, Zhao J, Huang C, Li X, Liu J, Zheng Z, Li S, 2016 Novel nonquaternary reactivators showing reactivation efficiency for soman-inhibited human acetylcholinesterase. *Toxicol. Lett* 246, 1–6. [PubMed: 26809136]
- Wilhelm CM, Snider TH, Babin MC, Jett DA, Platoff Jr. GE, 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. [PubMed: 25448441]
- Winter M, Wille T, Musilek K, Kuca K, Thiermann H, Worek F, 2016 Investigation of the reactivation kinetics of a large series of bispyridinium oximes with organophosphate-inhibited human acetylcholinesterase. *Toxicol. Lett* 244, 136–142. [PubMed: 26210933]
- Worek F, Thiermann H, 2013 The value of novel oximes for treatment of poisoning by organophosphorus compounds. *Pharmacol. Ther* 139 (2), 249–259. [PubMed: 23603539]
- Worek F, Koller M, Thiermann H, Wille T, 2016 Reactivation of nerve agent-inhibited human acetylcholinesterase by obidoxime, HI-6 and obidoxime+HI-6: kinetic in vitro study with simulated nerve agent toxicokinetics and oxime pharmacokinetics. *Toxicology* 350–352, 25–30.
- Zurer P, 1998 Japanese cult used VX to slay member. *Chem. Eng. News* 76 (35), 7.

Fig. 1. Baseline (blue bar) and Comparative (red bar) Protective Ratio of MMB4 DMS, (*) Indicates statistical significance at the 0.05 level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 2.

Baseline (blue bar) and Comparative (red bar) Protective Ratio of HLö-7 DMS, (*) Indicates statistical significance at the 0.05 level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 3.
Mean proportion of survival for animals treated with MMB4 DMS (n =44/group) after challenge with VX (0.016 mg/kg), GB (0.096 mg/kg), and PHO (3.84 mg/kg).

Fig. 4. Mean proportion of survival for animals treated with HLö-7 DMS after challenge with VX (0.016 mg/kg; n = 66), GB (0.096 mg/kg; n = 61), and PHO (3.84 mg/kg; n = 44).

Fig. 5.
Mean proportion of survival for animals treated with 2-PAM Cl after challenge with VX (0.016 mg/kg; n = 66), GB (0.096 mg/kg; n = 44), and PHO (3.84 mg/kg; n = 44).

Table 1

Summary of the baseline Protective Ratios against subcutaneous challenge of selected organophosphate challenge.

OP	Oxime Therapy [^] (with atropine)	24-h Lethality	24-h Toxicity (mg/kg)			Slope	Baseline Protective Ratio	p value
			LD ₅₀	LCL	UCL			
GB	None	26/40	0.048	0.043	0.051	19.46		
	2-PAM Cl	15/36	0.332	0.139	1.495	1.28	6.97	< 0.0001 *
	HLö-7 DMS	17/36	0.268	0.103	1.081	1.24	5.62	0.0001 *
	MMB4 DMS	14/36	0.673	0.234	5.046	1.04	14.1	< 0.0001 *
VX	None	15/30	0.0081	0.007	0.0092	14.52		
	2-PAM Cl	11/24	0.0308	0.0164	0.0812	5.24	3.79	< 0.0001 *
	HLö-7 DMS	10/24	0.144	0.0282	0.3468	2.22	17.7	< 0.0001 *
	MMB4 DMS	10/24	0.1163	0.0379	0.3269	1.98	14.3	< 0.0001 *
PHO	None	13/28	1.92	1.53	2.28	9.44		
	2-PAM Cl	8/16	3.93	3.14	4.85		2.05	< 0.0001 *
	HLö-7 DMS	7/16	3.85	3.1	4.83		2	< 0.0001 *
	MMB4 DMS	8/16	5.05	3.85	6.84		2.63	< 0.0001 *

* Indicates significance at the 0.05 level.

[^] Oxime doses were delivered in equimolar to 2-PAM Cl (25.7 mg/kg) levels at HLö-7 DMS = 75.9mg/kg and MMB4 DMS = 65.3 mg/kg.

Table 2

Comparative Oxime Protective Ratio Relative to 2-PAM Cl.

OP	Treatment Comparison	Comparative Protective Ratio [^]	95% Delta Confidence Interval	P-value of Comparison
GB	2-PAM Cl vs. HLö-7 DMS	0.81	(0.23, 2.84)	0.7389
	2-PAM Cl vs. MMB4 DMS	2.03	(0.49, 8.35)	0.3266
VX	2-PAM Cl vs. HLö-7 DMS	4.67	(2.01, 10.82)	0.0003 *
	2-PAM Cl vs. MMB4 DMS	3.77	(1.57, 9.06)	< 0.0001 *
PHO	2-PAM Cl vs. HLö-7 DMS	0.98	(0.75, 1.28)	0.8799
	2-PAM Cl vs. MMB4 DMS	1.28	(0.94, 1.76)	0.1183

[^] Determined by dividing the baseline PRs of the indicated oxime by the corresponding baseline PR of 2-PAM Cl.

* Indicates significance at the 0.05 level.

Table 3.

Treatment Range by Oxime.

	OP	Oxime Dose Range (mg/kg)
MMB4 DMS	GB	0.4 – 65.5
	VX	1.0 – 65.5
	PHO	11.0 – 200.0
HLö-7 DMS	GB	3.1 – 180
	VX	1.0 – 150
	PHO	18.8 – 265
2-PAM Cl	GB	3.0 – 100.7
	VX	1.0 – 130.0
	PHO	6.4 – 100.0

Table 4.

Parallel Slope Models for OP x Oxime Treatments.

OP	Treatment	# of Animals	Slope	P-value Slope	ED ₅₀ (mg/kg)	95% Confidence Interval	Therapeutic Index ^a
GB	MMB4 DMS	44	1.07	<0.0001	6.8	(2.5, 19)	100
	HLö-7 DMS	61(*)			38	(18, 86)	8
	2-PAM Cl	44			26	(11, 70)	7
VX	MMB4 DMS	44	0.95	<0.0001	5.3	(1.8, 15)	128
	HLö-7 DMS	66			66	(29, 183)	8
	2-PAM Cl	66			62	(28, 184)	3
PHO	MMB4 DMS	44	2.3	<0.0001	29	(17, 47)	23
	HLö-7 DMS	44			96	(61, 150)	3
	2-PAM Cl	44			31	(19, 50)	6

* Data from the highest oxime dose group of 180 mg/kg was omitted.

^aThe therapeutic index is the ratio of the LD50 to the ED50 for a given oxime. The LD50 values for the oximes are summarized in Snider et al. (2016).