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EFFECTS OF 1,1′-OXYDIMETHYLENE BIS-(4-*TERT*-BUTYLPYRIDINIUM CHLORIDE) (SAD-128) AND DECAMETHONIUM ON REACTIVATION OF SOMAN- AND SARIN-INHIBITED CHOLINESTERASE BY OXIMES

LARREL W. HARRIS, WILLIAM C. HEYL, DAVID L. STITCHER and CLARENCE A. BROOMFIELD
Physiology/Pharmacology Branch, Biomedical Laboratory, Chemical Systems Laboratory, Aberdeen Proving Ground, MD 21010, U.S.A.

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Abstract—The effects of 1,1′-oxydimethylene bis-(4-*tert*-butylpyridinium chloride) (SAD-128) and decamethonium on reactivation by oxime of Soman- and Sarin-inhibited erythrocyte acetylcholinesterase (AChE; EC 3.1.1.7) are reported. The inclusion of SAD-128 or decamethonium (10^{-3} M) together with either TMB-4 or Toxogonin markedly augmented the reactivation of Soman- or Sarin-inhibited AChE; the recovery of enzyme activity was more than doubled in each instance when compared to the recovery in the presence of oxime alone. No reactivation was observed with SAD-128 or decamethonium in the absence of oxime. The i.v. LD_{50} of SAD-128 in rabbits is 11.9 mg/kg. When 5 mg/kg of SAD-128 was given i.v. to rabbits 2 min before poisoning with Soman, 15–20 per cent of the blood cholinesterase was protected from inhibition by Soman. The aging rate *in vitro* of Soman-inhibited erythrocyte AChE was decreased by a factor of 1.9 with 10^{-3} M SAD-128. A concentration of 1.4×10^{-4} M SAD-128 reduced the rate of acetylcholine hydrolysis by erythrocyte AChE *in vitro* to 50 per cent of control. We suggest that SAD-128 protects against inhibition by Soman due to its ability to function as a reversible inhibitor and that SAD-128 and decamethonium might be allosteric modifiers of the inhibited enzyme, rendering it more susceptible to reactivation by TMB-4 or Toxogonin.

While oxime derivatives of pyridinium salts are well established as reactivators of phosphonylated acetylcholinesterase (AChE) [1, 2], several of the compounds studied by Olidges and Schoene [3] and Schoene *et al.* [4] that were useful in the treatment of organophosphorus intoxication did not possess the oxime moiety. One such compound was 1,1′-oxydimethylene bis-(4-*tert*-butylpyridinium chloride) (SAD-128). This compound was shown to be beneficial in Soman poisoning when administered either prophylactically or therapeutically. Since the therapeutic administration was intramuscular (i.m.) 1 min after poisoning with Soman subcutaneously (s.c.), we theorized that, due to different routes of administration, SAD-128 could have been absorbed into the blood and carried to the enzyme more rapidly than Soman, and as a result some of the enzyme was shielded from phosphonylation. The possibility that SAD-128 protects against inhibition by Soman due to its ability to function as a reversible inhibitor of cholinesterase was tested. In addition, we explored the effects of SAD-128 on the aging rate of Soman-inhibited erythrocyte AChE, and the effects of SAD-128 and decamethonium (C_{10}) in conjunction with oximes on reactivation of Soman- or isopropyl methylphosphonofluoridate (Sarin)-inhibited AChE. The results are reported herein.

MATERIALS AND METHODS

Materials. 1,1′-Trimethylene bis-(4-formyl pyridinium bromide) dioxime (TMB-4), 1,1′-(oxydimethylene) bis-4-formyl pyridinium chloride dioxime (Toxogonin), pyridine-2-aldoxime methochloride (PAM), and atropine were obtained from commercial sources; SAD-128 was obtained from Research Division of Chemical Systems Laboratory. All experiments *in vivo* were conducted with male albino rabbits weighing 2.5 to 3.5 kg.

Blood cholinesterase assays. The method was similar to that reported by Siakotos *et al.* [5]. Whole blood (usually 0.6 ml) was withdrawn from the ear vein into a heparinized syringe. In animal studies, assays were made both before and after anti-AChE administration so that the enzyme level of each animal before poisoning served as the baseline for calculating subsequent inhibition. The blood was expressed into a disposable 10 × 75 mm glass tube, mixed, and assayed within 0.5 min for cholinesterase activity. A typical assay mixture consisted of 200 μl of whole blood, 50 μl of 0.2 M sodium phosphate, pH 7.6, with 0.03% Saponin and 50 μl of 3×10^{-2} M [^{14}C]acetylcholine (ACh). When human erythrocytes were used, the assay mixture consisted of 100 μl of lysed cells in Lubrol buffer (0.1 M sodium phosphate, pH 7.8,

containing 0.3 M NaCl and 1% Lubrol WX), 150 μl of double-distilled water, and 50 μl of 3×10^{-2} M ACh. The mixtures were incubated for 5 min at 37°. The reaction was terminated by addition of 5 ml of dioxane/resin. The samples were made up to 10 ml with dioxane, mixed, and centrifuged; 5 ml of the supernatant was transferred to 10 ml of the scintillation mixture and counted.

Effects of SAD-128 on inhibition of whole blood cholinesterase by Soman. The rabbits were protected with 8 mg/kg of atropine i.m. 7 min before poisoning with 13 μg/kg of Soman ($1.2 \times LD_{50}$), i.v.; SAD-128, 5 mg/kg, i.v., was injected 2 min before Soman. Whole blood was removed for cholinesterase determination at 5 min after atropine and at 5, 30 and 60 min after Soman.

Reactivation of Soman-inhibited rabbit whole blood cholinesterase by TMB-4 (alone and with SAD-128). Rabbits were given 8 mg/kg of atropine i.m. 5 min before poisoning with $1.2 \times LD_{50}$ Soman i.v. Three min after poisoning, 8.9 mg/kg of TMB-4 (alone or with 5 mg/kg of SAD-128) was administered i.v.; blood samples were collected for cholinesterase assay before, 2 min after Soman, and 57 min after oxime administration.

Effects of SAD-128 on human erythrocyte AChE undergoing aging after inhibition with Soman in vitro. Heparin-treated or EDTA-treated blood was centrifuged and erythrocytes were washed with 0.15 M NaCl containing 1 USP unit heparin/ml. Aging studies were performed as described by Fleisher *et al.* [6]. Erythrocytes were added to 0.01 M sodium borate–0.15 M NaCl, pH 8.8; the cell-bound AChE was inhibited with 5×10^{-8} M Soman for 10 min at 2°. During this time they were centrifuged for 5 min at 2000 rpm in an International model PR-2 centrifuge. Acetylcholinesterase inhibition was carried out at pH 8.8 and 2° to preclude premature aging [6]. In order to determine the level of reactivatable enzyme at the beginning of the study, 0.2 ml of sedimented erythrocytes was transferred into 0.05 M phosphate-buffered saline (PBS) composed of sodium phosphate–0.15 M NaCl, pH 7.3, and containing 0.1 M PAM. Controls, including Soman-treated cells incubated in the absence of PAM and cells not treated with Soman, were carried through the same experimental procedure. Aging of the remaining erythrocytes (2 ml) was immediately started by adding the cells to 16 ml of 0.1 M PBS, pH 7.3 and 37°, alone or with 10^{-3} M SAD-128 (final concn). Two-ml aliquots of the aging mixture were then removed at various time intervals up to 8 min into an equal volume of 0.2 M PAM in 0.15 M NaCl, pH 7.3. The cells were permitted to incubate with PAM for 1 hr at 25°. After centrifuging and removing the supernatant, erythrocytes were washed three times with 50 vols of 0.15 M NaCl–heparin/wash to remove excess oxime; 50 μl of erythrocytes was then mixed with 200 μl water + 200 μl Lubrol buffer for ChE assay.

Effects of SAD-128 or C_{10} on reactivation of unaged Soman- or Sarin-inhibited human erythrocyte AChE by oximes in vitro. Washed erythrocytes were treated with Soman and centrifuged as described above. Aliquots (0.2 ml each) of cells were put into 0.05 M PBS containing 1 USP unit heparin/ml, pH 7.3 and 37°, or buffer containing 10^{-3} M SAD-128 alone, 10^{-3} M TMB-4 (alone or together with 10^{-3} M SAD-128 or C_{10}), or 10^{-3} M Toxogonin, with and without SAD-128. Erythrocytes not exposed to Soman were carried through the treatments described above to serve as controls. The cells were permitted to incubate for 8 min at 37° followed by 52 min of incubation at room temperature. After centrifuging and removing the supernatant, each sample was washed free of excess pyridinium salts and processed for AChE activities as described above.

Sarin-inhibited erythrocyte AChE ages slowly with a half-time of 6 hr [7]. Thus, no precautions were taken to preclude premature aging. Erythrocytes were suspended in 0.05 M PBS, pH 7.3, and treated with 1×10^{-7} M Sarin for 15 min at 25°. After centrifuging, the sedimented cells were washed four times with 10 vols of 0.15 M NaCl/wash to remove any unreacted inhibitor. Six ml of sedimented cells then was transferred into 24 ml of 0.05 M PBS–heparin, pH 7.3, and divided into four aliquots and incubated at 37°. One hundred μl of 6×10^{-4} M TMB-4 or 100 μl of 6×10^{-4} M TMB-4 containing either 1.2×10^{-1} M SAD-128 or 1.2×10^{-1} M C_{10} in 0.05 M PBS–heparin, pH 7.3, was added to each of the three 12-ml aliquots of incubation mixture; the remaining solution was incubated in buffer only. One-ml aliquots from each were removed at 1, 2, 4, 8, 12, 16 and 32 min. The sedimented cells were washed and processed for enzyme activity as described earlier.

Inhibition of human erythrocyte AChE by SAD-128. One-tenth ml of erythrocytes was put into 6 ml of twice-distilled water; 0.1 ml of the resulting lysed cell suspension was placed into each 12-ml graduated centrifuge tube containing 0.05 ml of 0.2 M sodium phosphate buffer, pH 7.6. To each was added sufficient SAD-128 in a volume of 0.05 ml to produce a final concentration (in 0.3 ml) of 1, 10, 100, 200 and 1000 μM. After incubating for 10 min at 37°, 0.1 ml of 3×10^{-3} M ACh was added to each tube; after 5 min of incubation with ACh at 37°, the reaction was stopped with dioxane/resin and samples were processed for counting as described earlier.

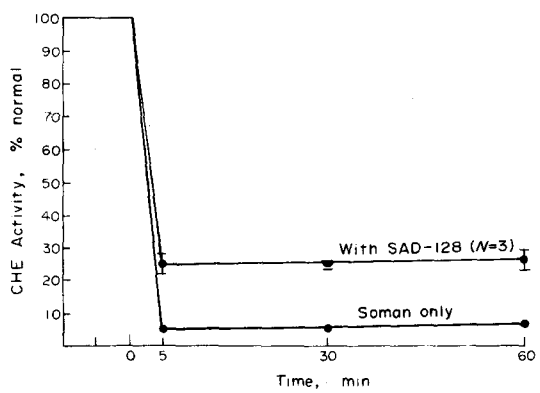

Fig. 1. Effects of SAD-128 on inhibition of whole blood cholinesterase (ChE) by Soman. SAD-128, 5 mg/kg, was administered i.v. 2 min before poisoning with Soman.

Table 1. Effects of TMB-4 alone and with SAD-128 on reactivation of Soman-inhibited rabbit whole blood cholinesterase *in vivo*

Treatment*	Per cent of inhibited blood cholinesterase reactivated†
TMB-4	14.8 ± 3.1
TMB-4 + SAD-128	18.5 ± 2.1

* TMB-4 (8.9 mg/kg), with and without 5 mg/kg of SAD-128, was given i.v. to atropinized rabbits 3 min after 1.2 × LD_{50} of Soman i.v.
† Mean ± S. D., N = 4.

RESULTS

Effects of SAD-128 on inhibition of whole blood cholinesterase by Soman in vivo. The data plotted in Fig. 1 show that SAD-128 caused the enzyme to be less sensitive to inactivation by Soman; those animals which received SAD-128 before Soman exhibited 15–20 per cent more enzyme activity than controls. Administration of SAD-128 at 5 min after Soman caused no change in cholinesterase activity.

Reactivation of Soman-inhibited rabbit whole blood cholinesterase by TMB-4 (alone and with SAD-128) in vivo. Table 1 presents the results of a study of whole blood cholinesterase levels after poisoning of the rabbits with Soman and treatment with 8.9 mg/kg of TMB-4 alone or with 5 mg/kg of SAD-128. Approximately 15 per cent of the inhibited cholinesterase is reactivated after TMB-4. The reactivation by TMB-4 was slightly increased (to 18.5 per cent) with SAD-128.

Effects of SAD-128 on human erythrocyte AChE undergoing aging after inhibition with Soman in vitro. Figure 2 presents the results of a study of the effects of 1×10^{-3} M SAD-128 on the rate of aging of Soman-inhibited erythrocyte AChE. The half-time ($T_{1/2}$) for aging is the time required for 50 per cent of the enzyme to become resistant to reactivation by oximes when compared to the amount reactivated at the time aging was initiated. The $T_{1/2}$ was calculated from the least squares fit of the first-order plot of percentage of AChE reactivated vs time. The $T_{1/2}$ for aging with and without SAD-128 was calculated to be 2.5 and 1.3 min respectively; the $T_{1/2}$ for aging was significantly retarded ($P < 0.001$, two-tailed *t*-test) by SAD-128.

Effects of SAD-128 or C_{10} on reactivation of unaged Soman- or Sarin-inhibited human erythrocyte AChE by oximes in vitro. We chose to use 1×10^{-3} M SAD-128 or C_{10} in the studies, the results of which are shown in Table 2 and Fig. 3, because ancillary studies revealed that this concentration of SAD-128 was most effective in enhancing enzyme reactivation by oximes. The data in Table 2 show that TMB-4 reactivation of Soman-inhibited enzyme was signifi-

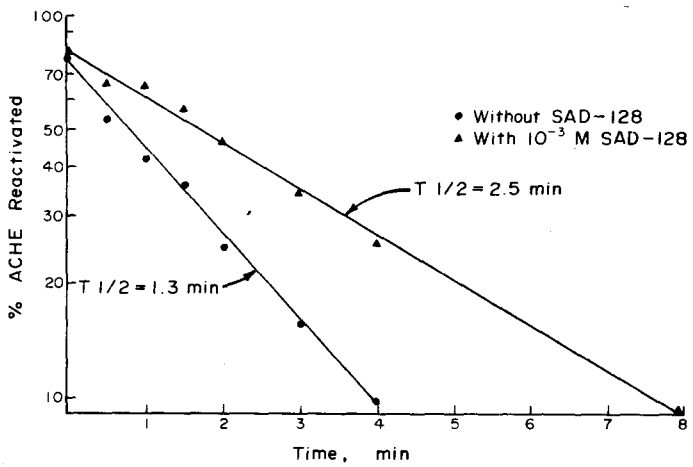

Fig. 2. Effects of SAD-128 on human erythrocyte AChE undergoing aging after inhibition with Soman *in vitro*. The study represents one of three separate experiments.

Table 2. Effects of oximes alone and with SAD-128 or C_{10} on reactivation of Soman-inhibited human erythrocyte AChE *in vitro*

Treatment*	No. of runs	Per cent AChE reactivated (mean ± S. D.)
SAD-128	12	1.9 ± 1.8
TMB-4	6	17.9 ± 7.2
TMB-4 + SAD-128	6	39.1 ± 8.6
TMB-4 + decamethonium (C_{10})	3	42.1 ± 10.7
Toxogonin	5	10.5 ± 2.1
Toxogonin + SAD-128	5	29.4 ± 1.5

* Soman-inhibited cells were incubated with 0.05 M phosphate buffer and 0.15 M NaCl, pH 7.3, containing 1×10^{-3} M oxime alone or with 1×10^{-3} M SAD-128 or C_{10}.

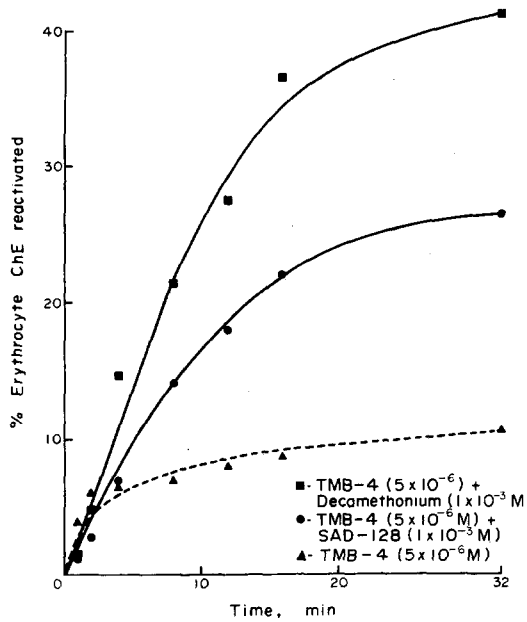

Fig. 3. Effects of SAD-128 or C_{10} on reactivation of Sarin-inhibited erythrocyte AChE by TMB-4 *in vitro*. Each point is the mean of two experiments.

cantly augmented ($P < 0.001$) by SAD-128 or C_{10}. The reactivation of the inhibited enzyme by Toxogonin was also enhanced by SAD-128. Figure 3 illustrates the effects of SAD-128 or C_{10} on the reactivation of Sarin-inhibited AChE by TMB-4; the percentage of AChE reactivated by TMB-4 was more than doubled when either SAD-128 or C_{10} was included with the oxime. Neither SAD-128 nor C_{10} produced any significant increase of enzyme activity in the absence of oxime.

Effects of SAD-128 on inhibition of erythrocyte AChE. A semi-logarithmic plot of percentage of AChE inhibited vs concentration of SAD-128 resulted in a straight line between 1 and 100×10^{-5} M SAD-128. The concentration (i_{50}) of SAD-128 required to inhibit 50 per cent of the AChE was estimated from the graph and was found to be 1.4×10^{-4} M.

DISCUSSION

As several selected pyridinium salts afforded some protective action in Soman intoxication [3, 4] and as SAD-128 was effective against Soman-poisoned mice both prophylactically and therapeutically [3], its action was studied more extensively in the present paper. The protective effect of SAD-128 cannot be explained by a direct reaction with Soman [3]. Our data show that SAD-128 cannot reactivate Sarin- or Soman-inhibited ChE. When SAD-128 is given prophylactically to Soman-poisoned rabbits, 15–20 per cent of the blood enzyme remains active, suggesting that some of the enzyme is protected from phosphonylation. SAD-128 is a reversible inhibitor of AChE *in vitro* with an i_{50} value of 1.4×10^{-4} M; it is, therefore, reasonable to assume that SAD-128 protects against inhibition by Soman due to its reversible anti-ChE properties. The findings of Schoene *et al.* [4] are in accord with our findings; they found that the compounds which proved beneficial against Soman *in vivo* were also able to protect the AChE from Soman *in vitro*.

The enchanced reactivation of the inhibited enzyme by oximes in the presence of quaternary amines was an unexpected observation. Pyridinium salts are inhibitors of AChE [4, 8], presumably by binding noncovalently to the anionic subsite. Thus, one might expect SAD-128 to compete with the pyridinium oximes and interfere with reactivation of the enzyme, because it is considered likely that the anionic subsite of the active center is involved in reactivation of the inhibited enzyme by pyridinium oximes [9]. However, our experiments produced no evidence of any interference by SAD-128 with reactivation by oximes (regardless of the oxime used). In fact, 10^{-3} M SAD-128 or C_{10} markedly ($P < 0.002$) enhanced reactivation of Sarin-inhibited AChE in the presence of as little as 5×10^{-6} M TMB-4 (Fig. 3).

It has been assumed for a long time that quaternary amines inhibit AChE by interaction at the anionic subsite of the active center. More recently, however, the kinetic data of Kuhnen [10] suggested that several pyridinium salts, including TMB-4 and Toxogonin, exhibit both competitive and noncompetitive inhibition of AChE. Wombacher and Wolf [11] and Roufogalis and Wickson [12] have provided evidence for a binding site on the enzyme separate and distinct from the catalytic (active) center. The kinetic data of Wombacher and Wolf [11] suggest that the "regulatory site" contains at least two negative charges and is devoid of catalytic activity. Furthermore, the binding of bis quaternary compounds to this site produced a conformational change at the active center. In light of these considerations, it is possible that SAD-128 and decamethonium also bind at the regulatory site and produce a conformational change at the active center of the inhibited enzyme, rendering it more susceptible to reactivation by oximes.

The pyridinium salts (2-PAM and SAD-128) have been shown to retard aging [6, 13]. The augmentative effect by SAD-128 on reactivation by oximes could be explained on this basis, because unaged, reactivatable enzyme would be available for a longer period of time. Aging, however, does not appear to play a prominent role in the enhanced reactivation of AChE by oximes in the presence of SAD-128, because SAD-128 also enhances the rate of reactivation of Sarin-inhibited erythrocyte AChE (Fig. 3). The $T_{1/2}$ for aging of Sarin-inhibited AChE at physiological pH and temperature is approximately 6 hr [7]. The dramatic increase in reactivation of Sarin-inhibited AChE by SAD-128–TMB-4 or C_{10}–TMB-4, when compared to TMB-4 alone, provides further support for the hypothesis that these bis quaternary compounds produce an allosteric modification of the AChE, rendering it more susceptible to reactivation by certain oximes. We have no explanation (except that C_{10}, due to its structure, might bind more readily to the regulatory site) for the markedly greater effect of C_{10} as compared to SAD-128 on enzyme reactivation by TMB-4. We should keep in mind that membrane-bound AChE might respond differently from solubilized enzyme, since the "modifiers" could also react with membranes and produce additional effects.

SAD-128 is rather toxic to rabbits; we have found the i.v. LD_{50} in rabbits to be 11.9 (6.8 to 20.9) mg/kg, $P = 0.05$). In view of the toxicity of SAD-128 it may not be possible to achieve an effective concentration *in vivo*. This could explain in part the contrast between the augmentive effects of SAD-128 on reactivation of Soman-inhibited AChE *in vivo* and *in vitro* by TMB-4. Moreover, the amount of reactivation by TMB-4 of the two phosphonylated blood enzymes (acetyl-ChE and butyryl-ChE) might be different; it is unlikely that this could account for the marginal effect of SAD-128 on TMB-4 reactivation *in vivo*, because inhibition of rabbit whole blood cholinesterase with BW284C51, a specific inhibitor of AChE [5], indicates that less than 10 per cent of the total hydrolysis of ACh is catalyzed by butyrylcholinesterase. Thus, the antidotal use of SAD-128 in combination with oximes *in vivo* may not be promising.

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