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Efficacy of a combination of acetylcholinesterase reactivators, HI-6 and obidoxime, against tabun and soman poisoning of mice

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Abstract. The bispyridinium oxime HI-6, 1-((((4-aminocarbonyl)pyridinio)methoxy) methyl)-2-(hydroxyimino)-methyl)pyridinium dichloride monohydrate, combined with atropine is an effective treatment for soman (pinacolyl methylphosphonofluoridate) poisoning but is relatively ineffective against tabun (ethyl N-dimethyl phosphoroamidocyanidate) poisoning in mice. This contrasts with those results obtained using the *bis*pyridinium oxime obidoxime[1,1'-(oxy *bis*(methylene)) *bis*(4-(hydroxyimino)-methyl) pyridinium dibromide]. The purpose of this study was to investigate the efficacy of the combination of HI-6 and obidoxime plus atropine against poisoning by tabun and soman in mice. The combination of ineffective single doses of obidoxime (5 or 10 mg/kg) and HI-6 (25 or 50 mg/kg) improved the treatment of tabun poisoning over either oxime alone. Combinations employing higher concentrations of obidoxime (25 or 50 mg/kg) and HI-6 (100 or 200 mg/kg) resulted in significant toxicity in the absence of organophosphate poisoning. Against soman poisoning the addition of obidoxime to HI-6 did not attenuate the efficacy of HI-6. The half-life of elimination and peak serum concentrations of HI-6 and obidoxime were not altered following administration of the combined injection. Reactivation of tabun-inhibited acetylcholinesterase was found consistently in the diaphragm but not in the brain. Using response surface methods it was possible to estimate the optimal therapy against soman and tabun poisoning (74.5 mg/kg HI-6 + 31.9 mg obidoxime against 1052 μg/kg challenge of tabun and 129 mg/kg HI-6 + 0 mg/kg obidoxime against 390 μg/kg challenge of soman). It is proposed that reactivation of tabun inhibited acetylcholinesterase at the diaphragm may be responsible for the increased efficacy of the combination of HI-6 and obidoxime against tabun poisoning in mice.

Key words: HI-6 1-((((4-aminocarbonyl)-pyridinio]methoxy]methyl)-2-((hydroxyimino)-methyl)pyrindium dichloride – Obidoxime 1,1'-(oxy*bis*(methylene))*bis*(4-hydroxyimino)-methyl) pyridinium dibromide – Soman (pinacolyl methylphosphonofluoridate) – Tabun (ethyl N-dimethylphosphoramidocyanidate) – *Bis*pyridinium – Oximes – Reactivation

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Introduction

In recent years the oxime HI-6, 1-((((4-aminocarbonyl)pyridinio) methoxy) methyl)-2-(hydroxyimino)methyl) pyridinium dichloride monohydrate, when combined with the cholinolytic atropine, has emerged as a very effective therapeutic drug in treatment of poisoning by soman (pinacolyl methylphosphonofluoridate; Wolthuis and Kepner 1978; Maksimović et al. 1980; Bošković 1978; Clement 1981, 1983; Inns and Leadbeater 1983; Bošković et al. 1984) sarin (isopropyl methylphosphonofluoridate; Schoene and Oldiges 1973; Maksimović et al. 1980; Inns and Leadbeater 1982; Clement 1983) and VX (O-ethyl S-[2-(diisopropylamino)ethyl]methylphosphonothioate; Wilhelm et al. 1979; Maksimović et al. 1980; Inns and Leadbeater 1983). However HI-6 was relatively ineffective against poisoning by tabun (ethyl N-dimethyl phosphoroamidocyanidate; Maksimović et al. 1980; Clement 1983; Inns and Leadbeater 1983; Cetković et al. 1984). HI-6 is a powerful reactivator of *unaged* soman- (Bošković 1979; Maksimović et al. 1980; Benschop et al. 1984; De Jong and Wolring 1984), sarin- (Maksimović et al. 1980; Clement 1982) and VX-inhibited acetylcholinesterase (Maksimović et al. 1980). However, HI-6 either does not or is a *very weak* reactivator of tabun inhibited acetylcholinesterase (Clement 1982; Cetković et al. 1984).

The efficacy and reactivation properties of HI-6 against extremely toxic organophosphates combined with its relatively low toxicity and high safety (Maksimović et al. 1980; Clement 1983; Bošković et al. 1984) and the fact that HI-6 was well tolerated in man (Kusić et al. 1985) make it an attractive compound for inclusion in the therapy of nerve agent poisoning. From the military point of view, it is important that the nerve agent therapy employed, for instance the oxime reactivator, be effective against all potential threat nerve agents, of which tabun is still considered one (Meselson and Robinson 1980). Obidoxime [1,1'-)-oxy*bis*(methylene))*bis*)4-(hydroxyimino)methyl) pyridinium dibromide] is a very effective reactivator of tabun-inhibited acetylcholinesterase (Heilbronn and Sundwall 1964; Puu et al. 1986) and when combined with atropine is one of the best therapies against tabun poisoning (Maksimović et al. 1980; Clement 1983). However, obidoxime is ineffective in treatment of soman poisoning (Clement 1983).

The purpose of this study was to investigate the efficacy of a combination of HI-6 and obidoxime against tabun

and soman poisoning in mice. By utilizing response surface methods (RSM; Carter et al. 1985) it was possible to optimize HI-6 and obidoxime therapy against either tabun or soman poisoning. Consideration of confidence regions about the optimum provided information about the efficacy of the optimum therapy combination against tabun and soman poisoning in mice.

Materials and methods

Toxicology. Male mice (CD-1^R; 25–30 g) were obtained from Charles River Canada Ltd., St. Constant, Quebec. The mice were acclimatized in our vivarium for at least 1 week following their arrival prior to use in these experiments. Mice had free access to food and water before and after drug exposure. The light-dark photoperiod was 12 h in duration with lights on at 0700 hours.

Mice were pretreated with atropine (17.4 mg/kg) plus oxime(s) by intraperitoneal (i. p.) injection 5 min prior to receiving tabun (1052 μg/kg) or soman (390 μg/kg) by subcutaneous (s. c.) injection. The 24 h s. c. LD_{50} for tabun and soman in our laboratory was 292 μg/kg and 130 μg/kg, respectively. The oximes plus atropine were administered 5 min prior to organophosphate. Significant differences ($p < 0.05$) were determined using Fisher's Exact test.

Acetylcholinesterase determination. Acetylcholinesterase activity was determined by the radiometric procedure of Siakotos et al. (1969) as described in Clement (1982). Significant differences were calculated using the Newman-Keuls method of multiple comparisons. A $p < 0.05$ was considered significant.

Pharmacokinetics. Mice were injected i. p. with either HI-6 (50 mg/kg), obidoxime (50 mg/kg) or HI-6 plus obidoxime (50 + 50 mg/kg). At various times after injection (3, 7, 10, 15, 20, 30, 45 and 60 min) mice were sacrificed by decapitation and blood was collected directly into Microtainer (Becton Dickenson) serum separator tubes. These were centrifuged at 3000 *g*, immediately frozen and stored at −20° C until analysis for HI-6 and obidoxime by HPLC (Clement et al. 1987).

Statistical analysis. As a result of the agents used, it would be expected that the proportion of animals surviving would increase to a point where treatment toxicity would exceed therapeutic effect, and then decrease as treatment levels increase beyond that point. The logistic model, quadratic in its argument, is used to describe such a relationship, where

$$P(\text{survival}) \frac{1}{1 + \exp^{-f(x)}} \qquad (1)$$

where

$$f(x) = \beta_0 + \beta_1 \chi_1^2 + \beta_2 \chi_2 + \beta_{11} \chi_1^2 + \beta_{22} \chi_2^2 + \beta_{12} \chi_1 \chi_2$$

and

χ_1 = dose of HI-6 (mg/kg)
χ_2 = dose of obidoxime (mg/ kg)
β_0 = an unknown parameter associated with the proportion of untreated survivors
β_1 = an unknown parameter associated with the effect on survival of HI-6
β_2 = an unknown parameter associated with the effect on survival of obidoxime

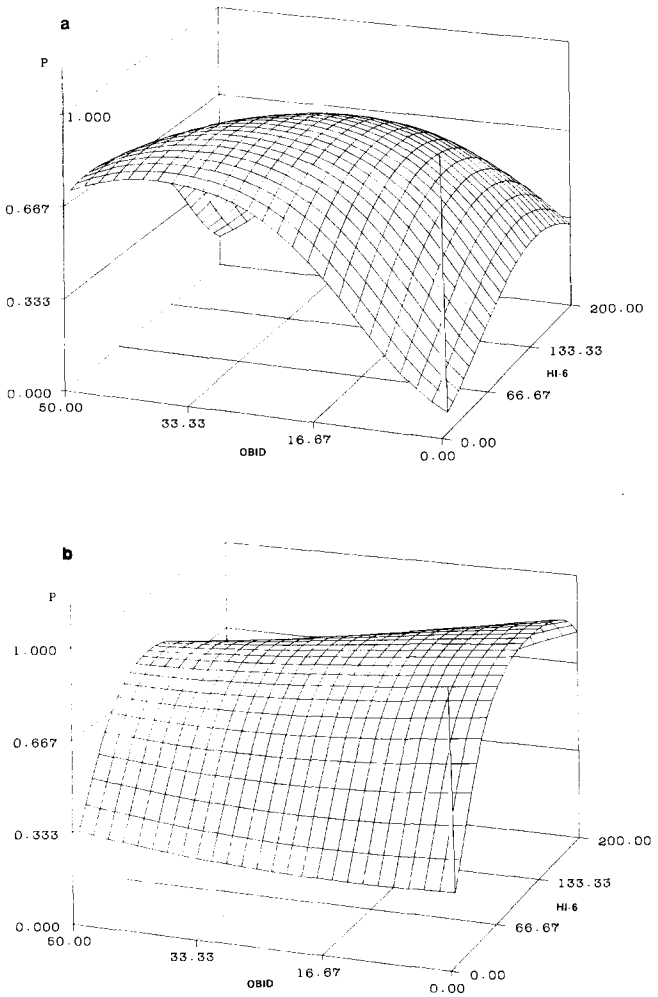

Fig. 1. Three-dimensional representation of survival (P axis) data as a function of HI-6 and obidoxime for fixed levels of atropine (17.4 mg/kg) and either tabun (1052 μg/kg) **(a)** or soman (390 μg/kg) **(b)**

β_{11} = an unknown parameter associated with the toxicity of HI-6
β_{22} = an unknown parameter associated with the toxicity of obidoxime
β_{12} = an unknown parameter associated with the interaction of HI-6 and obidoxime.

The model parameters were estimated from the experimental data by the method of maximum likelihood. The estimated surfaces are graphed in Fig. 1 (a, b). The *p*-values for the likelihood ratio test for the significance of the models are both < 0.0001. The parameter estimates, for both analyses, are given in Table 5. It should be noted that, with this model, positive coefficients are associated with agents of effects that tend to increase the probability of survival, while negative co-efficients are associated with agents of effects that tend to decrease the probability of survival.

Materials. Tabun, soman, HI-6 (Batch # DRES-32) and obidoxime were synthesized at DRES. All were in excess of 98% pure when examined by NMR and/or HPLC. Other chemicals used were obtained from common commercial sources.

Results

The results in Table 1 show the efficacy of HI-6 and obidoxime alone and in combination against tabun poisoning in mice. In the efficacy studies in Tables 1 and 2 atropine (17.4 mg/kg) was present in the same solution with the oxime(s). As was found by others, obidoxime was much more effective than HI-6 in the treatment of tabun (1052 μg/kg; s. c) poisoning. Addition of HI-6 (25, 50 or 100 mg/kg) increased significantly ($p < 0.05$) the efficacy of the lower doses of obidoxime (5 or 10 mg/kg) against tabun poisoning. At the higher obidoxime doses (25 or 50 mg/kg) addition of HI-6 did not significantly increase the antidotal effect. Conversely, addition of obidoxime to HI-6 significantly increased ($p < 0.05$) the antidotal effect. At the higher doses of HI-6 and obidoxime an increase in the toxicity of the treatment mixture in the absence of tabun poisoning was evident (Table 1).

In contrast to HI-6, obidoxime was ineffective against soman poisoning (*loc. cit.*). Thus, the effect of the addition of obidoxime on the efficacy of HI-6 against soman poisoning was investigated (Table 2). Addition of obidoxime did not significantly alter the efficacy of HI-6 against soman poisoning. The combinations of obidoxime (25 or 50 mg/kg) and HI-6 (100 or 200 mg/kg) produced an increase in lethality which was probably due to toxicity of the oxime combination as found in Table 1.

Following poisoning by soman and tabun in oxime-pretreated mice there did not appear to be any difference in the condition of the animals. They all exhibited marked signs of poisoning, e. g. tremors and fasciculations for hours after poisoning. By 24 h they appeared to have recovered completely.

Table 1. Efficacy of combination of HI-6 and obidoxime against tabun poisoning[a]

mg/kg	% Survivors				
HI-6 / Obidoxime	0	25	50	100	200
0	0	0	0	20	10
5	10	20	60[c, d]	70[c, d]	50
10	20	70[c, d]	70[c, d]	90[c, d]	60[d] (0)[b]
25	50[e]	60[d]	80[d]	60 (10)	40 (10)
50	90[e]	70[d]	80[d] (10)	30[c] (0)	30[c] (50)

[a] Mice were injected with atropine (17.4 mg/kg) or atropine (17.4 mg/kg) and HI-6 and/or obidoxime, i.p., 5 min before tabun (1052 μg/kg; s.c.). Mortality was assessed 24 h later. Each group was composed of ten animals
[b] Toxicity (%) resulting from a combination of both oximes + atropine (17.4 mg/kg) in the absence of exposure to tabun
[c] Significantly different ($p < 0.05$) from appropriate control group containing obidoxime + atropine only
[d] Significantly different ($p < 0.05$) from appropriate control group containing HI-6 + atropine only
[e] Significantly different ($p < 0.05$) from group receiving atropine only

Table 2. Efficacy of combination of HI-6 and obidoxime against soman poisoning[a]

mg/kg	% Survivors				
HI-6 / Obidoxime	0	25	50	100	200
0	0	70[b]	60[b]	80[b]	70[b]
5	10	70[c]	70[c]	80[c]	90[c]
10	0	70[c]	90[c]	80[c]	70[c]
25	0	70[c]	70[c]	40	60[c]
50	0	60[c]	90[c]	80[c]	20[d]

[a] Mice were injected with atropine (17.4 mg/kg) or atropine (17.4 mg/kg) and HI-6 and/or obidoxime, i.p., 5 min prior to soman (390 μg/kg, s.c.). Mortality was assessed 24 h later. Each group was composed of ten animals
[b] HI-6 plus atropine groups which were significantly different ($p < 0.05$) from control group receiving atropine only
[c] Significantly different ($p < 0.05$) from control group receiving obidoxime + atropine only
[d] Significantly different ($p < 0.05$) from control group receiving HI-6 + atropine only

Table 3. Effect of HI-6 and obidoxime on acetylcholinesterase activity following tabun poisoning[a]

Group	Dose mg/kg	Acetylcholinesterase activity[b]									
		Striatum	% Con	Pons-Medulla	% Con	Cortex	% Con	Diaphragm	% Con	Serum	% Con
Untreated control	–	29.02 ± 0.74[c]	100	7.02 ± 0.93	100	3.56 ± 0.17	100	0.81 ± 0.09	100	932 ± 113	100
Treated control	–	0.20 ± 0.02	0.69	0.06 ± 0.04	0.85	0.02 ± 0.01	0.56	0.04 ± 0.01	4.94	17 ± 9	1.8
Obidoxime + HI-6 + Atropine	50 50 17.4	0.38 ± 0.04	1.31	0.33 ± 0.03[d]	4.7	0.11 ± 0.02[d]	3.09	0.23 ± 0.02[d]	28.4	301 ± 48[d]	32.3
Obidoxime + Atropine	50 17.4	0.53 ± 0.12[d]	1.83	0.13 ± 0.01[e]	1.9	0.03 ± 0.01[e]	0.84	0.17 ± 0.02[d]	21.0	228 ± 32[d]	24.5

[a] Mice were injected with oxime(s) and atropine 5 min before receiving tabun (1052 mg/kg; s.c.). Mice were sacrificed 15 min after tabun and tissue homogenates prepared. With the group receiving tabun only, tissues were removed immediately upon cessation of breathing or following sacrifice at 15 min, whichever came first
[b] Acetylcholinesterase activity expressed as nmoles acethylcholine hydrolysed/mg tissue/min except for serum where activity expressed as nmoles acetylcholine hydrolysed/mL serum/min
[c] Mean ± SEM ($N = 4-5$)
[d] Significantly different ($p < 0.05$) from the group receiving tabun only
[e] Significantly different ($p < 0.05$) from the group receiving obidoxime (50) + HI-6 (50) + atropine (17.4)

Table 4. Pharmacokinetic parameters of HI-6 and obidoxime administered alone or as combined injection in mice[a]

Parameter	Alone		Combination	
	HI-6	Obidoxime	HI-6	Obidoxime
$t_{1/2}$[b] (min)	11.2	8.1	10.9	8.5
Peak level (μg/ml)	65.8 ± 13.7[c]	32.8 ± 13.1	79.8 ± 8.4	25.4 ± 11.9
Time to peak (min)	7	7	7	10

[a] Mice were injected with HI-6 (50 mg/kg) or obidoxime (50 mg/kg), i.p., alone or HI-6 plus obidoxime (50 + 50 mg/kg; i.p.) as a combined injection

[b] $t_{1/2}$ is the elimination half-life of the oximes

[c] Mean ± SD ($N = 5$)

In the case of tabun poisoning, reactivation of acetylcholinesterase in brain (cortex, pons-medulla and striatum), diaphragm and serum was evaluated (Table 3). Administration of obidoxime (50 mg/kg) only resulted in significantly higher acetylcholinesterase activity in striatum, diaphragm and serum compared to the tabun group in the absence of treatment. The addition of HI-6 (50 mg/kg) to obidoxime (50 mg/kg) resulted in significantly higher acetylcholinesterase activity in pons-medulla, cortex, diaphragm and serum following tabun poisoning. However, only in the pons-medulla and cortex did the addition of HI-6 result in significantly ($p < 0.05$) higher acetylcholinesterase activity over that obtained with obidoxime (50 mg/kg) alone.

The pharmacokinetics of HI-6 and obidoxime administered alone or as a combined injection was evaluated. The results in Table 4 show that elimination half-life ($t_{1/2}$) and the peak serum level of each oxime administered alone or in a combined solution was not altered significantly. The time to peak level was prolonged slightly in the case of obidoxime in the combined injection, however this was not considered to be significant as the mean serum level of obidoxime was 22 μg/ml at 7 min as compared to 25.4 μg/ml at 10 min.

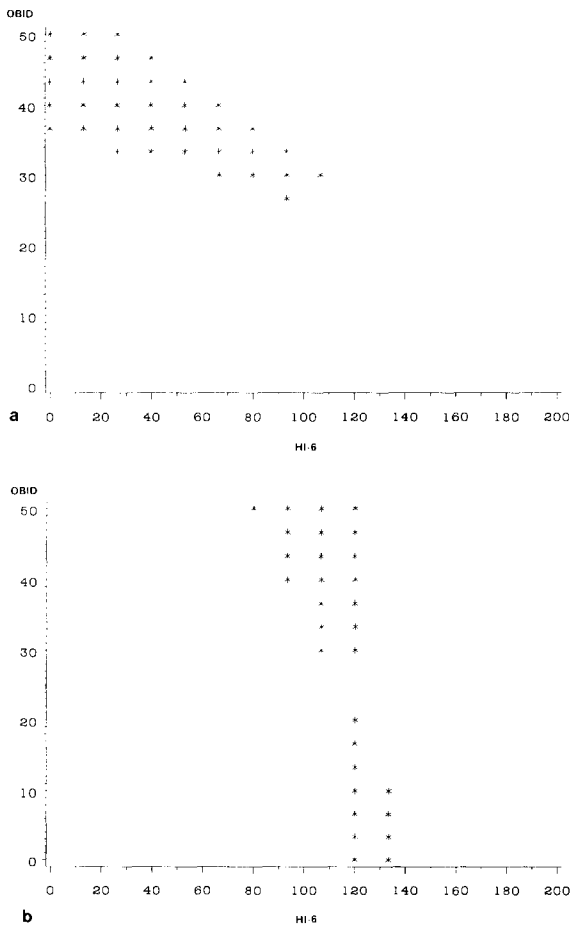

Fig. 2. Estimated 95% confidence regions of optimum treatment regimens as a function of HI-6 and obidoxime for fixed levels of atropine and either tabun **(a)** or soman **(b)**

Response surface analysis

The estimated surfaces are presented in Fig. 1 (a, b) and the 95% confidence limits in Fig. 2 (a, b).

The toxicities of HI-6 and obidoxime alone and in combination were also indicated in the response surface analysis. As shown in Table 5, the coefficients associated with the toxic effects of HI-6 and obidoxime alone and the interaction between them, β_{11}, β_{22} and β_{12}, respectively, were all negative and significant.

The ineffectiveness of obidoxime against soman poisoning, within the experimental region considered, was also indicated in the response surface analysis. As shown in Table 5, the coefficients associated with the therapeutic and toxic effects of obidoxime, β_2 and β_{22}, are not significant in modeling soman exposure and treatment. Further, the interaction term between obidoxime and HI-6, β_{12}, is negative and significant, indicating a toxic synergism from the combination treatment.

Discussion

The results of this study demonstrate that the addition of obidoxime significantly improved ($p < 0.05$) the efficacy of HI-6 against tabun poisoning and did not compromise the efficacy of HI-6 against soman poisoning. Against tabun poisoning the observed combination of obidoxime plus HI-6 (10 + 100 mg/kg, respectively) provided optimal ther-

Table 5. Modeling probability of survival logistic regression analysis of HI-6 and obidoxime in the presence of either tabun (1052 μg/kg) or soman (390 μg/kg)[a]

Variable	Tabun study		Soman study	
	Parameter estimate	p value	Parameter estimate	p value
Intercept	−2.7959	<0.0001	−1.5442	0.0001
X1	0.0325	0.0002	0.0629	<0.0001
X2	0.2027	<0.0001	−0.0046	0.8912
X1SQ	−0.0001	<0.0023	−0.0002	<0.0001
X2SQ	−0.0026	<0.0001	−0.0003	0.6781
X1X2	−0.0005	0.0002	−0.0003	0.0163

[a] For definitions, see Eq. (1)

apeutic benefit. Estimated optimal treatment levels were obtained from the fitted dose-response models by the direct optimization method of Nelder and Mead (1965). The results are presented in Table 6. In order to assess the variability contained in the data and thus in these results, it is necessary to consider the confidence regions about the location of the optimal treatments. These are given in Fig. 2 (a, b). In Fig. 2a it can be seen that the confidence region about the optimum includes zero doses of HI-6, indicating the obidoxime alone is sufficient in treating tabun exposure. For further discussion the reader is referred to Carter et al. (1982). Further, from the confidence region shown in Fig. 2b, it is apparent that HI-6 is necessary in treating soman exposure. The level of obidoxime treatment has minimal effect on the probability of survival, as indicated by the presence of the ridge in Fig. 1b, the range of the confidence region shown in Fig. 2b, and the lack of significant coefficients in Table 5.

Therefore, based on the shape of the surfaces in Fig. 1, and the confidence regions in Fig. 2, it is apparent that the addition of obidoxime improved the efficacy of HI-6 against tabun poisoning and obidoxime does not compromise the efficacy of HI-6 against soman poisoning. At the higher doses of oximes, HI-6 (100 and 200 mg/kg) and obidoxime (25 and 50 mg/kg), an increase in the mortality was evident in the absence of organophosphate poisoning. This was probably due to the toxicity of the oxime mixture (Table 1). The i. p. LD_{50} of obidoxime and HI-6 in mice was 111 and 588 mg/kg, respectively (Clement 1983). Since both oximes, HI-6 and obidoxime, were present and both excreted primarily by renal filtration, the pharmacokinetics of the oximes were examined. It was found that elimination of the oximes and peak concentrations attained were not significantly different when administered as a combined injection.

The relationship between organophosphate poisoning, acetylcholinesterase inhibition and lethality is not totally clear. In the classical sense, extremely toxic organophosphates such as soman, sarin, tabun and VX phosphorylate acetylcholinesterase. It is proposed that the resulting cholinergic overstimulation leads to disruptions of normal phasic discharge of medullary respiratory related units which eventually progress in severity until there is a loss of rhythmic discharge and central respiratory drive ceases resulting in respiratory arrest (Rickett et al. 1986).

Reactivation of unaged phosphorylated acetylcholinesterase is undoubtably the function of the oxime and the rationale for its use. However, the relationship between efficacy against organophosphate poisoning and reactivation of brain acetylcholinesterase is equivocal (Clement 1979; Heffron and Hobbiger 1980; Lundy and Shih 1983). In this study the optimal observed combination of obidoxime and HI-6 (10 + 100 mg/kg) against tabun poisoning resulted in brain acetylcholinesterase activities which were not significantly different from the mice receiving tabun only (data not shown). Even though at the dose of obidoxime and HI-6 used (50 + 50 mg/kg) small but significant increases in brain acetylcholinesterase activity were found, it is doubtful that reactivation of brain acetylcholinesterase was the primary antidotal effect.

Lack of measurable reactivation as evidenced by examining tissue acetylcholinesterase activity from organophosphate-treated animals may be misleading. Oxime reactivation of phosphorylated acetylcholinesterase may actually occur *in vivo*, however; due to the organophosphate depot (Van Helden and Wolthuis 1983), re-inhibition of acetylcholinesterase may occur. Thus, when tissues from animals poisoned with organophosphates are examined for acetylcholinesterase activity it appears that reactivation has not occurred. This explanation would be plausible if HI-6 is considered to be a reactivator of tabun-inhibited acetylcholinesterase *in vitro*. However, HI-6 does not, or only weakly reactivates tabun-inhibited acetylcholinesterase *in vitro* (*loc. cit.*), making this explanation untenable. Thus, if reactivation is not apparent then some other effect or action of the oxime at another locus must be responsible for the antidotal effect. After oxime treatment, significant ($p < 0.05$) reactivation of acetylcholinesterase in the diaphragm was found consistently in the combinations examined (Table 3), whereas there was no consistency in the acetylcholinesterase activity in the various brain regions and efficacy against tabun poisoning. This suggests that in the mouse, the primary therapeutic effect may result from reactivation of acetylcholinesterase at a peripheral site, namely the diaphragm (Clement 1982).

Alternatively, the increased efficacy found in the use of a combination of HI-6 and obidoxime against tabun poisoning may be an extension of the dose-dependent synergism of cholinolytic and oxime noted by Borbely et al. (1975), the mechanism of which remains an enigma.

Table 6. Estimation of optimal therapy: Nelder Mead optimization results

Probability of survival	Tabun dose (μg/kg)	Optimum combination	
		HI-6 (mg/kg)	Obidoxime (mg/kg)
0.839	1052	74.53	31.89
Probability of survival	**Soman dose (μg/kg)**	**Optimum combination**	
		HI-6 (mg/kg)	Obidoxime (mg/kg)
0.926	390	129.24	0

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