e) In rat study E-1, the effect of P on the regeneration of AchE activity after Soman administration was studied. Atropine and PAM alone caused no regeneration; adding P to this regimen caused significant regeneration but was not efficacious in protecting against Soman-induced lethality. (Results shown under description of study, earlier.). Although this result does not speak to the question of a correlation between P-induced AchE inhibition and efficacy, it does show a dissociation between post-Soman enzyme regeneration and efficacy. (The hypothesized mechanism of action of P is that the AchE inhibition due to P will result in a net decrease in inhibition due to Soman).

In summary, there is little evidence for a correlation between the degree of P-induced AchE inhibition and efficacy against Soman-induced lethality. This is particularly evident in the monkey studies, where the data are most reliable since enzyme inhibition was measured in the same animals given Soman. One monkey study showed similar efficacy with P doses ranging from one causing 26% inhibition at the time of Soman exposure down to one causing no demonstrable inhibition. In general, the lack of correlation in the animal studies reflected a lack of correlation between the dose of P and degree of efficacy; the correlation between the dose of P and degree of enzyme inhibition was generally good.

There was also no clear evidence for a relationship between P-induced AchE inhibition and efficacy in several "supporting" studies in monkeys and guinea pigs where more than 1 dose of P was used, e.g. vol. 1.6 p. 64, vol. 1.34 p. 214, and Leadbetter et.al., Fundam. Appl. Toxicol. 5: S225-S231, 1985).

The reasons for the lack of correlation between P-induced enzyme inhibition and efficacy are not clear. Possibilities include:

a) RBC or plasma enzyme is not an accurate marker for AchE activity at critical sites in the nervous system needed for efficacy.

b) Only a very small amount of AchE needs to be protected for maximal efficacy, and this was achieved even at the dose of P tested causing the lowest amount of enzyme inhibition. (However, demonstration of efficacy at a dose of P causing no inhibition, as seen in monkeys, would seem to argue against this possibility).

c) Although inhibition of AchE might be necessary for the efficacy of P, it is not the degree of inhibition at the time of Soman exposure (i.e., the measure being proposed as the surrogate marker) which is critical. For example, note the study cited earlier (vol 1.7, p. 65+) in which a series of physostigmine homologs were less efficacious than physostigmine despite the fact that all drugs were given at a

dose causing the same AchE inhibition at the time of Soman administration; the interpretation of this result was that the rate of decline of enzyme inhibition ("decarbamylation rate") was too slow in the case of the homologs to allow for the availability of enough free enzyme needed for survival. (As also noted earlier, it has been suggested that a decarbamylation rate which is too fast would also lead to loss of efficacy). Of course, there are also many other events which occur after the initial P-induced AchE inhibition at the time of Soman exposure which could be involved in the efficacy of P, e.g., sequentially, protection of the enzyme, changes in acetylcholine levels, changes in cholinergic receptor activation, changes in organ function, etc. The quantitative relationship between any of these events would affect the relationship between the degree of P-induced AhcE inhibition at the time of Soman exposure and the degree of efficacy of P.

d) Inhibition of AchE is not involved (or not significantly involved) in the mechanism of protective action of P. Actions of nerve agents aside from AchE inhibition, e.g. direct cholinergic receptor interactions or noncholinergic actions such as GABA antagonism, have been noted as possibly being contributory to poisoning (Sidell and Borak, Annals of Emergency Medicine 21(7): 865-871, 1992). Likewise, actions of P aside from AchE inhibition have been noted, e.g. various cholinergic receptor interactions and decreased neurotransmitter release. (Cited in NDA vol 1.3, p. 99-101, also in vol 1.33, p. 232+, Albuquerque et.al. FAT 4: S27-S33, 1984, Adler et.al., J. Appl. Toxicol. 12(1): 25-33, 1992, and Anderson and Chamberlain, NeuroToxicol. 9(1): 89-96, 1988). In one paper (Kawabuchi et.al., Synapse 2: 139-147, 1988), both the (+) and (-) enantiomers of physostigmine protected rats from sarin-induced lethality, despite the fact that the (+) enantiomer is a very weak inhibitor of AchE (although note that enzyme inhibition was apparently not measured in vivo in this study); the authors suggest the protective effect was thus not primarily due to protection of AchE but to some other action of physostigmine, i.e. nicotinic channel blockade.

4) Animal Toxicity Studies

Dr. Sparenborg's review of these studies is attached.

A discussion of the more recently conducted studies on the interaction between pyridostigmine with DEET and permethrin is presented in the Evaluation section below.

EVALUATION:

A) Efficacy

Pyridostigmine (P) was effective in reducing the lethality induced by the organophosphorus nerve agent Soman when given prior to Soman administration, in combination with atropine and 2-PAM given post-Soman, in monkeys and to a lesser extent (as shown in non-GLP studies) in guinea pigs. (A limited amount of data suggested that P had no beneficial effect when given without atropine/2-PAM. Some studies also showed that P given without atropine and PAM did not sensitize animals to the lethal effects of Soman [as has been seen regarding some sublethal effects of Soman in some studies], although data on this point were relatively limited.). Little or no beneficial effect of P was seen in mice, rats, and rabbits. A relatively small beneficial effect of P against Tabun-induced lethality was seen in a small numbers of studies but overall the data were too minimal to establish a clear drug effect. No beneficial effect of P was seen in a small number of studies using the nerve agents Sarin or VX; in one study in mice P reduced (but did not eliminate) the effect of atropine + PAM against VX, but only at a dose of P causing a relatively high degree of AchE inhibition (60%).

Since, in contrast to some other agents, lethality due to Soman is only slightly reduced by the "standard" treatment of atropine + 2-PAM in animals (in part thought to be due to the rapid aging of Soman-inhibited acetylcholinesterase [AchE], rendering it refractory to reactivation by 2-PAM), a beneficial effect of P would represent a therapeutic advance in protection from Soman-induced lethality. However, it is not clear if the results in monkeys and guinea pigs can be extrapolated to humans, or if they can, what would be the dosage of P necessary for efficacy in humans. In considering these problems, the areas of concern which need to be addressed are as follows: (1) relevance of the animal model used to the human situation, (2) the fact that there were species differences in efficacy (efficacy seen in monkeys and guinea pigs but little or no efficacy seen in mice, rats, and rabbits), and (3) the "surrogate marker" for efficacy proposed by the sponsor, i.e. P-induced RBC AchE inhibition at the time of Soman exposure, did not show a clear relationship to efficacy.

(1) Relevance of animal model

The animal model used, i.e. lethality following exposure to Soman, is in general similar to the anticipated human situation (more so than most of the animal models of human disease generally encountered in the evaluation of neurologic/psychiatric drugs). It is likely that the biochemical mechanism of Soman-induced toxicity (i.e., AchE inhibition, although other actions have been proposed as noted earlier) is the same across species. The changes in cholinergic function expected from irreversible inhibition of AchE would be expected to be similar across species, at least qualitatively. (See sponsor's discussion in volume 2.1, p. 406-410). However, note that, as discussed earlier, the routes of Soman

administration in animals (various parenteral routes) were not the same as the anticipated human exposure (dermal/inhalation), and the likely differences in Soman tissue distribution and time course would likely result in different patterns of toxic effects with possibly differing sensitivities to the beneficial effects of P. (In addition to differences in the pattern of toxicity, different routes of Soman administration, along with possible differences in intrinsic Soman PK parameters across species regardless of route, could result in species differences in the rate of elimination of Soman from blood and tissues; this variable would be predicted to have an effect on the efficacy of P in that, according to the proposed mechanism of action, for efficacy to occur the rate of elimination of Soman should be faster than the rate of regeneration of P-inhibited AchE). (It is also noted that the route of administration of P in several animal studies was i.m. whereas that proposed for human is p.o. This might not be a significant problem if it is assumed that the mechanism of the protective effect of P is AchE inhibition since the degree of such inhibition, rather than dose or plasma levels of P, was generally used as the measure of P exposure in these studies; however, if other actions of P or its metabolites were involved in its protective effects such actions and consequent protection from Soman might differ significantly with different routes of administration).

Also note that in the animal "efficacy" studies, Soman was administered at a fixed time after the administration of P. According to the proposed hypothesis regarding the mechanism of action of P it should only be effective when its degree of AChE inhibition is declining (i.e. when enzyme activity is regenerating), i.e. when the Soman administration occurs near or after the Tmax for P-induced AChE inhibition (although this has not been well-established empirically as noted earlier). In the pivotal monkey studies, Soman was given either after (study A-1) or at (study A-2) this Tmax; in the other animal studies there was generally little information given on this point. It is not clear how well the time interval between the last dose of P and exposure to Soman can be controlled in humans, but it might be predicted that P would not be effeactive if Soman exposure occurred relatively soon after the last dose, i.e. at a time when P-induced AChE inhibition was increasing.

(Also note that the benefits of P were only shown with lethal doses of Soman; limited animal data indicate that P may produce additive effects with sublethal doses of nerve agents).

2) Species differences in efficacy

The sponsor hypothesizes that humans will respond to the beneficial effects of P more like monkeys and guinea pigs than like rats, mice, and rabbits, based on 2 arguments discussed in detail earlier, i.e. that the species differences in sensitivity to P are explained by species differences in (1) the activity of

carboxylesterase, an enzyme which detoxifies Soman, and (2) the decarbamylation rate of P-inhibited AchE, and that the carboxylesterase activity and decarbamylation rate in humans are more similar to those in the animal species in which P was effective. However, as also discussed in detail earlier, the data presented in support of these arguments do not clearly establish such species differences, alternative explanations for the results obtained were not always adequately ruled out, and, importantly, it has not been well established how or even if these variables are significant determinants of the efficacy of P.

3) Use of P-induced RBC AchE inhibition as a "surrogate marker" for efficacy

The sponsor is proposing that P, given to humans at a dose which causes a degree of RBC AchE inhibition (at the time of exposure to Soman) which is associated with "efficacy" in animals, will be efficacious in humans.

(Note that there are really 2 types of surrogacy implicit in this argument, i.e. [1] P-induced AchE inhibition as a surrogate for efficacy, and [2] efficacy [and the quantitative relationship between P-induced AchE inhibition and efficacy] in animals as a surrogate for efficacy [and the quantitative relationship between P-induced AchE inhibition and efficacy] in humans. [Also note that the proposed surrogate marker, i.e. P-induced AchE inhibition, is not a marker for the severity of the "disease" being treated (e.g., as CD4 count is used as a surrogate marker for AIDS) but rather a pharmacological effect of P, albeit one which is hypothesized to be related to its mechanism of action for efficacy (a hypothesis which is plausible but not proven). A surrogate marker for the severity of the "disease" in this case might be Soman-induced AchE inhibition; paradoxically in this situation this is the same action as is produced by the treatment (i.e., pyridostigmine), with the hypothesis being that P pre-treatment results in Soman causing less net AchE inhibition than if P were not given. (There were little or no data submitted to show a relationship between this protection of AchE and protection from lethality in animals exposed to Soman [e.g., showing survival was correlated with less net Soman-induced AchE inhibition]; at any rate the use of Soman-induced AchE inhibition as a surrogate marker for the effect of P in humans would have to involve Soman exposure in humans.)]).

To support the use of P-induced RBC AchE inhibition as a surrogate marker for efficacy it is necessary to show a relationship between such inhibition and efficacy. However, the animal data provide little support for such a relationship. As discussed in detail earlier, in most of the studies in which more than one dose level of P (causing varying degrees of AchE inhibition) was used there was no relationship seen, particularly in monkeys, the species in which the greatest efficacy was seen and in which the AchE measurements were most valid (i.e. were made in the same animals receiving Soman). (The sponsor seems to be in agreement with this lack of correlation between degree of AchE inhibition and

efficacy - see volume 2.5, p. 61-62). Possible reasons for this lack of relationship were discussed earlier.

Even if no quantitative relationship were seen between enzyme inhibition and efficacy, it might be argued that any inhibition over a certain level will be associated with efficacy; this seems to be the sponsor's argument set forth in volume 2.5, p. 60-63, where it is implied that any dose of P causing at least 10% RBC AchE inhibition should be effective. (It is stated that the proposed human dosage regimen of P will result in 20-40% RBC AchE inhibition). However, the basis for this argument is not clear. The minimum amount of enzyme inhibition associated with efficacy varied across studies and across species. In guinea pigs it ranged from 16 to 70%. In rats, mice, and rabbits, little or no efficacy was seen despite inhibition of up to 70%. (As discussed earlier, the sponsor has hypothesized that humans should respond better than mice, rats, and rabbits, although, as also discussed, the basis for this was not well-established). At the other extreme, in monkeys a no-effect threshold was not established, and in fact efficacy was seen at a dose of P causing no demonstrable RBC AchE inhibition, further calling into question the relevance of such inhibition as a surrogate marker for efficacy.

Furthermore, even assuming that (1) the animal model for efficacy is relevant to humans, (2) that humans will respond to P more like monkeys and guinea pigs than like rats, mice, and rabbits, and (3) that P-induced RBC AchE inhibition is in some way a marker for efficacy within a species, the question still remains of what degree of enzyme inhibition is needed in humans for clinically relevant efficacy, i.e., what the recommended dosage regimen should be. (This problem would not be as crucial for a new symptomatic treatment intended to be given after Soman exposure, in which case the dose could be determined empirically by adjusting it as needed until efficacy were seen). The basic question is whether the quantitative relationship between AchE inhibition and protection from Soman-induced lethality is the same across species, i.e. does the same amount of inhibition result in the same amount of protection. The inhibition of AchE by P, assuming this is its primary mechanism of action, would be an early step in a chain of events leading to efficacy, e.g. inhibition of AchE → protection of AchE from Soman-induced inhibition → partial reversal of Soman-induced increase in acetylcholine levels → changes in cholinergic receptor activation → change in end organ function → survival of animal. Species differences in the quantitative relationships (including time course) between any of these pairs of events (and the many in between, as well as compensatory effects [e.g. receptor desensitization], cholinergic or noncholinergic effects unrelated to AchE inhibition which could affect efficacy either positively or negatively, etc.) would affect the overall quantitative relationship between P-induced AchE inhibition and efficacy. (As discussed earlier, the sponsor has discussed some species differences which could affect degree of efficacy e.g. differences in the

rate of regeneration of P-inhibited AchE, which is relevant to the question of whether equal amounts of P-induced AchE inhibition will result in equal amounts of AchE protection from Soman across species. However, there are many other possible steps in the sequence noted above where species differences could occur.) There might also be species differences in the minimum amount of AchE activity necessary for survival.

Another potential source of quantitative differences in the relationship between P-induced RBC AchE inhibition and efficacy across species is the relationship between AchE activity in RBC (the proposed surrogate marker) and that in the nervous system (the hypothesized site of protective effect). Although for many cholinesterase inhibitors AchE activity in RBC has been used to track toxic effects in the nervous system, examples of a dissociation also exist (cited in volume 1.1, p. 129-130, also see Sidell, Clin. Toxicol. 7(1): 1-17, 1974). Little or no data were submitted regarding the quantitative relationship (including time course) between AchE inhibition in RBC and that at critical sites in the nervous system related to efficacy following the administration of P across species. Species differences in the ability of RBC AchE to correspond to nervous system AchE could arise from intrinsic differences in enzyme properties as well as species differences in P distribution and other PK parameters.

Another problem in extrapolating the quantitative relationship between P-induced AchE inhibition and efficacy across species is that, as discussed earlier, the expected routes of Soman exposure in humans are different from those used in the animal studies, and that this variable might affect the efficacy of P. (As also noted earlier, intrinsic species differences in the rate of Soman elimination, regardless of route of administration, would also be expected to affect the efficacy of P.). Intrinsic species differences in the PK and metabolic pattern of P, and the fact that the route of administration of P was (sometimes) different in the animal studies from that proposed in humans, could also present a problem for quantitative extrapolation. Yet another problem is that although atropine + PAM were shown to be required for the efficacy of P in animals, it is not clear if this permissive effect is quantitatively similar across species (i.e. will the atropine and PAM doses to be given to humans provide the same magnitude of permissive effect as was provided at the doses used in the animal studies).

A final problem with the use of RBC AchE inhibition as a surrogate marker for quantitative extrapolation across species is that it is not clear how similar the methods for sampling and assaying for enzyme activity were in the animal studies to those proposed for use in humans. Many factors can affect measured enzyme activity including time between sampling and assay and degree of dilution of sample (since the inhibition by P is reversible), substrate used (different substrates have different affinities for AchE vis a vis butyrylcholinesterase), and the various other parameters of the assay. In some

cases whole blood was used rather than RBC. In some cases the methods used were not described. For quantitative extrapolation of results form animals to humans it is important that the methods used to measure the surrogate marker be similar if not identical. We requested at the meeting of 4/6/95 that the sponsor address this question, but this has not as yet been addressed. (At this meeting we also requested that the sponsor address the problem noted by DSI regarding monkey study #A-1, i.e. that this study specified a 3 minute interval between blood sampling and AchE assay, whereas a DSI audit indicated that the actual times were closer to — minutes. This has also not been addressed by the sponsor).

4) Conclusion

Pyridostigmine (P) was shown to be effective (when atropine + 2PAM are also given) in a model of Soman-induced lethality in monkeys and (to a lesser extent) guinea pigs. Little or no efficacy was seen in rats, mice, and rabbits. As discussed above, the data and arguments presented in support of the hypothesis that humans will respond more like monkeys and guinea pigs were not convincing. Regarding the proposed use of P-induced AchE inhibition as a surrogate marker for efficacy, examples were noted where efficacy was seen without an effect on the surrogate marker, and where an effect on the surrogate marker was seen without efficacy. Even when P had an effect on both the surrogate marker and efficacy, there was in most cases no quantitative correlation between the two. Furthermore, even if it could be assumed that P will be effective in humans, and that some association exists between the surrogate marker and efficacy in animals, it is not known what degree of effect on the surrogate marker would be needed in humans for clinically relevant efficacy. In sum, it is not possible to predict with reasonable certainty from the animal data if P will be effective in humans, what the degree of efficacy would be, or what the recommended dosage regimen should be.

B) Animal toxicity studies

Dr. Sparenborg's review of these studies is attached. As indicated, animal toxicity studies have gone up to 3 months' duration in rats and dogs; a 6 month rat study was also done but was suboptimal in that the sample sizes were small, only males were used, and the doses used were too low for adequate characterization of toxicity. Dr. Sparenborg's review indicates that the major toxic finding from these studies was various observed signs indicative of cholinergic activation; for other results see his review. As also indicated, there have been various reports of neuromuscular junction pathology (detected by light microscopy and/or EM) due to P in rats. This was not seen consistently across all studies (it was not seen in the 3 month rat toxicity study). Reversibility of the changes after drug discontinuation was reported (vol 1.31, p. 120; vol. 1.7, p. 174). Functional changes at the neuromuscular junction due to P have been reported (e.g. vol 1.21, p. 1; vol. 1.4, p. 1); it is not known if these changes are related to the histological changes seen or whether or not they are extensions of the pharmacological actions of the drug. The latter reference showed that continuous s.c. infusion of P in rats for 14 days caused altered twitch tensions; recovery was essentially complete by 1 day after stopping treatment. Neuromuscular junction and skeletal muscle pathology have been reported for other cholinesterase inhibitors and cholinergic agonists and is thought to be possibly secondary to increased synaptic acetylcholine levels resulting in excessive receptor stimulation and calcium entry. (Laskowski and Dettbarn, Ann. Rev. Pharmacol. Toxicol. 17: 387-409, 1977; Meshul, Brain Res. 497: 142-148, 1989).

As indicated in Dr. Sparenborg's review, P was considered to be positive in a segment II study in rabbits, producing an increase in visceral abnormalities, primarily hydronephrosis and arterial variations.

As also indicated in Dr. Sparenborg's review, the genotoxicity battery performed with P is suboptimal. An Ames test was negative, but did not use an A-T-detecting strain such as TA 102 or E. coli WP2. A rat micronucleus test was done and was said to be negative, but was lacking in adequate detail. No other studies were performed. This problem was communicated to the sponsor by me by phone on 12/11/95. I noted that the currently recommended genotoxicity battery includes bacterial mutagenicity (Ames Test), in vitro clastogenicity, and in vivo clastogenicity. The sponsor is "currently conducting an Ames Test, a Chromosome Aberrations Assay in Chinese Hamster Ovary Cells, a Mouse Lymphoma Mutagenesis Assay, and a Mouse Micronucleus Test, the results of which will be reported when available." (volume 2.1, p. 285).

The animal toxicity studies performed with P are adequate for NDA approval considering the expected short-term and sporadic use of the drug for its proposed indication, the life-threatening nature of the proposed indication and absence of satisfactory alternative treatment, and the long marketing history of P.

Three animal studies were performed which examined the toxic interactions between pyridostigmine (P), permethrin (an insecticide), and DEET (an insect repellant), since claims have been made that various illnesses experienced by veterans of the Persian Gulf War (sometimes considered to be a "Gulf War Syndrome") were caused by exposure to some combination of these compounds. The studies were (1) an acute oral lethality study in rats, (2) an interaction study in hens using doses of each drug causing minimal clinical effects when given alone, and measuring various parameters including motor function and histological exam of spinal cord and peripheral nerve, and (3) an acute lethality study in cockroaches using topical application. All 3 studies reported some interaction (i.e. greater-than-additive effects) between P and the other compounds as described earlier (although the cockroach study cannot be considered reliable as discussed earlier). However, there are several problems encountered in attempting to extrapolate these results to conclude that the drug combinations should be suspected as causes of a "Gulf War Syndrome" (or any other illness) experienced by Gulf War veterans, e.g.:

(1) It is not known how or if the endpoints used relate to the illnesses reported in humans. This is obviously true in the studies which used acute lethality as an endpoint. In the hen study lower doses were used and various neurologic signs and histopathology were evaluated; unfortunately reversibility was not studied which would have been useful since the human illnesses have been reported as being lingering.

(2) Regarding the studies which used lethality as an endpoint, it is not clear that the interactions seen at the very high doses used would be likely to have occurred at the lower exposures of the compounds likely to have been received in humans. (As noted earlier, the dose of P given to the soldiers was relatively low, and the sponsor has estimated that exposure to DEET and permethrin was also relatively low). The mechanisms of the lethal interactions observed were not elucidated (various PK and PD possibilities were discussed); knowledge of such mechanisms might have been useful for extrapolating results to lower doses. (Even in the case of the hen study, which used lower doses, the validity of extrapolating the results to Gulf War veterans depends on their degree of exposure to the test compounds relative to that in the test animals).

(3) Another question regarding exposure is that it was not shown that the effects seen with the various combinations would not also have been seen at higher doses of the individual compounds given singly (this depends on the shape of the dose-response curve of the individual compounds), and thus it is possible that the effects in Gulf War veterans, assuming they are related to these compounds, could be due not to any particular combination but to one of the compounds by itself if exposure were high enough. Human neurotoxicity from high doses of DEET, including tremor, weakness, stumbling, muscle cramps, restlessness, slurred speech, seizures, impaired cognition, irritability, paranoia, delusions, and aggressive behavior, has been reported. (cited in Abou-Donia paper and in

Ellenhorn, MJ, Medical Toxicology, Elsevier, The Netherlands, 1988.). DEET has also been reported to be a demyelinating agent and to cause spongiform myelinopathy in rat brain (cited in NDA volume 2.9 [sponsor's #2.82], p. 184).

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RECOMMENDATIONS:

The animal toxicity studies submitted are adequate to support the proposed use of pyridostigmine. However, as discussed in detail above, there is a great deal of uncertainty in attempting to extrapolate the animal "efficacy" data to humans both in a qualitative and quantitative manner, i.e. concerning whether pyridostigmine will be effective, what the degree of efficacy would be, or what the recommended dosage regimen should be; it is thus recommended that this NDA not be approved, at least based on the data submitted.

The following two points should be addressed by the sponsor:

1) As indicated in the submission of 7/18/96, errors were made regarding the numbers of monkeys which were said to have survived through 10 days post-Soman administration in phase V of the study described in item 5.1.5.58B. The sponsor should audit the data for all phases of this study in order to see if additional mistakes were made.

2) In the rat micronucleus assay (item 5.2.10.28) it was indicated that this study was done in conjunction with the 180 day toxicity study. However, the high dose in the latter study was 10 mg/kg, whereas the high dose in the micronucleus assay was said to be 30 mg/kg. The sponsor should explain this discrepancy.

/S/

Barry N. Rosloff, Ph.D.

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ATTACHMENT

REVIEW AND EVALUATION OF PHARMACOLOGY AND TOXICOLOGY DATA

NDA 20-414

TOXICITY STUDIES

Reviewer:	Steven Sparenborg, Ph.D.
Dates:	Written Review - September 18, 1995 Filing Date - sometime in 1996 User Fee Due Date - n/a
Sponsor:	Office of the Surgeon General Dept. of the Army Falls Church, VA
Drug:	pyridostigmine bromide
Chemical name:	3-{[(dimethylamino) carbonyl]oxy}-1-methylpyridinium bromide
Category:	reversible cholinesterase inhibitor
Indication:	pretreatment for nerve gas poisoning

Table of Contents:

GLP

All toxicity studies were conducted in accordance w/FDA GLP, excapt as noted in the review of individual studies.

Note: Portions of this review excerpted from the sponsors submission are enclosed within quotation marks.

"Three month toxicity study of pyridostigmine bromide in rats."

1. Study Parameters
Test species - rat, Sprague-Dawley, VAF
Supplier -
Initial body weight - males 210-260 g; females 130-170 g
Initial age - 6-7 weeks
Route of administration - oral
Vehicle - apparently distilled water
Drug Batch or Lot number - Lot no. 525013
Study performed at -

Dates -
Grouping - 10/s/d
Doses - 0, 5, 15, 30, 60 mg/kg

2. Results

Observed effects - The types of observations were limited to tremors, rough coat hair, dark material around eyes, and decreased activity. These signs were generally more prevalent in males than females. Tremors would be expected as a result of extreme cholinesterase inhibition. They were not observed in the 5 mg/kg groups and tended to subside over the course of the experiment in those rats dosed with 15 mg/kg or more.

Dark material around the eyes was noted in 9 HDf, but not in the lower dose female groups. 8 HDm had this condition also, but it was also noted in every other male treated group. Only 1 male in the 5 mg/kg group showed this.

Decreased activity and rough coat hair were observed in a dose-related manner with most rats at the higher doses affected, but only a few at the lower doses.

Mortality - Two HDm died during the study, apparently related to the test material.

Body Weight; Food and water consumption - At the end of the first week of dosing, weight gains in the 30 mg/kg groups were 48% (males) and 100% (females) of their respective control groups. In the high-dose groups, gains were 9% (males) and 50% (females). There was strong evidence of recovery of weight gains in the second week. By the end of the study, body weights of the high-dose groups were within 4% of the control groups. Food consumption was similarly affected.

Hematology - The normal hematological measures were not affected by pyridostigmine. Measured at the end of the study, red blood cell AChE was inhibited by -2, 43, 34, and 49% of the control value in the 5, 15, 30 and 60 mg/kg male groups. Female

RBC AChE inhibition was 5, 19, 42 and 29% of controls. Due to large variation, only the male 15 and 60 mg/kg groups were statistically different from controls. The sponsor claimed that low control values for RBC AChE activity was a factor in the low inhibition in the test groups. Younger animals (8-9 weeks old) in a 2-week study had about 1.00 units/ml of AChE compared to the 0.3 units/ml in the 19-20 week-old controls in this study.

parameters measured

Hb	MCH		thrombocytes	
HCR	MCHC	MCV	total leuco	erythrocytes
			differ leuco	

Blood chemistry - Three male rats of the high dose group had triglyceride or cholesterol levels that were 1.2 to 1.8 times the upper range of controls. The group means for these measures were not significantly different from the control group.

The 5 and 60 mg/kg female groups were statistically different from controls in the levels of cholesterol. The group means were 1.3 times the control group mean. In each of these two groups, the difference was mostly attributable to 2 or three rats with values that were 1.2 to 1.8 times the upper range of controls. None of these rats exhibited any unusual findings in histopathology.

parameters measured

BUN	ASAT	Ca	bilirubin	cholesterol
ALAT	albumin	Na	globulin	inorg. phosphorus
AFOS		K	glucose	total proteins
		Cl	creatinine	triglycerides

Organ weights - adrenals, brain, heart, kidney, liver, spleen, gonads were weighed.

No effects

Pathology exams

Macro - no effects noted at necropsy
Micro - one of the rats that died had hypospermia of the epididymis. Two other HDm rats also had this condition plus degeneration of the seminiferous tubules.

tissue examined

adrenals	ileum	salivary gland
aorta	jejunum	sciatic nerve
bone-femur	kidney	skeletal muscle
bone-marrow	liver	spinal cord
brain	lung/bronchi	spleen
cecum	lymph node	stomach
colon	(mandibular)	testes
duodenum	mammary glands	thymus
epididymides	ovary	thyroid
esophagus	pancreas	trachea
eyes	parathyroids	urinary bladder
heart	pituitary	uterus
	prostate	vagina
	rectum	

Only the control and high-dose groups were examined.

"Ninety day subchronic oral toxicity study of pyridostigmine bromide in rats - with EM study of NMJ."

1. Study Parameters
Test species - Sprague-Dawley rats albino
Supplier - [redacted]
Initial body weight - 151-287 grams
Initial age - 8 weeks at start of study
Route of administration - oral
Vehicle - in diet
Drug Batch or Lot number - lot 590034
Study performed at - Letterman Army Institute of Research, Presidio of San Francisco
Dates - Oct 86 to Mar 87
Grouping - 15/s/d (5/s/d were sacrificed after Day 28)
additional 6/s/d used for EM study of NMJ
Doses - 0, 1, 10, 30, 60, 90 mg/kg

Dietary mixtures were prepared and adjusted weekly. Actual dosing ranged from 91.5 to 102% of target.

2. Results

Observed effects -

The most dramatic sign was a strongly dose-dependent increase in the startle reflex. Although 2 control males and 8 females increased their startle reflex, nearly every rat treated with 60 or 90 mg/kg increased their reflex. Increases in the 10 and 30 mg/kg groups were intermediate between the controls and higher dose groups. The lowest dose, 1 mg/kg was not different from controls. Other noticeably drug-related effects were stains and material around the nose, affecting 8-9 rats in the higher groups versus 6 per control group; rough coat or alopecia (5x controls in the high-dose group); stains around mouth and anus (only in 3-4 in the 2 highest groups). There was also a mild increase in the level of aggressiveness in the higher dose groups. Only one HDm exhibited tremors. One to four females in most treated groups were afflicted with conjunctivitis or stained material around the eyes.

Mortality - none

Body Weight; Food and water consumption - Body weights of the 60 and 90 mg/kg male groups were only 83-85% of the controls at the end of the first week of dosing. This difference persisted in the highest dose male group through the second week, but no differences were found at any other time of the study. Food and water consumption was similarly affected. Female treated groups

tended to be heavier than their controls throughout the study, a difference which developed prior to dosing.

Hematology -
MCHC levels were increased in treated males and females by about 3% at either the 28 day or 90 day sampling time, but not both times. These increases, though statistically significant, appear to be chance variations. No other effects appear to be more drug-related than MCHC.

parameters measured

Hb	MCH		thrombocytes	reticulocytes
HCR	MCHC	MCV	total leuco	erythrocytes
			differ leuco	

Blood chemistry - The chloride level in the highest dose male group was significantly above the control group. Lower dose groups were not significantly different, but there appears to be a trend:
for the 0, 1, 10, 30, 60, 90 mg/kg groups, chloride levels at the 90th day were 101.4, 102.1, 104.1, 104.6, 105.9, 107.2 Meq/l. Females were very similarly affected. These changes were not due to outliers and were not noticeable at 28 days. No other changes could be considered drug related.

AChE inhibition ranged from 25-67% in males and from 18-60% in females at Day 28. Inhibition increased by Day 90 with ranges of 30-89% in males and 74-95% in females. General ChE inhibition ranged from 0-76% in males and did not seem to increase over time. Female ChE inhibition was from 0-37% at Day 28 and from 12-65% at Day 90.

parameters measured

BUN	ASAT	Ca	bilirubin	cholesterol
ALAT	albumin	Na		inorg. phosphorus
AFOS	LD	K	glucose	total proteins
Mg	Fe	Cl	creatinine	triglycerides
				creatin kinase
				AChE
				ChE
				uric acid

Organ weights -
Organs weighed: adrenals, brain, heart, kidneys, liver, spleen,

gonads.

Organ weights were reported as absolute, relative to body weight, and relative to brain weight. The number of statistically significant differences in comparing treated groups to the controls was fewer than expected by chance alone. HDm at the 90-day sacrifice had adrenal weights (relative to body w.) that were 130% of controls ($p < .05$), but there was no trend across treatment groups. Essentially, there is no evidence of any drug-related effect on organ weights.

Pathology exams

Macro - no effects

Micro - At the final sacrifice, three HDm rats had atrophy of acinar cells in the pancreas, whereas only one control male had this condition. Five control males had chronic inflammation of the pancreas vs. only two HDm with inflammation. Females were not affected with either of these conditions at all, and neither were males at the interim sacrifice.

Three HDm had chronic inflammation of the heart vs. only one control male, at the final sacrifice. Females were not affected, and neither were males at the interim sacrifice.

These findings are probably not related to a drug effect, but rather to chance variation, as they are common to rats. No other effects appeared to be more drug-related than these. The sponsor's pathologists felt these to be common effects and not drug-related.

tissue examined

adrenals
aorta
bone-femur
sternum
brain
cecum
colon
diaphragm*
duodenum
ear
esophagus
eyes w/ON
Harderian gl.
heart
ileum
jejunum
kidney*
liver*
lung*
lymph nodes
(mesenteric)
(submandibular)
mammary glands
nose/turbinates
ovary
pancreas
pituitary
rectum
salivary glands
sciatic nerve
skeletal muscle
skin
spinal cord
spleen
stomach
testes
thymus
thyroid w/para
tongue
trachea
urinary bladder
uterus
male accessory sex organs
extensor digitorum longus muscle*
soleus muscle*

* tissues examined in all rats, the remainder only in control and high-dose groups

NMJ study -
3/s/d were sacrificed on Day 28 and 3 more on Day 90. 5 neuromuscular junctions from their diaphragm were examined for rarefaction or vacuolation of presynaptic and post synaptic mitochondria, vacuolization of postsynaptic organelles, focal retraction of nerve terminal from primary cleft, and irregularities and disruption of z bands.

No effects were found.

APPEARS THIS WAY
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"One hundred eighty day subchronic oral toxicity study of pyridostigmine bromide in rats"

1. Study Parameters
Test species - male albino Sprague-Dawley
Supplier -
Initial body weight - males 101-142 g 14 days before dosing
Initial age - 6-7 weeks
Route of administration - oral
Vehicle - in diet (mixed weekly)
Drug Batch or Lot number - lot 590034
Study performed at -

Dates - Oct86-Jun 87

Treatment Group	Dose (mg/kg/day)	No. of Animals* M
control	0	10 + 5
LD	1	10
HD	10	10 + 5
HD5†	10	10 + 5

* number treated for 180 days plus the number continued through 30-day recovery period
† treated for only five days per week

2. Results

Observed effects -

The numbers of rats with increased startle reflex were 4, 3, 7, 8 in the C, L, H, and H5 groups, respectively. No other signs were treatment related, except possibly decreases among treated rats in the frequency of occurrence for stained fur.

Mortality - none

Body Weight; Food and water consumption -
There was evidence of a reduction in weight gain during the first

week only, for the groups treated only 5 days a week, but not for the group treated with the same daily dose for 7 days a week. The effect in the 5 day/week group was not significant and is probably not related to drug treatment. No other effects were noted.

Hematology - no effects AChE inhibition was 21%, 49% and 31% in the LD, HD, and HD5 groups, respectively. ChE was inhibited 25%, 63% and 30%. AChE was also inhibited 36% at the end of the recovery period in the HD5 group. There was no other inhibition at the end of the recovery period. This late inhibition was most likely due to sampling bias brought on by comparing 5 treated rats to 5 untreated rats. Statistical differences were not computed. Inhibition was calculated according to this formula: [(mean baseline activity minus normalized mean treated activity) divided by mean baseline activity] times 100%.

parameters measured

Hb	MCH		thrombocytes	
HCR	MCHC	MCV	total leuco	erythrocytes
			differ leuco	

Blood chemistry - no effects

parameters measured

BUN	ASAT	Ca	bilirubin	cholesterol
ALAT	albumin	Na		inorg. phosphorus
AFOS	LD	K	glucose	total proteins
Mg	Fe	Cl	creatinine	triglycerides
				creatin kinase
				AChE
				ChE
				uric acid

Organ weights - no effects
Organs weighed: adrenals, brain, heart, kidneys, liver, spleen, gonads.

Pathology exams

Macro - no effects
Micro - The numbers of rats with cortical lymphocyte aggregates in the kidney were 5, 4, 9, 5 for the C, L, H and H5 groups, respectively. After recovery, these numbers were 3, 2, 0 for the C, H, H5 groups. This is not strong evidence for a drug effect. There was progressive renal disease in 4, 3, 8, and 2

rats of the C, L, H and H5 groups at the end of the dosing period. After recovery the numbers were 0, 2, 3.

Chronic multifocal inflammation of the liver was found in 3, 3, 9, and 4 rats of the C, L, H and H5 groups at the end of the dosing period. The H group was significantly different from controls. After recovery, the numbers were 2, 3, 1 for the C, H, and H5 groups.

tissue examined

adrenals
aorta
bone-femur
sternum
brain
cecum
colon
diaphragm
duodenum
esophagus
eyes w/ON
heart
ileum
jejunum
kidney
liver
lung
lymph nodes
(mesenteric)
nose/turbinates
pancreas
pituitary
rectum
salivary glands
sciatic nerve
skeletal muscle
skin
spinal cord
spleen
stomach
testes
thymus
thyroid w/para
tongue
trachea
urinary bladder
male accessory
sex organs
extensor digitorum longus muscle
soleus muscle

APPEARS THIS WAY
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"90-Day Oral Dose Toxicity Study of Pyridostigmine in Dogs"

1. Study Parameters
Test species - beagle dogs
Supplier - [redacted]
Initial body weight - 7.4 to 12.4 upon arrival
Initial age - 10-13 months at start of dosing
Route of administration - oral gelcaps
Vehicle - "Mestinon equivalent buffer"
Drug Batch or Lot number - WR250710AS
Study performed at - [redacted]
Dates - Mar 87 to Jun 87

Treatment Group	Dose† (mg/kg)	No. of Animals* M	F
control	0	4 + 1	4 + 1
LD	0.05	4	4
MD	0.5	4 + 1	4 + 1
HD	2.0	5 + 2	5 + 2

† Each dog was dosed 3 times a day, so that the HD group received 6 mg/kg/day.

* number treated for 90-98 days plus the number continued through 90-day recovery period

The diluted (liquid) drug solution was analyzed for stability after 8 days and was found to be acceptable. Therefore, each drug solution was used within 4 days of its preparation. The high-dose actually used the undiluted Mestinon formulation.

2. Results

Observed effects - There was dose-related frequency of occurrence of soft feces/diarrhea, red feces and mucoid diarrhea. At the high-dose, these signs were observed in most dogs. Occasional emesis was noted in dogs of all groups.

Mortality - a HDf was sacrificed in moribund condition on Day 23

and a LDf on Day 31. It was stated that because of abnormal chemistry values pre-dosing, this dog should never have been used in the study. There was evidence of moderate chronic interstitial nephritis and the kidney was small. The blood values suggested nephropathy.

In the HDf, "Palpitations of the abdominal area prior to sacrifice indicated a firm loop of bowel consistent with intussusception, a pyridostigmine-related abnormality found in a previous pyridostigmine study (Page and Emmerling, 1986, 14-day Pilot Dose Range Oral Toxicity Study). While the small intestine appeared to be red and thickened at necropsy, no changes were apparent on histological analysis. Since this was a high dose dog and pyridostigmine-induced intussusception was found in another study, it is probable that moribundity in this dog was due to pyridostigmine-induced intestinal intussusception. The occurrence of several episodes of emesis in this dog [in Weeks 1, 2 and 3] prior to its death is consistent with an effect of pyridostigmine on the gut."

Body Weight; Food consumption - no significant effects

Ophthalmic exam - no effects

Cholinesterase levels -

The asymptote of cholinesterase inhibition was reached by Day 2 in the HD and by Day 5 in the other two dose level groups and remained at that level throughout the dosing period and quickly recovered after dosing stopped.

Low - decreased by 15%

Mid - decreased by 45-55%

High - decreased by 70-80%

Hematology - blood drawn from jugular vein on Days -12, 45, 89, 180.

no effects predose values not reported

parameters measured

Hb	thrombocytes	prothrombin time
HCR	total leuco	erythrocytes
	differ leuco	

Blood chemistry -

No clearly drug-related effects. The mean value for BUN in HDf was significantly greater than in control females. A large contributor to this difference was #417. It also had values for total protein, albumin and haptoglobin that were above the range of controls. Its levels of sodium, potassium and chloride were 6-20% below the means of HDf and controls. It was the only animal with abnormal values, which still were not very abnormal. Magnesium was 25% higher than the group mean for controls and HDf.

Lactate dehydrogenase was dose-dependently reduced in all treated male groups, but the control group appears to be higher than normal. The LDf group also appears to be 40% higher than the average of the other female groups.

parameters measured

BUN	ASAT	Ca	bilirubin	cholesterol
ALAT	albumin	Na	globulin	phosphorus
AFOS	LD	K	glucose	total proteins
	Mg	Cl	creatine pk	triglycerides
				haptoglobin
				pyruvate kinase

Urinalyses -

The above mentioned HDf #417 had occult blood in the urine at the end of the recovery period. There were other scattered incidences of occult blood, but they did not reflect any relationship to drug treatment.

parameters measured

		blood (Hb)	epi.
protein	bilirubin		crystals
glucose	sediment		erythrocytes
volume		leukocytes	

Organ weights - no effects

Organs weighed: adrenals, brain, heart, kidneys, liver, spleen, thyroid w/para, gonads.

Pathology exams

Macro - no effects
Micro -

PITUITARY - cysts were noted in the pars distalis of 1/4 LDm, 1/5 HDm, 2/4 LDf, 1/4 MDf, 2/5 Hdf but not in any controls. This is probably not related to drug treatment, but it showed the strongest dose-related incidence of any pathology.

THYMUS - Cysts were also noted in the thymus. For the C, L, M and H groups, the incidences were 2, 3, 1 and 1 for males and 1, 0, 4 and 2 for females.

tissue examined

adrenals
bone-rib junction
bone-marrow
brain
(cerebrum)
(midbrain)
(medulla)
cecum
colon
diaphragm
duodenum
esophagus
eyes
heart
ileum
jejunum
kidney
liver
lung
lymph nodes
(mesenteric)
(precapsular.)
ovary
pancreas
parathyroids
pituitary
prostate
salivary gland
sciatic nerve
soleus muscle
skin
spinal cord
spleen
stomach
testes
thymus
thyroid
trachea
urinary bladder
uterus
gall bladder
tongue
tonsil
ureter
epididymides
EDL muscle

SEGMENT I

"Fertility and General Reproductive Performance Study of Pyridostigmine Bromide in Female Rats"

Sprague-Dawley VAF female rats from ——— (30 per dose) were given pyridostigmine Br (lot no. 88-0095) from two weeks prior to mating with untreated males through lactation Day 20 at doses of 0, 5, 15, 45 mg/kg p.o. Ten dams from each group were sacrificed on gestation Day 13 and the uterus and ovaries were examined. The remaining dams were allowed to deliver their pups at term and raise them until Day 21 post partum, except those pups culled at Day 4 to reduce litter sizes to ten pups of equal sex distribution whenever possible.

Adverse F0 Effects -

Tremors, dark material around the eyes, and rough coat hair were seen in all HD dams and many MD dams throughout the dosing period. Two HD dams died after only a few days of dosing. They exhibited wheezy breathing. Low dose rats were free of any clinical signs.

HD dams reduced food consumption and lost an average of 17 grams during the first week of dosing. Their body weights were only 89% of controls at the end of the first week, but they gained weight rapidly in the next two weeks and were equal to controls thereafter.

Adverse F1 Effects -

Body weight gains of the HD pups lagged behind those of the other groups during lactation (9% less than controls). The birth weights were not different from controls. Although not statistically significant, the number of early resorptions was slightly increased in the HD group. The means with standard deviations for the C, L, M, and H groups were: 4.2±3.7, 3.6±4.6, 3.8±5.2, 5.5±7.5. The dose-related increases in SD suggest that there may have been larger numbers of resorptions in a few dams in the higher groups.

There were no effects on fertility. The concentration of drug in solution was assayed and found to be appropriate.

"Fertility and General Reproductive Performance Study of Pyridostigmine Bromide in Male Rats"

Male Sprague-Dawley rats (30/d) were given pyridostigmine Br by gavage for at least 70 days prior to mating at doses of 5, 15 or 45 mg/kg. Females were not dosed and each one was mated with one male. Testes and epididymides were weighed at sacrifice of the males after mating period. Ten females per group were sacrificed on gestation Day 13, the remainder were allowed to give birth and raise pups until Day 21 pp.

Adverse F_0 effects - 4 HD males died within 23 days of start of dosing, after exhibiting tremors, rough coat hair and dark material around eyes. But most HD animals had these same signs. Some MD and LD rats had these signs also. No tremors in the LD group, though. HD males weight gain was 89% of controls, most of the difference came in the first week of dosing. Their rate of gain was equal to controls after the first week but they never completely made up the difference. Food consumption in HD rats was reduced to 60% of controls during Week 1. The other treated groups were not different from controls in weight gain or food consumption.

Adverse F_1 effects - none

This was a thorough study, but did not find any reproductive measure to have been altered by the drug.

"Effect of pyridostigmine bromide on fertility in male and female rats"

This study was not GLP, sponsored by the —— chemical defense group at ——
The stability of drug was not tested, but cholinesterase levels were monitored during the study and proved that drug was working because the activity of this enzyme was reduced in a dose-related manner.

The drug (Mestinon) was given in drinking water at a rate that averaged to 9, 26, and 79 mg/kg/day, although consumption was greater earlier in the study than later. Males were dosed for 11 weeks before mating and females for 15 days prior to mating. Treatment continued through Day 20 of gestation for the females. Half were killed that day and half allowed to deliver.
Adverse F0 Effects - Hdm exhibited tremor and body twitching, Hdf just tremors. These started after about 2 weeks of dosing. Hdf lost weight during the first week of dosing, but regained enough to be equal with all other groups. Hdm did not gain as much as

controls during the first three weeks of dosing and out gained the others later, but not enough to equal them.
Adverse F1 Effects - mortality was 25%, 12% and 1% in the low, mid and hi dose groups. The sponsor attributed pup loss to maternal neglect, but could not explain the reverse dose-ordered trend in deaths.

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SEGMENT II

"Developmental Toxicity Study of Pyridostigmine Bromide in Rats"

Sprague-Dawley VAF female rats from ——— , (25 per dose) were given pyridostigmine Br from Day 6-15 of gestation at doses of 0, 3, 10, 30 mg/kg p.o. plus a positive control group received acetylsalicylic acid (600 mg/kg p.o.) on Day 10 of gestation. One-third of fetuses from each litter were examined for visceral alterations using Wilson's technique. The remainder were stained with Alizarin Red-S for skeletal alterations.

Adverse F0 Effects -

The HD group dams lost an average of 10 grams ($p < .01$) in the first two days of dosing, while all other groups gained at least 10 grams. The HD group began to gain weight again at the same rate as controls, but did not catch up to the Body weights of the controls. Weight gain in the positive control group was reduced ($p < .01$) for 3 days after dosing, but their Body weights were not different from controls at the end of gestation.

Tremors and increased incidence of dark material around the nose and eyes and rough coat were observed in the MD and HD groups. One control was sacrificed in moribund condition on Day 10. The cause of death was related to a leukemia involving a mononuclear cell type.

Adverse F1 Effects -

There was a significant increase (2x controls and other treated groups) in the number of early resorptions, defined as the presence of the remains of an embryo that did not undergo organogenesis, in the HD group ($p < .05$). The group means and standard deviations for the C, L, M, H and positive control groups were: 2.1±2.6, 1.8±2.3, 2.1±2.0, 4.1±5.0, and 2.3±2.4.

"Effect of Pyridostigmine Bromide on Pregnancy in the Rat"

This study was conducted for the ——— chemical defense group at ——— . Groups of 20-21 pregnant Wistar rats received Mestinon in drinking water at actual average doses of 12, 38 and 87 mg/kg from Day 6-16 of gestation. They were sacrificed on Day 20. Tremors and twitching were observed in dams of the high-dose group. Spiky coat and increased reflex responses were observed in the mid-dose rats. Food and water intake was sharply reduced in the high-dose group, resulting in lost weight during the first 3 days of dosing, which was not fully recovered. The fetuses were examined according to a standard protocol, but no effects were found. Not GLP, done in 1984. Cholinesterase levels (plasma and blood) were properly reduced in a dose-related manner.

"Developmental Toxicity Study of Pyridostigmine Bromide in Rabbits"

20 pregnant female NZW rabbits per group were given pyridostigmine Br at doses of 5, 15, 45 mg/kg by gavage on gestation Days 6-18. All females were sacrificed on Day 29. Body weights were not affected by the drug. The rabbits used in this study were one month younger than what the sponsor ordered. The pregnancy rate was rather low, 61%, 55%, 68% and 68% for the C, L, M, H groups.

A fifth group of 20 does received 6-aminonicotinamide at 2.5 mg/kg on Day 9 only as a positive control. The pregnancy rate here was the same as in other groups.

Adverse F0 Effects - All but one HD rabbits had tremors and diarrhea throughout dosing. Decreased activity and salivation was observed in some of these HD rabbits. One HD and one MD died after convulsing. Two controls died of non-drug-related causes.

Adverse F1 Effects -

Malformations, the total number of visceral malformations was statistically increased in the HD group compared to the control group, $p < .05$; 6.5%, 3.6%, 5.6%, 18.2% for the C, L, M, and H groups, respectively based on individual fetuses. The proportion of litters affected was 30%, 20%, 36% and 55%. No single visceral malformation was significantly increased. It appeared that hydronephrosis was the main cause for an apparent increase in malformations, the rates of incidence of this being the same ratio as the total.

Variations in carotid arteries were increased in all treated groups in a non-dose-related manner, $p < .05$; 2%, 18%, 14%, 10% for the C, L, M, H groups. The innominate arteries were also affected in a similar manner

Fetuses from the 6-aminonicotinamide group did not have hydronephrosis at all. The rate of arterial variations was similar to other treated groups. 8% of positive control fetuses had folded retina vs. zero percent on all other groups.

"Mestinon Teratology Study in the New Zealand White Rabbit."

Pyridostigmine Br formulated as Mestinon was dissolved in the drinking water of 16 pregnant does from Day 6 thru 18 of gestation. The average daily dose was 5.3, 15.4, and 35.1 mg/kg. All were sacrificed on Day 28. This study was apparently not GLP

because there was no claim for that and the stability of test article was never measured. The study was performed by _____ _____ in 1984 for _____. Cholinesterase levels were measured and were dose-relatedly decreased. There was a high level of pre-implantation loss in the MD group (43%), but this was attributed to chance factors occurring before dosing began. One high-dose litter had tail malformations. No other effects were noted.

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SEGMENT III

"Perinatal and Postnatal Study of Pyridostigmine Bromide in Rats"

Sprague-Dawley VAF female rats from ———— (25 per dose) were given pyridostigmine Br from Day 15 of gestation through lactation Day 20 at doses of 0, 3, 10, 30 mg/kg p.o. Day 0 of lactation was the day delivery of pups was completed for that particular dam.

Adverse F0 Effects -

All MD and HD dams had tremors throughout the dosing period. The severity of these tremors was dose-related and they were caused by the cholinergic properties of pyridostigmine. The occurrence of dark material around the eyes and nose was dose-related but only occurred in the MD and HD groups. Rough coat hair was seen in 3 LD dams and in 21 and 25 dams in the MD and HD groups.

One HD dam died on Day 21 of gestation. The pups were dead also. No explanation for the death was offered.

The HD dams severely curtailed eating for the first two days of dosing, but returned to normal daily weight gains by Day 20 of gestation, but their body weights didn't catch up to controls until Day 6 of lactation. The HD dams weighed 92% of controls at Day 21 of gestation.

Adverse F1 Effects -

The HD and MD pups showed very slight evidence of reduced weight gain during the first 4 days of life, but were not different from controls by Day 7. This was probably due to difficulty nursing from a dam with tremors. The same finding was made in the Segment I study.

Special Studies - Genotoxicity Tests

A. The Ames test

Pyridostigmine Bromide was tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, TA 1537 R+, and TA1538. Six plates per strain per concentration of test compound and control substance, with and without S-9, were tested. The concentrations of test drug used went up to 1,600 ug/plate. The positive controls used were: 2-aminofluorene (TA 98 & 1538); N-methyl-N'-nitro-N-nitrosoguanidine (TA 100 & 1535); 9-aminoacridine (TA 1537). All of these positive control substances increased the numbers of revertants, as expected. Pyridostigmine did not increase revertants under any condition. This study is not claimed to be GLP.

a second Ames test was performed years later at LAIR.

After performing a toxicity test, it was determined that the test drug could be applied up to 5,000 µg/plate. The usual strains, i.e. TA 98, TA 100, TA 1535, TA 1537, and TA 1538 were used with and without S-9. The same positive control substances were used as in the previous study, with the addition here of benzo (a) pyrene. These produced increases in the reversion rate, but pyridostigmine did not.

B. Rat bone marrow micronucleus test -

Bone marrow of Sprague-Dawley rats was sampled at an unreported time after an unreported duration of dosing. Daily doses of 1, 10 and 30 mg/kg in the diet were used. Mitomycin C, the positive control, was injected i.p. at doses of 0.5 mg/kg and 0.75 mg/kg. Only one rat per dose was used and they were sacrificed at 35 hours after the injection. Percentages of polychromatic erythrocytes were not affected by drug exposure, or the positive control. The numbers of micronucleated PCE's was increased by 4x with the high dose of mitomycin C, but they were not affected by pyridostigmine. The test-drug-treated rats may have been dosed for months. They came out of quarantine in November of 1986 but were not sacrificed until February (females) or June (males) of 1987. The highest dose resulted in cholinesterase inhibition of 60% at an unreported sampling time. The animals showed typical cholinergic signs in a dose-related manner.

Special Studies - The Neuromuscular Junction

"The Effects of Pyridostigmine Bromide on the Rat Diaphragm. Morphological Observations" R.E. Foster, 1983

and

"Ultrastructural Effects of Pyridostigmine on Neuromuscular Junctions in Rat Diaphragm" Hudson, Foster, and Kahng, 1986

The second title above is the published report of this work. Albino rats were treated acutely with PB at a dose ranging from 3.6 mg/kg sc (1 x LD_{50}) to 0.0036 mg/kg. They were perfused 10-30 minutes later. There was considerable within-animal variation in the severity of effects in NMJ's and even variations in the presynaptic parts of the junction. Variability was greatest at the lowest dose, which resulted in 5% cholinesterase inhibition in whole blood, which was not significantly different from controls. This suggests that these variations were probably not different from variations seen in controls. At 50% cholinesterase inhibition (0.036 mg/kg), small separations between pre- and post-synaptic elements were seen frequently. At 72-93% inhibition of cholinesterase (0.36 and 3.6 mg/kg) effects in the mitochondrial matrix ranged from small rarefactions to complete disruption or absence of the matrix. Large and dense vesicles were found in some terminals. The synaptic cleft varied in width and was invaded by fingers from neighboring Schwann cells.

Subacute treatment of rats was done with —— minipumps containing 1.5 or 10 mg/ml. Sacrifices took place at 2, 7 or 14 days after implantation. Cholinesterase inhibition was 30% at the lower dose and 70% at the higher dose. The nature of effects seen with this dosing method differed from the acute method in that whole nerve terminals were completely separated from their post-synaptic membranes. This was probably the result of continued growth of Schwann cell processes into the cleft which did not have time to develop with acute methods. At the longer dosing times, some presynaptic terminals degenerated. These effects showed dose and time dependency.

In the earlier military report, Foster reported disrupted banding patterns (A and I bands) and necrosis in the muscle fibers near NMJ's in rats dosed with 3.6 mg/kg sc.

"Effects of Single Intravenous and Oral Doses of Pyridostigmine Bromide on Neuromuscular Junction Morphology in Sprague-Dawley Rats" Page & Kluwe, 1988

This study was done according to FDA GLP. No other study in this section of this review was GLP, although the NMJ issue was examined as part of the 90-day (dietary) rat toxicity study, which was GLP.

After a preliminary study in which the i.v. and p.o. LD_{50} values were determined, the following doses were used, 3 rats/sex/dose:

i.v. - 0.01 x LD_{50} - 0.01 mg/kg males
0.015 mg/kg females = 0-10 % inhib.
0.1 x LD_{50} - 0.1 mg/kg males
0.15 mg/kg females = 15-42% inhib.

p.o. - 0.01 x LD_{50} - 1.25 mg/kg males & females
= 46-63% Chol. inhib.
0.1 x LD_{50} - 12.5 mg/kg males & females
= 80% Chol. inhib.

Focal vacuolization and rarefaction of mitochondria, retraction of nerve terminal from junctional folds, enlarged vesicles in the nerve terminal and Schwann cell intrusions in the cleft were noted in all dose groups, including controls, and were therefore considered non-drug-related effects due to fixation artifact, plane of section differences, or active synaptic metabolic differences. The only drug-related effect was found only in the high oral dose group. This finding consisted of dilated mitochondria and ER in muscle cells near the junctions, sometimes associated with irregularities of the Z band. This effect was noted at Day 1 but not Days 28 or 56.

"Acute Toxicity of Pyridostigmine in Rats: Histological Findings" Gebbers et al., 1986

Tif: RAI rats were given pyrido at 20 or 40 mg/kg by gavage and killed 24 hrs later. Plasma ChE levels were inhibited by 50% in the low dose and 75% in the high dose. Histological changes were more severe in the low dose than in the high dose! The researchers found a mean value of 8 necrotic foci per mm^2 in the low dose animals and 5/mm^2 in the high dose. These foci consisted of single fibers or groups of fibers. Some foci were accompanied by infiltrations of PMN neutrophilic granulocytes, lymphoid, and histiocytic cells, many with 10-20 leucocytes and some very severe with 50 leucocytes.

"Calcium Channel Blocker Reverses Anticholinesterase-induced Myopathy" Meshul, 1989

This study was independently financed (by the V.A.) and published in *Brain Research*. The author gave pyridostigmine bromide to Wistar rats at 3 mg/kg twice a day, subcutaneously for 1-8 days. It produced damage to the soleus muscle in the immediate vicinity of the endplate. Postjunctional folds were mildly disrupted in some junctions and Z-discs and A- and I-banding was disrupted. The duration of dosing did not affect the severity of these findings. The terminal was not affected at all. Since the same effects occur when a cholinergic agent is applied, it was thought that excessive activation by unhydrolyzed ACh was causing the damage. Diltiazem, a calcium channel blocker was added and this reduced the severity of the damage, showing that receptor-activated calcium influx was a step in the mechanism of damage. Cholinesterase inhibition was not monitored in this study.

In a similar study (or the same one), this researcher reports in an abstract that there was also separation of the terminal from the endplate, an accumulation of dark and lucent membrane-bound vesicles/vacuoles and a pronounced loss of muscle sarcoplasmic reticulum membrane. These changes persisted to Day 7. The dose of pyridostigmine was the same. The same effects were noted after administration of sarin, an irreversible cholinesterase inhibiting nerve gas.

Summary

The following toxicity studies were contracted by the U.S. Army with either 1) [redacted] 2) [redacted]; or were done by Army personnel at the Letterman Army Institute of Research, Presidio, CA:

type	route	high dose
3-mo rat	gavage	60 mg/kg
90-day rat	dietary	90 mg/kg
180-day rat	dietary	10 mg/kg males only
90-day dog	gelcaps	2 mg/kg every 8 hrs

All were under GLP regulations.

The clinical signs caused by pyridostigmine in rats were dose-dependent and consisted of tremors in high-dose rats dosed by gavage, rough coat hair, dark material around the eyes, and decreased activity. The tremors are attributable to excessive cholinergic stimulation. The startle reflex was measured in the two rat dietary studies. Even though some controls increased their startle reflex over the course of the study, a greater proportion of the treated animals did so at doses of 10 mg/kg or more. The only sign observed in dogs was a dose-related increase in the frequency of soft feces/diarrhea.

Two rats dosed with 60 mg/kg via gavage died of apparently drug-related causes. One dog dosed with 2 mg/kg t.i.d. was sacrificed in moribund condition It had abnormal blood chemistry values before dosing suggesting nephropathy and moderate chronic interstitial nephritis was found upon necropsy. It also had a red and thickened small intestine that was probably related to the test drug. This dog had frequently vomited.

Rats, especially males, in the gavage study had reduced weight gains during the first week of dosing. High-dose females gained only half as much as controls and the HD males gained only one-tenth as much. They regained their appetites in the second week and continued then to gain as much as controls. The effects was similar in the 90-day dietary study, but much less severe. There were no effects on weight gain or food consumption in the 180-day rat study (because the doses were so low) nor in the dog study.

There were no effects on hematology in any of these studies. There was a clear trend of increased blood chloride levels in male rats dosed via diet for 90 days. The highest group was

significantly greater than the control group; 101.4 vs. 107.2 Meq/l with the other groups falling in between. There was a similar effect in females, though not as pronounced. This trend was not noticeable at 28 days and was not due to outliers. Chloride levels in other rat studies were not increased, however. One high-dose female dog had increased values for BUN, albumin, haptoglobin, magnesium, and total protein. Its values for Na, K, and Cl were decreased and it had occult blood in the urine, but no histopathological correlates.

A urinalysis was performed in the dog study, but not in any of the rat studies. No effects were noted in the dogs except the dog mentioned above with occult blood. There were no organ weight effects in any study.

One of the two rats that died in the 90-day gavage study had hypospermia of the epididymis. Two other high-dose rats had this condition along with degeneration of the seminiferous tubules. There is some suggestion of a drug-related histological effect in the 180-day rat study. The numbers of rats with cortical lymphocyte aggregates in the kidney and of rats with progressive renal disease were doubled in the high-dose group (10 mg/kg) compared to the control group (not significant). The incidence of chronic multifocal inflammation of the liver was triple in the high-dose group over that of the control group ($p < .05$). There appeared to be recovery of this effect after dosing had been stopped for 30 days. Although the doses were very low, this is the longest toxicity study of pyridostigmine. Perhaps the duration of dosing is related to the appearance of kidney and liver problems. These increases were found in the group dosed every day of the week, but the group dosed only five days per week at the same dose level did not have increased liver and kidney problems. Dogs did not show clear drug-related effects in pathology. Cysts were noted in the pars distalis of the pituitary of half or fewer of the treated dogs in a non-dose-related manner, but not in any controls. This still may be chance variation. No signs of the effects of these cysts were reported.

The following toxicity studies were performed without compliance to GLP standards:

type	route	high dose
2-wk rat	dietary	40 mg/kg
100-day rat	drinking water	4.0 mg/kg
21-wk rat	dietary	64 mg/kg
4-wk rabbit	i.m.	1.0 mg/kg

4-wk rabbit	i.v.	1.0 mg/kg	
2-wk dog	?	40→8 mg/kg	
4-wk dog	i.m.	1.0 mg/kg	
4-wk dog	i.v.	1.0 mg/kg	
19-wk monkey	gavage	4.0 mg/kg	5 days/week

These studies were performed during the period from the mid-fifties to the mid-eighties. These studies did not report toxic findings different from those covered by the GLP studies.

Reproduction toxicity studies were conducted by They used doses of up 45 mg/kg in the Segment I studies (separate for males and females) and up to 30 mg/kg in the Segment II and III studies. Rabbits were dosed at up to 45 mg/kg in a Segment II study. The high-dose groups in all rat studies lost weight during the first week of dosing and later regained most of the loss. Weight gain in rabbits was not affected by the drug, although all the high-dose does had tremors. One or two adult rats died during the course of dosing as a result of the drug. Adult rats also had tremors, dark material around the eyes and rough coat, just as in the toxicity studies.

Fertility was not affected by the drug. The number of early resorptions was significantly ($p < .05$) increased (2x) in the high-dose group of the Segment II study over controls. A similar effect was noted in the Segment I study, but the variance was too great to allow for significance. Pups born to high-dose mothers were of normal body weight at birth but gained weight at a slightly slower rate than controls. I guess its hard to nurse from a mother who's shaking. In a Segment I study performed by the British for their army, doses of 9, 26 and 79 mg/kg in drinking water resulted in mortality of 25%, 12% and 1% in the L, M, H groups respectively. No account of this inverse order of mortality was given.

The total number of visceral malformations in high-dose rabbit offspring was increased to 3 times that of controls ($p < .05$). It was clear that the type of malformation most increased was hydronephrosis, although not it nor any other type was significantly increased. Arterial branching variations were moderately increased in all treated groups, but in a non-dose-related manner ($p < .05$). The pregnancy rate in this study was only 55-68%, perhaps due to the supplier providing rabbits that were one-month younger than what was ordered. The supplier was to have provided pregnant rabbits. In a British Segment II study (non-GLP) pyridostigmine was given to rabbits in the drinking water at up to 35 mg/kg and no effects were reported.

Two Ames tests were performed, one with up to 1600 µg/plate and a later one with up to 5000 µg/plate. Both studies were negative. A rat *in vivo* micronucleus test was negative. Daily doses of up to 30 mg/kg were used but the duration of dosing was not specified and the interval between the termination of dosing and sacrifice was not reported.

The issue of toxicity at the myoneural junction was a focal point of study. The earliest investigation reported by the sponsor was an ultrastructural study done at ——— by ——— in the early 1980's. In a report to a NATO study group in 1983, he reported a plethora of findings, both pre- and post-junctional. He administered pyridostigmine bromide to rats in a broad dose range that produced cholinesterase inhibition up to 93% at the highest dose. Thirty minutes after single injections, he found mitochondria within terminals to have rarified, disrupted or absent matrices. There were large, dense vesicles and the synaptic cleft varied in width and had finger-like projections from neighboring Schwann cells protruding into the cleft. Post-junctionally, he found necrosis and disruption of banding patterns in A and I bands with Z-line alterations, also.

Other rats were implanted with —— mini-pumps and received pyridostigmine for up to 14 days. The actual amount of pyridostigmine received by these rats was not reported, but their cholinesterase was inhibited by up to 70%. Complete separation of nerve terminals from the junctions were observed under this condition and some terminals were degenerating in the rats exposed for the longer durations. Foster stated that there was much variability in the severity of effects within dose groups. This work was later published with a non-military collaborator as the first author. The published version focused on the terminal findings and did not mention the muscle cell necrosis or band disruptions.

——— reported in an Army sponsored GLP study that the only drug-related effect of inhibiting cholinesterase at up to 80% with a single oral dose of 12.5 mg/kg of pyridostigmine was dilated mitochondria and endoplasmic reticula in muscle cells. These disruptions were sometimes associated with irregularities of the Z band. They found vacuolization and rarefaction of mitochondria, retraction of the nerve terminal from junctional folds, enlarged vesicles in terminals and Schwann cell intrusions into clefts, but they saw them in all dose groups. They interpreted these effects as artifacts of fixation, plane of section differences or active metabolic differences.

The Army conducted a 90-day dietary toxicity study (GLP) in which rats received up to 90 mg/kg/day of pyridostigmine. This produced inhibition of cholinesterase of up to 60-70%. The main study rats, treated identically, had cholinesterase inhibition of up to 90%. The authors claimed that sampling methods may have reduced the ability to measure all the inhibition that might have

taken place. They examined NMJ's after 28 and 90 days of dosing, looking for all of the effects reported by Foster, but found none of them. They pointed out that those effects were likely to have been produced by ischemia as a result of the anesthetic and perfusion methods used by others. They decapitated their rats and used a different fixative.

Gebbers et al. found necrosis at the endplates of muscle cells, some of which were infiltrated with leucocytes. Meshul et al. found disrupted band patterns, but not necrosis. These published, non-GLP studies used doses that inhibited ChE by 75% or more. Meshul also found large vesicles and separation of the terminal from the endplate. These rats were anesthetized.

The different findings of these studies seem to be based on the different methods used for preparing the tissue. A side-by-side comparison, performed in the same laboratory by the same people, would help to resolve the issue.

AUTHOR	GLP Study Date	HIGHEST DOSE (mg/kg)	DURATION OF DOSING	% ChE Inhib	FINDINGS
Foster Foster & Hudson	No 1983 1986	3.6 s.c. and 10 mg/ml mini-pump	single or 14 days via mini-pump	90 or 70	acute: pre-synap.- mitochond. matrix disruption, large vesicles, Schwann cell growth into cleft, post-synap.- disrupted bands and necrosis subacute: degenerated nerve terminals, complete separation of pre- and post membranes
Gebbers	No 1986	40 p.o.	single	75	necrotic foci around endplate with leucocytic infiltration
———	Yes 1988	12.5 p.o.	single	80	dilated mitochondria and ER in muscle with disrupted bands
90-day toxicity study	Yes 1988	90 dietary	90 days	90	no drug-related findings
Meshul	No 1989	3 twice a day	1-8 days	? (probably 90)	disruption of postjunctional folds and bands

Evaluation

The toxicity studies submitted were performed with doses of pyridostigmine that produced observable signs indicative of marked cholinergic activation. Furthermore, inhibition of cholinesterase was near maximal in the high dose groups. A few rats died after being dosed with the high doses of the drug. Weight loss or reduced gain resulted in each rat study except the 180-day toxicity study, which used exceptionally low doses of the test drug. These signs indicate that, overall, sufficiently high doses were used to reveal potential toxicities. There were no major problems as a result of pyridostigmine administration at high-doses for lengthy periods of time.

Additional genetic toxicity studies should be performed, such as the mouse lymphoma and chromosome aberration studies and the reverse mutation assay of WP2 tester strains of *E. coli*. I believe that it would be of benefit to the Army to conduct as many mutagenicity assays as possible. The more thoroughly they demonstrate that this drug is non-genotoxic, the less subject this drug will be to blame for possible future adverse outcomes. The rat micronucleus test was not adequately reported. A full explanation of methods used was not provided. It was not clear whether just males were tested or if females were also tested, how long dosing continued and how long after dosing stopped that the rats were sacrificed. In a telephone conversation with Dr. Clawson of USAMRDC in Ft. Detrick, I learned that the records of the micronucleus study were put in storage when the Letterman Army Institute of Research was closed. When this issue is clarified by the sponsor, we may need to request that this study be repeated or that a similar assay be conducted.

Reports of effects at the myo-neural junction were submitted from 5 different laboratories. Two of these were performed according to GLP, and it was these two that reported very slight or no effects. The other studies reported substantial changes in either pre-synaptic terminals or in the muscle tissue or both. Each study utilized a unique dosing regimen. The only covariate that seems consistent among the studies was the inhibition of cholinesterase to more than 50% of normal. No two studies reached the same conclusion about the effects of pyridostigmine.

Recommendations

1. The chronic toxicity and reproductive studies submitted herein do not suggest that pyridostigmine will cause undesirable effects in humans when used for less than 90 days.

2. The sponsor should conduct additional mutagenicity assays to more thoroughly examine the potential of the drug to cause the initiation or promotion of tumors. I recommend the mouse lymphoma assay, the bacterial reverse mutation assay using *E. coli*, and one or two other types of assays.

/S/

STEVEN SPARENBORG, Ph.D.

cc: NDA 20-414
HFD-120
HFD-120/GFitzgerald
/SSparenborg

END OF ATTACHMENT