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MMB-4 Inhibition of Acetylcholinesterase Is Similar across Species

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14. ABSTRACT
MMB-4, an oxime that can reactivate acetylcholinesterase (AChE) after inhibition by some nerve agents, is currently in advanced development for potential human use as a replacement for the currently fielded oxime 2-PAM. It has long been known that while certain oximes can be therapeutically effective against OP intoxication at appropriate doses, they can also act as reversible inhibitors of AChE at higher doses. This study was designed to test the hypothesis that the unexpected toxicity of MMB-4 in rabbits is due to increased sensitivity of rabbit AChE to inhibition by MMB-4 compared with AChE from other species.

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Introduction/Overview

MMB-4, an oxime that can reactivate acetylcholinesterase (AChE) after inhibition by some nerve agents, is currently in advanced development for potential human use as a replacement for the currently fielded oxime 2-PAM. It has long been known that while certain oximes can be therapeutically effective against OP intoxication at appropriate doses, they can also act as reversible inhibitors of AChE at higher doses. This study was designed to test the hypothesis that the unexpected toxicity of MMB-4 in rabbits is due to increased sensitivity of rabbit AChE to inhibition by MMB-4 compared with AChE from other species.

We measured AChE activity from red blood cell (RBC) ghosts generated from rabbit, guinea pig, monkey, human and rat whole blood. The concentration of MMB-4 that resulted in 50% inhibition of AChE activity (IC_{50}) for RBC ghosts from each species was found to be very similar, differing by roughly two-fold; of note, rabbit AChE was the *least* sensitive to MMB-4-mediated inhibition. When examined for the capacity to regain activity after inhibition by MMB-4, inhibited rabbit AChE was found to fully reactivate within 20 seconds of dilution of the oxime. Together, the results suggest that the unexpected toxicity of MMB-4 in rabbits is not due to highly efficient inhibition of AChE in rabbits relative to other species.

Materials and Methods

Reagents

Acetylthiocholine (ATCh) and 5, 5' dithiobis 2-nitrobenzoic acid (DTNB) were obtained from Sigma-Aldrich (St. Louis, MO). MMB-4 (1,1-methylenebis 4-hydroxyiminomethyl-pyridinium dimethanesulfonate) was obtained from the stock supply available at the USAMRICD.

Biological Samples

Guinea pig (Hartley; Charles River Laboratories, Wilmington, MA) and rat (Sprague Dawley, Charles River Laboratories) whole blood was collected at the USAMRICD in heparinized vials and immediately stored at 4°C until use. Human, monkey (rhesus macaque), and rabbit (New Zealand White) whole blood (heparinized) was purchased from Bioreclamation IVT (Baltimore, MD) and stored at 4°C until use. No individual patient information was provided for the human samples.

Red Blood Cell Ghost Preparation

Red blood cell ghosts were prepared from whole blood samples from each animal species using a previously published method (1). In brief, heparinized whole blood stocks (30ml) were centrifuged at 3,000 x g for 10 minutes at 4°C, and plasma was decanted from the cell pellet. Cell pellets were re-suspended and washed consecutively with two volumes of ice cold

phosphate buffer (0.1 M, pH 7.4) followed by centrifugation. Packed erythrocytes were then re-suspended and diluted in 20 volumes of hypotonic phosphate buffer (6.7 mM, pH 7.4) to promote hemolysis. Suspended erythrocytes were ultra-centrifuged at 50,000 x g for 30 minutes at 4°C. Supernatants were removed and pellets were re-suspended in hypotonic phosphate buffer for two additional washing cycles followed by 50,000 x g for 30 minutes at 4°C. Erythrocyte ghosts were re-suspended in phosphate buffer and then concentrated by centrifugation at 100,000 x g for 30 minutes at 4°C. Red blood cell (RBC) ghosts were pooled and diluted to a final volume of 10 ml with ice cold phosphate buffer (0.1 M, pH 7.4). Aliquots of the erythrocyte ghosts were stored at -80°C until use.

AChE Inhibition by MMB-4

AChE activity was measured using 0.2 mM ATCh and 0.5 mM DTNB in 0.1 M phosphate buffer (pH 7.4) at 25°C, with detection of the hydrolyzed DTNB product TNB determined by measuring absorbance at 435 nm. AChE activity was determined either in the absence or in the presence of MMB-4 (at 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19, 0.097, 0.048, 0.024, 0.012, 0.006, 0.003 mM). Assays conducted in the presence of MMB-4 were initiated with a mixture of ATCh, DTNB, and MMB-4. Aliquots of RBC ghosts were quickly thawed and diluted with ice cold 0.1 M phosphate buffer (pH 7.4). A sufficient concentration of RBC ghosts was added in the absence of MMB-4 to provide a measurable initial velocity (linear response in accumulation of TNB) over a 5-minute reaction period using a SpectraMax Plus (Molecular Devices, Sunnyvale, CA). Initial velocities determined for AChE in the absence of MMB-4 were set at 100% and used to normalize velocities measured in the presence of MMB-4. Assays were conducted in triplicate using a 96-well format with data acquisition via SoftMax Pro (version 5.4). An IC_{50} value was determined for AChE from each animal species by fitting the percent of AChE activity with respect to MMB-4 concentration in GraphPad Prism (version 5) using a nonlinear regression dose-response model for inhibition (normalized response with variable slope).

Assessing the Rate of Recovery of Activity of Rabbit AChE after Inhibition by MMB-4

Rabbit AChE activity (using rabbit red blood cell ghosts) was measured (as above, at 435 nm) in the absence or presence of MMB-4 (3 mM) for ~2.5 minutes with 0.2 mM ATCh and 0.5 mM DTNB in 0.1 M phosphate buffer (pH 7.4) at 25°C. To determine if MMB-4 inhibition of rabbit AChE is readily reversible, a fraction of each reaction was diluted 4-fold into fresh 0.2 mM ATCh and 0.5 mM DTNB and reaction rates were monitored for an additional ~2 minutes.

Results

UV/Vis Spectrum of MMB-4 and TNB

MMB-4 absorbs light maximally at ~400 nm, with significant absorbance at 412 nm (Figure 1A). TNB, the reaction product of DTNB, has a maximum of absorbance at 412 nm (Figure 1B). Spectrophotometric assays are typically designed to measure the loss or accumulation of a single chemical species in a reaction system. At 435 nm the absorbance profile of MMB-4 is significantly depressed (see Figure 1A), while the absorbance of TNB at 435 nm is only modestly lower than at 412 nm. Therefore, AChE activity and inhibition studies were carried out at 435 nm to reduce interference from MMB-4.

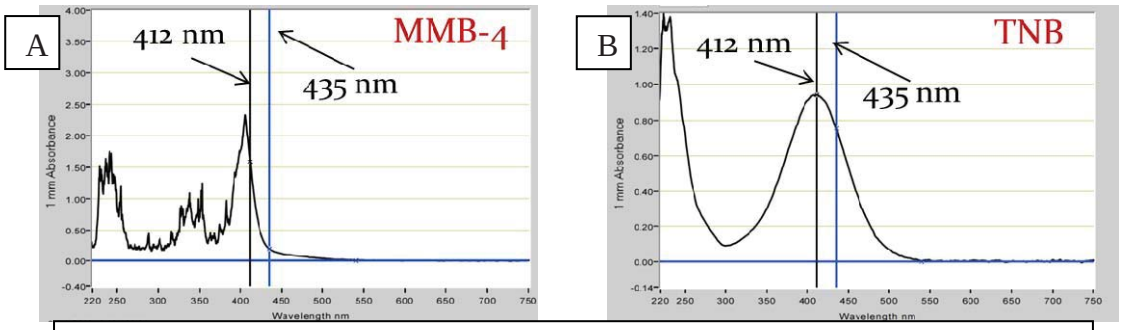

Figure 1. Full spectrum profiles (220-750 nm) measured for both MMB-4 and TNB. MMB-4 absorbs significantly less at 435 nm compared to 412 nm. TNB has a maximum absorbance at 412 nm with ~80% maximal absorbance at 435 nm.

Comparison of IC_{50} Values for MMB-4 with AChE from Different Species

As shown in Figure 2, MMB-4 was found to be an inhibitor of AChE for each of the species tested, where inhibition ranged from 0 to 100% over a roughly 2-log MMB-4 concentration range. IC_{50} values (and associated error) for MMB-4 with each AChE sample were calculated from these curves, as shown in Table 1. Note that the lower the IC_{50} value, the stronger the inhibition. The IC_{50} values were very similar for AChE from each species, differing by roughly 2-fold. Rhesus macaque AChE was found to be the most sensitive to inhibition by MMB-4, while rabbit AChE was the least sensitive.

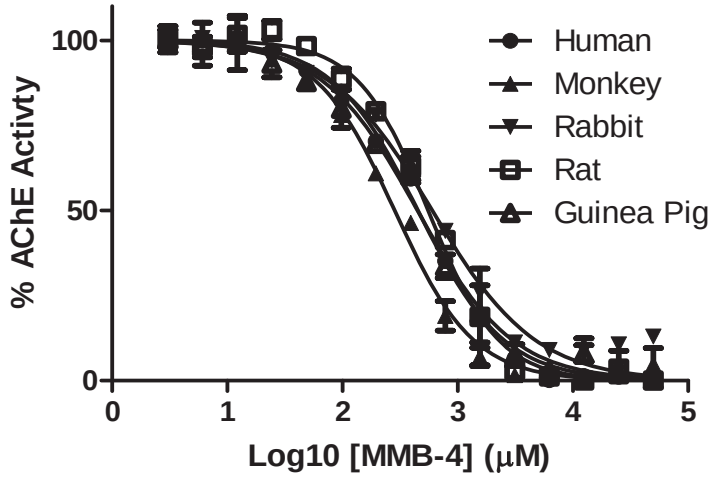

Figure 2. Percent AChE activity relative to MMB-4 concentration for each animal species. The concentration of MMB-4 that reduces AChE activity to 50% is the IC_{50} value.

Table 1. IC_{50} values for MMB-4

Animal Species	IC_{50} [95% CI] (μM)
Human	452.5 [426.2 - 480.3]
Monkey (Rhesus macaque)	283.9 [259.1 - 311.0]
Rabbit (New Zealand White)	600.5 [534.4 - 674.9]
Guinea Pig (Hartley)	448.3 [380.3 - 528.4]
Rat (Sprague Dawley)	554.4 [482.3 - 637.4]

Assessing the Capacity of Rabbit AChE to Recover Activity after Inhibition by MMB-4

To determine if MMB-4 inhibition of rabbit AChE is readily reversible, a modified Ellman's assay (2) was used. For these experiments, AChE activity from rabbit RBC ghosts was determined in the presence of MMB-4 at 3 mM (well above the IC_{50} value). As shown in Figure 2, this concentration of MMB-4 was sufficient to cause substantial inhibition of AChE activity. If MMB-4 dissociates slowly from the AChE active site, inhibition is expected to persist following dilution of MMB-4. After initial incubation of rabbit AChE with 3 mM MMB-4, samples were diluted 4-fold and immediately assessed for activity. As shown in Figure 3, the inhibition of rabbit AChE by MMB-4 is very rapidly reversed when the oxime is diluted; the activity level of

MMB-4-inhibited rabbit AChE was indistinguishable from uninhibited rabbit AChE within 20 seconds of dilution of MMB-4.

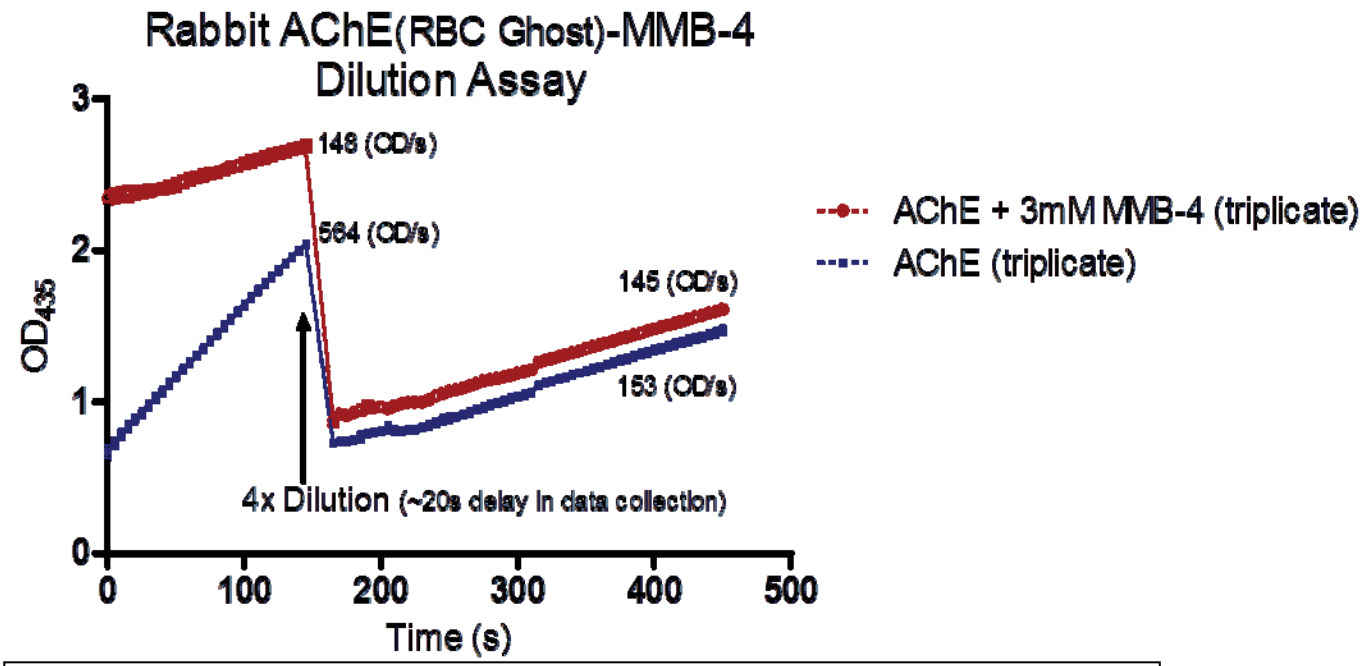

Figure 3. Reversal of Inhibition of Rabbit AChE by MMB-4 after Dilution. AChE activity was measured in the presence (3 mM) and absence of MMB-4 for ~120 seconds. After a 4-fold dilution (and 20-second gap in data collection) of the samples, AChE was assessed again. Rabbit AChE activity was restored to uninhibited levels very rapidly following sample dilution (and reduction in MMB-4 concentration).

Discussion

We examined the capacity of MMB-4 to inhibit AChE activity in RBC ghosts from human, monkey (rhesus macaque), rabbit (New Zealand White), guinea pig (Hartley), and rat (Sprague Dawley). Monkey AChE was the most sensitive to MMB-4 inhibition, with the lowest IC_{50} value measured (~284 uM) for any of the animal species. Rabbit AChE was the least sensitive to inhibition by MMB-4, with an IC_{50} of ~600 uM. This result provides strong evidence to reject the hypothesis that the unexpected toxicity of MMB-4 in rabbits is due to direct inhibition of rabbit AChE by MMB-4.

Even though the IC_{50} values for MMB-4 were remarkably similar (differing by only 2-fold) among all species tested, it remained possible that the increased sensitivity of rabbits to MMB-4 could be mediated by an extremely slow off-rate of MMB-4 from rabbit AChE; the IC_{50} values presented were generated in the presence of excess MMB-4, and thus the dissociation rates of MMB-4 from the enzymes could not be determined in these experiments. We examined the

rate of recovery of rabbit AChE activity after inhibition by MMB-4 and subsequent dilution to reduce the MMB-4 concentration. The results indicate that rabbit AChE recovers activity very rapidly (within 20 seconds) after reduction in MMB-4 concentration, providing further evidence to reject the hypothesis that differential sensitivity of the rabbit to MMB-4 toxicity is mediated by interaction with AChE.

Together, the data suggest that the enhanced sensitivity of rabbits to MMB-4 is not due to inhibition of AChE, but rather has origins in an alternate mechanism of toxicity.

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