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***In vitro* P-glycoprotein activity does not completely explain *in vivo* efficacy of novel centrally effective oxime acetylcholinesterase reactivators**

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Abstract

Novel substituted phenoxyalkyl pyridinium oxime acetylcholinesterase (AChE) reactivators (US patent 9,227,937) that showed convincing evidence of penetration into the brains of intact rats were developed by our laboratories. The oximes separated into 3 groups based on their levels of brain AChE reactivation following exposure of rats to the sarin surrogate nitrophenyl isopropyl methylphosphonate (NIMP). P-glycoprotein (P-gp) is a major blood brain barrier (BBB) transporter and requires ATP for efflux. To determine if P-gp affinity screening could be used to reduce animal use, we measured *in vitro* oxime-stimulated ATPase activity to see if the *in vivo* reactivation efficacies related to the oximes' functions as P-gp substrates. High efficacy oximes were expected to be poor P-gp substrates, thus remaining in the brain longer. The high efficacy oximes (24–35% brain AChE reactivation) were worse P-gp substrates than the low efficacy oximes (0–7% brain AChE reactivation). However the oxime group with medium *in vivo* reactivation of 10–17% were even worse P-gp substrates than the high efficacy group so their reactivation ability was not reflected by P-gp export. The results suggest that *in vitro* P-gp ATPase activity can remove the low efficacy oximes from *in vivo* testing, but is not sufficient to differentiate between the top two tiers.

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Keywords

P- glycoprotein; oxime; acetylcholinesterase; reactivator; BBB; organophosphate

Introduction

The organophosphate (OP) anticholinestese class includes both insecticides (e.g., chlorpyrifos, diazinon, malathion) and the nerve agents (e.g., sarin, VX, tabun). Since these chemicals persistently inhibit acetylcholinesterase (AChE), excess acetylcholine accumulates in synapses resulting in excitotoxicity, seizures, permanent brain damage, and even death. Because of the large quantities of OPs present worldwide, they pose a great chemical threat. The OP nerve agent, sarin, was manufactured by a cult named “Aum Supreme Truth” and released in 1994 near the district court in Matsumoto and in 1995 in the subway near the National Police Agency in Tokyo (Yanagisawa *et al.* 2006) in terrorist attacks that resulted in 19 deaths and 6,100 exposures. More recently, the Assad regime deployed sarin in Syria in 2013 and 2017 resulting in many civilian deaths (Loveluck 2017). VX was used to assassinate the half-brother of North Korean leader Kim Jong Un (Swenson 2017) and Novichok, which is thought to be a new and extremely toxic OP nerve agent, was used in the March 4th, 2018 attempted assassination of a former Russian spy and his daughter (Hay 2018). OP insecticide poisoning has killed 5 million people during the last thirty years and continues to kill at the rate of 200,000 per year, mostly by deliberate ingestion during suicide attempts (Eddleston and Chowdhury 2015). Because of such risks, therapies to counteract OP poisoning effects, both physiological and cognitive, continue to be of paramount importance.

Present therapy involves atropine, which acts as a competitive inhibitor at autonomic cholinergic receptors, benzodiazepines to prevent seizures and pyridinium oxime administration to reactivate the inhibited AChE. In the United States this oxime is pralidoxime chloride (2-PAM), which cannot effectively cross the blood-brain barrier (BBB) and therefore fails to prevent excitotoxicity and its subsequent brain damage that can lead to long term behavioral changes and cognitive deficits (Clement *et al.* 1979, Sakurada *et al.* 2003). To meet this critical need, our laboratories invented a series of substituted phenoxyalkyl pyridinium oxime acetylcholinesterase reactivators (US patent 9,227,937) (Figure 1 and Table 1), some of which have provided convincing evidence of penetrating the rat brain in *in vivo* tests using nerve agent surrogates that leave AChE inhibited with the same chemical moiety as sarin or VX (Meek *et al.* 2012, Chambers *et al.* 2013, Chambers *et al.* 2016). These novel oximes are investigational at present.

The BBB is formed by brain-capillary endothelial cells that have little pinocytotic ability, few fenestrations and are linked by tight junctions (Tamai and Tsuji 2000). Because of this morphology, the BBB physically prevents the movement of chemicals into the brain from the blood by going around the endothelial cell layer (Reese *et al.* 1967, Pardridge 1988). Chemicals must move through the endothelial cells to enter and affect the brain. This requirement to pass through cells means that the BBB should be more permeable to lipophilic than hydrophilic chemicals and much effort has gone into increasing the

lipophilicity of drugs which need to enter the brain (Tamai and Tsuji 2000). Although our novel oximes displayed a huge range in lipophilicity (3 orders of magnitude), they were all more lipophilic than traditional oximes such as the currently FDA-approved oxime antidote 2-PAM (Chambers *et al.* 2013) and therefore more likely to cross the BBB.

Prior to the start of *in vivo* testing, prevailing theory on the BBB suggested that the most lipophilic novel oximes would best enter the brain and therefore be the best reactivators of brain AChE. However higher oxime lipophilicity did not correlate directly to increased reactivation efficacy in our previous rat *in vivo* tests (Chambers *et al.* 2013). For this reason *in vitro* lipophilicity testing is not a suitable screen for *in vivo* brain AChE reactivation efficacy.

This disconnect led us to consider the possibility that some of the novel oximes were interacting with one or more of the BBB transporters. The P-glycoprotein (P-gp) efflux transporter is a membrane glycoprotein member of the ATP binding cassette (ABC) superfamily which is encoded by the *MDR1* gene in humans but 2 genes, *Mdr1a* and *Mdr1b*, in rodents (Schwab *et al.* 2003). P-gp spans the luminal membrane of brain capillary endothelial cells and its central portion is a channel through which substrates are pumped out of the BBB endothelial cell back into the bloodstream. Substrates can enter the transport channel either from the interior of the cell or from its membrane but in all cases transport from the intracellular space requires ATP (Edwards 2003).

Increased P-gp activity results in reduced drug accumulation in the brain and is the main reason it is so difficult to deliver small molecule drugs to treat brain disease (Miller 2014). Since P-gp actively moves lipophilic chemicals out of the endothelial cells and back into the blood (Tsuji *et al.* 1992), perhaps it prevents some of our novel oximes from entering or remaining in the brain to reactivate the inhibited AChE.

Our hypothesis was that the high efficacy oximes from the *in vivo* tests would be the worst P-gp substrates. This would allow the oxime to remain in the brain longer and have more opportunity to reactivate the AChE. Conversely the low efficacy oximes should be the best P-gp substrates and be rapidly removed from the brain before having a chance to reactivate the AChE.

If an oxime's efficacy as a P-gp substrate correlates with its *in vivo* AChE reactivation efficacy, we could use an established *in vitro* test to screen our large number of candidates. This would result in a significant reduction in animal use.

Materials and Methods

Reactivator Groups

Oximes were sorted into 3 groups based on their level of efficacy in reactivating rat brain AChE during prior *in vivo* research (Chambers *et al.* 2016). The high efficacy group resulted in 24 to 35% *in vivo* reactivation of rat brain AChE, the medium group reactivated 10 to 17% and the low efficacy group caused less than 7% reactivation.

Testing for P-gp Affinity

Since substrate transport from the brain-capillary endothelial cells requires ATP, the amount of oxime-stimulated ATPase activity was measured using the Corning® Gentest™ ABC Transporter Membrane ATPase Assay (Cat. #459006 Tewksbury, MA) per the manufacturer's instructions. The colorimetric assay is based on determination of ATPase activity by measuring the release of inorganic phosphate (Pi) in the presence of the suspected P-gp substrate. Higher amounts of Pi production correlate with a faster rate of efflux through the P-gp transporter indicating that the test chemical is a good P-gp substrate.

Since the *in vivo* testing upon which the efficacy of the oximes was judged utilized rats, Rat Multi-Drug-Resistance 1a (MDR1A) (Gentest™ # 453440) and 1b (MDR1B) (Gentest™ # 453434) membranes were selected. Prior research (Schinkel *et al.* 1995) suggested that both proteins are required in rodents to fulfill the same function as the single human MDR1. The P-gp protein is produced from rat *Mdr1a* or *b* cDNA expressed by a baculovirus system infecting High Five, BTI-TN5B1–4 insect cells. Microsomes were then prepared from these to yield membrane bound P-gp protein derived from either rat *Mdr1a* or *b* genes. Since the rest of the membrane is of insect origin, no other mammalian transport systems were present. Microsomes from wild-type baculovirus infected High Five, BTI-TN5B1–4 insect cells (Gentest™ # 453200) were used as controls. These microsomes have no transporters. These techniques were modified from (Sarkadi *et al.* 1995).

The Gentest™ ATPase Assay itself is a modification of (Druekes *et al.* 1995) designed for use with a 96 well plate. Each 60 µl experimental reaction mixture contained: (final concentrations) 20 µg of MDR1A, MDR1B, or control microsome preparation; 4 mM MgATP, and 20 µM verapamil (positive control) or 5mM test oxime in an assay buffer comprised of 50 mM Tris-MES, 2 mM EGTA, 50 mM potassium chloride, 2 mM DTT, and 5 mM sodium azide.

A control reaction mixture had 100 µM sodium orthovanadate (NAOV) added to the above solution in order to measure non-P-gp ATPase activity since orthovanadate inhibits P-gp by trapping MgADP in the nucleotide binding site. This amount was subtracted from the experimental value to yield vanadate-sensitive (P-gp specific) ATPase activity. Two more reaction mixtures both lacking MgATP, with and without NAOV, were also prepared to act as time zero controls.

All 4 reaction mixes were incubated in parallel for 30 min. at 37°C with shaking and stopped with 30 µl of 10% SDS. The zero-time control mixes were then supplemented with MgATP. Next 200 µl of colorimetric reagent (35 mM ammonium molybdate in a 1 to 4 mix of 15 mM zinc acetate/10% ascorbic acid) was added. The plate was returned to 37°C for an additional 20 min. incubation with shaking. Absorbance at 800 nm was measured and the amount of Pi released was determined by comparison to a phosphate standard curve.

Two assays were done for each oxime, the vehicle (DMSO), the positive control (verapamil) and a water blank. Each assay contained 4 technical replicates. The Grubbs' test was used to remove outliers prior to averaging the results. Calculations were as follows: Average Pi release for the test chemical minus release for same with NAOV / 30 min / 0.02 mg =

nmol/min/mg protein which is the *in vitro* ATPase activity rate (*i.e.*, ability to be transported by P-gp). Next the oxime ATPase rates had the DMSO vehicle rate subtracted and the positive control (verapamil) had the water blank rate subtracted. This gave the final *in vitro* ATPase activity rate. Each novel oxime's final *in vitro* ATPase activity rate was compared to its *in vivo* brain AChE reactivation capability. Due to the lack of randomness in assignment of the oximes to reactivation groups and the small sample number (6 or less) per group, the nonparametric Kruskal-Wallis one-way ANOVA was used to test for significant differences. When a statistically significant difference was found, the Wilcoxon rank sum test with a Bonferroni correction was used to determine which pairs of groups differed.

Results

The median AChE reactivation percentages from the *in vivo* tests were significantly different ($P < 0.001$) with the high efficacy group's median being 29%, the medium group's 13% and the low group's 0%. The reactivation efficacies of all the groups were significantly different from each other with the medium to low groups at $P < 0.01$, high to medium at $P < 0.03$, and high to low at $P < 0.02$ (Table 2).

As before, lipophilicity failed to correlate with reactivation efficacy as there was no statistically significant difference found amongst the three groups with respect to their median octanol/water coefficients (High = 0.183, Medium = 0.259, Low = 0.291; $P = 0.55$).

The median MDR1A associated ATPase activity for the high reactivating oxime group was 4 nmol/min/mg, 0 for the medium group and 21 for the low reactivating oximes (Table 2). For comparison, the median MDR1A value for the positive control, verapamil was 33 nmol/min/mg. The oxime group medians were significantly different from each other with $P < 0.03$. Intergroup comparisons showed that the high and medium groups were not significantly different ($P < 0.4$). Neither were the high and low groups ($P < 0.2$). Prior to applying the Bonferroni correction, the medium and low groups were significantly different with a $P < 0.03$ but after the correction the value rose to $P < 0.08$.

The median MDR1B associated ATPase activity for the high reactivating oxime group was -1 nmol/min/mg, 1 for the medium group and 4 for the low reactivation oximes. None of the three groups were significantly different with a $P < 0.2$. The median MDR1B value for verapamil was 10 nmol/min/mg. For several oximes, the value for ATPase activity was negative (Table 2). This means that the rate of transport for these oximes through P-gp was lower than the rate for orthovanadate which is an inhibitor of P-gp and functioned as the assay's negative control.

The total ATPase activity (MDR1A and B) showed a pattern similar to that of MDR1A alone. The three groups were significantly different at $P < 0.04$ but intergroup analysis showed no significant difference between high and low ($P < 0.2$) or high and medium ($P < 1.2$). As before, the medium and low groups were significantly different with a $P < 0.03$ but after the Bonferroni correction the value rose to $P < 0.08$ (Table 2).

Discussion

In spite of the low statistical significance, different distinct patterns are discernible when *in vivo* reactivation efficacy is compared to *in vitro* ATPase activity (Table 2). The low efficacy oximes generally had the highest rates of ATPase activity, which would indicate higher potential efflux via the P-gp transporter. The medium reactivation oxime group, however, generally showed lower rates of ATPase activity, suggesting lower efflux than the most effective oxime group. This means that the medium reactivation oximes were worse P-gp substrates than those with the highest reactivation percentages.

As our hypothesis suggested, the low efficacy *in vivo* AChE reactivators had higher affinities for P-gp as shown by their high rates of *in vitro* ATPase activity. At 21 nmoles/min/mg the median for the low group was over five times the median rate of the high group and twenty-one times the medium efficacy group rate (Table 2). This would suggest that these oximes, as good substrates for P-gp, were quickly transported back to the bloodstream and were either unable to reach the inhibited brain AChE or failed to stay within the brain long enough to reactivate AChE. The low efficacy group's much larger ATPase activity rate makes it feasible to use this method to cull the poorest oximes from further *in vivo* examination.

Contrary to our hypothesis, the novel oximes which were the high efficacy *in vivo* AChE reactivators did not have the lowest affinity for P-gp but rather fell between the low efficacy reactivators and those that had medium activity. Also contrary to our hypothesis, the oximes with medium *in vivo* AChE reactivation ability generally had the lowest affinity for the P-gp efflux transporter which should mean that they were able to stay within the brain longer and had more time to reactivate AChE than the other two groups.

The inverted results found for the high and medium oxime reactivators indicate that another transport system and/or aspect of pharmacokinetics is likely influencing the *in vivo* reactivation. The breast cancer resistance protein (BCRP) efflux transporter is found in the BBB, uses ATP and transports many of the same substrates as P-gp (Bircsak *et al.* 2013). The medium reactivators may be substrates of BCRP which removes them quickly enough to reduce their effectiveness. The capillary endothelial cells of the BBB are known to possess Phase 1 and Phase 2 metabolic enzymes but little is known regarding their effects or any possible interaction they may have with BBB transporters (Miller 2014). Their presence, however, means that metabolites of the oximes may be being transported or rejected by P-gp instead of the original chemicals.

It is also possible that some of the oximes have the ability to block the P-gp transporter so that efflux stops and along with it consumption of ATP. This would explain the negative ATPase rates. It was recently determined that for a drug to cause P-gp associated ATPase activity specific disulfide bonds must be formed following the binding of the ATP molecule (Loo *et al.* 2013). It is possible that the oximes exhibiting ATPase activity less than orthovanadate have inhibited the formation of these bonds.

Another possibility may be that many substrate molecules enter the P-gp binding site but only a few are expelled (Loo and Clarke 2005). These authors suggest that substrates can

bind in many orientations but only one results in ATPase activity and transport. They also indicate that the binding pocket is large enough to bind two different drugs at the same time. This bottleneck wherein many chemicals enter the binding site but few are expelled led Tran *et al.* (2005) to suggest that the drugs that were not expelled must eventually move back into the inner monolayer of the apical membrane where they can presumably migrate around until they reenter the P-gp binding site through one of two transmembrane domain portals (Subramanian *et al.* 2016) and possibly assume a new orientation conducive to ATPase activity and transport. In this manner the medium efficacy oximes could be trapped in a traffic jam between the apical membrane and the binding site resulting in both a low ATPase value and yet an inability to enter the brain to reactivate any AChE.

P-gp affinity was able to screen out the least effective oxime reactivators but it could not differentiate between the best and the second tier. Other researchers have found that P-gp assays based on ATPase activity are suitable only as screens (Bircsak *et al.* 2013). This may be due to the fact that the ATPase activity assay cannot distinguish P-gp substrates from inhibitors (Zhang *et al.* 2006). The complexity of P-gp transport with its numerous substrates, modulators, inhibitors, competitive and non-competitive interactions (Subramanian *et al.* 2016), may make it impossible to replicate completely with a single assay of any format. Researchers are starting to report that only the use of multiple transporter assays is sufficient to completely evaluate a chemicals' P-gp susceptibility (Dickens *et al.* 2013). The best options would include measurement of substrate accumulation using a bidirectional transmembrane system and pharmacokinetic studies (Bircsak *et al.* 2013, Dickens *et al.* 2013). This would be the next step to take with the oximes of medium reactivation efficacy.

While pharmacokinetics is certainly an important consideration on *in vivo* efficacy, the paradigm used in our earlier studies was designed to convincingly demonstrate which novel oximes could enter the brain. Extensive discussion of our oximes' reactivation efficacies (*in vitro* and *in vivo*), lipophilicities and central bioavailabilities can be found in earlier works (Meek *et al.* 2012 and Chambers *et al.* 2013, 2016). These experiments administered the oxime at the time of peak brain AChE inhibition when the OP compound was in the process of being cleared; this strategy reduced or eliminated the potential for reinhibition of reactivated AChE in the periphery that could artificially reduce the circulating levels of OP available to enter the brain. Therefore any decrease in brain AChE inhibition following oxime administration should have been the result of oxime entry into the brain and the results would not have been confounded by the disposition of the OP's. The oximes were all administered at the same time post OP challenge and at the same molar equivalent so that comparison of the efficacy of the oximes could be made as parallel as possible. While we are not ignoring the potential contributions of the metabolism and disposition differences in the oximes, the present study was set up to determine whether interaction with P-gp could predict some of our *in vivo* results, and to an extent these results were predictive.

Conclusion

In vitro P-gp affinity was able to screen out the low efficacy *in vivo* oxime reactivators but it could not differentiate between the high efficacy group and the second tier. Nevertheless it is

feasible to use the assay to eliminate the least effective compounds from *in vivo* testing and therefore reduce animal use.

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Disclosure of Interest

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Figure 1.
Generic structure of substituted phenoxyalkyl pyridinium oximes.

TABLE 1:

Novel substituted phenoxyalkyl pyridinium oximes. n is the number of C's in the alkyl chain (n in Figure 1). R is the substitution on the phenoxy moiety (R in Figure 1).

OXIME	n	R
MSU 1	4	4-Cl-
MSU 6	4	3-CH=CHCH=CH-4
MSU 13	3	3-CH=CHCH=CH-4
MSU 17	4	4-Ph-C(:O)-
MSU 18	5	4-Ph-C(:O)-
MSU 20	4	4-Ph-CH ₂ -O-
MSU 29	4	3,4-Cl ₂ -
MSU 30	3	2,4,6-Cl ₃ -
MSU 32	5	3-O-C(:O)CH=C(CH ₃)-4
MSU 33	3	3,4-Cl ₂ -
MSU 37	4	3-CH ₃ -4-Cl-
MSU 38	4	2,6-Cl ₂ -4-O ₂ N-
MSU 39	4	2,4,6-Cl ₃ -
MSU 42	4	4-Cl-3,5-(CH ₃) ₂ -
MSU 44	3	4-Br-
MSU 47	5	2,3,5-(CH ₃) ₃ -

Table 2:

Comparison of the novel oximes *in vivo* rat brain AChE reactivation efficacy (%) to their corresponding *in vitro* ATPase activity (nmoles/min/mg). The highest efficacy group resulted in more than 20% *in vivo* reactivation of rat brain AChE, the medium group reactivated 10 to 20% and the least effective group resulted in less than 10% reactivation. ATPase activities are shown for rat MDR1A and MDR1B both separately and combined (TOTAL). AChE reactivation efficacies for oximes marked with ◀ have been reported previously (Chambers et al. 2013).

OXIME	<i>In Vivo</i> AChE Reactivation (%)	MDR1A ATPase Activity nmoles/min/mg	MDR1B ATPase Activity nmoles/min/mg	TOTAL (A + B) ATPase Activity nmoles/min/mg
<u>HIGHEST EFFICACY</u>				
MSU 20 ◀	35	12	6	18
MSU 6 ◀	30	13	-1	12
MSU 1 ◀	29	2	-1	1
MSU 13 ◀	28	4	6	10
MSU 33 ◀	24	4	-2	2
MEDIAN	29	4	-1	10
<u>MEDIUM EFFICACY</u>				
MSU 37	17	-0.2	4	4
MSU 38	14	2	1	3
MSU 30 ◀	13	-9	-4	-13
MSU 44	10	-2	-9	-11
MSU 29 ◀	10	16	2	18
MEDIAN	13	0	1	3
<u>LOWEST EFFICACY</u>				
MSU 39	7	21	10	31
MSU 42	5	17	6	23
MSU 32	0	27	5	32
MSU 17 ◀	0	23	2	25
MSU 18 ◀	0	21	1	22
MSU 47	0	-1	2	1
MEDIAN	0	21	4	24