BIOCHEMICAL PHARMACOLOGY 73 (2007) 1807–1817

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# Kinetic analysis of reactivation and aging of human acetylcholinesterase inhibited by different phosphoramidates

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ARTICLE INFO

*Article history:*
Received 23 January 2007
Accepted 13 February 2007

*Keywords:*
Acetylcholinesterase
Organophosphates
Oximes
Reactivation
Aging
Structure-activity relationship

ABSTRACT

The high number of fatalities due to poisoning by organophosphorus compound-based (OP) pesticides and the availability of highly toxic OP-type chemical warfare agents (nerve agents) emphasize the necessity for an effective medical treatment. Acute OP toxicity is mainly caused by inhibition of acetylcholinesterase (AChE, EC 3.1.1.7). Reactivators (oximes) of inhibited AChE are a mainstay of treatment. However, human AChE inhibited by certain OP, e.g. the phosphoramidates tabun and fenamiphos, is rather resistant towards reactivation by oximes while AChE inhibited by others, e.g. the phosphoramidate methamidophos is easily reactivated by oximes. To get more insight into a potential structure-activity relationship human AChE was inhibited by 16 different tabun analogues and the time-dependent reactivation by 1 mM obidoxime, TMB-4, MMB-4, HI 6 or HLö 7, the reactivation kinetics of obidoxime and the kinetics of aging and spontaneous reactivation were investigated. A clear structure-activity relationship of aging, spontaneous and oxime-induced reactivation kinetics could be determined with AChE inhibited by *N*-monoalkyl tabun analogues depending on the chain length of the *N*-alkyl residue. *N*,*N*-dialkyl analogues bearing ethyl and *n*-propyl residues were completely resistant towards reactivation while *N*,*N*-di-i-propyl tabun was highly susceptible towards reactivation by oximes. AChE inhibited by phosphonoamidate analogues of tabun, bearing a *N*,*N*-dimethyl and *N*,*N*-diethyl group, could be reactivated and had comparable reactivation kinetics with obidoxime. These results in conjunction with previous data with organophosphates and organophosphonates emphasizes the necessity for kinetic studies as basis for future work on structural analysis with human AChE and for the development of effective broad-spectrum oximes.



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*Abbreviations:* AChE, acetylcholinesterase (E.C. 3.1.1.7); BChE, butyrylcholinesterase (E.C. 3.1.1.8); ATCh, acetylthiocholine iodide; BTCh, S-butyrylthiocholine iodide; DTNB, 5,5′-dithio-bis(-2-nitrobenzoic acid); obidoxime, 1,1′-(oxybis-methylene)bis[4-(hydroxyimino)methyl] pyridinium dichloride; pralidoxime, 2-[hydroxyimino methyl]-1-methylpyridinium chloride; HI 6, 1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]pyridinium dichloride monohydrate; HLö 7, 1-[[[4-(aminocarbonyl)pyridinio]methoxy]methyl]-2,4-bis-[(hydroxyimino)methyl] pyridinium dimethanesulfonate); TMB-4, 1,3-trimethylene-bis(4-hydroxyiminomethylpyridinium) dibromide; MMB-4, 1,1′-methylene-bis[4-(hydroxyimino)methyl] pyridinium dibromide

0006-2952/$ – see front matter 
doi:10.1016/j.bcp.2007.02.008

## 1. Introduction

Organophosphorus compounds (OP) still pose a major problem in toxicology. The use of OP pesticides for pest control and for attempting suicide causes huge numbers of intoxications and several hundreds of thousands of fatalities per year especially in developing countries [1,2]. In addition, the availability and use of highly toxic OP based nerve agents represents a pertinent hazard to the population [3,4]. The main mechanism of action of OP is a progressive inhibition of acetylcholinesterase (AChE) by phosphylation (denotes phosphorylation and phosphonylation) of the active site serine leading to an inactive enzyme species [5,6]. The failure of inhibited AChE to hydrolyze the neurotransmitter acetylcholine results in an endogenous acetylcholine intoxication followed by an over-stimulation of cholinergic receptors, a massive disturbance of numerous body functions and finally in death by respiratory failure [7,8].

Presently, standard treatment of OP poisoning includes the administration of a muscarinic antagonist, e.g. atropine, and of an oxime to reactivate inhibited AChE. Hereby, antimuscarinic drugs act only symptomatically while oximes may restore the enzyme function. Due to the fact that the established oximes obidoxime, pralidoxime and TMB-4 (Fig. 1) are considered to be rather ineffective against various nerve agents, numerous new oximes were synthesized in the past decades [9]. Presently, several bispyridinium oximes (e.g. HI 6, HLö 7, MMB-4; Fig. 1) are under investigation to replace the established compounds.

Recently, a kinetic analysis of interactions between human AChE, structurally different OP and oximes revealed marked differences in the ability of oximes to reactivate OP-inhibited AChE, depending on the OP and the oxime [10]. One finding of this study was that tabun-inhibited AChE was rather resistant towards reactivation by oximes, a result which is in agreement with a low antidotal efficacy in vivo [11]. Moreover, it could be shown that small modifications of the tabun structure, i.e. a diethylamido instead of a dimethylamido group at the phosphorus atom, resulted in a complete resistance of inhibited AChE towards reactivation by oximes. On the other hand, methamidophos-inhibited AChE (Table 1) was highly susceptible towards reactivation by oximes.

In order to get more insight into potential structural requirements for the reactivatability of AChE inhibited by phosphoramidates the present study was undertaken. Hereby, the time-dependent reactivation of human AChE inhibited by different tabun analogues, bearing *N*,*N*-dialkyl and *N*-monoalkyl groups (Table 1), was tested with obidoxime, TMB-4, HI 6, HLö 7 or MMB-4 (1 mM) and the kinetics of reactivation by obidoxime was determined. Since oxime-induced reactivation inevitably leads to the formation of highly reactive phosphylated oximes [12] which may re-inhibit reactivated AChE [13] additional experiments were performed to investigate a potential effect of such reaction products on reactivation.

Phosphylated AChE may undergo spontaneous dealkylation through alkyl-oxygen bond scission ('aging'), resulting in an irreversibly inactivated enzyme, or spontaneous dephosphylation ('spontaneous reactivation'), both reactions being strongly dependent on the structure of the OP [10]. Therefore, the kinetics of aging and spontaneous reactivation were determined as well.

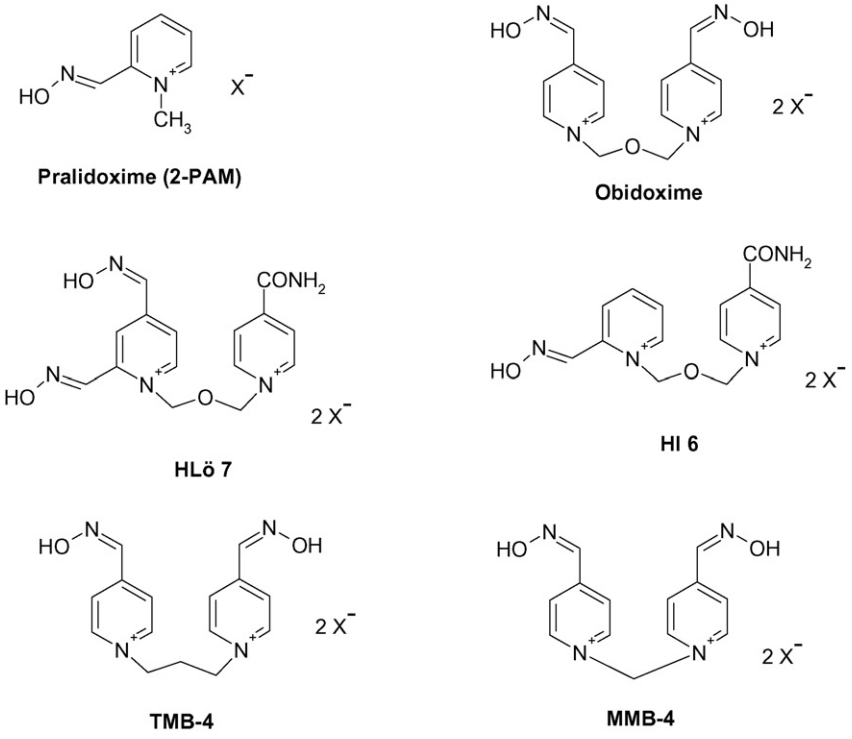
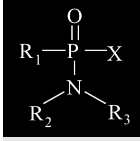



**Fig. 1 – Structures of oximes addressed in this study.**

**Table 1 – Structural formulae of the organophosphorus compounds used in this study**



| Compound | $R_1$ | $R_2$ | $R_3$ | X |
|---|---|---|---|---|
| 1 (Tabun) | O-Ethyl | Methyl | Methyl | CN |
| 2 | O-Ethyl | Ethyl | Ethyl | CN |
| 3 | O-Ethyl | *n*-Propyl | *n*-Propyl | CN |
| 4 | O-Ethyl | i-Propyl | i-Propyl | F |
| 5 | O-Methyl | Methyl | Methyl | CN |
| 6 | O-Methyl | Ethyl | Ethyl | CN |
| 7 | O-Methyl | *n*-Propyl | *n*-Propyl | CN |
| 8 | O-Methyl | i-Propyl | i-Propyl | F |
| 9 | O-Ethyl | Methyl | H | F |
| 10 | O-Ethyl | Ethyl | H | F |
| 11 | O-Ethyl | *n*-Propyl | H | F |
| 12 | O-Methyl | Methyl | H | F |
| 13 | O-Methyl | Ethyl | H | F |
| 14 | O-Methyl | *n*-Propyl | H | F |
| 15 | Methyl | Methyl | Methyl | F |
| 16 | Methyl | Ethyl | Ethyl | F |
| Methamidophos | O-Methyl | H | H | S-Methyl |
| Fenamiphos | O-Ethyl | i-Propyl | H | 3-Methyl-4-methylthiophenyl |

X indicates the leaving group.

## 2. Materials and methods

### 2.1. Materials

Acetylthiocholine iodide (ATCh), S-butyrylthiocholine iodide (BTCh), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) and TMB-4 dibromide (TMB-4) were obtained from Sigma and obidoxime dichloride (obidoxime) was purchased from Duphar. HI 6 dichloride (HI 6) was kindly provided by Dr. Clement (Defence Research Establishment Suffield, Ralston, Alberta, Canada), MMB-4 dichloride (MMB-4) was a gift from Professor Fusek (Purkyne Military Medical Academy, Hradec Kralove, Czech Republic) and HLö 7 dimethanesulfonate (HLö 7) was a custom synthesis by Dr. Braxmeier. Tabun and its analogues (>98% by GC–MS, $^{1}$H-NMR and $^{31}$P-NMR) were made available by the German Ministry of Defence, all other chemicals were from Merck Eurolab GmbH.

Stock solutions of compounds 1–16 (1% v/v; Table 1) were prepared in 2-propanol, stored at 4 °C and appropriately diluted in distilled water just before the experiment. Oximes (200 mM) were prepared in distilled water, stored at −60 °C and diluted as required in distilled water at the day of the experiment. All solutions were kept on ice until the experiment.

Hemoglobin-free erythrocyte ghosts in 0.1 M phosphate buffer, pH 7.4, were prepared as described before [14]. Aliquots of the erythrocyte ghosts with an AChE activity adjusted to that found in whole blood, were stored at −60 °C until use. Prior to use, aliquots were homogenized on ice with a Sonoplus HD 2070 ultrasonic homogenator (Bandelin electronic, Berlin, Germany), three-times for 5 s with 30 s intervals, to achieve a homogeneous matrix for the kinetic studies.

In order to prevent AChE denaturation during long-term experiments at 37 °C AChE was stabilized by addition of plasma with completely inhibited and aged BChE [15]. Plasma was obtained as described above and inhibited by soman (100 nM) for 30 min at 37 °C to ensure complete inhibition and aging of BChE. The treated plasma was dialyzed (phosphate buffer, 0.1 M, pH 7.4) overnight at 4 °C to adjust pH and to remove residual inhibitor.

### 2.2. Enzyme assays

AChE and BChE activities were measured spectrophotometrically (Cary 3Bio, Varian, Darmstadt) at 436 nm with a modified Ellman assay [16,17]. The assay mixture (3.16 ml) contained 0.45 mM ATCh (AChE) or 1.0 mM BTCh (BChE) as substrate and 0.3 mM DTNB as chromogen in 0.1 M phosphate buffer (pH 7.4). Assays were run at 37 °C.

### 2.3. Inhibition of human AChE by tabun analogues

Ghosts were incubated with a small volume (1%, v/v) of appropriate concentrations of compounds 1–16 (Table 1) for 30 min at 37 °C to achieve an AChE inhibition by >80%. Thereafter, the treated samples were dialyzed (phosphate buffer, 0.1 M, pH 7.4) overnight at 4 °C to remove residual inhibitor. Then, the absence of inhibitory activity was tested by incubation of treated and control ghosts (30 min, 37 °C).

### 2.4. Reactivation of OP-inhibited AChE

OP-inhibited AChE was incubated with obidoxime, TMB-4, MMB-4, HI 6 or HLö 7 (1 mM, 37 °C; Fig. 1) and the residual AChE activity was measured at specified time intervals (2–30 min). Enzyme activities were referred to control activity and the % reactivation was calculated according to de Jong and Wolring [18]. Experiments were performed in duplicate.

In order to investigate the effect of enzyme concentration on reactivation of inhibited AChE concentrated and diluted (300-fold in phosphate buffer) OP-inhibited AChE was incubated with obidoxime (20 μM) and the enzyme activity was determined at specified time intervals (1–15 min).

### 2.5. Kinetics of oxime reactivation

The reactivation kinetics (37 °C) were determined by two different procedures depending on the reactivating potency of the oximes. In case of expected high reactivating potency the reactivation kinetics were determined with the continuous procedure presented by Kitz et al. [10,14,19]. Hereby, 10 μl OP-inhibited AChE was added to a cuvette containing phosphate buffer, DTNB, ATCh and specified oxime concentrations (final volume 3.16 ml). ATCh hydrolysis was continuously monitored over 10 min. Activities were individually corrected for oxime-induced hydrolysis of ATCh.

In case of low reactivating potency a discontinuous procedure was applied [10,20] which allowed use of higher oxime concentrations (up to 5 mM). 60 μl OP-inhibited AChE was incubated with 2 μl oxime solution (different concentrations) and 1 μl ATCh (450 μM final concentration). Aliquots (10 μl) were transferred to cuvettes after specified time intervals (1–9 min) for measurement of AChE activity.

The dissociation constant $K_D$, the reactivity rate constant $k_r$ and the second-order rate constant $k_{r2}$ were calculated by standard procedure as described before [10]. Eight to ten different oxime concentrations were used for the determination of the reactivation rate constants.

### 2.6. Determination of rate constants for aging ($k_a$) and spontaneous reactivation ($k_s$)

OP-inhibited AChE was mixed with equal volumes of soman-treated human plasma to prevent denaturation of AChE during long-term experiments at 37 °C. Aliquots were taken after various time intervals for determination of AChE activity (spontaneous reactivation) and of the decrease of oxime-induced reactivation (aging). Hereby, small aliquots were incubated with obidoxime (1 mM) for 15 min (compounds 4, 9, 10, 11, 12, 13, 14) or 30 min (compounds 15, 16) or with TMB-4 (1 mM) for 60 min (compounds 1, 5). Data from duplicate experiments were referred to control activities and the percentage reactivation (% react) was calculated. The pseudo-first-order rate constants $k_s$ (spontaneous reactivation) and $k_a$ (aging) were calculated by a non-linear regression model [10].

## 3. Results

The inhibition of human AChE by tabun analogues required in part large concentrations of inhibitor, ranging from 50 nM (compound 1) to 180 μM (4). Hereby, phosphoramidates bearing an *O*-methyl group were generally less potent inhibitors of AChE compared to *O*-ethyl analogues. AChE could not be inhibited by compound 8 despite of using 5 mM final concentration.

### 3.1. Reactivation of OP-inhibited AChE by oximes

The time-dependent reactivation of OP-inhibited human AChE by oximes was tested with 1 mM obidoxime, TMB-4, MMB-4, HI 6 and HLö 7. Tabun-inhibited (1) AChE could be partially reactivated by oximes, the extent of reactivation decreased in the order TMB-4 > obidoxime > HLö 7 > MMB-4 (Fig. 2). HI 6 was completely ineffective. A similar pattern was observed with the *O*-methyl tabun analogue (5) although the maximum increase of AChE was lower compared to tabun (Fig. 2). AChE inhibited by *N*,*N*-diethyl and *N*,*N*-di-*n*-propyl tabun analogues (2, 3, 6, 7) was completely resistant towards reactivation by all tested oximes (Fig. 2). Interestingly, the reactivation of AChE inhibited by the *N*,*N*-di-i-propyl tabun analogue (4) followed a completely different pattern (Fig. 2). All oximes were able to reactivate the inhibited enzyme and after 30 min incubation the increase in AChE activity was in the range of 65–75%.

AChE inhibited by the two phosphonoamidates bearing a *N*,*N*-dimethyl (15) or a *N*,*N*-diethyl group (16) could be reactivated by all tested oximes (Fig. 3). The increase of AChE activity was fast but incomplete with only minor differences between the oximes.

All oximes were able to reactivate human AChE inhibited by *N*-monoalkyl tabun analogues (Fig. 4). There was only little difference in the velocity and extent of reactivation between *O*-ethyl and *O*-methyl analogues. Compounds bearing a *N*-methyl group (9 and 12; Fig. 4) tended to be slightly more susceptible towards reactivation with some oximes compared to compounds having an *N*-ethyl group (10 and 13; Fig. 4) but there was a marked reduction in reactivatability with *N*-*n*-propyl compounds (11 and 14; Fig. 4).

The potential effect of phosphyloxime (POX), formed during the reactivation of inhibited AChE by oximes, on net reactivation was tested by incubation of concentrated and highly diluted (300-fold) OP-inhibited AChE with 20 μM obidoxime. No difference in pattern and extent of increase of AChE activity was observed with tabun-inhibited (1) AChE indicating no effect of POX on net reactivation. However, reactivation of AChE inhibited by *N*-monoalkyl analogues (9–14) or by phosphonoamidates (15, 16) resulted in a, in part marked, acceleration of reactivation with diluted AChE (Fig. 5). These data indicate the formation of stable and reactive POX species and re-inhibition of reactivated AChE in case of physiologic AChE concentrations.

### 3.2. Reactivation kinetics of obidoxime with OP-inhibited AChE

The reactivation kinetics of human AChE inhibited by compounds 1, 4, 5 and 9–16 was determined with obidoxime (Table 2). No kinetics could be determined with *N*,*N*-dialkyl tabun analogues 2, 3, 6 and 7 due to the inability of oximes to reactivate AChE and with compound 8 due to the failure to inhibit AChE. With *O*-methyl tabun (5) obidoxime had a more than 10-fold lower second-order reactivation rate constant compared to tabun (1). Surprisingly, $k_{r2}$ of obidoxime with *N*,*N*-di-i-propyl tabun-inhibited AChE (4) was more than 36-fold higher compared to tabun.

There was little difference in the reactivation kinetics of obidoxime with human AChE inhibited by phosphonoamidates

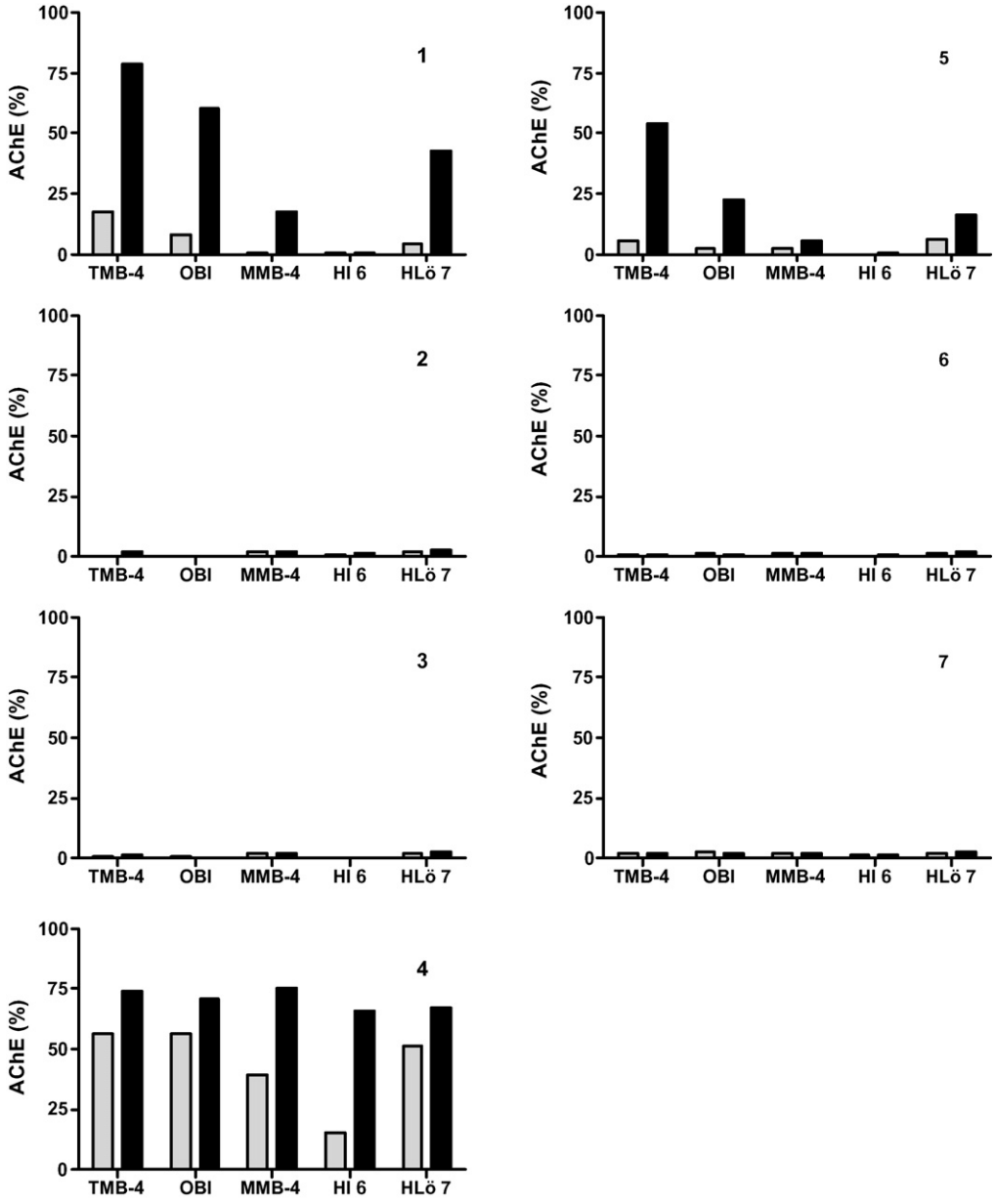



**Fig. 2 – Time-dependent reactivation of OP-inhibited AChE by TMB-4, obidoxime (OBI), MMB-4, HI 6 or HLö 7 (1 mM). Human AChE inhibited by compounds 1, 2, 3, 4, 5, 6 or 7 (Table 1) was incubated with oxime and the AChE activity was determined after 2 (gray column) and 30 min (black column). Data are given as % reactivation.**

(15, 16; Table 2), i.e. the *N*,*N*-alkyl residue (methyl versus ethyl) had almost no effect on the reactivating potency.

The reactivation kinetics of obidoxime with *N*-monoalkyl tabun analogues revealed a dependence of the dissociation constant $K_D$, the reactivity rate constant $k_r$ and the second-order rate constant $k_{r2}$ on the chain length of the *N*-alkyl residue (Table 2). Fig. 6 visualizes the structure-activity relationship of the reactivation kinetics of *N*-monoalkyl tabun analogues. The respective data [10] on the reactivation of methamidophos ($NH_2$) and fenamiphos (*N*-i-propyl) were included for comparison. Accordingly, the affinity and reactivity of obidoxime decreases with the chain length of the *N*-alkyl residue resulting in a dramatic difference of the second-order reactivation rate constant, while there is only little difference between *O*-methyl and *O*-ethyl analogues.

### 3.3. *Aging and spontaneous reactivation of OP-inhibited AChE*

Spontaneous reactivation and aging of OP-inhibited human AChE followed pseudo-first-order kinetics and was strongly dependent on the structure of the tabun analogues (Table 2): As an example Fig. 7 shows the respective data of tabun-inhibited AChE. There was a substantial difference in the aging and spontaneous reactivation kinetics between tabun (1) and its *O*-methyl analogue (5). Aging proceeded almost three-fold faster with tabun, on the other hand spontaneous reactivation

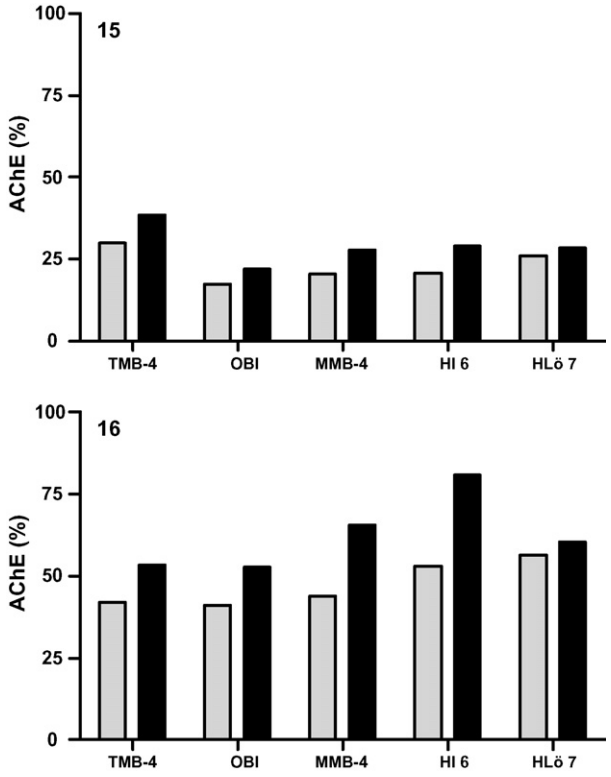



**Fig. 3 – Time-dependent reactivation of OP-inhibited AChE by TMB-4, obidoxime (OBI), MMB-4, HI 6 or HLö 7 (1 mM). Human AChE inhibited by compounds 15 or 16 was incubated with oxime and the AChE activity was determined after 2 (gray column) and 30 min (black column). Data are given as % reactivation.**

outweighed aging with *O*-methyl tabun while no spontaneous reactivation occurred with tabun-inhibited enzyme. The velocity of aging and spontaneous reactivation was almost similar with *N*,*N*-di-i-propyl tabun (4).

Aging of human AChE inhibited by the phosphonoamidate compounds 15 and 16 followed almost identical kinetics (Table 2). Spontaneous reactivation was markedly slower with both compounds.

AChE inhibited by *N*-monoalkyl tabun analogues did not reactivate spontaneously (Table 2). Aging velocity decreased with increasing chain length of the *N*-alkyl residue. Fig. 8 shows the relation between aging rate constant and chain length, again data of methamidophos ($NH_2$) and fenamiphos (*N*-i-propyl) were included for comparison. Aging proceeded slightly faster with *O*-methyl compared to *O*-ethyl analogues.

## 4. Discussion

### 4.1. *Reactivation of OP-inhibited AChE by oximes*

The oxime-induced reactivation of human AChE inhibited by tabun and its analogues was strongly dependent on the structure of the phosphyl moiety (Figs. 2–4, Table 2). The reactivation by oximes was mainly affected by the different residues at the amido group and to less extent by modifications at the *O*-alkyl group. Marked differences were recorded between *N*,*N*-dialkyl and *N*-monoalkyl compounds. For example, obidoxime had a 100-fold higher second-order reactivation rate constant with *N*-monomethyl tabun (9) compared to tabun (1), a comparison of the respective *O*-methyl analogues (12 versus 5) resulted in a more than 700-fold difference.

Examination of the reactivation of human AChE inhibited by a homologous series of *N*-monoalkyl analogues revealed a

**Table 2 – Rate constants for the spontaneous dealkylation ($k_a$) and reactivation ($k_s$) and for the obidoxime-induced reactivation ($K_D$, $k_r$, $k_{r2}$) of OP-inhibited AChE[a]**

| Compound | $K_D$ (μM) | $k_r$ ($min^{-1}$) | $k_{r2}$ ($mM^{-1}\,min^{-1}$) | $k_a$ ($h^{-1}$)[b] | $k_s$ ($h^{-1}$)[b] |
|---|---|---|---|---|---|
| 1 | 97.3 | 0.04 | 0.41 | 0.036 | ∅[d] |
| 2[b] | ∅ | ∅ | ∅ | ∅[c] | ∅[d] |
| 3[b] | ∅ | ∅ | ∅ | ∅[c] | ∅[d] |
| 4 | 12.8 | 0.19 | 15.0 | 0.018 | 0.019 |
| 5 | 453.0 | 0.018 | 0.039 | 0.013 | 0.04 |
| 6[b] | ∅ | ∅ | ∅ | ∅[c] | ∅[d] |
| 7[b] | ∅ | ∅ | ∅ | ∅[c] | ∅[d] |
| 8 | n.t. | n.t. | n.t. | n.t. | n.t. |
| 9 | 7.5 | 0.3 | 40.6 | 0.024 | ∅[d] |
| 10 | 34.1 | 0.24 | 6.9 | 0.014 | ∅[d] |
| 11 | 143.7 | 0.046 | 0.32 | 0.006 | ∅[d] |
| 12 | 14.3 | 0.42 | 29.1 | 0.038 | ∅[d] |
| 13 | 57.0 | 0.21 | 3.8 | 0.017 | ∅[d] |
| 14 | 240.2 | 0.04 | 0.17 | 0.016 | ∅[d] |
| 15 | 9.3 | 0.075 | 8.1 | 0.19 | 0.019 |
| 16 | 10.9 | 0.08 | 7.4 | 0.19 | 0.008 |

n.t.: human AChE could not be inhibited by compound 8.
[a] For structural details see Table 1.
[b] Failure of oximes to reactivate inhibited AChE.
[c] Not feasible.
[d] No spontaneous reactivation of inhibited AChE activity during the observation period.

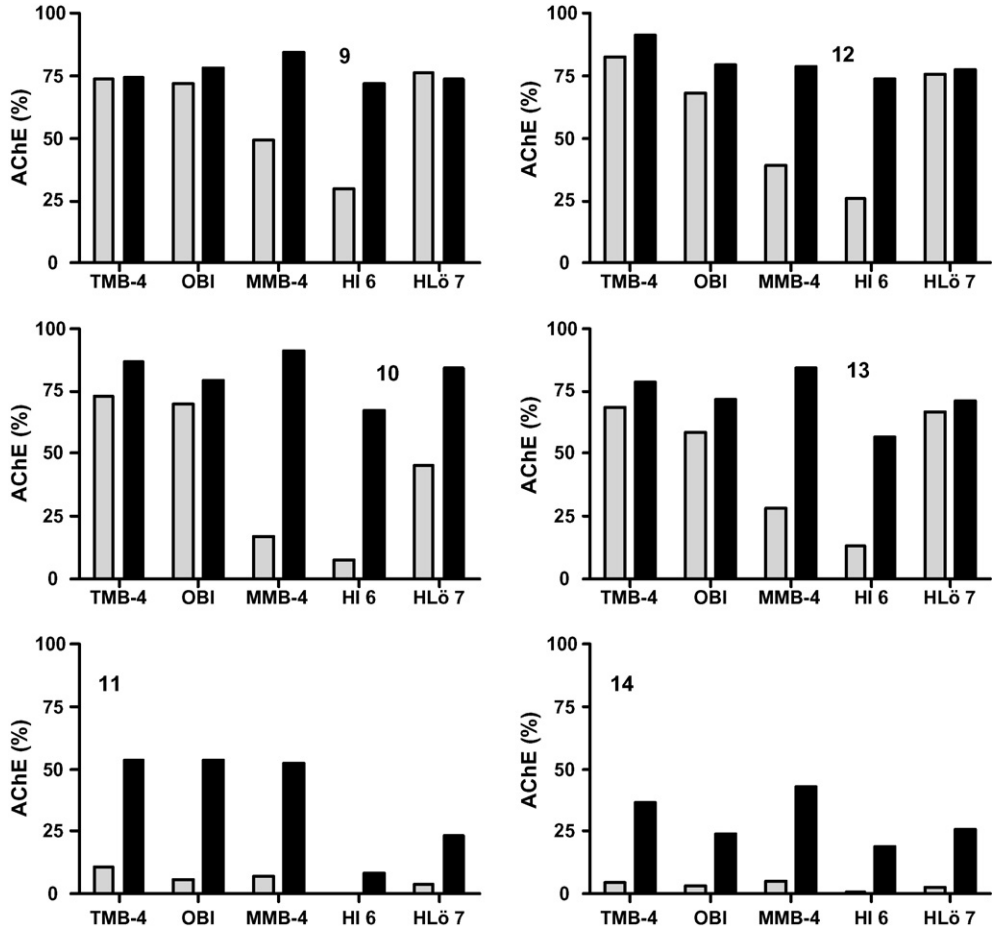



**Fig. 4 – Time-dependent reactivation of OP-inhibited AChE by TMB-4, obidoxime (OBI), MMB-4, HI 6 or HLö 7 (1 mM). Human AChE inhibited by compounds 9, 10, 11, 12, 13 or 14 was incubated with oxime and the AChE activity was determined after 2 (gray column) and 30 min (black column). Data are given as % reactivation.**

structure-activity relationship depending on the chain length of the residues (Table 2, Fig. 4). There was a consistent decrease in reactivatability as well as in reactivating potency, determined with obidoxime, with increasing chain length of the residue (Figs. 4 and 6). The decline of $k_{r2}$ was a result of decreasing both affinity and reactivity of the oxime. Inclusion of literature data of methamidophos- and fenamiphos-inhibited AChE [10] indicates that this relationship is at least valid from unsubstituted amido to *N*-i-propyl group. These findings are in agreement with limited data from previous studies. De Jong et al. [21] investigated the reactivation of electric eel AChE inhibited by various phosphoramidates and found a decrease in reactivity of benzyl-P2A comparing methamidophos ($NH_2$) and crufomate (*N*-methyl). Reactivation of phosphoramidate-inhibited human AChE by oximes resulted in a dramatic decrease in reactivatability with compounds bearing a *N*-*n*-butyl residue compared to agent with a $NH_2$ group [22].

Previously, it was shown that human AChE inhibited by *N*,*N*-diethyl tabun (2) was completely resistant towards reactivation by oximes while tabun-inhibited AChE could be reactivated to some extent [10]. Now, the present study extends the database by including *N*,*N*-di-*n*-propyl tabun (3) and the respective *O*-methyl analogues (5–7). It turned out that AChE inhibited by *O*-methyl and *O*-ethyl tabun analogues bearing an *N*,*N*-diethyl or *N*,*N*-di-*n*-propyl residue could not be reactivated by oximes (Table 2). Presently, it is unknown whether this fact is due to an inability of oximes to attack the phosphyl-AChE complex or due to an extremely rapid aging of the inhibited enzyme. In view of these results it was most surprising that AChE inhibited by *N*,*N*-di-i-propyl tabun (4) was readily accessible towards reactivation by oximes (Fig. 2) giving a moderately high second-order reactivation rate constant with obidoxime (Table 2). At present it is not known if a similar phenomenon could be observed with the respective *O*-methyl analogue (8) since this compound failed to inhibit AChE to a relevant extent (the identity of compounds 4 and 8 was re-checked during the experiments by $^1$H-NMR).

The reactivation of *O*-methyl tabun (5), its *N*,*N*-diethyl analogue (6) in comparison with the phosphonoamidates 15 and 16 revealed marked differences (Table 2, Figs. 2 and 3). AChE inhibited by both phosphonoamidates was susceptible towards reactivation and obidoxime resulted in a comparable reactivation kinetics indicating that the substitution of the *O*-methyl by a methyl group had a dramatic effect on the accessibility of the oxime to the phosphyl-AChE complex.

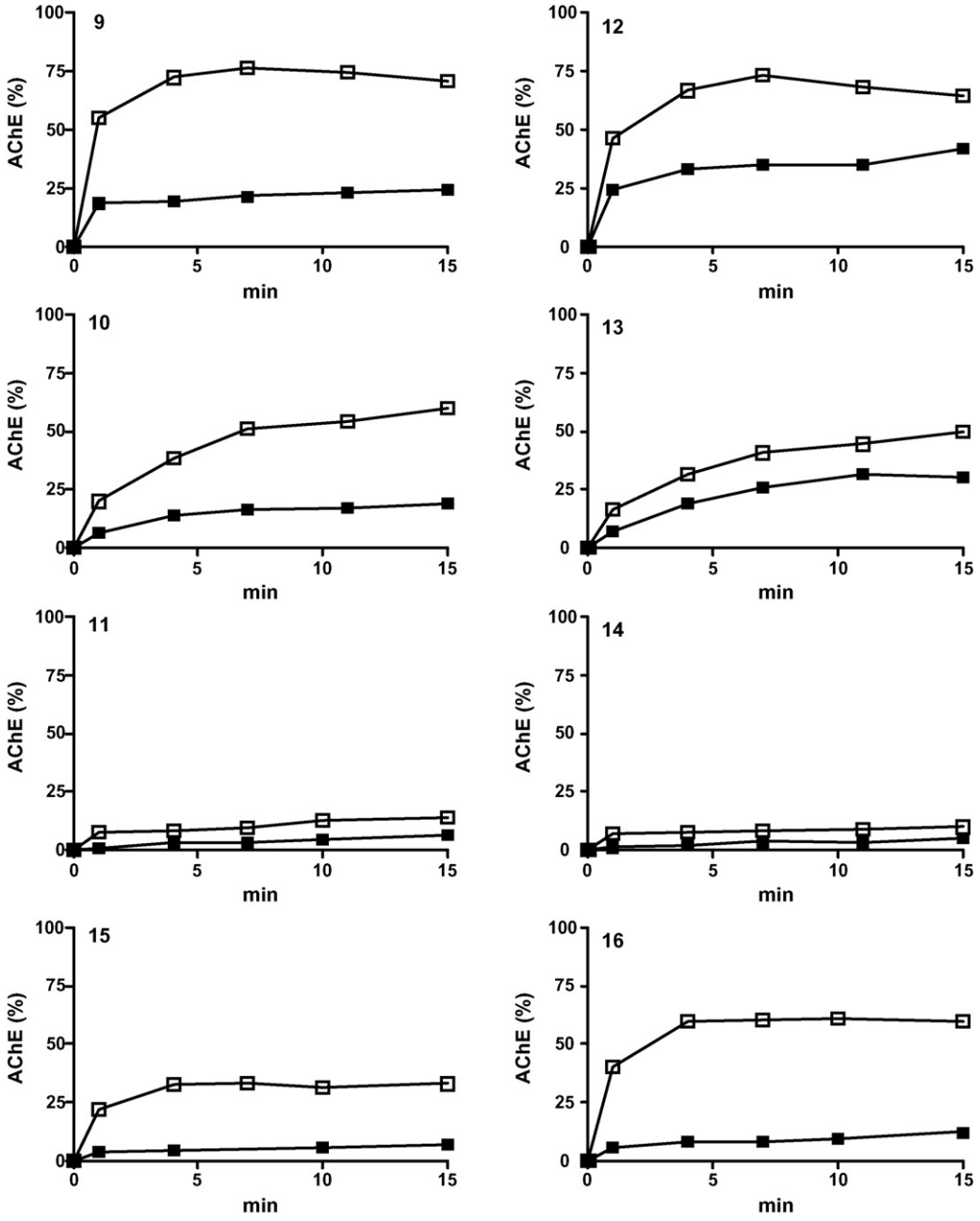



**Fig. 5 – Effect of enzyme concentration on the reactivation of OP-inhibited AChE by obidoxime. Human AChE was inhibited by compounds 9, 10, 11, 12, 13, 14, 15 or 16. Concentrated (■) and diluted (300-fold in phosphate buffer; □) AChE was incubated with obidoxime (20 μM) and the enzyme activity was determined at specified time intervals. Data were referred to control and are given as % reactivation.**

Apart from structure-related differences in the reactivation kinetics by obidoxime of inhibited AChE a differential pattern of reactivation by individual oximes was noted. The ability of oximes (1 mM) to reactivate tabun- (1) and *O*-methyl tabun-inhibited AChE (5) decreased in the order TMB-4 > obidoxime > HLö 7 > MMB-4, HI 6 being completely ineffective (Fig. 2) [10,22,23]. In contrast, HI 6 was able to reactivate AChE inhibited by *N*,*N*-di-i-propyl tabun, phosphonoamidates and *N*-monoalkyl tabun analogues (Figs. 2–4). With these compounds some differences between oximes related to the reactivation velocity were recorded but the maximum increase in AChE activity after 30 min oxime incubation was comparable.

Reactivation of OP-inhibited AChE by oximes inevitably leads to the formation of highly reactive phosphyloximes (POX) which may re-inhibit reactivated enzyme [13,24]. According to the reaction mechanism the generation of POX is dependent on the concentration of inhibited AChE. Therefore, POX, if stable, may affect net reactivation. Previous studies showed the formation of reactive and stable POX during the reactivation of AChE inhibited by different OP preferentially with oximes bearing an oxime function at

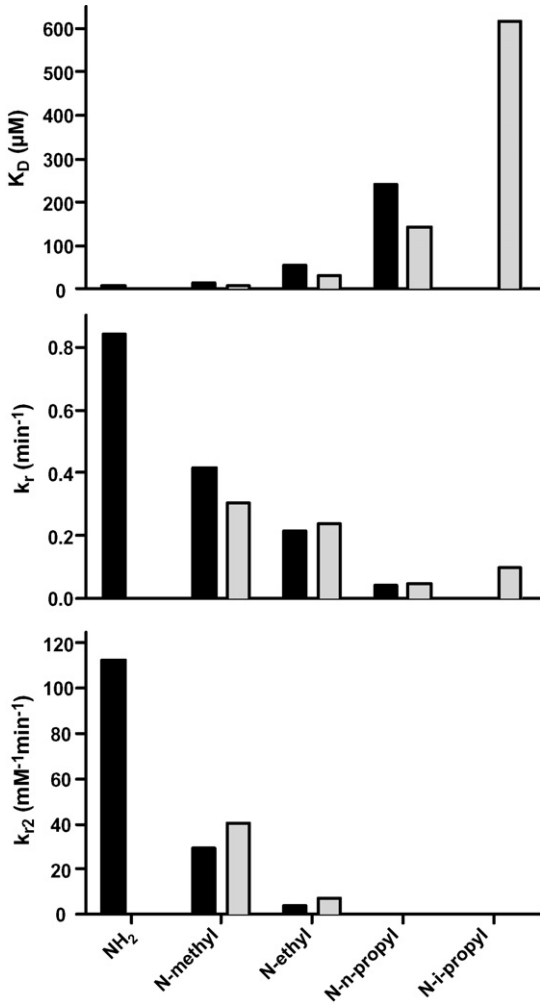



**Fig. 6 – Structure-activity relationship in the reactivation by obidoxime of human AChE inhibited by different *N*-monoalkyl tabun analogues. Abscissa labeling indicates the *N*-alkyl residues. Gray columns resemble *O*-ethyl and black columns *O*-methyl analogues ($R_1$, cf. Table 1). Data are shown for the dissociation constant $K_D$ (top), reactivity rate constant $k_r$ (middle) and second-order rate constant $k_{r2}$ (bottom). Data of methamidophos ($NH_2$) and fenamiphos (*N*-i-propyl) were included for comparison [10].**

position 4 at the pyridinium ring, e.g. obidoxime [13,24–29]. Hence, the potential impact of POX on reactivation was tested with OP-inhibited AChE and obidoxime (20 μM). By using a biological assay, i.e. activity of reactivated AChE, only a semi-quantitative evaluation of a potential POX effect was possible. Nevertheless, the differences in reactivation of concentrated and diluted AChE indicate formation of POX sufficiently stable to lead to a re-inhibition of reactivated concentrated enzyme, especially in case of compounds 9, 12, 15 and 16.

### 4.2. *Aging and spontaneous reactivation of OP-inhibited AChE*

The investigation of the aging kinetics of N-monoalkyl tabun-inhibited AChE resulted in an increase in aging half-time

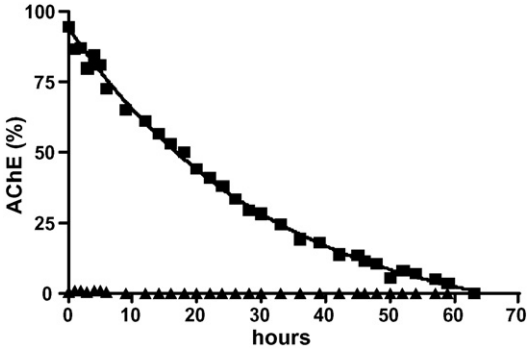



**Fig. 7 – Kinetics of aging and spontaneous reactivation of tabun-inhibited human AChE. Stabilized AChE preparations were treated as described in Methods. Triangles represent the increase of AChE activity due to spontaneous reactivation and squares represent the decrease of oxime-induced reactivatability ("aging"). Data were referred to control and are given as % reactivation.**

depending on the chain length of the *N*-alkyl residue (Table 2). Hence, the decrease of reactivation (Fig. 6) and aging kinetics (Fig. 8) with increasing chain length of the *N*-alkyl group indicates an augmented stabilization of the phosphyl-AChE complex. A similar relationship was observed with tabun and its *O*-methyl analogue (5), i.e. a substantially lower reactivation rate and aging constant with compound 5 (Table 2). Furthermore, reactivation and aging kinetics of AChE inhibited by the phosphonoamidate 15 was substantially higher compared to *O*-methyl tabun (5). On the other hand, aging of *N*-i-propyl tabun-inhibited AChE proceeded two-fold slower compared to tabun but its reactivation rate constant was 36-fold higher.

Spontaneous reactivation of inhibited AChE was recorded only with some compounds (4, 5, 15, 16) and no structure-activity relationship could be derived. In contrast to tabun

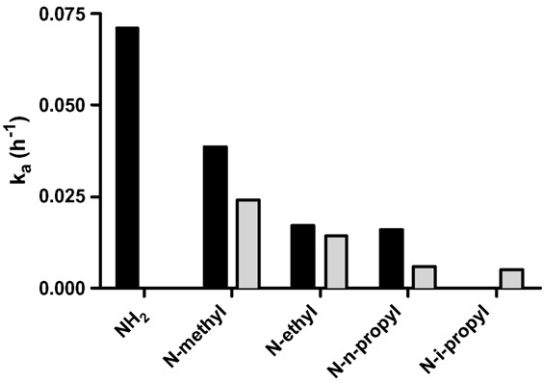



**Fig. 8 – Structure-activity relationship in aging of human AChE inhibited by different *N*-monoalkyl tabun analogues. Abscissa labeling indicates the *N*-alkyl residues. Gray columns represent *O*-ethyl and black columns *O*-methyl analogues ($R_1$, cf. Table 1). Data are given as aging rate constant ($k_a$). Data of methamidophos ($NH_2$) and fenamiphos (*N*-i-propyl) were included for comparison [10].**

O-methyl tabun-inhibited AChE showed a spontaneous reactivation which was much faster compared to aging.

### 4.3. *Structural aspects for the reactivation of AChE inhibited by tabun analogues*

In the past decades there have been attempts to derive structural requirements of oximes from kinetic studies with OP-inhibited AChE. De Jong et al. pointed to the importance of the position of the oxime group at the pyridinium ring and suggested that an oxime group in position 4 may be important for the reactivation of tabun-inhibited AChE [30]. In fact, the present data with tabun- and O-methyl tabun-inhibited AChE support and extend this view (Fig. 2). In view of data on reactivation kinetics determined previously [10,31] it is apparent that oximes bearing an oxime group in position 4, e.g. TMB-4, obidoxime, HLö 7, are superior to oximes with a position 2 group, e.g. pralidoxime and HI 6. Hereby, the bispyridinium oxime MMB-4, having an oxime group at position 4 at both pyridinium rings (Fig. 1), was a surprisingly weak reactivator indicating that the bridge, ether versus methylene, connecting both pyridinium rings may be of importance as well.

Now, the data on the time-dependent reactivation of AChE inhibited by N-monoalkyl tabun analogues and phosphonoamidates with a fixed oxime concentration indicate that with these compounds the structural requirements for an oxime able to reactivate the phosphylated AChE are different. Still, 4-oximes (TMB-4, obidoxime, HLö 7) are superior reactivators but in contrast to tabun-inhibited enzyme HI 6 showed some effect.

Recently, the crystal structure of wild-type and mutant mouse AChE was used as a basis for computational analysis of interactions between AChE, OP and oximes in order to define structural requirements for the reactivation of inhibited AChE by oximes [23,32–34]. In studies using methylphosphonates with O-alkyl groups of different size the analysis indicated that spatial constraints and steric limitations are major factors for the oxime access to the phosphonyl residue and its ability for nucleophilic attack [33,34]. Furthermore, it was suggested that an increasing size of the alkyl group impairs oxime reactivation. This assumption is apparently in line with part of the kinetic data of the present study, i.e. a decrease of $k_{r2}$ of AChE inhibited by N-monoalkyl analogues depending on the size of the N-alkyl residue (Table 2). However, the determination of the reactivation kinetics with human AChE inhibited by sarin homologues with O-alkyl groups from methyl to i-butyl and a study with different methylphosphonothioates did not show such a relation [35,36].

Ekström et al. investigated the crystal structure of tabun-inhibited mouse AChE and found a displacement of the side chain of Phe338 narrowing the active site gorge [32]. The authors suggested that this structural modification may affect the oxime entry and may contribute to the resistance of tabun-inhibited AChE towards reactivation. Moreover, it was assumed that the orientation of oximes in the active site as well as binding of oximes in the peripheral anionic site of the enzyme is different and may explain the distinctive reactivating potency of obidoxime and HI 6 [23].

The major limitation of the significance of these studies is the use of mouse AChE for structural analysis which may be different from human AChE. In addition, the assumption that spatial constraints, steric limitations and differential orientation of oximes are major factors for defining the reactivating potency of oximes is not reflected by kinetic data with various OP and oximes.

### 4.4. *Conclusions*

The kinetic analysis of interactions between human AChE and structurally different tabun analogues demonstrates that the oxime-induced reactivation was strongly dependent on the structure of the phosphyl moiety. There was a marked difference in reactivatability by the tested oximes and the reactivating potency of obidoxime between tabun analogues bearing a N,N-dialkyl or a N-monoalkyl group. With N-monoalkyl analogues a structure-activity relationship of oxime-induced reactivation and aging of the inhibited AChE depending on the chain length of N-alkyl residue was observed. No such relationship could be derived with data from N,N-dialkyl tabun analogues and selected phosphonoamidates. These results in conjunction with previous data with organophosphates and organophosphonates emphasizes the necessity for kinetic studies as basis for future work on structural analysis with human AChE and for the development of effective broad-spectrum oximes.

## Acknowledgement

The authors are grateful to T. Hannig and J. Inwich for their skilful and engaged technical assistance. The authors thank M.M. Blum for valuable suggestions and discussion.

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