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ORIGINAL ARTICLE

DESIGN, CHEMICAL SYNTHESIS, AND *IN VITRO* EVALUATION OF NOVEL UNCHARGED CHOLINESTERASE REACTIVATOR

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Summary

The primary toxicological mechanism of organophosphorus compounds (OPCs) is the irreversible inhibition of acetylcholinesterase (AChE), leading to the accumulation of acetylcholine and subsequent cholinergic crisis, which can result in fatal respiratory failure. Conventional oxime reactivators, such as pralidoxime and obidoxime, are limited by their inability to permeate the blood-brain barrier (BBB) and inconsistent efficacy across different OPC types. To address these limitations, we designed a novel non-quaternary oxime reactivator codenamed **K1396**, with enhanced lipophilicity for improved BBB penetration and dual-binding capability at both the peripheral anionic site (PAS) and the catalytic active site (CAS) of AChE. This study compares the *in vitro* reactivation potency, cytotoxicity, and BBB penetration potential of **K1396** with standard oximes. **K1396** demonstrated comparable or superior reactivation potency, particularly against VX-inhibited AChE, and showed lower cytotoxicity in specific cell lines. Furthermore, **K1396** exhibited favorable permeability across the lipid layer, suggesting potential CNS availability. The findings support the therapeutic potential of **K1396** as an effective and broad-spectrum reactivator for OPC poisoning.

Key words: reactivator; A-agents; acetylcholinesterase; cytotoxicity; enzyme kinetics; uncharged reactivator; blood-brain barrier; organophosphorus compounds; nerve agents

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1. Introduction

Insecticides (e.g., paraoxon, chlorpyrifos) and nerve agents (NAs; sarin, tabun, VX) represent two classes of organophosphorus compounds (OPCs) (1). Misuse of NAs is a permanent threat to the population, especially in war conflicts (e.g., Gulf War, Syria 2013) or terrorist attacks (e.g., assassination of Kim Jong-nam 2017, Skripal case 2018) (2–4). The irreversible inhibition of acetylcholinesterase (AChE; E.C.3.1.1.7) is the main toxicological mechanism of action caused by OPC. The essence of this inhibition is the formation of a strong covalent bond between the enzyme and an OPC in the active site of the enzyme (5). OPC poisoning results in the accumulation of the neurotransmitter acetylcholine (ACh) at cholinergic synapses and neuromuscular junctions. Subsequent excessive stimulation of cholinergic receptors leads to convulsive seizures, respiratory problems, and cholinergic crisis and can result in death through suffocation. Antidote therapy can be managed via anticholinergic compounds like atropine that can antagonize the action of ACh on its receptors, anticonvulsant drugs (e.g. diazepam), and oxime reactivators that can restore the activity of inhibited AChE. Their therapeutic potential is constantly debated (5–7). Although hundreds of reactivators have been synthesized during the past decades, no universal reactivator is effective against all types of OPCs. Another drawback of oxime-based reactivators is their limited bioavailability in the brain due to the presence of permanent positive charge as a part of the pyridinium scaffold (8).

Thus, the concept of uncharged non-quaternary reactivators has been introduced (9). Such compounds should more easily target CNS areas thanks to higher lipophilicity and expected BBB permeation. Another drawback of the standard pyridinium-based oxime reactivators is that they do not exert optimal binding to the catalytic part of the enzyme; their activity depends on the presence of specific NA, and resulting phosphyloxime residue can cause re-inhibition of AChE, all of can be circumvent or improved in case of some non-quaternary reactivators (9).

Based on the literature survey, we designed a reactivator codenamed **K1396** (Fig. 1). The design reflected the dual-binding site strategy, meaning that the reactivator should be able to interact with both the peripheral anionic site (PAS) and catalytic active site (CAS) of AChE. In 2011, the group of prof. Renard (9) described the first non-quaternary reactivator based on 3-hydroxy-2-pyridinaldoxim moiety, capable of cleaving a non-aged complex formed between NA and hydroxyl serine from AChE (9). The reactivation moiety (also known as CAS ligand, i.e. 3-hydroxy-2-pyridinaldoxime), is able to stabilize phosphyloxime after reactivation. The group of de Koning *et al*. pioneered a new way of rational reactivator development with a higher affinity to the PAS of AChE (10). In accordance with this study, diphenylmethoxypiperidine was selected as peripheral site ligand (PSL; highlighted in red Fig. 1). This PSL was linked to the reactivating moiety of 4-pyridineldoxime using five methylene tether linker, optimal length between (CAS) and PAS of the enzyme (11). Thus, the structure of **K1396** was designed to exert an increased affinity to OP-inhibited enzyme, showing higher lipophilicity, thus increasing the potential to permeate through BBB.

The aim of this study was to compare *in vitro* data of novel non-quaternary oxime **K1396** with two standards (pralidoxime and obidoxime). Their reactivation potency, cytotoxicity, and estimation of BBB penetration are summarized here.

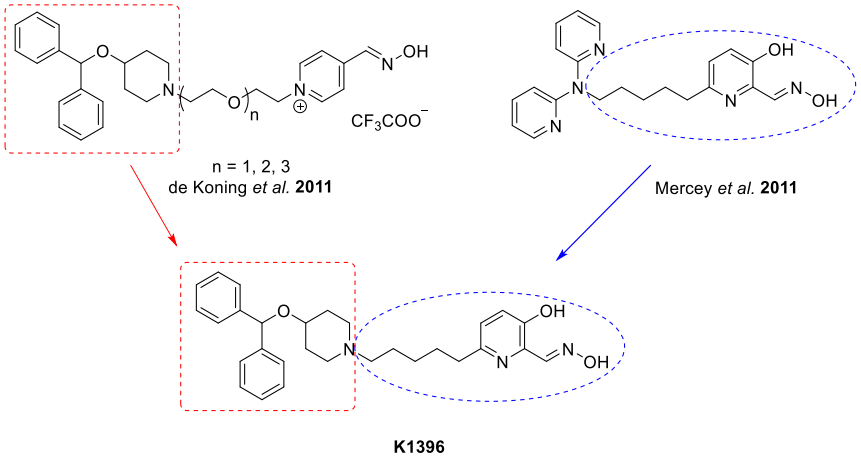


Figure 1. Molecular design of new nonquaternary reactivator **K1396**.

2. Material and Methods

2.1. Chemistry

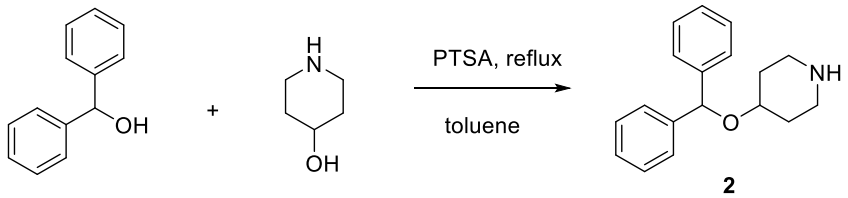
2.1.1. General Chemistry

All reagents and solvents were purchased from Sigma-Aldrich (Prague, Czech Republic) or Fluorochem - Doug Discovery (United Kingdom) and were used in the highest available purity without further purification. The reactions were monitored by thin layer chromatography (TLC) performed on aluminum sheets precoated with silica gel 60 F_{254} (Merck, Prague, Czech Republic). The spots were visualized by ultraviolet light (at wavelength 254 nm). Purifications of crude products were performed by column chromatography on silica gel 100 (particle size 0.063–0.200 mm, 70–230 mesh ASTM, Fluka, Prague, Czech Republic). Nuclear magnetic resonance (NMR) spectra were recorded in deuterated chloroform-*d* ($CDCl_3$-*d*; 7.26 (H) or 77.16 (C) ppm) or dimethylsulfoxide-d_6 (DMSO-d_6; 2.50 (H) or 39.7 (C) ppm) on a Varian S500 spectrometer operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C. Chemical shifts (δ) are reported in parts per million (ppm) and spin multiplicities are given as singlet (s), broad singlet (bs), triplet (t), triplet of triplets (tt), doublet (d), doublet of doublet of doublets (ddd), or multiplet (m). Coupling constants (*J*) are reported in Hz. A CEM Explorer SP 12 S was used for the MW-assisted reaction. The final compounds were analyzed by LC-MS with a Dionex Ultimate 3000 RS UHPLC system coupled with and Q Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) to obtain high-resolution mass spectra (HRMS). Gradient LC-MS analysis confirmed > 95% purity.

2.1.2. Preparation of 4-(diphenylmethoxy)piperidine (2)

4-(diphenylmethoxy)piperidine (2): α,α-Diphenylmethanol (710 mg, 3.9 mmol) was dissolved in toluene (35 mL), and 4-hydroxypiperidine (394 mg, 3.9 mmol) was added to this solution (Scheme 1). *p*-Toluenesulfonic acid monohydrate (PTSA; 807 mg, 4.2 mmol) was added immediately after. The resulting reaction mixture was stirred overnight under reflux using Dean-Stark apparatus. After cooling to room temperature, the mixture was washed with 5% solution of NaOH and water. The organic part was dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography using mobile phase DCM/MeOH/NH_3 (9/1/0.1). Intermediate **2** was isolated as colorless oil in 51% yield.

^{1}H NMR ($CDCl_3$-$_d$): δ 7.22–7.28 (m, 8H), 7.15–7.18 (m, 2H), 5.46 (s, 1H), 3.44 (tt, J = 8.3, 3.8 Hz, 2H), 3.04 (ddd, J = 12.5, 6.0, 4.4 Hz, 2H), 2.88 (bs, 1H), 2.55 (ddd, J = 12.6, 9.4, 3.2 Hz, 2H), 1.83–1.89 (m, 2H), 1.49–1.56 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 142.8, 128.4, 127.4, 127.1, 80.1, 72.6, 43.9, 32.5. NMR spectra were in good agreement with previously published data (12).

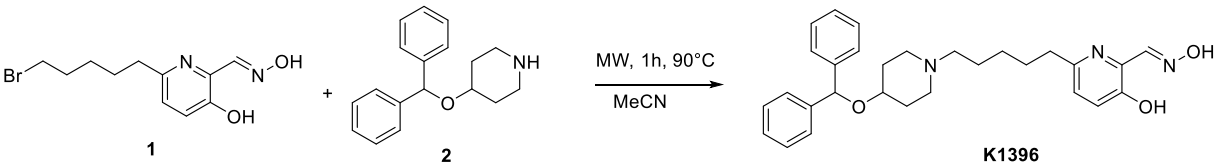


Scheme 1. Chemical synthesis of 4-(diphenylmethoxy)piperidine (**2**).

2.1.3. *N*-alkylation reaction between 2 and key intermediate 1

6-{5-[4-(diphenylmethoxy)piperidin-1-yl]pentyl}-2-[(*E*)-(hydroxyimino)methyl]pyridin-3-ol (K1396): The alkylating agent **1** prepared as previously described (13). Intermediate **1** (121 mg; 0.42 mmol) and the piperidine derivative **2** (113 mg; 0.42 mmol) were dissolved in anhydrous acetonitrile (MeCN), followed by the addition of *N*,*N*-diisopropylethylamine (DIPEA; 146 µL; 0.84 mmol) under argon atmosphere (Scheme 2). The reaction mixture was challenged to MW irradiation with the following settings: a dynamic curve, maximum power 100 W, maximum pressure cap 300 PSI, and 90 °C for 1 hour. After the reaction, the solvent was evaporated under reduced pressure, and the crude reaction mixture was purified by column chromatography using mobile phase dichloromethane/methanol (DCM/MeOH = 9:1) to afford the title product **K1396** as a white amorphous solid in 45% yield.

^{1}H NMR (500 MHz, DMSO-d_6) δ 11.83 (s, 1H), 10.09 (s, 1H), 8.26 (s, 1H), 7.37 – 7.28 (m, 8H), 7.27 – 7.19 (m, 3H), 7.12 (d, *J* = 8.4 Hz, 1H), 3.46 – 3.21 (m, 2H), 2.68 – 2.59 (m, 4H), 2.19 (t, *J* = 7.3 Hz, 2H), 1.97 (s, 2H), 1.85 – 1.77 (m, 2H), 1.64 – 1.57 (m, 2H), 1.56 – 1.47 (m, 2H), 1.44 – 1.35 (m, 2H), 1.29 – 1.19 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 153.36, 151.86, 151.56, 143.67, 135.80, 128.72, 127.56, 127.07, 124.49, 124.22, 79.40, 72.76, 58.16, 51.18, 36.90, 31.62, 29.78, 27.06, 26.83. HRMS (ESI$^+$): $[M+H]^+$: calculated for $C_{13}H_{24}N_5O_4^+$ (m/z): 474.27512; found: 474.27350; LC MS > 97%.



Scheme 2. Chemical synthesis of **K1396**.

2.2. Dissociation constant (pK_a) determination

The pK_a measurement depends on the direct determination of a ratio between the neutral and ionized molecule. The protonation of ionizable groups in studied compounds was monitored using UV-visible spectrophotometry.

Twelve 20 mM phosphate-pyrophosphate buffers (pH from 5 to 13) were prepared according to the methodology published by Radic *et al* (14). All chemicals used were supplied by Merck (Darmstadt, Germany). pH instrumentation (Metrohm, Prague, Czech Republic) was used in the experiment. To a 900 µL buffer solution (containing 0.1 M NaCl) a 100 µL of 1mM solution of the tested compound (**K1396**) in suitable solvent was added (most often distilled water).

The UV spectrum for each pH point was recorded on Synergy 2 (BioTek, Germany) in the range of 200 to 650 nm wavelength in triplicate. Differences in maximal absorbance were used for pK_a determination. Absorbance values of the solvent were subtracted from the absorbance of the tested compound. The resulting pK_a values were calculated in GraphPad Prism5 (GraphPad Software, San Diego, CA, USA) by nonlinear regression. The measured values were compared with predicted values in Marvin Sketch software (http://www.chemaxon.com).

2.3. Cholinesterases inhibition

Human recombinant acetylcholinesterase (3.1.1.7; *Hss*AChE) and human recombinant butyrylcholinesterase (3.1.1.8; *Hss*BChE) were prepared according to previously public protocol (15). The inhibitory activity of the tested drugs was determined using Ellman´s method and is expressed as IC_{50} values, i.e., the concentration that reduces the cholinesterase activity to 50% of original activity (16,17). The assay mixture contained 1 mM acetylthiocholine iodide (ATCh) as substrate and 0.25 mM 5,5´-dithiobis(2-nitrobenzoic acid) (Ellman´s reagent, DTNB) as a chromogen in 0.1 M phosphate buffer (pH 7.4). The activity was determined by measuring the increase in absorbance at 412 nm at 37° C in 2 min intervals using multi-mode microplate reader Synergy 2 (Vermont, USA). Assayed solutions of the target compound (10-2 – 10-8 M) were preincubated at the 96-well plate for 5 min at 37° C. Each concentration was assayed in triplicate. Software Microsoft Excel (Redmont, WA, USA) and GraphPad Prism version 5.02 for Windows were used for the statistical data evaluation. *In vitro*, the BChE assay was similar to the method described above; butyrylthiocholine (BTCh) was used as a substrate at 1 mM concentration. All chemicals used were supplied by Merck (Prague, Czech Republic).

2.4. *In vitro* reactivation potemcy

The following chemicals were obtained from Merck (Prague, Czech Republic): ATCh, BTCh, DTNB, dimethylsulfoxide (DMSO), paraoxon methyl, paraoxon ethyl. The OP compound were made available from Military Research Institute (Brno, Czech Republic) with ≥ 95 % purity. Tested reactivators were synthesized at the Department of Toxicology and Military Pharmacy, University of Defence (Hradec Kralove, Czech Republic). The compounds were analyzed by LC-MS consisting of UHLPC Dionex Ultimate 3000 RS coupled with Q Exactive Plus orbitrap

mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) to obtain high-resolution mass spectra. Gradient LC analysis confirmed > 95% purity. The microplate reader Synergy 2 was used in the experiments.

The reactivation potency of two standards and **K1396** was evaluated on *Hss*AChE and *Hss*BChE. The enzyme was inhibited by a solution of the appropriate cholinesterase inhibitor – sarin, VX, tabun, A-234, paraoxon ethyl or paraoxon methyl – in propan-2-ol at a concentration of their IC_{50}. The excess inhibitor was subsequently removed using an octadecylsilane-bonded silica gel SPE cartridge. The inhibited enzyme was incubated for 10 min with a solution of reactivator at concentrations 10^{-4} and 10^{-5} M at 37 °C. The reaction was started by the addition of the substrate ATCh and BTCh in case of using *Hss*AChE and *Hss*BChE, respectively. The activity of *Hss*AChE/*Hss*BChE was then measured spectrophotometrically at 412 nm by the modified method of Ellman. Each concentration of reactivator was assayed in triplicate. The obtained data were used to compute reactivation potency (R; Equation 1). Results were corrected for oximolysis and inhibition of *Hss*AChE/*Hss*BChE by reactivator.

$$R = \left(1 - \frac{\Delta A_0 - \Delta A_r}{\Delta A_0 - \Delta A_i}\right) \times 100 \text{ [\%]} \quad \text{(Eq. 1)}$$

ΔA_0 – change in absorbance caused by intact cholinesterases (phosphate buffer instead of AChE/BChE inhibitor), ΔA_i – change in absorbance provided by AChE/BChE-exposed to inhibitors, and ΔA_r – change in absorbance caused by *Hss*AChE/*Hss*BChE incubated with the solution of reactivator.

2.5. *Cytotoxicity study*

The cytotoxic effect of tested compound **K1396** was evaluated using three different cell lines of human origin, namely ACHN, SH-SY5Y, and HepG2 (ACHN and HepG were obtained from ATCC, USA, SH-SY5Y from ECACC, UK). All cell lines were cultivated in Dulbecco's modified Eagle´s medium (Biosera, Nuaille, France) supplemented with 10% fetal bovine serum (Biosera), 1% penicillin (10,000 U/mL) – streptomycin (10,000 µg/mL) antibiotic solution (Sigma-Aldrich) at 37°C in CO_2 incubator (Binder CO_2 Incubator CB160, Tuttlingen, Germany) and routinely passaged by trypsinization at 75 – 85% confluence. For SH-SY5Y cells, the medium was further supplemented with 1% non-essential amino acid solution (Sigma-Aldrich).

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma–Aldrich, Prague, Czech Republic) reduction assay was used for measurement of **K1396** cytotoxicity. Cell viability was detected after 24-hour incubation with the tested compounds. For the assay, ACHN, SH-SY5Y, and HepG2 cells were seeded into 96-well plates in 100 µL volume and density of 8 × 103, 20 × 103, and 15 × 103, respectively. Cells were allowed to attach overnight before the treatment. The stock solution of the tested compound was prepared in DMSO (Sigma-Aldrich, Prague, Czech Republic), which was further serially diluted in the appropriate culture medium and added to the cells in a 96-well culture plate. The final concentration of DMSO was less than 0.25% per well.

After 24 h incubation, the medium containing serially diluted compound was aspirated from each well and replaced by 100 µL of fresh medium containing MTT (0.5 mg/mL). Plates were subsequently incubated at 37 °C in a CO_2 incubator for 1 hour. Medium containing MTT was then aspirated, and formazan was dissolved in 100 µL of DMSO. The optical density of each well was measured using Synergy 2 at wavelength 570 nm.

The cytotoxicity of **K1396** was expressed in IC_{50} value, which was calculated using 4-parametric nonlinear regression with the statistic software GraphPad Prism. Data were obtained from three independent experiments performed in triplicates. The IC_{50} value was expressed as mean ± SEM.

2.6. PAMPA assay

PAMPA (the parallel artificial membrane permeability assay) is a high-throughput screening tool applicable for predicting the passive transport of potential drugs across the blood-brain barrier (BBB) (18,19). In this study, it has been used as a non-cell-based *in vitro* assay carried out in a coated 96-well membrane filter. The filter membrane of the donor plate was coated with PBL (polar brain lipid, Avanti, USA) in dodecane (4 µl of 20 mg/ml PBL in dodecane) and the acceptor well was filled with 300 µl of phosphate buffer saline (PBS; pH 7.4; V_A).

The tested compounds were dissolved first in DMSO and subsequently diluted with PBS (pH 7.4) to final concentrations of 100 μM in the donor wells. The concentration of DMSO did not exceed 0.5% (v/v) in the donor solution. About 300 μL of the donor solution was added to the donor wells (V_D), and the donor filter plate was carefully put on the acceptor plate so that the coated membrane was "in touch" with both the donor solution and acceptor buffer. In principle, the test compound diffused from the donor well through the lipid membrane (*Area* = 0.28 cm^2) to the acceptor well. The concentration of the drug in both donor and the acceptor wells was assessed after 3, 4, 5, and 6 h of incubation in quadruplicate using the UV plate reader Spark (Tecan Group Ltd, Switzerland) at the maximum absorption wavelength of each compound. Besides that, the solution of theoretical compound concentration, simulating the equilibrium state established if the membrane were ideally permeable, was prepared and assessed as well. The concentration of the compounds in the donor and acceptor well and equilibrium concentration were calculated from the standard curve and expressed as the permeability (*Pe*) according to the equation:

$$Pe = C \times -ln\left(1 - \frac{[drug]_{acceptor}}{[drug]_{equilibrium}}\right)$$

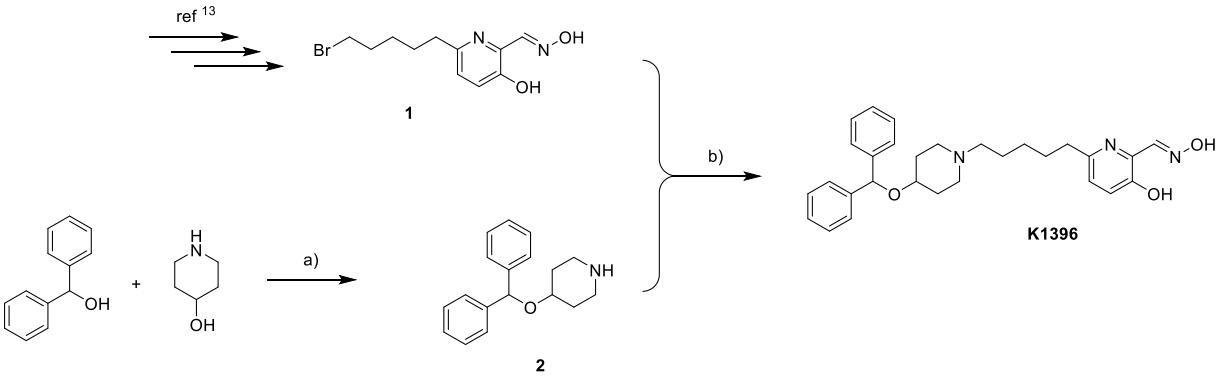
Where

$$C = \left(\frac{V_D \times V_A}{(V_D + V_A) \times Area \times Time}\right)$$

3. Results and Discussion

3.1. Chemistry

The chemical synthesis of **K1396** comprised three parts (Scheme 3). Firstly, key intermediate **1** was prepared according to the already published procedure (13). Secondly, piperidine intermediate **2** was formed from benzhydrol and 4-hydroxypiperidine under acidic conditions using Dean-Stark apparatus. Finally, **K1396** was obtained by microwave (MW) assisted *N*- alkylation reaction between corresponding intermediates **1** and **2**. All the compounds were characterized by ^{1}H and ^{13}C NMR, HRMS data, and their purity was verified by LC-MS analysis (UV detection), which showed a purity of over 95% (Supplementary Information).



Scheme 3. The chemical synthesis of **K1396**. Reagents and conditions: a) p-toluenesulfonic acid, toluene, Dean-Stark, 24h, 130 °C; b) MW, *N*,*N*-diisopropylethylamine, acetonitrile, 1 h, 90 °C.

3.2. Dissociation constant (pK_a) determination

The pK_a values indicate the ratio between ionized and unionized forms in a given pH. pK_a determination of cholinesterase reactivators is important because the required nucleophilic attack is mediated through the dissociated oxime moiety, i.e. oximate anion at physiological pH. Therefore, we have determined pK_a of standard oximes and **K1396** (Table 1). The effective pK_a range was usually found between 7.0-8.5, whereas values over 8.5 are considered incapable of reactivation (20). Moreover, pK_a is a limiting factor for penetration through the biological membranes (20). According to the chemical structure, Marvin software detected two pK_a values for obidoxime (two oxime groups) and **K1396** (oxime and hydroxyl groups). These compounds were software-predcited to have pK_a values out of the recommended range.

However, experimentally determined pK_a values are slightly different. For obidoxime, pK_a 7.96 was found, which is comparable with the literature value (7.82) (20). pKa values of **K1396** are out of optimal range (21). However, other factors must also be considered when dealing with reactivation properties, like appropriate orientation into the enzyme's active site, presence and position of the oxime group, molecular size and flexibility of the ligand, and overall lipophilicity, among others.

Table 1. Predicted and determined pKa values of standard reactivators and novel compounds.

compound	**pK_a software predicted**	**UV-vis determined pK_a**
2-PAM[a]	**7.63**	**7.91 ± 0.05**
obidoxime[a]	**8.56** **9.16**	**7.96 ± 0.02**
K1396	**8.09** **9.4**	**8.72 ± 0.19** **10.8 ± 0.04**

3.3. Cholinesterases Inhibition

The IC_{50} values were determined to reveal the interaction between compounds and native cholinesterases according to the method of Ellman *et al.* (Table 2) (16). It is expected that the compound needs to maintain a certain balance between exerting affinity for the enzyme and not revealing an extremely potent inhibition profile. Indeed, potent inhibition of native enzymes could be the reason for excluding the compound from the follow-up reactivation potency assay. As a result, all the compounds under the study revealed moderate affinity to *Hss*AChE in the micromolar range, while 2-PAM and obidoxime turned out to be two to three orders less potent inhibitors of *Hss*BChE compared to **K1396**. This feature reflects the nature of **K1396**, which is bulkier compared to standard oximes and thus can better accommodate a larger pocket of *Hss*BChE. From the results obtained, **K1396** can be classified as a moderate inhibitor with a balanced affinity towards both cholinesterases. The IC_{50} values do not discriminate the compound from the follow-up reactivation studies.

Table 2. The inhibition profile of the oxime reactivators under the study was expressed as IC_{50} values for *Hss*AChE and *Hss*BChE.

Compound	*Hss*AChE IC_{50} (μM) ± SEM[a,b]	*Hss*BChE IC_{50} (μM) ± SEM[a,b]
2-PAM	**453 ± 19**	**11,300 ± 1,300**
obidoxime	**468 ± 29**	**1590 ± 119**
K1396	**83.3 ± 3.8**	**45.9 ± 2.1**

[a] IC_{50} values measured by modified Ellman´s assay

[b] Results are the mean of at least three independent experiments

3.4. *In vitro* reactivation study

Next, we determined the reactivation potencies of the newly discovered oxime reactivator **K1396** and compared them with standard oximes 2-PAM and obidoxime. For the screening purpose, we used sarin, tabun, VX and A-234 from the group of NAs, and ethyl-paraoxon and methyl-paraoxon from the group of pesticides. Representative data are displayed in Figure 2 with a reactivator's screening concentration of 10^{-4} M (Fig. 2 A and B) and 10^{-5} M (Fig. 2 C and D). A-234 data were omitted from the graphical representation due to the lack of reactivation ability of any oxime used in the study. Such data are consistent with our recent findings (unpublished data), revealing that none of the standard or experimental nucleophiles can reactivate A-234-inhibited *Hss*AChE/*Hss*BChE. Reactivation data for obidoxime against pesticides are considetent with the literature (22). Notably, **K1396** revealed promising reactivation potency in the case of VX-inhibited *Hss*AChE, which was even more pronounced at lower compound concentrations. While negligible reactivation of **K1396** was observed in higher compound concentrations against sarin and tabun for both cholinesterases, the reactivation profile improved significantly in lower concentrations. Moreover, **K1396** retained high reactivation potency against VX-inhibited *Hss*AChE/*Hss*BChE at all concentrations,

comparable to 2-PAM and obidoxime at higher concentrations and even outperforming them at lower concentrations. Notably, **K1396** demonstrated the highest reactivation potency against methyl-paraoxon-inhibited cholinesterases.

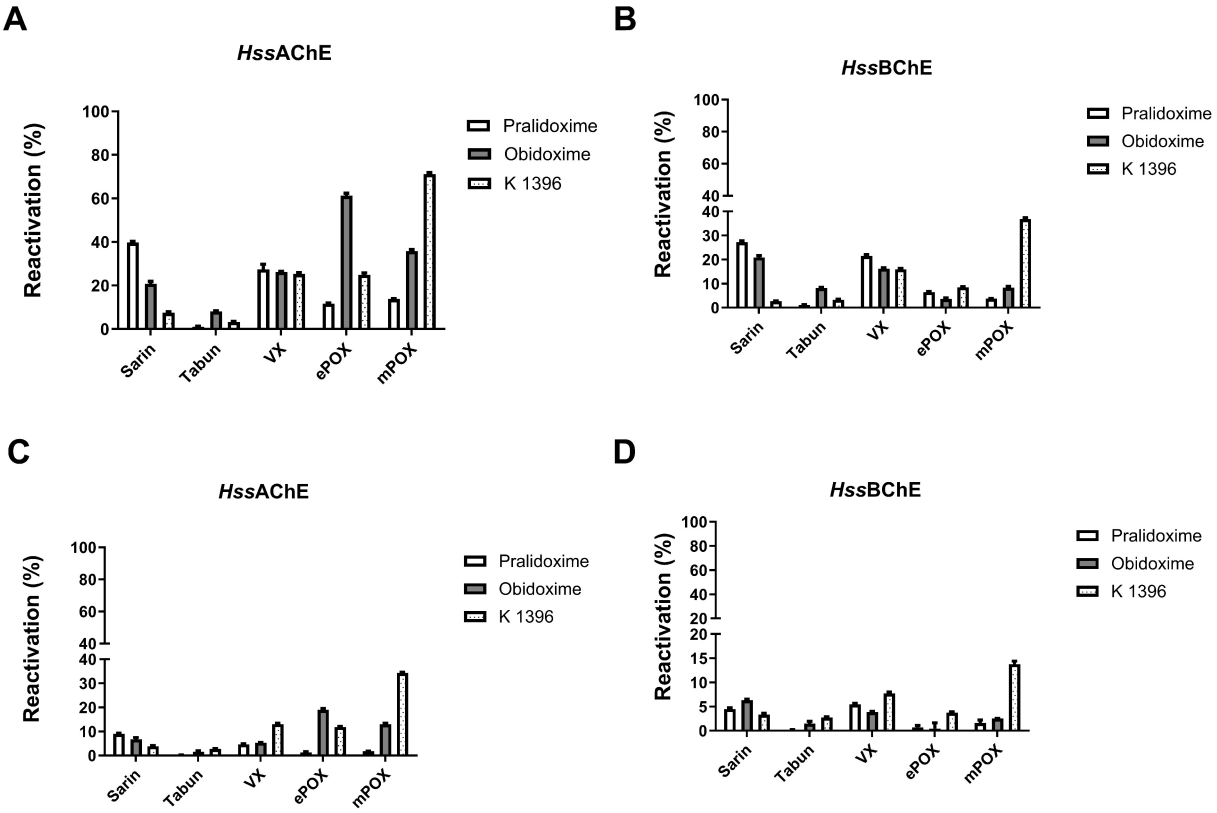


Figure 2. The potency of oximes to reactivate *Hss*AChE and *Hss*BChE (% of reactivation) inhibited by sarin, tabun, VX, ePOX, and mPOX (mean ± SEM). Results from the screening concentration of 10^{-4} M and 10^{-5} M are displayed in A and B, and C and D figures, respectively. All the experiments were performed in triplicate and repeated three independent times.

3.5. Cytotoxicity study

To elaborate on the cytotoxic profile of the reactivators, we evaluated their cytotoxicity against ACHN, SH SY5Y and HepG2 cell lines via MTT assay (Table 3). Interestingly, **K1396** displayed a lower cytotoxicity profile than 2-PAM and obidoxime when challenged against the adenocarcinoma cell line (ACHN). The IC_{50} values for neuronal (SH-SY5Y) and hepatic (HepG2) cell lines are in the same order of magnitude for all three tested compounds. These data preclude a similar safety profile to standard oximes, although they must be taken with precaution as other factors may contribute to the toxicity of the compound (the conversion of the compound into toxic species via CYP-mediated metabolism, cardiotoxicity via hERG channel etc.).

Table 3. Comparison of IC_{50} values of tested compounds in the ACHN, SH SY5Y and HepG2 cell measurements using colorimetric MTT assay.

Compound	ACHN IC_{50} (μM) ± SEM[a]	SH-SY5Y IC_{50} (μM) ± SEM	HepG2 IC_{50} (μM) ± SEM
2-PAM	**14.2 ± 0.3[c]**	**19.5 ± 0.9[b]**	**22.8 ± 0.8[c]**
obidoxime	**5.33 ± 0.23[c]**	**3.25 ± 0.03[b]**	**4.02 ± 0.28[c]**
K1396	**>32**	**15.1 ± 1.5**	**20.2 ± 0.4**

[a] Results are the mean of at least three independent experiments

[b] IC_{50} were taken from ref. Hepnarova et al.(23)

[c] IC_{50} were taken from ref. Muckova et al.(24)

3.6. PAMPA assay

PAMPA assay is a useful tool in primary drug development screening to distinguish between the compounds capable of crossing biological barriers. In line with the design consideration, **K1396** proved to permeate the lipid layer under experimental conditions. As expected, 2-PAM and obidoxime did not permeate through the barrier, given the presence of a permanent change in their structure (Table 4). From this standpoint, **K1396** is predicted to permeate BBB by passive diffusion, although other aspects may hinder the compound CNS availability *in vivo* (e.g. P-gp efflux system.)

Table 4. List of tested compound and their prediction of BBB penetration.

Compound	*Pe* ± SEM ($\times 10^{-6}$ cm s^{-1})	n	CNS (+/-)
2-PAM[a]	**0.36 ± 0.16**	**2**	**CNS -**
obidoxime[a]	**0.68 ± 0.09**	**2**	**CNS -**
K1396	**8.94 ± 0.93**	**2**	**CNS +**

n = number of measurements

CNS + (high BBB permeation predicted): Pe (×10-6 cm s-1) > 4.0

CNS - (low BBB permeation predicted): Pe (×10-6 cm s-1) < 2.0

CNS +/- (BBB permeation uncertain): Pe (×10-6 cm s-1) from 4.0 to 2.0

[a] *Pe* values were taken from ref. Soukup et al.(25)

4. Conclusion

The present study introduces **K1396**, a novel uncharged cholinesterase reactivator designed to address the limitations of existing oxime reactivators, such as inadequate blood-brain barrier (BBB) penetration and limited efficacy against various OPCs. The design strategy focused on enhancing lipophilicity and enabling dual binding at both the CAS and PAS of AChE. *In vitro* evaluations demonstrated that **K1396** exhibits comparable or superior reactivation potency against OPC-inhibited AChE, particularly with VX, and shows promising potential in penetrating the BBB, as indicated by the PAMPA assay results. Moreover, **K1396** displayed a favorable cytotoxicity profile across different human cell lines, indicating a potential for improved safety compared to traditional oxime reactivators. While **K1396** demonstrated limited reactivation potency against certain NAs like sarin and tabun, its significant efficacy against VX and specific pesticides positions it as a versatile candidate for further development. These findings suggest that **K1396** is a promising broad-spectrum reactivator for OPC poisoning, with potential advantages in terms of CNS availability and safety. Further research, including *in vivo* studies, will be necessary to validate its efficacy and safety profile, ultimately contributing to the development of more effective treatments for OPC intoxication.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Adherence to Ethical Standards

This article does not contain any studies involving animals or human participants performed by any of the authors.

Supporting Information

Supporting Information are available online. It contains 1H and 13C NMR spectra, LC-MS and HRMS records.

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