View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Bioorganic & Medicinal Chemistry 21 (2013) 5923–5930

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

NMR determination of *Electrophorus electricus* acetylcholinesterase inhibition and reactivation by neutral oximes

Sibelle Feitosa da Cunha Xavier Soares, Andréia Aguiar Vieira, Reinaldo Teixeira Delfino, José Daniel Figueroa-Villar *

Medicinal Chemistry Group, Chemistry Department, Military Institute of Engineering, Praça General Tiburcio 80, 22290-270 Praia Vermelha, Rio de Janeiro, Brazil

ARTICLE INFO

Article history:
Received 12 March 2013
Revised 21 May 2013
Accepted 29 May 2013
Available online 19 June 2013

Keywords:
NMR
Acetylcholinesterase inhibition
Neutral oximes
Paraoxon
Alzheimer disease
Organophosphorus intoxication
AChE reactivation

ABSTRACT

Neurotoxic organophosphorus compounds (OPs), which are used as pesticides and chemical warfare agents lead to more than 700,000 intoxications worldwide every year. The main target of OPs is the inhibition of acetylcholinesterase (AChE), an enzyme necessary for the control of the neurotransmitter acetylcholine (ACh). The control of ACh function is performed by its hydrolysis with AChE, a process that can be completely interrupted by inhibition of the enzyme by phosphylation with OPs. Compounds used for reactivation of the phosphylated AChE are cationic oximes, which usually possess low membrane and hematoencephalic barrier permeation. Neutral oximes possess a better capacity for hematoencephalic barrier permeation.

NMR spectroscopy is a very confident method for monitoring the inhibition and reactivation of enzymes, different from the Ellman test, which is the common method for evaluation of inhibition and reactivation of AChE. In this work ^{1}H NMR was used to test the effect of neutral oximes on inhibition of AChE and reactivation of AChE inhibited with ethyl-paraoxon. The results confirmed that NMR is a very efficient method for monitoring the action of AChE, showing that neutral oximes, which display a significant AChE inhibition activity, are potential drugs for Alzheimer disease. The NMR method showed that a neutral oxime, previously indicated by the Ellman test as better in vitro reactivator of AChE inhibited with paraoxon than pralidoxime (2-PAM), was much less efficient than 2-PAM, confirming that NMR is a better method than the Ellman test.

© 2013 Published by Elsevier Ltd.

1. Introduction

Organophosphorus compounds (OPs) are important molecules used as pesticides, covering about 40% of this market.[1] Unfortunately, the inadequate use of organophosphorus pesticides causes intoxication in humans and animals, several suicides and considerable environmental damages.[2–5] According to the World Health Organization (WHO), the OPs cause about 3 million intoxications and 200,000 deaths in the whole world every year.[6] In fact, the biological effects of OPs depend on the interaction time, concentration and type of OP to which the victim was exposed.[7,8] The toxicity of OPs varies significantly depending on their structures, and the most dangerous are the chemical warfare agents.[9,10] The OPs toxicity differences are related with their structural characteristics, such as the type of functional groups bound to the phosphorus atom.[9,10]

The main action of neurotoxic OPs is the inhibition of acetylcholinesterase (AChE).[11–13] The function of this enzyme is the control of nerve impulse transition by hydrolysis of the neurotransmitter acetylcholine (ACh), which is liberated on the neuronal synapse at the autonomous and central nervous system, as well as at neuromuscular junctions.[14–17] The hydrolysis of ACh by human AChE happens at the enzyme's active site, with participation of the amino acid residues Ser203, Glu334 and His447.[9,18] The inhibition of AChE by OPs occurs by phosphylation of Ser203, which is the amino acid directly involved in the ACh hydrolysis process, leading to cholinergic crisis and death.[18,19]

The reactivation of the AChE function in patients intoxicated with OPs is actually performed by administration of cationic pyridinic oximes.[10,20–23] These oximes act by dephosphylation of Ser203.[18,24–32] Nowadays there are many reports of new oximes with good efficiency as AChE reactivators.[33–48] However, there are still several problems with this type of drugs. For example, most of these oximes are not good hematoencephalic barrier permeators, thus leading to restrictions on their brain activites.[41,49] Also, it has not been discovered a single oxime that is effective to reactivate AChE inhibited with any type of neurotoxic OP.[10,20,23]

The treatment of Alzheimer disease (AD), which is an important brain problem that affects about 30 million people in the whole world, is also related to AChE activity.[50] AD usually leads to

* Corresponding author. Tel.: +55 21 25467057; fax: +55 21 25467059.
E-mail addresses: daniel.figueroa@pesquisador.cnpq.br, jdfv2009@gmail.com (J.D. Figueroa-Villar).

0968-0896/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmc.2013.05.063

problems on the transmission of nerve impulses because it causes a serious decrease of the number of neural transmitters, like ACh. Therefore, competitive inhibitors of AChE are used for the treatment of AD patients, because its inhibition leads to brain concentration increase of ACh, re-establishing the neuron functions.[51]

It has been shown that neutral oximes, which normally have a better hematoencephalic barrier permeation, display a potential to function as inhibitors of AChE or reactivators of phosphylated AChE.[41,49–52]

The physical–chemical phenomena related to ligand-receptor intermolecular interactions are fundamental for understanding biological processes. In this context, nuclear magnetic resonance spectroscopy (NMR) is one of the most powerful techniques for ligand–protein interaction studies, being an efficient method to determine and quantify intermolecular interactions.[53–57] NMR is also a very confident technique for the detection and identification of different compounds, thus making it a very secure monitoring method for kinetics studies of inhibition and reactivation of enzymes.[58]

Enzyme inhibition studies are normally conducted using UV–visible spectroscopy methods.[59] However, in some cases, these methods require the use of a second enzyme to transform the substrate of the tested enzyme into a secondary product with a very different λ_{max}. In this case, the tested enzyme inhibitor could also interact with the second enzyme, leading to wrong results. Also, some UV–visible methods require the use of a different substrate to test the enzyme. For example, the Ellman test needs the use of acetyl thiocholine instead of acetylcholine (ACh) as substrate for acetylcholinesterase (AChE).[60] Despite the fact that acetyl thiocholine functions well as a substrate for AChE, not using ACh as substrate could lead to variations on the inhibition and reactivations tests. A good alternative to UV–visible methods is the use of Nuclear Magnetic Resonance (NMR). The disadvantages of NMR in comparison with UV–visible methods are the fact that the former is a less sensitive method, requiring the use of relatively higher concentrations and longer testing times. The main advantage of NMR is its precision, especially on the definitive detection and differentiation of enzyme substrates, products and inhibitors. For this reason, enzyme inhibition tests with NMR do not need substrate exchange or the use of another enzyme to modify the product.

The objective of this work was to use ^{1}H NMR spectroscopy to study the capacity of neutral oximes as inhibitors of *Electrophorus electricus* (*Ee*AChE) and reactivators of *Ee*AChE inhibited by paraoxon (**1**), in comparison with 2-PAM (**2**) (Fig. 1). These two topics were selected to determine the efficiency of NMR in both methods.

In this work, despite 2-PAM not being the most effective reactivator for paraoxon-inhibited AChE, it was selected as positive standard compound because, in comparison with the other effective cationic oximes, its structure is the most similar to the tested neutral oximes, allowing better comparison of the results due to differences on molecular properties but less with molecular structure differences.

Several enzymes can be used for kinetics, inhibition and reactivation studies of AChE.[61] The active site of *Ee*AChE is basically identical with the one of human acetylcholinesterase (*Hu*AChE).[62] The selection of *Ee*AChE for this work was based on its better reactivation by pralidoxime when inhibited with paraoxon when compared with *Hu*AChE. This difference is not based on the amino acid sequence of the active sites but on non-reactivation process, denaturation or aging, of the human enzyme.[63]

2. Materials and methods

2.1. Material solutions

2.1.1. Phosphate buffer pH 7.4

All monitoring processes by NMR were performed using phosphate buffer at pH 7.4 in 98% D_2O as solvent. The buffer solution was prepared at 0.01 M concentration with dry sodium mono-basic phosphate (NaH_2PO_4) and dry sodium di-basic phosphate (Na_2HPO_4).

2.1.2. EeAChE solution

In a 5.00 mL volumetric balloon, 710 μL of *Electrophorus electricus* AChE (100 UN/mL, quaternary form, CAS number 0009000811, C2888 from Sigma Aldrich) and 0.5 mg of bovine serum albumin (BSA, A2153) were dissolved in 4.00 mL of phosphate buffer (pH 7.4) and the solution volume was adjusted to 5.00 mL. The solution was kept in storage at 4 °C, with a EeAChE concentration of 0.33 nM.

2.1.3. Solution of substrate: acetylcholine (ACh)

In a 10.00 mL volumetric balloon, 546 mg of ACh (Sigma–Aldrich) were dissolved in 8.00 mL of phosphate buffer (pH 7.4) and the solution volume was adjusted to 10.00 mL, leading to a ACh concentration of 0.30 M.

2.1.4. Oximes solutions

Each oxime solution was prepared in a 1.00 mL volumetric balloon using 12.0 μmol of the oxime, 125 μL of DMSO-d_6 and the necessary amount of phosphate buffer (pH 7.4) to complete the volume. The final concentration of each oxime was 12.0 mM.

2.1.5. Ethyl-paraoxon solution

The basic solution of ethyl-paraoxon was prepared by dissolving 100 μL of pure-ethyl-paraoxon (127.4 μg) (density 1.274 g/mL) in a 50.00 mL volumetric balloon, using distilled water as solvent. 2.0 μL of the basic solution ware poured into a 5.00 mL volumetric balloon and the volume completed with phosphate buffer (pH 7.4), leading to a final solution of 3.70 nM concentration, which was used for inhibition of EeAChE.

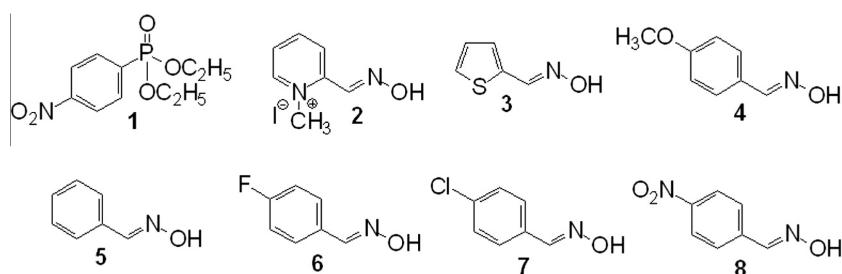


Figure 1. AChE inhibitor and oximes tested as inhibitors and reactivators of EeAChE: Paraoxon (**1**), pralidoxime (**2**), thiophene-2-aldoxime (**3**), 4-methoxy-benzaldoxime (**4**), benzaldoxime (**5**), 4-fluor-benzaldoxime (**6**), 4-chloro-benzaldoxime (**7**) and 4-nitro-benzaldoxime (**8**).

2.2. NMR experiments

All NMR experiments were performed at 20.0 ± 0.1 °C in the Varian Inova 500 MHz spectrometer of the LNBio (National Bioscience Laboratory) in Campinas/SP (Brazil). The samples were prepared in 5 mm NMR tubes using phosphate buffer in D_2O (pH 7.4) as solvent. All samples were prepared exactly 1 min before their introductions into the magnet, and the lock and shimming process was carried in all samples during 4 min, in order to have exactly the same processing time in all experiments. The number of transients for each spectrum was 16 and the acquisition time was 3.75 s for each transient.

2.2.1. NMR monitoring of EeAChE inhibition by neutral oximes

Monitoring ACh hydrolysis by EeAChE was performed with NMR by integration of the singlet signal of the ACh methyl group at 2.24 ppm and of the produced acetic acid at 2.16 ppm. The signal intensity in all tests was calculated by integration of a 0.3 ppm zone centralized on each methyl signal.

In each case, the different solution components were added in the following order: firstly 2.00 μL of the basic solution of EeAChE, followed by 5.00 μL of the respective oxime solution, then 5.00 μL of ACh solution and the necessary volume of phosphate buffer to adjust the total solution volume to 600 μL. Each experiment was performed with the following concentrations: 2.5 mM of ACh, 1.09 pM of EeAChE and 0.1 mM of oxime. The hydrolysis process was monitored by collection of ^{1}H NMR spectra in intervals of 5 min. Each spectrum was obtained using 30° pulses and 16 scans, with d1 of 0.5 s and at 20 °C (NMR room temperature). The process was also executed with EeAChE and ACh without oximes, in order to use these results as the positive comparative data. All experiments were executed in duplicate.

2.2.2. Inhibition of EeAChE with ethyl-paraoxon

AChE inhibition was first conducted by treatment of 2.0 μL of the AChE solution (0.33 nM) with 1.5 μL of the ethylparaoxon (diethyl-4-nitrophenyl phosphate) solution (3.70 nM) for 1 h at 25 °C. After 1 h, the inhibited enzyme solution was mixed with 5.0 μL of ACh solution and monitored by NMR for 60 min, indicating that ACh was completely not hydrolyzed, confirming the AChE inhibition. Monitoring the reactivation process of the inhibited AChE was conducted with the same conditions used for the inhibition studies of this enzyme (Section 2.2.1), but 5.00 μL of the respective oxime solution were added before adjusting the final volume to 600 μL. Pralidoxime (2-PAM) was used as positive control.

2.2.3. NMR monitoring neutral oximes reactivation of EeAChE inhibited with paraoxon

To analyze the process of reactivation of EeAChE inhibited with ethyl-paraoxon by NMR, it was used the previously described procedure applied for the oxime inhibition monitoring (Section 2.2.1), with the pure EeAChE fraction (2.0 μL) replaced by the ethylparaoxon inhibited EeAChE solution (2.0 μL) on the same concentration.

2.3. Synthesis of oximes

All used oximes, which are known compounds, were prepared by reaction of the respective aldehydes (2.0 mmol) with hydroxylamine hydrochloride (4.0 mmol) in a 50 mL round bottom flask, using a mixture of 3.0 mL of water and 10 mL of 95% ethanol as solvent.[41] The reaction mixture was kept under stirring at 25 °C for 24 h. The precipitated products were separated by filtration and recrystallized from methanol, leading to the pure oximes with yields from 60% to 85%. The structure and purity of each prepared oxime was confirmed by ^{1}H and ^{13}C NMR spectra (See Supplementary Material).

2.4. Molecular modeling

All oximes structures were optimized on Intel dual 2.66 GHz processor with 2048 Mb RAM using the Spartan06 package with the functional density (DFT) B3LYP method and the 6-311++G** basis set. The obtained molecular properties were relative energy (kJ/mol), molecular mass (atomic units – amu), molecular area (MA), PSA (polarization surface area), Log *P* and dipole moment (Debye).

3. Results and discussion

^{1}H NMR spectroscopy was chosen to monitor the inhibition and reactivation of AChE in order to develop a simple and more effective method, which could be applied on further studies for the development of potential compounds for treatment of Alzheimer disease (AD) and defense agents against organophosphorus chemical weapons. Inhibition and reactivation o AChE were executed in order to determine the efficiency of NMR in both methods. The other motive for working in these two topics is that development of AChE inhibitors for Alzheimer disease and reactivators of AChE inhibited by organophosphorus compounds, are two important topics for health problems.

The fact that cationic oximes, which are usually pyridine derivatives, are used as reactivators of AChE confirms that they interact with AChE active site, suggesting that they could also act as AChE competitive inhibitors. One of the problems of AD is the small production of the neurotransmissor acetylcholine (ACh) on the patients. For this reason, AChE inhibitors are used to increase ACh concentration, resulting in better brain performance.[64,65] Accordingly, oximes could also have potential for the treatment of AD, especially the neutral ones, which display a better permeation through the hematoencephalic barrier than the cationic oximes.

Some simple neutral oximes were reported as potential reactivators of human AChE inhibited with paraoxon.[41] Some of these compounds, shown in Figure 1, were selected for NMR testing in this work, in order to compare this method with the Ellman test and to evaluate the effect of the *para*-substituent of benzaldoximes (**4**–**8**) on their capacity to inhibit or reactivate AChE. For example, thiophene-2-aldoxime (**3**) was selected because, when tested as human AChE reactivator using the Ellman test, it gave better results (93% reactivation) than 2-PAM (83% reactivation).[41] As the positive active reference compound we selected pralidoxime (**2**), which is the simplest commercial cationic oxime with reasonable AChE reactivating capacity, but usually insufficient against very toxic organophosphorus warfare agents.[66] There are other cationic oximes with better reactivation capacity than pralidoxime,[67] but this compound was selected as control for two reasons. The first motive for selecting 2-PAM is its structural similarity to the tested neutral oximes, a condition that allows a better comparison of the obtained results. The second motive is the comparison of obtained NMR results with the Ellman test previously executed with these compounds, a process that was also executed using 2-PAM as positive control.[41] Also, because the Ellman test was executed with AChE inhibited with paraoxon, this compound was selected as organophosphorus inhibitor in this work.

3.1. Inhibition of EeAChE

The main problem of monitoring enzyme activity by NMR is the necessity of manipulation of the samples after mixing all components in a 5 mm NMR tube. For this reason, the addition of the enzyme substrate needs to be performed as the last sample

preparation step. Once the substrate is added, AChE starts the hydrolysis process. Because AChE is a very fast enzyme, with a turnover number of 7.4×10^5 molecules/min,[68] the substrate could be completely hydrolyzed in few minutes, preventing NMR monitoring. To solve that problem, the concentration of the enzyme must be very low, in order to make it act during a much longer time. So, AChE with a concentration of 1.1 pM was used, leading to a slow hydrolysis process that could be monitored by ^{1}H NMR during several minutes.

In order to avoid differences among the experiments, the NMR process was followed using exactly the same sample preparation procedure and NMR sample lock and shimming processing time. Therefore, all samples were prepared directly in the 5 mm NM tube during 1 min, with the introduction of ACh as last step. Also, the introduction of the sample in the NMR magnet, followed by locking and shimming processes, was conducted in exactly 4.0 min for all samples. This procedure was necessary in order to establish appropriate conditions to compare the results obtained in all experiments, which were carried out in duplicate. In this form, analyses of all samples were started exactly 4.0 min after the addition of ACh. Each experiment required 16 scans and an acquisition time of 3.75 s, leading to a total time of 1.0 min for each spectrum. That procedure confirmed the initiation of each spectrum after 5.0 min in all experiments. The hydrolysis of acetylcholine by AChE initially leads to formation of choline (Ch) and acetylated AChE on Ser203, which is then transformed by water to AChE and acetic acid (AcOH), as shown in Figure 2. The complete hydrolysis process was monitored by integration of the methyl signals of the ACh acetyl group (δ 2.24), which decreases with time, and of acetate (δ 2.16), which increases with time.

The ^{1}H NMR spectra of methyl groups of acetylcholine (ACh, left signal, δ 2.24) and acetate (AcOH, right signal, δ 2.16) along the *Ee*AChE reaction time in the presence of benzaldoxime (**5**) is shown in Figure 3. When the integration of each signal is determined using the same spectral width it can be observed, as shown in Figure 4, that the two methyl signals are in direct correlation, indicating that these data can be correctly used to monitor enzyme kinetics. In order to obtain the data for Figure 4 at time zero, it was necessary to obtain the spectrum of pure ACh at 2.5 mM concentration without the presence of AChE, while the other spectra with reaction time different from zero were determined in the presence of *Ee*AChE at 1.1 pM concentration.

The determination of all signal intensities was accomplished by using an integration width of 0.3 ppm centered on each signal, and these data was correlated with the ACh and Ac concentrations. In Figure 5 it is shown the variation of the concentration of ACh with time in the presence of pure AChE and also in the presence of all oximes tested in this work as potential inhibitors.

Figure 5 shows that thiophene-2-aldoxime (**3**) is the best inhibitor of all tested oximes, including 2-PAM (**2**). Interestingly, 2-PAM possesses a AChE inhibition capacity similar to benzaldoxime (**5**), and can be observed that the neutral oximes with a strong electron extractor (NO_2, **8**) or a strong electron donator group (OMe, **4**) at the *para* position of the aromatic ring are very inefficient AChE inhibitors. The results with compounds **4** and **8** suggest that

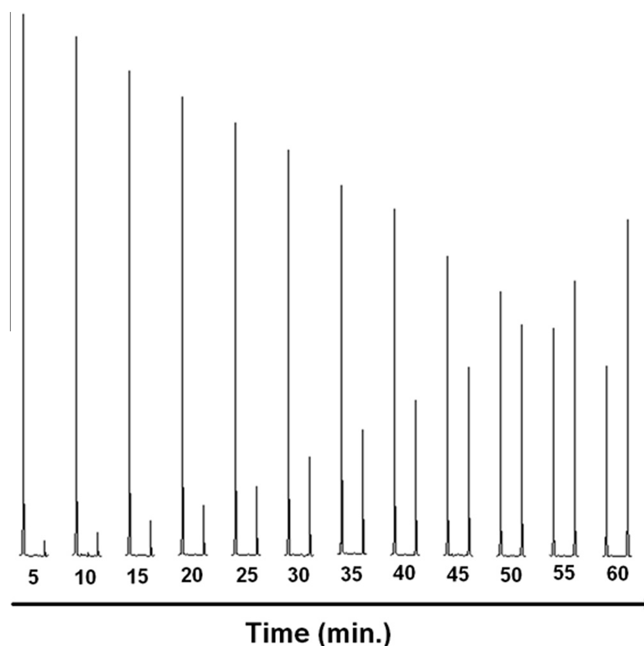


Figure 3. Example of the methyl signal variation of acetylcholine (left signal) and acetate (right signal) under the effect of EeAChE in the presence of benzaldoxime (**5**).

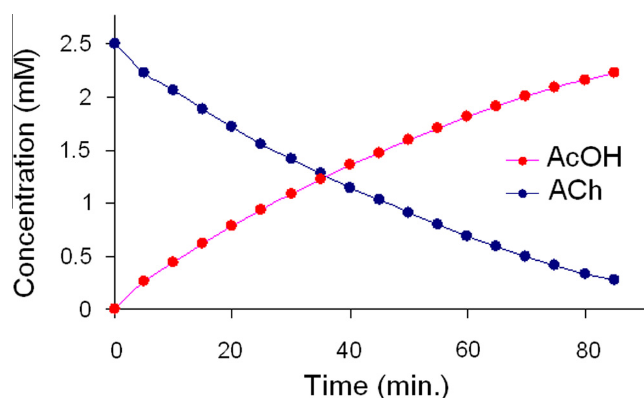


Figure 4. Comparison of the methyl signal intensity of acetylcholine (ACh) with acetic acid (AcOH) during the EeAChE action in the presence of benzaldoxime (**5**).

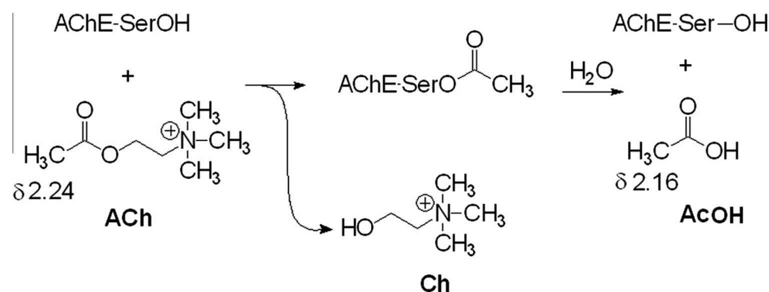


Figure 2. Hydrolysis of acetylcholine (ACh) by acetylcholinesterase (AChE) and chemical shifts of the methyl groups used for NMR monitoring.

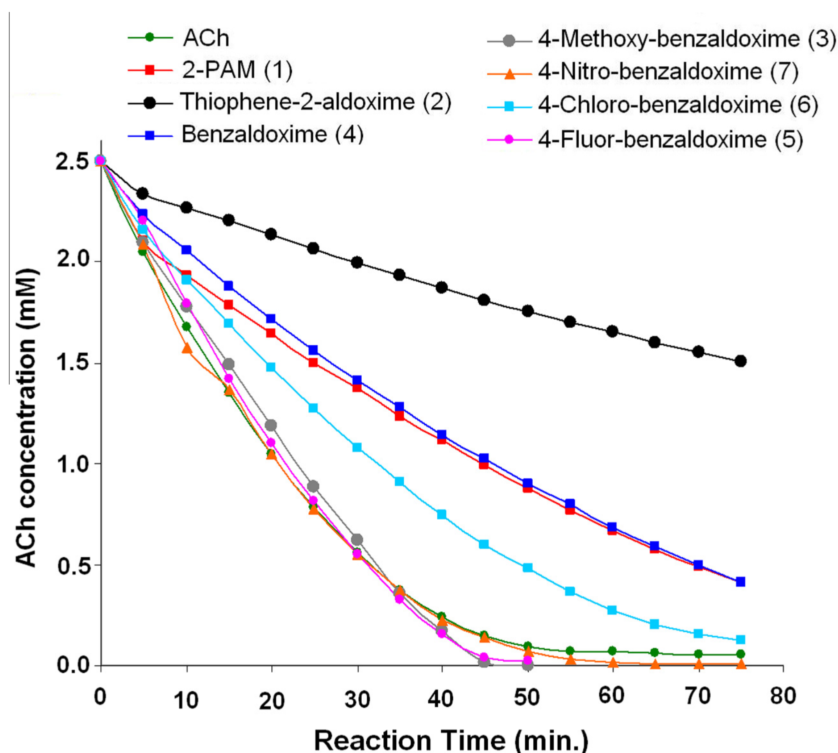
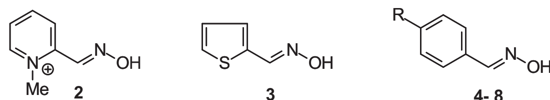


Figure 5. Variation of the concentration of ACh (μM) with time monitored by ^{1}H NMR through integration of the methyl signal of the acetyl group (δ 2.24). The experiment was conducted with pure AChE (black), and with the presence of oximes: pralidoxime (red), thiophene-2-aldoxime (blue), benzaldoxime (violet), 4-methoxy-benzaldoxime (magenta), 4-nitro-benzaldoxime (orange), 4-chloro-benzaldoxime (pink) and 4-fluor-benzaldoxime (green).

Table 1
Molecular Properties of 2-PAM and the neutral oximes obtained by Molecular Modeling (B3LYP, 6-311++G**)



Oxime	R	MM (amu)	MA (Å^2)	PSA (Å^2)	DM (D)	Log*P*	Δ*E* (au)	AI (%)
2	–	136.17	173.27	20.25	2.53	−2.31[71]	−0.406	34
3	–	127.17	142.93	32.96	1.07	0.4064	−0.560	61
4	OCH_3	151.17	182.31	39.32	2.92	2.0427	−0.575	4
5	H	121.14	152.84	32.65	1.02	2.1691	−0.570	37
6	F	138.14	163.60	19.84	2.15	2.3272	−0.567	1
7	Cl	154.60	173.56	19.83	2.38	2.7273	−0.563	22
8	NO_2	166.14	177.98	71.14	5.14	2.2030	−0.539	0

MM = molecular mass in atomic units (amu), MA = molecular area, PSA = polarization surface area, DM = dipole moment in Debye (D), $\Delta E = E_{RNOH}-E_{RNO}$ (atomic units, au), AI = AChE inhibition in 30 min (%).

oxime's acidity is not well related to its AChE inhibition capacity. On the other hand, the comparison between the fluorinated and chlorinated oximes (**6** and **7**, respectively) shows that oxime **7** is a much better AChE inhibitor than **6**.

In an attempt to discover which molecular properties are related to the oximes AChE inhibition capacity, we carried out a simple molecular modeling study of their structures and properties. Moreover, since neutral oximes could be used as drugs for AD, their structural properties could also be related to their capacity for permeation of the hematoencephalic barrier (or blood–brain barrier, BBB), a characteristic we want to favor. In general terms, molecules with molecular mass (MM) lower that 450 Da, polarization surface area (PSA) lower than 140 Å^2 (with even better performance when the PSA is lower than that 60 Å^2) and a controlled hydrophilicity, would be better BBB permeators.[69] The comparison of these properties obtained by molecular modeling analysis indicates that cationic and neutral oximes possess appropriate molecular mass and PSA; however, cationic oximes, which are salts, are more hydrophilic than neutral oximes. These results are shown in Table 1 for pralidoxime (2-PAM, **2**) and the neutral oximes (**3**–**9**), and compared with their percentage of AChE inhibition during a period of 30 min. The acidities of the oximes were estimated using the energy difference (Δ*E*) between their neutral and anionic forms. Oximes with the lowest values of Δ*E* are the most acidic ones.

These results show that none of the calculated parameters displays a good correlation with AChE inhibitory capacity. The best correlation between the inhibition capacity (%) and the calculated parameters occurs with MA (R^2 = 0,60), Log *P* (R^2 = 0.53), DM (R^2 = 0,51) and MM (R^2 = 0,49), but none of these correlations is satisfactory. Considering the PSA data, it can be observed that there is no correlation between the PSA values and the AChE inhibitory

activity of the oximes ($R^2 = 0.09$). The worst correlation is for ΔE ($R^2 = 0.04$).

Since 2-PAM was the only cationic oxime tested, in order to better understand the properties of the neutral oximes, we extracted the data of 2-PAM from the correlation process. When 2-PAM data are extracted, the only important improvement on the correlation of AChE inhibition capacity was with MA, which increases to $R^2 = 0.74$, indicating that molecular area is somehow related to AChE inhibition efficiency of neutral oximes. Aside from that, some other observations are important. For example, in the case of neutral oximes, the ones that possess dipole moment close to 1.0 D are the best inhibitors (**3** and **5**). The other observation is that when the dipole moment increases the neutral oximes are less effective AChE inhibitors. It can be observed that 4-nitro-benzaldoxime (**8**), with the highest MD (5.14), is the least effective neutral oxime (0%). The only different result is for the 4-fluor-benzaldoxime, which is a very ineffective AChE inhibitor (1%), but displays a dipole moment of 2.15 D, lower than oximes **7** (2.38 D) and **4** (2.92 D), which are medium (22%) and low (4%) AChE inhibitors, respectively. Pralidoxime possesses a medium DM value (2.53 D) and a medium inhibitory capacity (34%), very similar to benzaldoxime (**5**, 37%), which has a much lower DM value (1.02 D). The results suggest that the behavior of cationic oximes is different from neutral oximes.

The Log *P* values, without the 2-PAM data, display $R^2 = 0.53$ on correlation with AChE inhibition percentage, indicating that there is not good correlation of samples solubility with their AChE inhibitory activity. Independently, the most effective AChE inhibitor, thiophene-2-aldoxime (**3**), displays de lowest Log *P* value (0.4064), being the neutral oxime most soluble in water. The second better AChE inhibitor, benzaldoxime (**5**), which Log *P*, even displaying the second lowest Log *P* of the neutral oximes (2.1691), is very similar in solubility to the other tested compounds, especially to 4-nitro-benzaldoxime, which is the worst AChE inhibitor of all. On the other hand, the Log *P* of 2-PAM reported in the literature,[70] is −2.31, being the most soluble of all the tested compounds, even much more than thiophene-2-aldoxime. These results conclude that the oximes solubility in water does not display correlation with AChE inhibition. The good point on the reported Log *P* values of the neutral oximes, in comparison with 2-PAM, is an indication of their potential better capacity for BBB permeation than the cationic oximes.

On the other hand, the fact that the neutral oximes with the most effective AChE inhibition capacities are 2-thiophenyl oxime and benzaldoxime, which possess the lowest molecular mass and the lowest molecular area, indicates that these parameters have some impact on AChE inhibition.

3.2. Reactivation of EeAChE inhibited with paraoxon

For monitoring the capacity of neutral oximes as EeAChE reactivators it was necessary to inhibit the enzyme without using an excess of paraoxon. Accordingly, there was used the appropriate volume (0.5 μL) of the paraoxon solution (3.33 mM) to inhibit 4.0 μL of the EeAChE solution (0.33 nM).

The inhibition was performed during 60 min. To confirm the complete EeAChE inhibition, 5.00 μL of the ACh solution were added to the inhibited enzyme and the solution was monitored by ^{1}H NMR for 85 min, without any variation of ACh concentration. This solution was then mixed with 5.00 μL of pralidoxime solution and monitored by NMR, showing an effective hydrolysis of ACh. To confirm that pralidoxime did not hydrolyze ACh, a mixture of ACh and pralidoxime without EeAChE was also monitored, showing that the concentration of ACh did not decrease during 85 min in the presence of pralidoxime. This procedure was repeated with all neutral oximes, showing that none of them

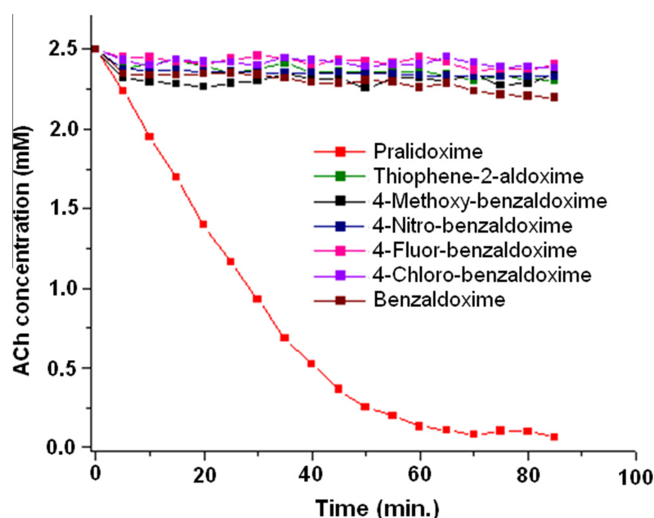


Figure 6. Graphic of the variation of ACh concentration in the presence of EeAChE inhibited with paraoxon with the presence of the oximes. Pralidoxime (red), thiophene-2-aldoxime (green), 4-methoxy-benzaldoxime (black), 4-nitro-benzaldoxime (blue), 4-flour-benzaldoxime (pink), 4-chloro-benzaldoxime (light blue) and benzaldoxime (brown).

Table 2
Percentage of reactivation of EeAChE inhibited with paraoxon by the tested oximes in 85 min

Oxime	EaAChE–paraoxon reactivation (%)	Oxime	EaAChE–paraoxon reactivation (%)
2	97.40	**6**	4.04
3	8.02	**7**	4.42
4	7.60	**8**	6.61
5	10.00		

was an effective EeAChE–Paraoxon reactivator. The results are shown in Figure 6.

The results are shown in Table 2, where it is evident that the studied neutral oximes are poor *Ee*AChE–paraoxon reactivators, when compared to pralidoxime. In fact, all neutral oximes displayed a very similar reactivation capacity, with benzaldoxime being the most effective one, followed by thiophene-2-aldoxime.

From the molecular modeling data of Table1, it is clear that pralidoxime is the most acidic oxime, displaying a much lower Δ*E* value than all neutral oximes, which are very weak reactivators. This analysis indicates that oximes acidity is an important factor for their potential effect as AChE reactivators.

In order to compare the reactivation capacity of the two best neutral oximes, benzaldoxime (**4**) and thiophene-2-aldoxime (**3**), the experiment was repeated with these oximes and monitored by NMR during 6 h. These results are shown in Figure 7.

Numerically, the results of Figure 7 indicate that the percentage of EeAChE reactivated by benzaldoxime during 375 min corresponds to 58.6%, while for 2-thiophenyl oxime it is 15.5%. These results confirm that neutral oximes have some potential to act as reactivators of AChE inhibited by neurotoxic organophosphorus compounds, a process that certainly needs to be very much improved in order to obtain new neutral oximes as effective agents for defense against chemical weapons.

These NMR results are opposite to the data obtained with neutral oximes using the Ellman test in a previously published article.[41] The data reported with the Ellman test indicated thiophene-2-aldoxime (**3**) as a better reactivator of AChE (93%)

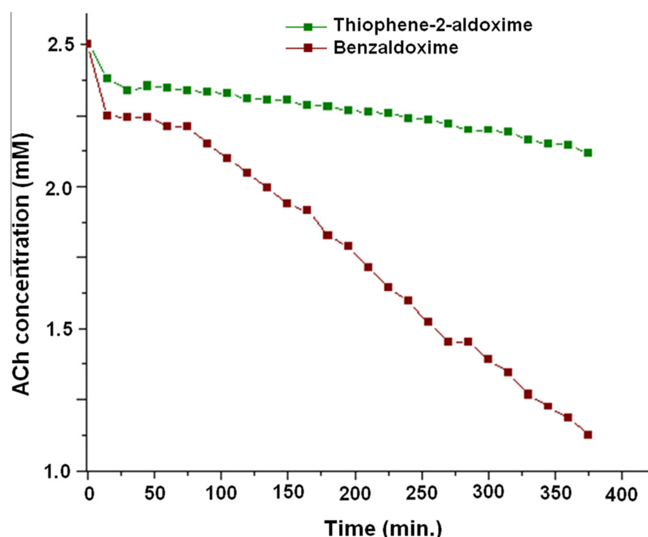


Figure 7. Reactivation of EeAChE–paraoxon with thiophene-2-aldoxime (green) and benzaldoxime (brown) during 6 h.

than 2-PAM (83%), but the NMR method showed that 2-PAM is much more effective (97.4%) than compound **3** (8.0%).

4. Conclusions

The obtained results of AChE inhibition indicates that neutral oximes (which possess a better capacity to permeate the hemato-encephalic barrier than cationic oximes) with small aromatic rings (low molecular mass and molecular surface) are effective AChE inhibitors. These compounds have potential for the treatment of Alzheimer disease by improving the concentration of the neurotransmissor acetylcholine in the patients.

Our results show that 2-PAM is only the second best EeAChE inhibitor, indicating that its cationic form, despite being effective for its interaction with the active site, is not the most important characteristic for inhibition of the enzyme. These results suggest that the presence of neutral, small and unsubstituted aromatic rings is an important factor for AChE inhibition.

The experiments of reactivation of AChE inhibited with paraoxon showed that pralidoxime is more effective than all neutral oximes tested, probably due to its greater acidity, that allows an easier formation of its anionic form inside the active site of the phosphylated AChE. The anionic form of oximes is necessary to perform the effective nucleophilic attack on the phosphate group linked to Ser203 in the enzyme active site.

Despite the low AChE reactivation capacity shown by neutral oximes, our experiments confirmed that some of these compounds could reactivate more than 50% of the inhibited enzyme in 6 h. These results indicate that structural variation on neutral oximes, especially looking for more acidic compounds, could lead to the development of new AChE reactivators with better permeability of the hematoencephalic barrier, a characteristic that is important to improve the efficiency of antidotes against intoxication with organophosphorus compounds.

The obtained information of this work can be used for design of new AChE inhibitors and reactivators, however using other acetylcholinesterases, pesticides or nerve agents, the tested results could be different. For example, if *Ee*AChE is inhibited by another organophosphorus compound, it is possible that pralidoxime could display a lower effectiveness, while one of the inactive neutral oximes could increase its reactivation capacity. In the future it would be especially important to discover general AChE inhibitors and reactivators.

The Ellman test certainly works quite well for many experiments, leading to real results. However, because this test uses acetyl thiocholine instead of ACh as substrate in the presence of 5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid (DTNB) and is based on UV–visible spectroscopy, it could lead to wrong results in some cases. In this test, acetyl thiocholine is hydrolyzed to thiocholine, which reacts with DTNB to produce 5-mercapto-2-nitrobenzoic acid, a yellow product detected by UV–visible spectroscopy. So, if some enzyme inhibitors or reactivators display a λ_{max} similar to 5-mercapto-2-nitrobenzoic acid, the Ellman test is flawed. Also, despite acetyl thiocholine being a substrate of AChE, its difference to ACh could lead to different results. For these reasons, the use of the NMR method is very appropriate.

In this work it was shown that NMR is a very effective and simple method to study and monitor inhibition and reactivation processes related to acetylcholinesterase. We believe NMR is better than Ellman test. This conclusion is based on the fact that, despite compound **3** is the best inhibitor of EeAChE, its action for reactivation of the enzyme inhibited by paraoxon is certainly inferior to 2-PAM as shown by NMR, which is a result completely different from the one obtained by the Ellman test.

There exists several methods for enzyme kinetics and inhibition studies, mainly by spectrophotometry and radiometric assays, for example, UV–Visible spectroscopy, mass spectrometry, fluorescence, temperature and pressure jump methods.[71] Methods that led to certain problems could be improved by some modifications, for example, the Ellman test with changes on the values of λ_{max} sometimes leads to better results.[72] However, NMR is especially important for these studies because is a very effective method to determine and distinguish the structure, conformation and concentration of enzyme substrates, products and inhibitors. Even more, besides the enzyme kinetic and inhibition studies, NMR provides much other information, like molecular diffusion coefficients, relaxation times and inter-atomic distances, which are important to study the interaction of these compounds with their bio-molecular targets.[57,73]

Acknowledgments

Our work was financed by CAPES/Pro-defense and Instituto Nacional de Ciência e Tecnologia de Biologia Estrutural e Bioimagem (INBEB). We also thank the opportunity given by the National Bioscience Laboratory (LNBio) to use their Varian Inova 500 MHz spectrometer in this work.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.05.063.

References and notes

1. Elhalwagy, M. E. A.; Zaki, N. I. *Environ. Toxicol. Pharmacol.* **2009**, *28*, 219.
2. Eddleston, M.; Street, J. M.; Self, I.; Thompson, A.; King, K.; Williams, N.; Naredo, G.; Dissanayake, K.; Yu, L. M.; Worek, F.; John, H.; Smith, S.; Thierman, H.; Harris, J. B.; Clutton, R. E. *Toxicology* **2012**, *294*, 94.
3. Brown, M. A.; Brix, K. *J. Appl. Toxicol.* **1998**, *18*, 393.
4. Eskenazi, B.; Bradman, A.; Castorina, R. *Environ. Health Perspect.* **1999**, *107*, 409.
5. Gershon, S.; Shaw, F. H. *Lancet* **1961**, *1*, 1371.
6. Sogorb, M. A.; Vilanova, E.; Carrera, V. *Toxicol. Lett.* **2004**, *151*, 219.
7. Abdollahi, M.; Karami-Mohajeri, S. *Toxicol. Appl. Pharmacol.* **2012**, *245*, 309.
8. Marrs, T. C. *Pharmacol. Ther.* **1933**, *58*, 51.
9. Delfino, R. T.; Ribeiro, T. S. E.; Figueroa-Villar, J. D. *J. Braz. Chem. Soc.* **2009**, *20*, 407.
10. Marrs, T. C. In *Toxicology of Organophosphate Nerve Agents*; Marrs, T. C., Maynard, R. L., Sidell, F. R., Eds.; John Wiley & Sons: Chichester, 2007; Vol. 8, pp 191–221.
11. Sidell, F. R. *J. Appl. Toxicol.* **1994**, *14*, 111.
12. Worek, F.; Koller, M.; Thiermann, H.; Szinic, Z. L. *Toxicology* **2005**, *214*, 182.
13. Mallender, W. D.; Szegletes, T.; Rosenberry, T. L. *J. Biol. Chem.* **1999**, *274*, 8491.

14. Quinn, D. M. *Chem. Rev.* **1987**, *87*, 955.
15. Pundir, C. S.; Chauhan, N. *Anal. Biochem.* **2012**, *429*, 19.
16. Taylor, P. *J. Biol. Chem.* **1991**, *266*, 4025.
17. Dvir, H.; Silman, I.; Harel, M.; Rosenberry, T. L.; Sussman, J. L. *Chem. Biol. Interact.* **2010**, *187*, 10.
18. Sussman, J. L.; Harel, M.; Frolow, F.; Oefner, C.; Goldman, A.; Toker, L.; Silman, I. *Science* **1991**, *253*, 872.
19. Axelsen, P. H.; Harel, M.; Silman, I.; Sussman, J. L. *Protein Sci.* **1994**, *3*, 188.
20. Sidell, F. R. Nerve agents In *Medical Aspects of Chemical and Biological Warfare. Textbook of military medicine*; Sidell, F. R., Takafuji, E. T., Franz, D. R., Eds.; Office of the Surgeon General, US Army: Washington, 1997; Vol. 3, pp 129–179.
21. Black, R. M.; Harrison, J. M. In *The Chemistry of Organophosphorus Compounds*; Hartley, F. R., Ed.; John Wiley & Sons: Chichester, 1996; Vol. 4, pp 781–840.
22. Eyer, P. A.; Worek, F. In *Toxicology of Organophosphate Nerve Agents*; Marrs, T. C., Maynard, R. L., Sidell, F. R., Eds.; John Wiley & Sons: Chichester, 2007; Vol. 2, pp 30–329.
23. Jokanovič, M. *Curr. Top. Med. Chem.* **2012**, *12*, 1775.
24. Soreq, H.; Seidman, S. *Nat. Rev. Neurosci.* **2001**, *2*, 294.
25. Silman, I.; Sussman, J. L. *Curr. Opin. Pharmacol.* **2005**, *5*, 293.
26. Ashani, Y.; Radic, Z.; Tsigelny, I.; Vellom, D. C.; Pickering, N.; Quinn, D. M.; Doctor, B. P.; Taylor, P. *J. Biol. Chem.* **1995**, *270*, 6370.
27. Eddleston, M.; Szinicz, L.; Eyer, P.; Buckley, N. Q. *J. Med.* **2002**, *95*, 275.
28. Eyer, P. A.; Worek, F. In *Chemical warfare agents – toxicology and treatment*; Marrs, T. C., Maynard, R. L., Sidell, F. R., Eds., 2nd ed.; John Wiley & Sons: West Sussex, 2007; pp 305–329. Vol. 15.
29. Grosfeld, H.; Barak, D.; Ordentlich, A.; Velan, B.; Shafferman, A. *Mol. Pharmacol.* **1996**, *50*, 639.
30. Kuca, K.; Jun, D.; Musilek, K. *Mini-Rev. Med. Chem.* **2006**, *6*, 269.
31. Schwarz, I. M.; Loewenstein, Y.; Glick, D.; Liao, J.; Norgaardpederson, B.; Soreq, H. *Mol. Brain Res.* **1972**, *31*, 101.
32. Gonçalves, A. S.; França, T. C. C.; Silva, A. W. S.; Figueroa-Villar, J. D. *J. Braz. Chem. Soc.* **2006**, *17*, 968.
33. Kim, T. H.; Kuca, K.; Jun, D.; Jung, Y. S. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2914.
34. Kuca, K.; Jun, D.; Cabal, J.; Hrabinova, M.; Bartosova, L.; Opletalova, V. *Basic Clin. Pharmacol.* **2006**, *98*, 389.
35. Kuca, K.; Jun, D.; Kim, T.; Cabal, J.; Jung, Y. B. *Korean Chem. Soc.* **2006**, *27*, 395.
36. Worek, F.; Aurbek, N.; Koller, M.; Becker, C.; Eyer, P.; Thiermann, H. *Biochem. Pharmacol.* **1807**, *2007*, 73.
37. Worek, F.; Thiermann, H.; Szinicz, L. *Arch. Toxicol.* **2004**, *78*, 212.
38. Kassa, J.; Karasova, J. Z.; Krejciova, M. *J. Appl. Biomed.* **2013**, *11*, 7.
39. Braundhuber, F.; Zengerle, M.; Porwol, L.; Tenberken, O.; Thiermann, H.; Worek, F.; Kubik, S.; Reiter, G. *Toxicology* **2012**, *302*, 163.
40. Mercey, G.; Renou, J.; Verdelet, T.; Kliachyna, M.; Baati, R.; Gillon, E.; Arboléas, M.; Loiodice, M.; Nachon, F.; Jean, L.; Renard, P. Y. *J. Med. Chem.* **2012**, *55*, 10791.
41. Ribeiro, T. S.; Prates, A.; Alves, S. R.; Oliveira-Silva, J. J.; Riehl, C. A. S.; Figueroa-Villar, J. D. *J. Braz. Chem. Soc.* **2012**, *23*, 1216.
42. Mercey, G.; Renou, J.; Verdelet, T.; Renou, J.; Kliachyna, M.; Baati, R.; Nachon, F.; Jean, L.; Renard, P. Y. *Acc. Chem. Res.* **2012**, *45*, 746.
43. Berend, S.; Vrdoljak, A. L.; Kuca, K. *Chem. Biol. Interact.* **2008**, *175*, 413–416.
44. Jeong, H. C.; Kang, N. S.; Park, N. J.; Yum, E. K.; Jung, Y. S. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1214.
45. Kuca, K.; Cabal, J.; Musilek, K.; Jun, D.; Bajgar, J. *J. Appl. Toxicol.* **2005**, *25*, 491.
46. Acharya, J.; Rana, H.; Kaushik, M. P. *Eur. J. Med. Chem.* **2011**, *46*, 3926.
47. Rao, C. S.; Venkateswarlu, V.; Achaiah, G. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2134.
48. Bharate, S. B.; Guo, L.; Reeves, T. E.; Cerasoli, D. M.; Thompson, C. M. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5101.
49. Dadparvar, M.; Wagner, S.; Wien, S.; Kufleitner, J.; Worek, F.; von Briesen, H.; Kreuter, J. *Toxicol. Lett.* **2011**, *206*, 60.
50. Tayeb, H.; Yang, H. D.; Price, B. H.; Tarazi, F. I. *Pharmacol. Ther.* **2012**, *134*, 8.
51. Small, G.; Bullock, R. *Alzheimers Demen.* **2011**, *7*, 177.
52. Gonçalves, A. S.; França, T. C. C.; Wilter, A.; Figueroa-Villar, J. D. *J. Braz. Chem. Soc.* **2006**, *17*, 968.
53. Meyer, B.; Peters, T. *Angew. Chem., Int. Ed.* **2003**, *42*, 864.
54. Skinner, A. L.; Laurence, J. S. *J. Pharm. Sci. (US)* **2008**, *97*, 4670.
55. Moore, J. M. *Biopolymers* **1999**, *51*, 221.
56. Fielding, L. *Curr. Top. Med. Chem.* **2003**, *3*, 39.
57. Figueroa-Villar, J. D.; Tinoco, L. W. *Curr. Top. Med. Chem.* **2009**, *9*, 811.
58. Exnowitz, Franziska.; Meyer, Bernd.; Hackl, Thomas. *Biochim. Biophys. Acta* **2012**, *1824*, 443.
59. Bisswanger, H. *Enzyme Kinetics: Principles and methods*; Wiley-VCH: Weinheim, 2002. Chapter 3.
60. Oliveira-Silva, J. J.; Alves, S. R.; Inacio, A. F.; Meyer, A.; Sarcinelli, P. N.; Mattos, R. C.; Ferreira, M. F. A.; Cunha, J. C.; Moreira, J. C. *Hum. Exp. Toxicol.* **2000**, *19*, 173.
61. Wiesner, J.; Kriz, Z.; Kuka, K.; Jun, D.; Koca, J. *J. Enzyme Inhib. Med. Chem.* **2007**, *22*, 417.
62. Ziemianin, A.; Ronco, C.; Dolé, R.; Jean, L.; Renard, P.-Y.; Lange, C. M. *J. Pharm. Biomed.* **2012**, *70*, 1.
63. Bharate, S. B.; Guo, L.; Reevens, T. E.; Cerasoli, D. M.; Thompson, C. M. *Bioorg. Med. Chem.* **2010**, *18*, 782.
64. Benzi, G.; Moretti, A. *Eur. J. Pharmacol.* **1998**, *346*, 1.
65. Liston, D. R.; Nielsen, J. A.; Villalobos, A.; Chapin, D.; Jones, S. B.; Hubbard, S. T.; Shalaby, I. A.; Ramirez, A.; Nason, D.; White, W. F. *Eur. J. Pharmacol.* **2004**, *486*, 9.
66. Worek, F.; Reiter, G.; Eyer, P.; Szinicz, L. *Arch. Toxicol.* **2002**, *76*, 523.
67. Worek, F.; Von der Wellen, J.; Musilek, K.; Kuka, K.; Thiermann, H. *Arch. Toxicol.* **2012**, *86*, 1379.
68. Wilson, I. B.; Harrison, M. A. *J. Biol. Chem.* **1961**, *236*, 2292.
69. Fischer, H.; Gottschlich, R.; Seelig, A. *J. Membr. Biol.* **1998**, *165*, 201.
70. Petroianu, G. A.; Lorke, D. E. *Mini-Rev. Med. Chem.* **2008**, *8*, 1328.
71. Bisswanger, H. *Enzyme Kinetics. Principles and Methods*; WILEY-VCH: Weinheim, 2002. Chapter 3.
72. Worek, F.; Mast, U.; Kiderlen, D.; Diepold, C.; Eyer, P. *Clin. Chim. Acta* **1999**, *288*, 73.
73. Coles, M.; Heller, M.; Kessler, H. *Drug Discovery Today* **2002**, *8*, 803.