THE REACTIVATORS OF ISOPROPYLMETHYL-PHOSPHONYLATED ACETYLCHOLINESTERASE DERIVED FROM 2-HYDROXYIMINOMETHYLPYRIDINE

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Summary

Four new 4-substituted pyridinium-2-aldoxime methoiodides were prepared and their physico-chemical properties were estimated, including their pKa values. Compounds were tested as reactivators of acetylcholinesterase inhibited by isopropyl-methylphosphonofluoridate in vitro. Their acute toxicities were estimated in mice after i.m. administration. Results show that these compounds are less toxic than 2-pyridinium-aldoxime methiodide (2-PAM) alone, but only one compound was better reactivator than 2-PAM.

KEY WORDS: Acetylcholinesterase; Organophosphates; Inhibition; Pyridinium-Aldoximes; Reactivators.

Introduction

The class of chemical warfare agents known as nerve gases are organophosphorus compounds that are extremely potent inhibitors of acetylcholinesterase (1), an enzyme that terminates the action of the neurotransmitter acetylcholine by catalysing its hydrolysis (2). Irreversible inhibition by organophosphorus compounds results in the accumulation of endogenous acetylcholine in synaptic cleft and paralysis of nerve impulse transmission in the central and peripheral nervous system. The toxic manifestations of nerve agent exposure are therefore those of excess acetylcholine at muscarinic and nicotinic acetylcholine receptors in synapses and nerve-muscle endings (3) Military countermeasures to nerve agent intoxication include a muscarinic acetylcholine receptor antagonist, to block the overstimulation of the receptor by endogenous acetylcholine, and an oxime, to reactivate the inhibited acetylcholinesterase. Atropine has been universally adopted as acetylcholine receptor antagonist (4). Oximes are known to be successfully used as therapeutics against intoxication with many organophosphates. These compounds are able to reactivate the phosphorylated acetylcholinesterase by nucleophilic dephosphorylation (5). 2-Hydroxy-

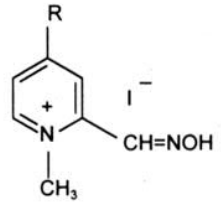
iminomethylpyridinium methiodide (2-PAM) was the first useful pyridinium aldoxime *(6, 7)*.2-PAM is used by several countries, including the USA *(8)* and the UK *(9)*. Some other countries prefer the different types of oximes, for instance bis-(4-hydroxyiminomethylpyridinium)-propane dibromide (TMB-4, trimedoxime) *(10)*, bis-(4-hydroximino-methylpyridinium) -2-oxa-propane dichloride (LüH-6, obidoxime, Toxogonin) *(11, 12)* or (2-hydroxyimino-methylpyridinium-1-methyl-4-carbamoylpyridinium)-1-methylether dichloride (HI-6) *(13)*. Many other oximes are synthesized and tested as new potential acetylcholinesterase reactivators *(14)*.

The present work concerned the study of the reactivating effect of four oximes, derivatives of 2-PAM, on rat brain acetylcholinesterase inhibited by isopropylmethyl-phosphonofluoridate.

Experimental

Enzyme preparation. As a source of acetyl-cholinesterase a homogenate of whole rat brains (the Wistar strain without sex preference of individuals weighing 200-220 g) were used. The animals were killed in narcosis by cutting the carotids and the brains were excised, rinsed in saline, homogenized in an Ultra-Turrax (Germany) in distilled water to 10 % (w/v) homogenate and stored at -18 °C in refrigerator. Immediately before the use of samples of enzyme were thawed.

Reagents: The following compounds were tested as reactivators of isopropylmethylphosphonylated acetylcholinesterase: 2-PAM (I), 1-methyl-2-hydroxy-iminomethyl-4-carboxylpyridinium iodide (II), 1-methyl--2-hydroxyiminomethyl-4-methoxycarbonyl-pyridinium iodide (III), 1-methyl-2-hydroxyiminomethyl-4-carbamoyl-pyridinium iodide (IV), and 1-methyl-2,4-bis-hydroxy-iminomethylpyridinium iodide (V). Compound I, m.p. 219 °C (decomp.), ref, *(15)* gives a m.p. of 218-219 °C (decomp.) was made by Léčiva, Prague, and was used as a reference reactivator. Compounds II, III, IV, and V were synthesized in Military Medical Academy, Hradec Králové, and were in excess of 98 % pure. Tested oximes were dissolved in a distilled water to 0.01 mol.l^{-1} solutions and were processed the same day. Isopropyl-methylphosphonofluoridate was obtained from the Military Maintenance Basis, Zemianské Kostolany, the Slovak Republic. All other chemicals used were of analytical grade.



I R = H
II R = COOH
III R = $COOCH_3$
IV R = $CONH_2$
V R = CH=NOH

The dissociation constants of the oxime group (pK_A) were determined potentiometrically by titration their 0.001 mol.l^{-1} solutions by 0.01 mol.l^{-1} NaOH. Automatic titrator RTS 822 (Radiometer, Copenhagen, Denmark) was used for all measurements. The pK_A values were estimated according to ref. *(16)*.

The activity of acetylcholinesterase was measured by an automatic titrator RTS 822 working in a pH-stat regime. The substrate used was 0.005 mol.l^{-1} acetylcholine iodide and acetic acid released by enzymatic hydrolysis was titrated by 0.01 mol.l^{-1} NaOH. All measurements were made at 25 °C and pH 8.0 under a nitrogen atmosphere. The activity of the enzyme was expressed as initial rate of substrate hydrolysis.

The preparation of isopropylmethylphosphonylated acetylcholinesterase was performed *in situ* by 30 min preincubation of enzyme at pH 8.0 with 5x10^{-8} mol.l^{-1} isopropylmethylphosphonofluoridate. The reactivation of phosphonylated acetylcholinesterase was carried out over the range of reactivators concentration from 10^{-6} to 10^{-3} mol.l^{-1} at pH 8.0. Each experiment was carried out with 5 and at the most 12 different reactivator concentrations. The procedure used for the determination of kinetic constants of reactivation was essentially the same as that described by Main and Iverson *(17)*.

The acute toxicity of all reactivators was made in male mice (Velaz, Prague). Compounds were administered i.m. and LD_{50} values were estimated according to Schaper et al. *(18)*.

Results and Discussion

All compounds prepared were found to be stable as substances and relatively stable in neutral aqueous solutions, except of compound III, which is hydrolyzed to compound II. The hydrolysis of ester III was alkaline catalyzed first-order reaction. The half-life time at pH 8.0 was 56 min, at pH 9.0 14 min, and at pH 10.0 only 1 min. By this procedure, i.e. by alkaline hydrolysis, compound II was prepared from compound III. Physicochemical data obtained for the compounds described are listed in Table I.

Table I

Physicochemical parameters of compoundds used

Compound	M.W.	M.p., °C	pK_{A1}	pK_{A2}
I	264.16	274	7.90	
II	295.22	-	8.11	3.81 [a]
III	310.16	-	7.54	
IV	307.07	208-213	7.66	
V	307.32	169-171	7.66	8.77 [b]

[a] the dissociation constant of carboxyl group
[b] the dissociation constant of the second carbaldoxime group

The reaction of phosphonylated acetylcholinesterase (EI) with the reactivator (R), i.e. the oxime-induced reactivation of enzyme inhibited by organophosphate, may be represented by the scheme *(19)*.

$$EI + R \underset{}{\overset{K_R}{\rightleftharpoons}} EIR \xrightarrow{k_R} E + P$$

where EIR is the intermediary complex of phosphonylated enzyme with reactivator, P the reaction product, and E the regenerated enzyme. K_R is the dissociation constant of complex EIR and k_R the decomposition rate constant of this complex. The ratio of the two constants k_R/K_R is the bimolecular rate constant (k_r) which defines the overall efficiency of the reactivator. The kinetic constants of reactivation of *in vitro* tested compounds on the model of isopropylmethylphosphonylated rat brain acetylcholinesterase are presented in Table II.

Table II

Kinetic constants of reactivation of isopropylmethyl-phosphonylated acetylcholinesterase and acute toxicities of compound used

Compound	K_R $\mu mol.l^{-1}$	k_R min^{-1}	k_r min^{-1}	LD_{50} (mouse) i.m. $(mg.kg^{-1})$
I	354	0.143	403.1	419
II	4990	0.163	32.7	-
III	72	0.039	541.7	1178
IV	758	0.179	236.1	1289
V	511	0.102	199.6	132

If we use bimolecular rate constant of reactivation to determine the reactivating potency of these oximes, only compound III is better reactivator than 2-PAM. Unfortunately, ester III is only little stable in aqueous solutions and hydrolysed to the acid II, which is the worst reactivator of whole series. These great differences in reactivation effect of ester III and its hydrolytic product II are interesting. The presence of carboxylic group in the molecule of compound II, characterised by low pK_a value, result in a strong decrease of affinity to phosphonylated enzyme. Meanwhile 2-PAM and other reactivators of this type are good probes for the anionic subsite of acetylcholinesterase owing to their positive charge on quaternary nitrogen (20), negative charge of carboxylic group in compound II has opposite effect. Therefore K_R value of acid II is 70 times smaller than ester III. This is the main reason of different efficacy of both these compounds.

Nevertheless, the preliminary experiments with compound III and IV in the role of antidotes against organophosphate poisoning showed hopeful results when against mevinphos intoxication in mice were used (21). Observed good antidote effect against organophosphate poisoning *in vivo* may be only partially explained by low toxicity of compound III. Further experiments are needed.

References

1. KOELLE, GB. (Ed.): Cholinesterases and Anti-cholinesterase Agents. Vol. XV. Handbuch der experimentellen Pharmakologie. Berlin, Göttingen and Heidelberg. Springer-Verlag, 1963.
2. ROSENBERRY, TL.: Biophys. J., 1979, vol. 26, p. 263-290.
3. HOLMSTEDT, B.: Ann. N. Y. Acad. Sci. USA, 1959, vol. 144, p. 433-453.
4. DUNN, MA. - Sidell, FR.: J. Am. Med. Assoc., 1989, vol. 262, p. 649-652.
5. WILSON, IB. - GINSBURG, S. - MEISLICH, EK.: J. Am. Chem. Soc., 1955, vol. 77, p. 4286-4291.
6. CHILDS, AF., et al.: Brit. J. Pharmacol., 1955, vol. 10, p. 462.
7. WILSON, IB. - GINSBURG, S.: Biochim. Biophys. Acta, 1955, vol. 18, p. 168.
8. DUNN, MA. - SIDELL, FR.: J. Am. Med. Assoc., 1989, vol. 262, p. 649-652.
9. LEADBEATER, L.: Chem. Br., 1988, vol. 24, p. 683-688.
10. HOBBIGER, F. - SADLER, PW.: Brit. J. Pharmacol. Chemother., 1959, vol. 14, p. 192-201.
11. LÜTTRINGHAUS, A. - HAGEDORN, I.: Arzneimittel-Forsch., 1964, vol. 14, p. 1-5.
12. ERDMANN, WD. - ENGELHARD, H.: Arzneimittel-Forsch., 1964, vol. 14, p. 5-11.
13. CLEMENT, JG.: Biochem. Pharmacol., 1982, vol. 31, p. 1283-1287.
14. DAWSON, RM.: J. Appl. Toxicol., 1994, vol. 14, p. 317-331.
15. GREEN, AL. - SEVILLE, B.: J. Chem. Soc., 1956, p. 3887-3890.
16. ALBERT, A. - SERJEANT, EP.: Ionisation Constants of Acids and Bases. A Laboratory Manual. London. Methuen Co., Ltd., 1962.
17. MAIN, AR. - IVERSON, F.: Biochem. J., 1966, vol. 100, p. 525-530.
18. SCHAPER, MM. - THOMPSON, RD. - WEIL, CS.: Arch.Toxicol., 1994, vol. 68, p. 332-338.
19. DAVIES, DR. - GREEN, AL.: Biochem. J., 1956, vol. 63, p. 529-536.
20. FU FH, et al.: Acta Pharmacol. Sinica, 1997, vol. 18, p. 444-446.
21. KASSA, J. - BIELAVSKÝ, J.: Pharmacol. Toxicol., 1997, vol. 81, p. 144-146.

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