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IN VITRO REACTIVATION POTENCY OF NEWLY DEVELOPED OXIMES K027 AND K048

Kamil KUCA[1] Jiri CABAL[1] Daniel JUN[1]
Martina HRABINOVA[1] Jiri KASSA[1]

ABSTRACT

In 2003, we have developed two promising acetylcholinesterase (AChE; EC 3.1.1.7) reactivators – K027 and K048. Both of them were designed as derivatives of HI-6 and trimedoxime (TMB-4). They consist of two quaternary pyridinium rings, one oxime group in the position four at the first pyridinium ring, one carbamoyl group at the position four at the second pyridinium ring and they differ just in the length of the connection chain between both pyridinium rings (K027 – three-methylene bridge, K048 – four-methylene bridge). In our study, we would like to show their potency to reactivate *in vitro* AChE inhibited by nerve agent tabun (GA). We have used rat and human brain cholinesterases as the appropriate source of the enzyme. As resulted from this work, there are differences in the course of the reactivation process between rat and human species. Owing to this fact, reactivation test with human species should be included in the AChE reactivator developmental process.

Key Words: Acetylcholinesterase reactivator, acetylcholinesterase reactivator I (K027), acetylcholinesterase reactivator II (K048), tabun

YENİ GELİŞTİRİLEN K027 VE K048 OKSİMLERİNİN *İN VİTRO* REAKTİVASYON POTENSİ

ÖZET

2003 yılında, K027 ve K048 adlı iki yeni ümit verici asetilkolinesteraz reaktivatörü (AChE; EC 3.1.1.7) geliştirilmiştir. Her ikisi de HI-6 ve trimedoxime (TMB-4) türevleri olarak tasarlanmıştır. Bunlar birinci piridin halkasında dördüncü pozisyonda bir oksim grubu, ikinci piridin halkasında dördüncü pozisyonda bir karbomil grubu olmak üzere iki kuarterner piridin halkası içermekte ve sadece iki piridin halkası arasındaki bağlantı halkasının uzunluğu bakımından farklılık göstermektedirler (K027 – üç metilen köprüsü, K048 – dört metilen köprüsü). Bu çalışmada, söz konusu reaktivatörlerin sinir gazı tabun (GA) ile inhibe edilen AchE'ı *in vitro* olarak tekrar aktifleştirme güçleri gösterilmek istenmiştir. Uygun enzim kaynağı olarak sıçan ve insan beyni kolinesterazları kullanılmıştır. Bu bulguya dayanarak insanlarla ilgili reaktivasyon testlerinin AChE reaktivite gelişim yöntemlerini de içermesi gerektiği düşünülmektedir.

Anahtar Kelimeler: Asetilkolinesteraz reaktivatör, asetilkolinesteraz reaktivatör I (K027), asetilkolinesteraz reaktivatör II (K048), tabun

INTRODUCTION

Organophosphorus compounds (OPCs) are utilized in industry as softening agents, hydraulic liquids, lubricant additives, plasticizers, antioxidants and for antiflammable modifications. They are also used in veterinary and human medicine as drugs or chemicals for the study of nervous function and, at last but not least, these compounds are, unfortunately, usable (and used) for military purposes as chemical warfare agents (nerve agents) and as poisons exploited by terrorists (Bajgar 2004; Kuca et al. 2006).

The toxicity of OPCs, especially nerve agents, is mainly due to their ability to inhibit enzyme acetylcholinesterase [AChE; EC 3.1.1.7] by phosphorylation or phosphonylation of serine hydroxy group in its active site (Marrs 1993; Patocka et al. 2004).

[1]Department of Toxicology, Faculty of Military Health Sciences, University of Defense, Czech Republic
Yazışma Adresi: Dr.Kamil KUCA, Department of Toxicology, Faculty of Military Health Sciences, University of Defense, Czech Republic
Tel: +420 973 251 523 Fax: +420 495 518 094 e-posta : kucakam@pmfhk.cz

Owing to the fact, that duration of spontaneous reactivation is comparable with natural lifetime of AChE in the body, the inhibition is designed as irreversible (Patocka et al. 2005). After the inhibition process, enzyme is not able to serve its mission to cleave neuromediator acetylcholine (ACh). The resulting accumulation of ACh at synaptic junctions over-stimulates cholinergic pathways and subsequently desensitizes cholinergic receptor sites (Kassa 2006).

Standard treatment of intoxications consists of combination of anticholinergic drugs to counteract the effect of accumulated ACh (e.g. atropine) and AChE reactivators (called oximes) to reactivate OPCs-inhibited AChE. Monoquaternary pralidoxime (2-PAM; 1-methyl-2-hydroxyiminomethylpyridinium chloride) and bisquaternary obidoxime (Toxogonin®; 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxa-propane dichloride) and oxime HI-6 (1-(4-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)–2-oxa-propane dichloride) are typical members of the family of AChE reactivators (Figure 2) (Kassa 2002).

In the present time, attention of scientists working in this research area is focused mainly to the treatment of tabun-poisonings, because of very bad potency of current treatment means in the case of tabun intoxications. Its deleterious effects are extraordinarily difficult to counteract due to the existence of a lone electron pair located on the amidic group that makes the nucleophilic attack almost impossible (Eto 1976; Koplovitz et al. 1995; Cabal & Bajgar 1999).

In 2003, two new reactivators of tabun-inhibited AChE, oximes K027 and K048, were developed in our department (Figure 1) (Kuca et al. 2003a,b). In this work, we would like to present their potency to reactivate *in vitro* tabun-inhibited AChE on two different species (rat – experimental animal, human – reality). Their reactivation potency is compared with currently used AChE reactivators – pralidoxime, obidoxime and HI-6.

MATERIAL AND METHODS

Chemical

Both new oximes (K027 and K048) were prepared earlier (Kuca et al., 2003a,b). HI-6 (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)–2-oxa-propane dichloride), pralidoxime, respectively obidoxime were purchased from Léãiva (Czech Rep) respectively Merck (Germany). Purities of the tested AChE reactivators were detected using 1H-NMR spectra. Nerve agent (tabun; GA) was obtained from the Military Facility Brno in 94% purity. All other chemicals used were of reagent grade (Sigma-Aldrich, Czech Republic).

Enzyme

Rat brain AChE was chosen as the source of the enzyme. Its preparation was as follows. Lightly ether-narcotized animals were killed by bleeding from a carotid artery and the brains were removed, washed with saline and homogenized in an Ultra-Turrax homogenizer in a distilled water to make a 10 % homogenate.

In vitro measurement

Reactivation efficacy of the oximes was tested *in vitro* on the model of AChE inhibited by tabun using standard reactivation test with electrometric instrumentation (Kuca and Kassa, 2003). The AChE homogenate (0.5 ml) was mixed with 0.5 ml of tabun in dry isopropanol and incubated for 30 min (25°C). Then 2.5 ml of 3M NaCl was added and supplied by distilled water to a volume of 23 ml. After that, 2 ml of 0.02 M acetylcholine bromide was added and enzyme activity was assayed titrimetrically at pH 8.0 and 25°C on the Autotitrator RTS 822 (Radiometer, Denmark).

The activities of intact (a_o) and tabun-inhi bited (a_i) AChE were determined. When tabun-inhibited AChE was incubated 10 min with solution of reactivator, the activity of reactivated AChE (a_r) was obtained. The activity values a_o, ai and ar were calculated from the slopes of the initial part of titration curves. Each value represents arithmetic mean from two independent measurements.

RESULTS AND DISCUSSION

All obtained results are shown in Table 1 and in Figures 2-3. As it can be seen just three

oximes – obidoxime, K027 and K048 were able to reactivate tabun-inhibited AChE of both rat and human species.

Rat brain reactivation : The highest affinity towards inhibited rats AChE and also highest velocity of the whole reactivation process was obtained for obidoxime. Unfortunately, its potency to reactivate tabun-inhibited AChE did not surpass 20%, which could be enough to save intoxicated organism. On the contrary, oxime K048 achieved maximal percentage of reactivation potency (28%) of all tested AChE reactivator. However, its maximal reactivation potency was reached at relatively high oxime concentration. At for human relevant doses (10^{-5} and 10^{-4} M), reactivation potency of obidoxime and K048 are similar (10^{-4} M) or favours obidoxime (10-5 M).

Human brain reactivation : Quiet different results were obtained in human brain. As it can be seen in Table 1, oxime K048 surpassed all other effective AChE reactivators. Its affinity towards inhibited AChE is the highest compared to others and its second order rate constant characterizing the whole reactivation process is more than five times higher compared to

Table 1. Reactivation potency of tested AChE reactivators

Reactivator	Species	K_R [μM]	k_R [min^{-1}]	k_R[min^{-1} M^{-1}]
Pralidoxime	Rat	-*	-*	-*
Obidoxime	Rat	3.2	0.020	6250
HI-6	Rat	-*	-*	-*
K027	Rat	54	0.0148	273
K048	Rat	93	0.0324	348
Pralidoxim	Human	-*	-*	-*
Obidoxime	Human	1412	0.06	42
HI-6	Human	-*	-*	-*
K027	Human	2511	0.05	20
K048	Human	251	0.06	239

KR, dissociation constant of inhibited enzyme-reactivator complex; kR, the first order rate constant of reactivation; kr, the second order rate constant of reactivation

* we were not able to measure values of the kinetics constants, because of very low ability of this oxime to reactivate tabun-inhibited AChE

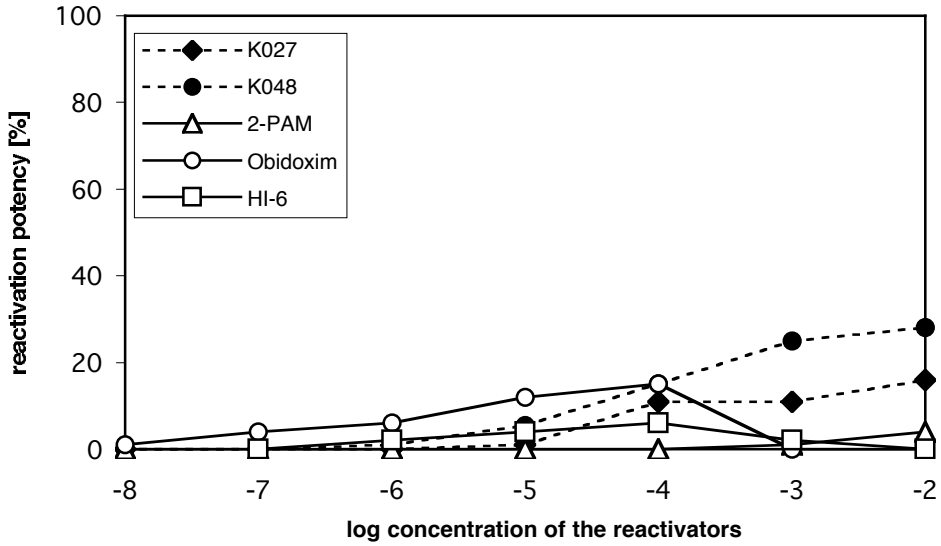

Figure 1. Concentration-reactivation relationship of the new oximes K027 and K048 in comparison with currently used oximes to tabun-inhibited AChE (AChE source – rat brain, inhibition time – 30 min, reactivation time – 10 min, pH – 7.6, 25°C)

obidoxime. The same results were obtained in dependence of reactivation potency on oxime concentration shown in Figure 3. Oxime K048 reached reactivation potency higher than all other tested oximes, and moreover, with the maximal value at the lowest concentration compared to obidoxime and K027.

The reactivating efficacy of oximes has been mainly investigated in rodents (Dawson 1994; Kassa 2002). Nevetheless, the structural and functional differences between human and animal AChE may result in a different affinity and reactivity of oximes. There are studies indicating species depending marked differences in the ability of oximes to reactivate organophosphate-inhibited AChE (Clement and Erhardt, 1994; Worek et al., 2002; Kuca et al. 2005). In addition, the results showing marked differences in the effective oxime concentrations for reactivating sarin-inhibited human, guinea-pig and rat AChE were recently presented (Worek et al., 2001). Our study confirmed these rules and showed that there is necessity to test all newly developed AChE reactivators not only on laboratory animals but also on human tissues.

Our results also demonstrate that there are new AChE reactivators able to sufficiently reactivate AChE inhibited by tabun – K027 and K048. These oximes are promising not only against tabun but also in the case of pesticide-poisoning (Petroianu et al. 2006 a,b). Further investigation demonstrate their in vivo toxicity and protective ration (Calic et al. 2006; Kassa and Kunesova 2006a,b; Kassa 2006). After we summarize these results, we could think over their implemetation into the army as new antidotal mean against tabun intoxication.

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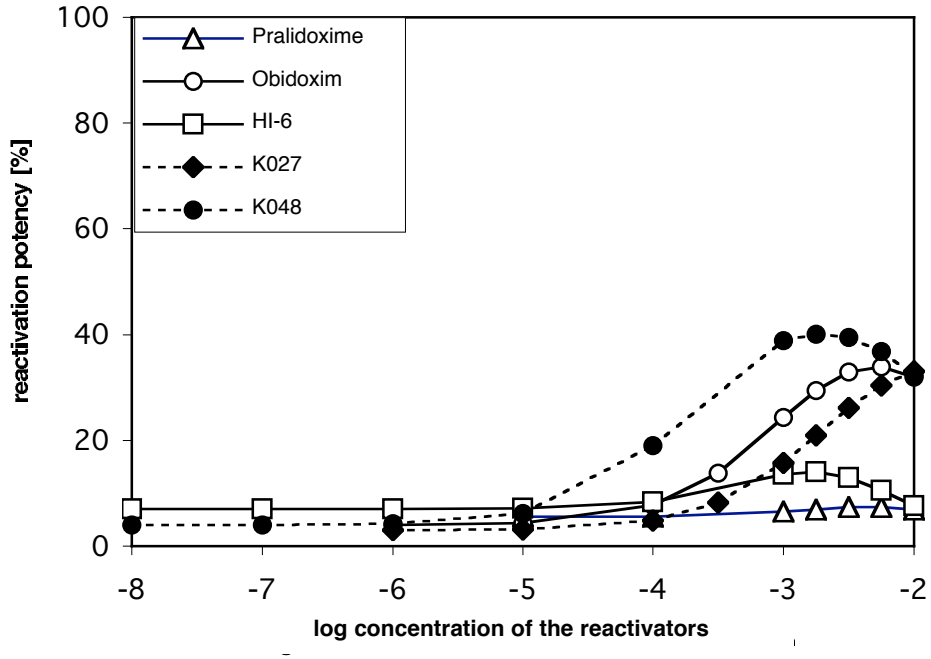

Figure 2. Concentration-reactivation relationship of the new oximes K027 and K048 in comparison with currently used oximes to tabun-inhibited AChE (AChE source – human brain, inhibition time – 30 min, reactivation time – 10 min, pH – 7.6, 25°C)

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