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In vitro reactivation of sarin-inhibited brain acetylcholinesterase from different species by various oximes

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Abstract
In vitro as well as *in vivo* evaluation of the reactivating efficacy of various oximes against nerve agent-inhibited acetylcholinesterase has been usually done with the help of animal experiments. Nevertheless, previously published data indicate that the reactivation potency of oximes may be different in human and animal species, which may hamper the extrapolation of animal data to human data. Therefore, to better evaluate the efficacy of various oximes (pralidoxime, obidoxime, HI-6, K033) to reactivate brain acetylcholinesterase inhibited by sarin by *in vitro* methods, human, rat and pig brain acetylcholinesterase were used to calculate kinetic parameters for the reactivation. Our results show differences among the species, depending on the type of oxime, and indicate that data from animal experiments needs to be carefully evaluated before extrapolation to humans.

Keywords: *Sarin, oximes, reactivation, species differences, acetylcholinesterase,* in vitro

Introduction

Highly toxic organophosphorus compounds (OPs), called nerve agents, are still considered to be the most dangerous chemical warfare agents. They pose potential threats to both military and civilian populations as evidenced in recent terroristic attacks[1]. Their acute toxicity is based on the irreversible inhibition of the enzyme – acetylcholinesterase (AChE; EC 3.1.1.7) and subsequent accumulation of the neuromediator acetylcholine at peripheral and central cholinergic sites[2,3]. AChE plays a key role in the physiological function of the cholinergic nervous system and, therefore, its inhibition is a life-endangering factor. The inhibitory effect is based on phosphorylation or phosphonylation of the serine hydroxy group at the esteratic site of the active centre of the enzyme[2].

The antidotal treatment of acute poisoning with nerve agents is based on the administration of anticholinergic drugs to antagonize the overstimulation of the cholinergic receptors caused by the accumulated acetylcholine and acetylcholinesterase reactivators to reactivate phosphonylated or phosphorylated AChE[4]. According to experimental data, quaternary pyridinium aldoximes have been found to be the most promising to reactivate nerve agent-inhibited AChE[5]. Monoquaternary pralidoxime (2-PAM, 2-hydroxyiminomethyl-1-methylpyridinium chloride) or more extended bisquaternary compounds such as obidoxime [1,3-bis(4-hydroxyiminomethyl-pyridinium)-2-oxa-propane dichloride] and H-oxime HI-6 [1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride] are the basic representatives of these aldoximes (Figure 1). Unfortunately, currently used oximes are not sufficiently effective against some nerve agents; they are unable to sufficiently reactivate nerve agent-inhibited AChE regardless of the type of nerve agent used[6,7]. While pralidoxime and obidoxime have very low potency to reactivate soman and cyclosarin-inhibited AChE[8,9], the oxime HI-6 seems to be practically ineffective in reactivating tabun-inhibited AChE[10]. Therefore, numerous oximes have been synthesised to find another bispyridinium oxime sufficiently able to

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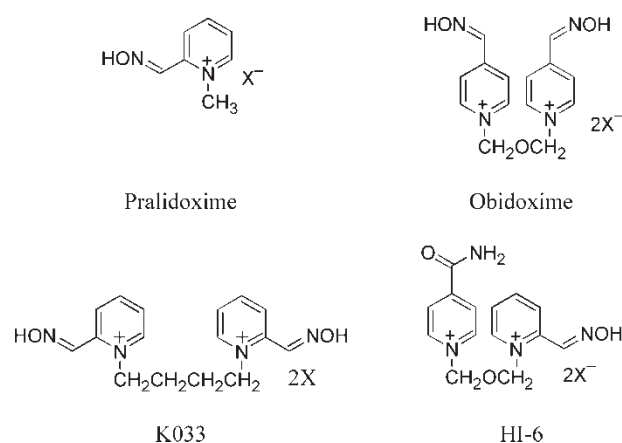
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Figure 1.   Chemical structures of the tested acetylcholinesterase reactivators.

reactivate nerve agent-inhibited AChE regardless of the type of nerve agent. One of oximes newly developed at our Department of Toxicology is a symmetric bisquaternary oxime, called K033 [1,4-bis(2-hydroxyiminomethylpyridinium) butane dibromide][11] (Figure 1).

The reactivating efficacy of oximes has been mainly investigated in rodents[4,5]. Nevertheless, the structural and functional differences between human and animal AChE may result in a different affinity and reactivity of oximes. There are studies indicating that there are species-dependent marked differences in the ability of oximes to reactivate organophosphate-inhibited AChE[12,13]. In addition, the results showing marked differences in the effective oxime concentrations for reactivating sarin-inhibited human, guinea-pig and rat AChE have been recently presented[14].

For ethical reasons, the reactivating efficacy of new oximes against nerve agent poisoning cannot be investigated in humans. Therefore, an exact analysis of species differences can be crucial for the assessment of oxime efficacy in humans. In order to provide a valid database, experiments were undertaken to determine kinetic parameters, including the reactivation rate constants and percentage of reactivation, for pralidoxime, obidoxime, HI-6 and the oxime K033 for human, rat and pig AChE inhibited by sarin. Brain AChE was selected as the enzyme source because brain AChE is the main target for acute neurotoxicity of nerve agents.

Materials and methods

Enzymes and chemicals

The human, pig or rat brains were used as a source of AChE after homogenization. Sarin was obtained from the Military Technical Institute (Brno, Czech Republic) and was 95% pure as evaluated by acidimetric titration. The monopyridinium and bispyridinium oximes (pralidoxime, obidoxime, the oxime HI-6 and the oxime K033) were earlier synthesised at the Department of Toxicology of the Military Medical Academy (Czech Republic). Their purities were determined using a HPLC technique. All other drugs and chemicals of analytical grade were obtained commercially and used without further purification.

In vitro experiments

Reactivation effectivity of the oximes was tested *in vitro* on the model of AChE inhibited by sarin using a standard reactivation test with electrometric instrumentation[15,16].

Calculation of percentage of reactivation

The percentage of reactivation (%R) was calculated from the measured activities of the intact enzyme a_0, nerve agent-inhibited enzyme a_i, and reactivated enzyme a_r, using Equation (1). The enzyme activity was measured at room temperature (25° C) and pH 7.6.

$$\%R = [1 - (a_0 - a_r)/(a_0 - a_i)] \times 100 \qquad (1)$$

Determination of activity of intact AChE (a_0)

Human, pig or rat brain homogenate (0.5 ml) was added to a 0.3 M sodium chloride solution (20 ml). Then 0.02 M solution of acetylcholine iodide (2.0 ml) was added and the system was adjusted with 0.3 M sodium chloride solution to a total volume of 25.0 ml. The liberated acetic acid was titrated with 0.01 M sodium hydroxide on an RTS 822 titrator in the pH-stat mode (pH 8.0) at room temperature (25°C). The ionic strength of the solution was rendered with sodium chloride to a constant value $I = 0.25$ M. The slope of the linear part of the time dependence of the sodium hydroxide used represents the activity of the intact enzyme (in fact, the initial rate of the enzymatic reaction).

Determination of activity of inhibited AChE (a_i)

Human, pig or rat brain homogenate (0.5 ml) was treated with 5.10^{-8} M aqueous solution of sarin (0.5 ml) for 30 min, which resulted in about 95% inhibition of the enzyme. 0.02 M solution of acetylcholine iodide (2.0 ml) was added and the mixture was adjusted to 25.0 ml with 0.3 M sodium chloride solution. The activity of the inhibited enzyme (a_i) was immediately determined as described in the previous experiment.

Determination of activity of reactivated AChE (a_r)

Reactivation of the enzyme, inhibited in the above mentioned experiment, was performed immediately after the inhibition. A solution (1.0 ml) of the reactivator was added to the enzyme in an appropriate concentration range of $1.10^{-7}-1.10^{-2}$ M. After 10 min reactivation at $25°$ C, 0.02 M solution of acetylcholine iodide (2.0 ml) was added. The mixture was adjusted to 25.0 ml with 0.3 M sodium chloride solution and immediately afterwards the activity of the reactivated enzyme was determined as described in the previous experiments. According to our observation, the possibility of nerve agent-induced inhibition of released free enzyme during the reactivation of the enzyme is negligible.

Calculation of rate and dissociation constants

The measured values of enzyme activity in relationship to the used concentrations of reactivator were used for the calculation of the rate and dissociation constants with the help of Equation (2):

$$\%A = (100{}^\star(1 - EXP(-t{}^\star k_R{}^\star C_R/(C_R + K_R))){}^\star(1 - a_i/a_0) + 100{}^\star a_i/a_0)/(1 + (C_S/K_M + 1){}^\star C_R/(K_{dis}{}^\star f_d)) \quad (2)$$

where: $\%A$ = percentage of enzyme activity after reactivation, when 100% = activity of intact enzyme, t = time of the reactivation, k_R = pseudofirst order-rate constant of reactivation for $C_R = \infty$, C_R = molar concentration of reactivator, K_R = dissociation constant of the enzyme-inhibitor-reactivator complex, a_i = activity of inhibited enzyme, a_0 = activity of intact enzyme, C_S = molar concentration of substrate, K_M = dissociation constant of enzyme - substrate complex (Michaelis constant), K_{dis} = dissociation constant of enzyme - reactivator complex, f_d = dilution constant calculated from the ratio of volume of substrate reaction and volume of reactivator reaction, $\star$ = multiplication.

The first part of the above equation $(1 - EXP(-t{}^\star k_R{}^\star C_R/(C_R + K_R)))$ describes an increase in enzyme activity due to dephosphonylation of the inhibited enzyme and the second part $(1 + (C_S/K_M + 1){}^\star C_R/(K_{dis}{}^\star fd))$ describes a decrease in enzyme activity due to a competitive reaction of reactivator and substrate at the binding site of the enzyme. Fraction a_i/a_0 introduces correction for subtotal inhibition of enzyme in Equation (2). A computer program for non-linear regression was used to calculate reactivation parameters. Calculated parameters were the dissociation constant of the enzyme-reactivator complex (K_{dis}), dissociation constant of the enzyme-inhibitor-reactivator complex (K_R) and the first-order rate constant for reactivation (k_R).

Other parameters of the equation were introduced as constants.

The second-order rate constant of reactivation (k_r) was calculated using Equation (3):

$$k_r = k_R/K_R \quad (3)$$

Determination of the dissociation constant of enzyme-reactivator complex

Appropriate volume of 0.01 M solution of the oxime was added to human, pig or rat brain homogenate (0.5 ml) so as to achieve the desired oxime concentration C_R in the range $5.10^{-5} - 5.10^{-2}$ M. Then 0.02 M solution of acetylcholine iodide (2.0 ml) was added, the mixture was adjusted to 25.0 ml with 0.3 M sodium chloride and the rate of the enzyme reaction (v) was immediately determined analogously as in the previous experiments. The dissociation constant (K_{dis}) was obtained by non-linear regression from the dependence of v on C_R using Equation (4):

$$v = v_{max} \: x \: C_s/(C_s + K_M x(1 + C_R/K_{dis})) \quad (4)$$

where, in addition to the above-defined symbols, v_{max} denotes the maximum (limiting) rate of the enzymatic reaction, C_s is the substrate concentration and K_M is the Michaelis constant for hydrolysis of acetylcholine by AChE ($1.9.10^{-4}$ M).

Statistical evaluation

Statistical significance was determined by the use of Student's t-test and differences were considered significant when $P < 0.05$. Statistical evaluation was determined with the relevant computer programs[17].

Results

The ability of tested monopyridinium and bispyridinium oximes to reactivate AChE from three species inhibited by sarin *in vitro* as characterized by percentage of reactivation and kinetic parameters is summarized in Table I and Figures 2–5.

Kinetics parameters shown in Table I characterize the ability of all studied oximes to reactivate sarin-inhibited AChE *in vitro*. The values of the dissociation constant (K_{dis}) indicating the affinity of oximes toward the non-inhibited AChE show that the oximes K033 and HI-6 have a higher affinity for the human, pig as well as rat intact enzyme than the commonly used obidoxime and pralidoxime ($P < 0.05$). In addition, the oxime K033, HI-6 and pralidoxime have a higher affinity to human AChE compared to pig and rat AChE while obidoxime has a markedly lower affinity to human AChE compared to pig and rat enzyme ($P < 0.05$). The dissociation constant K_R, which characterizes affinity of oximes to the inhibited

Table I. Kinetic parameters for the reactivation of sarin-inhibited AChE in human, rat and pig brain homogenate *in vitro*.

Oxime	Species	K_{DIS} [μM]	k_R [min^{-1}]	K_R [μM]	k_r [min^{-1}M^{-1}]
Pralidoxime	Human	59 ± 2.0	0.19 ± 0.02	2810 ± 205	68
Pralidoxime	Rat	210 ± 12.0^{X}	0.29 ± 0.01^{X}	4360 ± 1223^{X}	67
Pralidoxime	Pig	100 ± 8.0^{X}	0.23 ± 0.03^{X}	4670 ± 560^{X}	50
Obidoxime	Human	$1047 \pm 49.0^{*}$	$0.03 \pm 0.01^{*}$	$144 \pm 13^{*}$	208
Obidoxime	Rat	$280 \pm 29.0^{*X}$	$0.12 \pm 0.02^{*X}$	$2450 \pm 317^{*X}$	49
Obidoxime	Pig	$20 \pm 2.3^{*X}$	$0.10 \pm 0.02^{*X}$	$354 \pm 110^{*X}$	282
K033	Human	$3 \pm 0.2^{*}$	$0.10 \pm 0.01^{*}$	$120 \pm 18^{*}$	833
K033	Rat	$29 \pm 3.0^{*X}$	$0.05 \pm 0.01^{*X}$	$62 \pm 20^{*X}$	811
K033	Pig	$4 \pm 0.8^{*X}$	$0.08 \pm 0.03^{*}$	$380 \pm 87^{*X}$	211
HI-6	Human	$12 \pm 1.6^{*}$	$0.08 \pm 0.02^{*}$	$181 \pm 44^{*}$	441
HI-6	Rat	$24 \pm 3.0^{*X}$	$0.39 \pm 0.08^{*X}$	$389 \pm 89^{*X}$	1003
HI-6	Pig	$17 \pm 0.7^{*X}$	$0.08 \pm 0.02^{*}$	$98 \pm 10^{*X}$	816

K_{DIS}- dissociation constant of the enzyme-reactivator complex; K_R - dissociation constant of inhibited enzyme-reactivator complex; k_R - the first-order rate constant of reactivation; k_r - the second-order rate constant of reactivation.
Statistical significance:
*- HI-6, obidoxime and K033 compared to pralidoxime for all sources of enzyme.
^{X} – rat and pig AChE compared to human AChE for all oximes.

AChE (inhibitor-enzyme complex), indicates that the affinity of HI-6, obidoxime and the oxime K033 to the enzyme-inhibitor complex is comparable (with the exception of the affinity of obidoxime to the rat enzyme-inhibitor complex) and markedly higher compared to pralidoxime (P < 0.05). Obidoxime and pralidoxime have higher affinity to the human enzyme-inhibitor complex compared to the pig and rat
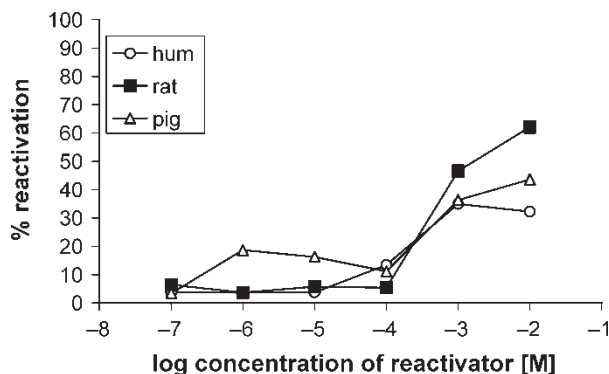

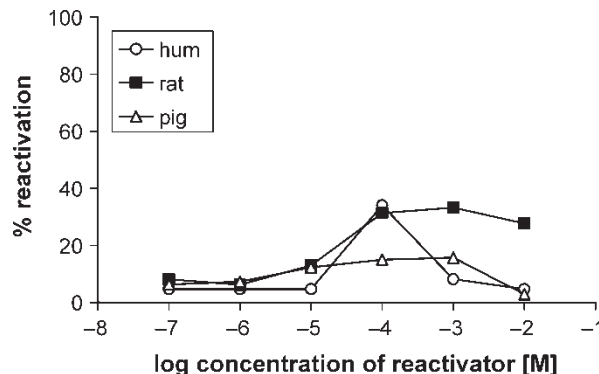Figure 2. Reactivation–concentration relationships of **pralidoxime** to sarin-inhibited AChE.

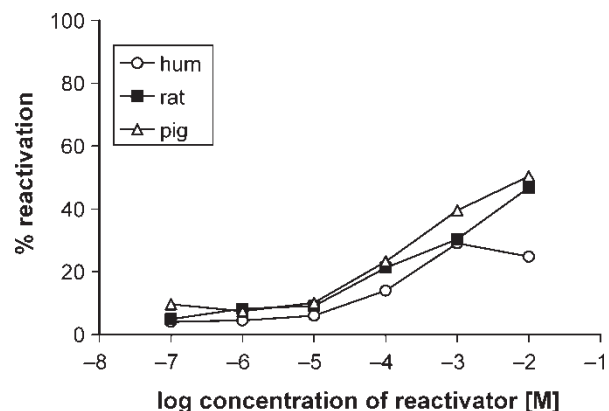Figure 4. Reactivation–concentration relationships of **K033** to sarin-inhibited AChE.

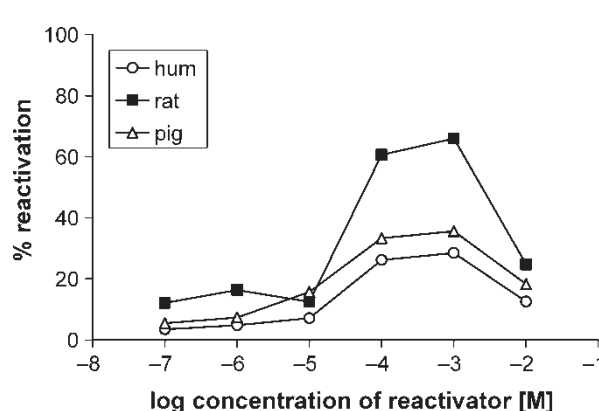Figure 3. Reactivation–concentration relationships of **obidoxime** to sarin-inhibited AChE.

Figure 5. Reactivation–concentration relationships of **HI-6** to sarin-inhibited AChE

enzyme-inhibitor complex ($P < 0.05$). On the other hand, K033 has lower affinity for the human enzyme-inhibitor complex than the rat enzyme-inhibitor complex and the oxime HI-6 has lower affinity for the human enzyme-inhibitor complex compared to the pig enzyme-inhibitor complex ($P < 0.05$). The differences in the affinity of the studied oximes to intact or inhibited enzyme correspond to the differences in the second-order rate constant for reactivation of sarin-inhibited AChE (k_r). The velocity of HI-6 - induced reactivation of sarin-inhibited rat and pig AChE is markedly higher compared to the other studied oximes but the rate of HI-6 - induced reactivation of sarin-inhibited human AChE is 2-fold lower compared to K033. Pralidoxime shows the lowest velocity of reactivation of sarin-inhibited AChE among all the studied oximes with the exception of rat brain AChE where the lowest velocity of reactivation of sarin-inhibited AChE was found for obidoxime.

The concentration-reactivation relationship for all the studied oximes is expressed in Figures 2–5. According to our results shown in Figure 2, pralidoxime shows very low potency to reactivate sarin-inhibited AChE at 10^{-4} M, a concentration that should be safe for human use regardless of the source of enzyme. The potency of obidoxime to reactivate sarin-inhibited AChE at 10^{-4} M is higher compared to pralidoxime (Figure 3) but the difference between the efficacy of pralidoxime and obidoxime to reactivate sarin-inhibited AChE is relatively small especially in the case of reactivation of sarin-inhibited human AChE. Pralidoxime as well as obidoxime are able to increase their reactivation potency at 10^{-3} M, nevertheless, this concentration is too high and toxic for human use. The oxime K033 shows markedly higher efficacy in reactivating sarin-inhibited human and rat AChE at 10^{-4} M in comparison to pralidoxime and obidoxime (Figure 4). The efficacy of the oxime HI-6 to reactivate sarin-inhibited AChE at 10^{-4} M is the highest among all the studied oximes but just for rat AChE (Figure 5). In the case of reactivation of sarin-inhibited human AChE, the potency of HI-6 decreases markedly compared to the reactivation of sarin-inhibited rat AChE and it is lower compared to that of K033.

Discussion

The results demonstrate remarkable differences in the reactivation kinetics of inhibited brain AChE depending on the oxime and species. The efficacy of AChE reactivators depends on their reactivity and affinity to nerve agent-inhibited enzyme. Their reactivity is derived from the nucleophilic activity of the oxime anion that is attached on the pyridinium ring[18]. The reactivity of all oximes studied (k_R) is similar regardless of the source of AChE because their basic

structure is very similar. They differ from each other by the number of pyridinium rings (monopyridinium vs bispyridinium oximes), position of the oxime group on the pyridinium ring and, in the case of bispyridinium oximes, by the chemical structure of the bridge between both pyridinium rings (K033 vs HI-6 and obidoxime) only.

The affinity of oximes for intact enzyme, characterized by K_{dis}, and for nerve agent-inhibited enzyme, characterized by K_R, is derived from various physical features (steric compatibility, electrostatic effects, hydrophobic interactions) and from the shape and the size of the whole molecule as well as functional groups[19]. The affinity of reactivators for nerve agent-inhibited AChE is considered to be the most important factor for their reactivating efficacy. Our results demonstrate that the strength of reactivator binding to AChE is usually decreased regardless of the source of enzyme because of reduction of a space in the cavity of the AChE molecule following enzyme phosphonylation[15] with the exception of the binding of obidoxime to human AChE. It means that sarin generally reduces the strength of binding of oximes to AChE[20]. Nevertheless, there are marked differences depending on the source of the enzyme, especially in the case of obidoxime-induced reactivation of sarin-inhibited AChE. Sarin markedly reduces the strength of binding of obidoxime to rat and pig AChE but increases the strength of its binding to human AChE.

The strength of binding of all studied oximes to nerve agent-inhibited AChE and the rate of its reactivation generally corresponds to the *in vitro* potency of oximes to reactivate nerve agent-inhibited AChE[15,21,22]. It depends not only on the type of oxime but also on the species (the source of the enzyme). The reactivating efficacy of bispyridinium oximes strongly depends on the chemical structure of the bridge connecting both pyridinium rings, the position of the oxime groups and the chemical structure of the substituent situated on the second pyridinium ring[10,23]. Generally, the oximes HI-6 and K033 show higher *in vitro* potency to reactivate sarin-inhibited AChE at human-relevant concentrations compared to obidoxime and especially pralidoxime regardless of the source of the enzyme. Nevertheless, there are big differences between the efficacy of all bispyridinium oximes studied to reactivate sarin-inhibited AChE if various sources of AChE are used[13]. Alternatively, the monopyridinium oxime pralidoxime does not show any differences in its potency to reactivate sarin-inhibited human, rat an pig AChE because its efficacy to reactivate sarin-inhibited AChE is very low regardless of the species. While the oxime K033 shows a higher efficacy to reactivate sarin-inhibited human and rat AChE compared to pig AChE, the oxime HI-6 shows lower potency to reactivate sarin-inhibited human AChE compared to rat and pig AChE. Thus, the oxime HI-6

is the most efficacious reactivator of sarin-inhibited rat and pig AChE but its potency to reactivate human AChE is a little less compared to the oxime K033. It is very difficult to find the real reason for the different efficacy of oximes to reactivate sarin-inhibited AChE for various species. The difference in chemical structure of oximes tested could be one of the possible reasons for the dependence of their reactivation potency on species[24].

In conclusion, we should keep in our mind that some kinetic parameters of oximes, especially affinity to nerve agent-inhibited AChE and the rate of its reactivation, as well as the potency of oximes to reactivate nerve agent-inhibited AChE can depend on species. Thus, the reactivation efficacy of currently available oximes seems to be worse for humans compared to experimental data obtained from animal experiments. The presented data on reactivating properties of oximes provide a basis for a reliable evaluation of data from animal experiments and may allow a better extrapolation of animal data to humans.

Acknowledgements

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