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PROTECTIVE ACTIVITY OF PYRIDINIUM SALTS AGAINST SOMAN POISONING *IN VIVO* AND *IN VITRO*

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Abstract—The protective effects of a series of pyridinium salts against soman poisoning were studied using female mice. From the most active compound, 130 nmoles/kg (i.m.) reduced the toxic effect of a LD_{95} of soman (s.c.) down to the 50 per cent mortality level. The compounds with protective activity *in vivo* were able to protect acetylcholinesterase (AChE) from soman *in vitro*. The protective efficiency was evaluated by comparing the rates of AChE inhibition in the absence and in the presence of 1 mM pyridinium salt. The most effective substances decreased the inhibition rate down to about 1/100 of the unprotected value. Additionally, the influence of these compounds upon the acetylcholine hydrolysis by AChE was investigated. A striking correlation exists between the protective efficiencies *in vitro* and the competitive inhibition constants of the pyridinium salts.

Soman, *O*-(1,2,2-trimethylpropyl)-methyl-fluorophosphonate, is known as a highly toxic and powerful inhibitor of acetylcholinesterase (AChE; EC. 3.1.1.7), acting by phosphonylation of the active site of the enzyme. In a secondary step, the phosphonylated AChE undergoes rapid dealkylation ("aging") by elimination of the 1,2,2-trimethylpropyl residue. The resulting methylphosphonyl-AChE is resistant against the nucleophilic attack of oximes, which are commonly used as cholinesterase reactivators [1–3]. *In vivo*, the half-time of the aging-process was found to be about 5 min [1, 2]. Consequently, in the case of an intoxication by soman the application of reactivators is ineffective [4, 5].

Nevertheless it could be shown that with certain pyridinium salts, a moderate protection against soman poisoning in mice can be achieved [5]. By continuing these investigations, we found several other compounds with, in part, remarkable protective efficiencies *in vivo*. In order to get some informations about the biochemical background of the protective mechanism, we studied the influence of the biologically active substances upon (1) the rate of phosphonylation of acetylcholinesterase (AChE) by soman and (2) the steady state kinetics of the AChE/acetylcholine system.

MATERIALS AND METHODS

The pyridinium compounds HP 71, HP 73, HH 60, HH 65, HH 69, HH 70, HY 10 were gifts from Prof. Dr. I. Hagedorn, Freiburg/Br.; P 141 and SAD 128 were prepared by Dr. R. Reiner, Frankfurt/M.; HS 8 [6], HH 38, HH 39 and HH 54 [7] were resynthesized according to the cited literature. The alkylene homologues P 60 to P 66 were prepared by reacting the corresponding α,ω-alkylene-dihalogenides with a 4-fold excess of 4-*tert* butylpyridine in boiling acetonitrile overnight. After recrystallisation from isopropanol–ether the yields were 40–60 per cent. The melting points and analytical data are shown in Table 1. Melting points were determined on a "Kofler–Mikroheiztisch" (for the hygroscopic compounds P 63, P 64, P 66 in a closed tube); halogenide determinations were carried out potentiometrically, the CHN-analysis were performed by the Microanalytic Laboratory Beller, Göttingen.

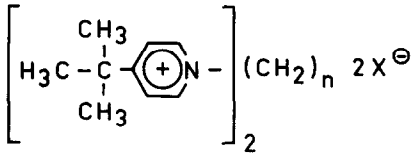
For the experiments *in vitro*, acetylcholinesterase from bovine red cells (Serva, Heidelberg) was used; the content of active sites was determined according to Schoene [8] as to be 6.1×10^{-12} moles per mg.

Soman was used in appropriate dilutions in absolute ethanol. The solvent for all other stock solutions, buffers and experiments was distilled water containing 0.1 M NaCl and 0.02 M $MgCl_2$. Enzymatic activities were determined by pH-stat titration under nitrogen in a Metrohm Combititrator 3 D with acetylcholineiodide as substrate. For the determination of the Michaelis constant and the inhibition equilibrium constants a double-syringe arrangement for substrate and titrant was used. This modification of the automatic Metrohm burette is described by H. Kuhnen elsewhere [9].

Evaluation of the ED_{50}. After administration of 10 mg/kg of atropinium sulfate a LD_{95} of soman (0.280 mg/kg; aq solution, containing 0.9% NaCl and 2% ethanol) was given s.c. to female NMRI-mice (av. body weight 23 g). One min after intoxication the pyridinium salt was given i.m. (aq solution containing 0.9% NaCl). Collectives of 8 mice were used in each experiment. Based upon the number of deaths within the following 24 hr, the ED_{50} was evaluated according to the method of Litchfield and Wilcoxon [10].

Rate constants of AChE-inhibition by soman. Equimolar amounts of soman (considering only its two fast reacting enantiomers [11]) and AChE were mixed at time zero in 5 ml Tris buffer (1 mM; pH 7.60, 20°C). The concentration of each reactant was 2.4×10^{-9} M. One-ml samples were withdrawn at suitable time intervals to determine the enzymatic activity by pH-stat titration with 55 mM acetylcholine. Because the inhibition proceeds very fast, only a few samples could be taken. So the inhibition experiment was repeated up to fifteen times in order to get a reliable average result. The inhibition rate was controlled each day.

Table 1. Analytical data of the newly synthesized α,ω-bis-[*tert* butylpyridinium-(1)]-alkylenedihalogenides



Compound	n	X	Formula	Mol Wt	m.p. (°C)	Calculated %				Found %			
						C	H	N	X	C	H	N	X
P 60	1	Br	$C_{19}H_{28}Br_2N_2$	444.27	272	51.36	6.35	6.31	35.98	51.12	6.57	6.19	35.52
P 61	2	Br	$C_{20}H_{30}Br_2N_2$	458.30	>350	52.41	6.60	6.11	34.88	51.98	6.57	6.04	34.70
P 62	3	I	$C_{21}H_{32}I_2N_2$	566.30	268–9	44.54	5.70	4.95	44.82	45.22	5.80	5.00	45.00
P 63	4	Br	$C_{22}H_{34}Br_2N_2$	486.35	255	54.33	7.05	5.76	32.87	54.13	7.20	5.56	32.20
P 64	5	Br	$C_{23}H_{36}Br_2N_2$	500.37	215–7	55.21	7.25	5.60	31.94	55.07	7.42	5.46	31.80
P 65	6	Br	$C_{24}H_{38}Br_2N_2$	514.40	299–300	56.03	7.45	5.45	31.07	55.97	7.47	5.37	31.40
P 66	7	Br	$C_{25}H_{40}Br_2N_2$	528.43	223–4	56.82	7.63	5.30	30.25	57.18	7.62	5.19	29.70

The inhibition of AChE by soman in presence of pyridinium salt was carried out under exactly the same conditions except the addition of 1 mM (final concentration) of pyridinium salt prior to the introduction of soman. Each inhibition experiment was performed three times. The control run contained AChE and pyridinium salt in the same concentration, thus taking account of the inhibitory effect of the latter.

The average second-order rate constants k' of the AChE inhibition by soman were calculated according to the equation

$$k' = 1/t_{1/2} \times c$$

with $t_{1/2}$ = half time of the reaction and c = the (identical) concentrations of AChE and soman.

As a measure for the protective efficiencies of the pyridinium salts the ratio k'/k'_p was calculated, with k'_p = rate constant in presence of pyridinium salt.

Determination of K_M, V_{max}, K_I *and* K'_I. The experiments were carried out in the combititrator equipped with a double-syringe arrangement [9]. The concentrations of the titrants acetylcholine iodide and sodium hydroxide were carefully adjusted to 0.01 N. The equimolarity was checked by a control run, giving a straight line over a period of 20 min.

For determination of the Michaelis constant K_M and the maximal velocity V_{max}, enzyme stock solution corresponding to 1.25 mg of the enzyme preparation was added to 24 ml of the reaction medium. Sufficient substrate acetylcholine iodide was introduced to make a final concentration of 1.2×10^{-4} M. The rate of hydrolysis was measured by pH-stat titration at pH 7.00, 20°. After about 10 min, another portion of substrate was added to bring the concentration up to 2.4×10^{-4} and the titration was started again. This procedure was repeated with 4 and 6×10^{-4} M substrate.

In order to ensure a proper function of the experimental arrangement, this operation was performed before each inhibition experiment.

For the determination of the inhibition constants K_I and K'_I, the experimental arrangement was the same as described above except the addition of pyridinium salt prior to the first introduction of substrate. The application of three different concentrations of inhibitor and repeating each experiment three times proved to be sufficient to give reliable results. The inhibitor (pyridinium salt) concentrations were varied within one or two orders of magnitude, depending upon the respective inhibitory strength.

K_M and V_{max} were determined in the usual way [12] from the intercepts in the double reciprocal plot obtained from the inhibitor free experiments.

The equilibrium constants K_I and K'_I denote the competitive and noncompetitive portion of inhibition, respectively. According to Wilson [13], Kitz [14] and Krupka [15], the kinetics of reversible inhibition reactions of this type can be described by the equation

$$\frac{1}{V} = \frac{1}{V_{max}}\left[1 + \frac{(I)}{K'_I}\right] + \frac{K_M}{V_{max}}\left[1 + \frac{(I)}{K_I}\right] \cdot \frac{1}{(S)}, \quad (1)$$

which describes the linear relationship between the reciprocals of initial velocity V and substrate concentration (S). The competitive inhibition constant K_I was calculated from the slope, the noncompetitive K'_I from the intercept on the ordinate.

Decomposition of soman in presence of pyridinium salt. These control experiments were to be done to ensure that the measured protective effects could not be caused by a chemical reaction between soman and the pyridinium salts, which would result in a detoxification of the reaction mixture.

To a 1 mM solution of pyridinium salt in Tris buffer (10 mM, pH 7.60), thermostated to 20°, soman was added to give a final concentration of 2.75×10^{-5} M. At appropriate time intervals 1-ml samples were withdrawn and extracted with 1 ml *iso*-octan. From the organic layer 5-μl aliquots were transferred into a Perkin-Elmer GC 900 Gaschromatograph (column: Chromosorb G/phenylsilicon oil, 145°). For the detection of soman the flame ionization detector was used.

From the decrease of the soman concentration vs time the decomposition rate constant was calculated. The rate constant for the decomposition of soman in absence of pyridinium salt was determined in the same way using Tris buffer alone as a solvent.

RESULTS AND DISCUSSION

1. *Protective effects* in vivo. The ED_{50} data are compiled in Table 2. With the exception of HH 60,

HH 65 and HH 70, for all compounds an ED_{50} value could be established. This means, that these substances are more or less effective against the soman intoxication.

In contrast to the typical AChE reactivator Toxogonin® which does not show any protective effect against soman [4, 5], a remarkable low ED_{50} could be achieved with its bis-*tert* butylether HY 10. The ED_{50} values of the α,ω-bis-(*tert* butyl-pyridinium)-alkylene halogenides decrease with increasing length of the alkylene chain. The compound P 65 containing a hexamethylene bridge plays an exceptional role: its ED_{50}, 0.013×10^{-5} moles/kg, indicates the highest protective efficiency we have found in this series. In the group of urea derivatives the unsymmetrical 3,4-isomer is the most effective compound.

2. *Decomposition of soman.* For the spontaneous hydrolysis of soman in Tris buffer (pH 7,60 and 20°) the rate constant $k_s = 4.8 \times 10^{-4}\,min^{-1}$ was obtained, corresponding to a half life of about 24 hr. A comparable reference from the literature is the value $5.3 \times 10^{-4}\,min^{-1}$ determined by Grochowski [16] in 0.05 M KCl at pH 7.6, 20°. In presence of the pyridinium salts no increase of the decomposition rate was found.

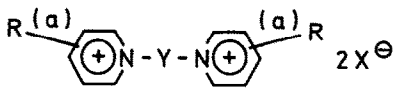
It follows from these experiments that the protective effects of the pyridinium salts are not caused by a detoxification of soman, resulting from a direct attack of the pyridinium compound on the soman molecule.

3. *Rate constants for AChE-inhibition by soman.* The average rate constant for the AChE-inhibition by soman in absence of pyridinium salt was found to be $k' = 6.7 \times 10^7\,M^{-1}\,min^{-1}$ with $\pm 1.2 \times 10^7\,M^{-1}\,min^{-1}$ as the maximal absolute deviation.

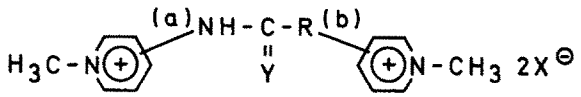
This value determined at pH 7.60, 20° is to be compared to the finding of Keijer and Wolring [11] (pH 7.5, 25°), that two in the four stereoisomers of soman have the rate constants 12×10^7 and $2.8 \times 10^7\,M^{-1}\,min^{-1}$, whereas the other two are relatively poor inhibitors of AChE with rate constants $\leq 10^4\,M^{-1}\,min^{-1}$.

Under our experimental conditions the portion of inhibition contributed by the latter poor inhibitors can be neglected. Moreover, for our purposes it seemed not necessary to distinguish between the two

Table 2. ED_{50} against a LD_{95} of soman, rate constants k'_p for AChE inhibition by soman in presence of pyridinium salts and dissociation constants K_I, K'_I of the complexes between AChE and pyridinium salts



	a†	R	Y	X	$ED_{50} \times 10^5$ (moles/kg)		$k'_p \times 10^{-7}$ ($M^{-1}\,min^{-1}$)	k'/k'_p	$K_I \times 10^5$ [M (±S.D.)]		$K'_I \times 10^5$ [M (±S.D.)]	
HS 8	3	$CONH_2$	CH_2OCH_2	Cl	11.40	(8.97–14.46)	6.1	1.1	~16*			
HY 10	4	$CHNO\text{-}C(CH_3)_3$	„	I	0.56	(0.47–0.66)	1.6	4.2	1.74	(0.40)	10.5	(0.7)
HP 71	4	$CHNNHCONH_2$	„	Cl	3.31	(2.80–3.92)	3.7	1.8	3.29	(0.50)	4.9	(0.7)
HP 73	3	$NHCOCH_3$	„	Cl	8.38	(6.52–11.00)	2.6	2.6	2.71	(0.47)	12.8	(1.5)
P 60	4	$C(CH_3)_3$	CH_2	Br	2.52	(2.11–3.00)	6.4	1.04	~100*			
P 61	4	„	$(CH_2)_2$	Br	2.01	(1.34–3.02)	5.9	1.1	~51*			
P 62	4	„	$(CH_2)_3$	I	1.76	(1.50–2.07)	5.7	1.2	12.8	(1.0)	119	(56)
SAD 128	4	„	CH_2OCH_2	Cl	2.15	(1.82–2.53)	3.8	1.8	3.36	(0.42)	44.6	(8.3)
P 141	4	$CH(CH_3)C(CH_3)_3$	$(CH_2)_3$	Br	0.95	(0.75–1.22)	2.5	2.7	5.00	(0.21)	48.9	(2.7)
P 63	4	$C(CH_3)_3$	$(CH_2)_4$	Br	0.62	(0.49–0.78)	1.7	3.9	1.56	(0.25)	5.6	(1.0)
P 64	4	„	$(CH_2)_5$	Br	0.58	(0.50–0.69)	0.42	16.0	0.35	(0.04)	1.33	(0.02)
P 65	4	„	$(CH_2)_6$	Br	0.013	(0.011–0.015)	0.084	79.8	0.055	(0.01)	0.46	(0.14)
P 66	4	„	$(CH_2)_7$	Br	0.13	(0.11–0.15)	0.057	118	0.034	(0.003)	0.10	(0.03)



	a†	b†	Y	R	X	$ED_{50} \times 10^5$ (moles/kg)	$k'_p \times 10^{-7}$ ($M^{-1}\,min^{-1}$)	k'/k'_p	$K_I \times 10^5$ [M (±S.D.)]		$K'_I \times 10^5$ [M (±S.D.)]	
HH 38	3	3	O	—NH—	I	1.04 (0.87–1.22)	0.30	22.3	0.22	(0.04)	0.80	(0.20)
HH 39	4	4	O	„	I	1.02 (0.82–1.27)	0.080	83.8	0.057	(0.009)	0.19	(0.03)
HH 54	3	4	O	„	I	0.38 (0.29–0.50)	0.13	51.2	0.090	(0.011)	0.33	(0.01)
HH 60	4	4	H_2	„	I	—	1.3	5.1	1.31	(0.10)	9.0	(1.4)
HH 65	3	3	S	„	I	—			8.45	(0.75)	44.8	(10.7)
HH 69	3	3	NH	„	I	6.73 (5.60–8.09)	3.4	2.0	5.88	(0.60)	29.5	(10.9)
HH 70	3	3	O	—$NHCH_2$—	I				4.00	(0.11)	23.4	(9.8)

* Estimated from Dixon plot.

† *a* and *b* denote the positions of substituents in the pyridine nuclei.

The ED_{50} of pyridinium salt (i.m.) reduces the toxic effect of a LD_{95} of soman (s.c., mice) to a level of 50 per cent mortality; 95 per cent confidential limits in parentheses. With HH 60, HH 65 and HH 70 no ED_{50} could be established.

k'_p was determined by inhibition of AChE with soman in presence of 1 mM pyridinium salt; k', the rate constant in absence of pyridinium salt, was $6.7 \times 10^7\,M^{-1}\,min^{-1}$ (pH 7.60; 20°; 1 mM Tris buffer). k'/k'_p represents a measure for the protective efficiency *in vitro*.

K_I and K'_I are the competitive and noncompetitive equilibrium constants for inhibition of AChE by the pyridinium salts in presence of acetylcholine ($1.2\text{–}6 \times 10^{-4}$ M), obtained at pH 7.0; 20°; Standard deviation is given in parentheses.

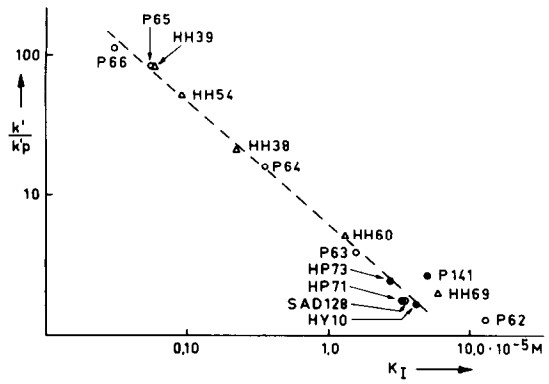


Fig. 1. Double-logarithmic plot of the protective efficiency k'/k'_p versus the competitive part of reversible AChE inhibition by several pyridinium salts.

fast-reacting components of soman. Therefore we determined an average rate constant k' as described above, including the inhibitory effects of both enantiomers.

In order to get a measure for the protective action of the pyridinium salts, we determined the average rate constant k'_p for the inhibition of AChE by soman in presence of these compounds. Additionally, in Table 2 the ratio k'/k'_p is given as an expression for the protective efficiency.

4. *Equilibrium constants* K_M, K_I, K'_I. K_M and V_{max} were determined to be 1.58×10^{-4} M (± 0.16) and 0.475 (± 0.022) μmoles acetylcholine per min, respectively. These values are in good agreement with the data reported in the literature [12, 17].

All inhibition experiments showed both competitive and noncompetitive inhibition of AChE by the pyridinium salts. Eq. 1 was used for the calculations of K_I and K'_I, except the weak inhibitors HS 8, P 60 and P 61, the inhibition constants of which were estimated from a Dixon plot [18]. The results are included into Table 2.

In each case the noncompetitive part of inhibition (K'_I) is smaller than the competitive one. The gradation of the K'_I-values resembles the sequence of the K_I-data, but there is no linear correlation between them.

The double-logarithmic plot of the protective efficiency k'/k'_p versus the competitive inhibition constant K_I (Fig. 1) shows an excellent correlation between these two parameters. Concerning the K'_I-values this correlation is by far not so significant.

Lengthening of the alkylene bridge in the group of bis-*tert* butylpyridinium-alkylene halogenides results in an increase of protective activity; P 66 is the most effective compound *in vitro*. The urea derivatives show an interesting gradation between the 4,4-, 3,4- and 3,3-isomers (HH 39, HH 54, HH 38).

These results do not fully correspond to the *in vivo* data: there we found indeed a gradation with respect to the length of the alkylene bridge, but as a significant exception P 65 exhibits the highest biological activity; among the urea compounds the unsymmetrical 3,4-isomer HH 54 proved to be the best protector. Thus, no clear correlation can be stated between the *in vivo* and *in vitro* results. Whether the shielding of AChE observed *in vitro* is of any relevance for the protection by pyridinium salts *in vivo*, requires further investigation.

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