Prevention of physostigmine-, DFP-, and diazinon-induced acute toxicity by monoethylcholine and N-aminodeanol

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1 Choline, and the choline analogues monoethylcholine (MEC) and N-aminodeanol (NAD) were examined for prophylactic activity in acute acetylcholinesterase inhibitor toxicity in mice. The rank order of potency of the compounds was MEC > NAD > choline.

2 Simultaneous administration of MEC (60 mg kg⁻¹) or NAD (200 mg kg⁻¹) with physostigmine reduced lethality to 17 and 13% respectively. MEC (60 mg kg⁻¹) completely protected against diisopropylfluorophosphate (DFP) and diazinon toxicity, and NAD reduced lethality to 17% for both agents. Choline (200 mg kg⁻¹) exhibited only negligible antidotal activity against the inhibitors.

3 *In vitro* concentrations of choline, MEC, and NAD, similar to the estimated concentration obtained *in vivo* in the acute toxicity study, produced mixed inhibition of mouse brain acetylcholinesterase. The inhibition was dose-related and was additive to the inhibition produced by the cholinesterase inhibitors.

4 All three analogues reduced ligand binding at the nicotinic, M₁, and M₂ receptors. The rank order of potencies for the analogues at each receptor was nicotinic: (choline > MEC > NAD), M₁: (MEC > choline > NAD), and M₂: (MEC > choline > NAD).

5 It is proposed that the analogues prevent acetylcholinesterase inhibitor toxicity peripherally by interacting with acetylcholinesterase, and/or by competing with acetylcholine for binding to cholinergic receptors.

Introduction

Cholinesterase inhibitors primarily inhibit acetylcholinesterase (AChE) and secondarily inhibit other esterases that hydrolyze choline esters (Wills, 1970; Chemnitz *et al.*, 1988). After inhibition of these enzymes acetylcholine accumulates at all cholinergic nerve endings and cholinergic receptors (Holmes, 1953) and in human plasma (Okonek & Kilbinger, 1974). Toxic doses of these agents may produce cardiovascular and respiratory depression mediated by excessive central and peripheral acetylcholine receptor stimulation. Although the probable cause of death is anoxia (Clement & Lee, 1980) it is unclear

whether central respiratory depression (Meeter & Wolthuis, 1968) or peripheral respiratory paralysis (Clement & Lockwood, 1982) is responsible. Central or peripheral toxicity also appears to vary with the nature of the acetylcholinesterase inhibitor (van Helden *et al.*, 1983).

A standard clinical antidote for AChE inhibitor toxicity is available which consists of atropine to block the muscarinic effects of acetylcholine and pralidoxime to reactivate acetylcholinesterase. However, the nicotinic effects of acetylcholine (ganglionic and diaphragmatic stimulation) remain untreated. The effectiveness of the antidote also depends on the type of inhibitor (i.e. organophosphate or carbamate)

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responsible for the toxicity as well as the time lag between exposure and treatment (Fleisher & Harris, 1965). The combination of an oxime and anticholinergic agent is effective for organophosphate toxicity (Leadbeater *et al.*, 1985). However, in carbamate intoxication, atropine, but not pralidoxime, is recommended since the latter is ineffective against some carbamates and may actually increase toxicity in the case of carbaryl (Natoff & Reiff, 1973). Experimental antidotal approaches have included the use of soman simulator 'Som-Sim' (Wolthuis *et al.*, 1981), choline acetyltransferase inhibitors (Schoene *et al.*, 1977), reversible cholinesterase inhibitors (Harris *et al.*, 1978), and hemicholinium-3 (HC-3) (Lin *et al.*, 1987).

Since the available treatment of AChE inhibitor toxicity is less than satisfactory for carbamates, it was the purpose of the present study to examine analogues of choline as possible antidotes. The rationale for this approach was based on the premise that the analogues will compete with acetylcholine for binding to acetylcholine receptors. A reduction in acetylcholine-mediated toxicity should result since the analogues possess both reduced affinity and intrinsic activity for these receptors. Mechanisms of action of the analogues with respect to their effect on the kinetics of inhibition of AChE and their affinity for M_1 , M_2 , and nicotinic receptors are also presented.

Methods

Virus free male ICR mice (15–20 g) and Sprague Dawley rats (175–200 g) were obtained from Harland Industries (Prattville, AL and Indianapolis, IN, U.S.A., respectively). All animals were housed in a temperature-controlled environment (25°C) with a 12 h light/dark cycle. Mouse brain or rat heart tissue was obtained following decapitation with a guillotine.

Prevention of acetylcholinesterase inhibitor toxicity

The ability of choline, monoethylcholine (MEC), or N-aminodeanol (NAD) to prevent acetylcholinesterase inhibitor toxicity was determined by simultaneous administration (i.p.) with acetylcholinesterase inhibitor in mice. The observation period for the acute toxicity of physostigmine was 30 min, and 6 h for diisopropylfluorophosphate (DFP) or diazinon. Physostigmine (0.8 mg kg⁻¹) and diazinon (60 mg kg⁻¹) were administered intraperitoneally and DFP (6 mg kg⁻¹) was administered subcutaneously.

Estimation of in vivo concentration

Initial *in vivo* concentrations of the analogues after intraperitoneal administration were estimated on the following assumptions: (1) 80% of the weight of the animal constituted total body water, (2) the dose was distributed through total body water. The initial concentration was then estimated to be the dose, divided by the total body water.

Acetylcholinesterase inhibition

A modification of the spectrophotometric method of Ellman *et al.* (1961) was used to determine acetylcholinesterase activity. A 25% homogenate of whole mouse brain (free of cerebellum and lower brain stem) in 0.1 M sodium potassium phosphate buffer (pH 7.9) containing 1% Triton-X-100 was prepared using a Polytron (setting = 5). The homogenate was centrifuged at 40,000 *g* for 30 min, and the supernatant used for determining cholinesterase activity. Acetylcholinesterase inhibitor or choline analogue was pre-incubated with enzyme for 2 min before addition of substrate. All enzyme velocities were determined at 20°C during the initial 1–2 min of reaction.

Binding assays

The affinity of choline, MEC and NAD for three types of acetylcholine receptors was examined by variations of a filtration assay (Aronstam *et al.*, 1978). Affinity was determined from the ability of the analogue to inhibit the binding of ligand to the receptor. The ratio of the concentration of labelled ligand to its K_d was 0.5 for [³H]-pirenzepine, 1.0 for [³H]-N-methylscopolamine, and 230 for [¹²⁵I]- α -bungarotoxin. Data analysis was performed using the programme LIGAND.

The affinity of the analogues for the M_1 receptor was examined in mouse brain cerebral cortex tissue by use of [³H]-pirenzepine. Cerebral cortex tissue was homogenized in 10 volumes of sodium potassium phosphate buffer (pH 7.4, 0.05 M) in an Elvehjem apparatus with a teflon pestle. The homogenate was then centrifuged at 1000 *g* for 10 min, the supernatant recentrifuged at 40,000 *g* for 1 h and the pellet resuspended in buffer. The reaction volume was 1 ml and contained 200 μ g protein, 100 μ M DFP, 3 nM [³H]-pirenzepine, choline analogue, and sodium potassium phosphate buffer. Choline analogue and ligand were added 20 min after the addition of DFP, and incubated an additional 40 min at 20°C. The assay was terminated by filtration through Whatman GF/B filters. Non-specific binding was determined with 1 μ M atropine.

Table 1 Reversal of acetylcholinesterase inhibitor toxicity by monoethylcholine (MEC), N-aminodeanol (NAD) and choline (Ch)

Treatment	n	Observation period (min)	Time to death (min)	% deaths
Physostigmine (0.8 mg kg ⁻¹)	21	30	6.0 ± 3.0	87.7
Physostigmine + MEC (60 mg kg ⁻¹)	6	30	27.0*	16.7
Physostigmine + NAD (200 mg kg ⁻¹)	8	30	7.4*	12.5
Physostigmine + Ch (200 mg kg ⁻¹)	6	30	5.9	83.3
DFP (6 mg kg ⁻¹)	26	360	12.1 ± 0.8	96.2
DFP + MEC (60 mg kg ⁻¹)	8	360	0	0
DFP + NAD (200 mg kg ⁻¹)	9	360	20.2 ± 3.4	100
DFP + NAD (600 mg kg ⁻¹)	6	360	22.5*	16.7
DFP + Ch (200 mg kg ⁻¹)	9	360	16.7 ± 2.8	88.9
Diazinon (60 mg kg ⁻¹)	29	360	33.1 ± 8.8	93.1
Diazinon + MEC (60 mg kg ⁻¹)	6	360	0	0
Diazinon + NAD (400 mg kg ⁻¹)	6	360	35.0*	16.7
Diazinon + NAD (600 mg kg ⁻¹)	6	360	7.0 ± 2.2	50.0
Diazinon + Ch (200 mg kg ⁻¹)	9	360	22.1 ± 3.1	77.8

Male ICR mice (18–25 g) were administered inhibitor alone or concurrently with choline analogue. All agents were administered i.p. except for DFP (administered s.c.). Solvents used were: water (physostigmine, MEC, NAD, Ch); 0.15 M NaCl (DFP); and propyleneglycol (diazinon). Average time to death does not include survivors.

n = Number of animals; ± = standard error of mean

* n = 1

Rat heart was the tissue source for M₂ muscarinic receptor material. After rinsing in buffer, the tissue was minced with scissors, and homogenized in 10 volumes of sodium potassium phosphate buffer (pH 7.4, 0.05 M) in a Polytron (2 × 30 s, speed 6), and further homogenized in an Elvehjem apparatus with a teflon pestle. The homogenate was then centrifuged at 1000 g for 10 min, the pellet rehomogenized with fresh buffer as above and centrifuged at 1000 g. The 1000 g supernatants were pooled and centrifuged at 40,000 g for 35 min, and the pellet resuspended in buffer. The reaction volume used was 2 ml and contained 500 µg protein, 100 µM DFP, 0.3 nM [³H]-scopolamine methyl chloride, choline analogue, and sodium potassium phosphate buffer. The remaining assay procedure was the same as for the M₁ receptor assay.

The source of nicotinic receptor material was the electric organ of *Torpedo californica* (Pacific Bio-marine, California). Frozen tissue was homogenized in 13 volumes of NaCl-Tris buffer (0.4 M NaCl:0.01 M Tris, pH 7.2) in a Waring blender for 1 min (high speed). The homogenate was centrifuged at 2000 g for 10 min, the supernatant recentrifuged at 30,000 g for 30 min, and the pellet resuspended in buffer. The reaction volume used was 1 ml and contained 25 µg of protein, 100 µM DFP, 1 nM [¹²⁵I]-α-bungarotoxin, choline analogue, and NaCl-Tris buffer. Incubation conditions were similar to the M₁ assay above.

Methylated bovine serum albumin (BSA) was added at the end of the incubation period (final concentration 3 mg ml⁻¹) to reduce non-specific filter binding to Whatman GF/C filters. Non-specific binding was defined with unlabelled α-bungarotoxin (1 µM).

Reagents and isotopes

Physostigmine sulphate (Sigma) and choline chloride (Sigma) were prepared in distilled water and diisopropylfluorophosphate (DFP, Sigma) was prepared in 0.15 M sodium chloride. Diazinon (Ciba-Geigy) was dissolved in propylene glycol and diazoxon (Ciba-Geigy) was dissolved in water. All cholinesterase inhibitor solutions were prepared fresh immediately before use. [N-methyl-³H]-scopolamine methyl chloride (72 Ci mmol⁻¹) was purchased from Amersham, U.K. [N-methyl-³H]-pirenzepine (85 Ci mmol⁻¹) and [¹²⁵I]-α-bungarotoxin (17.3 µCi µg⁻¹) were purchased from New England Nuclear.

Monoethylcholine iodide (MEC) was prepared by reacting N,N-dimethyl-aminoethanol and ethyl iodide. N-aminodeanol perchlorate (N-amino-N,N-dimethylaminoethanol) was prepared according to the method of Newton *et al.* (1983). All synthesized compounds were recrystallized and their structures confirmed using IR, n.m.r., and melting points.

Results

Prevention of acetylcholinesterase inhibitor toxicity

Table 1 summarizes the ability of several choline analogues to prevent the acute toxicity of reversible and irreversible acetylcholinesterase inhibitors. Physostigmine produced fatalities in ~88% of mice tested with an average time to death of 6 min. Simultaneous administration of monoethylcholine (MEC, 60 mg kg⁻¹) or N-aminodeanol (NAD, 200 mg kg⁻¹) reduced the deaths to ~17% and ~13% respectively. Simultaneous administration of choline did not appear to alter toxicity or the time to death. DFP was lethal in ~96% of mice tested. Simultaneous administration of MEC (60 mg kg⁻¹) afforded complete protection against DFP. NAD, inactive at 200 mg kg⁻¹, reduced lethality to ~17% when administered at 600 mg kg⁻¹. Choline (200 mg kg⁻¹) reduced lethality to 89%. The acute toxicity of diazinon was completely blocked by simultaneous administration of 60 mg kg⁻¹ MEC. NAD (400 mg kg⁻¹) reduced deaths to ~17% but increased toxicity when administered at 600 mg kg⁻¹. Choline at 200 mg kg⁻¹ reduced deaths to ~78%.

Acetylcholinesterase inhibition

The choline analogues were also examined for their ability to affect the kinetics of acetylcholinesterase (AChE) in the presence and absence of AChE inhibitors. The cuvette concentration of the choline analogues was selected to match the estimated *in vivo* concentration achieved in the acute toxicity studies. In Figure 1, MEC60 corresponds to a cuvette concentration of 0.33 mM monoethylcholine (MEC) which was approximated *in vivo* with a dose of 60 mg kg⁻¹ (i.p.). MEC alone produced a mixed inhibition of AChE. In the presence of the reversible inhibitor physostigmine, MEC60 produced a mixed inhibition which was additive to the effect of physostigmine. Further inhibition was observed with a two fold higher concentration of MEC (0.66 mM, MEC120). In the presence of the irreversible inhibitor DFP, MEC also produced a dose-related mixed inhibition which was additive to the effect of DFP. The *in vitro* AChE inhibitor activity of the irreversible agent diazinon was determined with its biologically active metabolite, diazoxon (Kreuger *et al.*, 1960). In the presence of diazoxon, MEC also produced a dose-related inhibition which was additive to the effect of diazoxon. In Figure 2, NAD200 corresponds to a cuvette concentration of N-aminodeanol (NAD) of 1.8 mM which was

Figure 1 Effect of monoethylcholine (MEC) and inhibitor on acetylcholinesterase (AChE). AChE activity was determined with 40,000 *g* mouse brain supernatant at 20°C by the Ellman method. Inhibitor and analogue were preincubated for 2 min before addition of substrate. Velocity (V) is expressed as $\mu\text{M min}^{-1} \text{mg}^{-1}$ protein, and (S) as mM^{-1} . Phys and P refer to physostigmine; DFP and D refer to diisopropyl fluorophosphate; Diaz and Dz refer to diazinon (diazoxon). Additional experimental details appear in the legend to Table 2 and in the methods section.

approximated *in vivo* with a dose of 200 mg kg⁻¹ (i.p.). NAD alone produced a mixed inhibition of AChE. In the presence of physostigmine, DFP, or diazoxon, NAD also produced a dose-dependent

Figure 2 Effect of N-aminodeanol (NAD) and inhibitor on acetylcholinesterase (AChE). AChE activity was determined with 40,000 g mouse brain supernatant at 20°C by the Ellman method. Inhibitor and analogue were preincubated for 2 min before addition of substrate. Velocity (V) is expressed as $\mu\text{M min}^{-1}\text{mg}^{-1}$ protein, and (S) as mM^{-1} . Phys and P refer to physostigmine; DFP and D refer to diisopropylfluorophosphate; Diaz and Dz refer to diazinon (diazoxon). Additional experimental details appear in the legend to Table 2 and in the methods section.

mixed inhibition of AChE which was additive. Mixed inhibition of AChE was also observed for choline at 1.8 mM (CH200) in the presence or absence of all three AChE inhibitors (Figure 3).

Table 2 contains the kinetic constants obtained in the AChE studies. Choline, MEC, and NAD all decreased the velocity of AChE, as shown by increased slopes, alone or in the presence of the AChE inhibitors. In the absence of inhibitor or in the presence of physostigmine, choline, MEC, and NAD decreased V_{max} . However in the presence of DFP, MEC decreased V_{max} , whereas NAD and choline increased V_{max} . In the presence of diazoxon, choline, MEC, and NAD all increased V_{max} . With the exception of the effect of MEC in the presence of physostigmine, the choline analogues appeared to increase K_i for each AChE inhibitor.

Affinity of choline analogues for cholinceptors

The affinity of the choline analogues for cholinceptors was determined by competition binding assays. MEC appeared to be the most potent at the M_1 receptor displacing [^3H]-pirenzepine with an IC_{50} of 1.2 mM (Figure 4, Table 3). A similar pattern was observed at the M_2 receptor for MEC, displacing [^3H]-methylscopolamine with an IC_{50} of 0.51 mM. At the nicotinic receptor, the effect of the analogues was less clear due to intersection of the binding curves near their IC_{50} values. Choline appeared to be the most potent in displacing [^{125}I]- α -bungarotoxin with an IC_{50} value of 26 mM. For both the M_1 and M_2 receptor there is fairly close agreement (see Table 3) between the IC_{50} value of the analogue and its binding affinity (K_i). The IC_{50} and K_i values are less similar for the nicotinic receptor and may reflect covalent attachment of α -bungarotoxin to the receptor (Bennet, 1978), and the higher ligand/ K_d ratio used in the assay. As shown by the B_{max} values, there is approximately 50 times more receptor material/mg protein in nicotinic than in M_1 and M_2 tissue sources.

Discussion

Choline, and the choline analogues monoethylcholine (MEC) and N-aminodeanol (NAD) were examined for their ability to prevent acute acetylcholinesterase (AChE) inhibitor toxicity in mice. The AChE inhibitors used included a reversible agent (physostigmine) and two irreversible organophosphates (DFP and diazinon). Diazinon is a thionophosphate and was included as an example

Figure 3 Effect of choline (Ch) and inhibitor on acetylcholinesterase (AChE). AChE activity was determined with 40,000 *g* mouse brain supernatant at 20°C by the Ellman method. Inhibitor and analogue were preincubated for 2 min before addition of substrate. Velocity (*V*) is expressed as $\mu\text{M min}^{-1} \text{mg}^{-1}$ protein, and (*S*) as mM^{-1} . Phy and P refer to physostigmine; DFP and D refer to diisopropylfluorophosphate; Diaz and Dz refer to diazinon (diazoxon). Additional experimental details appear in the legend to Table 2 and in the methods section.

Figure 4 Binding affinity of choline (Ch), monoethylcholine (MEC) and N-aminodeanol (NAD) at M_1 (a), M_2 (b) and nicotinic (c) cholinergic receptors. Assay parameters (tissue source, ligand, incubation volume) for each procedure were: nicotinic (torpedo electric organ, [^{125}I]- α -bungarotoxin, 1 ml); M_1 (mouse brain cortex, [^3H]-pirenzepine, 1 ml); and M_2 (rat heart, [^3H]-methylscopolamine, 2 ml). After 20 min preincubation with DFP (100 μM), ligand was added and incubated an additional 40 min at 20°C. Additional experimental details appear in the Methods section. Data were analysed using the programme LIGAND.

of an agent that undergoes metabolic activation by the host to the more active phosphate analogue diazoxon (Kanazawa, 1983). Accordingly a slower onset in toxicity would be expected compared to physostigmine or DFP. The average time to death for diazinon was found to be approximately 3 times

Table 2 Effect of monoethylcholine (MEC), N-aminodeanol (NAD), or choline (Ch) on the kinetics of acetylcholinesterase inhibitors

Inhibitor ^a	Analogues ^b	Relative slope ^c	V _{max} (% of control)	K _i (M)
Physostigmine	—	1.7	81	5.0 × 10 ⁻⁵
	MEC (60)	2.0	64	4.9 × 10 ⁻⁵
	MEC (120)	2.3	55	5.1 × 10 ⁻⁵
	NAD (200)	2.7	78	1.3 × 10 ⁻⁴
	NAD (400)	3.9	88	2.3 × 10 ⁻⁴
	Ch (200)	2.7	74	1.3 × 10 ⁻⁴
DFP	—	1.3	68	5.1 × 10 ⁻⁵
	MEC (60)	1.5	59	5.7 × 10 ⁻⁵
	MEC (120)	2.3	62	8.6 × 10 ⁻⁵
	NAD (200)	2.3	76	1.3 × 10 ⁻⁴
	NAD (500)	3.9	87	2.1 × 10 ⁻⁴
	Ch (200)	4.5	100	2.8 × 10 ⁻⁴
Diazoxon ^d	—	1.5	61	6.6 × 10 ⁻⁵
	MEC (60)	3.1	73	7.3 × 10 ⁻⁵
	MEC (120)	3.8	67	8.0 × 10 ⁻⁵
	NAD (200)	4.0	100	3.1 × 10 ⁻⁴
	NAD (500)	4.3	72	2.2 × 10 ⁻⁴
	Ch (200)	4.3	96	2.5 × 10 ⁻⁴
—	MEC (60)	1.7	57	9.7 × 10 ⁻⁵
—	NAD (200)	1.7	88	1.1 × 10 ⁻⁴
—	Ch (200)	2.7	94	1.6 × 10 ⁻⁴

Acetylcholinesterase activity was determined with 40,000 *g* mouse brain supernatant at 20°C by the Ellman method. Inhibitor and analogue were preincubated for 2 min before addition of substrate.

^a Concentrations used for physostigmine, diisopropylfluorophosphate (DFP), and diazoxon were 25 μM, 3 μM, and 1 μM, respectively.

^b The cuvette concentration of the analogue approximated the *in vivo* concentration achievable following parenteral administration. The number in parentheses corresponds to the dose administered in mg kg⁻¹. The cuvette concentration for MEC (60) was 0.33 mM, and was 1.8 mM for NAD (200) and Ch (200), respectively.

^c Compared to the uninhibited enzyme rate.

^d Biologically active metabolite of diazinon.

Table 3 Cholinoceptor binding constants for monoethylcholine (MEC), N-aminodeanol (NAD) and choline (Ch)

Receptor	Agonist	η _H	B _{max} (fmol mg ⁻¹)	IC ₅₀ (M)	K _i (M)
Nicotinic	ACh	0.40	2,530	2.5 × 10 ⁻⁷	1.1 × 10 ⁻⁹
	MEC	0.67	2,430	3.3 × 10 ⁻²	1.5 × 10 ⁻⁴
	NAD	0.63	2,580	4.8 × 10 ⁻²	2.1 × 10 ⁻⁴
	Ch	0.40	2,250	2.6 × 10 ⁻²	1.1 × 10 ⁻⁴
Muscarinic M ₁	ACh	0.56	37	1.6 × 10 ⁻⁵	7.9 × 10 ⁻⁶
	MEC	0.44	38	1.2 × 10 ⁻³	5.8 × 10 ⁻⁴
	NAD	0.71	41	4.3 × 10 ⁻³	2.2 × 10 ⁻³
	Ch	0.48	40	1.5 × 10 ⁻³	7.7 × 10 ⁻⁴
Muscarinic M ₂	ACh	0.67	53	7.0 × 10 ⁻⁷	3.4 × 10 ⁻⁷
	MEC	0.89	58	5.1 × 10 ⁻⁴	2.5 × 10 ⁻⁴
	NAD	0.79	58	2.6 × 10 ⁻³	1.3 × 10 ⁻³
	Ch	0.83	62	9.2 × 10 ⁻⁴	4.5 × 10 ⁻⁴

Assay parameters (tissue source, ligand, incubation volume) for each procedure were: nicotinic (torpedo electric organ, [¹²⁵I]-α-bungarotoxin, 1 ml); M₁ (mouse brain cortex, [³H]-pirenzepine, 1 ml); and M₂ (rat heart, [³H]-methylscopolamine, 2 ml). After 20 min preincubation with DFP (100 μM), ligand was added and incubated an additional 40 min at 20°C. Additional experimental details appear in the legend to Figure 4 and in the Methods section.

that of DFP (Table 1), and correlates with the time of maximal diazoxon formation in the mouse (Kreuger *et al.*, 1960).

The rank order of effectiveness for the analogues against AChE inhibitor toxicity appears to be MEC $\gg$ NAD $>$ choline. MEC was approximately three times more active than NAD in physostigmine toxicity, and 6 and 10 times more active in diazinon and DFP toxicity respectively. The higher dose of NAD used (600 mg kg^{-1}) exacerbated diazinon toxicity but was maximally effective in DFP toxicity. This may reflect an interaction with diazinon rather than toxicity of the analogue itself since doses of NAD alone up to 800 mg kg^{-1} were non-toxic. The slight activity observed with choline agrees with data reported by Stovner (1956). In the present study, the maximal antidotal dose of choline was limited to 200 mg kg^{-1} . Administration of choline at 400 or 600 mg kg^{-1} ($n = 3$, data not shown), produced a dose-related synergistic toxicity with DFP or diazinon. The toxicity data suggest that the prevention of toxicity observed reflects a structure-activity relation of the analogues and is not based solely on a non-specific quaternary function modality. Since choline, MEC, and NAD are quaternary agents with limited distribution to the CNS, it is probable that the antidotal action observed against the AChE inhibitors occurs primarily on the periphery.

The *in vitro* data presented demonstrate that choline, MEC, and NAD interfere with the kinetics of acetylcholinesterase (AChE). A similar inhibitory action for choline on AChE and serum cholinesterase has been observed (Adams & Whittaker, 1950). The analogues produced a dose-related mixed inhibition of AChE in the presence or absence of each of the three AChE inhibitors (Figures 1, 2, 3). Based on the cuvette concentrations used, MEC appeared to be the most active with NAD and choline approximately equal to each other in potency. In general, the presence of the analogue and inhibitor increased the K_i values compared to the inhibitor alone. However, the effect of the analogues was more complex with respect to the effect on V_{max} (Table 2). Depending on the AChE inhibitor, V_{max} values were observed to increase and decrease. The data suggest that the analogues temporarily bind to the enzyme, causing AChE velocity to slow. It is proposed, therefore, that the rate of carbamylation or phosphorylation of the enzyme would also slow. A similar effect has been observed for the effect of tetra-N-alkylammonium ions on the rate of phosphorylation of AChE by the organophosphate O-ethyl S-n-propyl methyl phosphonothiolate (Sellers *et al.*, 1983). The *in vivo* reduction in toxicity observed could then be explained in terms of the additional time that would then be allowed for inactivation of the AChE inhibitor by mixed-function

oxidases (Smith *et al.*, 1974; Cremlyn, 1978). A similar protective effect has been reported for the reduction in toxicity of organophosphates by reversible AChE inhibitors (Harris *et al.*, 1978) and clonidine (Aronstam *et al.*, 1986). It is also possible that the observed reduction in physostigmine toxicity is due to an increase in the rate of decarbamylation of AChE. A parallel effect has been noted for the ability of tetra-N-alkyl ammonium ions to alter the rate of deacylation of AChE (Sellers *et al.*, 1983) and for excess free carbamate to increase decarbamylation of AChE (Watts & Wilkinson, 1977). This hypothesis is supported by the observation that the amount of AChE inhibition produced by the combination of inhibitor and analogue was not additive when compared to the sum of the effects of either agent alone.

Choline, MEC, and NAD exhibited binding affinities for M_1 , M_2 and nicotinic receptors (Figure 4). At M_1 and M_2 receptors, the rank order of potency was MEC $>$ choline $>$ NAD, whereas at the nicotinic receptor, the order of potency was choline $>$ MEC $\cong$ NAD. Although different ligands were used in the present study, similar K_i values and rank order potencies were obtained for choline and MEC on M_1 and nicotinic receptors (Buccafusco & Aronstam, 1988). Based on the *in vivo* concentrations achieved and the IC_{50} values obtained in the binding experiments, the analogues should compete *in vivo* to a greater extent with acetylcholine for binding to M_1 and M_2 receptors than to nicotinic receptors. Although less competition with acetylcholine would occur at the nicotinic receptor with the *in vivo* doses used, the extent of acetylcholine displacement at the diaphragm during AChE inhibitor toxicity may still be significant.

The present study demonstrates that choline analogues prevent acetylcholinesterase inhibitor toxicity if administered at the time of exposure to the inhibitor. A more practical treatment would be one that could be instituted after exposure to the inhibitor. Preliminary data suggests that post treatment with MEC may be an effective method of reducing DFP toxicity. Administration of MEC (60 mg kg^{-1}) 2 min after DFP administration increased the time to death from 12.1 ± 1.2 ($\pm$ s.e.) to 19.3 ± 3.8 ($\pm$ s.e.) min and reduced fatalities from 96% to 85% ($n = 7$, data not shown). It should be noted that the pharmacokinetics of MEC are unknown at present and further studies with respect to the dose of MEC and the interval of post-treatment are needed to evaluate fully post-treatment efficacy. Data are not available for the post-treatment use of NAD or choline.

It is unclear why choline was the least effective antidote for the three cholinesterase inhibitors examined, yet exhibited receptor binding and AChE interactions with a potency similar to NAD. Also,

differences in activity and potency were observed for the analogues, suggesting that a structural-activity relationship exists for the pharmacological parameters investigated. From the data obtained, it is proposed that the mechanism of antidotal activity for MEC and NAD in acute cholinesterase inhibitor toxicity is peripheral rather than central. It is further proposed that the analogues bind reversibly to AChE to prevent covalent attachment of the inhibi-

tor and/or compete with acetylcholine for binding to muscarinic and nicotinic receptors.

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