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CHOLINERGIC AND GABAERGIC NEUROTOXICITY OF SOME ALKYLATING AGENTS

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Abstract—A series of nitrogen mustard derivatives was tested for neurotoxic effects on cholinergic and GABAergic markers at three rat brain regions: hippocampus, striatum and cortex. All compounds were administered intracerebroventricularly, and the enzymatic activities were measured 7 days after treatment. The effects of synthesised nitrogen mustard derivatives with indole, quinoline and hemicholinium backbone structures were compared. Of these compounds, only the hemicholinium analogue showed some preferential neurotoxicity to cholinergic neurones, thus offering a basis for designing novel, more specific cholinergic neurotoxins.

A great deal of evidence has accumulated, particularly in the last few years, suggesting that a central cholinergic deficit may be a primary biochemical indicator in senile dementia of the Alzheimer's type and some other mental disorders [1–5]. In particular, the activities of choline acetyltransferase (ChAT) in the cerebral cortices of patients suffering from senile dementia of Alzheimer's type correlate well with the occurrence of neuritic plaques [6]. The availability of an animal model of cholinergic hypofunction would be a great help to test the so-called "cholinergic hypothesis" in this and related disorders. ChAT is a fairly stable enzyme which does not demonstrate marked post-mortem changes [7], making it a suitable index of cholinergic function for experimental studies.

To develop an animal model of cholinergic deficiency it would be essential to obtain *in vivo* inhibitors of ChAT or neurotoxins which specifically affect cholinergic markers. Among the most extensively studied *in vitro* inhibitors of ChAT are some halogenated substances which probably acylate the active site of ChAT that is normally acylated by AcCoA. Analogues of styrilpyridine presumably exhibit inhibitory effects due to Van der Waals' forces and hydrophobic binding [8]. Quinones, primarily naphthaquinones, react similarly to halogenated substrate derivatives with sulfhydryl (SH) groups within the enzymatic structure [9]. However, none of these compounds appears to produce an appreciable effect on ChAT activity *in vivo*.

One of the most powerful inhibitors of ChAT activity is hemicholinium Hc-3. This molecule is also a highly specific and potent inhibitor of sodium-dependent high-affinity uptake for choline [10, 11]. However, its inhibition is reversible [12].

In an attempt to induce a prolonged irreversible effect on cholinergic neurones, the hemicholinium molecule was modified by introducing an alkylating group which could covalently bind with nucleophilic sites in the choline uptake and acetylating system. To assess the specificity of potential ChAT inhibitory and neurotoxic effects of the hemicholinium structural analogue, and several other alkylating agents with the same functional alkylating group, the enzymatic activities of ChAT and, as a control, glutamic acid decarboxylase (GAD) were investigated in three main areas of the rat brain.

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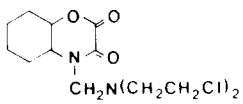
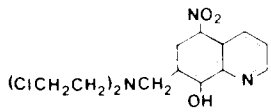
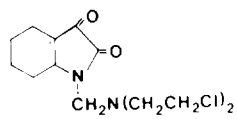
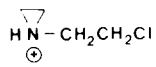
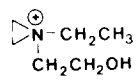
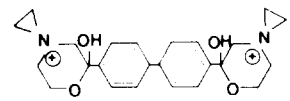
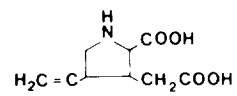
MATERIALS AND METHODS

Compounds. The structures of the compounds used in this study are presented in Table 1. Solutions were prepared as previously described by Tagari *et al.* [27] and diluted with saline to a final concentration of 4 nmoles/μl, as was kainic acid (KA) (Sigma Chemical Co., UK). All solutions were maintained at 4° and used within 4 hr of preparation. [^{14}C]DL-Glutamic acid and [^{14}C]acetyl-CoA were purchased from Amersham International (UK). All other reagents were of the highest available purity.

Neurotoxin administration. Male Wistar rats (300 g) were anaesthetized with Equithesin (250 μl/kg) and prepared for unilateral (left) stereotactic intracerebroventricular (i.c.v.) injection of the compounds presented in Table 1. Vehicle or drug solution (2 μl) was infused into the left ventricle (co-ordinates 0.6 mm posterior and 2.0 mm lateral from bregma, 3 mm ventral from dura, adjusted from Konig and Klippel [28]). Coordinates were initially confirmed by injecting an aniline dye. After 7 days the animals were decapitated.

Tissue sampling. Brains were rapidly removed and placed on ice, and the entire striatum and hippocampus from each side and a slice of cortex extending 3 mm rostral and 3 mm caudal from the coronal plane of the injection co-ordinate were dissected out. The tissues were rapidly weighed and mechanically homogenised in 10 vol. (w/v) of homogenising medium [phosphate buffer (pH 7.4) containing 10 nmoles disodium EDTA (BHD Chemicals Ltd., UK) and 0.5% (v/v) Triton X-100 (Sigma Chemical Co., UK)].

Table 1. List of compounds tested in this study.

	Formula	Abbreviation	Reference
1		BzM	13
2		QnM	14
3		IdM	14
4		M	15
5		AF64A	16–24
6		HcM	25*
7		KA	26

* B. Zorc, D. Maysinger and I. Butula, data presented at the Congress on Chemistry, Zagreb, Yugoslavia, 12–14 Feb. 1985.

Biochemical determinations. Aliquots (5 μl) of homogenates were taken for the determination of protein concentration as measured by a dye binding method [29]. ChAT and GAD enzymatic activities were estimated [30–32] in freshly thawed aliquots of homogenates that had been maintained at −70°. All determinations were performed in duplicate with experimental samples and their appropriate vehicle control samples in the same assay.

Statistical analyses. Results are expressed as group mean ± S.E.M. The statistical comparisons between control and mustard-treated animals were performed applying an unpaired Student's *t*-test.

RESULTS

ChAT activities in three brain regions (hippocampus, striatum and cortex) 7 days after the drug treatment are shown in Fig. 1. A marked decrease in activity (approximately 40%) was observed in the hippocampus after treatment with the hemicholinium analogue HcM. Of the other compounds tested, only the benzoxazine derivative BzM and nor-nitrogen mustard M exhibited considerably smaller reductions of ChAT activity (about 26%). HcM was slightly more effective in the striatum than in the hippocampus. Other compounds did not modify ChAT activity significantly below the 5% level in the striatum. The losses in enzymatic activity of ChAT in the cortex were analogous to those observed in the striatum; thus, a greater loss of ChAT activity was observed after the administration of HcM, whereas isatine and quinoline derivatives failed to produce changes in ChAT activity in the investigated brain areas.

In vivo effects of GAD enzymatic activity are shown in Fig. 2. No loss of GAD enzymatic activity was observed with QnM or IdM. Nor-nitrogen mustard M weakly affected GAD activity in the hippocampus but not in the other two brain areas. The benzoxazine derivative BzM reduced GAD activity in the hippocampus by 44%, in the cortex by 55%, and in the striatum by approximately 20%, while the hemicholinium derivative HcM considerably decreased GAD activity in the hippocampus (42%) and even more in the striatum (67%). Preliminary observations by Tagari *et al.* [27] indicated that ethylcholine mustard aziridinium ion (AF64A) under comparable conditions decreases ChAT activity 47% in the hippocampus, 21% in the striatum and 31% in the cortex. GAD activity was not changed significantly in any of these brain areas using AF64A.

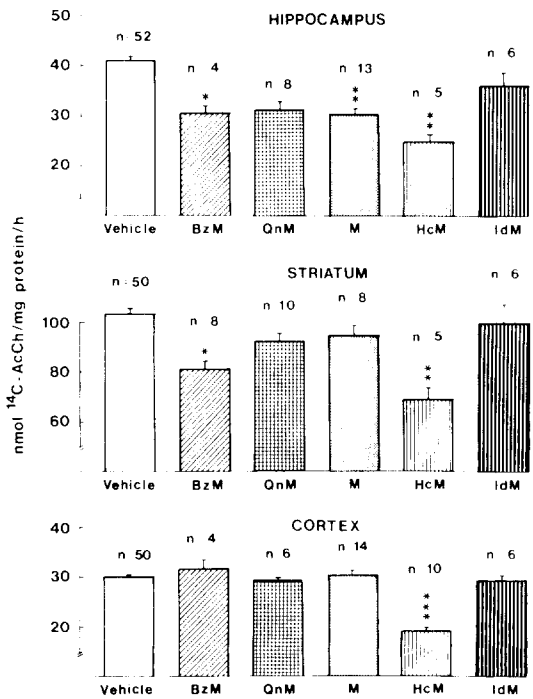


Fig. 1. *In vivo* effects of various nitrogen mustards on choline acetyltransferase activity (ChAT) in three brain regions of the rat. Ordinate: ChAT activities in hippocampus, striatum and cortex are expressed as means ± S.E.M.; n = number of animals. Enzymatic activities were determined as described previously [30, 31]. Key: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.005$. Abscissa: Abbreviations of the compounds (structures presented in Table 1) injected i.c.v. (8 nmoles/animal).

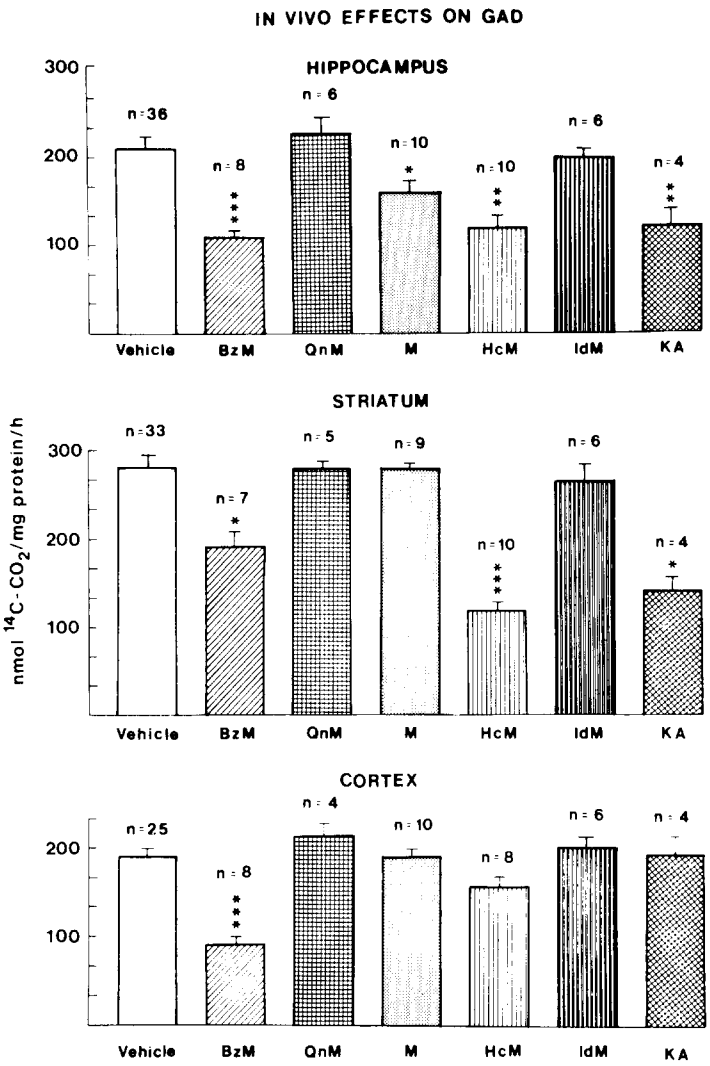


Fig. 2. *In vivo* effects of nitrogen mustard derivatives and kainic acid on glutamic acid decarboxylase (GAD) in the rat brain. Ordinate: GAD activities in hippocampus, striatum and cortex are expressed as means ± S.E.M.; n = number of animals. Enzymatic activities were determined as described previously [29, 32]. Key: (*) $P < 0.05$; (**) $P < 0.01$; and (***) $P < 0.005$. Abscissa: Abbreviations of the compounds injected i.c.v. (8 nmoles/animal) (structures of the compounds are presented in Table 1).

DISCUSSION

There have been attempts to develop a specific cholinergic neurotoxin in order to obtain an animal model for the study of central cholinergic deficiencies. So far, the most thoroughly investigated putative cholinergic neurotoxin is AF64A [23, 24]. Controversial results have been obtained with this compound depending on the applied doses, routes of administration, and biochemical methods utilised for the assessment of results [16–24]. According to Mantione *et al.* [24], Sandberg *et al.* [17] and Walsh *et al.* [33] AF64A may be used to produce a selective and persistent central cholinergic deficit. Injection of 8 nmoles of AF64A directly into striatum of the rat caused a 46% reduction in ChAT activity without affecting GAD or tyrosine hydroxylase [16]. In addition, behavioral studies by Walsh *et al.* [33] show that after the administration of this compound passive avoidance and radial arm deficits in rat can occur. Our preliminary results [27] support earlier reports indicating that AF64A can be used for production of a limited central cholinergic deficit in the rat, although this was also accompanied by a discrete loss of GABAergic marker.

The present studies suggest that some congeneric structures of hemicholinium may be more suitable candidates for selective cholinergic neurotoxins. Furthermore, the results from this study show clearly that the selection of a "carrier molecule" to which an alkylating group is attached (such as the chloroethylamino group) is crucial. A marked depletion of ChAT activity can be achieved with i.c.v. application of nor-nitrogen mustard (M), but this effect is not specific, as another enzymatic system (GAD) is also affected.

It seems that the most attractive and the most promising backbone structure for designing an effective and selective neurotoxic agent for cholinergic neurones is hemicholinium. This compound is a potent, reversible inhibitor of the high-affinity choline transport system thought to be located in nerve terminals [10, 11, 25]. The highly reactive bi-ethylenimine derivative of hemicholinium (HcM) caused a substantial decrement in the activity of ChAT, the cholinergic marker enzyme, in one area of the brain (cortex) without significantly affecting other non-cholinergic markers. The specificity of this compound is nevertheless relative as it has some effects on GAD in the hippocampus and striatum. The relative ineffectiveness of nitrogen mustard derivatives with isatine and quinoline structures may be at least partly ascribed to their instability in aqueous media [34] and to pharmacokinetic factors which can vary with structural properties of the molecules, e.g. lipophilicity and topology, and possibly other factors. From these experiments it seems that only the hemicholinium molecule, of the series investigated here, offers possibilities for structural modifications which may render a useful probe for specific and irreversible disruption of cholinergic neurotransmission. We are currently developing some new hemicholinium analogues with changed aromatic and N-aliphatic moieties to test this hypothesis.

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