The cholinergic pharmacology of tetrahydroaminoacridine *in vivo* and *in vitro*

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1 The effect of tetrahydroaminoacridine (THA) on cholinergically mediated behaviour in the rat and mouse has been investigated. In addition the actions of this compound on cholinesterase activity and on muscarinic and nicotinic receptors has also been examined.

2 Administration of THA (5–20 mg kg⁻¹, i.p.) produced a dose-dependent increase in tremor, hypothermia and salivation in both rats and mice. A similar profile of activity was seen following physostigmine (0.1–0.6 mg kg⁻¹) administration.

3 THA was approximately fifty fold less potent than physostigmine in inducing behavioural change but its effects persisted for over twice as long as those of physostigmine. For example THA-induced hypothermia was still present at 4 h in the mouse and 8 h in the rat.

4 *In vitro* THA was a potent non-competitive inhibitor of rat brain cholinesterase (IC₅₀: 57 ± 6 nM) and bovine erythrocyte acetylcholinesterase (IC₅₀: 50 ± 10 nM) but was a more potent inhibitor of horse serum butyrylcholinesterase (IC₅₀: 7.2 ± 1.4 nM).

5 Radioligand binding studies indicated that THA binds non-selectively but with moderate potency to both M₁ (K_i: 600 nM) and M₂ (K_i: 880 nM) muscarinic receptors. THA also interacted with the allosteric site present on cardiac M₂ receptors.

6 It is concluded that THA is a reversible non-competitive inhibitor of cholinesterase with a long half life (compared with physostigmine). It also may antagonize muscarinic receptors at high doses. The long half life may account for its reported efficacy in the treatment of Alzheimer's disease.

Introduction

The central cholinergic deficits found in patients with clinically diagnosed Alzheimer type dementia (ATD) have been reported to correlate with the degree of cognitive impairment (Perry *et al.*, 1978; Francis *et al.*, 1985). Therefore many attempts have been made to reverse the cognitive deficits by increasing cholinergic activity (reviewed by Brinkman & Gershon, 1983; Kopelman, 1986). In general these attempts have met with little success. Directly acting agonists such as bethanechol or arecoline produce little or no improvement (Christie *et al.*, 1981; Penn *et al.*, 1988) although the short acting cholinesterase inhibitor physostigmine has been reported in some studies to induce small, transient improvements in cognitive function in ATD patients (Smith & Swash, 1979; Mohs *et al.*, 1985). Recently a more substantial improvement in a small group of ATD patients has been reported with another cho-

linesterase inhibitor, tetrahydroaminoacridine (THA) (Summers *et al.*, 1986).

The reason for the greater efficacy of THA in comparison with other cholinergic treatments is not clear. It has been reported that THA has direct actions on potassium channels in both neuronal and cardiac tissue (Osterrieder, 1987; Stevens & Cotman, 1987). THA has also been reported to have effects on monoamine uptake and release (Drukarch *et al.*, 1988; De Souza *et al.*, 1989). However, these effects occur at relatively high concentrations and little appears to be known about the direct actions of THA at muscarinic receptors or about its pharmacology, pharmacokinetics and duration of action, factors which may be important in accounting for the differences in therapeutic efficacy between THA and other esterase inhibitors such as physostigmine. In the present series of experiments we have characterized the cholinergic actions of THA *in vivo* and compared them to those produced by physostigmine,

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the behavioural actions of which are due primarily to its inhibition of cholinesterase. We have also examined the interactions of THA with cholinergic system enzymes and receptor binding sites *in vitro*. Some of this work has been reported in preliminary form to the British Pharmacological Society (Hunter *et al.*, 1988).

Methods

Animals and drug administration

Male C57BL/6J mice (20–25 g) or male Lister Hooded rats (250–300 g) supplied by Harlan Olac, Bicester, were used in these experiments. They were housed in groups of 6 under a 12 h light/12 h dark cycle with food and water available *ad libitum*. THA was dissolved in a solution containing 0.9% saline and 20% dimethylsulphoxide (DMSO). Scopolamine hydrobromide and physostigmine sulphate were dissolved in distilled water. Vehicle alone was administered as the control for each experiment and the dosage volumes were 10 ml kg⁻¹ for mice and 1 ml kg⁻¹ for rats. All weights were expressed as base.

Comparison of the acute behavioural effects of THA and physostigmine

Groups of animals (6 animals per group) were injected (i.p.) with vehicle or various doses of THA (5–20 mg kg⁻¹) and observed 30 min later for effects on the cholinergic system. In the mouse these were tremor (scored 0–3), salivation (0–3) and rectal temperature. In the rat these were tremor (scored 0–3), salivation (weight in 10 mg units), analgesia as measured by tail flick latency and rectal temperature. Other behavioural effects in the rat such as chewing were also recorded. The effects of various doses of physostigmine (0.1–0.6 mg kg⁻¹) were studied in the same way. To confirm that these effects were mediated via muscarinic receptors, scopolamine (2.0 mg kg⁻¹, s.c.) was administered immediately before THA in a separate experiment and behavioural observations made at 30 min post-injection.

Comparison of the duration of action of THA and physostigmine

Two series of observations were made, one each in rats and mice. In each series, one experiment was carried out with THA (20 mg kg⁻¹) and one with physostigmine (0.4 mg kg⁻¹ in the mouse and 0.6 mg kg⁻¹ in the rat). In each experiment six groups of animals were injected with vehicle and six groups with drug, with 6–8 animals in each group.

Tremor, salivation and rectal temperature were measured at 30 min and 1, 2, 3, 4, 8 and 24 h post-injection in the experiments with THA and at 15 min, 30 min and 1, 2, 3, and 4 h in the experiments with physostigmine.

Measurement of acetylcholinesterase activity

Rat striata were homogenized in 40 volumes of 20 mM sodium phosphate buffer pH 7.7. Acetylcholinesterase activity was assessed by the colourimetric method of Ellman *et al.* (1961) with acetylthiocholine as substrate. In some experiments bovine erythrocyte acetylcholinesterase was used as the enzyme source. Butyrylcholinesterase was assayed by a similar method with butyrylthiocholine iodide as substrate. Drugs were added immediately before assay and the reaction was initiated by the addition of enzyme.

[³H]-pirenzepine and [³H]-N-methyl scopolamine binding

Rat heart was minced, homogenized in 50 volumes of ice cold sodium phosphate (10 mM; pH 7.4) in an Ultra-Turrax rotating blade homogenizer and filtered through muslin to remove connective tissue (10,000 r.p.m.). Rat cerebral cortex was homogenized as above but it was not necessary to filter the homogenate through muslin. Homogenates were centrifuged at 15,000 g for 15 min at 4°C, and the membrane pellets frozen at -20°C before use. Pellets were resuspended in 50–80 volumes (original wet weight) of sodium phosphate (10 mM; pH 7.4) before assay.

For [³H]-pirenzepine binding, incubations contained [³H]-pirenzepine (1 nM), displacing agents and 160 µl of cortical membrane suspension in a total volume of 200 µl of buffer. Following incubation at 30°C for 1 h, bound ligand was separated by filtration over glass fibre filters using a Skatron cell harvester. For [³H]-N-methyl scopolamine ([³H]-NMS) binding 160 µl of heart membranes was incubated with [³H]-NMS (1 nM) and displacing agents in a total volume of 200 µl for 90 min at 30°C. Bound ligand was separated by filtration over glass fibre filters.

For both ligands, non-specific binding was defined as that not displaced by atropine (10 µM). To study the dissociation of [³H]-NMS from heart membrane binding sites, membranes were incubated with 1 nM [³H]-NMS for 90 min at 30°C. Dissociation was initiated by the addition of 10 µM atropine sulphate plus drug under investigation and incubations were filtered at various times following addition of the atropine.

[³H]-oxotremorine-M binding

Rat cortex was homogenized in 20 volumes of 20 mM Tris-HCl containing 3 mM MgCl₂ pH 7.4 in an Ultra-Turrax homogenizer. The homogenate was centrifuged at 15,000 *g* for 15 min, the resulting pellet was resuspended in Tris MgCl₂ buffer and incubated at room temperature for 15 min. The preparation was then centrifuged at 15,000 *g* for 15 min, resuspended in 40 volumes of Tris MgCl₂ buffer containing 0.1% dichlorodivinyl phosphate (DDVP), and used immediately.

Incubations contained 160 μ l of membrane preparation, [³H]-oxotremorine-M (1.5 nM) and displacing agents in a total volume of 200 μ l. Following incubation at 25°C for 1 h, bound ligand was separated by filtration over glass fibre filters which had been presoaked in 0.05% polyethyleneimine. Non-specific binding was defined as that not displaced by atropine (10 μ M).

[³H]-nicotine binding

Rat brain minus cerebellum was homogenized in 50 μ M Tris-HCl pH 7.4 in an Ultra-Turrax homogenizer. The homogenate was centrifuged at 15,000 *g* for 15 min at 4°C and the pellet washed once by rehomogenization and centrifugation. The final membrane pellet was resuspended in 50 mM Tris-HCl pH 7.4 containing 100 mM KCl₂, 40 mM CaCl₂ and 20 mM MgCl₂. Incubations contained 160 μ l membrane preparation, 4 nM [³H]-nicotine and displacing agents in a total volume of 200 μ l. Following incubation at room temperature for 1 h, bound ligand was separated by filtration over glass fibre filters presoaked in polyethyleneimine (0.1%). Non-specific binding was defined as that not displaced by nicotine (10 μ M).

Data analysis

Behavioural data were analysed by analysis of variance where applicable, followed by protected *t* tests. For non parametric data analysis was by Kruskal Wallis tests or chi squared tests. Inhibition of ligand binding was calculated from Hill plots and linear regression analysis.

Drugs and chemicals

[³H]-pirenzepine (80–90 Ci mmol⁻¹), [³H]-N-methylscopolamine (80–90 Ci mmol⁻¹), [³H]-(-)-nicotine (80 Ci mmol⁻¹) and [³H]-oxotremorine-M (80–90 Ci mmol⁻¹) were obtained from New England Nuclear, U.K. Dichlorodivinylphosphate (DDVP) was purchased from Greyhound Chemicals, U.K. Acetylcholinesterase (bovine erythrocyte, type XII-S),

butyrylcholinesterase (horse serum, type XI), physostigmine and scopolamine hydrobromide were all obtained from Sigma, U.K. THA.HCl was purchased from Aldrich Chemical Company.

Results

Acute behavioural effects

THA and physostigmine both produced a dose-dependent increase in all the above measures of cholinergic function in both rat (Figure 1) and mouse (data not shown). In the rat and mouse doses of THA greater than 20 mg kg⁻¹ were fatal. In the rat physostigmine and THA both produced a dose-dependent increase in tremor and salivation and a decrease in temperature (Figure 1). These compounds also produced analgesia, decreased rearing in an open field and increased frequency of chewing behaviour (Figure 1).

In the mouse THA produced a much larger decrease in temperature than physostigmine and maximal doses also produced higher levels of tremor and salivation (data not shown). In both species THA was approximately 50 fold less potent than physostigmine in producing cholinergic effects, which were blocked by scopolamine (2 mg kg⁻¹) given immediately before THA (10 mg kg⁻¹).

Duration of action of THA and physostigmine

In the rat the effect of physostigmine on tremor and salivation was maximal 30 min post-injection and had disappeared by 2 h and 3 h respectively (Figure 2), although at this time there was still a significant decrease in temperature, compared to controls. Although THA produced a maximal effect on tremor and salivation at 30 min post-injection, some salivation and tremor were still present at 4 h and 8 h respectively (Figure 2). The temperature decrease produced by THA was maximal at 4 h and there was still a significant decrease compared to controls at 8 h (Figure 2).

In the mouse a similar pattern was observed except that the effects of physostigmine disappeared by 2 h, whereas THA-treated animals still showed a significant drop in temperature and some tremor. The duration of action of THA in the mouse was approximately twice as long as physostigmine (data not shown) but the effect of both drugs was considerably shorter in the mouse than in the rat.

Inhibition of acetylcholinesterase by THA

THA was found to be a potent inhibitor of both rat brain cholinesterase (IC₅₀ = 57 $\pm$ 6 nM) and bovine

Figure 1 The effects of (a) tetrahydroaminoacridine (THA) and (b) physostigmine in the rat on (i) temperature, salivation and tremor; (ii) chewing and rearing; (iii) latency to tail flick. In (ai) and (bi), A = temperature change, B = salivation, C = tremor; in (aii) and (bii) D = chewing, E = rearing. Data for salivation, tremor and chewing are expressed as the median, all other data are shown as the mean with s.e.mean shown by vertical bars.

erythrocyte acetylcholinesterase ($IC_{50} = 50 \pm 10$ nM) with very similar potency. THA was eight fold more potent as an inhibitor of horse serum butyrylcholinesterase ($IC_{50} = 7.2 \pm 1.4$ nM). The kinetic analysis of the interaction of THA with rat brain acetylcholinesterase indicated that inhibition is apparently non-competitive (Figure 3) with a calculated K_i value of 80 nM. The inhibition of acetylcholinesterase by THA was unaltered after a 30 min preincubation period. In addition, incubation of tissue homogenate

with 10^{-7} M THA (sufficient to inhibit > 50% activity) followed by a 10 fold dilution immediately before assay did not result in significant inhibition of cholinesterase.

Interaction of THA with muscarinic receptors

[3 H]-pirenzepine was used to label muscarinic M_1 receptors in cerebral cortex, and [3 H]-NMS to label M_2 receptors in heart. THA had significant activity

Table 1 Interaction of tetrahydroaminoacridine (THA) with muscarinic and nicotinic cholinceptor binding

Receptor ligand	Nicotinic ([3 H]-nicotine)	M_1 ([3 H]-pirenzepine)	M_2 ([3 H]-NMS)	Agonist binding ([3 H]-oxo-M)
Tissue	Whole brain	Cerebral cortex	Heart	Cerebral cortex
THA	3400	600 ± 86	880 ± 180	770
Atropine	—	0.77 ± 0.15	1.01 ± 0.15	1.72
Carbachol	—	14500	—	13.1
Pilocarpine	—	4900	—	73.4
Nicotine	3.5	—	—	—

Values are apparent $K_i \times 10^{-9}$ M, expressed as the mean $\pm$ s.e.mean of at least 3 determinations, or the mean of 2 determinations. Apparent K_i values were calculated using the equation $K_i = IC_{50}/(1 + ([L]/(K_d)))$ where [L] = ligand concentration and K_d = dissociation constant of the ligand.
 [3 H]-NMS = [3 H]-N-methylscopolamine; [3 H]-oxo-M = [3 H]-oxotremorine-M

Figure 2 The duration of action of tetrahydroaminoacridine (THA 20 mg kg^{-1} , closed symbols), physostigmine (0.6 mg kg^{-1} , open symbols) in the rat: (a) shows the effects on temperature. By 24 h THA-treated animals were not significantly different from controls. Data are the mean with s.e.mean shown by vertical bars. In (b) and (c) are shown the duration of action on tremor and salivation respectively. Data are expressed as medians.

* $P < 0.01$, ** $P < 0.001$ compared to controls.

at both binding sites. It can be seen from Table 1 that THA did not distinguish between M_1 and M_2 receptors. THA was a weak inhibitor of [^3H]-oxotremorine-M binding, the ratio of K_i values in [^3H]-pirenzepine and [^3H]-oxotremorine-M binding was similar to that of atropine (Table 1) and considerably less than that of the agonist carbachol or the partial agonist pilocarpine (Table 1).

Kinetic experiments were performed *in vitro* on the dissociation of [^3H]-NMS from rat heart binding sites. In the presence of either gallamine

Figure 3 Kinetic analysis of the inhibition of brain acetylcholinesterase by tetrahydroaminoacridine (THA). The graph shows a double reciprocal plot of acetylcholinesterase activity in the presence of increasing concentrations of THA. Data are from a single experiment which was replicated three times: (□) control; (●) $2 \times 10^{-8} \text{ M}$; (■) $5 \times 10^{-8} \text{ M}$; (○) $10 \times 10^{-8} \text{ M}$.

($3 \times 10^{-4} \text{ M}$) or THA ($3 \times 10^{-4} \text{ M}$), the dissociation rate of [^3H]-NMS was markedly reduced (Figure 4a). Concentration-response curves for this effect (Figure 4b) suggested that THA was equipotent with gallamine, although neither compound produced a maximum effect.

Three hours following administration to rats of either gallamine (20 mg kg^{-1}) or THA (20 mg kg^{-1}) both drugs had produced a significant reduction in [^3H]-NMS binding to heart preparations (Figure 5a). In addition, the dissociation of [^3H]-NMS from heart preparations from gallamine- and THA-treated rats was significantly lower than tissue prepared from control rats (Figure 5b).

Discussion

In vivo THA had a similar profile of action in the rat and mouse with the effects on the cholinergic system being comparable with those produced by physostigmine in both species. These results are in agreement with previous studies in rodents which have indicated that physostigmine and THA produced similar improvements in memory in poorly trained or lesioned mice (Flood *et al.*, 1986; Davis *et al.*, 1987).

The main difference between the two compounds was in the duration of action and potency. Physostigmine was some 50 fold more potent than THA, but its duration of action was much shorter. The short duration of action of physostigmine behaviourally corresponds well with the time course of its inhibition of brain cholinesterase which is maximal between 5 and 20 min after injection (Hallak & Giacobini, 1986; 1987). Unfortunately due to the rapid

Figure 4 (a) Dissociation of bound $[^3\text{H}]\text{-N}$ -methylscopolamine ($[^3\text{H}]\text{-NMS}$) from rat heart membranes. The graph shows a semilogarithmic plot of the dissociation of $[^3\text{H}]\text{-NMS}$ under control conditions ($\blacktriangle$) and in the presence of either gallamine ($\blacksquare$, $3 \times 10^{-4}\text{ M}$) or tetrahydroaminoacridine (THA) ($\square$, $3 \times 10^{-4}\text{ M}$). (b) Effects of increasing concentrations of gallamine ($\square$) or THA ($\blacklozenge$) on the dissociation of bound $[^3\text{H}]\text{-NMS}$ from rat heart membranes. The dissociation of $[^3\text{H}]\text{-NMS}$ is given as the percentage of bound $[^3\text{H}]\text{-NMS}$ remaining 30 min after dissociation was initiated by the addition of $10\ \mu\text{M}$ atropine.

reversibility of the inhibition of acetylcholinesterase by THA (see below), the degree of brain cholinesterase inhibition could not be measured directly in our study. Nevertheless it seems reasonable to assume that the duration of behavioural change reflects the duration of enzyme inhibition.

The biochemical actions of the two compounds also appear different. Although both THA and physostigmine are potent inhibitors of both central and peripheral cholinesterase they appear to bind to different sites on the enzyme. Kinetic analysis demonstrated that THA was a non-competitive inhibitor of rat brain cholinesterase. It is clear that THA is not an irreversible inhibitor of acetylcholinesterase, as the inhibition was not time-dependent and was rapidly reversible by dilution. The results indicate that THA does not bind to the catalytic ('esteratic') site and it has been suggested by others (Steinberg *et al.*, 1975) that THA might interact with a hydrophobic binding site adjacent to the

catalytic site and exert its inhibitory effects either via an allosteric mechanism or via some steric effect. The present data would support an allosteric interaction as no change in the affinity of cholinesterase for acetylcholine was observed in the presence of THA. Physostigmine on the other hand, interacts with the catalytic site to produce a covalently modified enzyme (Aldridge, 1971).

In addition, THA, unlike physostigmine, bound with moderate potency to muscarinic receptors. THA does not distinguish between M_1 and M_2 receptor subtypes, since the calculated K_i values of THA for cortical M_1 and cardiac M_2 receptors were 600 nM and 880 nM respectively. Although these affinities are considerably less than those of atropine, they may still be relevant to the actions of THA at high doses. It has been estimated that about 80% inhibition of brain cholinesterase is required to induce a significant increase in central cholinergic function (Holmstedt *et al.*, 1967). At this level of cholinesterase inhibition, 30% of central M_1 receptors would be occupied by THA. It is possible therefore

Figure 5 The interaction of tetrahydroaminoacridine (THA) and gallamine with heart $[^3\text{H}]\text{-N}$ -methylscopolamine ($[^3\text{H}]\text{-NMS}$) binding *ex vivo*. Animals were treated as described in the methods: (a) shows the amount of $[^3\text{H}]\text{-NMS}$ binding in heart homogenates containing 3.2 mg of tissue, $P^* < 0.05$ compared to control; (b) shows the dissociation of $[^3\text{H}]\text{-NMS}$ from heart membranes prepared from THA ($\blacksquare$) or gallamine ($\blacklozenge$)-treated animals; ($\square$) control. Data are mean with s.e. mean shown by vertical bars; $n = 6$ per group.

that at high doses of THA some antagonism of its cholinergic effects may occur. However, at the maximum dose used in the present study this was not apparent.

The ligand binding data provide no information on the agonist/antagonist action of THA. However the ratio of THA potencies in [³H]-pirenzepine binding versus [³H]-oxotremorine-M binding was similar to that of the antagonist atropine, suggesting that THA may well be an antagonist (see Freedman *et al.*, 1988).

THA also interacted with the allosteric site present on rat heart M₂ receptors. The physiological significance of this interaction is unclear, although it has been suggested that the cardiac effects of the nicotinic agent, gallamine may be mediated by an inter-

action with this site (Stockton *et al.*, 1983). In the present study THA appeared to be at least as active as gallamine *in vivo* suggesting that cardiac muscarinic M₂ receptor antagonism could occur following administration of high doses of this drug.

In conclusion the present studies show that THA is a potent, reversible, non-competitive inhibitor of acetylcholinesterase *in vitro*, albeit some 50 fold weaker at producing effects on the cholinergic system than physostigmine *in vivo*. THA interacts with muscarinic receptors at high concentrations. It also has a much longer duration of action *in vivo* than physostigmine and this factor may contribute to the alleged enhanced effectiveness of THA over physostigmine in the treatment of dementia (Summers *et al.*, 1986).

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(Received October 13, 1988

Revised February 17, 1989

Accepted April 21, 1989)