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INHIBITION OF RAT BRAIN HISTAMINE-*N*-METHYLTRANSFERASE BY 9-AMINO-1,2,3,4-TETRAHYDROACRIDINE (THA)

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Abstract—9-Amino-1,2,3,4-tetrahydroacridine (THA), an inhibitor of acetylcholinesterase, has been proposed as a treatment for Alzheimer's disease on the basis of its ability to increase cerebral levels of acetylcholine. THA shares structural features with aminoquinoline compounds known to be inhibitors of histamine-*N*-methyltransferase (HNMT). THA was found to be a potent competitive inhibitor of rat brain HNMT *in vitro*, with a K_i of 35 nM with respect to both histamine and *S*-adenosyl-L-methionine, the co-substrate. Two hours after systemic administration of THA (5 and 10 mg/kg, i.p.), HNMT from rat brain was largely inhibited. The levels of histamine in striatum and cerebral cortex were elevated by this treatment. Thus, THA at moderate doses is able to alter histamine metabolism in the central nervous system.

The presence of histamine-containing neurons in the caudal hypothalamus of the rat has been demonstrated by immunohistochemical studies employing antibodies raised against histidine decarboxylase [1] and histamine [2]. Neurotransmitter histamine in the mammalian brain has been implicated in the regulation of a wide variety of functions including arousal, water balance, body temperature and analgesia [3]. The cerebral metabolism of histamine differs from that of other amine neurotransmitters in that a high-affinity, saturable neuronal uptake mechanism appears to be absent, although some uptake may occur in glia [4]. Diamine oxidase, which is mainly responsible for the metabolism of histamine in peripheral tissues, is virtually absent from the rat brain [5]. Brain is rich in histamine-*N*-methyltransferase (HNMT) which specifically catalyzes methylation of the imidazole ring of histamine, with *S*-adenosyl-L-methionine (SAM) serving as the co-substrate. Because of the absence of catabolic pathways other than N-methylation [6], and the lack of a robust histamine uptake mechanism, cerebral histamine concentrations are potentially sensitive to HNMT inhibition.

9-Amino-1,2,3,4-tetrahydroacridine (THA) is similar structurally to the quinacrine-derived antimalarial alkaloids, which are potent inhibitors of HNMT [7]. THA has been proposed recently as a therapeutic drug in Alzheimer's disease [8]. Inhibition of HNMT by molecules with an aminoacridine nucleus has been reported, but not characterized [9]. Therefore, a radioenzymatic assay was employed to study the inhibition of rat brain HNMT activity by THA. This inhibition was compared to that produced by three other compounds: metoprine, a potent HNMT inhibitor [10], 4-aminopyridine (4-AP) and physostigmine. The degree of HNMT inhibition produced in rat cerebral cortex was determined 2 hr after peripheral administration of metoprine and THA. The effects of these drugs on the histamine content of cortex, striatum and hypothalamus were determined.

MATERIALS AND METHODS

Reagents, unless specified, were from Sigma. [^{3}H]SAM was from New England Nuclear (10 Ci/mmol). The inhibition assay was conducted as follows: The 45–70% ammonium sulfate fraction prepared from five Sprague–Dawley rat brains was dialyzed against 1 mM sodium phosphate and stored at −20° [11]. Standard reaction conditions consisted of 40 μg protein (Biorad assay) in 300 μL sodium phosphate buffer (80 mM, pH 7.9) which was incubated in the presence of 10 μM [^{3}H]SAM (0.1 μCi) and 6.7 μM histamine at 37° for 20 min, after determination of linearity. Metoprine (Burroughs–Wellcome), physostigmine, THA-hydrochloride (Aldrich) and 4-AP were included at various final concentrations ranging between 10^{-3} and 10^{-8} M. Metoprine was prepared initially as a 0.1 M solution in 5% lactic acid. Reactions were terminated by the addition of 120 μL sodium borate (2.5 M, pH 11). Tritiated product was extracted into 6 mL toluene/isoamyl alcohol (3:1) by mixing for 1 min on a vortex shaker and then back-extracted into 250 μL sodium phosphate (1 M, pH 7.1). The aqueous phase was washed with 3 mL toluene/isoamyl alcohol, and after centrifugation, 200 μL was added to 10 mL ACS (Amersham) containing 2% bis(2-ethylhexyl)hydrogen phosphate (Aldrich) for liquid scintillation counting.

Values for histamine-free blanks were less than 0.1% of total radioactivity. Inhibitor concentrations for 50% inhibition (IC_{50}) were determined by inter-

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polation. Kinetic parameters for the inhibition by THA were determined by Lineweaver–Burk analysis [12] with substrate concentrations ranging from 1 to 10 μM and THA at four concentrations between 50 and 200 nM. Statistics, where reported, are ± SE of (N) determinations. K_i values were calculated from the apparent K_m values in the presence of various concentrations of THA according to the expression $K_m^{[I]}/K_m = \langle 1 + [I]/K_i \rangle$ where $K_m^{[I]}$ is the apparent K_m in the presence of the inhibitor [13]. Replotting the data by the method of Dixon yielded almost identical results [14].

For *in vivo* experiments, groups of five male Long–Evans rats weighing between 200 and 250 g were administered saline or THA (5 and 10 mg/kg) or metoprine (10 mg/kg) by intraperitoneal injection and killed 2 hr later by cervical dislocation. Samples of cortex, striatum and hypothalamus were dissected and stored at −70° until analysis.

For the determination of cortical HNMT activity after peripheral administration of inhibitors, 100-mg samples of parietal cortex were sonicated in 4 vol. of ice-cold sodium phosphate buffer (100 mM, pH 7.9). Portions (80 μL) of the homogenates were incubated in a final volume of 150 μL under conditions otherwise identical to those used for the kinetic studies. Enzyme activity in control samples was calculated as nanomoles product formed per minute per 20 mg tissue and reported as the mean ± SE of five determinations. Activity in samples containing HNMT inhibitors was calculated as a percentage of the mean activity in control cortex samples.

Concentrations of histamine in cortex, striatum and hypothalamus were determined employing a radioenzymatic assay using HNMT purified 150-fold from porcine kidney [15]. Tissue samples were sonicated in 10 vol. of sodium phosphate buffer (100 mM, pH 7.9), heated to the boiling point for 8 min, chilled on ice, and then centrifuged for 15 min at 15,000 *g*. To estimate the concentrations of histamine in extracts containing HNMT inhibitors, the method of standard addition was employed. Portions (5–25 μL) of the supernatant fraction were incubated for 1 hr at 4° in the presence of 1 μg of the enzyme preparation, and 0.2 μCi [^{3}H]SAM in a final volume of 100 μL buffer. Tritium-labeled product was extracted as described above.

The linearity of the histamine assay was determined at six points ranging from 0 to 250 pg histamine. The pooled extracts of two whole rat brains were spiked with between 0 and 250 pg histamine, as were the pooled extracts from two whole rat brains at 2 hr after administration of either THA (10 mg/kg, i.p.) or metoprine (10 mg/kg, i.p.). The histamine assay was found to be linear in both the presence and absence of inhibitors. The slopes of the standard curves determined in the presence of inhibitors were reduced considerably with respect to the slope of the assay of histamine standards alone.

The apparent histamine content of each experimental sample was determined. Each sample was also spiked with 100 pg histamine and re-analyzed. The recovery of the spike was quantitative in the case of samples from control animals and ranged from 30 to 75% in the samples containing HNMT inhibitors. Histamine concentrations were corrected for individual spike recoveries and reported as ng/g. Total histamine contents were always within the linear range of the assay.

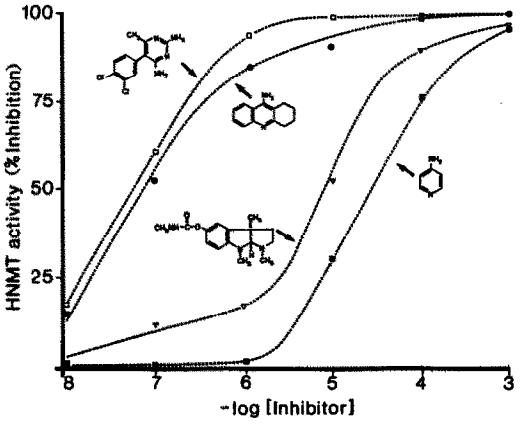

Fig. 1. Inhibition of HNMT in the presence of 6.7 μM histamine and 10 μM [^{3}H]*S*-adenosylmethionine (0.1 μCi) and various inhibitors. Inhibitors, with structures indicated from left to right, are metoprine, THA, physostigmine and 4-aminopyridine. Each point is the mean of at least three determinations. SEs, omitted for clarity, were always less than five percentage units. Enzyme activity in the absence of inhibitors (V_0) was 150 pmol·min^{-1}·(mg protein)$^{-1}$.

RESULTS AND DISCUSSION

Under standard conditions, the assay employing rat brain HNMT was linear for at least 30 min. The inhibition of HNMT by various compounds is illustrated in Fig. 1. Metoprine was the most potent inhibitor tested, with an IC_{50} of 56 nM, while THA had an IC_{50} of 74 nM. Physostigmine inhibited HNMT with an IC_{50} of 8 μM and 4-AP with an IC_{50} of 28 μM.

The K_m of the rat brain enzyme preparation for histamine was 3.3 ± 0.3 μM (Fig. 2A) and the K_m for SAM was 4.3 μM (Fig. 2B). These data are the means of, respectively, three and four determinations, of which two are illustrated. The V_{max} was 197 ± 4 pmol·min^{-1}·(mg protein)$^{-1}$. HNMT was subject to substrate inhibition at histamine concentrations above 10 μM. These kinetic properties are comparable to those reported for purified guinea pig brain HNMT [16]. THA inhibited HNMT in a manner that was competitive with respect to histamine [Fig. 2A, $K_i = 35 \pm 6$ nM (N = 3)] and displayed mixed competition with respect to SAM [Fig. 2B, $K_i = 39 \pm 5$ nM (N = 4)].

The inhibition of cortical HNMT activity by THA and metoprine is illustrated in Table 1. THA produced a dose-dependent inhibition of cortical HNMT. Metropine was more potent than THA, producing nearly complete inhibition. This could be due to its slightly greater intrinsic potency or to greater penetration into brain tissue. The concentrations of THA and metoprine present in the *in vitro* assay following *in vivo* administration could be estimated by interpolation of the percentage inhibition from the inhibition curves for rat brain HNMT

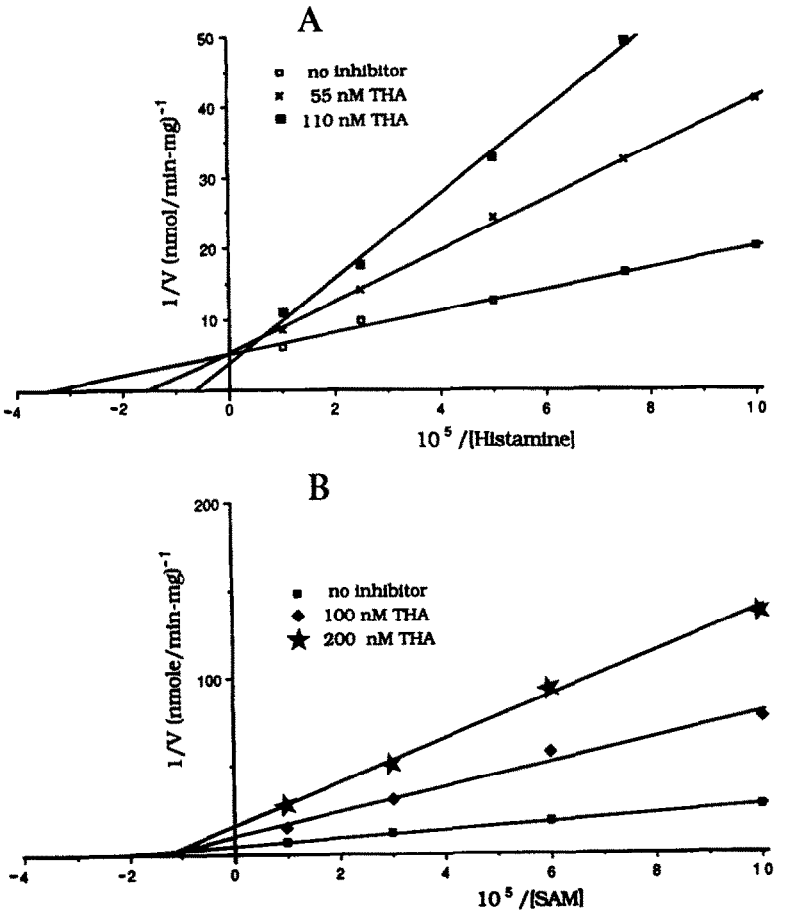

Fig. 2. (A) Double-reciprocal plots of the inhibition of HNMT by 55 and 110 nM THA with [^{3}H]*S*-adenosylmethionine held at 10 μM and histamine varied between 1 and 10 μM. Each point is the mean of three determinations which varied by less than 5%. (B) Double-reciprocal plots of the inhibition of HNMT by 100 and 200 nM THA with histamine held at 6.7 μM and [^{3}H]*S*-adenosylmethionine varied between 1 and 10 μM. Each point is the mean of three determinations which varied by less than 5%.

Table 1. Inhibition of cortical HNMT activity

Treatment	Activity (nmol·min^{-1}·20 mg^{-1})	Inhibition *in vitro* (%)	Estimated inhibition *in vivo* (%)
Control	14.5 ± 0.9		
THA (5 mg/mg)	8.9 ± 0.4*	39	75
THA (10 mg/kg)	6.4 ± 0.8*	56	85
Metoprine (10 mg/kg)	1.9 ± 0.2*	87	97

Two hours after i.p. drug injections, HNMT activity was determined in cortical homogenates. Values are means ± SE (N = 5). Inhibition *in vitro* is reported as a percentage of control activity. Inhibition *in vivo* was estimated from the inhibition curves in Fig. 1 as follows: To correct for 10-fold dilution of the inhibitors during the assay, the concentrations of inhibitors *in vitro* were estimated by interpolation of the inhibition curves. The *in vivo* inhibitions were taken from interpolations of the curves at concentrations one log unit to the right.

* Significantly different from control, $P < 0.001$.

Table 2. Effect of THA and metoprine on brain histamine

Treatment	Histamine (ng/g)		
	Cortex	Striatum	Hypothalamus
Control	36.7 ± 1.0	36.5 ± 1.6	349 ± 25
THA (5 mg/kg)	61.8 ± 4.2* (168%)	48.9 ± 3.1† (134%)	423 ± 31 (121%)
THA (10 mg/kg)	79.4 ± 5.9* (216%)	54.2 ± 4.6† (148%)	477 ± 68 (132%)
Metoprine (10 mg/kg)	104.9 ± 5.8* (286%)	84.1 ± 7.0* (230%)	506 ± 65‡ (145%)

Animals (N = 5) were killed at 2 hr after saline, THA (5 and 10 mg/kg, i.p.) or metoprine (10 mg/kg, i.p.). The concentrations of histamine were determined in cortex, striatum and hypothalamus. Values are means ± SE are also reported as percentages of control values. *–‡ Significantly different compared to control: *$P < 0.001$, †$P < 0.025$, and ‡$P < 0.1$.

(Fig. 1). Given a 10-fold dilution of inhibitors in cortex under the conditions of the assay, an approximation of the inhibition *in vivo* could be made by interpolation at one $\log_{10}$ unit to the right on the inhibition curves. This provides only an estimation of the inhibition *in vivo*, but it is apparent that substantial inhibition of HNMT in cerebral cortex occurs at 2 hr after i.p. administration of moderate doses of the HNMT inhibitors.

The results of the determination of histamine concentrations in brain tissues are illustrated in Table 2. Histamine concentrations for control rat brain areas are similar to those reported previously [17]. THA produced an approximately 2-fold increase in cortical histamine that was dose dependent. Metoprine produced a nearly 3-fold increase in cortical histamine, consistent with its greater potency as an HNMT inhibitor. Results were similar in striatum but the magnitude of the increases were lower. In hypothalamus, a trend towards increased histamine levels did not reach significance in the THA groups and was marginally significant in the metoprine group.

Metoprine, a dihydrofolate reductase inhibitor derived from diaminopyrimidine, is also a potent inhibitor of HNMT [10]. Peripheral administration of 10 mg/kg metoprine has been shown previously to inhibit HNMT in the rat brain by more than 80% [18] and to produce a long-lasting 2-fold increase in whole rat brain histamine levels [19]. The rates of accumulation of histamine after metoprine may be calculated from the data in Table 2. This yields rates of 0.31 $nmol \cdot g^{-1} \cdot hr^{-1}$ for cortex, 0.21 $nmol \cdot g^{-1} \cdot hr^{-1}$ for striatum, and 0.71 $nmol \cdot g^{-1} \cdot hr^{-1}$ for hypothalamus. These turnover rates are similar to, but somewhat lower than, those rates calculated from the accumulation of *tele*-methylhistamine subsequent to pargyline [17, 20]. The inhibition of monoamine oxidase (MAO) by pargyline, an irreversible inhibitor, may be more rapid and complete than the inhibition of HNMT by metoprine, resulting in a probable underestimation of turnover as calculated from the accumulation of histamine after partial inhibition of HNMT.

The present *in vitro* study shows THA and metoprine to be nearly equipotent HNMT inhibitors; few compounds are more potent. Among these are quinacrine, which inhibits HNMT from guinea pig skin with a K_i of 20 nM [21] and TMQ, an aminoquinazoline, which inhibits bovine brain HNMT with a K_i of 7 nM [10]. The mechanism of HNMT inhibition by THA presumably involves structural features common to these molecules. HNMT is inhibited by a plethora of compounds with different structural features, but many of the most effective inhibitors have in common an aromatic nitrogen and a basic nitrogen separated by one or more carbons [7, 10, 21]. These are not sufficient conditions for HNMT inhibition, since 4-AP was 400-fold less potent than THA. For high-affinity inhibition, a side-chain on the aromatic ring also seems to be necessary.

The ability of a compound to inhibit HNMT *in vitro* does not necessarily indicate that it will influence histamine metabolism *in vivo* [22]. The above data indicate that THA is able to inhibit cerebral HNMT and greatly increase cerebral histamine levels at 2 hr after a peripheral dose. Given that THA is a lipophilic molecule, it seems likely that repeated doses of THA could produce a cumulative inhibition of HNMT in the central nervous system.

Alzheimer's disease is a dementia often associated with degeneration of the cortical cholinergic innervation [23] in addition to the classical neuropathological features such as neuritic plaques and tangles. The belief that a cholinergic deficit contributes to the clinical state in Alzheimer's disease had lead to attempts at neurotransmitter replacement therapy, in analogy to the use of L-DOPA for Parkinson's disease. Recent interest has focused on inhibition of acetylcholinesterase (AChE), the enzyme which inactivates acetylcholine. THA inhibits AChE with an IC_{50} on the order of 100 nM [24]. Improved cognitive performance has been reported in some mildly demented Alzheimer's patients undergoing clinical trials with THA [8].

It is not certain that the inhibition of AChE is the only property of THA pertinent to its reported clinical efficacy and/or side-effects. For example, THA shares with its structural fragment 4-aminopyridine (4-AP) the ability to block certain classes of potassium channels [25]. THA and related molecules

are also inhibitors of MAO [24]. These effects, occuring at concentrations on the order of 100 μM, seem unlikely to arise after oral THA doses employed in clinical studies which ranged from 100 to 200 mg *per diem* [8].

Oral physostigmine is apparently without benefit for Alzheimer's disease [26]. If THA is a superior treatment to other AChE inhibitors, additional physiological and biochemical properties of THA could be responsible. The K_i of THA for HNMT is well within the therapeutic range of plasma THA concentrations, which was on the order of 100 nM [8]. The low dose of THA in this study was able to inhibit HNMT substantially in the cerebral cortex and also to produce robust increases in cerebral histamine levels.

The cholinergic deficit may be *sine qua non* for Alzheimer's disease, but degeneration of other nonthalamic cortically-projecting neuron systems, such as the noradrenergic locus coeruleus, is well documented [27]. In Alzheimer's cases, high densities of neurofibrillary tangles have also been found in the vicinity of the cortically-projecting histamine neurons [28]. Cortical histamine levels have been reported to be either reduced [29] or increased [30] in studies of Alzheimer's cases. The reason for this disagreement is not clear at this time. However, it is possible that a histaminergic dysfunction may contribute to aspects of the clinical condition. Potentiation of cerebral histaminergic transmission by THA, especially in conjunction with AChE inhibition, may improve cognitive performance. In the present study, THA was found to be among the most potent inhibitors of HNMT yet described and to be capable of interfering with cerebral histamine metabolism at peripheral dose close to those employed in clinical studies.

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