Biochemical Pharmacology, Vol. 57, pp. 1059–1066, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00378-5

Effects of Selected Histamine H_3 Receptor Antagonists on *tele*-Methylhistamine Levels in Rat Cerebral Cortex

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ABSTRACT. The H_3 antagonist thioperamide is thought to act on brain H_3 autoreceptors to increase both the release and metabolism of neuronal histamine (HA). Our studies investigated the effects of several new brain-penetrating H_3 antagonists on rat cerebral cortical levels of the HA metabolite *tele*-methylhistamine (*t*-MH). Animals were pretreated with H_3 antagonists (0.3 to 30 mg/kg; 1–4 hr; i.p.) in the presence or absence of the monoamine oxidase inhibitor pargyline to prevent metabolism of *t*-MH. Cortical *t*-MH levels were measured by both radioimmunoassay (RIA) and gas chromatography–mass spectrometry (GC–MS). Pargyline (60 mg/kg; 1 hr; i.p.) produced an ~2-fold increase in *t*-MH levels as measured by either GC–MS or RIA. Thioperamide (± pargyline) increased *t*-MH levels as measured by both GC–MS and RIA. In contrast, neither 5-cyclohexyl-1-(4-imidazol-4-ylpiperidyl)pentan-1-one (GT-2016) (± pargyline), 4-(6-cyclohexylhex-*cis*-3-enyl)imidazole (GT-2227) (± pargyline), nor clobenpropit (minus pargyline) increased *t*-MH levels as measured by GC–MS. A good agreement was found between *t*-MH levels as determined by either RIA or GC–MS except after treatment with GT-2016, which increased apparent *t*-MH brain levels according to the former but not the latter method. Subsequent studies suggest the *in vivo* formation of a GT-2016 metabolite, which can cross-react in the *t*-MH RIA. Although all H_3 receptor antagonists studied to date seem capable of enhancing brain HA release, only thioperamide presently was found to enhance cortical *t*-MH levels. Thus, H_3 receptor antagonists may differentially affect HA release and turnover, and brain *t*-MH levels may not be reliable predictors of H_3 agonist, partial agonist, or antagonist *in vivo* activity. BIOCHEM PHARMACOL **57**;9:1059–1066, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. *tele*-methylhistamine; histamine H_3 receptor antagonists; thioperamide; GT-2016; gas chromatography–mass spectrometry; radioimmunoassay

The synthesis and release of HA§ in the CNS are thought to be under the tonic inhibitory control of presynaptic HA H_3 autoreceptors [1]. Selective pharmacological tools have been developed that have aided in the characterization of the HA H_3 receptor. Selective H_3 agonists, such as (*R*)-α-methylhistamine and imetit, decrease HA release and synthesis [2, 3], whereas selective H_3 antagonists, such as thioperamide, clobenpropit (VUF-9153), and 5-cyclohexyl-1-(4-imidazol-4-ylpiperidyl)pentan-1-one (GT-2016), enhance HA release and/or synthesis [4–6].

The predominant clearance mechanism for HA in the CNS is through metabolism to *t*-MH by HA methyltransferase and subsequent oxidation by MAO B [7]. Since most HA methylation is thought to occur outside of histaminergic neurons [8], and because there is a strong correlation between the rate of HA turnover and the levels of *t*-MH [9], an increase in the rate of neuronal HA release in the CNS is thought to result in a concomitant increase in the CNS levels of *t*-MH. Furthermore, previous studies using the prototype H_3 antagonist thioperamide have shown an enhancement of *in vivo* brain HA release [4, 10] and an elevation of CNS levels of *t*-MH [11, 12]. These studies have led to the presumption that all H_3 antagonists will have effects similar to those of thioperamide, promoting increases in CNS HA release and *t*-MH production. To this end, several laboratories have used brain *t*-MH levels as an index of *in vivo* H_3 pharmacological activity [3, 13, 14]. For example, the inability of some new H_3 antagonists to enhance brain *t*-MH levels after systemic administration has led to the inference that such compounds have poor brain penetration [13–15].

Although the HA-releasing properties of newer H_3 blockers (e.g. clobenpropit and GT-2016) have been established [5, 6], less is known about the ability of these compounds to increase tissue levels of *t*-MH. In the present

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§*Abbreviations*: HA, histamine; MAO, monoamine oxidase; [^{3}H]NAMHA, [^{3}H]N^{α}-methylhistamine; RIA, radioimmunoassay; *t*-MH, *tele*-methylhistamine; and SPE, solid phase extraction.

Received 14 May 1998; accepted 28 September 1998.

studies, the effects of several new brain-penetrating H_3 antagonists on the levels of *t*-MH in the rat cerebral cortex have been investigated after systemic administration. Two methods for measuring tissue *t*-MH levels were compared, a GC–MS method and RIA.

MATERIALS AND METHODS

Animals

Adult male Sprague–Dawley rats (150–250 g) were purchased from Harlan Sprague–Dawley and housed two per cage on a 12-hr light/dark schedule with *ad lib*. access to Teklad Mouse/Rat Diet 7012 (Harlan Sprague–Dawley) and water in accordance with the Animal Welfare Act of 1970 and amendments. Animals were acclimated to laboratory conditions for a minimum of 1 week prior to initiation of experiments.

Animal Treatments and Ex Vivo *H_3 Receptor Binding Analysis*

Rats were acutely treated with a single i.p. injection of compound (N = 4/group). Drugs were administered in a final volume of 1 mL/kg. All doses specified are reflective of the base. Rats were euthanized by a lethal injection of sodium pentobarbital (Nembutal®, Abbott Laboratories; 150 mg/kg; i.p.) at the indicated times post-administration. Following euthanasia, the upper torso was perfused transcardially through the aortic arch with 60 mL of 0.9% saline to remove potential vascular drug contamination. Brains were removed, dissected, and frozen on dry ice. The tissue was stored at −80° prior to conducting the binding studies. *Ex vivo* H_3 receptor occupancy was determined in rat cortical membranes with [^{3}H]NAMHA as previously described [6, 16]. Briefly, on the day of binding experiments, the tissue was homogenized using a motor-driven tissue grinder (Omni 1000) in 9 vol. (w/v) of 50 mM sodium phosphate buffer (pH 7.4). *Ex vivo* binding was carried out in a total volume of 0.2 or 0.4 mL of 50 mM sodium phosphate buffer containing ~1 nM [^{3}H]NAMHA and 0.15 to 1 mg protein. Nonspecific binding was determined using 10 μM thioperamide. Samples were incubated for 40 min at 25° and subsequently filtered through Whatman GF/C glass fiber filters pre-soaked in binding buffer with 0.3% polyethyleneimine, using an Inotech cell harvester (Inotech Biosystems International). The filters were washed rapidly three times with Tris–NaCl buffer (25 and 145 mM, respectively, pH 7.4, 4°). Samples were quantitated using Ecolume scintillation fluid (ICN Biomedicals) and a Packard model 1900TR liquid scintillation analyzer (Packard Instrument Co.). The ED_{50} values (doses that produced 50% inhibition of [^{3}H]NAMHA binding) in milligrams per kilogram were determined by linear regression analysis of the data on a log-linear plot.

Preparation of t-MH Samples

On the day of experiments, the contralateral cerebral cortex from animals used in the *ex vivo* binding studies was homogenized in ~10 vol. (w/v) of 0.1 N $HClO_4$ and assayed for *t*-MH by both RIA and GC–MS methods.

RIA Measurement of t-MH

Perchlorate homogenates were centrifuged in a microfuge (16,000 *g* for 20 min at 4°), and the supernatants were collected. An aliquot of $HClO_4$ extract (200 μL) was neutralized with 0.15 N Na_2HPO_4 (600 μL) and assayed for *t*-MH using a commercial RIA (Immunotech) according to the kit instructions. *t*-MH values were normalized to wet weight of tissue.

GC–MS Measurements of t-MH

Tissue extracts were stored frozen at −80° prior to GC–MS analysis. This assay was performed as described previously [17], except that trideuteromethylhistamine was used as the internal standard. $HClO_4$ extracts were made alkaline, extracted with *n*-butanol:chloroform (1:1), back-extracted with HCl (0.01 N), and evaporated to dryness. Residues were derivatized with heptafluorobutyric anhydride and pyridine. Derivatives were extracted into toluene and assayed by selected ion monitoring of *m/e* 304 and 307 for *t*-MH and its internal standard, respectively. Ions 517 and 520 were also monitored as confirming ions. Gas chromatography was performed with an HP5890A GC operating in splitless mode with a temperature-programmed DB-5MS column (30 m, 0.25 mm i.d., 0.1 μm film thickness, helium). Electron impact mass spectra were obtained with an HP5790A mass selective detector at −70 eV.

HPLC-UV Measurement of GT-2016 and GT-2035

Cerebral cortex, kidney, and liver extracts from the RIA studies (see above) were allowed to come to room temperature. A 200-μL sample of tissue extract was injected onto an SPE cartridge (Oasis HLB, Waters Corp.). All SPE cartridges were preconditioned with 1 mL of methanol immediately followed by 1 mL of purified HPLC-grade water while under constant vacuum at 20 kPa. The sample was drawn through the SPE cartridge under vacuum and then washed with 500 μL of water. The SPE cartridge was eluted with 1 mL of methanol into a 5 mL glass reaction vial. The methanol was evaporated under forced air in an 80° oven for 30 min. Samples were reconstituted with 200 μL of mobile phase and filtered (0.45 μm nylon). The samples were analyzed by HPLC using an acetonitrile:100 mM acetate buffer (pH 7.0) gradient and UV detection at 220 nm. Quantitation of GT-2016 and 4−4(piperidylimidazoyl) (GT-2035) in tissue extracts was accomplished by comparing the peak area of the analyte with a previously

run standard curve (0.01 to 50 μg/mL) of GT-2016 or GT-2035, respectively.

In Vitro *Metabolism of GT-2016*

GT-2016 free base (10.0 mg) was dissolved in 400 μL of a 1:1 solution of DMSO:PEG 400. This solution was added to 4.6 mL of phosphate-buffered saline (pH 7.2) containing 118 U of recombinant amidase (*Pseudomonas aeruginosa*; Sigma) and incubated at 37°. The disappearance of GT-2016 and the appearance of GT-2035 were monitored by HPLC and UV detection as described above.

Data Analysis

Data were analyzed by ANOVA followed by Newman-Keuls post-hoc analyses or Student's *t*-test.

Chemicals

GT-2016 maleate and free base, GT-2035 2HCl, 4-(6-cyclohexylhex-*cis*-3-enyl)imidazole (GT-2227) maleate, and thioperamide free base were synthesized by Gliatech chemists. [^{3}H]NAMHA (81.5 Ci/mmol) was purchased from DuPont NEN Research Products. Clobenpropit 2HBr was provided by Dr. Timmerman at the Leiden/Amsterdam Center for Drug Research.

RESULTS

Treatment of rats with thioperamide (10 mg/kg; i.p.) and/or pargyline (60 mg/kg; i.p.) for 2 hr resulted in significant increases in cortical *t*-MH levels as measured by either RIA or GC–MS methods (Fig. 1). Both the H_3 receptor antagonist and the MAO inhibitor alone produced an approximate doubling of cortical *t*-MH levels compared with vehicle-treated groups. Further, the combination of both thioperamide and pargyline produced an additional increase in cortical *t*-MH levels over that of either treatment alone as measured by either RIA or GC–MS methods. This dose of thioperamide provided maximal HA H_3 receptor occupancy as measured by *ex vivo* binding (ED_{50} = 1.5 ± 0.6 mg/kg). Thioperamide, at concentrations up to 10 μM, did not cross-react in the *t*-MH RIA (Table 1).

In subsequent studies, rats were dosed with GT-2016 (3, 10, and 30 mg/kg; i.p.) for 1 hr in the presence or absence of pargyline (60 mg/kg; i.p.). These doses of GT-2016 provided *ex vivo* ED_{50} values of 15.5 ± 10.2 and 12.0 ± 5.5 mg/kg (mean ± SEM, N = 4) in the absence and presence of pargyline, respectively. Pargyline produced a 2-fold increase in cortical *t*-MH levels as measured by both RIA and GC–MS (Fig. 2). There was no effect of GT-2016 on cortical *t*-MH levels as measured by GC–MS in the absence or presence of pargyline (Fig. 2). However, GT-2016 (10 and 30 mg/kg) produced a significant dose-dependent increase in cortical *t*-MH levels in the absence of pargyline as measured by RIA. In the presence of pargyline, GT-2016 produced no additional increase in *t*-MH levels above that induced by pargyline alone. As shown in Table 1, GT-2016 did not cross-react in the *t*-MH RIA at concentrations up to 10 μM.

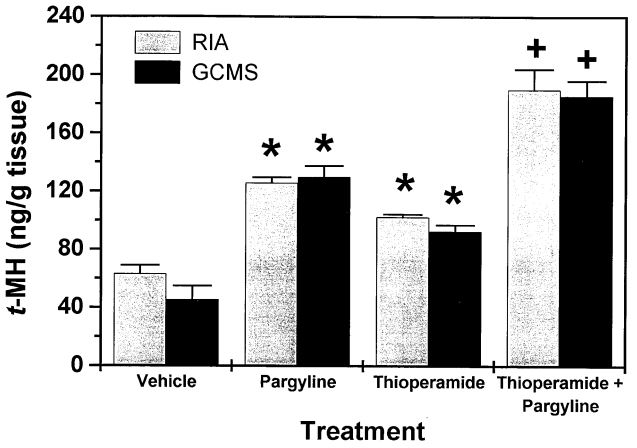
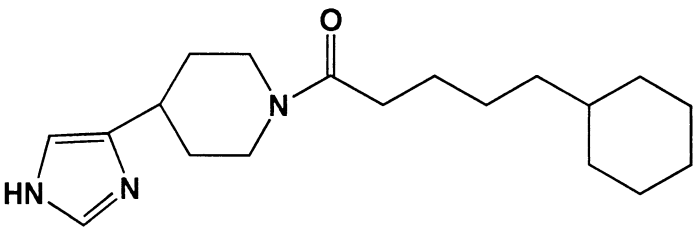
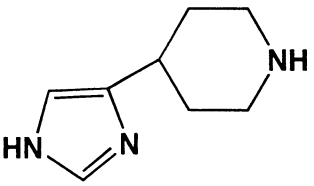
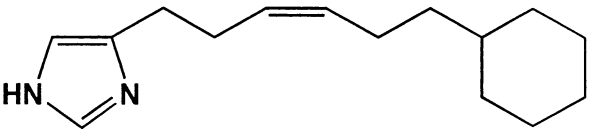
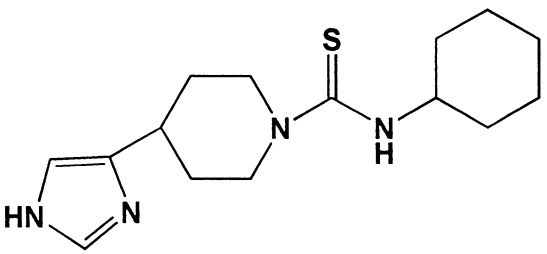
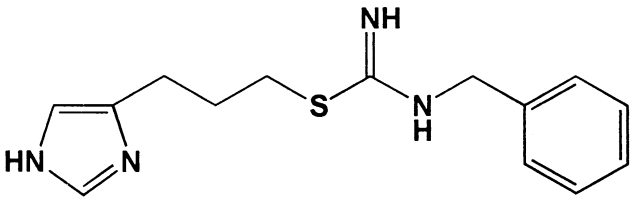


FIG. 1. Levels of *t*-MH in rat cortex following thioperamide and/or pargyline. Thioperamide (10 mg/kg) and/or pargyline (60 mg/kg) and the respective vehicles were administered i.p. 2 hr before the animals were euthanized. Cortical *t*-MH levels were determined by RIA or GC–MS and expressed as nanograms per gram wet weight tissue (mean ± SEM; N = 4/group). Key: (*) all drug-treated groups were significantly different from their respective vehicle-treated group as measured by RIA or GC–MS ($P < 0.01$); and (+) the combined treatment with thioperamide and pargyline was significantly different from all other groups as measured by RIA or GC–MS ($P < 0.01$).

In subsequent studies, rats were dosed with the HA H_3 receptor antagonist GT-2227 (0.3, 1, and 3 mg/kg; i.p.) in the presence or absence of pargyline (60 mg/kg) for 1 hr. These doses of GT-2227 provided *ex vivo* ED_{50} values of 0.7 ± 0.1 and 0.6 ± 0.1 mg/kg (mean ± SEM, N = 4) in the absence and presence of pargyline, respectively. Pargyline produced an approximate 2-fold increase in cortical *t*-MH levels as measured by both RIA and GC–MS (Fig. 3). GT-2227 had no effect on cortical *t*-MH levels in the presence or absence of pargyline as measured by either method. Similarly, clobenpropit (3, 10, and 30 mg/kg; i.p.) in the absence of pargyline produced only a slight increase in cortical *t*-MH levels at the 10 mg/kg dose as measured by RIA (Fig. 4). There was no significant increase in cortical *t*-MH levels induced by clobenpropit as measured by GC–MS. Clobenpropit was found to have an *ex vivo* ED_{50} value of 3.9 ± 4.4 mg/kg (mean ± SEM, N = 4). As shown in Table 1, GT-2227 did not cross-react in the *t*-MH RIA at concentrations up to 10 μM, and clobenpropit showed only slight antibody cross-reactivity (0.4%).

To further evaluate the GT-2016-induced increases in cortical *t*-MH levels observed with the RIA (Fig. 2), rats were dosed with GT-2016 (30 mg/kg; i.p.) over a 4-hr time course, and apparent *t*-MH levels were evaluated in the cerebral cortex, liver, and kidney. GT-2016 produced a time-dependent increase in cortical *t*-MH levels (Fig. 5) similar to what was observed in Fig. 2. Furthermore, GT-2016 produced a time-dependent increase in apparent

TABLE 1. Structures of compounds and cross-reactivities in the *t*-MH RIA

Compound	Structure	Cross-reactivity *
GT-2016		< 0.1% at 10 μM
GT-2035		45 ± 9% at 0.1 μM
GT-2227		< 0.1% at 10 μM
Thioperamide		< 0.1% at 10 μM
Clobenpropit (VUF-9153)		0.4 ± 0.1% at 10 μM

*Cross-reactivity was determined by adding a specific concentration of compound to the RIA and determining the degree of apparent *t*-MH detection. Values for clobenpropit and GT-2035 are presented as means ± SEM (N = 4).

t-MH levels in both the liver and kidney. For comparison, animals were dosed with thioperamide (10 mg/kg) over a 4 -hr time course. Thioperamide produced a time-dependent increase in cortical *t*-MH levels (Fig. 5) similar to what was observed with 10 mg/kg thioperamide in Fig. 1. There was, however, no increase in *t*-MH levels in either liver or kidney following treatment with thioperamide. Thus, H_3 receptor antagonist activity is not likely to account for the observed increase in apparent *t*-MH levels induced by GT-2016 in liver and kidney. The time-dependent increase in apparent *t*-MH levels measured by RIA in the liver and kidney would be consistent with the formation of a GT-2016 metabolite that could potentially cross-react in the *t*-MH RIA. As shown in Table 1, a potential metabolite of GT-2016 (GT-2035) had 45% cross-reactivity in the *t*-MH RIA. The presence of GT-2035 in cerebral cortex, liver, and kidney extracts was verified by HPLC analyses. In kidney extracts, the levels of GT-2035 were approximately 200-fold higher than the levels of GT-2016 (Table 2). GT-2016 and GT-2035 also were detected in cerebral cortex and liver extracts but were below the quantitation limits of the present methodology. Furthermore, *in vitro* incubation of GT-2016 with amidase for 1 hr resulted in 95% metabolism of GT-2016 to GT-2035, as measured by HPLC (data not shown).

Finally, to further establish the validity of the RIA and

GC–MS methodologies, control *t*-MH levels from the GT-2016, clobenpropit, and GT-2227 studies were pooled. Control cortical *t*-MH levels from Figs. 2–4 were compiled. The *t*-MH levels for control vehicle-treated animals as measured by GC–MS were 52.7 ± 3.4 (mean ± SEM, N = 12) and 61.1 ± 3.9 (mean ± SEM, N = 12) as measured by RIA. This analysis showed that there was no significant difference in *t*-MH levels as measured by RIA or GC–MS ($P > 0.05$), indicating a good general agreement between these methods for *t*-MH analysis. To further compare the RIA and GC–MS methodologies, the data from Figs. 1–4 (minus data from GT-2016-treated animals) were compiled, and a correlation analysis was performed. This analysis showed that there was a significant correlation ($r = 0.89$; $P < 0.0001$; N = 70) between *t*-MH levels measured by RIA and those measured by GC–MS (Fig. 6).

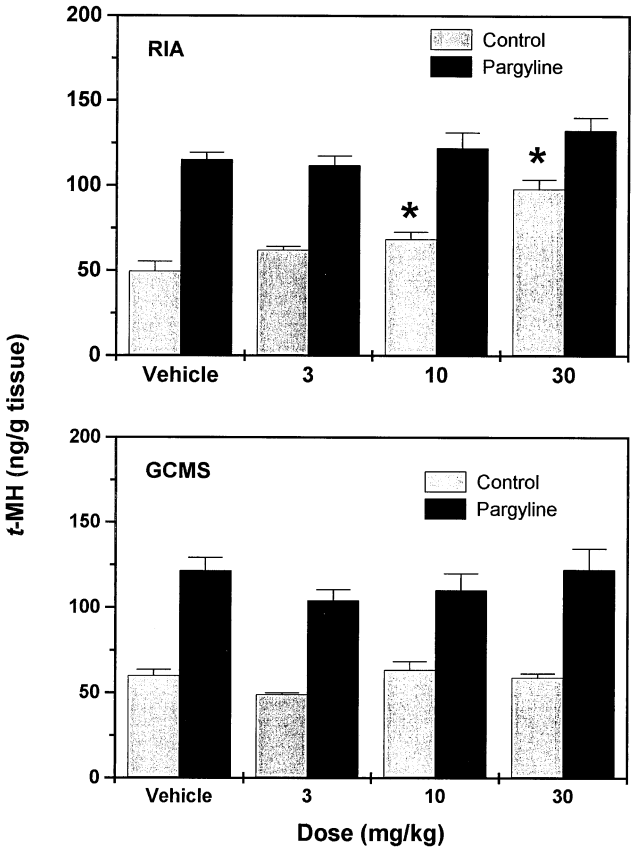


FIG. 2. Levels of *t*-MH in rat cortex following GT-2016 and/or pargyline. GT-2016 (3, 10, and 30 mg/kg) and/or pargyline (60 mg/kg) and the respective vehicles were administered i.p. 1 hr before the animals were euthanized. Cortical *t*-MH levels were determined by RIA (top) or GC–MS (bottom) and expressed as nanograms per gram wet weight tissue (mean ± SEM; N = 4/group). Key: (*) GT-2016-treated groups were significantly different from the vehicle-treated group as measured by RIA ($P < 0.05$). There were no significant effects of GT-2016 on *t*-MH levels as measured by GC–MS ($P > 0.5$). All pargyline-treated groups were significantly different from the corresponding vehicle- or GT-2016-treated groups in the absence of pargyline as measured by either RIA or GC–MS ($P < 0.01$).

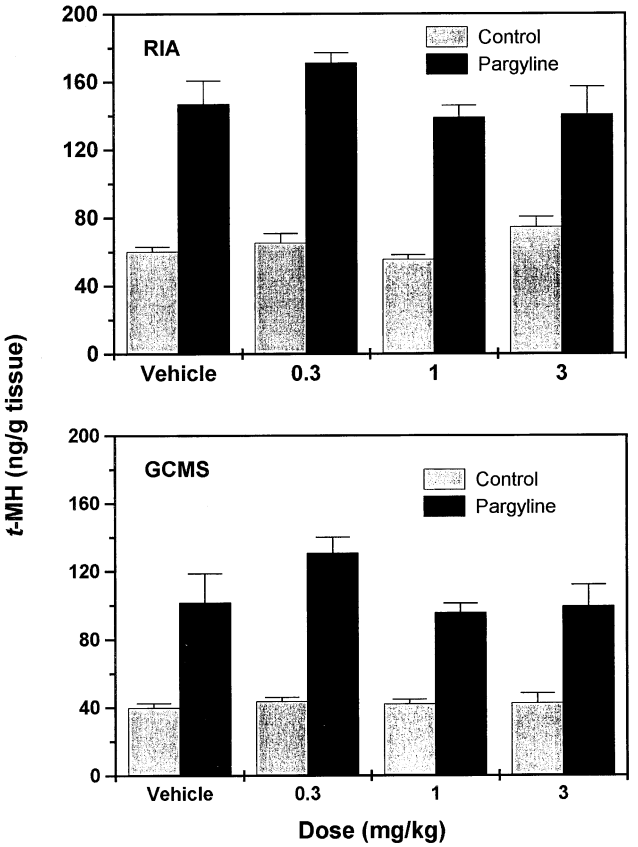


FIG. 3. Levels of *t*-MH in rat cortex following GT-2227 and/or pargyline. GT-2227 (0.3, 1, and 3 mg/kg) and/or pargyline (60 mg/kg) and the respective vehicles were administered i.p. 1 hr before the animals were euthanized. Cortical *t*-MH levels were determined by RIA (top) or GC–MS (bottom) and expressed as nanograms per gram wet weight tissue (mean ± SEM; N = 4/group). There were no significant effects of GT-2227 on *t*-MH levels as measured by RIA or GC–MS ($P > 0.5$). All pargyline-treated groups were significantly different from the corresponding vehicle- or GT-2227-treated groups in the absence of pargyline ($P < 0.01$) as measured by either RIA or GC–MS.

DISCUSSION

In the past several years, studies of HA H_3 receptor antagonists have been limited to using thioperamide [2]. The recent development of several diverse classes of HA H_3 receptor antagonists has enabled further pharmacological characterization of the effects of these compounds on the release and metabolism of HA. These series include clobenpropit [18], iodoproxyphan [19], and GT-2016 [6], as well as several newer chemical series, which include GT-2227 and GT-2331 [20–22]. Studies have now shown that, like thioperamide, the newer H_3 receptor antagonists also enhance HA release as measured by *in vivo* microdialysis [6] or tissue HA content [5]. However, while the metabolism of HA released following thioperamide treatment has been characterized [12, 16], the effects of newer HA H_3 receptor antagonists on *t*-MH levels have not been studied in detail. The present studies were designed to evaluate the effects of several selective HA H_3 receptor antagonists on cortical

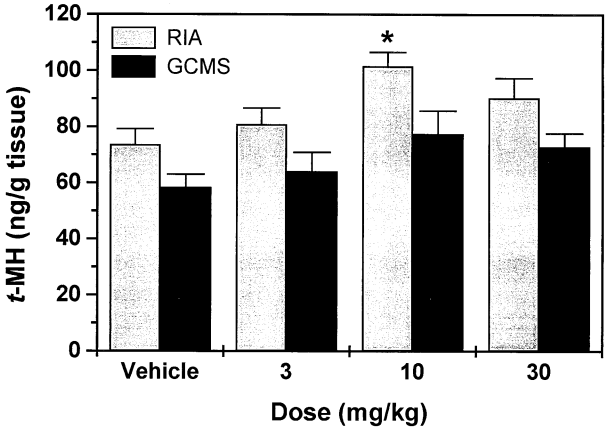


FIG. 4. Levels of *t*-MH in rat cortex following clobenpropit. Clobenpropit (3, 10, and 30 mg/kg) and vehicle were administered i.p. 1 hr before the animals were euthanized. Cortical *t*-MH levels were determined by RIA or GC–MS and expressed as nanograms per gram wet weight tissue (mean ± SEM; N = 4/group). Key: (*) clobenpropit-treated group was significantly different from the vehicle-treated group as measured by RIA ($P < 0.05$). There was no significant effect of clobenpropit on *t*-MH levels as measured by GC–MS ($P > 0.05$).

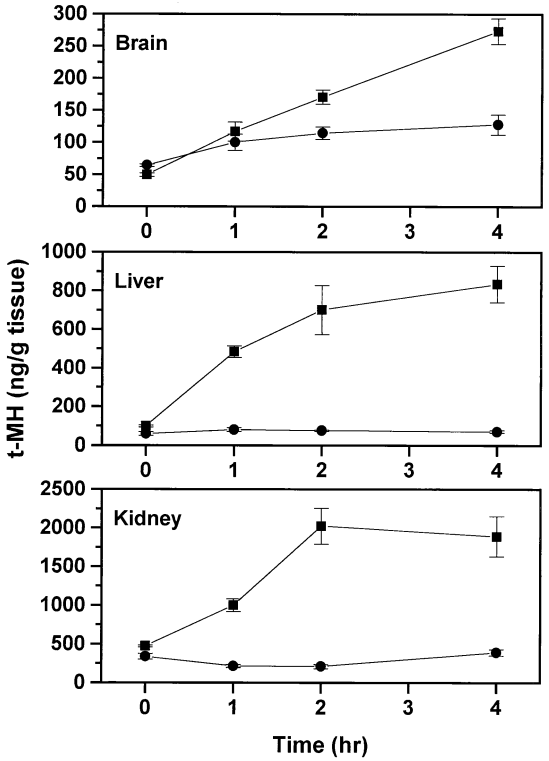


FIG. 5. Apparent levels of *t*-MH in rat cortex, liver, and kidney following GT-2016 (■) or thioperamide (●). GT-2016 or thioperamide (30 or 10 mg/kg, respectively) and the respective vehicles (time 0) were administered i.p. for 1–4 hr before the animals were euthanized. Levels of *t*-MH in the cortex, liver, and kidney were determined by RIA and expressed as nanograms per gram wet weight tissue (mean ± SEM; N = 4/group). In the cortex, both GT-2016 and thioperamide produced a significant increase in apparent *t*-MH levels at all time points ($P < 0.05$). However, in the liver and kidney, only GT-2016 produced a significant increase in apparent *t*-MH ($P < 0.05$, all time points).

t-MH levels, and to compare RIA and GC–MS methodologies for the determination of *t*-MH.

Overall, there was good agreement between the RIA and GC–MS results for *t*-MH levels in the present studies. The data demonstrate that there was not a significant difference in the control levels of *t*-MH as measured by RIA or GC–MS over several experiments, thus providing evidence that either method is suitable for the measurement of basal levels of CNS *t*-MH. Furthermore, there was a highly significant correlation between pooled *t*-MH values as measured by RIA or GC–MS for all of the data (excluding the GT-2016 experiments) in the present study (Fig. 6). These observations provide further validation of the RIA in the assessment of *t*-MH levels.

The H_3 receptor antagonists used in these studies represent a broad range of chemical diversity with good CNS penetration and duration profiles. Thioperamide produced an increase in cortical *t*-MH levels, which was increased further in the presence of pargyline as measured by either the RIA or GC–MS method. These results are in agreement with previous reports showing that thioperamide induces both an increase in HA release and an increase in *t*-MH [4, 12]. However, GT-2016, GT-2227, and clobenpropit had no effect on *t*-MH levels as measured by GC–MS. There was initial concern that the lack of an observable increase in *t*-MH was the result of subsequent metabolism of *t*-MH by MAO. However, even in the presence of the MAO inhibitor pargyline, neither GT-2016 nor GT-2227 produced an increase in *t*-MH as measured by GC–MS (clobenpropit studies were not performed in the presence of pargyline). These findings are somewhat puzzling, since both clobenpropit and GT-2016 promote the release of HA [5, 6], particularly since the prototype H_3 antagonist thioperamide, which also promotes HA release, further induces an increase in *t*-MH synthesis.

The brain levels of the HA metabolite *t*-MH have been suggested to be an index of histaminergic neuronal activity [9]. Methods for the measurement of *t*-MH include GC–MS [17], HPLC [23], and RIA [16, 24]. The present results show a good general agreement between the GC–MS and RIA methods for samples from vehicle-treated animals, as well as from rats treated with pargyline, thioperamide, GT-2227,

TABLE 2. Quantitation of GT-2016 and GT-2035 levels in kidney extracts

	Compound (μg/g wet weight tissue)			
Compound	0 hr	1 hr	2 hr	4 hr
GT-2016	ND	10.8 ± 0.9	6.2 ± 1.5	6.1 ± 0.3
GT-2035	ND	1700 ± 72	1366 ± 86	1693 ± 194

GT-2016 (30 mg/kg) and vehicle (time 0) were administered i.p. for 1–4 hr before the animals were euthanized. Levels of GT-2016 and GT-2035 in the kidney were determined by HPLC (mean ± SEM; N = 4/group). ND indicates non-detectable levels of GT-2016 or GT-2035.

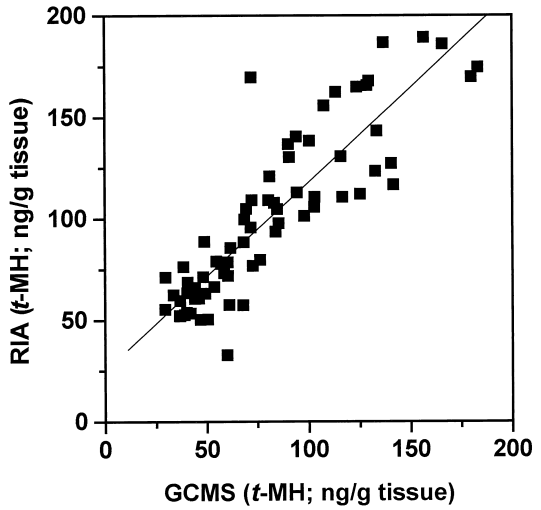


FIG. 6. Correlation between cortical *t*-MH levels measured by RIA and GC–MS. The cortical *t*-MH values as measured by both RIA and GC–MS shown in Figs.1–4 (minus data from GT-2016-treated animals) were compiled. There was a significant correlation between *t*-MH levels measured by RIA and GC–MS ($r = 0.89$; $P < 0.0001$; $N = 70$).

or clobenpropit. However, GT-2016 increased apparent CNS *t*-MH levels in the absence of pargyline as measured by RIA, but not by GC–MS. The lack of an agreement between the RIA and GC–MS methodologies for this particular antagonist suggested the possibility of drug cross-reactivity in the RIA. However, since high concentrations of GT-2016 did not cross-react with the *t*-MH antibody (Table 1), the *in vivo* formation of a GT-2016 metabolite that cross-reacted in the RIA was suspected. This theory is supported by the dramatic increase in apparent *t*-MH levels in liver and kidney tissues as measured by RIA. This increase in liver and kidney *t*-MH levels is not apparent with thioperamide, consistent with the known absence of H_3 receptors in the liver [25]. GT-2016, a piperidine amide, could be envisioned to undergo metabolism by nonspecific amidases to form GT-2035, as well as an array of other metabolites. Such analogs or their methylated derivatives would be potential candidates for antibody cross-reactivity. In fact, GT-2035 shows significant cross-reactivity in the *t*-MH RIA. Furthermore, GT-2035 was detected by HPLC in the cerebral cortex, liver, and kidney extracts from rats treated with GT-2016, and the metabolism of GT-2016 to GT-2035 was demonstrated *in vitro*. That a GT-2016 metabolite is responsible for the increase in apparent *t*-MH in the brain is further supported by the studies performed in the presence of pargyline (Fig. 2). In those studies, there was no further increase in apparent *t*-MH in the presence of pargyline as measured by RIA. These studies indicate that the metabolite in question is not formed in the presence of an MAO inhibitor, and further suggest a role for MAO activity in the metabolic pathway of GT-2016.

The present results show that all brain-penetrating H_3 antagonists do not increase brain levels of the HA metabolite *t*-MH, as seen with thioperamide. The fact that thioperamide is the only HA H_3 antagonist in the present work that induces an increase in *t*-MH levels may be suggestive of a non-HA H_3-mediated activity of thioperamide similar to recent reports [26]. Recently, several new H_3 antagonists with good *in vitro* potency were reported to be inactive in the CNS based on their failure to elevate brain *t*-MH levels, while other compounds were reported to be active based on their ability to elevate brain *t*-MH levels [13–15]. These negative results were attributed to potential metabolism, lack of CNS penetration, and/or pharmacokinetic/metabolic variables. Current studies clearly establish that GT-2016, GT-2227, and clobenpropit penetrate the blood–brain barrier, block brain H_3 receptors, and increase the release of neuronal HA (unpublished observations for GT-2227), but do not increase brain *t*-MH levels. Furthermore, H_3 agonists (data not shown) and potential histaminergic metabolites can cross-react with *t*-MH antibodies in the RIA. Consequently, the classification of H_3 agonist, partial agonist, and antagonist activities should not be based solely on *in vivo* drug-induced changes in *t*-MH levels. The mechanisms by which some H_3 antagonists can increase brain HA release, but not raise tissue levels of its methylated metabolite, require further investigation.

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