Biochemical Pharmacology, Vol. 37, No. 24, pp. 4707–4711, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

ACTIONS OF THE BRAIN-PENETRATING H_2-ANTAGONIST ZOLANTIDINE ON HISTAMINE DYNAMICS AND METABOLISM IN RAT BRAIN

LINDSAY B. HOUGH,* STEPHEN JACKOWSKI, NANCY EBERLE, KATHLEEN R. GOGAS, NANCY A. CAMAROTA and DIANE CUE

Department of Pharmacology and Toxicology, Albany Medical College, Albany, NY 12208, U.S.A.

(*Received* 9 *November* 1987; *accepted* 6 *May* 1988)

Abstract—The effects of zolantidine, the first brain-penetrating H_2-receptor antagonist, on the brain levels of histamine (HA) and the HA metabolite *tele*-methylhistamine (t-MH), the activity of histamine methyltransferase (HMT) and the brain HA turnover rates were investigated in rats. Zolantidine dimaleate (0.1 to 100 mg/kg, s.c.) had no effect on whole brain levels of HA or t-MH and no effect on brain HMT activity, when measured 30 min after administration. Furthermore, brain t-MH levels in pargyline-treated animals were unaffected by zolantidine (0.1 to 25 mg/kg), indicating the absence of an effect on brain HA turnover. *In vitro*, zolantidine was a potent competitive inhibitor of both brain and kidney HMT, with K_i values of 2.3 and 2.7 μM respectively. These results show that, despite the ability of zolantidine to inhibit HMT *in vitro*, large doses of this drug did not alter brain HA methylation or turnover *in vivo*, and they imply that blockade of post-synaptic H_2-receptors does not change brain HA dynamics.

Even though there is now overwhelming evidence that histamine (HA) in brain is synthesized, stored, and released by cells of a specific widely-projecting neuron system [1–4], little attention has been paid, until recently, to understanding the regulation of the activity of these neurons. Although it has been demonstrated that brain HA turnover rates (believed to reflect the activities of these cells) can be modulated pharmacologically [e.g. Refs. 5–7], the relationship between the activation of HA receptors and the activity of histaminergic neurons has remained unclear. Work by Arrang, Schwartz and colleagues [8, 9] adds considerably to this area by demonstrating that activation of a novel HA receptor leads to a decrease in the release of neuronal HA. Confirmation of the existence of this pre-synaptic "H_3-" autoreceptor was achieved recently by the development of selective, brain-penetrating H_3-agonists and -antagonists capable of altering histaminergic dynamics *in vivo* as well as *in vitro* [10].

In contrast to the advances made in understanding pre-synaptic regulation of histaminergic activity, much less is known about the possible post-synaptic regulation of this activity by H_1- or H_2-receptors. Such phenomena may be of regulatory importance for other transmitter systems [11]. This dearth of information is no doubt partially attributable to the poor brain penetration exhibited by H_2-receptor antagonists [12, 13]. Recently, however, the first potent, selective, brain-penetrating H_2-receptor antagonist was developed [14]. Zolantidine (2-[3-[3-(piperidinomethyl) phenoxy] propylamino]-benzthiazole, SKF-95282) exhibits a brain/blood ratio of 1.4 after intravenous infusion (cf. 0.036 for cimetidine) and is 7–22 times more potent than cimetidine on H_2-receptors [14, 15]. Zolantidine was also reported to be a competitive inhibitor of guinea pig brain histamine methyltransferase (HMT, the main enzyme of brain HA catabolism) in concentrations about 10-fold greater than its dissociation constant for blockade of H_2-receptors [15]. We have shown recently that low doses (e.g. 5 mg/kg) of peripherally-administered zolantidine dimaleate inhibit naloxone-insensitive stress-induced analgesia [16], consistent with our other studies suggesting a role for HA and H_2-receptors as mediators of endogenous non-opiate analgesia [17, 18]. Since brain levels of the drug have not been measured after subcutaneous injection, however, it is difficult to know whether behaviorally-effective doses of this drug act via H_2-blockade and/or HMT inhibition. Thus, to investigate the relationship between brain H_2-receptor blockade and HA turnover, and to assess the potency of zolantidine as an inhibitor of HA metabolism in rat brain *in vivo*, we studied the effects of zolantidine on the levels of brain HA and its metabolite *tele*-methylhistamine (t-MH), on brain HA turnover rates, and on HMT activity *in vivo* and *in vitro*.

METHODS

Male Sprague–Dawley albino rats (200–300 g, Taconic Farms, Germantown, NY) were maintained in 12-hr light–dark cycles and used for all experiments. Beginning 3 hr into the light cycle, animals received s.c. injections (doses are salts) of either zolantidine dimaleate or vehicle (see Table 1). Thirty minutes later, animals were decapitated, and whole brains were weighed and homogenized in 4 vol. of ice-cold deionized water. Aliquots (0.1, 0.35, and 0.5 ml) were taken to measure HA, t-MH and HMT activity, respectively, as described previously [7, 19–22]. Brains and kidneys from untreated animals were homogenized in 4 and 9 vol.

* Author to whom correspondence should be sent.

of water, respectively, and used for *in vitro* HMT experiments.

To measure HA, aliquots were diluted with an equal volume of 0.1 N sodium phosphate buffer (pH 7.9), mixed, heated in boiling water for 10 min, cooled and centrifuged. Supernatant fractions were assayed by the single-isotope radioenzymatic assay as recently described [7]. All samples were incubated at 0° [23] in the presence and absence of 0.05 ng exogenous HA for recovery measurements, which were 96 ± 3%. All histamine levels were uncorrected for recovery, and recoveries from zolantidine-treated animals were not different from controls.

To measure the brain metabolite t-MH, aliquots were diluted with an equal volume of 0.8 N perchloric acid and taken to 1 ml with 0.4 N perchloric acid made to contain 10 ng trideuterated t-MH. The samples were centrifuged, extracted, derivatized and analyzed by combined gas chromatography–mass spectrometry according to the method developed and described previously [21], with minor modifications recently described [7].

The inhibition of HMT by zolantidine *in vivo* was investigated by assessing the HMT activity in brain homogenates from zolantidine-treated animals. Aqueous homogenates (prepared as described above) were mixed with an equal volume of 0.2 N sodium phosphate buffer (pH 7.9) and centrifuged (50,000 *g* for 11 min). Supernatant fractions (0.1 ml) were incubated with HA (20 μM final concentration unless noted otherwise) and [^{3}H]*S*-adenosyl-L-methionine (SAM, 9.8 μM) in a total volume of 0.46 ml sodium phosphate buffer (0.1 N) at 37° for 15 min, similar to the assay described in detail by Taylor and Snyder [24]. The reaction was stopped with 0.2 ml of 0.4 N perchloric acid, and labeled t-MH was extracted and counted exactly as described by Beaven *et al.* [25]. Activity is expressed in $\text{pmol} \cdot (15\ \text{min})^{-1} \cdot (\text{g tissue homogenized})^{-1}$. The counts contributed by blanks, determined by incubation of vials without enzyme, were subtracted from each sample before calculating results. The results of the effects of zolantidine on the above parameters were assessed by one-way analysis of variance (ANOVA).

The effect of zolantidine on HMT activity was also determined *in vitro* by incubation of homogenates from untreated animals with or without zolantidine in the presence of 10, 20 and 50 μM HA. HMT activities measured in the absence of zolantidine were fitted to the Michaelis–Menten equation with the RS/1 procedure $FITFUNCTION [26], an iterative algorithm utilizing non-linear regression to yield estimates of K_m and V_{max} for each enzyme preparation. These values were also estimated by linear regression applied to double-reciprocal plots ($1/[S]$ vs $1/V$ [27]). Results from these two independent methods were in excellent agreement, and the data given (Table 2) are from the double-reciprocal method. Zolantidine inhibition data are expressed as Dixon plots (Fig. 1A, [28]), and K_i values were estimated by re-plotting the slopes of Dixon curves vs 1/[HA] (Fig. 1B), as recommended by Segel [29]. Each homogenate yielded a separate estimate of K_m, V_{max}, and K_i, the means of which are given in Table 2.

RESULTS

Zolantidine dimaleate (0.1 to 100 mg/kg, s.c.) had no effect on whole brain HA or t-MH levels when determined 30 min after administration (Table 1). Similarly, HMT activity in these brain homogenates was unaffected (Table 1). As observed previously, the monoamine oxidase (MAO) inhibitor pargyline elevated whole brain t-MH levels; we [e.g. Ref. 22] and others [e.g. Ref. 30] have shown the amount of this elevation to be an estimate of the rate of brain HA turnover. In the present results, zolantidine treatment had no effect on the magnitude of the pargyline-induced t-MH accumulation (Table 1).

Zolantidine clearly inhibited HMT activity *in vitro* (Fig. 1, Table 2). A dose-related inhibition of both kidney and brain enzymes was observed from 0.3 to 100 μM. Dixon plots of the inhibition in the presence of various HA concentrations revealed a surmountable antagonism (Fig. 1A). When the slopes of the Dixon plot associated with each substrate concentration were re-plotted vs 1/[HA], a straight line was obtained with an intercept not significantly different from zero [fitted intercept (mean ± SD) was 0.0146 ± 0.019], showing the zolantidine-induced inhibition of HMT to be competitive with respect to HA [28, 29]. When calculated from the slopes of Dixon re-plots for individual enzyme preparations, mean K_i values for zolantidine were 2.3 and 2.7 μM for brain and kidney, respectively, not significantly different from each other (Table 2).

DISCUSSION

It was considered to be highly likely that zolantidine would change brain HA dynamics, either by blockade of brain H_2-receptors, with a resulting regulatory change in HA turnover, or by inhibition of HA methylation, since it was reported to inhibit HMT *in vitro* [15]. Surprisingly, when tested over a wide range of doses, zolantidine had no effect on the brain levels of HA or t-MH, or on the activity of HMT (Table 1). Furthermore, no dose of drug tested exerted a significant effect on whole brain HA turnover rates, estimated from the pargyline-induced increase in brain t-MH levels [22, 30].

No data have yet established the potency of zolantidine (given s.c.) as a brain H_2-receptor antagonist. However, considering that: (1)an i.v. dose of 0.8 mg/kg inhibited HA-stimulated gastric acid secretion by 50% in the anaesthetized rat,* (2) the compound appears to be more potent as an antagonist of non-gastric H_2-receptors (including brain) than of gastric H_2-receptors,* and (3) the brain/blood ratio of zolantidine is greater than 1.0 [15], it is highly likely that a significant blockade of brain H_2-receptors occurs after a dose of 25 mg/kg. It is also likely that brain H_2-receptors were blocked 30 min after administration. Although brain drug levels have not been measured, zolantidine inhibits

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Table 1. Effects of zolantidine on whole brain levels of HA, t-MH and HMT activity

Dose (mg/kg)	HA (ng/g)	t-MH (ng/g)	PRG-induced t-MH (ng/g)	HMT [pmol·(15 min)$^{-1}$·g^{-1}] Expt. 1	Expt. 2
0	43.0 ± 2.5	33.7 ± 3.6	95.5 ± 5.6	1741 ± 141	1377 ± 22
0.1	48.9 ± 5.5	34.4 ± 2.5	102.0 ± 3.0	1670 ± 20	
1	44.0 ± 7.2	33.4 ± 2.3	98.8 ± 4.6	1747 ± 114	
5	38.8 ± 9.0	43.3 ± 7.9	87.1 ± 9.0	1587 ± 160	
25	45.5 ± 7.6	28.0 ± 2.7	100.3 ± 8.7	2106 ± 165	
100	43.2 ± 3.6	38.8 ± 1.8			1369 ± 38

Animals received the indicated dose of zolantidine dimaleate (s.c.) and were decapitated 30 min later. Whole brain homogenates were assayed for their contents as described (N = 5–11, except for HMT, where N = 3–5). Control animals (corresponding to a dose of 0) received either saline, or disodium maleate (12.8 mg/kg, the molar equivalent in maleate to 25 mg/kg of zolantidine dimaleate); results from these treatments did not differ, and thus were combined. In a separate experiment to estimate HA turnover ("PRG-induced t-MH", N = 5–6), whole brain t-MH levels were assayed from animals that received pargyline hydrochloride (75 mg/kg, i.p.), followed 1 hr later by zolantidine or vehicle, and were then killed after a second hour. Values are means ± SEM.

Table 2. Inhibition of HMT by zolantidine

Enzyme source	V_{max} [nmol·g^{-1}·(15 min)$^{-1}$]	HA K_m (μM)	Zolantidine K_i (μM)
Rat whole brain	19.2 ± 1.9	41.0 ± 5.5	2.3 ± 0.7
Rat kidney	326.9 ± 30.6	69.6 ± 20.1	2.7 ± 0.3

HMT activity was determined in homogenates from naive animals as described. Aliquots of each homogenate were incubated in triplicate in the presence of various concentrations of HA and zolantidine, as depicted in Fig. 1. For each homogenate, separate K_m and V_{max} values were estimated from the activity observed in the absence of zolantidine. Each homogenate also yielded a separate Dixon plot (as in Fig. 1A); the slopes of these lines were then re-plotted vs 1/[HA] (as in Fig. 1B). Zolantidine K_i values for each homogenate were calculated according to the formula in Fig. 1B, from the slope of the Dixon replot, along with the estimated K_m and V_{max} found for that enzyme preparation. Values shown are the mean ± SEM of three separate homogenates.

stress-induced analgesia from 30 to 60 min after s.c. administration, with a maximum effect after 30 min [16].

The results of Table 1 therefore suggest that blockade of H_2-receptors does not induce a regulatory change in brain HA turnover rate. If, as some data suggest, H_2-receptors mediate post-synaptic histaminergic transmission [3, 4], then these results imply that HA turnover rate is not regulated post-synaptically, a phenomenon which may occur in other transmitter systems [11]. The affinity of zolantidine for the pre-synaptic H_3-receptor is presently unknown. If, as suggested by Arrang *et al.* [10], blockade of H_3-receptors increases HA turnover, then our results would predict a low affinity of zolantidine for this receptor.

Zolantidine inhibited both rat kidney and rat brain HMT *in vitro* (Fig. 1, Table 2), with similar K_i values (about 2.5 μM, Table 2). The inhibition was competitive with respect to HA; the type of inhibition with respect to SAM was not investigated. These results are similar to those of Calcutt *et al.* [15], who reported zolantidine to be a competitive inhibitor of guinea pig brain HMT. Their K_i value (0.25 μM) is 10-fold lower than that found presently. This is not attributable to the SAM concentrations used in the respective studies (which were nearly identical), but may be due to differences in assay pH (7.4 vs 7.9) or species. Although there is evidence that HMT exists in more than one form [31–33], recent physical biochemical characterizations of the purified enzymes from rat and guinea pig brain show very strong similarities [34, 35]. The same seems to be true for rat kidney and rat brain HMT, as immunochemical studies suggest that these enzymes share several antigenic determinants [36]. Our results (Table 2), showing the similarity in the affinity of zolantidine for these two enzymes, also suggest that the enzymes bear resemblance. They may not be identical, however, as some inhibitors strongly discriminate between rat kidney and rat brain enzymes [33].

Other H_2-antagonists inhibit [37] (and sometimes activate [38]) HMT, depending on substrate concentrations and/or enzyme source, although the concentrations in which they are active are in the range of 60 to 30 μM. The differences in our estimated K_i values and those of Calcutt *et al.* [15] notwithstanding, it seems clear that, among H_2-antagonists, zolantidine is the most potent inhibitor of HMT

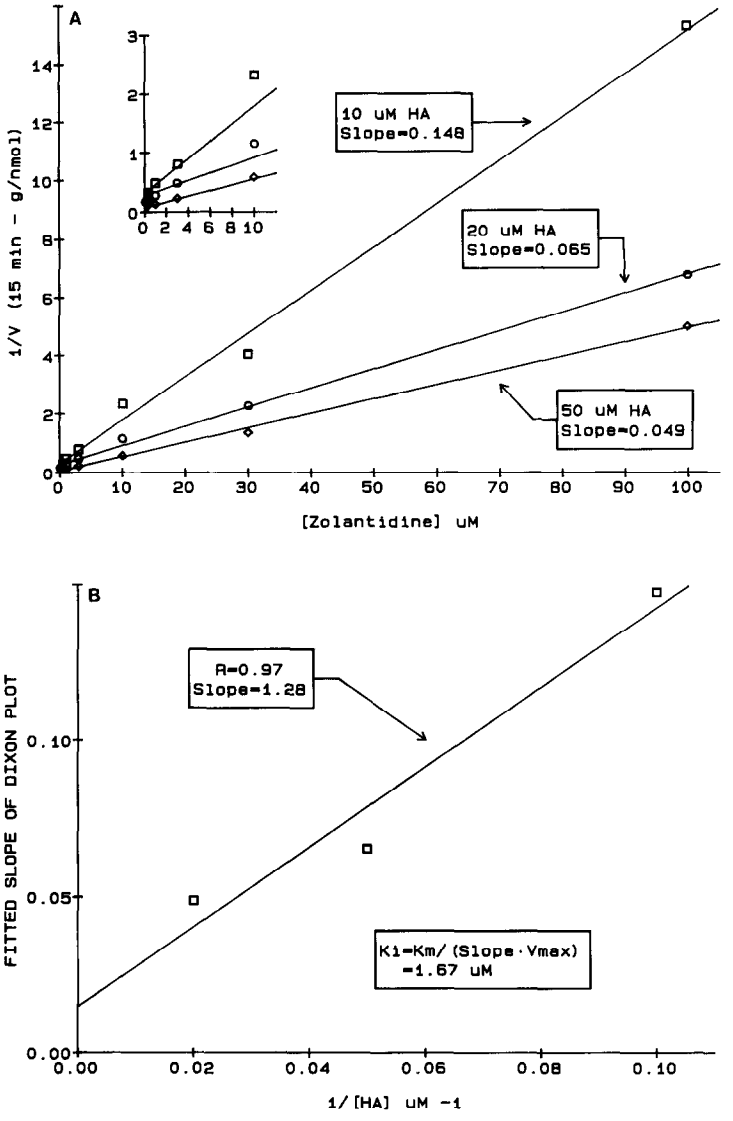

Fig. 1. Inhibition of HMT by zolantidine. HMT activity was determined in triplicate in homogenates of rat brain in the presence of various concentrations of zolantidine and HA. Results are shown as a Dixon plot. (A, ordinate = 1/HMT activity), with mean values from three separate homogenates shown as a function of zolantidine concentration (abscissa), determined at the three substrate concentrations identified. Standard error of the means ranged from 3 to 16% of the mean values. (Inset for Fig. 1A: expanded scales for lower portions of x and y axes.) For each HA concentration, the best line was determined by linear regression. The slope of each line was then re-plotted (B) against the reciprocal of substrate concentration (1/[HA]). The fitted intercept of the regression line of this plot (Fig. 1B) did not differ significantly from zero, indicating competitive inhibition. The slope of the line was used to calculate the K_i value (1.67 μM) as shown in the figure (see [29]). This estimate of K_i determined for the brain enzyme is slightly different than the mean value calculated from individual experiments (2.3 μM, Table 2) because the calculation in the figure used mean V_{max} and mean K_m values, whereas the value in the Table used the V_{max} and K_m values from each homogenate, removing the inter-homogenate variation in HMT activity from the K_i determination.

identified to date. Its greater affinity for H_2-receptors allows it to still be considered a selective H_2-antagonist, however [15].

Considering the potency of zolantidine on HMT *in vitro*, its lack of inhibition of HA methylation *in vivo* was surprising (Table 1). Whole brain HMT activity was unaffected by all doses of drug administered. However, since the brain was diluted by homogenization, caution must be used in making conclusions about the inability of the drug to inhibit the enzyme. More persuasive is the absence of effects of zolantidine on brain HA and metabolite levels (Table 1). Because drugs capable of inhibiting HMT *in vivo* raise HA levels and decrease brain levels of

HA metabolites [20], our results imply that zolantidine dimaleate, in doses up to 100 mg/kg, has no significant effects on brain HA methylation.

There are other examples of compounds that seem to inhibit HA methylation *in vitro*, but not *in vivo* [39, 40]. Compounds such as metoprine are effective *in vivo* [20], whereas other compounds with equal potency are not [40]. Similarly, lipid solubility (a crude estimate of brain penetration) also seems not to explain the discrepancy, since zolantidine appears to be considerably more lipophilic than metoprine [14, 39].

We recently observed that inhibition by zolantidine of stress-induced analgesia exhibited a biphasic dose–response curve, with doses greater than 5 mg/kg exhibiting less effect than lower doses [16]. We speculated that higher doses of zolantidine might be inhibiting HMT *in vivo*, and thus be counteracting the effects of H_2-blockade. The present results show that this is not the case, since doses up to 100 mg/kg had no effect on brain HA methylation 30 min after administration (Table 1). The investigation of this and other functions for HA and H_2-receptors in brain [4, 41] will be facilitated by the use of zolantidine.

Acknowledgements—This work was supported by grants from the National Institute on Drug Abuse (DA-03816) and the Sterling Winthrop Research Institute. K. R. G. is the recipient of a Sterling Drug Pharmacology Fellowship. We thank Smith, Kline & French, Ltd. for the sample of zolantidine.

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