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ACCELERATED COMMUNICATION

Identification of Two H_3-Histamine Receptor Subtypes

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SUMMARY

The H_3-histamine receptor provides feedback inhibition of histamine synthesis and release as well as inhibition of other neurotransmitter release. We have characterized this receptor by radioligand binding studies with the H_3 agonist N^{α}-[^{3}H]methylhistamine ([^{3}H]NAMHA). The results of [^{3}H]NAMHA saturation binding and NAMHA inhibition of [^{3}H]NAMHA binding were consistent with an apparently single class of receptors (K_D = 0.37 nM, B_{max} = 73 fmol/mg of protein) and competition assays with other agonists and the antagonists impromidine and dimaprit disclosed only a single class of sites. In contrast, inhibition of [^{3}H]NAMHA binding by the specific high affinity H_3 antagonist thioperamide revealed two classes of sites (K_{i_A} = 5 nM, B_{max_A} = 30 fmol/mg of protein; K_{i_B} = 68 nM, B_{max_B} = 48 fmol/mg of protein.) Burimamide, another antagonist that, like thioperamide, contains a thiourea group, likewise discriminated between two classes of sites. In addition to differences between some antagonist potencies for the two receptors, there is a differential guanine nucleotide sensitivity of the two. The affinity of the H_{3A} receptor for [^{3}H]NAMHA was reduced less than 2-fold, whereas [^{3}H]NAMHA binding to the H_{3B} receptor was undetectable in the presence of guanosine 5′-*O*-(3-thiotriphosphate). The distinction between H_{3A} and H_{3B} receptor subtypes, the former a high affinity and the latter a low affinity thioperamide site, draws support from published *in vitro* data.

The H_3-histamine receptor is a high affinity receptor reported to inhibit central nervous system histamine synthesis (1, 2) and release (1, 3, 4) and serotonin release (5), as well as peripheral norepinephrine (6), acetylcholine (7–9), and neuropeptide release (10) and histamine synthesis (1). Early evidence for such a receptor derived from high affinity binding of [^{3}H]histamine to rat brain membranes (11). More recently, a specific high affinity H_3 agonist, RAMHA, and an H_3 antagonist, thioperamide, have been reported (1).

The binding of [^{3}H]RAMHA shows complex kinetics, characteristic of ligand binding either to two discrete sites or to a single site that undergoes a change in affinity subsequent to ligand binding (12). We report here that the specific high affinity antagonist thioperamide discriminates between two discrete H_3 receptor subtypes at equilibrium.

Experimental Procedures

Materials. [^{3}H]NAMHA (80 Ci/mmol) was from Dupont NEN. NAMHA was from Calbiochem, histamine and pyrilamine from Sigma, impromidine from Smith, Kline, and French Laboratories, and GTPγS from Boehringer Mannheim. All other compounds were from Schering-Plough Research. Male Wistar rats (250 g) were from Charles River Breeding Farms.

Tissue preparation. Freshly dissected rat brains were disrupted with a Polytron in 20 volumes of ice-cold Tris·HCl (pH 7.5 at 25°). Debris was sedimented by 10 min of centrifugation at 1000 × *g*, after which membranes were sedimented at 50,000 × *g* for 10 min. Membranes were resuspended and washed twice. They were stored as pellets at −20° for up to 3 months with no apparent change in [^{3}H]NAMHA binding.

Radioligand binding assay. Tissue (0.2–0.3 mg of protein) was incubated 40 min in 0.5 ml of 50 mM Tris·HCl at 30°, with or without added compounds, and then filtered over 0.3% polyethylenimine-presoaked (13) Whatman GF/C filters (Brandel, Gaithersburg, MD). Filters were washed with three 4-ml volumes of buffer and counted at 48% efficiency. Nonspecific binding was determined in the presence of 2 μM RAMHA or 1 μM NAMHA. For competition binding assays, the [^{3}H]NAMHA concentration was 0.2 nM (16,000 dpm). At equilibrium, less than 10% of free ligand was bound (1,100 dpm) and, of this, 6% was nonspecific binding (50–70 dpm). Protein was assayed with a bicinchoninic acid reagent (14) (Pierce).

Data analysis. Saturation and competition binding experiments were individually analyzed with the LIGAND, weighted, nonlinear, least-squares, curve-fitting program (15). Curves were fit to saturation binding data with the equation

$$[B] = \sum_{i=1}^{i=n} \left(\frac{B_{max_i}[L]}{K_{D_i} + [L]} \right) + N[L]$$

where K_{D_i} and B_{max_i} are constants for ligand binding to site i of n sites, $[L]$ is the concentration of free radioligand, and N is the ratio of nonspecific to specific binding at infinite radioligand concentration. Competition curves were fit to the equation modified for multiple

ABBREVIATIONS: RAMHA, (*R*)-α-methylhistamine; NAMHA, N^{α}-methylhistamine; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

ligands. Goodness of fit was tested for $n = 1$, 2, and 3 with an F test of residual sums of squares corrected for differences in degrees of freedom.

Results

[^{3}H]NAMHA saturation binding data (Fig. 1A) subjected to Scatchard analysis disclosed an apparently single class of sites at equilibrium (K_D = 0.37 ± 0.037 nM, B_{max} = 73 ± 3 fmol/mg of protein, four experiments (Fig. 1B). Analysis of competition binding data for most compounds yielded Hill coefficients (n_H) close to 1.0, consistent with an apparently single class of binding sites (Table 1). These compounds include the agonists NAMHA, RAMHA, and histamine and the antagonists impromidine and dimaprit.

However, analysis of competition by the specific high affinity

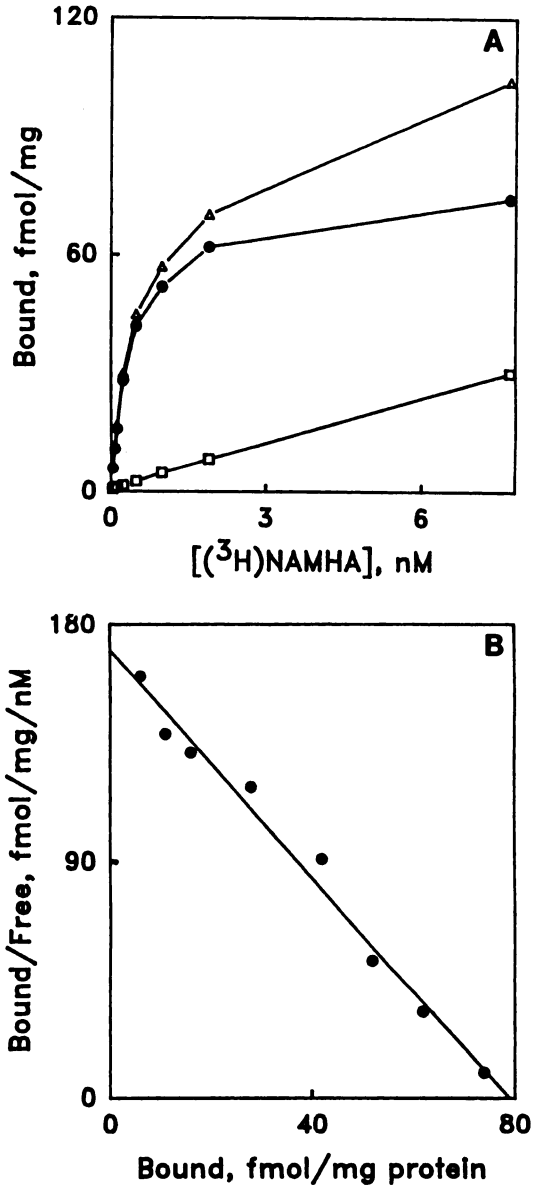

Fig. 1. [^{3}H]NAMHA saturation binding. A, Binding isotherm. ●, Specific binding; △, total binding; □, nonspecific binding. *Points* are the averages of triplicate determinations. B, Scatchard plot. Each of four saturation binding experiments was analyzed individually with the LIGAND program (15). Shown is the monoexponential fit from one experiment.

TABLE 1

Inhibition of [^{3}H]NAMHA binding

[^{3}H]NAMHA (0.2 nM) was incubated 40 min with 0.3 mg of tissue and 10 concentrations of each compound in duplicate. Nonspecific binding, 5% of total, was the same for all these drugs. K_i values were determined with the LIGAND program, based on 0.37 nM as the K_D value for [^{3}H]NAMHA. Values are mean ± range from two assays. The following compounds inhibited binding 20% or less at 1 μM: cimetidine, chlorpheniramine, ranitidine, pyrilamine, tiotidine, and metiamide.

Compound	K_i	n_H
	nM	
NAMHA	0.36 ± 0.22	1.0
RAMHA	1.2 ± 0	0.9
Histamine	4.4 ± 0.1	1.0
Impromidine	45 ± 1	1.0
Dimaprit	520 ± 150	1.0

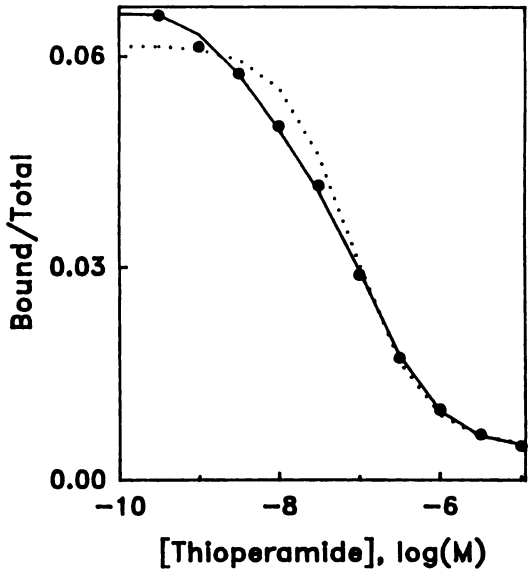

Fig. 2. Thioperamide competition for [^{3}H]NAMHA binding sites. . . . . , One-site fit; ———, two-site fit. Shown is one of six experiments. In all six experiments, a two-site fit was significantly better than a one-site fit (p = 0.001).

TABLE 2

Inhibition of [^{3}H]NAMHA binding by compounds that discriminate two sites

Assay details are as for Table 1. Values are mean ± standard error (six experiments for thioperamide, four experiments for burimamide). Hill coefficients were 0.7 for thioperamide and 0.6 for burimamide. For only these two compounds was a two-site fit an improvement over one site (thioperamide, p = 0.001; burimamide, p = 0.02).

Compound	K_{i_A}	K_{i_B}
	nM	nM
Thioperamide	4.9 ± 1.6	64 ± 11
Burimamide	97 ± 38	3500 ± 1800

H_3 antagonist thioperamide for [^{3}H]NAMHA binding sites (Fig. 2, Table 2) revealed two classes of H_3 sites (K_{i_A} = 4.9 ± 1.6 nM, B_{max_A} = 30 ± 5 fmol/mg of protein; K_{i_B} = 64 ± 11 nM, B_{max_B} = 45 ± 6 fmol/mg of protein; six experiments), based on p = 0.001 for a two- relative to one-site fit and $p > 0.05$ for a three- relative to a two-site fit. Burimamide, another antagonist that, like thioperamide, contains a thiourea group in the side chain, also discriminated between two classes of [^{3}H]NAMHA binding sites (Table 2).

The possibility that one of these sites might arise from an agonist-induced conformational change in the receptor was

TABLE 3

Densities of high and low affinity thioperamide sites at different times during [^{3}H]NAMHA steady state binding

Experimental details are summarized in Table 1. Values are mean ± standard error. Six experiments were performed for the 40-min assay, and three for the 60-min assay.

Length of incubation	K_{i_A}	B_{max_A}	K_{i_B}	B_{max_B}
min	nM	fmol/mg of protein	nM	fmol/mg of protein
40	4.9 ± 1.6	30 ± 5	64 ± 11	45 ± 6
60	8.6 ± 3.8	31 ± 3	123 ± 25	40 ± 6

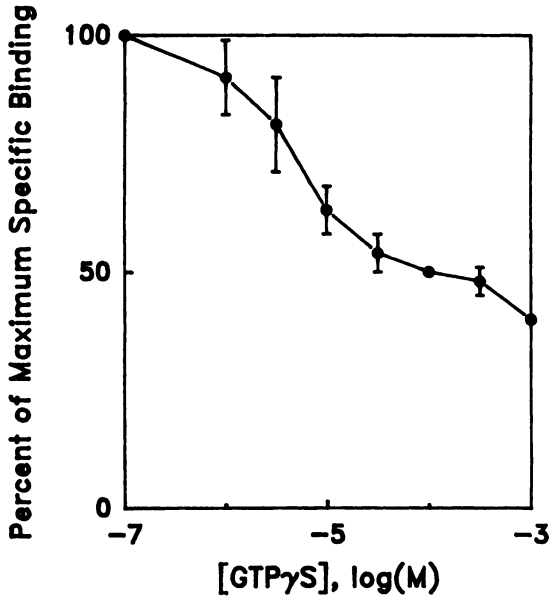

Fig. 3. GTPγS inhibition of specific [^{3}H]NAMHA binding. Binding of 0.2 nM [^{3}H]NAMHA in the absence and presence of 10^{-6} M NAMHA was determined with various concentrations of GTPγS. Shown is the average ± range of two experiments.

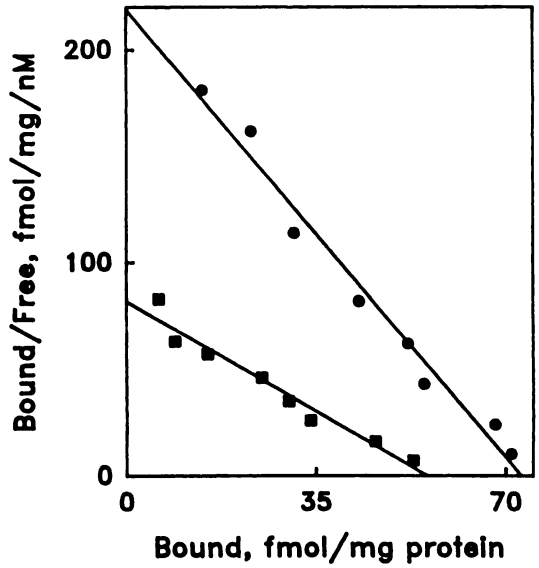

Fig. 4. Scatchard plot of [^{3}H]NAMHA saturation binding with and without GTPγS. ●, Control; ■, GTPγS. Shown is one of three paired experiments.

TABLE 4

Inhibition of [^{3}H]NAMHA binding in the presence of 10^{-4} M GTPγS

Details are as for Table 1, except total and nonspecific binding were determined in the presence of 10^{-4} M GTPγS.

Compound	K_i	n_H
	nM	
NAMHA	0.97 ± 0.33	0.9
RAMHA	1.6 ± 0.2	0.9
Histamine	6.7 ± 1.7	1.0
Thioperamide	10 ± 2	0.9
Impromidine	25 ± 5	1.0
Burimamide	240 ± 90	0.8
Dimaprit	370 ± 30	0.9

tested by running a thioperamide competition assay sufficiently beyond equilibrium to permit substantial conversion of an initial to a later conformation, if it were going to occur. Clearly, no change in the relative site numbers of high and low affinity sites occurred in the 20 min after steady state binding was reached, although the affinity of thioperamide for both sites appeared to decrease during this time (Table 3).

The specific guanine nucleotide sensitivity of H_3 receptor binding has already been demonstrated (11). We chose to characterize the nature of these effects with GTPγS. Maximum inhibition of [^{3}H]NAMHA binding (50% of control) occurred at 3.2×10^{-5} M GTPγS (Fig. 3). A concentration of 10^{-4} M GTPγS was used for subsequent experiments. Two effects of GTPγS were apparent in Scatchard plots of saturation binding (Fig. 4). One was a reduction of apparent site density, an average of 22 ± 4% in three experiments; the other was a 1.7 ± 0.1-fold decrease in apparent affinity of the remaining sites.

To determine whether these effects of GTPγS distinguished between high and low affinity thioperamide sites, competition binding assays were run in the presence of GTPγS (Table 4). Inhibition of [^{3}H]NAMHA binding in the presence of GTPγS was best characterized for all competitors by a one-site fit. For thioperamide and burimamide, this corresponded to the high affinity site. The average B_{max} from these experiments was 37 ± 2 fmol/mg of protein (14 experiments).

Discussion

We have found with the specific high affinity agonist [^{3}H]-RAMHA that binding to rat brain membranes is characterized by biexponential kinetics (12). Additionally, we found that the affinity of NAMHA for the H_3-histamine receptor was higher in a [^{3}H]RAMHA competition binding assay than had been reported for a [^{3}H]histamine binding assay (11). In our hands, NAMHA was a more potent competitor than RAMHA itself, so we tested the utility of [^{3}H]NAMHA as an H_3-histamine receptor radioligand. Studies in the guinea pig showed that [^{3}H]NAMHA bound with high affinity to a class of sites typical of an H_3-histamine receptor (16). In the rat, it binds to the same density of sites (Fig. 1B) as [^{3}H]RAMHA (12). The inhibitor profile of this binding is characteristic of an H_3 receptor. The H_2 compounds that compete with it for binding are those that are known to bind to an H_3 receptor. Although NAMHA may be less selective than RAMHA for the H_3 receptor (1, 3), the affinities of H_1 and other H_2 compounds make it clear that, at the concentrations of radioligand employed in this assay, binding is specifically to H_3 receptors (Tables 1 and 2). An advantage of [^{3}H]NAMHA (Fig. 1A) (16) over [^{3}H]-

RAMHA (12) as an H_3 radioligand is the much lower nonspecific binding with [^{3}H]NAMHA.

An argument for multiple receptor subtypes based only on the kinetics of radiolabeled agonist binding is bedeviled by the possibility that rapid agonist binding might induce a slow conformational change or enzymatic modification that is recognized as a second component in kinetic studies. Indeed, neither [^{3}H]NAMHA equilibrium saturation binding nor competition by NAMHA or other agonists for [^{3}H]NAMHA binding sites gave any evidence for multiple agonist-differentiated sites at equilibrium. However, competition by certain antagonists, notably thioperamide, for [^{3}H]NAMHA binding sites makes it clear that agonists bind to two H_3 receptor subtypes at equilibrium. Thus, a distinction between an H_{3A} site with high affinity for thioperamide (K_i = 5 nM) and an H_{3B} site with lower affinity for thioperamide (K_i = 64 nM) can be drawn.

Another recent study (17), as well as this one (Fig. 4), describes two effects of guanine nucleotides on H_3 receptor agonist binding. The dual effect of GTPγS on [^{3}H]NAMHA binding is consistent with two receptor subtypes. Agonist binding to the H_{3A} receptor is only slightly affected by GTPγS, whereas binding to the H_{3B} receptor is undetectable in the presence of GTPγS (Table 4).

Ample evidence already exists for functional distinctions between H_3 receptor subtypes, although it has not been interpreted as such. An 8-fold difference between the potencies of thioperamide to block RAMHA inhibition of histamine synthesis and release in rat brain has been ascribed to an artifact (1, 2). Because the duration of the release assay was 2 min and that of the synthesis assay 30 min, it was reasoned that accumulation of endogenous released histamine in the synthesis assay would result in an actual agonist concentration much greater than the nominal exogenous concentration. Because an antagonist IC_{50} value, from which the K_i is determined, is a function of agonist concentration in the assay (18), a substantial underestimation of thioperamide potency in the histamine synthesis assay would then result (1, 2). However, the same conditions should similarly affect tiotidine antagonism in the two assays, and yet tiotidine was reported to be more potent in the synthesis than in the release assay (2). Our data substantiate the difference between thioperamide potencies in the two assays as real. An H_{3A} receptor, one with high affinity for thioperamide, would have been detected in the release assay, whereas an H_{3B} receptor, one with low affinity for thioperamide, would have been detected in the synthesis assay.

Insofar as they have been characterized, the guinea pig ileum and mesenteric artery preparations contain H_3 histamine receptors. Of interest in the mesenteric artery preparation is a lack of metiamide antagonist effect, because in the rat brain histamine release assay, which we posit to involve an H_{3A} subtype, the K_i for metiamide is reported as 2.5 μM (2). As one of the compounds that contains a thiourea group in its side chain, metiamide might be expected to discriminate between two classes of sites. However, its very low potency ($IC_{50} \geq 10^{-4}$ M) precluded determination of a full dose-response curve in the binding assay. So it remains a possibility that the H_{3B} receptor is assayed in the guinea pig mesenteric artery. More detailed pharmacological characterization of the mesenteric artery and the ileum, with thioperamide and burimamide in particular, may distinguish between the H_3 receptors assayed in these tissues and, as such, provide receptor subtype-specific bioassays. Meanwhile, the rat brain [^{3}H]NAMHA binding assay may be useful for developing receptor subtype-specific drugs.

References

1. Arrang, J.-M., M. Garbarg, J.-C. Lancelot, J.-M. Lecomte, H. Pollard, M. Robba, W. Schunack, and J.-C. Schwartz. Highly potent and selective ligands for histamine H_3-receptors. *Nature (Lond.)* **327**:117–123 (1987).
2. Arrang, J.-M., M. Garbarg, and J.-C. Schwartz. Auto-inhibition of histamine synthesis mediated by presynaptic H_3-receptors. *Neuroscience* **23**:149–157 (1987).
3. Arrang, J.-M., M. Garbarg, and J.-M. Schwartz. Auto-inhibition of brain histamine release mediated by a novel class (H_3) of histamine receptor. *Nature (Lond.)* **302**:832–837 (1983).
4. Van der Werf, J. F., A. Bast, G. J. Bijloo, A. Van der Vliet, and H. Timmerman. HA autoreceptor assay with superfused slices of rat brain cortex and electrical stimulation. *Eur. J Pharmacol.* **138**:199–206 (1987).
5. Schlicker, E., R. Betz, and M. Gothert. Histamine H_3 receptor-mediated inhibition of serotonin release in the rat brain cortex. *Naunyn-Schmeideberg's Arch. Pharmacol.* **337**:588–590 (1988).
6. Ishikawa, S., and N. Sperelakis. A novel class (H_3) of histamine receptors on perivascular nerve terminals. *Nature (Lond.)* **327**:158–160 (1987).
7. Trzeciakowski, J. P. Inhibition of guinea-pig ileum contractions mediated by a class of histamine receptor resembling the H_3 subtype. *J. Pharmacol. Exp. Ther.* **243**:874–880 (1988).
8. Tamura, K., J. M. Palmer, and J. D. Wood. Presynaptic inhibition produced by histamine at nicotinic synapses in enteric ganglia. *Neuroscience* **25**:171–179 (1988).
9. Ichinose, M., and P. J. Barnes. Inhibitory histamine H_3-receptors on cholinergic nerves in human airways. *Eur. J. Pharmacol.* **163**:383–386 (1989).
10. Ichinose, M., and P. J. Barnes. Histamine H_3-receptor mediated inhibition of non-adrenergic, non-cholinergic bronchoconstriction in guinea-pig *in vivo*. *Am. Rev. Resp. Dis.* **139**:A236 (1989).
11. Barbin, G., J. M. Palacios, E. Rodergas, J.-C. Schwartz, and M. Garbarg. Characterization of the high affinity binding sites of [^{3}H]histamine in rat brain. *Mol. Pharmacol.* **18**:1–10 (1980).
12. West, R. E., Jr., A. Zweig, R. T. Granzow, M. I. Siegel, and R. W. Egan. Biexponential kinetics of (*R*)-α-[^{3}H]methylhistamine binding to the rat brain H_3 histamine receptor. *J. Neurochem.* **55**:1612–1616 (1990).
13. Bruns, R. F., K. Lawson-Wendling, and T. A. Pugsley. A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem.* **132**:74–81 (1983).
14. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85 (1985).
15. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for the characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
16. Korte, A., J. Myers, N.-Y. Shih, R. W. Egan, and M. A. Clark. Characterization and tissue distribution of H_3 histamine receptors in guinea pigs by N^{α}-methylhistamine. *Biochem. Biophys. Res. Commun.* **168**:979–986 (1990).
17. Schwartz, J.-C., J.-M. Arrang, M. Garbarg, and H. Pollard. A third histamine receptor subtype: characterisation, localisation and functions of the H_3-receptor. *Agents Actions* **30**:13–23 (1990).
18. Cheng, Y.-C., and W. M. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).

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