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Report

A Comparison of the Cholinergic Activity of Selected H_2-Antagonists and Sulfoxide Metabolites

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Famotidine and selected H_2-antagonists were evaluated with respect to toxicity and selected pharmacological activities. When administered intraperitoneally to mice at a dose equivalent to 10 times their respective H_2-antagonist ED_{50} values, no deaths were observed. Similarly, no alteration in brain ACh concentrations or overt pharmacological effects were noted. However, at 400 mg/kg, ranitidine produced 89% lethality, followed by cimetidine (11%) and famotidine. Only cimetidine and famotidine at this dose significantly elevated brain acetylcholine levels. These results do not correlate with the *in vitro* data, where ORF-17578 and ranitidine were the most potent entities with respect to acetylcholinesterase inhibition (~ 1–2×10^{-6} *M*), followed by nizatidine > cimetidine > famotidine. The sulfoxide metabolites of ranitidine and cimetidine were approximately one-tenth as potent as their parent compounds with respect to inhibition of acetylcholinesterase. Direct muscarinic stimulation or potentiation of acetylcholine-induced contraction in ileal tissue was not observed for any of the H_2-antagonists.

KEY WORDS: histamine-H_2-antagonists; acetylcholinesterase; acetylcholine—brain levels.

INTRODUCTION

The introduction of the H_2-antagonists, cimetidine and ranitidine, into clinical practice represented a major contribution to the treatment of peptic ulcer conditions (1). The development of H_2-antagonists has continued with the recent introduction of famotidine (Fig. 1). This sulfonylamidine bioisostere has demonstrated enhanced potency in reducing gastric acid secretion (2). Subsequently, other H_2-antagonists have been reported including nizatidine and ORF-17578, the thiazole and *N*-propargyl analogues of ranitidine, respectively. These compounds have demonstrated equivalent or increased antisecretory activity in preclinical and clinical trials (3,4).

The cholinergic activities of cimetidine and ranitidine have previously been documented in a number of *in vitro* systems (5,6). Although the extensive clinical utilization of these compounds has failed to demonstrate generalized cholinergic side effects at normal therapeutic doses, higher concentrations or intravenous administration have resulted in reports of bradycardia, flushing, and lacrimation as well as mental confusion. Concern therefore remains regarding the potential cholinergic, toxic manifestations of these agents at high doses and in those patients undergoing major surgical procedures or those with impaired renal function (7).

The purpose of the present study therefore, was to evaluate famotidine for its ability to elicit cholinergic side effects at high doses. Famotidine was also compared to ranitidine and cimetidine with respect to brain acetylcholine alteration, acetylcholinesterase inhibition, and ileal activity. Preliminary insights into the structure–activity relationships required for AChE inhibition were also determined utilizing nizatidine, ORF 17578, 2-*N*,*N*-dimethylaminomethyl-5-methylfuran, and the sulfoxide metabolites of the two most frequently used H_2-antagonists, ranitidine and cimetidine.

MATERIALS AND METHODS

Animals and Chemicals

Male ICR mice weighing between 18 and 25 g were purchased from Harlan Sprague Dawley Industries, Indianapolis, Ind. Ranitidine hydrochloride and ranitidine sulfoxide were generously provided by Glaxo, Inc. ORF 17578 was provided by Ortho Pharmaceutical Co., famotidine by Merck Sharp and Dohme Research Laboratories, cimetidine sulfoxide by Smith Kline and French Laboratories, and nizatidine by Eli Lilly and Company. Cimetidine was purchased from Sigma Chemical Company. 2-*N*,*N*-Dimethylaminomethyl-5-methylfuran was synthesized according to the procedure of Holdren and Hixson (8).

Toxicity Studies

For the toxicity studies, the H_2-antagonists were dissolved in sodium acetate buffer (0.05 *M*, pH 4.0) and administered at a dose of 400 mg/kg i.p. An equivalent volume of

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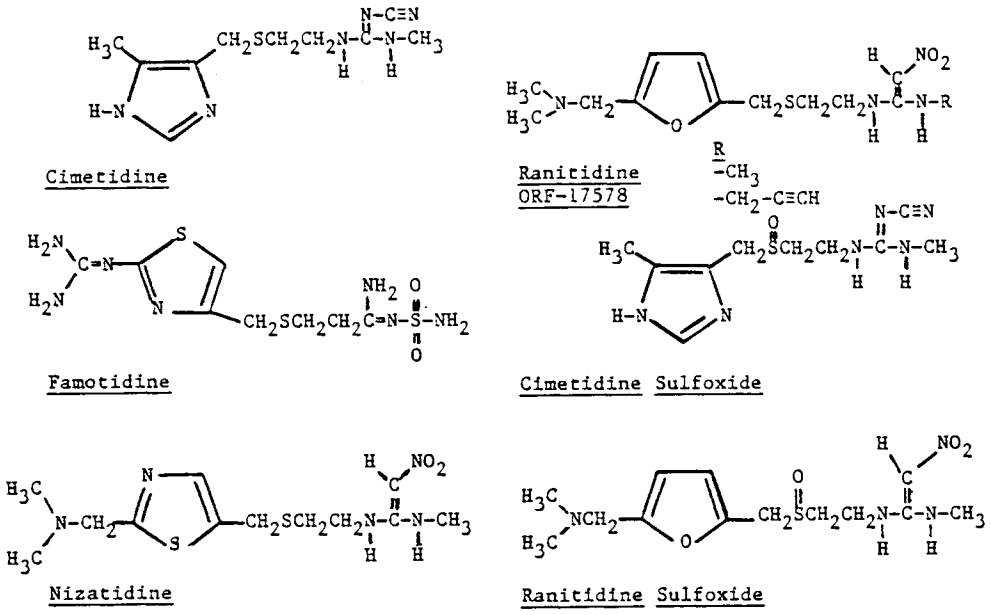

Fig. 1. Structure of the H_2-antagonists.

buffer was administered i.p. to control animals. Percentage deaths were determined 15 min after the administration of antagonist. Toxicity was also compared using 10 times the *in vivo* ED_{50} dose reported to block gastric acid secretion in rats. The effective doses reported for cimetidine, ranitidine, and famotidine are 25, 6.1, and 1.65 mg/kg, respectively (4,9). Therefore, intraperitoneal doses of 250, 61, and 16.5 mg/kg were also administered and evaluated as described above.

Acetylcholinesterase Assay

The acetylcholinesterase inhibitory activity of the analogues was assessed during the initial 1–2 min after the addition of substrate at 37°C using the method of Ellman (10). Acetylcholinesterase tissue sources included bovine and human red blood cells (Sigma Types XII-S and XIII, respectively) and mouse brain. Mouse brain acetylcholinesterase was prepared by homogenizing the whole brain (minus the cerebellum and brain stem) in 0.05 *M* phosphate buffer, pH 7.4, and centrifuging at 20,000*g* for 20 min. The supernatant was then used as a source of acetylcholinesterase. Inhibitors were preincubated for 2 min prior to the addition of substrate. The final substrate concentration (acetylthiocholine) was 5×10^{-5} *M*. IC_{50} values for the inhibitors were determined from a semilog plot of the percentage inhibition of enzyme velocity versus the substrate concentration.

Brain Acetylcholine Levels

Acetylcholine was determined in brain tissue 15 min after the administration of the H_2-antagonist or at the cessation of breathing. The tissue was obtained by decapitation and assayed by the gas chromatographic method of Kosh and Freeman (11). Briefly, the tissue was homogenized in formic acid:acetonitrile, then centrifuged, and the supernatant ion pair extracted with dipicrylamine. After chemical demethylation and back extraction, the final chloroform extract was injected into a Hewlett Packard 5880 gas chromatograph equipped with a Triton X-100:OV-17 packed column and a nitrogen phosphorous detector.

Smooth Muscle Activity

Smooth muscle activity was determined in isolated rat ileal tissue attached to a Narco Bio-System myograph transducer and amplified for chart recording. Two-centimeter ileal strips were obtained from Sprague Dawley rats after decapitation and were bathed in Tyrode's solution aerated with 100% oxygen at 37°C. The ileal strip was allowed to equilibrate in the muscle bath for 5 min prior to drug administration. Direct agonist activity was determined by adding the H_2-antagonist alone followed by a 2- to 5-min observation period. Indirect acetylcholinesterase inhibition or muscarinic blocking activity was then determined by adding acetylcholine (8.5×10^{-6} *M* final concentration) to the bath.

RESULTS

The purpose of the initial phase of the research was to compare three clinically available H_2-antagonists with respect to their cholinergic side effects in mice. Due to the poor distribution of cimetidine, ranitidine, and famotidine into the CNS [CSF/plasma concentrations = 0.07, 0.06, and 0.09, respectively, in humans (12,13)], a nontherapeutic dose of 400 mg/kg i.p. was utilized in an attempt to obtain adequate brain levels of the H_2-antagonists for AChE inhibition. Ranitidine was the most toxic of the three agents at this dose, producing death in approximately 89% of the animals, with an average time to death of 9 min (Table I). The onset of ranitidine toxicity was associated with a crouched position and slowed breathing at 2–3 min after administration, labored breathing at 5 min, and tremors and convulsions at 7–8 min. In comparison, cimetidine at the same dose was lethal for 11% of the animals. The predominant effect seen with cimetidine was a sedative and calming action, which

Table I. Comparative Toxicity of H_2 Blockers and Effects on Brain Acetylcholine in Mice

Treatment	Dose (mg/kg)[a]	N	Percentage deaths[b]	Time to death (min ± SE)[c]	ACh (nmol/g)[d]
			(A) 400 mg/kg		
Control	—	9	—	—	15.5 ± 0.8
Ranitidine	400	9	88.8	9.1 ± 0.8	16.6 ± 1.0
Cimetidine	400	9	11.1	14.5[e]	18.6 ± 1.0*
Famotidine	400	10	—		19.9 ± 1.0**
			(B) 10 × ED_{50}		
Control	—	6	—	—	11.5 ± 1.0
Ranitidine	61	6	—	—	13.8 ± 0.9
Cimetidine	250	6	—	—	13.6 ± 0.7
Famotidine	16.5	5	—	—	14.2 ± 1.0

[a] All compounds were dissolved in sodium acetate buffer (0.05 *M*, pH 4.0) and administered i.p. An equivalent volume of acetate buffer was administered i.p. to control animals.
[b] Percentage deaths was calculated 15 min after treatment with vehicle or H_2 blocker.
[c] Survivor times *not* included.
[d] Brain tissue was obtained by decapitation 15 min after administration of the H_2 blocker or at the cessation of breathing.
[e] $N = 1$; standard error not calculated.
* $P \leq 0.05$, compared to control (Student's *t* test).
** $P \leq 0.005$ compared to control (Student's *t* test).

was preceded by convulsions when death occurred. Famotidine was nontoxic at 400 mg/kg and was associated with extreme ptosis. At 10 times the ED_{50} dose reported to block gastric acid secretion (4,9), no deaths or overt pharmacological effects were observed for any of the H_2-antagonists.

Brain acetylcholine levels were also determined at the time of death or at 15 min after administration of the H_2-antagonist. Ranitidine, the most toxic of the agents at 400 mg/kg, did not increase acetylcholine levels (Table I). However, cimetidine and famotidine significantly ($P \leq 0.05$ and 0.005, respectively) increased acetylcholine concentrations in the brain. Administration of the H_2-antagonists at 10 times their ED_{50} dose appeared to increase brain ACh levels compared to controls but the alterations were not significant. The control brain ACh levels in Table IB were lower than those in Table IA, presumably a result of method variations in animal sacrifice, littermate homogeneity, or reagent differences.

The side effects seen in the toxicity study and the changes in brain acetylcholine levels suggested that an inhibition of acetylcholinesterase (AChE) was involved. Therefore, ranitidine, cimetidine, famotidine, and nizatidine were examined for their ability to inhibit AChE obtained from mouse brain tissue (Table II). For comparative purposes, human and bovine red blood-cell AChE tissue sources were also used. Ranitidine was found to be the most potent inhibitor, producing an IC_{50} in the low micromolar range. Nizatidine was approximately one-fourth as active, cimetidine ~100 times less potent, and famotidine essentially inactive. IC_{50} values for famotidine could be obtained only in the millimolar range. Only slight differences in IC_{50} values for AChE isolated from the three sources were observed for the H_2-antagonists. However, mouse brain AChE appeared to be the most sensitive to the action of ranitidine and nizatidine.

Since the three enzyme sources exhibited similar inhibition characteristics, additional H_2-antagonists were compared using only AChE from human red blood cells (Table II). Only two sulfoxide metabolites were available for examination. ORF 17578 exhibited an IC_{50} comparable to that of ranitidine, whereas the sulfoxide metabolites appeared to be one-tenth as potent as their parent compounds. 2-*N*,*N*-Dimethylaminomethyl-5-methylfuran exhibited only

Table II. IC_{50} Values for H_2 Blockers on Selected Acetylcholinesterases[a]

Compound	Enzyme source[b]		
	Mouse	Bovine	Human
Ranitidine	7.0×10^{-7}	2.3×10^{-6}	2.4×10^{-6}
Cimetidine	5.4×10^{-4}	2.5×10^{-4}	2.3×10^{-4}
Famotidine	[74.5%][c]	2.9×10^{-3}	2.0×10^{-3}
Nizatidine	2.8×10^{-6}	1.1×10^{-5}	9.8×10^{-6}
ORF-17578	—	—	2.0×10^{-6}
Ranitidine sulfoxide	—	—	1.7×10^{-5}
Cimetidine sulfoxide	—	—	[67%][c]
2-*N*,*N*-Dimethyl aminomethyl-5-methylfuran	—	—	[53%][c]

[a] Acetylcholinesterase was assayed at 37°C using the Ellman method. Inhibitors were preincubated 2 min prior to addition of substrate. Final substrate concentration (acetylthiocholine) was 5 $\times 10^{-5}$ *M*.
[b] Bovine and human red blood-cell preparations (Sigma Types XII-S and XIII, respectively) were utilized. Brain tissue from HA/ICR mice was the source of the mouse enzyme.
[c] An IC_{50} was not obtained. Value in brackets represents enzyme activity remaining at the maximum inhibitor concentration assayed (1×10^{-3} *M*).

weak activity (IC_{50}, $\sim 10^{-3}$ *M*). For graphical comparisons, ranitidine, ORF 17578, nizatidine, and cimetidine are plotted in Fig. 2. The inhibition curves for ORF 17578, nizatidine, and cimetidine appear to have similar slopes and differ only in their IC_{50} values. Ranitidine, however, has a shallower slope of inhibition. From the IC_{50} values obtained in Fig. 2, the apparent rank order of potency of the compounds is ORF 17578 ≧ ranitidine > nizatidine > cimetidine > famotidine (not shown).

Ranitidine, cimetidine, and famotidine were also examined for cholinergic activity *in vitro*. All three agents appear inactive under our assay conditions since direct muscarinic activity or potentiation of acetylcholine induced ileal contractions were not observed at concentrations up to 10^{-3} *M*.

DISCUSSION

The results of the present study utilizing acetylcholinesterase isolated from mouse brain as well as from bovine and human erythrocytes show little interspecies variation and correlate well with previous reports utilizing these and other enzyme sources (5,14). However, no enhancement in AChE activity was noted at low doses of ranitidine, which is opposite to the effect reported by Mehta *et al.* (15). Famotidine was consistently weaker in AChE inhibitory activity relative to the other H_2-antagonists tested. These results, as well as the decreased inhibitory activity of the thiazole analogue of ranitidine, nizatidine, indicate that the 2-*N,N*-dimethylaminomethyl-5-methylfuran moiety may be necessary for optimal AChE inhibition as previously suggested by Re *et al.* (16). Interestingly, ORF 17578, the propargyl analogue of ranitidine which retains the furanyl moiety, was found to possess equivalent or slightly enhanced activity. The fact that 2-*N,N*-dimethylaminomethyl-5-methylfuran demonstrated only marginal *in vitro* activity (see Table II) suggests that binding to a secondary site by the diaminonitroethene-containing side chain or its equivalent greatly increases AChE inhibition. Sulfoxidation of ranitidine and cimetidine both resulted in a 10-fold decrease in AChE inhibitory activity, further indicating the importance of the thioether portion of the structure in enzyme inhibition. Metabolism of these H_2-antagonists to their respective sulfoxides may, therefore, actually decrease cholinergic toxicity.

Ranitidine, cimetidine, and famotidine all demonstrated insignificant activity in the rat ileum assay, suggesting little or no direct cholinergic activity. The inactivity of these compounds may be due to poor *in vitro* tissue penetration. These results contrast, however, with previous reports demonstrating significant increases in guinea pig ileal contraction with ranitidine (16,17).

The intraperitoneal administration of 400 mg/kg of cimetidine and famotidine resulted in significant increases in brain acetylcholine levels in mice. In contrast, ranitidine demonstrated an insignificant elevation in acetylcholine concentrations. These results do not correlate with the relative *in vitro* inhibitory activities of these agents on mouse brain acetylcholinesterase and indicate that additional factors mediate the observed increases. It should be noted, however, that the duration of drug action was limited to an average of only 9 min with ranitidine due to its lethality, which possibly mitigated any increase in brain acetylcholine levels. This possibility is strengthened by the observation that ranitidine as well as the other H_2-antagonists at a nonlethal dose (10 × ED_{50}) caused insignificant increases in brain ACh levels. Furthermore, the lethality observed with the H_2-antagonists in this study also cannot be correlated with the observed increases in brain acetylcholine levels, suggesting the presence of peripherally mediated toxicity.

Differences in the pharmacological profile and toxicity of ranitidine appear to be present when nontherapeutic doses are administered intraperitoneally, intravenously, or orally. In contrast to the results of the present study, lifetime administration of oral doses up to 2000 mg/kg in mice and rats has been reported previously to be well tolerated. Intravenous administration, however, resulted in an LD_{50} value of 77 mg/kg in mice (18,19).

In summary, the results of this study demonstrate that there is a wide variation in the *in vitro* acetylcholinesterase inhibitory activity of the H_2-antagonists. The relationship of acetylcholinesterase inhibition to the changes in brain acetylcholine levels and toxicity observed *in vivo* remains to be elucidated.

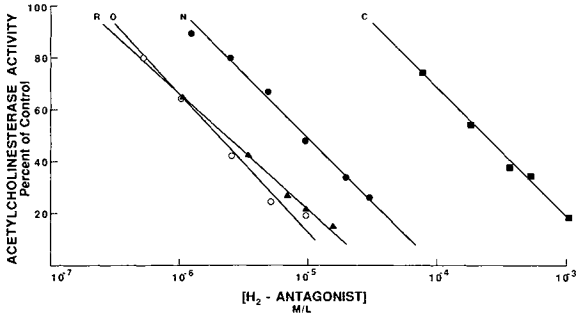

Fig. 2. The inhibition of human erythrocyte acetylcholinesterase by H_2-antagonists. R, ranitidine; O, ORF 17578; N, nizatidine; C, cimetidine. Inhibitory activity was determined by the method of Ellman (9). Final acetylthiocholine concentration = 5×10^{-5} *M*.

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