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ANTI-OXIDANT PROPERTIES OF H_2-RECEPTOR ANTAGONISTS

EFFECTS ON MYELOPEROXIDASE-CATALYSED REACTIONS AND HYDROXYL RADICAL GENERATION IN A FERROUS-HYDROGEN PEROXIDE SYSTEM

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Abstract—Ulcerogenesis of the gastroduodenal mucosa is caused by the digestive action of gastric juice and initially involves an inflammatory reaction with infiltration of phagocytes. The anti-inflammatory activity of many drugs have been attributed to the inhibition of the leukocyte enzyme, myeloperoxidase (MPO). In this study, the H_2-antagonists in clinical use were found to be potent inhibitors of MPO-catalysed reactions ($IC_{50} < 3\ \mu M$) under conditions resembling those in experiments with intact neutrophils. Since peak plasma concentrations of cimetidine, ranitidine and nizatidine are well within the micromolar range, after oral therapeutic dosing, our results may be of clinical relevance. The inhibitory actions of cimetidine and nizatidine were largely due to scavenging of hypochlorous acid (HOCl), a powerful chlorinating oxidant produced in the MPO–H_2O_2–Cl^- system. In contrast to famotidine, ranitidine was also a potent scavenger of HOCl, while both drugs inhibited MPO reversibly by converting it to compound II, which is inactive in the oxidation of Cl^-. The HOCl scavenging potencies of ranitidine and nizatidine were about three times higher than that of the anti-rheumatic drug, penicillamine, which had a potency similar to that of cimetidine. The rapid HOCl scavenging ability of penicillamine is thought to contribute to its anti-inflammatory effects. Using riboflavin as a probe, the H_2-antagonists were found to be inhibitors of hydroxyl radical (·OH) generated in a Fe^{2+}–H_2O_2 reaction mixture. Spectral analyses of the interaction of iron ions with the drugs and studies with chelators, suggest that the drugs were efficient chelators of Fe^{2+}, in addition to their ·OH scavenging abilities. Since the gastrointestinal tract can contain potentially reactive iron, the simultaneous presence of H_2-antagonists may help to suppress iron-driven steps in tissue damage.

Ulcerogenesis of the gastrointestinal tract appears to initially involve an inflammatory reaction with infiltration of phagocytic cells [1, 2]. The ability of phagocytes to injure cells and host tissues is dependent on the production of oxygen-derived reactive species and the ability of the target cells and tissues to detoxify the reactive metabolites. Reactive oxygen species generated by phagocytes include $O_2^{-\cdot}$, H_2O_2 and ·OH (hydroxyl radical) as well as hypochlorous acid (HOCl†) which is generated by the myeloperoxidase (MPO) system [3]. ·OH may be generated in a transition metal-catalysed Haber–Weiss reaction, but recent evidence suggests that it could also be generated by the MPO system in a transition metal-independent fashion [4].

MPO is an enzyme found in the azurophilic granules of polymorphonuclear leukocytes and is also a component of monocytes. Mature macrophages are normally devoid of MPO, but have been shown to readily take up the enzyme. This resulted in an increase in a number of biochemical activities, including an increase in the production of oxidants by the macrophage [5, 6]. Recently, it has also been shown that MPO can modulate the immune response through effects on macrophage function [7].

A major component of tissue damage is thought to result from the action of elastase and other proteolytic enzymes released by neutrophils, macrophages, and other secretory cells which partake in the inflammatory process. The actions of these proteolytic enzymes are in turn modified by α_1-protease inhibitor which inactivates elastase and other proteolytic enzymes. Various oxidants, including HOCl and ·OH, have been shown to inactivate α_1-antiprotease [8]. However, tissue injury by activated phagocytes cannot be entirely inhibited by anti-proteases, nor was tissue injury diminished in mice whose leukocytes were deficient in proteases. These findings suggest that alternative mechanisms, such as direct injury of tissues by reactive oxygen species, are also involved [3].

Since the direct involvement of oxygen-derived free radicals has been implicated in the mechanism of duodenal ulceration [9], we have investigated the antioxidative effects of various H_2-antagonists (Fig. 1) on MPO-catalysed reactions, and on ·OH generated in a ferrous-H_2O_2 reaction mixture.

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† Abbreviations: MPO, myeloperoxidase; HOCl, hypochlorous acid.

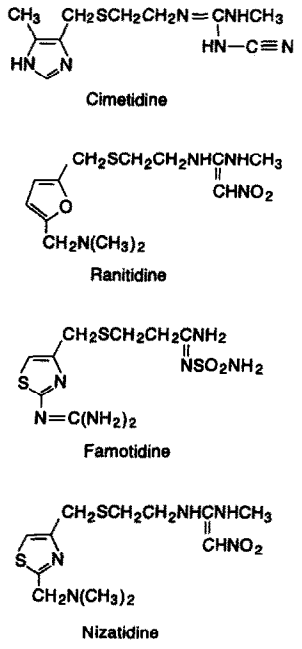

Fig. 1. Structural formulae of the H_2-antagonists used in this study.

MATERIALS AND METHODS

Reagents. MPO (donor H_2O_2 oxidoreductase, EC 1.11.1.7) from human neutrophils was prepared as described [10] and enzyme with a purity index ($R_Z = A_{423}/A_{280}$) of at least 0.73 was used. Phytic acid (dodecasodium salt hydrate) was from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and D-penicillamine from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The following H_2-antagonists were kindly donated as pure substances: cimetidine (SmithKline Beecham, Wynberg, JHB, R.S.A.); ranitidine (Glaxo, Midrand, R.S.A.); famotidine (Logos Pharmaceuticals, Midrand, R.S.A.) and nizatidine (Eli Lilly, Isando, R.S.A.).

Spectroscopic analyses. NADH fluorescence was measured on a Perkin–Elmer MPF-44A fluorescence spectrometer. UV absorption analyses were performed on Cary 219 and Beckman DU 640 spectrophotometers.

Oxidation of NADH. The decay of NADH fluorescence (excitation at 340 nm and emission at 450 nm) was used as an index of oxidation. Increasing concentrations of the compounds were incubated in mixtures containing 17 nM MPO and 10 μM NADH in 50 mM phosphate buffer (pH 7.4) in the presence of 150 mM NaCl. Reactions were initiated with H_2O_2 (50 μM in mixture) and the decay of NADH fluorescence was monitored. The slopes of the initial linear phase of the recording was used to calculate the rate of NADH oxidation. To determine the reversibility of MPO inhibition induced by famotidine, the decrease of NADH absorbance was monitored at 340 nm.

Scavenging of HOCl. In competition studies, reagent HOCl (30 μM in mixture) was added to solutions containing 10 μM NADH and increasing concentrations of the compounds. Relative HOCl scavenging abilities were determined from the inhibition of NADH fluorescence decay.

Hydroxyl radical detection. The ·OH-generating system consisted of 100 μM $FeSO_4$ and 100 μM H_2O_2 in 50 mM phosphate buffer (pH 6). Stock solutions of $FeSO_4$ were prepared in double-distilled water. Formation of ·OH was confirmed by the hydroxylation of benzoic acid. The 1- and 3-hydroxy benzoic acids formed have strong blue fluorescence at 408 nm (excitation at 305 nm) [11]. Since the H_2-antagonists interfered in some of the more generally employed ·OH detection systems, we have used riboflavin as acceptor molecule. Riboflavin has been reported to be a specific ·OH scavenger [12]. Scavenging of ·OH is accompanied by "irreversible" bleaching of the 445 nm absorbance band of riboflavin. Reduction of riboflavin also results in bleaching of the 445 nm band, but this reaction is easily reversible in the presence of oxygen. Both the hydroxylation and reduction reactions lead to a loss of riboflavin fluorescence. Addition of $FeSO_4$ alone did not decrease the fluorescence of riboflavin, i.e. the flavin was not reduced by Fe^{2+}. A typical 2-mL incubation mixture contained 100 nM riboflavin and a specific concentration of the H_2-antagonist in 50 mM phosphate buffer (pH 6). Reactions were initiated by adding $FeSO_4$, followed by H_2O_2. Generation of ·OH was detected by monitoring the decrease in riboflavin fluorescence.

Binding of ferrous ions to the drugs. The effects of 200 μM $FeSO_4$ on the UV spectra of the drugs (200 μM) in 50 mM phosphate buffer (pH 7.4) were investigated. Phytic acid, a unique Fe^{3+} chelator which keeps iron ions in a redox inactive form [13], was added to both reference and sample cuvettes (500 μM in mixture), before adding the $FeSO_4$ to the cuvettes. By this procedure, all free iron should have been removed from the incubation mixtures, since phytic acid can bind up to six iron ions.

RESULTS

Inhibition of MPO catalysis

The inhibition of NADH oxidation in the MPO–H_2O_2–Cl^- system by the H_2-antagonists and histamine, is shown in Fig. 2. All the H_2-antagonists were relatively good inhibitors of MPO-catalysed reactions. Concentrations at the 50% inhibitory level (IC_{50}) were less than 3 μM for all the drugs. Although low concentrations of cimetidine (Fig. 2A; bottom curve) seem to have a potency similar to that of the other drugs, it could not inhibit the MPO system by more than about 60%. Histamine (Fig. 2A; top curve) was a relatively inefficient inhibitor; at a concentration of 20 μM, more than 60% of the original NADH was still unoxidized.

Relative HOCl *scavenging abilities*

The relative HOCl scavenging abilities of the drugs are shown in Fig. 3. Ranitidine and nizatidine competed most efficiently with the detector molecule, NADH, for reaction with HOCl ($IC_{50} \approx 1$ μM). Since the molar ratio of scavenger to detector was only

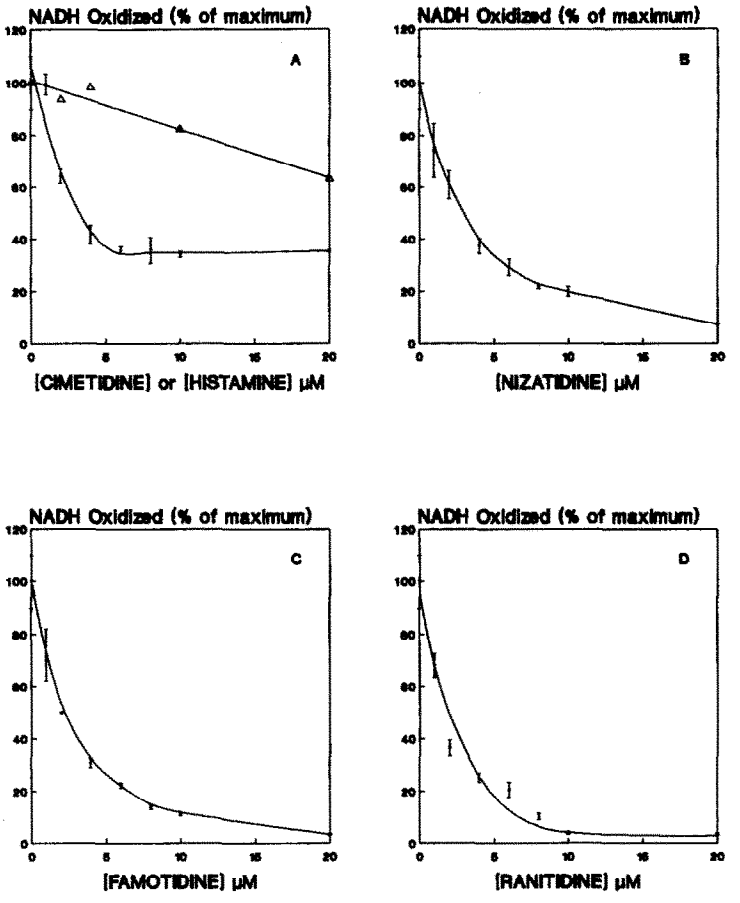

Fig. 2. Inhibition of MPO-catalysed reactions by the H_2-antagonists. Increasing concentrations of the drugs were added to solutions containing 17 nM MPO, 10 μM NADH and 150 mM NaCl in 50 mM phosphate buffer (pH 7.4). Reactions were initiated with the addition of H_2O_2 (50 μM total concentration) and NADH fluorescence monitored at 450 nm (excitation at 340 nm). The rate of NADH oxidation was determined from the initial slopes of the scans. Results are the means ± SD from four separate experiments. (A) The top and bottom curves are those for histamine and cimetidine, respectively.

about 0.1 at this point, it is evident that HOCl reacted much faster with the drugs than with NADH. Cimetidine ($IC_{50} \approx 2$ μM) and penicillamine ($IC_{50} \approx 3$ μM) were also good scavengers. Histamine and famotidine were relatively inefficient scavengers ($IC_{50} \approx 10$ μM).

Formation of MPO–hydrogen peroxide adducts

In the presence of H_2O_2 and Cl^-, MPO was converted to compound II (ferryl form) by famotidine (Fig. 4). Scan 2 was recorded 15 sec after adding H_2O_2 (200 μM in mixture) to 500 nM MPO and 200 μM famotidine in 50 mM phosphate buffer (pH 7.4), containing 150 mM NaCl. Under the same conditions, but in the absence of famotidine, all MPO–H_2O_2 adducts were reconverted to the native ferric enzyme (scan 1) within 15 sec. To compare the abilities of the different drugs to convert MPO to its compound II form, the change in absorbance at 455 nm with time was monitored (Fig. 5). Significant stabilization of compound II was observed only in the presence of famotidine (recording 1) and ranitidine (recording 2).

Figure 6 shows the reversibility of famotidine interaction with MPO. Recording 1 depicts the rate of NADH oxidation, 5 sec after adding H_2O_2 (100 μM in mixture) to a solution containing 80 nM MPO, 100 μM NADH and 150 mM NaCl in 50 mM phosphate buffer (pH 7.4). Recording 2 shows the effect of 100 μM catechol additionally added to the mixture before initiating the reaction with H_2O_2. The reaction rate was lower, presumably due to the fact that the electron donatory catechol was a competitive inhibitor which decreased the rate of HOCl production. Famotidine (100 μM), in the absence of catechol, completely blocked the reaction with H_2O_2 (recording 3). Addition of catechol (100 μM in mixture) 65 sec after the first addition of H_2O_2, had no effect on the oxidation of NADH, although the electron donor had reduced all compound II back to the native ferric enzyme. A second supplementation with H_2O_2, immediately after the catechol addition, was necessary to initiate a reversal of the famotidine effect (recording 4).

Effects of H_2-antagonists in the ·OH-generating system

All H_2-antagonists inhibited ·OH interaction with

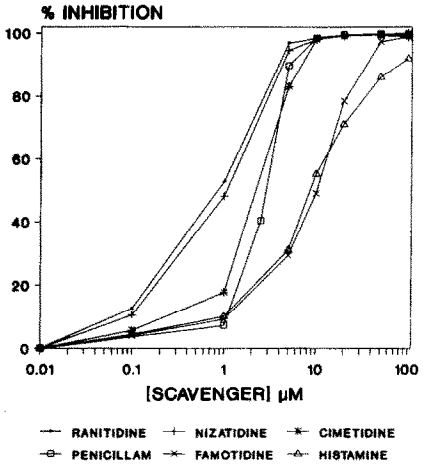

Fig. 3. Relative HOCl scavenging abilities of the drugs. Reagent HOCl (30 μM in mixture) was added to solutions of 10 μM NADH in 50 mM phosphate buffer (pH 7.4) containing increasing concentrations of the drugs. The inhibition of NADH oxidation was determined from the decrease in fluorescence at 450 nm. Each data point represents the means for three separate experiments in which the results differed by less than ±5%. PENICILLAM = penicillamine.

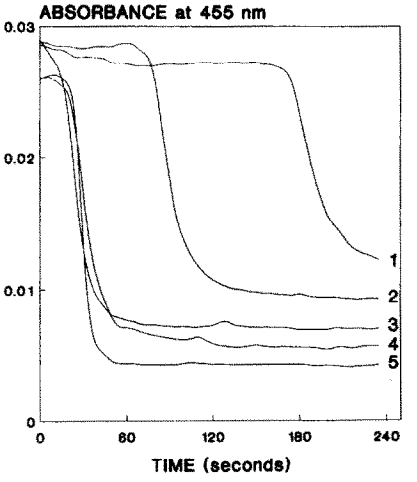

Fig. 5. Abilities of the drugs to stabilize compound II. The reaction mixtures contained 500 nM MPO, 150 mM NaCl and 200 μM of each drug in 50 mM phosphate buffer (pH 7.4). Recordings at a fixed wavelength of 455 nm were made directly after adding H_2O_2 (200 μM in mixture). Recordings, 1: famotidine; 2: ranitidine; 3: control without drug; 4: nizatidine; 5: cimetidine.

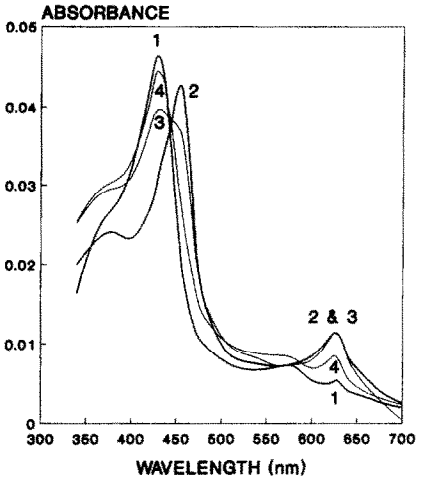

Fig. 4. Compound II accumulation induced by famotidine. Spectrum 1: 500 nM MPO and 200 μM famotidine in 50 mM phosphate buffer (pH 7.4); Spectrum 2: 15 sec after adding H_2O_2 (200 μM in mixture); Spectrum 3: 3 min after H_2O_2 addition; Spectrum 4: 4 min after H_2O_2 addition.

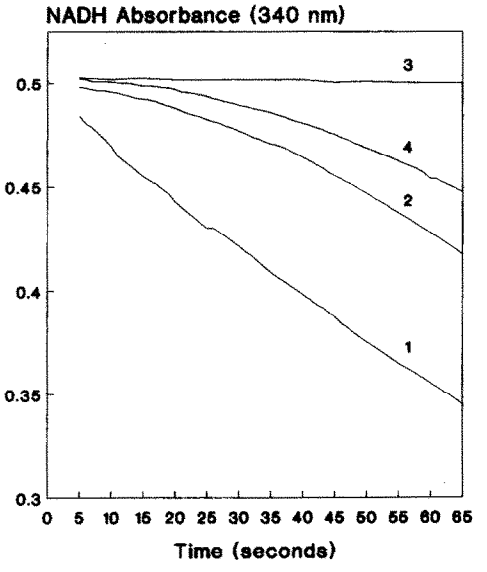

Fig. 6. Reversible famotidine-induced MPO inhibition. The incubation mixtures consisted of 80 nM MPO, 100 μM NADH and 150 mM NaCl in 50 mM phosphate buffer (pH 7.4). Recording 1: rate of NADH oxidation 5 sec after initiating the reaction with H_2O_2 (100 μM in mixture). Recording 2: same as for 1, but the incubation mixture contained 100 μM catechol additionally. Recording 3: same as for 1, but the incubation mixtures contained 100 μM famotidine additionally. Recording 4: after 65 sec, catechol (100 μM in mixture) was added to reaction mixture 3. The recording was started after initiating the reaction with H_2O_2 (100 μM in mixture).

riboflavin by 50% at concentrations less than 20 μM (Fig. 7). Histamine and the specific hydroxyl scavenger, mannitol, had potencies significantly lower ($IC_{50} \approx 40$ μM).

The effects of known iron chelators were also studied (Fig. 8). Phytic acid, a Fe^{3+} chelator which

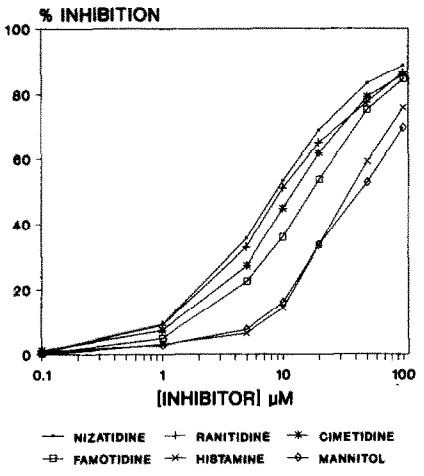

Fig. 7. Inhibition of riboflavin hydroxylation induced by the H_2-antagonists. Increasing concentrations of the drugs were added to solutions containing 100 nM riboflavin in 50 mM phosphate buffer (pH 6). Generations of ·OH were initiated by addition of $FeSO_4$ followed by H_2O_2 (final concentrations were 100 μM each). The decrease in riboflavin fluorescence was monitored at 520 nm (excitation at 455 nm). Each data point represents the means for four separate experiments in which the results differed by less than ±7%.

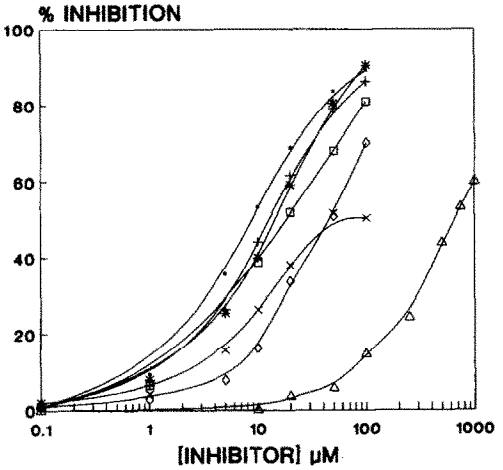

Fig. 8. Effect of iron chelators and ·OH scavengers on riboflavin hydroxylation. Experimental conditions were as described in the legend of Fig. 7. Each data point represents the means for four separate experiments in which the results differed by less than ±7%. NIZAT = nizatidine; CIMET = cimetidine; PENAM = penicillamine; DESFE = desferrioxamine; MANN = mannitol.

also stimulates the autoxidation of Fe^{2+} [13], was a poor inhibitor of riboflavin hydroxylation and inhibited by 50% only at a concentration of about 700 μM. In contrast, penicillamine, a Fe^{2+} chelator and powerful ·OH scavenger [8], had an IC_{50} of only about 15 μM. Its potency was similar to that of the H_2-antagonist, cimetidine, but lower than that of nizatidine ($IC_{50} \approx 9$ μM), the most potent inhibitor of the H_2-antagonists. In the presence of 30 μM EDTA, a Fe^{3+} chelator which do not prevent the interaction of iron ions with H_2O_2, the IC_{50} of nizatidine increased to about 20 μM. Mannitol, a specific ·OH scavenger with only weak iron binding capacity [8], was considerably less potent ($IC_{50} \approx 45$ μM). At low concentrations, the potency of the powerful Fe^{3+} chelator, desferrioxamine, which is also an excellent ·OH scavenger, was higher than that of mannitol.

Figure 9 shows the Fe^{2+}-induced effects on the UV spectra of the H_2-antagonists (1:1 molar ratio) at pH 7.4. Cimetidine (Fig. 9A) has no significant absorbance above about 250 nm (spectrum 1). Addition of $FeSO_4$ resulted in a significant increase in absorbance. A long trailing shoulder emerged at wavelengths above 250 nm. When the incubation mixture contained phytic acid to chelate free iron, a broad peak with maximum at 274 nm, was formed. Famotidine (Fig. 9B; spectrum 1) has peaks near 200 nm and one at 284 nm. Addition of $FeSO_4$ resulted in an increase in absorbance and a slight blue shift of the second peak to 282 nm (spectrum 2). In the presence of phytic acid and $FeSO_4$, the second peak remained at 282 nm and the absorbance increased slightly at the longer wavelengths (spectrum 3). Ranitidine (Fig. 9C; spectrum 1) has peaks at 226 nm and 312 nm. Addition of $FeSO_4$ showed an overall increase in absorbance and a slight blue shift of the second peak to 310 nm (spectrum 2). With phytic acid and $FeSO_4$, the peaks shifted to 224 nm and 308 nm, respectively (spectrum 3). Nizatidine (Fig. 9D; spectrum 1) has peaks at 254 nm and 314 nm. Addition of $FeSO_4$ resulted in a general increase in absorbance (spectrum 2) and when phytic acid was additionally present, the peaks shifted to 256 nm and 310 nm, respectively (spectrum 3). Fe^{3+}, or phytic acid alone, had no effect on the spectra of the H_2-antagonists.

DISCUSSION

We have previously used NADH as a detector molecule to monitor oxidation reactions in the MPO system [14, 15]. Oxidation of NADH was found to be predominantly induced by HOCl, which was formed in the MPO–H_2O_2–Cl^- system. Direct oxidation of NADH, or its chlorination, proceeded more slowly.

All the H_2-antagonists were relatively good inhibitors of MPO catalysis with IC_{50} values < 3 μM (Fig. 2). Conditions for MPO-catalysed oxidation of Cl^- were chosen to resemble those in experiments with intact neutrophils. The MPO content of 2×10^7 neutrophils/mL has been estimated to be 0.9 μM or 3×10^7 molecules/cell [16]. It was also estimated that 2–4 × 10^7 stimulated neutrophils utilized 100 μM H_2O_2 per hour for Cl^- oxidation [17]. In our system

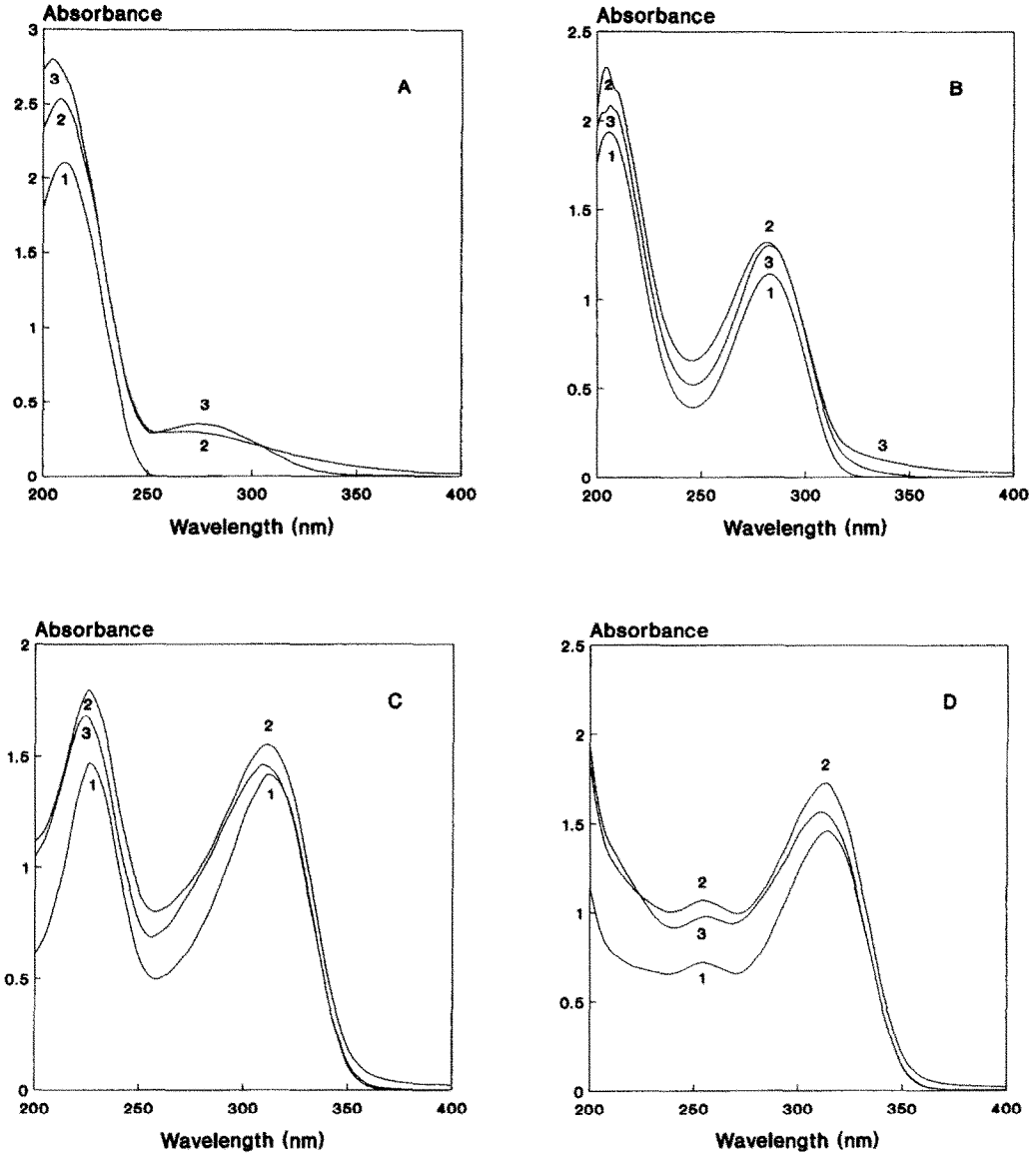

Fig. 9. Fe^{2+}-induced alterations of the drug UV spectra. Spectra 1: the incubation mixtures contained 200 μM of each drug in 50 mM phosphate buffer (pH 7.4). Spectra 2 were recorded 6.5 min after addition of $FeSO_4$ (200 μM) to the incubation mixtures. Spectra 3: same as for 1, but the incubation mixtures contained 500 μM phytic acid additionally. (A) Cimetidine; (B) famotidine; (C) ranitidine; (D) nizatidine.

the oxidation of 150 mM Cl^- by 50 μM H_2O_2 was catalysed by 17 nM MPO, which is equivalent to about 10^6 neutrophils/mL if 40% of the MPO content of the cells was secreted into the medium [18].

The concentration of HOCl (30 μM) used in our scavenging experiments was within the range expected to be generated *in vivo* [19]. Ranitidine, nizatidine and cimetidine were good scavengers of HOCl relative to the anti-rheumatic drug, penicillamine (Fig. 3), which is known to be an efficient scavenger [20]. Famotidine and histamine had similar HOCl scavenging abilities. However, relative to famotidine, histamine was a poor inhibitor of MPO-catalysed reactions (Fig. 2A and C). An additional inhibitory mechanism should thus be involved.

MPO reacts with H_2O_2 to form a highly unstable catalytically active complex, compound I, which is at an oxidized level two equivalents above the resting ferric enzyme. Compound I reacts with a variety of electron donors (including halides) to regenerate the ferric enzyme with oxidation of the electron donor. In the absence of a suitable electron donor, compound I can undergo a 1-electron reduction to its ferryl form (compound II) which is inactive as a catalyst of halide oxidation [21]. Compound II can

be identified spectrophotometrically (Fig. 4). Famotidine inhibited MPO-catalysed reactions primarily by promoting compound II accumulation (Figs 4 and 5). During the reaction of famotidine with MPO, no significant haem destruction occurred (Fig. 4) and inhibition of MPO seemed reversible. To strengthen this conclusion, catechol (an electron donor), was added to the famotidine-inhibited MPO reaction mixture (Fig. 6). On addition of H_2O_2, the oxidation of NADH proceeded again. This can be explained by the fact that catechol reduced MPO back to its native state and thus counteracted the effect of famotidine. Various other drugs have been found to be promoters of compound II accumulation [14, 15, 22].

Many anti-inflammatory drugs can react with HOCl, but only in the case of penicillamine and a few other drugs were the reactions fast enough to be relevant at clinical concentrations [20]. In our test system, all the H_2-antagonists, except famotidine, were at least as potent as penicillamine (Fig. 3). To be clinically relevant, the drug must also be present at a sufficient concentration at the target cells or tissues. In one study, a 400 mg and 80 mg oral dose of cimetidine resulted in an average blood peak concentration of 9.2 μM and 28.8 μM, 60–90 min after dosing, respectively [23]. After a 150 mg oral dose of ranitidine, mean peak plasma concentrations of about 400 ng/mL (i.e. 1.14 μM) were achieved within 1–2 hr [24]. The peak plasma concentrations after an oral dose of 300 mg of nizatidine were 1400–3600 ng/mL (i.e. 4.2–10.9 μM) [25]. The recommended daily dosages for cimetidine, ranitidine and nizatidine are 800, 300 and 300 mg for duodenal ulcer healing, respectively [26]. Thus, our results seem to be clinically relevant for these drugs.

Famotidine, however, was a relatively weak scavenger of HOCl in our *in vitro* system (Fig. 3). Clinically, it is given in much lower doses than the other H_2-antagonists (20–40 mg daily) and a plasma concentration of 20 μg/L (0.06 μM), which is about two orders of magnitude too low to be relevant as MPO inhibitor, was estimated to produce an intragastric pH of 4 [26]. Whether the ability of famotidine to accumulate inactive compound II of MPO is physiologically relevant, remains to be determined. It is possible, however, that higher local drug concentrations can be attained at the site of its absorption. A high rate of absorption was observed for cimetidine in the duodenum [23], which may also apply for the other H_2-antagonists.

Another reactive oxygen species which may be relevant in inflammatory conditions, is the hydroxyl radical. The intestine seems to be vulnerable, since it may contain large amounts of potentially reactive iron (particularly in the case of populations with diets rich in red meat). Dietary iron is taken up into the intestinal cells or stored in ferritin within the mucosal cells. Iron enters the protein shell of ferritin as Fe^{2+} and is deposited in the interior as Fe^{3+} after oxidation by the protein [8]. Various reducing agents, including reduced flavins, can release iron as Fe^{2+} from ferritin [27]. The released iron probably chelates to non-protein cellular constituents. It has been argued that minimizing the amount of this non-protein-bound iron is an important part of anti-oxidant defense [8].

Another source of iron available for *in vivo* ·OH generation could be haemoglobin released via intestinal bleeding. Many nonsteroidal anti-inflammatory drugs (inhibitors of prostaglandin synthesis) cause intestinal inflammation which may lead to ulceration and perforation [28]. Intestinal blood loss has been suggested to be the main cause of iron deficiency anaemia in patients with rheumatic disease receiving anti-inflammatory drugs [29]. It has been shown that iron can be released from haemoglobin by excess H_2O_2 and such released iron can catalyse formation of ·OH via the Haber–Weiss reaction [8].

Our Fenton ·OH-generating mixture consisted of $FeSO_4$ and H_2O_2 in 50 mM phosphate buffer (pH 6). Oxidized riboflavin, reported to be a specific ·OH scavenger [12], was employed as a detector molecule (Fig. 7). Since the concentration of iron was 100 μM, while the drugs inhibited by 50% at concentrations less than 20 μM, it is likely that the drugs acted as hydroxyl radical scavengers in our system, since most of the iron ions would have been free. It should, however, be emphasized that Fe^{2+} is rapidly oxidized in phosphate buffer [30], so that much of the "free" iron would have been catalytically inactive soon after adding an aliquot of the $FeSO_4$ stock solution (prepared in double distilled water) to the reaction mixture (50 mM phosphate buffer). Riboflavin possesses a chelating functionality at the N(5) nitrogen atom [30]. Chelators in which nitrogen atoms primarily bind the metal prefer the reduced forms of iron and tend to increase the redox potential of the metal [30]. The C(4a) position of the flavin isoalloxazine ring has been shown to be the site of derivatization during the catalytic reactions of flavoprotein hydroxylases [31, 32]. It is postulated that ·OH reacts site-specifically with riboflavin at the C(4a) position, proximate to N(5), the chelating site for Fe^{2+}. Hydroxylation will result in a loss of flavin fluorescence as in the case of the synthetically prepared hydroxylated FAD [31].

To investigate the possible chelating abilities of the drugs, the effects of known chelators were studied in the riboflavin system. Phytic acid, an established Fe^{3+} chelator which stimulates the autoxidation of Fe^{2+} [13], showed an effect only at high concentrations (Fig. 8). This, coupled to the observation that phosphate (buffer) also catalyses rapid Fe^{2+} autoxidation, strengthens the fact that riboflavin is primarily a Fe^{2+} chelator. Inhibition of riboflavin hydroxylation by nizatidine was decreased in the presence of EDTA, suggesting that iron chelation was involved. Although EDTA is primarily a Fe^{3+} binder, it also binds Fe^{2+} with lower affinity and, in contrast to phytic acid, it does not prevent the reaction of iron ions with H_2O_2. If only ·OH scavenging were involved, EDTA should have increased the effectiveness of nizatidine [11]. Penicillamine, primarily a Fe^{2+} chelator and a powerful ·OH scavenger [8], was a potent inhibitor. Mannitol, a specific ·OH scavenger with weak iron-chelating activity, had a considerably lower potency than the H_2-antagonists or penicillamine, while the potency of desferrioxamine, which is a Fe^{3+} chelator and powerful

·OH scavenger, was somewhat higher than that of mannitol at low concentrations. These results suggest that both iron chelation and ·OH scavenging are probably involved.

The formation of complexes of organic ligands with transition metals affects the ligand absorption spectrum [33]. Spectral analyses of the H_2-antagonists suggest that they form complexes with Fe^{2+}, but not with Fe^{3+} (Fig. 9). A 2.5-fold molar excess of phytic acid to Fe^{2+} ions could not prevent complex formation of Fe^{2+} with the drugs. Phytic acid can bind up to six divalent cations per molecule and accelerates the oxidation of Fe^{2+} by molecular oxygen, but not by H_2O_2, while the reduction of Fe^{3+} to Fe^{2+} remains unaffected. Thus phytic acid causes a substantial shift in the redox potential of iron, ensuring rapid removal of Fe^{2+} without the concomitant production of ·OH [13]. Since Fe^{2+} is also rapidly oxidized in phosphate buffer [30], our results suggest a high affinity of the H_2-antagonists for Fe^{2+}.

Iron-mediated oxidative damage in biological systems is well documented. Iron salts, particularly in the Fe^{2+} state, can degrade the protective layer of the gastrointestinal mucus and attack the cells underneath to cause erosion of the gastric mucosa [8]. One of the ways in which the toxicity of iron can be expressed, is when it acts as a catalyst in the Haber–Weiss reaction to generate ·OH or a similar species. Significant *in vivo* scavenging of the highly reactive hydroxyl radical by the H_2-antagonists seems unlikely, since the drug concentrations in the tissues will probably be too low. Inhibition of ·OH-induced cytotoxicity by chelating the catalytically active iron and hopefully directing the damage site-specifically to the chelator would obviously be more advantageous.

Thus, in addition to their inhibition of MPO-catalysed reactions, chelation of Fe^{2+} by the H_2-antagonists could be a useful supplementary *in vivo* antioxidative property of the drugs. This may be appreciated in light of the fact that a high rate of absorption of cimetidine was observed in the duodenum [23], which is also one of the most active sites of absorption of iron (in its ferrous form) [8].

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