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Hydroxyl radical scavenging reactivity of proton pump inhibitors

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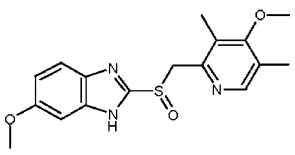
ABSTRACT

In addition to the established control of acid secretion of the class of proton pump inhibitors (PPI) reactivity from the pyridyl methyl sulphinyl benzimidazole type a second independent anti-inflammatory reactivity was observed in vitro. This inhibitory reactivity was clearly noticed using three different assays where the aggressive hydroxyl radicals were successfully trapped in a concentration dependent manner. There is unequivocal evidence that the proton pump inhibitors having the sulphoxide group are able to scavenge hydroxyl radicals which are generated during a Fenton reaction. By way of contrast, the corresponding thioethers were substantially less active. No detectable effect was seen in the superoxide radical scavenging system. In conclusion, pantoprazole as well as the other proton pump inhibitors have a pronounced inhibitory reactivity towards hydroxyl radicals.

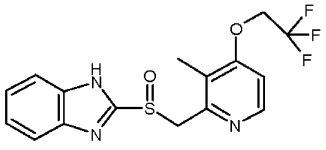
1. Introduction

Pantoprazole, omeprazole and lansoprazole, proton pump inhibitors from the benzimidazole type, are successful in the treatment of acid related diseases [1–3] which is based on the inhibition of the gastric H,K-ATPase [4–6]. However, acid related disorders are not necessarily associated with increased acid secretion [7].

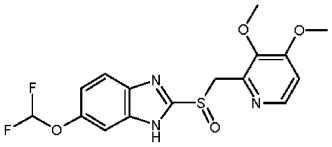
Omeprazole



Lansoprazole



Pantoprazole



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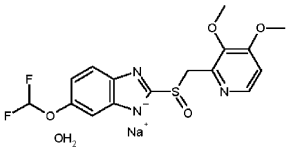

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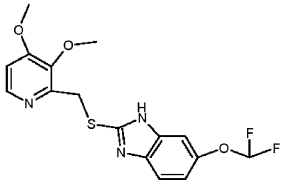
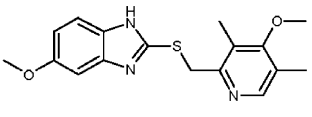


Omeprazole-thioether



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Acid acts as a noxious agent when physiological conditions have changed, e.g. during *Helicobacter pylori* infection, co-treatment using non-steroidal antiphlogistic drugs or increased reflux rates of gastric content into the oesophagus due to impaired lower oesophageal sphincter function. Infiltration of acid in tissue is normally accompanied by attraction of neutrophiles which release oxygen radicals thereby leading to the known signs of inflammation [8]. Efforts were undertaken to examine a possible role of proton pump inhibitors to inhibit neutrophile function in releasing superoxide radicals ($^{\bullet}O_2^-$). However, no clinically significant effects could be demonstrated [9,10], because inhibition of PMNs (chemotactic activity and $^{\bullet}O_2^-$ release) function was only successful in the presence of these inhibitors being 5–50 times higher (0.1–1.0 mmol/l) than transient peak levels achieved in plasma. In order to monitor this anti-inflammatory reactivity in vitro the inhibition of superoxide radicals was examined using an established superoxide dismutase assay [11]. Above all, the main focus was directed to the highly reactive hydroxyl radicals. It is well known that these radicals are able to destroy all kinds of biomolecules. These radicals may be generated in vivo and are the cause for a series of diseases including rheumatoid arthritis and other inflammatory processes and it was shown that the majority of the gastric lesions is caused by endogenous $^{\bullet}OH$, as revealed by almost complete protection by DMSO, a highly effective $^{\bullet}OH$ scavenger [15]. Hydroxyl radicals can easily be produced in vitro according to the Fenton reaction with transition metal ions in lower oxidation states, for example, either with iron(II) compounds or more efficiently with copper(I) in the presence of hydrogen peroxide or simply dioxygen [12]. The copper(I)-ions are readily formed upon reacting with ascorbic acid (AS) as an electron donor:

$$Fe(III)/Cu(II) + AS \rightarrow Fe(II)/Cu(I) + AS^{\bullet}$$

$$Fe(II)/Cu(I) + H_2O_2 \rightarrow Fe(III)/Cu(II) + {}^{\bullet}OH + OH^-$$

OH-radicals can also be formed by direct autoxidation of the metal ions in the presence of dioxygen to yield superoxide ($^{\bullet}O_2^-$) which is spontaneously dismutated into hydrogen peroxide and dioxygen. Thus, the above reactions can proceed. In order to examine the possible reactivity of a number of substituted benzimidazoles with hydroxyl radicals three different established methods were employed.

A suitable assay is the inhibition of the OH-radical-dependent degradation of the heme system of heme proteins [13]. The decline of the Soret band of the heme group can be conveniently quantified in the 350–450 nm region. An alternative assay used was the depolymerisation of hyaluronic acid by OH-radicals [14]. The progressive depolymerisation of this mucopolysaccharide was followed viscosimetrically and should be inhibited accordingly in the presence of the benzimidazoles.

In a third completely different method OH-radicals generated during a Fenton reaction decomposed 2-deoxy-D-ribose [15]. This reaction was monitored by measuring the formation of malondialdehyde-like compounds known to yield coloured thiobarbituric acid adducts which were eventually recorded at 532 nm. In the presence of OH-radical scavengers this dye formation is progressively inhibited.

The sulphoxide moiety and/or the thioether residue in the benzimidazoles were presumed to react with excited oxygen species known to be generated in the course of an inflammatory process. Two prominent reactive oxygen species including superoxide and hydroxyl radicals were expected to be scavenged by pyridyl sulphinyl benzimidazoles.

A crucial question was the survival of these benzimidazoles during Fenton type reactions. These stability measurements were conducted employing HPLC analysis allowing the detection of remaining intact primary compounds and products due to the attack by OH-radicals.

2. Materials and methods

2.1. Materials

Pantoprazole, omeprazole, lansoprazole and their corresponding thioethers were synthetized in analytical purity in the chemical laboratories at ALTANA Pharma, Konstanz, Germany. Potassium hyaluronate, thiobabituric acid and 2-desoxyribose were purchased from Sigma–Aldrich. Nucleosil C 8, 5 μm (125 mm × 4.6 mm, Fa. Grom, Herrenberg, Germany), precolumn LiCHROPREP RP-2 (12 mm × 4 mm, Fa. Merck, Darmstadt, Germany).

2.2. Methods

2.2.1. Hydroxyl radical assays

2.2.1.1. Degradation of the heme group in hemoglobin. Bovine erythrocytes were hemolysed and diluted with water to give an electronic absorption at 408 nm of 1.0. The assay was performed analogous to that of the myeloperoxidase enzymatic activity test where hypochlorite is produced which is known to degrade the heme group of heme proteins [13].

To 574 μl of aqueous hemoglobin solution in a thermostatted cuvette 6 μl of DMF or water and 10 μl of 10 mM aqueous copper sulphate were added for 5 min. The reaction was started after the addition of 10 μl 50 mM ascorbic acid and the absorption followed at 37 °C for 2 min in the control experiment. Depending on the solubility the respective inhibitor was added in a volume of 6 μl in DMF or water. The final concentrations in 600 μl were 160 μmol/l $CuSO_4$, 0.83 mmol/l ascorbic acid and 6.6 μmol/l hemoglobin. At the start of the experiment the pH of the hemolysate was 7.2 and 6.7 at the end of the reaction.

2.2.1.2. Depolymerisation of hyaluronic acid. Depolymerisation of hyaluronic acid was measured following viscosity change with time. All operations were performed at 22 °C. Relative viscosity was monitored by recording the time (seconds) required for a given volume (0.8 ml) of the reaction mixture to drain by gravity from the barrel of a plastic 1 ml syringe through a needle of appropriate size. The meniscus was timed as it passed between two calibration marks on the syringe barrel [14]. In the case of the benzimidazoles dissolved in DMF the reaction mixture was composed of 1.5 ml hyaluronate (1 mg/ml in 10 mmol/l phosphate buffer at pH 7.2) and 15 μl 10 mmol/l copper sulphate (final concentration 0.1 mmol/l). The reaction was started with 30 μl of 50 mmol/l ascorbic acid (final concentration 1 mmol/l) and incubated for

5 min. The same reaction mixture without the inhibitor compound but in the presence of DMF served as control. When the water-soluble sodium salt of pantoprazole was examined only 2.5 μl 10 mol/l copper sulphate and 5 μl 50 mM ascorbic acid were needed to yield an appropriate depolymerisation effect. The final concentrations in the 1.5 ml assay solution were 16.6 μmol/l $CuSO_4$, 166.6 μmol/l ascorbic acid and Na-pantoprazole 12 and 20 μmol/l.

2.2.1.3. *Thiobarbituric acid assay*. The thiobarbituric acid assay was performed according to the method described earlier [15]. Three hundred microliters 7.5 mM 2-desoxy-D-ribose were added to 1000 μl 10 mM potassium-phosphate buffer, pH 7.4 and incubated for 30 min at 37 °C in the presence of 100 μl inhibitor compound (final concentration 1.5 mmol/l) and 100 μl 10 mmol/l iron(II) ammonium sulphate (final concentration 0.66 mmol/l). The total volume was 1.5 ml. The reaction was stopped by the addition of 1 ml 1% (w/v) thiobarbituric acid dissolved in 50 mM NaOH and 1 ml of 2.8% (w/v) trichloro acetic acid and maintained for 15 min at 95 °C. The control reaction mixture contained the same volume of DMF as was present in the inhibitor compound. The dye was measured at 532 nm. A low absorption value indicates that deoxyribose is protected from •OH.

2.2.2. *Analysis of benzimidazoles by HPLC*

A 200 μl aliquot of the incubation mixture of Fenton reaction in presence of 10 μmol/l pantoprazole according to Section 2.2.1.2 was analysed by HPLC using the analytical column Nucleosil C 8, 5 μn (125 mm × 4.6 mm) and the precolumn was LiCHROPREP RP-2 (12 mm × 4 mm) in a reverse phase. A gradient of acetonitrile (10–45% (v/v)) and 10 mM phosphate buffer, pH 7.4, at a flow rate of 1 ml min^{-1} was used as effluent on the analytical column.

3. Results

It was of interest to examine pantoprazole as to which degree it is capable to scavenge reactive oxygen species. Pantoprazole did not display any detectable reactivity at all in the superoxide radical scavenging system. By way of contrast, an inhibitory effect was clearly seen with pantoprazole in three different assays where the highly reactive hydroxyl radicals were successfully trapped in a concentration dependent manner. The radicals were generated by both the Fe(II) and the much more potent Cu(II)/ascorbate-mediated Fenton reaction. Being aware of the situation that rather unphysiologically high Fe(II) or Cu(I) concentrations had to be used in the employed •OH-radical assays in vitro. This ascertained reliable and reproducible analytical data. Due to the exceedingly fast and efficient reactivity of the Cu(II)/Cu(I) system this transition metal ion was preferred during the heme degradation and hyaluronic acid depolymerisation studies. Likewise, elevated concentrations of benzimidazoles proved to be necessary to allow the convenient detection of the •OH-scavenging reactivity of all used drugs.

Biomolecules including the heme system of hemoglobin, hyaluronic acid and deoxyribose they all were effectively destroyed in the presence of this reactive oxygen species.

Bleaching of the heme group was measured by the decrease of the Soret band at 408 nm. Compared to the control 100 μM pantoprazole inhibited the degradation by 75% in this system. In the presence of 50 μM pantoprazole an inhibition of 36% was noticed. Due to its insolubility in water pantoprazole was dissolved in DMF. In the control experiment the same DMF concentration was added to the assay mixture.

To exclude a possible disturbing effect of DMF the water-soluble pantoprazole sodium salt was prepared and examined in the same system. Nearly identical results were obtained in this aqueous assay. Hundred micromolar inhibited by 74% and 50 μM by 34%, respectively. Even at a pantoprazole sodium concentration of 25 μM a distinct inhibition was seen (Fig. 1).

A similar mode of reaction of pantoprazole was seen in the assay of depolymerisation of hyaluronic acid which was viscosimetrically detected. At a concentration of 160 μM the drug had to be dissolved in DMF a nearly complete inhibition became apparent, whereas half of this concentration (80 μM) led to 48% of mucopolysaccharide degradation within 20 min.

Surprisingly, in the fully aqueous assay 12 μM of pantoprazole sodium caused a marked inhibition by 46% and 20 μM were needed to protect 90% of the biopolymer (Fig. 2). These results encouraged the use of the above aqueous test system as a sensitive tool to monitor the anti-inflammatory reactivity at low pantoprazole concentrations.

Further proof for an •OH-radical scavenging reactivity of pantoprazole, its sodium salt, omeprazole and lansoprazole was obtained in the thiobarbituric acid assay where deoxyribose served as substrate. However, 1500 μM of the compounds were required to inhibit the radical reaction and exhibited a quite similar inhibitory potential by approximately 50% (Table 1).

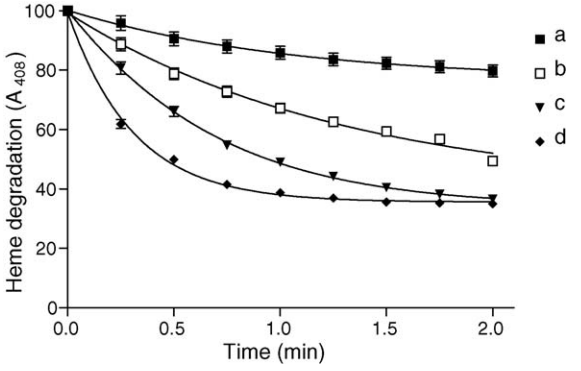

Fig. 1 – OH-radical induced bleaching of heme in the presence of water-soluble pantoprazole sodium salt. (a) 100 μM, (b) 50 μM, (c) 25 μM and (d) control. The time-dependent degradation was monitored at 408 nm at 37 °C for 2 min in a total volume of 600 μl. The reaction mixture was composed of 574 μl aqueous hemolysate (A_{408} ~1.0 in a 10 mm light path cuvette being the equivalent of 6.6 μmol/l), 6 μl aqueous inhibitor compound and finally 10 μl 10 mM $CuSO_4$ (final 0.16 mmol/l). The Fenton reaction was started with 10 μl 50 mM ascorbic acid (final 0.83 mmol/l). The control experiment was run in the absence of inhibitor. All experiments were performed in triplicate. The reproducibility was ±2.5%.

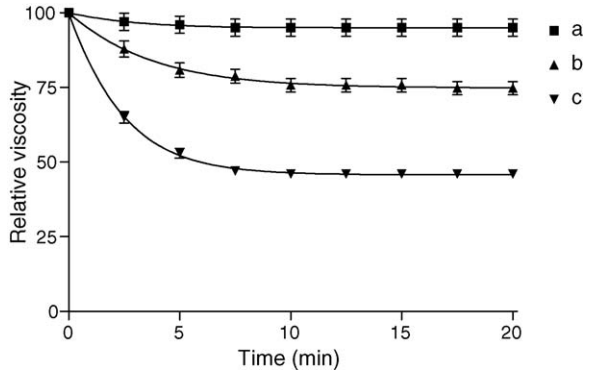

Fig. 2 – •OH-radical induced depolymerisation of hyaluronic acid in the presence of sodium pantoprazole. (a) 20 μmol/l, (b) 12 μmol/l and (c) control. Adequate concentrations of aqueous pantoprazole sodium salt were added to 1.5 ml of hyaluronic acid containing 2.5 μl 10 mmol/l $CuSO_4$. The relative viscosity was monitored at 22 °C after the addition of 5 μl 50 mmol/l ascorbic acid. The control was run in the absence of inhibitor compound. All other experimental conditions were the same as described in the legend to Fig. 1. The reproducibility was ±3%.

By way of contrast, the respective thioethers of the above benzimidazoles which are known to be metabolites of the sulphoxides displayed a significantly reduced •OH-radical scavenging activity.

A similar potency of the substituted benzimidazoles for inhibition of the •OH-radical induced reaction could also be observed in the heme bleaching system with the final concentration of 100 μmol/l of each compound (Table 2).

In all three employed assay systems for •OH production a time scale of up to 30 min was appropriate. During this time interval only the decrease of the parent compound was monitored. After 90 min 5% of pantoprazole sulphone could be detected in the Cu(I) driven Fenton reaction.

Table 1 – Desintegration of deoxy-D-ribose in the presence of •OH-radicals

Inhibition in % of control	
Pantoprazole	47 ± 1.0
Pantoprazole–thioether	22 ± 0.5
Omeprazole	46 ± 1.0
Omeprazole–thioether	24 ± 0.5
Lansoprazole	43 ± 0.9
Pantoprazole–sodium	58 ± 1.2

OH-radical scavengeing reactivity of different substituted benzimidazoles in the desoxyribose/thiobarbituric acid assay. The reaction mixture (total volume 1.5 ml) contained 300 μl 7.5 mM deoxy-ribose, 1 ml potassium phosphate buffer, pH 7.2, 100 μl inhibitor compound in DMF with the exception of the water-soluble sodium pantoprazole (final concentration 1.5 mM) and 100 μl 10 mM iron(II) ammonium sulphate as the Fenton reagent. After incubation for 30 min at 37 °C 1 ml 1% (w/v) thiobar-bituric acid dissolved in 50 mM NaOH and 1 ml of 2.8% (w/v) tri-chloro acetic acid were added and heated for 15 min at 95 °C. The red-coloured dye was measured at 532 nm and the absorption values expressed as percent of inhibition compared to the control. All experiments were performed in triplicate. The standard deviation was less than ±2%.

Table 2 – Heme bleaching in the presence of •OH-radicals

Inhibition in % of control	
Pantoprazole	75 ± 2.0
Pantoprazole–thioether	44 ± 1.1
Omeprazole	77 ± 2.0
Omeprazole–thioether	50 ± 1.0
Lansoprazole	71 ± 1.3
Pantoprazole–sodium	74 ± 1.9

OH-radical scavengeing reactivity of different substituted benzimidazoles in the heme bleaching assay. The values refer to the heme degradation after 2 min reaction time. The experimental conditions are the same as described in the legends to Fig. 1. The final concentrations of the substituted benzimidzoles were 100 μM, respectively. The data are mean values of three independent assays. The reproducibility was ±2.5%.

4. Discussion

It is intriguing to see that in addition to the established control of acid secretion a second acid independent reactivity of the class of substituted benzimidazoles is obvious. Acid infiltration into tissues may lead to an attraction of neutrophils and to an undesired inflammatory process. It can be concluded that the proton pump inhibitors from the benzimidazole type may have two issues of therapeutic action; one of inhibition of acid secretion and the other of possibly scavenging hydroxyl radicals and, therefore, they can be also considered as anti-inflammatory reagents. Compared to the inhibition of acid secretion the gastroprotective mechanism by pantoprazole was observed in morphometric studies after treatment of rats with ethanol–HCl and other tissue irritants showing lower efficacy for the latter. However, the mechanism of the observed gastroprotective effect was not elucidated [16]. Furthermore, in animal models the inhibition of acid secretion and reduction of mucosal damage was examined in the presence of omeprazole compared to the established reactivity of hydroxyl radical scavenging compounds including DMSO [17]. It was concluded that hydroxyl radicals might have been involved. Hydroxyl radicals are known to react more or less unspecifically in a diffusion-controlled manner with many an organic molecule. During the transient stage of hydroxyl radical formation many more reactive intermediates including ferryl species [24] or higher oxidation states of copper [25] may be formed. Likewise, excitation of the benzimidazole molecules should also be taken into consideration. Thus, the unspecific reaction of •OH-radicals and/or its precursors remains a challenging task.

All assays used in this study were transition metal driven oxidation reactions. Of course, one could explain the inhibition of these oxidation reactions via chelation of Cu and/or Fe ions. If this were the case then the control curves seen in Figs. 1 and 2 should not appear in their present stage. A time independent line remaining at the 100% level should be seen instead. For example, the many Cu-binding sites in the hemoglobin molecule certainly might bind the employed Cu(II)-ions. As stated before there will remain many open

coordination sites at the chelated copper allowing the oxygen triggered Fenton chemistry.

We have shown for the first time employing three different assays that pantoprazole is able to scavenge •OH-radicals. The scavenging activity of pantoprazole can be explained by the interaction with the •OH-radical generating system. In the case of lansoprazole this was also observed by Biswas et al. [17].

The maximal concentration of pantoprazole (C_{max}) in human plasma (5.7–7.2 μmol/l) [18,21] after oral administration of 40 mg is close to the range of the IC_{50} values for inhibition of hydroxyl radical generation. In comparison to that, C_{max}-values of 0.7–2.5 μmol/l after administration of 20–40 mg omeprazole [19,21,22], 2.1 μmol/l for 40 mg esomeprazole-MUPS [20], 2.4–4.3 μmol/l for 40 mg esomeprazole [21] and of 2.0–2.2 μmol/l for 30 mg lansoprazole [21,23] are obviously lower.

These results demonstrate a distinct specificity of pantoprazole as an anti-inflammatory reagent and underlines the second extraordinary function of this proton pump inhibitor in the treatment of acid related disorders which are generally accompanied by inflammation.

The therapeutic improvement achieved with the present proton pump inhibitors compared to histamine-2-receptor antagonists especially in reflux oesophagitis may effectively exceed the sole inhibition of acid secretion dealing with the oxygen free radical burst observed in inflammatory events. It should be emphasized when the histamine-2-receptor antagonist ranitidine was employed no OH-radical scavenging activity could be detected.

In conclusion emphasis should be placed on the fact that all results are derived from in vitro experiments. It is hoped that they will stimulate further studies in intact biological systems to support our suggestion of the dual reactivity of the proton pump inhibitors.

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