A Novel Antioxidant and Antiapoptotic Role of Omeprazole to Block
Gastric Ulcer through Scavenging of Hydroxyl Radical

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ABSTRACT
The mechanism of antiulcer effect of omeprazole was studied placing emphasis on its role to block oxidative damage and apoptosis during ulceration. Dose-response studies on gastroprotection in stress and indomethacin-induced ulcer and inhibition of pylorus-ligation induced acid secretion indicate that omeprazole significantly blocks gastric lesions at lower dose (2.5 mg/kg) without inhibiting acid secretion, suggesting an independent mechanism for its antiulcer effect. Time course studies on gastroprotection and acid reduction also indicate that omeprazole almost completely blocks lesions at 1 hr when acid inhibition is partial. The severity of lesions correlates well with the increased level of endogenous hydroxyl radical (\( \cdot \text{OH} \)), which when scavenged by dimethylsulfoxide, causes around 90% reduction of the lesions indicating that \( \cdot \text{OH} \) plays a major role in gastric damage. Omeprazole blocks stress-induced increased generation of \( \cdot \text{OH} \) and associated lipid peroxidation and protein oxidation indicating that its antioxidant role plays a major part in preventing oxidative damage. Omeprazole also prevents stress-induced DNA fragmentation suggesting its antiapoptotic role to block cell death during ulceration. The oxidative damage of DNA by \( \cdot \text{OH} \) generated \textit{in vitro}, is also protected by omeprazole or its analogue, lansoprazole. Lansoprazole when incubated in \( \cdot \text{OH} \) generating system scavenges \( \cdot \text{OH} \) to produce four oxidation products of which the major one in mass spectroscopy shows a molecular ion peak at m/z 385 which is 16 mass units higher than that of lansoprazole (m/z 369). The product shows no additional aromatic proton signal for aromatic hydroxylation in \( ^1\text{H} \) NMR. The product absorbing at 278 nm shows no alkaline shift for phenols, thereby excluding the formation of hydroxylansoprazole. The product is assigned to lansoprazole sulfone formed by the addition of one oxygen atom at the sulphur center following attack by the \( \cdot \text{OH} \). Thus omeprazole plays a
significant role in gastroprotection by acting as a potent antioxidant and antiapoptotic molecule.
INTRODUCTION

Proton pump inhibitors (1) such as omeprazole, lansoprazole, pantoprazole and rabeprazole are extensively used for therapeutic control of acid-related disorders including gastroesophageal reflux disease (GERD), Zollinger-Ellison syndrom and for peptic-ulcer disease caused by stress (stress-related erosive syndrom, SRES), nonsteroidal antiinflammatory drugs (NSAIDs) and by Helicobacter pylori infection (2-5). These compounds share a common structural motif contributed by a substituted pyridylmethylsulfinyl benzimidazole (Fig.1). Inhibition of gastric acid secretion by these compounds is considered as an important step to control the disorders (6). Proton pump inhibitors (PPIs) inhibit acid secretion by irreversibly interacting with the H^+-K^+-ATPase, the terminal proton pump of the parietal cell (7,8). In the acid space of the secreting parietal cell or in the vicinity of the enzyme, these compounds are converted to thiophilic sulfenamide or sulfenic acid which reacts mainly with the cys-813 residue in the catalytic subunit of the H^+-K^+-ATPase which is critical for enzyme inactivation (5). Although omeprazole, the primary member of the PPIs has been extensively used to control these disorders (2), lansoprazole, the second member of the substituted benzimidazole containing a trifluoroethoxy group, has also been used more recently (4).

The role of acid in gastroduodenal pathogenesis has been extensively studied. Although gastric ulcer patients show normal or reduced level of acid secretion, duodenal ulcer patients usually secrete more acid (9,10). In fact, ‘no acid, no ulcer’ is the dictum for duodenal ulcer. Since 30% of patients having duodenal ulcer and very few patients with gastric ulcer are hyperchlorhydric (9), clearly factors other than acid are involved in the pathogenesis of gastroduodenal ulcer. Although the secreted acid itself is not sufficient for ulcer formation, its corrosive property and increased
peptic activity is sufficient to aggravate the ulcer. Even the normal rate of acid secretion may cause ulceration in the breached mucosa when some gastroprotective factors are lost. Hence acid suppression by omeprazole is a common practice to control gastroduodenal lesions (2,5). Suppression of intragastric acid also helps in the healing of ulcer (11). In animals, the role of acid in gastric lesions has been studied using some animal models such as stress or NSAIDs-induced gastric ulcer. Stress itself inhibits gastric acid secretion through a central nervous reflex mechanism (12). Restraint-cold stress or restraint-water immersion stress induces gastric lesions which is associated with decreased or normal level of acid secretion (13,14). Since restraint or water-immersion stress significantly decreases acid secretion induced by pylorus ligation (14), acid plays a minor role in stress ulcer. Administration of antacids to neutralise secreted acid does not protect stress ulcer (15) suggesting that factors other than acid are involved in ulcer formation. However, in indomethacin-induced gastric damage, acidity may be increased due to decreased biosynthesis of prostaglandin (16,17). As acidity as high as 0.6 M HCl can experimentally produce gastric lesions (18), mild irritants like 0.35 M HCl prevents gastric damage caused by stronger necrotizing agent through “adaptive cytoprotection” mediated by increased formation of prostaglandin (19).

It is now generally agreed that gastric lesions develop when the delicate balance between some gastroprotective and aggressive factors is lost. Although the cellular and molecular basis of gastric mucosal defense against gastrodamaging factors are known (20), the mechanism of mucosal damage by the aggressive factors is not fully clear today. Stress (13,21,22), NSAIDs (23) and H. pylori (24) cause mucosal damage through a number of mechanism, of which some reactive oxygen species (ROS) such as $O_2^-$ and $\cdot OH$ are now considered to be one of the major causative
factors for mucosal lesions through oxidative damage (13,22,25-34). Lipid peroxidation, an important parameter for *OH-induced oxidative damage of membrane is increased in gastric lesions caused by ethanol (35), indomethacin (29) and water-immersion stress (36). Increased lipid peroxidation, increased protein oxidation and decreased glutathione level are also evident in restraint-cold stress-induced gastric lesions as a result of oxidative damage caused by the significant generation of *OH (13,22,37). Hydroxyl radical-mediated oxidative damage of membrane lipid and protein and depletion of glutathione have recently been reported in human gastric ulcer also (32). Hydroxyl radical is generated from O$_2^-$ and H$_2$O$_2$ in presence of trace amount of transition metal (released from protein by acidosis caused by ischaemia) by Haber-Weiss reaction (38) following alteration of the antioxidant enzymes of the gastric mucosa (13,22,32,34,37). Recent studies also indicate that programmed cell death or apoptosis plays a significant role in gastric ulceration. Gastric mucosal lesions caused by stress, indomethacin, ethanol and *H. pylori are also due to increased cell death by apoptosis (39-51). Apoptosis is promoted due to an imbalance between Bcl-2 family of antiapoptotic protein and apoptotic Bax protein in stress ulcer (39). Induction of tumor necrosis factor-α (TNF-α) and release of cytochrome c to activate caspase-3 like protease are involved in apoptotic cell death in indomethacin ulcer (41,42). Apoptosis also occurs due to nitric oxide production through induction of nitric oxide synthase by *H. pylori (45). Involvement of ROS and oxidative damage of DNA and DNA fragmentation have also been evident in apoptotic cell death in gastric mucosal injury (39,41,45-47).

Although omeprazole is believed to offer its antiulcer activity through acid suppression (1,2,5) by inactivating the H$^+\text{-}\text{K}^+$-ATPase (5,7,8,52), very little is known regarding its role in controlling oxidative damage and apoptotic cell death of the
Mechanism of antiulcer effect of omeprazole

gastric mucosa during ulceration. The role of acid suppression effect of omeprazole on gastroprotection against some necrotizing agents (ethanol, acidified aspirin, hypertonic saline, 0.6 M HCl) has been studied earlier (18,53), where evidence has been provided to show that acid inhibition plays no significant role on the gastroprotective effect of omeprazole. Moreover, omeprazole neither stimulates prostaglandin biosynthesis nor increases bicarbonate secretion to offer gastroprotection (18,53). Thus omeprazole exerts its antiulcer activity through some other mechanism which has not been explored yet. Using animal models of stress and indomethacin-induced gastric lesions and pylorus ligation-induced acid secretion, evidence has been presented in this paper to show that gastroprotective effect of omeprazole is not mediated through its acid inhibitory effect. Further evidence has been presented to show that endogenous \( \cdot \)OH plays one of the major roles in gastric lesions and omeprazole acts as a potent antioxidant to scavenge the endogenous \( \cdot \)OH thereby preventing the oxidative damage by increased lipid peroxidation and protein oxidation. Moreover, it offers antiapoptotic effect by blocking DNA fragmentation during ulceration. Evidence has also been presented to show that omeprazole or lansoprazole blocks \( \cdot \)OH-induced oxidative damage of DNA by scavenging \( \cdot \)OH in \textit{vitro}. Analysis of the major oxidation product of lansoprazole indicates that this antioxidant activity is due to scavenging of \( \cdot \)OH to form an oxygenated product which is assigned to lansoprazole sulfone. The studies thus provide new insights on the mechanism of antiulcer effect of proton pump inhibitors.

**MATERIALS AND METHODS**

**Drugs and chemicals** - Omeprazole was a kind gift from Dr. W. Beil of Medizinische Hochschule Hannover, Germany. Lansoprazole, melatonin, \( \alpha \)-phenyl
N-tert butyl nitrotrone (PBN), thiobarbituric acid (TBA), ethidium bromide, ascorbic acid, 2, 4 – dinitrophenylhydrazine, collagenase type 1A, pronase E, proteinase K, RNAase, catalase, guanidine-HCl, fast blue BB salt, tetraethoxypropane, benzenesulfonic acid and 5,5 – dimethyl – 1 - pyrroline N – oxide (DMPO) were purchased from Sigma, U.S.A. Desferrioxamine was obtained from Ciba Geigy Ltd, Switzerland. Vitamin E (α-tocopheryl acetate), dimethylsulfoxide (DMSO) and thin layer chromatography (TLC) plates coated with silica gel 60 F$_{254}$ were procured from Merck.

**Animals used** - Sprague – Dawley rats (200 – 250g) of both control and experimental groups kept separately in controlled condition were fasted for 24 hours with water *ad libitum*. The control group received the vehicle only while the experimental group received omeprazole intraperitoneally 30 min prior to restraint – cold stress or indomethacin administration for gastric ulceration or pylorus ligation for acid secretion. Animal experiments (n=8-30) were carried out following the guidelines of animal ethics committee of the institute. Human gastric mucosal biopsy specimens were obtained from Cancer Centre Welfare Home and Research Institute, Kolkata, following approval by the human ethics committee of the institute.

**Restraint-cold stress-induced gastric ulceration** - Rats were immobilised under light ether anaesthesia and subjected to cold (4 ± 1°C) stress for 3.5 hrs. (13). The severity of mucosal lesions was scored as ulcer index as follows: 0 = no pathology; 1 = a small ulcer (1-2 mm); 2 = a medium ulcer (3-4 mm); 4 = a large ulcer (5-6 mm) and 8 = larger ulcer (> 6 mm). The sum of the total scores divided by the number of animals was expressed as the mean ulcer index (13). Luminal acid content was determined by titration with 1mM NaOH using an autoburette pH-stat system from Radiometer, Copenhagen (13).
**Indomethacin-induced gastric ulceration** - Rats were orally fed with indomethacin at 48 mg/kg b.w. After 4 hrs, the animals were killed, gastric lesions in the mucosa (54) were scored and expressed as ulcer index as follows: 0 = no pathology; 1 = one pinhead ulcer. The sum of the total scores divided by the number of animals gives the ulcer index.

**Pylorus-ligation induced gastric acid secretion** - Hypersecretion was induced in rats by pylorus ligation (55) under light anaesthesia with ketamin (12 mg/kg b.w.). The animals were killed 2.5 hr after ligation, the clarified gastric fluid volume was determined and titrated for acid content with 1mM NaOH (13).

**Measurement of lipid peroxidation as an index of oxidative damage** - Lipid peroxidation products of the mitochondrial membrane fraction of fundic stomach homogenate were determined (22,32,56) as thiobarbituric acid - reactive substances (TBARS). The fundic stomach from control, stress-ulcerated and omeprazole (4 mg/Kg) pretreated stressed rats was homogenised in ice-cold 0.9% saline in a Potter-Elvehjem glass homogenizer for 45 sec to get 5% homogenate. One ml of the mitochondrial membrane fraction obtained after differential centrifugation (22) was allowed to react with 2 ml of TCA-TBA-HCl reagent containing 0.01% butylated hydroxytoluene, heated in a boiling waterbath for 15 min, cooled, centrifuged and the supernatant was used for TBARS determination at 535 nm using tetraethoxypropane as standard.

**Measurement of protein carbonyl content as an index of oxidative damage** - Protein oxidation was measured as carbonyl content (57) in the low speed supernatant of the fundic stomach homogenate (32). The fundic stomach from control, stress-ulcerated and omeprazole (8 mg/kg) pretreated stressed rats was homogenised in 50 mM sodium phosphate buffer (pH 7.4) in a Potter-Elvehjem...
glass homogenizer for 45 sec to get 10% homogenate. After centrifugation at 600 \( \times \) g for 10 min, proteins from 0.8 ml of the supernatant was precipitated with 5% TCA and allowed to react with 0.5 ml of 10 mM 2,4-dinitrophenylhydrazine for 1hr. After precipitation with 10% TCA, the protein was washed thrice with a mixture of ethanol-ethylacetate (1:1), dissolved in 0.6 ml of a solution containing 6 M guanidine-HCl in 20 mM potassium phosphate adjusted to pH 2.3 with trifluoroacetic acid, centrifuged and the supernatant was used for measurement of carbonyl content at 362 nm (\( \varepsilon = 22000 \, M^{-1} \, cm^{-1} \)).

**Measurement of endogenous \( \cdot \)OH** - Hydroxyl radical generated in the gastric mucosa was measured using DMSO as \( \cdot \)OH scavenger (58-60). Briefly, the control group was kept at room temperature without any stress after administration (ip) of 1 ml DMSO. The second group received the same amount of DMSO 30 min before the onset of restraint-cold stress. The third group received omeprazole (8mg/kg ip) 30 min prior to DMSO administration and then subjected to stress. After 3.5 hr of stress, the animals were killed and fundic stomach was processed for the extraction of methanesulfinic acid (MSA) formed by reaction of \( \cdot \)OH with DMSO. MSA was allowed to react with the fast blue BB salt to yield a yellow chromophore which was measured at 425 nm using benzenesulfinic acid as standard.

**Measurement of DNA damage in vivo as an index of apoptosis** - To study DNA fragmentation as an index of apoptosis, DNA was isolated from fundic mucosal surface epithelial cells of normal rats, rats subjected to restraint – cold stress without or after pretreatment with omeprazole (8 mg / kg). Fundic mucosa ( ~ 1.5 g ) from three animals was scraped, minced separately in PBS (50 mM sodium phosphate buffer containing 0.9% saline and 20 mM EDTA pH 8), washed twice with PBS and finally suspended in 2 ml PBS containing 0.5 mg/ml collagenase. The suspension was
incubated at 37°C for 1 hr with stirring, followed by addition of pronase E (1 mg / ml) and further incubated for 15 min at 37°C. It was centrifuged at 1000 rpm for 5 min. The pellet was dispersed and incubated with 2 ml of a lysis buffer containing 50 mM Tris Cl-, pH 8, 20 mM EDTA, 10 mM NaCl and 1% w/v SDS for 15 min. It was centrifuged at 14,000 g for 15 min and DNA was isolated from the lysate by phenol–chloroform extraction procedure (61). DNA was dissolved in 10 mM Tris Cl-, pH 8 containing 1 mM EDTA by gentle shaking at 65°C. Residual contaminating RNA was removed by incubating the DNA solution with 1 µg / ml DNAase free RNAase at 37°C for 1 hr followed by 0.1 mg / ml proteinase K for 3 hrs. Phenol-chloroform extraction was repeated to obtain purified DNA which was dissolved in 10 mM Tris Cl- buffer, pH 8 containing 1 mM EDTA. To study DNA fragmentation, DNA was loaded on to a 1.5% agarose gel. Electrophoresis was carried out at 100 V for 1.5 hr in TBE (Tris-borate 90 mM, EDTA 2 mM, pH 8) buffer and DNA was visualized by UV exposure after staining with ethidium bromide.

**Measurement of reactive oxygen species mediated oxidative damage of DNA in vitro and its protection by omeprazole or lansoprazole** - To study the antioxidant effect of omeprazole or lansoprazole, ·OH mediated oxidative damage of DNA isolated from rat mucosal surface epithelial cells or from human gastric mucosal biopsy specimen was studied in absence or presence of omeprazole or lansoprazole. For isolation of human gastric mucosal DNA, the minced mucosa (1 g) was digested with 12 ml of the digestion buffer (100 mM NaCl, 10 mM Tris Cl pH 8.0, 25 mM EDTA, 0.5% SDS, 0.1 µg/ml proteinase K and 1 µg/ml RNAase) by incubating in a shaker bath at 52°C for 15 hrs. DNA was extracted from the lysate after phenol–chloroform extraction as described (61). Rat DNA (~ 200 ng) or human DNA (~ 300 ng) was incubated in a ·OH generating system containing 100 mM sodium
phosphate buffer, pH 7.4, 0.2 mM CuSO₄ and 1 mM ascorbate (59) in a total volume of 30 µl for a period of 30 min at 37°C in presence or absence of omeprazole or lansoprazole. The reaction was stopped by the addition of 1 µg catalase and electrophoresis was carried out in 2% agarose gel.

**Scavenging of \( ^*\)OH by lansoprazole** - Hydroxyl radical was generated *in vitro* in the Cu²⁺-ascorbate system (59,60) and quantitated as described by Babbs and Steiner (58). The assay system contained in a final volume of 1 ml: 50 mM sodium phosphate buffer, pH 7.4, 0.2 mM CuCl₂, 2 mM ascorbate and 2 mM DMSO in absence and presence of lansoprazole. After incubation at 37°C for 1 hr, the reaction was stopped with 0.5 mM EDTA, methanesulfonic acid formed was extracted and allowed to react with fast blue BB salt for quantitation as described (58).

**Isolation of \( ^*\)OH mediated oxidation product of lansoprazole** - As crystalline lansoprazole is readily available commercially, this experiment was carried out with lansoprazole instead of omeprazole with the aim of isolating the \( ^*\)OH mediated oxidation product of lansoprazole, if the latter scavenges \( ^*\)OH. Lansoprazole (0.2 mM) was incubated at 37°C for 3.5 hr with 0.2 mM CuCl₂ and 2 mM ascorbate in the presence of 10 mM phosphate buffer, pH 7.4 in a final volume of 400 ml. The content was evaporated in a Eyela N-N series rotary vacuum evaporator and the residue was extracted repeatedly with chloroform followed by methanol. A control system containing 0.2 mM lansoprazole in 10 mM phosphate buffer was incubated under similar conditions without \( ^*\)OH generating system and subjected to the same extraction procedure to find out if any aerial oxidation occurs or not. The major oxidation product was isolated from the methanol extract after separation by preparative TLC on plate (8 × 18 cm) coated with silica gel 60 F₂₅₄ using chloroform : methanol (90 : 10) as the mobile phase. Compounds were detected by spraying
with iodine vapour. The major oxidation product was recovered from the TLC plate by elution with methanol and was further purified by Waters HPLC system using Waters 15 μ 100 Å Deltapak- C18 semipreparative column (7.8 × 300 mm) eluted with methanol:water (80:20) at a flow rate of 1 ml/min. The absorbance was monitored at 285 nm.

**Analysis of oxidation product of lansoprazole** - The HPLC purified major oxidation product of lansoprazole was dissolved in CDCl3 and 1H NMR spectrum was recorded in a Bruker 300 MHz NMR spectrometer. Molecular weight was determined by electron impact (EI+) mass spectrometry using Jeol JMS 600 mass spectrometer. UV-visible spectrum was recorded in a Shimadzu UV-1601 spectrophotometer.

**Statistical analysis** - All data were expressed as mean ± SEM (standard error of mean). Significance was calculated from Student’s t-test.

**RESULTS**

**Differential effect of omeprazole in blocking gastric ulcer and gastric acid secretion** - In order to investigate whether omeprazole blocks gastric lesions through an independent mechanism other than the inhibition of acid secretion, the dose-dependent effect of omeprazole was studied both on stress and indomethacin-induced gastric ulceration and pylorus-ligation-induced gastric acid secretion. Figure 2A indicates that omeprazole dose-dependently blocks both stress and indomethacin-induced gastric lesions showing nearly 90% inhibition at 8 mg/kg and 16 mg/kg respectively. More than 90% of the animals showed no gastric lesion at all. In contrast, omeprazole blocks pylorus-ligation induced acid secretion at a higher dose causing nearly 90% inhibition at 20 mg/kg (Fig. 2B). However, one significant
finding is evident from the dose-response patterns for blocking gastric damage and acid secretion. At the dose of 2.5 mg/kg, omeprazole cannot block acid secretion at all whereas at a slightly lower dose of 2 mg/kg, omeprazole blocks stress ulcer by 70% and indomethacin ulcer by 50%. The efficacy of omeprazole in blocking gastric damage and acid secretion can be more accurately determined by the potency (ED_{50}) calculation from the dose-response profiles. Whereas the ED_{50} values for inhibiting stress and indomethacin-induced gastric lesions are 0.8 and 2 mg/kg respectively, the same for the induced acid secretion is 3.25 mg/kg. The data indicate that omeprazole blocks gastric lesions through mechanism independent of its role on acid secretion. The relationship between gastroprotection and acid inhibition by omeprazole has been further clarified from the time-course studies of inhibition as shown in Table 1. The results indicate that at the initial period of 1 hr when acid secretion is inhibited by 50% only, gastroprotection by omeprazole is almost complete showing around 90% inhibition of the gastric lesions caused by stress or indomethacin. At later time periods of 2.5 and 3.5 hrs, gastroprotection remains more or less at the same level when acid inhibition is increased to 80%. It is thus clear that omeprazole can offer gastroprotection almost completely even when it cannot completely block acid secretion. In other words, omeprazole-induced gastroprotection is not decreased with relatively higher rate of acid secretion. Omeprazole thus protects gastric lesions through mechanisms other than acid inhibition.

**Role of hydroxyl radical on gastric ulceration** - In order to assess whether endogenous \( \cdot \)OH plays any significant role on the development of gastric lesions, the effect of DMSO, a specific \( \cdot \)OH scavenger (58) was studied on both stress and indomethacin induced gastric lesions. The data (Fig.3) indicate that DMSO causes 87% protection of stress ulcer (A) and 94% protection of indomethacin ulcer (B)
without affecting the luminal acid content. The results indicate that \( \cdot \text{OH} \) plays one of the major roles in stress or indomethacin induced gastric lesions. Figure 4 further shows that time-dependent severity of gastric lesions (ulcer index) correlates well with the increased generation of \( \cdot \text{OH} \) and not with the luminal acid content, suggesting that \( \cdot \text{OH} \) plays a significant role in the gastric damage.

**Effect of omeprazole on \( \cdot \text{OH} \)-mediated oxidative damage of the gastric mucosa** - As \( \cdot \text{OH} \) is one of the major causative factors for gastric ulceration and creates oxidative damage by increased membrane lipid peroxidation and protein oxidation (22), the effect of omeprazole was therefore studied on these two parameters. Table 2 shows that omeprazole blocks stress-induced generation of \( \cdot \text{OH} \) and at the same time completely prevents radical-induced increased lipid peroxidation and protein oxidation. Omeprazole thus blocks gastric oxidative damage by acting as an antioxidant through scavenging of endogenous \( \cdot \text{OH} \). The potency of omeprazole as an antioxidant to block gastric lesions was compared with some natural and synthetic antioxidants having antiulcer activity. The dose-response profiles (Fig.5) clearly indicate that omeprazole (A) is more potent than the naturally occurring antioxidants such as vitamin E or melatonin (B) or some synthetic antioxidants such as desferrioxamine (C), a transition metal ion chelator to prevent \( \cdot \text{OH} \) generation or PBN (D), a radical scavenger.

**Effect of omeprazole on DNA damage of the mucosal cell as an indication of apoptosis** - Apoptotic cell death is associated with DNA fragmentation and oxidative attack is thought to be one of the underlying mechanisms. As restraint-cold stress causes extensive damage of the surface epithelium with numerous cells sloughed off into the gastric lumen due to cell death (13), it is interesting to investigate whether this process is associated with apoptotic cell death or not. Figure 6
Mechanism of antiulcer effect of omeprazole shows that stress-induced gastric epithelial cell damage is associated with DNA fragmentation showing typical DNA ladder (lane 2), an index of cell apoptosis. However, omeprazole pretreatment can completely block stress-induced DNA fragmentation (lane 3) suggesting its antiapoptotic role to prevent cell death during ulceration.

**Antioxidant role of omeprazole and lansoprazole in blocking oxidative DNA damage in vitro** - Oxidative damage of DNA can be studied in vitro when incubated in a $\cdot$OH – generating system. Figure 7A shows that rat gastric mucosal cell DNA (lane 1) when incubated in the Cu$^{2+}$-ascorbate-mediated $\cdot$OH generating system, is completely fragmented into small pieces so that the main DNA band (lane 1) is not observed at all in lane 2. DNA can be completely protected from the oxidative damage by catalase (lane 7) suggesting the involvement of H$_2$O$_2$ in the process. Protection is also evident with the spin trap DMPO (lane 8) suggesting the generation of the radical species. The data indicate that DNA is oxidatively damaged by $\cdot$OH generated from H$_2$O$_2$ in presence of Cu$^{2+}$ and ascorbate (reducing equivalent of O$_2^-$) through metal catalyzed Haber-Weiss reaction (28). However, omeprazole blocks this oxidative damage in a concentration-dependent manner (lane 3-6) showing complete protection at 500 μM. Figure 7B shows similar effect of lansoprazole on the protection (lane 3-5) of oxidative damage of DNA. Lansoprazole completely prevents oxidative damage at 500 μM (lane 5). Lane 6 and 7 show the protective effect of catalase and DMPO respectively. Lansoprazole can also block oxidative damage of human gastric mucosal DNA (Fig.7C) in a concentration-dependent manner (lane 3-6) showing complete protection at 500 μM (lane 6).

**Effect of lansoprazole on the level of $\cdot$OH in vitro** - The protection of the oxidative DNA damage by omeprazole or lansoprazole may be explained as due to its
direct scavenging action on \( \cdot \text{OH} \) so that DNA is spared from the radical attack. In order to study the \( \cdot \text{OH} \) scavenging action, \( \cdot \text{OH} \) generation was measured \textit{in vitro} in the \( \text{Cu}^{2+} \)-ascorbate system in absence and presence of lansoprazole. Figure 8 indicates that lansoprazole can directly decrease the level of \( \cdot \text{OH} \) in a concentration-dependent manner showing 90% inhibition at 2 mM. As lansoprazole does not decompose \( \text{H}_2\text{O}_2 \) or chelate \( \text{Cu}^{2+} \) (data not shown), the effect is not due to the decreased generation of \( \cdot \text{OH} \) from endogenous \( \text{H}_2\text{O}_2 \) through metal-catalyzed Haber-Weiss reaction.

**Identification and isolation of oxidation product of lansoprazole** - In order to investigate the possibility for scavenging of \( \cdot \text{OH} \) by lansoprazole to form an oxidation product, lansoprazole was incubated in the \( \text{Cu}^{2+} \)-ascorbate system and the mixture after extraction with chloroform followed by methanol was subjected to TLC (Fig. 9A). Lane 1 shows the single spot of commercial lansoprazole used in this study. Lane 2, on the other hand, shows the formation of at least four oxidation products of lansoprazole of which spot 4 is the major oxidation product. None of these products were detected when lansoprazole was incubated only in phosphate buffer (not shown). This major oxidation product was isolated from the preparative TLC plate and subjected to HPLC (Fig. 9B). The chromatogram (tracing 1) shows a major peak of the product preceded by a number of small peaks probably contributed by some impurities from the silica gel. These impurities were removed by isolating the compound in the major peak by HPLC. The HPLC-purified product shows more than 95% purity as evidenced by HPLC profile shown in tracing 2. The product when run in TLC (Fig. 9A, lane 3) shows one single spot exactly matching with the \( R_f \) value of spot 4.
Characterization of the oxidation product of lansoprazole - The HPLC purified oxidation product of lansoprazole when analyzed by EI+ mass spectroscopy (Fig.10), shows a clear molecular ion peak at m/z 385, which was 16 mass units higher than that of lansoprazole (m/z 369 not shown). This indicates that the compound is an oxidation product of lansoprazole involving addition of one oxygen atom. The oxidation product absorbs at 278 nm (tracing 1) due to presence of the benzene ring (Fig. 11). Addition of alkali does not cause any alkaline shift to higher wave length (tracing 2) characteristic to phenol indicating that no hydroxylation occurs at the benzene ring to form hydroxylansoprazole. When compared with lansoprazole, no additional aromatic proton signal was detectable in the oxidation product by 1H NMR (not shown). Thus the oxidation product having molecular ion mass of 385 is an oxygenated species of lansoprazole, formed by scavenging of ·OH.

DISCUSSION

The salient points of the present studies are: omeprazole blocks stress and indomethacin-induced gastric lesions through mechanism independent of its role on acid secretion. Omeprazole can protect ulcer at a dose which does not inhibit acid secretion. Time course studies on gastroprotection and acid inhibition further indicate that omeprazole can almost completely block gastric lesions at the initial period when acid secretion is not completely inhibited. On the other hand, Larsson et al (18,53) showed that intravenous doses of omeprazole which block acid secretion cannot protect ethanol-induced gastric lesions suggesting that acid inhibition plays no significant role on gastroprotective effect of omeprazole, which is observed after oral administration of the drug presumably through its local action. Although this observation is opposite to ours because of the different experimental design and
different models of ulcer and acid secretion used, nevertheless, it is clear from both these studies that omeprazole offers gastroprotection through mechanism other than acid inhibition. Secondly, evidence has been provided to show that majority of the gastric lesions is caused by endogenous \( \cdot \text{OH} \), as revealed by almost complete (~90%) protection by DMSO, a specific \( \cdot \text{OH} \) scavenger (58). This is further supported by the finding that time-dependent severity of gastric lesions correlates well with the increase in endogenous \( \cdot \text{OH} \) and having no correlation with the luminal acid content. Almost complete protection by other antioxidants like melatonin, desferrioxamine and PBN further strengthens the view that \( \cdot \text{OH} \) plays one of the major roles in the development of gastric lesions. Thirdly, omeprazole scavenges the endogenous \( \cdot \text{OH} \) and thus blocks radical-induced oxidative damage of the membrane lipid and proteins. Fourthly, DNA damage and fragmentation, an indication of apoptotic cell death during ulceration is also protected by omeprazole. Omeprazole or lansoprazole also protects \( \cdot \text{OH} \) mediated oxidative damage of DNA in vitro. These studies indicate that omeprazole blocks gastric lesions by acting as an antioxidant and antiapoptotic compound. Finally, using lansoprazole as an analogue of omeprazole, evidence has been provided to show that lansoprazole scavenges \( \cdot \text{OH} \) to form lansoprazole sulfone as a major oxidation product.

Gastric mucosal integrity is maintained by a dynamic process of cell death and cell proliferation. Among various factors involved in gastric mucosal lesions, oxidative damage (13,22,25-37) and apoptotic cell death (39-51) play significant roles in the loss of gastric mucosal integrity caused by various aggressive factors. In other words, lesions develop when oxidative damage and apoptosis predominate over the healing process (62) by cell proliferation where the role of various growth factors, nitric oxide, endothelin, angiogenesis, mitogen-activated protein kinases and
Mechanism of antiulcer effect of omeprazole on oncogene (c-myc, c-Ha-ras and c-fos) expression has been demonstrated (63-73). The modern approach of understanding the mechanism of the antiulcer effect of omeprazole should therefore be directed towards exploring its plausible role in preventing oxidative damage and apoptosis as well as on the promotion of healing process by cell proliferation. As far our knowledge goes, this is the first evidence to show that omeprazole blocks gastric lesions by preventing oxidative damage and apoptosis of the gastric mucosal cells. Although omeprazole blocks ulceration at a lower dose (<2.5 mg/kg) without inhibiting acid secretion suggesting its independent antiulcer activity, at higher doses, its additional antisecretory action definitely exerts beneficial effect by preventing aggravation of the wound thereby helping the healing process by cell proliferation. The question arises as to how omeprazole offers antiulcer activity independent of acid secretion. Our studies indicate that omeprazole is highly effective in blocking membrane lipid peroxidation and protein oxidation which occur due to oxidative damage by ROS especially by $^\cdot$OH (13,22,25-37). Omeprazole can scavenge the endogenous $^\cdot$OH and thus prevents oxidative damage and gastric lesions. By blocking oxidative damage through lipid peroxidation and protein oxidation, omeprazole prevents loss of membrane permeability and dysfunction of the cellular proteins, leading to survival of the functionally active cells. Many natural and synthetic compounds are known to offer antiulcer effect by acting as antioxidants. Melatonin (a pineal hormone), vitamin E, PBN or desferrioxamine directly or indirectly decreases the endogenous level of $^\cdot$OH to block gastric ulcer (22,59). Comparative bioefficacy studies indicate that omeprazole is superior to these antioxidants in blocking gastric lesions. However, most important effect of omeprazole lies in its novel antiapoptotic role during ulceration, as evidenced by prevention of DNA fragmentation \textit{in vivo}. Apoptosis of mucosal cells occurs almost
in all types of gastric ulcer (39-51) where DNA damage and fragmentation occur by various aggressive factors (39,41,45-47). Using histological section and TUNEL staining technique (39), gastric mucosal cell apoptosis was detected upto 4 hrs after stress following which cell proliferation was found to be significantly increased to promote mucosal healing (39). Moreover, apoptosis is triggered by the upregulation of apoptosis-promoting Bax mRNA and downregulation of the antiapoptotic Bcl-2 mRNA expression (39). We have, however, directly demonstrated stress-induced DNA fragmentation in the surface epithelial cell and the beneficial role of omeprazole to block it thereby preventing apoptotic cell death and gastric lesions. It is not clear yet how differential expression of Bax and Bcl-2 proteins controls apoptosis. However, decreased gastric mucosal blood flow (39) leads to ischemic condition to generate ROS through alteration of antioxidant systems of gastric mucosa (13,22), which may cause apoptosis through oxidative damage of DNA (39,45,46). However, ischemia may also cause apoptosis through other mechanisms such as involvement of Bcl-2, Bax and c-fos proteins (74,75). Excessive generation of nitric oxide by gastric mucosal inducible nitric oxide synthase by stress also promotes apoptosis through increased formation of ROS (76,77). That ROS can cause oxidative damage of DNA isolated from both rat and human gastric mucosal epithelial cells has been evident from our in vitro studies where incubation of DNA with ·OH generating system (22,59,78) causes extensive DNA degradation which is sensitive to catalase and DMPO. Both omeprazole and lansoprazole have unique capacity to block this oxidative damage indicating its potent antioxidant role to protect DNA from the attack of ·OH. This could be achieved if omeprazole or lansoprazole can directly scavenge the ·OH to form oxidation product. Lansoprazole when incubated in the ·OH generating system, can in fact diminish the level of ·OH by its direct scavenging
action. This is evident by the observation that incubation of lansoprazole in the \textsuperscript{•}OH generating system produces four oxidation products of which the major one shows the addition of 16 mass units (m/z 385) over the mass of lansoprazole (m/z 369) indicating incorporation of an oxygen atom into lansoprazole. Omeprazole and lansoprazole undergo oxidation in cyt-P\textsubscript{450} systems to produce hydroxyomeprazole or hydroxylansoprazole and omeprazole sulfone or lansoprazole sulfone (79-81). Hydroxylation in the benzene ring of lansoprazole in our system does not occur as the oxidation product absorbing at 278 nm does not show the characteristic alkali shift for the formation of phenol. Absence of any additional aromatic proton signal in \textsuperscript{1}H NMR spectrum also negates the possibility of the formation of phenolic group in the oxidation product. Thus addition of an oxygen atom has occurred at the sulphur atom of the lansoprazole to form lansoprazole sulfone. The possible mechanism of formation of lansoprazole sulfone from lansoprazole by scavenging \textsuperscript{•}OH is shown in Fig.12. Lansoprazole is converted to a highly reactive sulphur centered radical intermediate by scavenging the \textsuperscript{•}OH and the intermediate is stabilized to form lansoprazole sulfone by further incorporation of \textsuperscript{•}OH at the sulphur radical with the elimination of one molecule of water.

The present study thus provides the first direct evidence for the antioxidant and antiapoptotic role of omeprazole in preventing gastric ulceration by scavenging endogenous \textsuperscript{•}OH. This is in contrast to the earlier observation that omeprazole neither protects indomethacin-induced gastric damage nor does it decrease apoptotic DNA fragmentation (41). Although a large number of reports (including the present study) indicate that omeprazole prevents indomethacin-induced gastric damage, the inability of omeprazole to block gastric lesions and associated apoptosis (41) is not clear and hence the conclusion that omeprazole does not possess antiapoptotic
property to block indomethacin ulcer (41) remains to be verified. However, a question may arise as to what percentage of total gastroprotection by omeprazole is mediated through block of apoptosis and its antioxidant action by scavenging \( \cdot \text{OH} \). Since quantitation of total gastroprotection by omeprazole is difficult to assess as it has morphological, cellular, biochemical and pharmacological parameters, extensive studies are required to answer this question. As gastric ulceration is a multifactorial process (ref.24,39,41), it is possible that gastroprotective effect of omeprazole may partially be mediated through other mechanisms also. Recently omeprazole has been shown to prevent compound 48/80 (mast cell degranulator)-induced gastric lesions (with no acid secretion) by acting as an antiinflammatory agent and also by preventing neutrophil infiltration, activation and associated mucosal damage (82). Thus, omeprazole may have multiple modes of action. Although no unifying concept has developed yet on the mechanism of gastric mucosal damage caused by various ulcerogens, it will be interesting to investigate whether omeprazole has a common molecular target for gastroprotection. As apoptosis (30-51) and reactive oxygen species (13,22,25-37,83,84) play significant roles in mucosal damage, it is conceivable that antiapoptotic and antioxidant role of omeprazole play a major part in the total gastroprotection. These novel actions of omeprazole are of particular clinical significance for the control of gastroduodenal ulcer by this class of proton pump inhibitors.

ACKNOWLEDGEMENT

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REFERENCES


TABLE 1

Time – course studies on gastroprotection and acid inhibition by omeprazole

Omeprazole (4 mg/kg) was administered (ip) 30 min prior to the onset of restraint- cold stress or oral administration of indomethacin or pylorus ligation. Animals were sacrificed at the desired time and gastric lesions or acid secretion was measured as described in the text.

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>Stress Ulcer</th>
<th>Indomethacin Ulcer</th>
<th>Induced Acid secretion (μ moles HCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ulcer index</td>
<td>% protection</td>
<td>Ulcer index</td>
</tr>
<tr>
<td></td>
<td>- Ome</td>
<td>+ Ome</td>
<td>- Ome</td>
</tr>
<tr>
<td>1.0</td>
<td>15 ± 3.1</td>
<td>1 ± 0.8*</td>
<td>93</td>
</tr>
<tr>
<td>2.5</td>
<td>39 ± 4.6</td>
<td>4 ± 1.8*</td>
<td>90</td>
</tr>
<tr>
<td>3.5</td>
<td>52 ± 5.9</td>
<td>4 ± 3.5*</td>
<td>92</td>
</tr>
</tbody>
</table>

* p < 0.001  § p < 0.05
TABLE 2

Effect of omeprazole on endogenous hydroxyl radical and oxidative damage as measured by lipid peroxidation and protein oxidation.

Omeprazole was injected (ip) 30 min before the onset of restraint-cold stress. Lipid peroxidation, protein oxidation and *OH were measured as described in the text. The data for *OH were presented after subtracting the control value which is 39±9 nmoles/g.

<table>
<thead>
<tr>
<th></th>
<th>*OH(nmoles/g)</th>
<th>Lipid peroxidation (nmoles TBARS/mg protein)</th>
<th>Protein carbonyl (nmoles/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.69±0.05</td>
<td>1.14±0.19</td>
<td></td>
</tr>
<tr>
<td>Control + stress</td>
<td>194±27</td>
<td>1.66±0.20*</td>
<td>2.42±0.19†</td>
</tr>
<tr>
<td>Control + omeprazole + stress</td>
<td>48±28†</td>
<td>0.64±0.09**</td>
<td>0.77±0.24†</td>
</tr>
</tbody>
</table>

*p<0.01 vs control; **p<0.02 vs stress; †p<0.001 vs control and control + stress.
**LEGENDS TO FIGURES**

Fig.1. Chemical structure of proton pump inhibitors.

Fig.2. Differential effect of omeprazole on gastric ulceration and gastric acid secretion. Omeprazole was administered (ip) 30 min before the onset of restraint-cold stress or oral administration of indomethacin or before pylorus ligation and the animals were sacrificed after 3.5, 4 and 2.5 hr respectively. The severity of gastric lesions was expressed as ulcer index. Acid secretion was measured by titrating the clarified gastric secretion with 1mM NaOH and the values represent the acid output for 2.5 hr.

*p<0.001 vs stress or pylorus ligated control; **p<0.001 vs indomethacin control; §p<0.05 vs indomethacin control.

Fig.3. Effect of dimethylsulfoxide on stress and indomethacin-induced gastric ulcer. Dimethylsulfoxide (1ml) was injected (ip) 30 min prior to the onset of stress or oral administration of indomethacin and ulcer index was determined after 3.5 and 4 hr respectively as described in the text. Luminal acid content was measured by titrating the clarified gastric secretion with 1mM NaOH.

*p<0.001 vs stress or indomethacin.

Fig.4. Correlation of the severity of gastric lesions with endogenous *OH and luminal acid content. The severity of gastric lesions was assessed as ulcer index at different times of stress. The endogenous *OH was measured after treatment with DMSO as described in the text. The intraluminal acid content was determined by titration with 1 mM NaOH.

Fig.5. Comparison of antiulcer effect of omeprazole with some natural and synthetic antioxidants. Omeprazole, melatonin, vitamin E,
Mechanism of antiulcer effect of omeprazole

desferrioxamine or PBN was administered 30 min prior to restraint-cold stress and ulcer index was scored as described in the text. *p<0.001 vs control; **p<0.01 vs control; ***p<0.02 vs control.

Fig.6. Effect of omeprazole on stress-induced DNA fragmentation as an index of apoptosis. DNA was isolated from the surface epithelial cells of gastric mucosa and subjected to agarose gel electrophoresis. Lane 1, control DNA, lane 2, DNA after restraint-cold stress and lane 3, DNA from stressed rat pretreated with omeprazole.

Fig.7. Effect of omeprazole and lansoprazole on oxidative damage of DNA in vitro. A, control DNA was incubated in Cu$^{2+}$-ascorbate system in absence or presence of varying concentrations of omeprazole. Lane 1, rat DNA; lane 2, DNA + Cu$^{2+}$-ascorbate; lane 3 to 6, DNA + 100, 250, 500 or 1000 μM omeprazole respectively + Cu$^{2+}$-ascorbate; lane 7, DNA + 1μg catalase + Cu$^{2+}$-ascorbate and lane 8, DNA + 20 mM DMPO + Cu$^{2+}$-ascorbate. B, lane 1, rat DNA; lane 2, DNA + Cu$^{2+}$-ascorbate; lane 3-5, DNA + 100, 250 or 500 μM lansoprazole respectively + Cu$^{2+}$-ascorbate; lane 6, DNA + 1 μg catalase + Cu$^{2+}$-ascorbate and lane 7, DNA + 20 mM DMPO + Cu$^{2+}$-ascorbate. C, lane 1, human DNA; lane 2, DNA + Cu$^{2+}$-ascorbate and lane 3-6, DNA + 25, 50, 100 or 500 μM lansoprazole respectively + Cu$^{2+}$-ascorbate.

Fig.8. Effect of lansoprazole on the level of ·OH in vitro. Varying concentrations of lansoprazole were incubated in a ·OH generating system as described in the text. Hydroxyl radical was quantitated by measuring the formation of methanesulfinic acid from DMSO, after complexation with fast blue BB salt.
Fig. 9. Thin layer chromatography and HPLC profiles of lansoprazole and its oxidation product(s). A, TLC profiles: lane 1 lansoprazole; lane 2, spot 1-4, lansoprazole oxidation products, spot 5, unreacted lansoprazole and lane 3, major oxidation product after isolation from TLC and purification by HPLC. B, HPLC profiles of purified major oxidation product of lansoprazole. Tracing 1, HPLC profile of major oxidation product after isolation from preparative TLC and tracing 2, the compound in the major peak in tracing 1 was isolated by HPLC and rechromatographed to show the purity of the compound.

Fig. 10. Electron-impact mass spectrum of the HPLC-purified major oxidation product of lansoprazole. The molecular ion mass was determined in a mass spectrometer using EI+ mode.

Fig. 11. UV-visible spectrum of HPLC-purified lansoprazole oxidation product in absence or presence of alkali. Tracing 1, spectrum of major oxidation product of lansoprazole and tracing 2 same after addition of 10 μl of 1N sodium hydroxide. The spectrum was taken in 1 ml methanol.

Fig. 12. Possible mechanism of formation of lansoprazole sulfone from lansoprazole and ·OH.
The abbreviations used are: $^1$H NMR, proton nuclear magnetic resonance; ROS, reactive oxygen species; PBN, $\alpha$-phenyl N-tert butyl nitrotrone; TBA, thiobarbituric acid; DMPO, 5-5-dimethyl-1-pyrol ne N-oxide; TCA, trichloroacetic acid; DMSO, dimethylsulfoxide; MSA, methanesulfonic acid; PBS, phosphate buffer saline with EDTA; SDS, sodium dodecyl sulphate.
Figure 1

Omeprazole

Lansoprazole

Pantoprazole

Rabeprazole
Figure 2

(A) Ulcer index vs. Omeprazole (mg/kg) for Stress and Indomethacin treatment groups.

(B) μmoles of HCl secreted vs. Omeprazole (mg/kg).

* indicates significant difference.

§ indicates non-significant difference.
Figure 3

(A) Urea index and (B) HCl production in the rat stomach.

- Stress: 60 ± 5 μmoles of HCl
- Stress + DMSO: 8 ± 1 μmoles of HCl
- Indomethacin: 30 ± 5 μmoles of HCl
- Indomethacin + DMSO: 5 ± 1 μmoles of HCl

*Significant difference compared to control.
Figure 4
Figure 5

[Graphs showing the mechanism of antiulcer effect of omeprazole]
Figure 6
Figure 7
Figure 8

[Graph showing the relationship between Lansoprazole concentration (mM) and OH generated (nmoles/hr).]
Figure 9
Figure 10
Figure 11
Figure 12

Lansoprazole

Lansoprazole sulfone
A novel antioxidant and antiapoptotic role of omeprazole to block gastric ulcer through scavenging of hydroxyl radical
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