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Gastroprotective Effect of 1-Phenyl-3-(4-hydroxy-3,5-di-*tert*-butylphenyl)prop-2-en-1-one in Rats

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Abstract

1-Phenyl-3-(4-hydroxy-3,5-di-*tert*-butylphenyl)prop-2-en-1-one (PHP), an antioxidant, has been studied for gastroprotective activity in-vitro and in-vivo, more specifically for its capacity to inhibit in-vitro iron- and copper-driven oxidant damage at acidic pH values mimicking intragastric conditions in the clinical setting.

Our studies showed significant inhibition of both iron- and copper-driven oxidant damage at pH 3·5 and 5·3. Intragastric and intraperitoneal administration of PHP (250 mg kg^{-1}) reduced gastric mucosal haemorrhagic lesions in stress-induced and ethanol-induced rat models. The gastroprotective effect of PHP against ethanol was reversed significantly by prior treatment with a dose of indomethacin that inhibits prostaglandin biosynthesis, indicating a possible role of prostaglandins in its gastroprotective effect. Treatment of PHP replenished reduced levels of gastric mucosal non-protein sulphydryls in ethanol-treated rats, suggesting the mediation of its effect through sulphydryls.

These results indicate that PHP was active at acidic pH. This is an interesting observation because highly acidic pH is known to be important in the development of gastric ulcers. Our study suggests that PHP might protect gastric mucosa by its capacity to scavenge free radicals.

Oxygen free-radicals are important in the pathogenesis of various disorders of the gastrointestinal system (Parks et al 1983; Itoh & Guth 1985). The role of free radicals in gastric damage mediated by lipid peroxidation is also well known (Takeuchi et al 1991). It has been shown that enzymes such as superoxide dismutase and catalase, and hydroxy-radical scavengers such as dimethylsulphoxide (DMSO) protect against tissue damage in the gastrointestinal tract (Parks et al 1983; Vaananen et al 1991). Recent studies have shown that omeprazole and rebamipide act as antioxidants in protecting against gastric damage (Yoshikawa et al 1993; Lapenna et al 1996). The role of *Helicobacter pylori* and non-steroidal anti-inflammatory agents in enhancing the neutrophil-dependent generation of reactive oxygen species, thereby aggravating gastric damage, has also been reported (Wallace et al 1990; Suzuki et al 1992). In our earlier studies PHP (1-phenyl-3-(4-hydroxy-3,5-di-*tert*-butylphenyl)prop-2-en-1-one) was found to have potent antioxidant activity (Rajakumar & Rao 1995, 1996). In this study we have investigated the effect of PHP on iron- and copper-induced gastric injury in-vitro, and stress, ethanol or indomethacin-induced gastric damage in rats.

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Materials and Methods

Reagents

Thiobarbituric acid, 5,5-dithiobis-2-nitrobenzoic acid and deoxyribose were purchased from Sigma (St Louis, MO). Other reagents used were of the highest analytical grade. PHP was synthesized in our laboratory by Claisen–Schmidt condensation (Rajakumar & Rao 1995).

Animals

Experiments, approved by the Institutional Animal Care and Use Committee, were performed on Wistar albino rats of either sex, 6–8 weeks, 210–240 g, fed on a normal laboratory diet. The animals

were deprived of food for 36 h before experimentation but were allowed free access to tap water throughout. All studies were performed on 6–10 rats per group.

Reactivity of PHP in iron- and copper-driven oxidant injury

Inhibition of iron- and copper-driven oxidant damage by PHP was assessed at pH 3·5 and pH 5·3 (Lapenna et al 1996). The reaction mixture contained deoxyribose (10 mM), $FeCl_3$ or $CuCl_2$ (1 mM), H_2O_2 (1 mM), ascorbic acid (100 μM), and different concentrations of PHP (10, 50, 100 or 500 μM) in acetate buffer (20 mM) pH 3·5 or 5·3 (final volume 1 mL). After 30 min incubation at 37°C, trichloroacetic acid (2·5%, 1 mL) and aqueous thiobarbituric acid (0·6%, 1 mL) were added and the mixture was heated for 15 min at 95°C. After cooling the absorbance was measured at 532 nm. The results were calculated as nmol thiobarbituric acid reactants mL^{-1} and expressed as percentage inhibition of thiobarbituric acid reactants, using a molar extinction coefficient of $1{\cdot}54 \times 10^5\ M^{-1}\ cm^{-1}$ for the calculation.

Effect of PHP on water-immersed and restraint-stress rats

The method of Lewis & Hanson (1991) was followed. PHP (250 mg kg^{-1}) was administered orally or intraperitoneally to rats fasted for 36 h. After 30 min the rats were placed in restraint cages and immersed in water at 23°C to the xyphoid level. After 5 h, the rats were killed by cervical dislocation and the stomach was cut open along the greater curvature and examined for erosions and ulcers in the glandular region. Ulcer index was calculated by following the standard scoring system (Motilva et al 1994). Mean score for each group was calculated and expressed as the ulcer index.

Effect of PHP on indomethacin pretreatment

Indomethacin was used to block the formation of prostaglandins by inhibition of prostaglandin cyclooxygenase (Tariq & Abdulrehman 1990). The rats were fasted for 36 h with free access to water and then treated with indomethacin (5 mg kg^{-1}) subcutaneously, 1 h before oral administration of PHP (250 mg kg^{-1}). After 20 min the animals were given 95% ethanol (0·5 mL $(100\ g)^{-1}$, orally). The animals were killed 1 h after administration of ethanol and the stomachs were excised and examined for lesions. The results were compared with those obtained from animals treated with PHP and ethanol but without indomethacin treatment.

Effect of PHP pretreatment on ethanol-induced gastric lesions

Animals were fasted for 36 h. One group was the control, the second was treated with PHP (250 mg kg^{-1}, orally), the third was treated with ethanol (95%) orally, and the last received ethanol after pretreatment with PHP. The animals were killed 1 h after administration of ethanol and each stomach was examined for lesions. The ulcer index was calculated as previously described (Motilva et al 1994).

Gastric mucosal non-protein sulphydryls were measured by homogenizing the glandular portion of the stomach in ice-cold EDTA (0·02 M) and mixing the homogenate (2 mL) with distilled water (2 mL) and trichloroacetic acid (50% w/v, 1 mL). The tubes were centrifuged at 3000 *g* for 10 min and the supernatant (1 mL) was mixed with Tris buffer (pH 8·9, 4 mL). A solution of 5,5-dithiobis-2-nitrobenzoic acid (1 mM) was added and the absorbance was measured at 412 nm against a reagent blank with no homogenate (Tariq & Abdulrehman 1990).

Lipid peroxide was determined as described previously (Keun et al 1996). Briefly, reaction mixture containing homogenate (0·5 mL), sodium dodecylsulphate (8·1% w/v), acetate buffer (pH 3·5, 20% w/v) and thiobarbituric acid solution (0·8% w/v) was mixed well and heated at 95°C for 60 min. The absorbance was measured at 532 nm and expressed as nmol malondialdehyde.

Statistical analysis

Results from in-vitro experiments are expressed as mean ± s.d. and the effects of the drugs were evaluated by two-way analysis of variance. Significant differences between results from in-vivo experiments were identified by the Mann–Whitney two-tailed *U*-test or Student's *t*-test for unpaired data.

Results

Effect of PHP on iron- and copper-driven oxidant injury

Table 1 shows the percentage inhibition of the degradation of deoxyribose at pH 3·5 or pH 5·3 by PHP in the presence of iron or copper. At pH 3·5, PHP had a significant antioxidant effect beginning at a drug concentration of 10 μM, which resulted in approximately 63% and 46% inhibition of iron- and copper-dependent deoxyribose degradation, respectively. At higher PHP concentrations the antioxidant effect on copper-dependent deoxyribose degradation was more pronounced, with almost 98% inhibition. PHP at pH 5·3 also significantly inhibited both iron- and copper-

Table 1. Effect of PHP on iron- and copper-dependent deoxyribose oxidation.

		Iron		Copper	
		pH 3·5	pH 5·3	pH 3·5	pH 5·3
Control		5·73 ± 0·19	7·30 ± 0·8	2·83 ± 0·23	2·40 ± 0·05
Control plus PHP	10 μM	2·1 ± 0·30*	1·07 ± 0·03†	1·5 ± 0·19‡	0·80 ± 0·07§
	50 μM	0·87 ± 0·05*	0·85 ± 0·04†	0·43 ± 0·09‡	0·70 ± 0·01§
	100 μM	0·77 ± 0·05*	0·71 ± 0·04†	0·17 ± 0·04‡	0·62 ± 0·03§
	500 μM	0·66 ± 0·12*	0·67 ± 0·07†	0·07 ± 0·03‡	0·45 ± 0·04§

The reaction mixture containing 10 mM deoxyribose, 1 mM $FeCl_3$ or $CuCl_2$, 1 mM H_2O_2 and 100 μM ascorbic acid and different concentrations of PHP (10, 50, 100 and 500 μM) in 20 mM acetate buffer, pH 3·5 or 5·3 (final volume 1 mL) was incubated for 30 min at 37°C and absorbance was measured at 532 nm. The results were calculated as nanomol thiobarbituric acid reactants mL^{-1} and expressed as mean ± s.d (n = 3). *$P < 0.001$, significantly different from control result (iron, pH 3·5), †$P < 0.001$, significantly different from control result (iron, pH 5·3), ‡$P < 0.001$, significantly different from control result (copper, pH 3·5), §$P < 0.001$, significantly different from control result (copper, pH 5·3).

dependent deoxyribose degradation with 85% and 67% inhibition, respectively, at a concentration of 10 μM. At a higher concentration of 500 μM, however, iron-mediated deoxyribose degradation was inhibited to a greater extent, inhibition being 91% compared with 87% inhibition of degradation in the presence of copper.

Effect of PHP on water-immersed restraint-stress rats

Table 2 shows the effects of pretreatment with different doses of PHP on stress-induced gastric mucosal damage. The ulcer index was reduced in a dose-dependent manner. At a dose of 50 mg kg^{-1} orally or intraperitoneally PHP afforded little protection; ulcer index 11·0 ± 3·2 on oral treatment and 8·5 ± 2·5 on intraperitoneal treatment compared with a control ulcer index of 12·8 ± 2·0. At an oral or intraperitoneal dose of 250 mg kg^{-1} there was a significant protection of over 60%. The ulcer indices were compared with that of the standard antiulcer drug ranitidine, which afforded 51% protection at 100 mg kg^{-1}, intraperitoneally.

Effect of PHP on indomethacin- and ethanol-induced gastric lesions

Macroscopic examination of the stomach showed (Table 3) a significant decrease in the ulcer index for the PHP-pretreated group (ulcer index 6·5 ± 0·5) compared with the ethanol-treated group (ulcer index 10·8 ± 1·5). Pretreatment of animals with

Table 3. Effect of PHP on gastric lesions induced by indomethacin and ethanol.

Treatment	Ulcer index
Control (vehicle)	0
Ethanol	10·8 ± 1·5
Indomethacin + ethanol	13·5 ± 1·8
PHP + ethanol	6·5 ± 0·5*
Indomethacin + PHP + ethanol	9·5 ± 1·4†§

Subcutaneous indomethacin (5 mg kg^{-1}) was given 1 h before oral PHP (250 mg kg^{-1}). Ethanol 95% (0·5 mL $(100 g)^{-1}$) was given orally 20 min after PHP. Results are expressed as means ± s.e.m. (n = 6–8). *$P < 0.01$, significantly different from result for ethanol-treated group. †$P < 0.05$, significantly different from result for ethanol + PHP-treated group. §$P < 0.05$, significantly different from result for indomethacin + ethanol-treated group (Student's *t*-test).

Table 2. Effect of pretreatment with PHP on susceptibility to stress-induced gastric mucosal damage.

Treatment	n	Dose (mg kg^{-1})	Mode of administration	Ulcer index	Protection (%)
Control	10	Vehicle	Oral or intraperitoneal	12·8 ± 2·0	–
PHP	6	50	Oral	11·0 ± 3·2	15·3
PHP	6	150	Oral	8·8 ± 2·8	32·4
PHP	8	250	Oral	5·1 ± 1·8*	61·0
PHP	6	350	Oral	4·6 ± 2·6*	65·6
PHP	6	500	Oral	1·6 ± 0·9*	88·0
PHP	6	50	Intraperitoneal	8·5 ± 2·5	34·5
PHP	6	150	Intraperitoneal	7·0 ± 1·4*	45·6
PHP	8	250	Intraperitoneal	4·2 ± 1·9*	68·2
PHP	6	350	Intraperitoneal	3·5 ± 1·1*	73·1
Ranitidine	8	100	Intraperitoneal	6·3 ± 0·8*	50·6

Results are expressed as means ± s.e.m. *$P < 0.05$, significantly different from control result (Mann–Whitney two-tailed *U*-test).

Table 4. Effect of PHP on non-protein sulphydryl level and lipid peroxidation in the glandular stomach of rats.

Treatment	n	Dose (oral, mg kg^{-1})	Non-protein sulphydryls (μmol (g tissue)$^{-1}$)	Malondialdehyde (μmol (g tissue)$^{-1}$)
Control	10	–	3·46 ± 0·21	164·2 ± 9·42
PHP	8	250	3·61 ± 0·06	148·5 ± 8·63
Ethanol	8	–	2·20 ± 0·13*	153·0 ± 8·90
PHP + ethanol	8	250	3·31 ± 0·19†	157·0 ± 9·60

Results are expressed as means ± s.e.m. *$P < 0.05$, significantly different from control result; †$P < 0.05$, significantly different from result for ethanol-treated group (Student's t-test).

subcutaneous indomethacin (5 mg kg^{-1}) aggravated 95% ethanol-induced gastric lesions. Oral administration of PHP (250 mg kg^{-1}) afforded significant protection against gastric mucosal damage induced by both indomethacin and ethanol.

Effect of PHP on non-protein sulphydryls and lipid peroxidation level in ethanol treated rat stomach

Table 4 shows the effect of PHP pretreatment on the level of non-protein sulphydryls in the glandular portion of the stomach of rats treated with ethanol. The gastric mucosal level was significantly reduced on treatment with ethanol (2·2 ± 0·1 μmol (g tissue)$^{-1}$) compared with the control (3·5 ± 0·2 μmol (g tissue)$^{-1}$) which was restored to normal (3·3 ± 0·2 μmol (g tissue)$^{-1}$) on oral pretreatment with PHP (250 mg kg^{-1}).

Table 4 also shows the effect of PHP on gastric lipid peroxidation as measured by malondialdehyde level in the stomachs both of treated and of untreated groups. The malondialdehyde level of the group treated with PHP alone was not significantly different from that of the control group with no treatment. However, the malondialdehyde level observed for the ethanol group was no higher than that of the control group.

Malondialdehyde levels in the PHP pretreated group were less than in the group treated with ethanol alone.

Discussion

It has been reported that both iron- and copper-mediated site-specific gastric damage play a major role in peptic ulcer, gastritis and other inflammatory diseases (Hiraishi et al 1991; Lapenna et al 1996). Our results indicate that PHP is capable of reducing both iron- and copper-mediated site-specific gastric damage. It is significant that PHP was active at acidic pH (3·5 and 5·3). Such high acidic pH values are known to play an important role in the development of gastric ulcers. Omeprazole, a well known anti-ulcer drug was also reported to have good antioxidant activity in acidic media (Lapenna et al 1996).

Reactive oxygen species are known to contribute to gastric damage induced by stress and shock (Itoh & Guth 1985; Li & Zhang 1993). In this investigation PHP reduced gastric mucosal damage drastically in a dose-dependent manner when administered orally or intraperitoneally. These results suggest that the anti-ulcer effect of PHP might be because of its capacity to scavenge hydroxyl radicals produced as a result of increased xanthine oxidase activity during stress. It has recently been shown that factors other than prostaglandin deficiency (e.g. free radicals) could be involved in the pathogenesis of non-steroidal anti-inflammatory drug-induced gastrointestinal damage (Soldato et al 1986). That prior depression of prostaglandin synthesis by treatment with indomethacin antagonized the protective effect of PHP against ethanol-induced gastric injury suggests that the gastroprotective effect of PHP might be mediated through endogenous release of prostaglandins and free-radical scavenging activity (Soldato et al 1986; Tariq & Abdulrehman 1990; Motilva et al 1994).

It has been reported that ethanol induces gastric mucosal damage by promoting the generation of reactive oxygen metabolites (Matsumoto et al 1993). Recent studies have shown that free-radical scavengers such as dipyridamole and rebamipide have a greater effect on ethanol-induced gastric damage (Tariq & Abdulrehman 1990; Keun et al 1996).

Reduced levels of endogenous sulphydryl have been associated with tissue damage by various chemical agents (Tariq & Abdulrehman 1990; Lopez et al 1996). Exogenous administration of sulphydryl-containing agents has been shown to prevent different forms of injury to the gastric mucosa in animal experiments (Lopez et al 1996). Our observations have shown a significant increase in levels of non-protein sulphydryls on pretreatment with PHP compared with the non-protein sulphydryl level in the glandular portion of the rat stomach treated with ethanol alone, suggesting that non-protein sulphydryls might be involved in PHP cytoprotection.

PHP inhibited ethanol-induced gastric haemorrhagic lesions, which might be attributed to its antioxidant properties in-vivo. However our studies indicate that the malondialdehyde level in the rat stomach after treatment with ethanol was not significantly higher than that reported for an untreated group (Keun et al 1996). This suggests that ethanol-induced gastric lesions do not lead to increased

levels of lipid peroxidation products, making it unlikely that the compounds are involved in the pathogenesis of the lesions (Kusterer et al 1987; Nalini & Balasubramanian 1993).

Our results indicate that PHP has potent gastroprotective activity. This is well supported by the inhibition by PHP of iron- and copper-driven oxidant injury, and of stress- and ethanol-induced gastric damage. The capacity of PHP to maintain non-protein sulphydryl levels in the glandular portion of the stomach during ethanol challenge suggests that the gastroprotective activity of PHP might be mediated by tissue sulphydryls.

We conclude that the high antioxidant activity of PHP might offer protection against a wide range of free-radical-mediated diseases of the gastrointestinal tract. Further studies might provide more information.

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