

Research Article

Synthesis of 2'-(5-Chloro-2-Hydroxybenzylidene) Benzenesulfanohydrazide Schiff Base and its Anti-Ulcer Activity in Ethanol-Induced Gastric Mucosal Lesions in Rats

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Abstract

Purpose: To study the anti-ulcer activity of 2'-(5-Chloro-2-hydroxybenzylidene) benzenesulfanohydrazide Schiff base against ethanol-induced gastric mucosal lesions in rats.

Methods: 2'-(5-Chloro-2-hydroxybenzylidene) benzenesulfanohydrazide (Cl-BzSO-HAP) was synthesized, and structurally characterized by elemental analysis, FT-IR, ¹H-NMR and ¹³C-NMR. Its preventive activity against ulcer induced by absolute ethanol in Sprague-Dawley rats were studied in vivo. Twenty four Sprague Dawley (SD) rats (12 males and 12 females) were assigned equally into 4 groups (n = 6), including negative control and positive control groups. The third group received a low oral dose of the compound (50 mg/kg) while the fourth group was administered a high oral dose (100 mg/kg). The degree of gastric lesion formation was assessed. Prior to dosing, the animals were fasted overnight.

Results: Cl-BzSO-HAP showed significant preventive activity against ulcer induced by absolute ethanol. Oral administration of the compound (both low and high doses) prior to ethanol administration inhibited gastric lesion formation by 88.0 and 94.9 %, respectively, compared to 82.7 % for the positive control, cimetidine.

Conclusion: Extensive gastric necrosis in rats induced by absolute ethanol was prevented by administration of Cl-BzSO-HAP resulting in reduced or total absence of lesions.

Keywords: Benzenesulfanohydrazone, Schiff base, Gastric ulcer, Histology.

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INTRODUCTION

Ulcer is an open sore, or lesion, usually found on the skin or mucous membranes of the body. Peptic ulcer is a lesion that occurs at the lining of the stomach or duodenum, where hydrochloric acid and pepsin are present. Previously, it was believed that lifestyle factors, such as stress and diet can cause ulcers. Subsequently, researchers determined that stomach fluid compounds – hydrochloric acid and pepsin – contribute to ulcer formation. Today, however, research shows that most ulcers (80 % of gastric ulcers and 90 % of duodenal ulcers) develop due to a bacterium called *Helicobacter pylori*. It is believed that, although all the three factors – lifestyle, acid and pepsin, and *H. pylori* – play a role in ulcer development, *H. pylori* is considered to be the primary cause.

Many of the past successes in medicinal chemistry have involved the fortuitous discovery of useful pharmaceutical agents from natural sources such as plants and microorganisms. One of the early examples of a rational approach to drug design was the development of the anti-ulcer drug, cimetidine [1]. There are literature reports on the antimicrobial activities of benzenesulfanohydrazones and their complexes [2]. Previous work on synthesis and characterization of Schiff bases ligands indicate the biological activity of these compounds against oxidation which was higher than that of vitamin E [3], as well as antibacterial [3], antiproliferative and antiviral activities [4]. This study extends this work by synthesizing a different Schiff base and evaluating its anti-ulcer activity.

EXPERIMENTAL

Materials and equipment

Perkin–Elmer FT-IR spectrometer, Joel JNM-LA400 FT-NMR system, Flash EA 1112 Series elemental analyzer were used in this study. The chemicals, 5'-chloro-2'-hydroxyacetophenone, benzenesulfanohydrazide and

acetic acid for acidifying ethanol, were obtained from Fluka and Aldrich and used as received without further purification. Schiff bases were synthesized by condensation reaction as described below. The reference anti-ulcer drug was obtained from University Malaya Medical Centre. Each tablet contained 200 mg of cimetidine. Tween 20 was manufactured by Merck Schuchardt OHG 85662 Hohen Brunn, Germany.

Synthesis of 2'-(5-chloro-2-hydroxybenzylidene) benzenesulfanohydrazide (figure 1)

Benzenesulfanohydrazide (0.690 g, 0.0035 mol) was accurately weighed on a digital balance in a 100 ml beaker and dissolved in 50 mL acidified ethanol (95:5, ethanol:acetic acid); 5'-chloro-2'-hydroxyacetophenone (0.60 g, 0.0035 mol) was also dissolved in another sample of acidified ethanol (50 mL). The two solutions were mixed together in a 500 mL flat bottom flask which was then clamped on a water bath and refluxed on a magnetic hot plate for 2 h while stirring. The resulting yellow precipitate was filtered into a conical flask and re-crystallized in ethanol to yield pale yellow crystals.

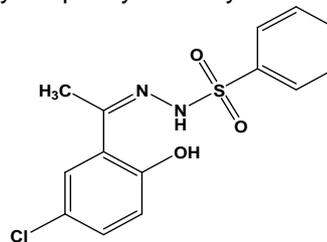


Figure 1: Chemical structure of 2'-(5-chloro-2-hydroxybenzylidene) benzenesulfanohydrazide

Elemental analysis

Approximately 1 - 10 mg of the compound was weighed accurately on a microbalance, and combusted at high temperatures in a stream of oxygen during which the products of combustion (carbon, hydrogen, and nitrogen) were recorded in a single analysis.

Fourier-transform infrared spectroscopy (FT-IR) analysis

Between 1 and 3 mg of ground Schiff base crystal was mixed thoroughly with about 350 mg of ground KBr. The mixture was transferred to a die that has a barrel diameter of 13 mm. This was then placed in a suitable press and pressed at around 12,000 psi for 2 min. Re-crystallization of the KBr resulted in a clear glassy disk about 1 mm thick. The KBr disk was analyzed by transmission at 4000 - 400 cm^{-1} on a Perkin-Elmer FT-IR spectrometer.

Fourier transform-nuclear magnetic resonance (FT-NMR) analysis

Approximately 10 mg of the compound was accurately measured into a vial and dissolved in 1 mL of deuterated dimethyl sulfoxide (DMSO). The liquid was transferred via a pipette filter into the NMR tube. ^1H and ^{13}C NMR spectra were recorded on a Jeol JNM-LA400 FT-NMR system. Trimethylsilane (TMS) was used as internal standard.

Ethics statement

Animal experiments were performed in accordance with the guidelines for animal experimentation issued by the Animal Care and Use Committee at the University of Malaya (Ethics no.: PM/27/07/2010/MAA). Throughout experiments, the animals received due care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health, USA.

Experimental animals

Sprague Dawley healthy adult male rats (200 - 225 g) were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya. The rats were divided randomly into 4 groups of 6 rats each. Each rat was placed individually in a separate cage (one rat per cage) with wide-mesh wire bottoms to prevent coprophagia during the

experiment. The animals were maintained on standard pellet diet and tap water. The study was approved by the Ethics Committee for Animal Experimentation, Faculty of Medicine University of Malaya, Malaysia.

Preparation of the oral dosage form

The Schiff base was dissolved in a mixture of tween 20 and distilled water in ratio of 1:10. Two doses of the extract were prepared: high dose (100 mg/kg), by dissolving 0.12 g of the solid Schiff base in 12 ml of 10 % Tween 20 and low dose (50 mg/kg), by dissolving 0.07 g the Schiff base in 12 ml of 10 % Tween 20. Dissolution was achieved with the aid of a vortex mixer (Vortex-2 Genie, Bohema, NY, USA).

Induction of gastric ulcer

Gastric ulcers were induced in the rats by administering absolute ethanol according to the method described by *Adulla et al.*[6]. The experimental animals were divided into four groups (n = 6). Control (negative control) received 5 ml kg^{-1} of 10 % Tween 20 orally via metal orogastric tube. Positive control group received orally 50 mg kg^{-1} cimetidine (suspended in 10 % Tween 20). The third group received 100 mg kg^{-1} of the extract (suspended in 10 % Tween 20) while the fourth group was administered 50 mg kg^{-1} of the extract. Sixty minutes later, 5 ml kg^{-1} of absolute ethanol was administered orally to the animals and then sacrificed 60 min thereafter by overdoses of xylazine and ketamine anaesthetics [6].

Macroscopic examination

The abdomen of each rat was opened and the pylorus ligated. The stomach was cut out, the stomach contents collected, centrifuged and the gastric juice separated from the mucus and its pH measured. The mucus was weighed and expressed in grams [5]. The inner surface area (mm^2) of each stomach covered by each lesion was measured using a microscope (x 1.8 magnification) according to the recommendation of Mahmood et al., 2010 [6] and the sum of erosion areas per rat

stomach was calculated. Ulcerated surface (US, %), was computed as in Eq 1.

$$US = (A/B) \times 100. \dots\dots\dots (1)$$

Where A is the total area covered by ulcers and B is the total corpus mucosal surface.

Ulcer index (UI) for each animal was then calculated as the mean ulcer score Inhibition (I, %) as in Eq 2 [7].

$$(I \%) = [(UI_{\text{control}} - UI_{\text{treated}}) / UI_{\text{control}}] \times 100 \dots\dots(2)$$

Microscopic examination (Histology)

The stomach was cut into small pieces and fixed in 10 % buffered formalin overnight [7]. The tissue was processed in an automated tissue processor (Leica, Germany) to achieve dehydration, cleaning and impregnation. It was then embedded in paraffin wax and sectioned into 5 μm thick sections with a rotary microtome. The sections were stained with haematoxylin and eosin and analyzed under light microscope at x10, 40 and 100 magnification to observe if any changes in the tissue structure when compared to the control group [6].

Statistical analysis

The results were expressed as mean \pm S.E.M (standard error of the mean). The statistical differences of each group of rats were calculated using student t-test SPSS for windows evaluation version 14. The significance difference was accepted at $p < 0.05$. Data were presented as mean \pm S.E.M.

RESULTS

Physical and chemical characterization of compound

Compound yield = 75%, m.pt=150°C. **Elemental analysis:** Actual: C(51.83%) ; H(4.33%) ; N(8.50%), Theoretical: C(51.72%) ; H(4.00%) ; N(8.62%). **IR spectra (KBr)** 3423 cm^{-1} (vAr-OH), 3232 cm^{-1} (vN-H), 1603 cm^{-1} (vC=N), 1324 cm^{-1} (vS=O), 1158 cm^{-1} (vS=O), 1074 cm^{-1} (vC-O), 1020 cm^{-1} (vN-N). **¹H NMR (DMSO)** 11.43ppm [δ (OH), 1H, s], 11.19ppm [δ (NH), 1H,s], 6.836-7.88ppm [δ (aromatic), m], 2.07ppm [δ (-CH₃), 3H, s]. **¹³C {¹H} NMR (DMSO)** 157.09ppm δ (C=N), 156.12ppm δ (C-O), 118.7-138.45ppm δ (aromatic), 14.86ppm δ (-CH₃).

Anti-ulcer activity of compound

The anti-ulcer activity of the compound is shown in Table 1. Ulcerated area of the stomach decreased from 840 mm^2 for control to 145 mm^2 for the cimetidine group and as low as 3.6 mm^2 for the test compound group. The ulcer inhibition capacity of the test compound (88.0 %) at a dose of 50 mg/kg was higher than that of cimetidine (82.7 %) inhibition. When the dose was increased to 100 mg/kg, ulcer inhibition increased to 99.6 %.. There was no significant difference ($p < 0.05$) between the effect of the two doses of the compound (50 mg/kg and 100 mg/kg) on the pH of gastric juice (4.25 ± 0.25 vs 3.21 ± 0.00) but both doses produced significantly ($p < 0.05$) lower pH than the cimetidine group ($\text{pH} = 6.20 \pm 0.49$).

Table 1: Anti-ulcer activity of the test compound, Cl-BzSO-HAP, in rats

Group	Pre-treatment (5 ml/kg dose)	Ulcerated area (mm^2)	Inhibition (%)	pH of gastric content	Weight of gastric mucus
1	Tween 20 (Ulcer Control)	840.0 \pm 4.6 ^a	-	3.78 \pm 0.15 ^a	0.73 \pm 0.07 ^a
2	Cimetidine (50 mg/kg)	145.0 \pm 4.9 ^b	82.7	6.20 \pm 0.49 ^b	1.18 \pm 0.07 ^b
3	Cl-BzSO-HAP (50 mg/kg)	100.8 \pm 4.3 ^c	88.0	4.25 \pm 0.25 ^a	1.07 \pm 0.06 ^b
4	Cl-BzSO-HAP (100 mg/kg)	3.6 \pm 0.2 ^d	99.6%	3.21 \pm 0.25 ^a	1.01 \pm 0.04 ^b

All values were expressed as mean \pm standard error mean. Means with different superscripts indicate significant difference at $p < 0.05$

DISCUSSION

The chemical structure of benzenesulfanohydrazones shows hydroxyl groups or a carbonyl compound with a reactive keto-group responsible for its hydrogen accepting property. Based on its structure and potentials to generate hydroxyl radicals, they can be described as oxidant and appears to increase the load of free radicals. However, the carbonyl group in the structure appears to achieve an antioxidant balance, thereby protecting the gastric mucosa from the injurious agents [8]. Therefore, this group can play the role of electrophilic acceptor in the structure. The mechanism of cytoprotective activity has been suggested to be mediated through a reaction between electrophilic acceptor and the sulfhydryl-containing groups of the mucosa. Furthermore, compounds with hydroxyl group can form hydroxyl radicals which are known to play a major role in the pathogenesis of gastric mucosal injury [8]. Reduced gastric acidity suggests that the cytoprotective mechanism of action of the synthesized compound may involve direct inhibition of gastric secretion and a simple neutralization of the acid secreted in the stomach. The effect of 2'-[5-Chloro-2-hydroxybenzylidene] benzenesulfanohydrazide compound on the acidity of gastric juice relative to that of the cimetidine group agrees with the findings of Murakamu & Njar [9,10].

The administration of ethanol to rats produces gastric mucosal lesions and erosions similar to those that occur in gastric ulcer. These lesions are produced because ethanol affects adversely the protective defense mechanisms of mucus [11]. Ethanol rapidly penetrates mucosa layer and causes extensive mucosal damage in rat stomach [6,12]. This is why only 60 min was needed to produce acute gastric ulceration in the rats that was visible as thick reddish-black lines in ulcerated control group (figure 2).

Histological analysis of the effect of ethanol on the stomach of the ulcer control rat revealed the presence of necrotic debris in the lamina propria of the mucosa. The lesions extended down the mucosa layer and involved the surface epithelium. The treated groups showed fewer lesions on the epithelial surface (figure 3). Mucosal blood flow has been claimed to be an important factor in mucosal damage by alcohol and is modulated by prostaglandin [13]. The ability of 2'-[5-Chloro-2-hydroxybenzylidene] benzenesulfanohydrazide (figure 1) to protect against mucosal damage by ethanol may be due to the effect of the former on prostaglandin.

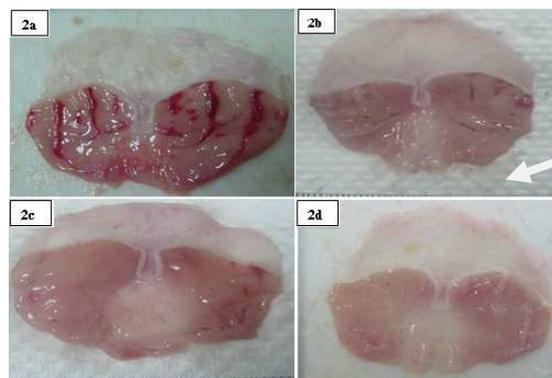


Figure 2: Gross appearance of the rat gastric mucosa in (2a) rats pre-treated with 5 ml/kg 10 % Tween 20 (ulcer control) and it shows extensive visible hemorrhagic necrosis on the mucosa; (2b) rats pre-treated with cimetidine (50 mg/kg) which shows milder damage to the gastric mucosa than ulcer control; (2c) rats pre-treated with Cl-BzSO-HAP (50 mg/kg) which also shows mild injuries on the gastric mucosa; (2d) rats pre-treated with Cl-BzSO-HAP (100 mg/kg) which showed no damage to the gastric mucosa.

2'-[5-Chloro-2-hydroxybenzylidene] benzenesulfanohydrazide exhibited a characteristic of histamine H_2 receptor antagonists, such as cimetidine which was used as standard antiulcer drug in the present study, in that it decreased acid output, but unlike cimetidine,

it also decreased the ulcerated area thus demonstrating its superiority to cimetidine.

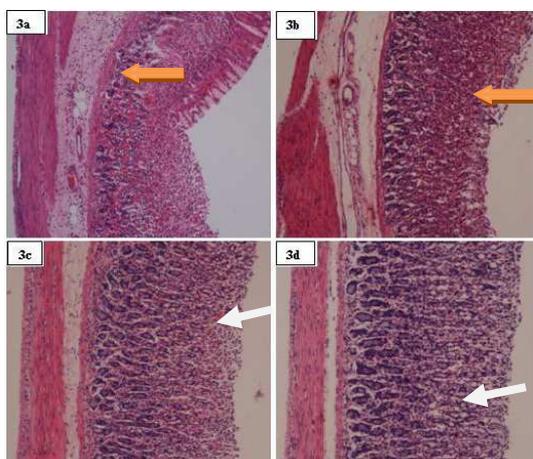


Figure 3: Histological examination of absolute ethanol-induced gastric mucosal damage in (3a) rats pre-treated with 5 ml/kg of 10% Tween 20 (ulcer control) which shows severe disruption to the surface epithelium and necrotic lesions penetrating deeply into mucosa (white arrow) as well as extensive edema of submucosa layer and leucocytes infiltration (orange arrow); (3b) rats pre-treated with cimetidine (50 mg/kg), showing mild disruption of the surface epithelial mucosa, and absence of deep mucosal damage, and also edema and leucocytes infiltration of the submucosal layer; (3c) rats pre-treated with Cl-BzSO-HAP (50 mg/kg), which show mild disruption of surface epithelium is present, absence of deep mucosal damage is absent and reduction of submucosal edema and leucocytes infiltration; (3d) rats pre-treated with Cl-BzSO-HAP (100 mg/kg), indicating no disruption to the surface epithelium as well as no edema and leucocytes infiltration of the submucosal layer (H&E stain, x10 magnification).

CONCLUSION

The synthesized compound, 2'-[5-Chloro-2-hydroxybenzylidene] benzenesulfanohydrazide, demonstrated potent anti-ulcer protection in absolute ethanol-induced ulcer model in rats that was superior to that of cimetidine thus underlining the need for its further evaluation as an anti-ulcer.

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