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Inhibitory Effects of 4-Guanidinobutyric Acid against Gastric Lesions

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Abstract

This study examined the inhibitory effects of 4-guanidinobutyric acid (4GBA), an alkaloid, against gastric lesions by assessing the inhibition of *Helicobacter pylori* (*H. pylori*) and gastric cancer cells. Acute and chronic gastritis were also observed using HCl/ethanol (EtOH) and indomethacin-induced gastric lesion models, respectively. 4GBA inhibited the growth of *H. pylori* in a dose dependent manner, and showed acid-neutralizing capacity. In the pylorus ligated rats, 4GBA decreased the volume of gastric secretion and gastric acid output slightly, and increased the pH. 4GBA at a dose of 100 mg/kg reduced the size of HCl/EtOH-induced gastric lesions (70.8%) and indomethacin-induced gastric lesions (38.8%). The antigastritic action of 4GBA might be associated with the acid-neutralizing capacity, anti-*H. pylori* action, and decreased volume of gastric secretion. These results suggest that 4GBA might be useful in the treatment and/or protection of gastritis.

Key Words: 4-guanidinobutyric acid, *Helicobacter pylori*, Cytotoxicity, Anti-oxidant, Gastric lesion

INTRODUCTION

4-Guanidinobutyric acid (4GBA), an alkaloid included in guanidino compounds, is present in the mammalian brain, herbal medicines, fish and shellfish (Tachikawa and Hosoya, 2011). 4GBA has stimulatory effects on monocytes and granulocytes (Schepers *et al.*, 2010) (Fig. 1). Recurring gastritis and gastric ulcers are generally caused by an imbalance between aggressive factors (*i.e.*, gastric acid, pepsin, stimulation of the vagus nerves, secretion of gastrin, and increasing the number of parietal cells) and protective factors (*i.e.*, bicarbonate ion, mucus productivity, mucus secretion, and prostaglandins) (Shay *et al.*, 1945). The gastric mucosal barrier is exposed to a range of aggressive factors, but is normally protected by unique protective mechanisms. Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin/chemotherapeutic agents and Aspirin, can cause gastric lesions, such as hemorrhages and ulcers by stimulating the gastric mucosal barrier directly (Eliakim *et al.*, 1995). Ethanol (EtOH) damages the stomach by accelerating the mucous membrane penetrability and inhibiting active transport. Reactive oxygen species (ROS), one of the aggressive factors, leads to acute and chronic inflammation in the stomach (Leirisalo-Repo *et al.*, 1993). *H. pylori* is an important pathogen associated with stomach cancer, chronic gastritis and ulceration in the stomach and duodenum by producing toxic agents (Leunk *et al.*, 1988; Sarosiek *et al.*, 1989; Correa, 1992; Slomiany 1992). The gastric mucosa infected with *H. pylori* has higher levels of ROS, which induce DNA damage (Drake *et al.*, 1998; Arend *et al.*, 2005). Antiacids are effective in accelerating the healing of duodenal and gastric ulcers due to the neutralization of gastric luminal acid (Tarnawski *et al.*, 1995). HCl/EtOH-induced gastric lesions appear to be produced by the direct irritation of the gastric mucosal barrier (Seiki *et al.*, 1990). EtOH induces long ulcers and petechial lesions within a relatively short period of time, which makes this technique suitable for screening anti-ulcer drugs. The continuous decrease in acid-neutralizing capacity and rapid acid movement into the duodenum, coupled with the hyper-secretion of pepsinogen, leads to abnormal acid secretion (Tarnawski *et al.*, 1985). Acute and chronic gastritis appear to be generated from the over-secretion of gastric juices. The inhibition of acid secretion is believed to be the most important factor for treating gastric ulcers and gastritis.

This study examined the effects of 4GBA using a range of methods including an evaluation of its anti-*H. pylori* activity, anti-oxidant effects using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, reducing power and acid-neutralizing capacity. The cytotoxicity of 4GBA was evaluated against human gastric cancer cell lines. The effects of 4GBA on HCl/EtOH- and indomethacin-induced gastritis models and on gastric secretion were also investigated.

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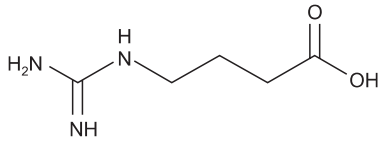

Fig. 1. Structure of 4-guanidinobutyric acid.

MATERIALS AND METHODS

Reagents and laboratory equipments

Brucella broth, bacto agar, horse serum, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT), sodium bicarbonate, positive control including ascorbic acid, hydrotalcite, ampicillin and cimetidine were obtained from Sigma (Sigma-Aldrich Inc., MO, USA). The cell culture medium and reagents, such as RPMI 1640, fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-EDTA were purchased from GIBCO (Invitrogen Inc., NY, USA). The other solvents were purchased from Duksan pure Chemical Co. Ltd. (Kyunggi-do, Korea). All other reagents were of pharmaceutical or analytical grade.

The equipment included a pH meter (IQ Scientific Instruments, Inc), clean Bench (Johnsam Co.), CO_2 incubator (Forma Scientific), water bath (Vision), inverted microscope (Olympus), autoclave (Duksan Chem. Co.), micropipette (Gilson Co.), centrifuge 5810R (Eppendorf), high speed centrifuge (Sorvall RT-6000), liquid nitrogen Dewars (CHART MVE), and UV-spectrophotometric plate reader (ASYS UVM340).

Anti-*H. pylori* activity

The inhibitory effect of 4GBA on the growth of *H. pylori* (ATCC, Rockville, MD, USA) was examined by modifying the method reported by Kim *et al.* (2003). 600 μl of the sample was mixed with 5.4 ml of brucella agar medium containing 7% horse serum in a petri dish. *H. pylori* (5×10^5 CFU) was seeded into the media and incubated for 3 days in a 37°C incubator (AnaeroPak Campylo: 85% N_2, 10% CO_2, 5%, O_2). The viability of *H. pylori* was determined from the colony counts after 3 days incubation. Ampicillin was used as the positive control.

Cell culture and cytotoxicity assay for gastric cancer cell lines

SNU638 and AGS gastric cancer cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured with RPMI-1640 containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a 5% CO_2 humidified incubator at 37°C. For the subculture, the SNU638 and AGS cells were rinsed twice with phosphate buffered saline (PBS, pH 7.4) to remove all traces of the serum (which can inhibit trypsin) and subdivided using 0.05% trypsin with 0.53 mM EDTA.

The cytotoxicity of 4GBA to SNU638 and AGS cells (gastric cancer cell lines) was examined using a MTT assay. The cells were seeded at 1×10^4 cells/well in a 96-well culture plate (Corning Inc., USA), and cultured for 24 hours at 37°C in a 5% CO_2 humidified incubator. The samples were added to the plate and incubated for a further 48 hours. MTT was added at a final concentration of 0.5 mg/ml and the samples were incubated for 4 hours at 37°C. After discarding all the media from the plates, 100 μl of DMSO was added to all wells. The plates were shaken for 5 minutes at room temperature to completely dissolve the formazan. The absorbance of the MTT formazan was determined at 540 nm using a UV-spectrophotometric plate reader (Choi *et al.*, 2004).

Table 1. Colonization inhibiting effect of 4-guanidinobutyric acid for *H. pylori*

Material	Dose (μM)	Colonization
Control	0	+++
	10	+++
4GBA	50	+++
	100	+
Ampicillin[a]	100	-

−: none, + : colonies (0-2×10^4 CFU), ++ : colonies (2-4×10^4 CFU), +++ : colonies (>4×10^4 CFU), [a]μg/ml.

Anti-oxidant effects

Free radical scavenging activity: One milliliter of DPPH (1.5×10^{-4} M) in MeOH was added to 4 ml of the samples, and the mixture was stirred. After incubation at room temperature for 30 minutes, the absorbance was read against a blank at 520 nm, and the level of scavenging DPPH free radical was monitored. The graph plotting the percentage inhibition shows the concentration providing 50% inhibition (IC_{50}). L- Ascorbic acid was used as the positive control (Lee *et al.*, 2005).

Reducing power: The reducing power was determined using the method reported by Oyaizu (1986). The samples were mixed with 500 μl of 0.05 M phosphate buffer (pH 6.6) and 500 μl of 1% $K_3Fe(CN)_6$, and the mixture was incubated at 50°C for 20 minutes. 500 μl of 10% trichloroacetic acid, (TCA) was then added, and the mixture was centrifuged at 3,000 rpm for 10 minutes. The supernatant layer (500 μl) was then added to 500 μl of distilled water and 100 μl of 0.1% $FeCl_3$. The absorbance of the mixture was determined at 700 nm by UV-spectrophotometry.

Acid-neutralizing capacity: The sample (0.5 mg) was added to 50 μl of 0.1 N HCl and incubated for 1 hour at 37°C with shaking. The acid-neutralizing capacity was determined by titration with 0.1 N NaOH using methyl orange as the indicator. Hydrotalcite was used as the positive control.

Animals

Male Sprague–Dawley rats, weighing 190 to 200 g, were purchased from Samtako, Kyunggi-do, Korea, and acclimatized to standard laboratory conditions (22 ± 2°C, 55 ± 5% humidity and 12 h light/dark cycle) for 14 days in the animal facility at Duksung Women's University. All experimental procedures for the rats were carried out in accordance with the Guidelines of the Care and Use of Laboratory Animals, Duksung Women's University. The animals were allowed access to food (standard pellet diet) and water ad libitum. The entire study was conducted in compliance with the Testing Guidelines for Safety Evaluation of Drugs (Notification No. 1999-61) and the Good Laboratory Practice Regulations for Non-clinical Laboratory Studies (Notification No. 2000-63) issued by the Korea Food and Drug Administration.

HCl/EtOH-induced mucosal membrane lesions

After 24 hours fasting with free access to water prior to the

experiment, the samples were administered orally to the rats. Thirty minutes later, 1 ml of a HCl/EtOH solution (150 mM HCl in 60% EtOH) was administered orally. After 1 hour, each animal was sacrificed by ether inhalation and its stomach was excised, inflated by injecting 2 ml of normal saline and then fixed for 30 minutes in a 2% formalin solution. The stomach was incised along the greater curvature and the glandular portion was examined for hemorrhage. The length (mm) of each lesion was measured under a dissecting microscope (10×), and the total value is expressed as the lesion index (Mizui and Doteuchi, 1983).

Indomethacin-induced gastric lesion

Using the method reported by Kasuya *et al.* (1979), the rats were fasted for 24 hours with free access to water prior to the experiment. The sample was dosed orally and 30 min later, indomethacin (35 mg/kg in 50 mM sodium biocarbonate solution) was injected subcutaneously. The animals were sacrificed 7 hours after the indomethacin injection. The excised stomach was placed in a 2% formalin solution for 30 minutes. The stomach was incised along the greater curvature and the glandular portion was examined for hemorrhage. Each lesion was measured and the total value is expressed as the lesion index. Cimetidine was used as the positive control drug.

Gastric secretion

After 24 hours fasting with free access to water prior to the experiment, the rats were administered with the samples intraduodenally (Shay *et al.*, 1945). Four hours after pyloric ligation, the animals were sacrificed, and the stomach contents were collected and centrifuged at 3,000 rpm for 10 minutes. The total volume of gastric juice and pH were measured, and the acid output (mEq/4 hrs) was determined by titration with 0.1 N NaOH using phenol red as an indicator.

Statistical analysis

All experiments were carrid out more than three times. The data was analyzed using a Student's *t*-test. *p*-values 0.05 were considered significant. When gastric lesions were induced by the various methods, the inhibitory effects of 4GBA on gastritis and gastric ulcers were determined as the inhibition ratio (%) as follows: Inhibition ratio (%)=lesion length (control)−lesion length (drug)/lesion length (control)×100

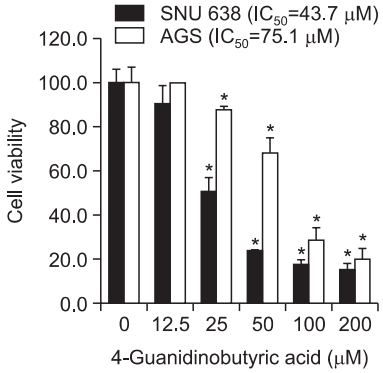

Fig. 2. Cell cytotoxicity against SNU 638 and AGS cells. Each value represents the mean ± S.E. of the data obtained from three independent experiments (■: SNU 638, □: AGS) Each value represents the mean ± S.E. Significantly difference, **p*<0.001 compared with control.

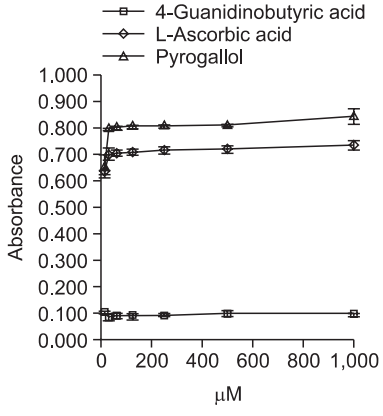

Fig. 3. Reducing power of 4-guanidinobutyric acid. Each value represents the mean ± S.E. of the data obtained from three independent experiments (⊟: 4-guanidinobutyric acid, ⟡: L-ascorbic acid, △: pyrogallol).

RESULTS

Anti-*H. pylori* activity

One of the aggressive factors is a *H. pylori* infection, which is a cause of gastritis and gastric cancer (Veldhuyzen van Zanten and Sherman, 1994; Kusters *et al.*, 2006). In addition, the gastric mucosa infected with *H. pylori* showed increases in the concentration of reactive oxygen species (ROS) inducing DNA damage (Drake *et al.*, 1998; Arend *et al.*, 2005). The colonization of *H. pylori* was investigated to determine the inhibitory effects of 4GBA on *H. pylori* (Table 1). 4GBA had inhibitory effects against the growth of *H. pylori* at a dose of 100 μM. 4GBA reduced the number of *H. pylori* colonies, demonstrating its potential to decrease the risk of *H. pylori* related pathogen-derived gastritis and inhibit the development of gastric cancer.

Table 2. Free radical scavenging effect of 4-guanidinobutyric acid

Material	IC_{50} (μM)
4GBA	>160
L-Ascorbic acid	<10

Table 3. Acid-neutralizing capacity of 4-guanidinobutyric acid

Material	NaOH consumption volume (μl)	Inhibition (%)
Control	56.3 ± 0.58	-
4GBA	48.7 ± 0.58*	13.6
Hydrotalcite	5.0 ± 0.77**	91.1

Each value represents the mean ± S.E. Significantly difference, **p*<0.05 compared to the control, ***p*<0.001 compared with control.

Cytotoxic effects on gastric cancer cells

The cytotoxic effects of 4GBA were investigated using SNU638 and AGS gastric cancer cell lines (Fig. 2). 4GBA exhibited cytotoxicity to SNU638 cells (IC_{50}=43.7) and AGS cells (IC_{50}=75.1 μM), indicating that 4GBA inhibits gastric cancer cell growth.

Antioxidant activities

ROS, one of the aggressive factors, leads to acute and chronic inflammation in the stomach (Leirisalo-Repo *et al.*, 1993). The DPPH radical scavenging activity and reducing power capacity were examined to determine the antioxidant activities involving ROS. 4GBA exhibited low antioxidant activities in the hydrogen-donating activity to the DPPH radical (IC_{50}>160 μM). 4GBA also exhibited low reducing power capacities, as determined by a Fe^{3+} reduction (Table 2, Fig. 3).

Acid neutralizing capacity

4GBA inhibited approximately 13.6% of NaOH consumption compared to the control (Table 3). The acid neutralizing capacity of 4GBA was relatively low, whereas hydrotalcite showed approximately 91.1% as a positive control. Although 4GBA had less acid neutralizing capacity than hydrotalcite, it might have gastroprotective effects as a neutraceutical. Antacids help accelerate the healing of duodenal and gastric ulcers by neutralizing the gastric luminal acid produced (Tarnawski *et al.*, 1995). 4GBA produced a slight increase in the pH of gastric content *in vitro*, and had potential gastroprotective effects.

Table 4. Effect of 4-guanidinobutyric acid on HCl/EtOH-induced gastric lesions in rats

Material	Dose (mg/kg)	Lesion index (mm)	Inhibition (%)
Control	0	120.6 ± 19.97	-
4GBA	50	95.0 ± 18.26*	21.2
	100	35.2 ± 11.32***	70.8
Cimetidine	150	71.3 ± 8.30**	40.9

Each value represents the mean ± S.E. of the rats (n=6). Significantly difference, *p<0.05 compared to the control, **p<0.01 compared to the control, ***p<0.001 compared to the control.

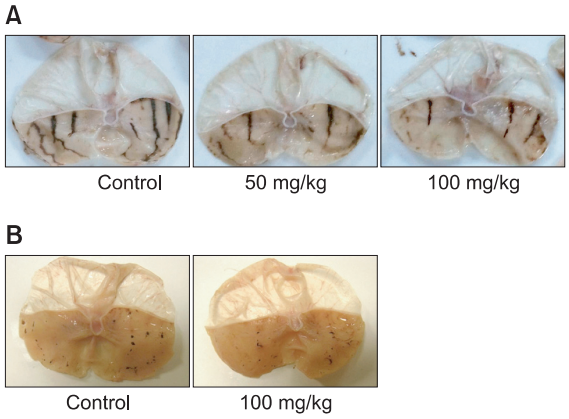

Fig. 4. Effect of 4-guanidinobutyric acid on HCl/EtOH and indomethacin-induced gastric lesions in rats (A: HCl/EtOH-induced gastritis, B: Indomethacin-induced gastric ulcer).

HCl/EtOH-induced gastric lesions

HCl/EtOH-induced gastric mucosal damage is associated with the overproduction of free radicals, which leads to an increase in lipid peroxidation. EtOH induces both wide ulcers and petechial lesions within a relatively short time, which makes this technique suitable as a screening method for assessing antiulcer drugs. The action of inhibiting HCl/EtOH-induced gastric lesions might be related to the antacid effects or cytoprotective properties in gastric mucus (Seiki *et al.*, 1990; Kahraman *et al.*, 2003). The simple acid neutralizing activity and cytoprotective effects against the gastric mucosa in EtOH-induced lesions can have cytoprotective action against EtOH-induced lesions. The mechanism of EtOH-induced lesions includes depletion of the gastric mucus content, damaged mucosal blood flow and mucosal cell injury.

As showed in Table 4 and Fig. 4A, the intra gastric administration of HCl/EtOH (150 mM HCl in 60% EtOH) caused multiple band-like lesions (120.6 ± 19.97 mm of the lesion index) in the gastric mucosa, whereas the normal rats did not show any gastric lesions (data not shown). The severity of these lesions was reduced dose-dependently by the p.o. administration of 4GBA (50 and 100 mg/kg). 4GBA (100 mg/kg) inhibited approximately 70.8% of the HCl/EtOH-induced gastric lesions, and was superior to cimetidine (150 mg/kg), a positive control (approximately 40.9% inhibition). Therefore, 4GBA has anti-gastritic activity. Overall, 4GBA has effective anti-ulcer activity against HCl/EtOH-induced stomach lesions.

Indomethacin-induced gastric lesions

NSAIDs can induce ulceration in the upper gastrointestinal tract (Wallace and Granger, 1992). In addition, NSAIDs cause a decrease in gastric mucosal blood flow (Ashey *et al.*, 1985). Indomethacin is a NSAID that induces severe gastric mucosal lesions. 4GBA (100 mg/kg) was administered orally to examine its inhibition effects on indomethacin-induced gastric lesions (Table 5, Fig. 4B). 4GBA reduced the indomethacin-induced gastric lesions in rats (38.8% inhibition).

Gastric secretion

The gastric-juice parameters, such as volume of gastric secretion and pH, were measured after submitting the rats to pylorus ligature with or without 4GBA intraduodenally, as listed in Table 6. 4GBA (100 mg/kg) decreased volume of gastric secretion slightly (5.7 ± 1.45 ml) compared to the control (6.4 ± 2.99 ml). Gastric ulcers appear to be caused by the oversecretion of gastric juice as well as an imbalance in the defensive and aggressive factors involved in maintaining the gastric

Table 5. Effect of 4-guanidinobutyric acid on indomethacin-induced gastric lesions in rats

Treatment	Dose (mg/kg)	Lesion index (mm^2)	Inhibition (%)
Control	0	19.0 ± 5.96	-
4GBA	100	14.2 ± 7.36	38.8
Cimetidine	200	9.0 ± 3.65*	52.6

Each value represents the mean ± S.E. of the rats (n=6). Significantly difference, *p<0.01 compared with control.

Table 6. Effect of 4-guanidinobutyric acid on gastric secretion in pylorus-ligated rats

Treatment	Dose (mg/kg)	Lesion index (mm)	Lesion index Inhibition (%)	Volume (ml)	pH	Total acid output (mEq/4 hrs)
Control	0	17.2 ± 5.98	-	6.4 ± 2.99	1.1 ± 0.22	0.48 ± 0.26
4GBA	100	12.0 ± 8.17	30.1	5.7 ± 1.45	1.2 ± 0.12	0.40 ± 0.23
Cimetidine	200	4.0 ± 2.00**	76.7	2.4 ± 0.15*	2.7 ± 0.30***	0.22 ± 0.02

Each value represents the mean ± S.E.of the rats (n=6). Significantly difference, *p<0.05 compared with control, **p<0.01 compared with control, ***p<0.001 compared with control.

mucosal integrity (McQuaid and Isenberg, 1992).

DISCUSSION

H. pylori is an important pathogen of stomach cancer after chronic gastritis and ulceration in the stomach and duodenum. The gastric mucosa infected with *H. pylori* show increases in ROS, inducing DNA damage. ROS, one of the aggressive factors, leads to acute and chronic inflammation in the stomach. The ulcer healing action of antiacids is believed to be due to the neutralization of gastric luminal acid. 4GBA exhibited inhibitory effects on the growth of *H. pylori* at a dose of 100 μM. The reduced *H. pylori* colonies demonstrated its potential to decrease the risk of *H. pylori*-related, pathogen-derived gastritis and inhibit the development of gastric cancer. 4GBA exhibited cytotoxicity in SNU638 cells (IC_{50}=43.7) and AGS cells (IC_{50}=75.1 μM). These results suggest that 4GBA inhibits gastric cancer cell growth. 4GBA inhibited approximately 70.8% of HCl/EtOH-induced gastric lesions at a dose of 100 mg/kg (acute gastritis model). The antigastritic effect of 4GBA on HCl/EtOH-induced gastric lesions might be related to the protection from direct irritation. The formation of gastric mucosal lesions by necrotizing agents, such as HCl/EtOH, is associated with the depression of the gastric defensive mechanisms (Kinoshita *et al.*, 1995). Indomethacin is a non-steroid anti-inflammatory agent known to induce severe gastric mucosal lesions (chronic gastritis models). 4GBA showed better inhibition of the gastric damage induced by the HCl/EtOH model than that observed in the indomethacin model. It was reported that in N-formyl-methionine-leucine-phenylalanine (fMLP)-stimulated monocytes, the percentage of ROS-producing monocytes was significantly higher when treated with 4GBA (Schepers *et al.*, 2010). In addition, it reported that in the brain, accumulation of guanidino compounds might induce epileptic discharge and convulsions (Tachikawa and Hosoya, 2011). Despite its side effects, 4GBA is expected to be a good candidate for the development of drugs with low toxicity and high effectiveness through modification of the structure or the addition functional groups that might improve the inhibition of gastric lesions..

In conclusion, 4GBA had inhibitory effects on the growth of *H. pylori* and cytotoxicity against SNU638 and AGS human gastric cancer cell lines. 4GBA also showed slight acid-neutralizing capacity. In addition, 4GBA inhibited HCl/EtOH-induced gastric lesions and Indomethacin-induced gastric lesions. The inhibitory effects of 4GBA against gastric lesions appear to be due to the acid-neutralizing capacities, anti-*H. pylori* actions and decreased volume of gastric secretion. Overall, 4GBA may be useful for the treatment and/or prevention of gastric lesions.

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