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Anti-*Helicobacter pylori* Effect of Costunolide Isolated from the Stem Bark of *Magnolia sieboldii*

Jong-Beak Park[1], Chong-Kyo Lee[1] and Hee Juhn Park[2]

[1]Pharmaceutical Screening Center, Korea Research Institute of Chemical Technology (KRICT), Taejon 305-343, Korea and [2]Department of Botanical Resources, Sangji University, Wonju 220-702, Korea

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Helicobacter pylori (*H. pylori*) infection is now established as the major pathogenic factor in chronic gastritis and peptic ulcer disease. In addition, there is accumulating evidence that *H. pylori* plays an important role in the process of gastric carcinogenesis. On the other hand, oriental traditional medicines have been used for stomach disease for thousands of years. In the present study, methanol extract from the stem bark of *Magnolia sieboldii* (*M. sieboldii*) and its components were investigated on their inhibitory effects against urease activity and growth of *H. pylori in vitro*. The methanol extract of *M. sieboldii* significantly inhibited the growth of *H. pylori* ATCC 43504 at 5 mg/ml. From the further fractionation, the chloroform fraction inhibited the bacterial growth dose-dependently. Among four fractions separated from the chloroform fraction by silica gel column chromatography, MS-C-2 was the most potent. Costunolide was isolated from the MS-C-2 subfraction by preparative TLC and recrystallization using n-hexane. Anti-*H. pylori* effect of costunolide was investigated using one commercial strain (*H. pylori* ATCC 43504) and three clinical strains (*H. pylori* 4, 43, 82548). Costunolide exhibited potent anti-*H. pylori* activity, and the MIC was around 100~200 μg/ml. However, costunolide had no inhibitory effect of *H. pylori* urease activity at the concentration used for the growth inhibition assay. From these results, we conclude that costunolide inhibits the growth of *H. pylori* by the independent manner of *H. pylori* urease inhibition.

Key word: *Helicobacter pylori*, Growth, Urease, *Magnolia sieboldii*, Costunolide

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative spiral-shaped bacterium that colonizes the mucus layer associated with the human gastric epithelium. Its presence in the gastric mucosa is associated with chronic gastritis, often accompanied by an active inflammatory component, and the formation of peptic ulceration is enhanced in certain infected individuals (Marshall *et al.*, 1988). Ingestion of the bacteria by human volunteers produces gastric infection and histologically demonstrable gastritis (Morris *et al.*, 1987). Eradication of the organism in ulcer patients results in resolution of the pathology, whereas reinfection is associated with greater recurrence of disease (Goodwin *et al.*, 1986). Retrospective seroepidemiological studies have demonstrated that individuals infected with *H. pylori* have an increased risk of developing adenocarcinoma (Nomura *et al.*, 1991). Long-term gastric colonization with *H. pylori* is thought to induce chronic atrophic gastritis (Parsonnet *et al.*, 1991), which is a precursor of gastric cancer (Correa, 1988). It has therefore been proposed that eradication of the bacterium, particularly within those populations in which an *H. pylori* infection is acquired at an early age, may reduce the incidence of this neoplasm (Leon-Barua *et al.*, 1993).

H. pylori is susceptible to most antimicrobial agents *in vitro* (Lambert *et al.*, 1986), however, *in vivo* eradication has been difficult. Eradication rates with most monotherapies have rarely exceeded 10% (Glupczynski *et al.*, 1987). Failure to eradicate relates to a number of potential problems including patient compliance, natural and acquired bacterial resistance to agents and poor delivery of drugs to the site of action (Goodwin *et al.*, 1988). The bacteria reside under and within gastric mucus, in gastric glands and in intercellular spaces as well as in the duodenal mucosa. These diverse sites mean that effective delivery of antimicrobial agents via either local or systemic routes may be difficult to achieve. Treatment of *H. pylori* infection has been best achieved with dual or tri-

Correspondence to: Jong-Beak Park, Pharmaceutical Screening Center, Korea Research Institute of Chemical Technology (KRICT), Taejon 305-343, Korea

ple therapy. Eradication rates of 60~80% were noted with dual therapy using a bismuth compound and metronidazole. Triple therapy combining bismuth and metronidazole with either tetracycline or amoxycillin has been the most effective regimen against *H. pylori* with eradication in 73~92% of subjects (Chiba *et al.*, 1992). Problems with triple antimicrobial therapy include a high incidence of side effects and poor patient compliance. The proton pump inhibitors (PPIs) omeprazole and lansoprazole combined with either clarithromycin or amoxycillin achieved eradication rates up to 90% (Adamek *et al.*, 1992; Rogan *et al.*, 1992; Bayerdorffer *et al.*, 1992). Combination therapy with omeprazole and an antibiotic results in ulcer healing, symptom resolution and *H. pylori* eradication, with minimal side effects, but it is expensive. In the initial two-year toxicity studies, administration of omeprazole to rats (but not mice) resulted in carcinoid tumors of the body of the stomach and hyperplasia of certain oxyntic mucosal endocrine cells, the enterochromaffin-like cells (Havu, 1986). The carcinoid tumors occurred more frequently in female rats given four different doses of omeprazole; they developed in 2% of rats given 1.7 mg per kg of body weight per day and in 40% of those given 140 mg per kg per day. These results led the Food and Drug Administration to restrict the length of omeprazole treatment to eight weeks, except in patients with the Zollinger-Ellison syndrome.

Research is now being carried out to determine which antibiotic and dose regimen will provide the most effective eradication of *H. pylori,* with the mildest and least common occurrence of side effects when given for the shortest period of time. In the present study, the methanol-extract of *Magnolia sieboldii* and its components were investigated on their anti-*H. pylori* effects *in vitro.*

MATERIALS AND METHODS

Bacterial cultures

Four strains of *H. pylori* were used in this study. *H. pylori* strain ATCC 43504 was a generous gift from Dr. K. Kobashi, Toyama Medical and Pharmaceutical University, Japan. Three clinical strains, 4, 43, and 82548 were donated from Youngjin Pharmaceutical Co., LTD. (Korea). Stock cultures of *H. pylori* strains were spread on Trypticase soy II agar with 5% sheep blood (BBL) and incubated at 37°C for three days. The plates were placed inside anaerobic jars, and Campypack system (Mitsubishi Gas Chemical Co., Japan) was used to generate the proper microoxic conditions. The bacteria were harvested and resuspended in saline. This suspension was used as the test inoculum.

Extraction and isolation of costunolide

Dried stem bark of *Magnolia sieboldii* (*M. sieboldii*) were pulverized and extracted three times with hot MeOH. The MeOH extract was evaporated to dryness and the residue (540 g) was suspended in water and fractionated by successive extractions with $CHCl_3$, EtOAc, and n-BuOH to give $CHCl_3$ soluble fraction (214 g), EtOAc soluble fraction (26 g) and n-BuOH soluble fraction (53 g). A part of $CHCl_3$ soluble fraction (20 g) was subjected to column chromatography on silica gel (Wakogel C200, Wako Pure Chemicals, Osaka, Japan). The column was eluted with n-hexane: EtOAc (3:1), and four fractions were collected. Fraction 2 was subjected to preparative TLC (silica gel) with n-Hexane:EtOAc (10:1) to give costunolide (122 mg).

Bacterial growth inhibition test

The effect on *H. pylori* growth was determined by the agar dilution method. A 2 ml assay in 10-mm plates (Falcon), each containing 1.8 ml of brucella agar (BBL) with 7% horse serum (Gibco). A range of costunolide solutions diluted in dimethylsulfoxide (DMSO) and each extract in methanol was added to the plates giving a final costunolide concentration of between 1 and 1,000 μg/ml and extrat concentration of 10 mg/ml. Each experiment consisted of three control plates to which 0.2 ml of DMSO or methanol and three test plates to which 0.2 ml of the appropriate costunolide/DMSO or extract/methanol solution had been added. One hundred microliters of each bacterial suspension was inoculated onto each plate. The plates were incubated at 37°C in a microaerobic environment. Readings were performed after three days of incubation. The inhibitory activities of the extracts on the growth of *H. pylori* was determined by judging visually with five grades as well (+++, over 70%), worse (++, 70~40%), slight (+, 40~10%), little (±, below 10%) and no(-). The MIC was defined as the lowest concentration of costunolide which inhibited visible growth. The viability was determined by preparing serial 10-fold dilutions in saline and inoculating 10 μl samples in triplicate onto brucella agar-supplemented with 7% horse serum. Colonies were counted after 3 days of incubation at 37°C under microaerophilic conditions and were expressed as CFU per milliliter.

Urease inhibition test

Bacterial cells were collected on ice and then were harvested by centrifugation (5,000×g, 20 min, 40°C) and washed once with saline, The pellet was drained well, and the cells were suspended in ice-cold 20 mM sodium phosphate buffer (pH 7.0) and disrupted

by ultrasonic vibration for 30 sec five times at intervals of 1 min in an ice bath. The lysate was centrifuged (20,000×g, 20 min, 4°C), and the supernatant was collected and used for urease assay. Urease activity was quantitated by the indophenol method with minor modification (Park *et al.*, 1996). After adding the phenol-nitroprusside reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and alkaline hypochlorite reagent (5.5% w/v $Na_2HPO_4 \cdot 12H_2O$, 0.5% w/v NaOH and 0.1% NaOCl), the quantity of ammonia liberated was determined from a standard curve correlating the A_{630} to the ammonium concentration of $(NH_4)_2SO_4$.

RESULTS AND DISCUSSION

Isolation of costunolide from *M. sieboldii*

Bioassay-directed fractionation by column chromatography of the chloroform fraction led to an isolation of costunolide (Tada, *et al.*, 1982). We previously reported the IR and ^{1}H-NMR spectrum of costunolide (Park, *et al.*, 1997).

Effects of *M. sieboldii* extracts on *H. pylori* growth

Traditional medicines have been used for many centuries, and some of them are knowm to be very effective for the stomach disease, however, a study has not been made on the gastroduodenal ulcer which is caused by *H. pylori*. In the present study, we investigated the effects of *M. sieboldii* methonal extract and its components on the growth of *H. pylori*. The effects of fractions from the methanol extract of *M. sieboldii* on the growth of *H. pylori* ATCC 43504 is shown in Table I. The methanol extract of *M. sieboldii* potently inhibited the growth of H. pylori at 5 mg/ml. In order to identify which components of *M. sieboldii* have the inhibitory effects on *H. pylori* growth, the methanol extract was fractionated as Fig. 1 to assay for the growh inhibition. As shown in Table I, the most effective fraction was a chloroform fraction, and the inhibition of the growth was dose-dependent. The other fractions did not inhibit the growth of *H. pylori* at the final concentration of 2.5 mg/ml media.

Table I. Effects of *Magnolia sieboldii* extracts on growth of *H. pylori* ATCC 43504

Extract	mg/ml	Growth
MeOH extract	5	-
n-BuOH	2.5	+++
EtOAc	2.5	+++
$CHCl_3$	0.25	+++
	1.0	++
	2.5	+

Anti-*H. pylori* effect of costunolide

Effects of four subfractions isolated from chloroform fraction of *M. sieboldii* on *H. pylori* growth is shown in Table II. Among them, MS-C-2 subfraction potently inhibited the growth of *H. pylori* at the concentration of 1 mg/ml. However, the other subfractions had no inhibitory at the concentration of 10 mg/ml. We have previously reported (Park, *et al.*, 1997) that MS-C-1 subfraction mainly contained (-)-germacrene and (+)-β-elemene, MS-C-2 subfraction (costunolide), MS-C-3 subfraction (β-sitosterol) and MS-C-4 (β-sitosterol-β-D-glucoside). Therefore, anti-*H. pylori* effect of costunolide was investigated using one commercial strain (*H. pylori* ATCC 43504) and three clinical strains (*H. pylori* 4, 43, 82548). As shown in Fig. 2, costunolide exhibited potent anti-*H. pylori* activity against these four strains in a dose-dependent manner, and the MIC was 100-200 μg/ml. We also investigated the inhibitory effect of costunolide on *H. pylori* urease activity, but this compound was not effective at the concentration used for the growth inhibition assay. From these results, we conclude that costunolide inhibits the growth of varying strains of *H. pylori* by the independent manner of *H. pylori* urease inhibition.

H. pylori infection is now established as the major pathogenic factor in chronic gastritis and peptic ulcer disease. Clarithromycin and omeprazole have been recommended for treatment of *H. pylori*. PPIs have

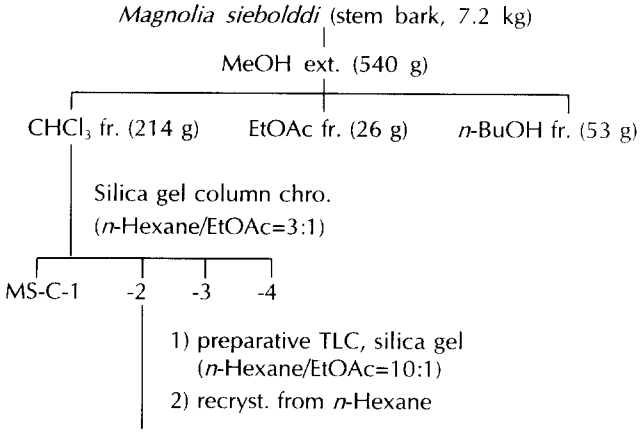

Fig. 1. Isolation of costunolide from the stem bark of Magnolia sieboldii.

Table II. Effects of *Magnolia sieboldii* subfractions on growth of H. pylori ATCC 43504

Subfractions	mg/ml	Growth
MS-C-1	10	+++
MS-C-2	10	-
	1	-
MS-C-3	10	+++
MS-C-4	10	+++

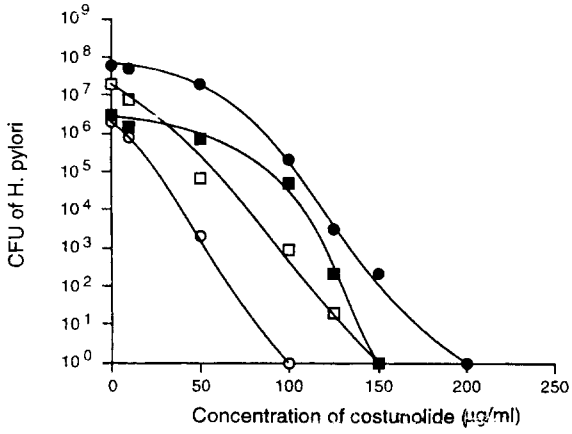

Fig. 2. Anti-*H. pylori* effect of costunolide against one commerical strain (*H. pylori* ATCC 43504, ○) and three clinical strains (*H. pylori* 4, ●; *H. pylori* 43, □; *H. pylori* 82548, ■).

multiple potential effects including suppression of gastric acid secretion, inhibtion of *H. pylori* urease activity and inhibition of *H. pylori* survival by urease-independent mechanisms. And we previously reported the kinetic studies of *H. pylori* urease inhibition by a novel PPI, rabeprazole (Park *et al.*, 1995, 1996). However, further studies determining the efficacy of this combination are needed since some strains of *H. pylori* may be highly resistant to clarithromycin (Malanoski *et al.*, 1993). While there is no universally accepted treatment, research is now being carried out to determine the most effective eradication regimen for *H. pylori*. And if the cost of therapy can be reduced, it may provide the potential for widespread *H. pylori* eradication and may become of considerable importance in prevention of peptic ulceration and possibly cancer. On the other hand, many kinds of traditional medicines have been used for the stomach disease, and some of them are known to be effective. However, only few studies have been made in connection with their anti-*H. pylori* and urease inhibition effects. Plaunotol from Plau-noi (Croton Sublyratus Kurz) was reported to have an anti-*H. pylori* effect (Shimoyama *et al.*, 1993) and ecabet sodium from Pini Resina to inhibit *H. pylori* urease activity and viability (Shibata *et al.*, 1993). Our study confirms the inhibitory effect of costunolide on the growth of *H. pylori in vitro*. Traditional medicines have been usually prepared not only as an extract of one kind herb but also a mixed extract of many kinds of different herbs, and a large dose of these extracts is usually challenged for humans. Thus, there is a potentially beneficial effect of dietary costunolide as one of the valuable antiulcer compounds.

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