財團法人明日醫學基金會專題研究計畫申請書

一、基本資料： 申請條碼：

本申請案所需經費(單選)	☑A 類(研究主持費及執行計畫所需經費) ☐B 類(研究主持費，限人文處計畫，不須填寫表 C002 及 C004 至 C009)				
計畫類別(單選)	☐一般型研究計畫 ☐特約研究計畫 ☑新進人員研究計畫 ☐其他				
研究型別	☑個別型計畫 ☐整合型計畫				
計畫歸屬	☐自然處 ☐工程處 ☑生物處 ☐人文處 ☐科教處 ☐永續會				
申請機構/系所(單位)	中國醫藥大學 醫學系				
本計畫主持人姓名	賴志河	職稱	助理教授	身分證號碼	
本計畫名稱 中文	中醫藥抑制幽門螺旋菌誘導胃上皮細胞發炎反應之探討				
本計畫名稱 英文	Investigation of inhibition of *Helicobacter pylori*-induced gastric epithelial cell inflammation by Chinese medicines				
整合型總計畫名稱					
整合型總計畫主持人				身分證號碼	
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研究學門(請參考本申請書所附之學門專長分類表填寫)	學門代碼	名稱(如為其他類，請自行填寫學門)			
	BI				
研究性質	☑純基礎研究 ☐導向性基礎研究 ☐應用研究 ☐技術發展				
本計畫是否為國際合作計畫	☑否； ☐是，合作國家：＿＿＿＿，請加填表 I001～I003				
本計畫是否申請海洋研究船	☑否； ☐是，請務必填寫表 C014。				
本計畫是否有進行下列實驗：(勾選下列任一項，須附相關實驗之同意文件) ☐人體實驗 ☐基因重組實驗 ☐動物實驗					
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二、研究計畫中英文摘要：請就本計畫要點作一概述, 並依本計畫性質自訂關鍵詞。

（一） 計畫中文摘要。(五百字以內)

幽門螺旋菌感染宿主後會引起強烈但無效的免疫反應，反而使細菌在宿主體內持續感染。臨床研究顯示幽門螺旋菌的感染會導致消化性潰瘍及胃癌等疾病，在世界許多地方也陸續發現對抗生素治療產生抗藥性的幽門螺旋菌。為了治療抗藥性的幽門螺旋菌，我們利用先前已建立的「快速體外分析中醫藥抗幽門螺旋菌的系統」，試圖從中醫藥庫之中篩選出抗幽門螺旋菌的替代藥物。在第一年的計畫中，我們將利用此平台來篩選 500 多種中醫藥，並分析其對幽門螺旋菌感染人類胃上皮細胞的作用。這些分析包括：中醫藥對細胞的毒性分析、抗幽門螺旋菌的活性、抑制細菌黏附及侵入細胞的活性、抑制幽門螺旋菌誘導 NF-κB、AP-1 訊息傳遞的活化的路徑及引起 IL-8 分泌之發炎反應等，都將一一被探討。最後，我們也將篩選出最具活性的中醫藥來申請專利，並擴大到體內分析系統。藉由本研究計畫之執行，我們將嘗試找尋合適且有效力的中醫藥來作為治療抗生素抗藥性之幽門螺旋菌及其引起的發炎反應的替代藥物。

關鍵詞：中醫藥、幽門螺旋菌感染、人類胃上皮細胞、細胞核因子 NF-κB、介白質-8

（二） 計畫英文摘要。（五百字以內）

Helicobacter pylori has emerged as an important persistent bacterial pathogen during the past two decades. The ability of the *H. pylori* to survive intracellularly within non-phagocytes and phagocytes is postulated to enhance the persistence of this pathogen in the gastric mucosa and cause chronic inflammation. Clinical evidence indicated that *H. pylori* is linked to a majority of peptic ulcers and to some types of gastric cancer, and its resistance to antibiotic treatment is now found worldwide. Our previous study has been established an *in vitro* screening system of Chinese medicine on anti-*H. pylori* activity. In the first year of this research plan, we will aim at evaluating the antimicrobial activity of more than 500 traditional herbs from School of Chinese medicine on *H. pylori*-infected human gastric epithelial AGS cells. We will also examine the inhibition of bacterial adhesion and invasion to AGS cells using *in vitro H. pylori*-infection model to select the most potent drug. Whether NF-κB and AP-1 signaling pathway are involved in Chinese medicine-inhibited mechanism will be also determined in our project. Finally, we will retrieve the patent protection of the candidate Chinese medicine, and based on this search to design around. The results from these studies will help us to select and understand the potent Chinese medicine on anti-bacterial activity and would be beneficial for the clinical treatment of *H. pylori*-induced pathogenesis.

Keywords: Chinese medicine; *Helicobacter pylori* infection; human gastric epithelial cells; NF-κB; interleukin-8 (IL-8)

三、研究計畫內容：

（一）近五年之研究計畫內容與主要研究成果說明。（連續性計畫應同時檢附上年度研究進度報告）

（二）研究計畫之背景及目的。請詳述本研究計畫之背景、目的、重要性及國內外有關本計畫之研究情況、重要參考文獻之評述等。本計畫如為整合型研究計畫之子計畫，請就以上各點分別述明與其他子計畫之相關性。

（三）研究方法、進行步驟及執行進度。請分年列述：1.本計畫採用之研究方法與原因。2.預計可能遭遇之困難及解決途徑。3.重要儀器之配合使用情形。4.如為整合型研究計畫，請就以上各點分別說明與其他子計畫之相關性。5.如為須赴國外或大陸地區研究，請詳述其必要性以及預期成果等。

（四）預期完成之工作項目及成果。請分年列述：1.預期完成之工作項目。2.對於學術研究、國家發展及其他應用方面預期之貢獻。3.對於參與之工作人員，預期可獲之訓練。4.本計畫如為整合型研究計畫之子計畫，請就以上各點分別說明與其他子計畫之相關性。

Introduction

Helicobacter pylori infection is a major etiological agent for chronic gastritis, which may lead to severe disorders including gastric ulcer, duodenal ulcer, and gastric adenocarcinoma (1,2). Eradication of *H. pylori* improves ulcer healing and reduces the recurrence of gastric and duodenal ulcers (3,4). The standard, recommended method of treating infected patients with severe symptoms was the combination of a proton pump inhibitor and two antibiotics: mainly clarithromycin, and amoxicillin or metronidazole (5,6). Indeed, an eradication rate of more than 90% was found in a number of reports based on this combination therapy (7-10). Given the widespread use of antibiotics in previous years, it was not unexpected that a few studies showed a relatively high failure rate (20% to 40%) (11-13). In parallel, antimicrobial resistance was found to be the main cause of therapy failure (14-16).

Several virulence factors are indicated to be crucial for persistent inhabitation of *H. pylori*, for instance: cell-surface adhesion molecules such as blood group antigen-binding adhesin (BabA)(17), vacuolating toxin (VacA) and cytotoxin-associated antigen (CagA)(18). The differential effects of bacterial virulence determinants along with host factors contribute to the development of distinct clinical sequelae during persistent infection of *H. pylori* (19). Accumulated reports have been found that functional CagA is capable not only of inducing actin rearrangement but also having their potential in induction of IL-8 secretion (20,21), thus contributing to inflammation in *H. pylori*-induced gastric diseases. With experiments

involving *H. pylori*-infection, IL-8 secretion from epithelial cells is directly dependent on the NF-κB signal pathway (20,22).

Despite combination of antibiotic therapy was powerful for eradication of *H. pylori* infection. However, the bacterial resistant rate was accompanied with increasing abuse of antibiotics in clinical. In this proposal, we will investigate whether chemical compounds extracted from Chinese traditional herbs have their ability to inhibit *H. pylori* infection of gastric epithelial cells. We also will investigate the effects of those isolated constituents on *H. pylori*-induced NF-κB or AP-1 activation and inflammation mediator IL-8 secretion from epithelial cells. Furthermore, we will select the useful candidate of Chinese Medicine for application of patents and for clinical trial.

Preliminary results

In our previous study, we have been established the standard method for screening Chinese Medicine on anti-*H. pylori* induced gastric epithelial cells pathogenesis. And parts of those studies were revised and accepted by Journal of Ethnophamacology on 2008. Briefly, we examined the anti-inflammatory effects of *Phyllanthus urinaria* chloroform (PUC) and methanol (PUM) extracts, and its eight isolates on *H. pylori*-infected human gastric epithelial AGS cells. *Phyllanthus urinaria* Linnea (Euphorbiaceae) is widely used in traditional Taiwanese medicine for the treatment of gastritis and ulcers. The results revealed that crude extracts PUC and PUM showed potent antimicrobial activity against *H. pylori* than pure isolates. On the other hand, *in vitro H. pylori*-infection model revealed that the inhibition of bacterial adhesion and invasion to AGS cells has dramatically reduced by treatment of extract PUC, while PUM has the same moderate effect. Furthermore, *H. pylori*-induced nuclear factor (NF)-κB activation, and the subsequent release of interleukin (IL)-8 in AGS cells were also inhibited by the extract PUC. These results open the possibility of considering *Phyllanthus urinaria* a chemopreventive agent for *H. pylori*-induced peptic ulcer or gastric cancer.

Specific aims and research design

Our previous studies have demonstrated some evidences for the association between bacteria virulence factors and host cells by *in vitro* infection systems. We also have established a rapid platform for screening effective Chinese Medicine on inhibited *H. pylori*-induced cell inflammatory responses. However, we do

not know that which the most powerful drugs on the anti-bacteria effect in Chinese Medicine bank and the molecular basis for inhibition mechanism still remains poorly understood. Hence, we propose to screening the useful Chinese Medicine and to investigate the roles of these drugs in the therapeutic effects on *H. pylori* infection. The proposed experiments are as follows (Figure 1).

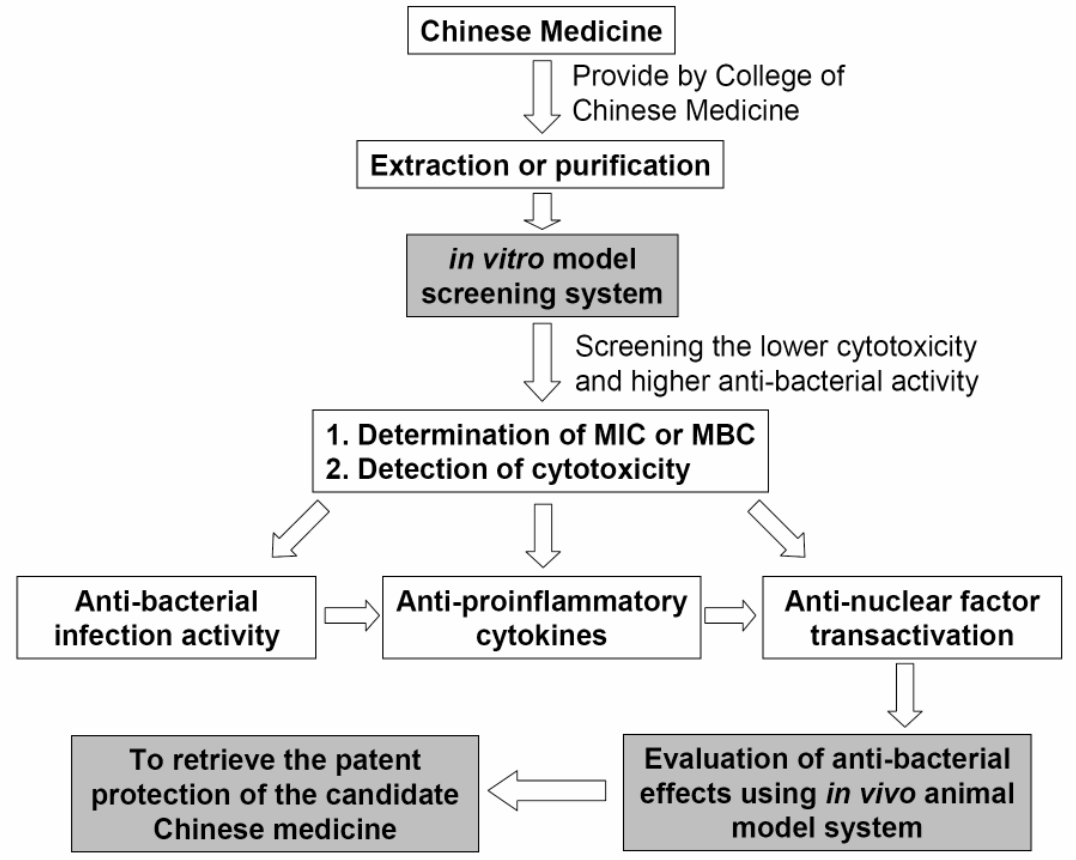

Figure 1. Schematic diagram for the overall research design using our established platform

1. To prepare the Chinese Medicine from College of Chinese Medicine

We first plan to get more than 500 drugs from Chinese Medicine bank which will provide by College of Chinese Medicine. We will than classify and do the proposed experiments as below.

2. To determine the minimum inhibition concentration (MIC) of each purified or extract compound

In an aim to screen the useful Chinese Medicine on bacterial viability, we will determine the MIC of those drugs against *H. pylori*. The lowest MIC of Chinese Medicine will be selected and tested for its cytotoxicity on gastric epithelial cells.

3. To analyze the cytotoxicity on gastric epithelial cells for each Chinese Medicine

In order to rule out the cytotoxicity of Chinese Medicine on host cells, mitochondrial respiration-dependent MTT assay was employed to determine their cytotoxicity.

4. To evaluate the activity of anti-bacterial adhesion and invasion of gastric epithelial cells

Our previous study using epithelial model, we suggested that *H. pylori* have a strategy to penetrate epithelial cells, to survive intracellularly and to evade antibiotic effects as well as host immune responses. In this proposal, we will test the selected Chinese Medicine in the inhibition of *H. pylori* adhesion and internalization of epithelial cells.

5. To investigate the inhibition in bacterial-induced nuclear factors transactivation

Recent reports had demonstrated that *Helicobacter pylori*-induced IL-8 release from AGS cells is mediated activation of NF-κB (20). To examine whether the candidate Chinese Medicine have their ability to inhibit inflammatory mediators in response to *Helicobacter pylori* infection, NF-κB-luciferase or AP-1 constructs will be used to determine luciferase expression following pretreatment of drugs and infection with *H. pylori*.

6. To identify the effects of Chinese Medicine on bacterial-induced proinflammatory cytokines secretion

A functional consequence of increased NF-κB activity is a parallel increase in IL-8 expression (21). To analyze whether the candidate Chinese Medicine could prevent the *H. pylori*-induced IL-8 production, AGS cells will either left untreated or pretreated with the drugs prior to *H. pylori* infection.

7. To analyze the inflammatory responses on macrophage for each Chinese Medicine

It has been shown that *H. pylori* might induce macrophage inducible nitric oxide (iNOS) expression (23). Nitric oxide (NO) production plays an important role in the gastric mucosal immune response to *H. pylori* and the associated inflammation. We next will test whether pre-treatment of macrophage with candidate Chinese Medicine would decreased NO production or iNOS expression.

8. To apply patents on the candidate Chinese Medicine

Using patent search system in intellectual property office, ministry of economic affairs, ROC (http://www.tipo.gov.tw/patent/search_patent/search_patent.asp) to retrieve the patent protection of the candidate Chinese medicine, and based on this search to design around.

Materials and methods

Bacterial and cell Culture

H. pylori 26695 (ATCC700392) was used as a reference strain. Bacterial strain was recovered from frozen stocks on Brucella agar plates (Becton Dickinson) containing 10 % sheep blood, 6 μg/ml vancomycin and 2 μg/ml amphotericin B under microaerophilic conditions for 2–3 days as described previously (24,25).

AGS cells (ATCC CRL 1739; human gastric adenocarcinoma epithelial cell line) were cultured in F12 (Hyclone) supplemented with 10% de-complement FBS (Hyclone). Penicillin and streptomycin (GIBCO BRL) were also added if needed. In the bacterial adhering, invading assay, and induced IL-8 secretion, the cell culture medium was not supplemented with antibiotics.

Nitric Oxide determination

In order to investigate whether Chinese Medicine can inhibit *H. pylori*–induced macrophage nitric oxide (NO) production. The production of NO was estimated from the accumulation of nitrite (NO_2^-), a stable end product of NO metabolism, in the medium using the Griess reagent as described previously (26). Briefly, cells were incubated with medium containing various types of Chinese Medicine and *H. pylori* wild type for 48 h.Equal volumes of culture supernatant or serum and Griess reagent (1:1 mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% α-naphthylethylenediamine dihydrochloride in distilled water) were mixed and incubated for 15 min at room temperature. The absorbance was measured at 540 nm on a spectrophotometer, and referred to a nitrite standard curve to determine the nitrate concentration in supernatants.

Cell viability assay

H. pylori wild type strain and candidate Chinese Medicine were co-incubated with AGS cells (2×10^6 cells/ml) in a 96-well plate for 48 h. Mitochondrial respiration-dependent MTT assay was employed to determine their cytotoxicity (27). Briefly, MTT in PBS (0.1 mg) was added into each well and then incubated at 37 °C for 4 h. The MTT formazan (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) crystals made due to dye reduction by viable cells were dissolved using acidified isopropanol (0.1 HCl) and mixed at room temperature. After 20 min, index of cell viability was calculated by measuring the optical density (OD) of the color produced by MTT dye reduction with a microplate reader (BIO-RAD, model 3550, USA) at 570 nm ($OD_{570–620}$). The mean OD value of the content of four wells was used for assessing the cell viability expressed as percentage of control.

Determination of anti-bacterial activity

All chemical compounds were evaluated for the antimicrobial activity dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich). The *in vitro* anti-bacterial activities of the chemical compounds were determined by disk agar diffusion method (28). Briefly, a total volume of 100 μl of *H. pylori* suspension (1×10^8 colony forming units (CFUs) /ml) was spread onto Mueller Hinton agar plates (BBL) containing 10% sheep blood. Sterile paper disks (6 mm, BBL) were placed on the agar surface with 10 μl of chemical compounds. DMSO was used as negative control and antibiotics (amoxicillin, clarithromycin, and metronidazole) were used as positive control. After 3 days for incubation at 37°C under the microaerophilic condition with humidity, the inhibition zone was determined in diameter.

Inhibitory of H. pylori adhere to and invasion into epithelial cells

H. pylori adhesion to and invasion of cultured AGS cells was done using a standard gentamicin assay as previous described (24). Chemical compounds and DMSO diluted in cell culture medium were added (to reach the indicated dilutions) directly to the cell culture medium for 10 min prior to inoculation of wells with *H. pylori* in log-phase. AGS cells were added with *H. pylori* at a MOI of 50 and incubated at 37°C for 6 hours. To determine the number of cell-adhesion bacteria, infected cells were washed three times to

remove unbound bacteria and then lysed with distilled water for 10 min. Lysates were diluted in PBS, plated onto Brucella blood agar plates and cultured for 4–5 days, after which the CFUs were counted. To determine the number of viable intracellular bacteria, infected cells were washed three times in PBS and incubated with 100 μg/ml of the membrane-impermeable antibiotic gentamicin (Sigma-Aldrich) for 1.5 hours at 37°C to remove extracellular bacteria, followed by the same procedures as above to obtain CFUs. The adhesion or invasion activity was determined as the mean of at least six experiments performed in duplicate. The activity to inhibit *H. pylori* adhesion and invasion was determined as: % of inhibition = (CFUs of *H. pylori* in the presence of compounds/ CFUs of *H. pylori* in the absence of compounds) × 100.

Transient transfection of NF-κB reporter gene

NF-κB-Luc reporter plasmid was given from Dr. Chih-Hsin Tang (Department of Pharmacology, China Medical University) (29). AGS epithelium was grown to 90% confluence in 12 well–plate and was transfected using Lipofectamine 2000 (Invitrogen). After 24 hr incubation, transfection was complete, and cells were incubated with various concentrations of PU-M, PU-C, and DMSO (to reach the indicated dilutions) and then infected with *H. pylori* strains for 6 hr. To prepare cell lysates, 100 μl of reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the cotransfected β-galactosidase expression vector.

Interleukin-8 measurement

To detect interleukin–8 (IL-8) released by gastric epithelial cells during *H. pylori* infection, the levels of IL-8 was measured. AGS cells were added with various concentrations of DMSO and Chinese Medicine (to reach the indicated dilutions) in cell culture medium before *H. pylori* infection. The treated cells then infected with *H. pylori* strain 26695 at a MOI of 1:100 in the presence of these agents. The supernatants were collected and stored at −80°C before analysis. The level of IL-8 in supernatants from AGS cell cultures was determined by using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D systems), according to the manufacturer's instruction.

Statistical Analysis

The correlation of Chinese Medicine on anti-bacterial adherence, invasion activity, and IL-8 secretion of treated AGS epithelial cells relative to DMSO treated control cells was determined by Student's *t*-test. $*P < 0.05$, $**P < 0.01$.

Anticipative results

In this proposal, we anticipate obtaining some valuable results regarding the inhibition of *H. pylori* pathogenesis by Chinese Medicine. These experiments are designed in the light of our previous study, which shall allow us to select useful drugs for therapy of *H. pylori* infection. Our results also allow us to develop the new strategies to prevent bacterial persistent infection in clinical therapy. In this project, we are confident that we will soon establish the experiment procedures and will also obtain interesting results. The expected progresses are as follows.

1. Determination of the MIC of each Chinese Medicine.

2. Analysis of the cytotoxicity on gastric epithelial cells for each Chinese Medicine.

3. Identification of candidate Chinese Medicine on anti-*H. pylori* induced gastric epithelial cells pathogenesis.

4. Evaluation of the activity of anti-bacterial adhesion and invasion of gastric epithelial cells.

5. Identification of the effects of Chinese Medicine on inhibition of bacterial-induced proinflammatory cytokines secretion.

6. Analysis of the inflammatory responses on macrophage for each Chinese Medicine

Our previous study has established a rapid and correct screening method for Chinese Medicine on anti-bacterial induced gastric epithelial cell pathogenesis. Our results showed that the traditional Chinese drug has its ability to inhibit *H. pylori* infection of epithelial cells and diminished the secretion of bacterial-induced inflammatory cytokine. Thus, indicates that this platform may be used and selected a new potent drug for anti-*Helicobacter pylori* infection of cells.

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