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Isoliquiritigenin Selectively Inhibits H_2 Histamine Receptor Signaling[S]

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ABSTRACT

Isoliquiritigenin, one of the major constituents of *Glycyrrhiza uralensis* (licorice), is a natural pigment with a simple chalcone structure 4,2′,4′-trihydroxychalcone. In this study, isoliquiritigenin showed selective H_2 histamine receptor (H_2R) antagonistic effect and remarkably reduced several H_2R-mediated physiological responses. Preincubation of U937 and HL60 hematopoietic cells with isoliquiritigenin significantly inhibited H_2R agonist-induced cAMP response in a concentration-dependent manner without affecting the viability of cells. Isoliquiritigenin also blocked the binding affinity of [^{3}H]tiotidine to membrane receptors in HL-60 cells. Isoliquiritigenin did not affect the elevation of cAMP levels induced by cholera toxin, forskolin, or isoproterenol, indicating that the action site of isoliquiritigenin is not G_s protein, effector enzyme, adenylyl cyclase, or β_2-adrenoceptor. Isoliquiritigenin affected neither H_1R- nor H_3R-mediated signaling. In molecular docking studies, isoliquiritigenin exhibited more favorable interactions with H_2R than histamine. Isoliquiritigenin prominently inhibited H_2R selective agonist dimaprit-induced cAMP generation in MKN-45 gastric cancer cell. Moreover, isoliquiritigenin reduced gastric acid secretion and protected gastric mucosal lesion formation in pylorus-ligated rat model. Taken together, the results demonstrate that isoliquiritigenin is an effective H_2R antagonist and provides the basis for designing novel H_2R antagonist.

Histamine is one of the aminergic neurotransmitters and plays an important role in the regulation of several pathophysiological processes (Jutel et al., 2005b). Histamine is found in every human tissue and can act as a local hormone, a mediator in processes related to allergy and inflammation, or a neurotransmitter (Jutel et al., 2005a). Histamine exerts its effect through H_1, H_2, H_3, and H_4 receptors (Gutzmer et al., 2005). In particular, the H_2 histamine receptor (H_2R) is coupled to the G_s protein/adenylyl cyclase system in a variety of tissues (e.g., brain, stomach, heart, gastric mucosa, lung) and produces intracellular cAMP (Alewijnse et al., 1998). Gantz et al. (1991) were the first to clone a cDNA encoding a 359-amino acid H_2R. Using degenerate primers based on the known sequence similarity of various G protein-coupled receptors, the H_2R sequence was obtained from canine gastric parietal cDNA by PCR. Soon thereafter, the intronless genes encoding the rat, human, guinea pig, and mouse H_2R were cloned by means of homology screening (Gantz et al., 1991). Many reports demonstrated the cellular function of H_2R and its importance. In immune system, the responses of T helper cells (Th1 and Th2) are negatively regulated by H_2R through the activation of different biochemical intracellular signals (Jutel et al., 2001). The presence of H_2R in U937 (promyelocytic lymphoma cell line; Shayo et al., 2004), HL-60 (promyelocytic leukemia cell line; Suh et al., 2001), and MKN-45 (gastric cancer cell line; Arima et al., 1991) cells has been reported.

Licorice root (*Glycyrrhiza uralensis*) is used as a harmonizing ingredient in many traditional herbal formulations. It is used in more formulations than any other herb in oriental medicine. Although it is considered to be the quintessential "servant" herb, it is often referred to as the "King of Herbs".

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ABBREVIATIONS: PCR, polymerase chain reaction; Ro 20-1724, 4-[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone; HE, hematoxylin and eosin; ILG, isoliquiritigenin; RT, reverse transcription.

Although the flavonoid-rich fraction from the extract of licorice has been used as a gastrointestinal disorder and an antiulcer medicine (Fukai et al., 2002), the mechanism is still elusive.

Since Black et al. (1972) first defined the H_2R and its involvement in gastric acid secretion, H_2R antagonists (e.g., cimetidine, ranitidine, famotidine) have been developed and used clinically as antiacid secretagogues. These histamine H_2R antagonists have revolutionized the treatment of peptic ulcers with their prominent therapeutic effects. The components of *G. uralensis* have a strong antipeptic ulcer effect (Shibata, 2000). Constituents of *G. uralensis* include glycyrrhizic acid, glycyrrhetic acid, and isoliquiritigenin (Shibata, 2000). Fukai et al. (2002) reported that the flavonoid-rich fraction of licorice extract had an antiulcer effect; therefore, from among the major constituents of licorice, we selected the isoliquiritigenin (4,2′,4′-trihydroxychalcone), which has basic chemical structure of a flavonoid, as a novel H_2R antagonist and examined its effect on H_2R activity. Isoliquiritigenin has been reported as a potent antioxidant, has cancer-preventing properties, and inhibits platelet aggregation (Baba et al., 2002). Isoliquiritigenin also reduce nitric oxide and suppress aberrant crypt foci development (Takahashi et al., 2004). In present study, we used U937 and HL-60 hematopoietic cell lines and the rat pylorus-ligated model (Shay et al., 1954) to evaluate the potential activity of isoliquiritigenin as H_2R antagonist and antipeptic ulcer agent against gastric ulcer. Through this study, we provide evidence that isoliquiritigenin negatively regulates most of the H_2R's activity and clearly acts as a specific, competitive H_2R antagonist in terms of blocking secretion of gastric acid and ulcer formation similar to the action of ranitidine.

Materials and Methods

Materials. Histamine, ranitidine, isoliquiritigenin, forskolin, cholera toxin, and Ro 20-1724 were obtained from Sigma (St. Louis, MO). Tiotidine was obtained from Tocris Cookson Inc. (Ballwin, MO). Dimaprit was purchased from Tocris (Bristol, UK). [^{3}H]cAMP and [^{3}H]tiotidine were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). RPMI-1640 and penicillin-streptomycin were purchased from Life Technologies (Gaithersburg, MD). Bovine calf serum was obtained from HyClone Laboratories (Logan, UT).

Cell Culture. U937, HL-60 cell line (American Type Culture Collection, Manassas, VA), and MKN-45 cell (Korean Cell Line Bank, Seoul, Korea) was cultured in suspension at 37°C in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated bovine calf serum and 1% (v/v) penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO_2. Primary cultures of bovine adrenal cortex cells were prepared according to the method of Reichenstein et al. (2004).

Measurement of [^{3}H]cAMP. Intracellular cAMP generation was determined by [^{3}H]cAMP competition assay in binding to cAMP binding protein using a cAMP kit (Neuronex, Pohang, Korea), according to the manufacturer's instructions.

RT-PCR for Evaluation of H_2R mRNA Expression. Total RNA was isolated from the U937 and HL-60 cells using Centrizol (Neuronex). Ten micrograms of total RNA was reverse-transcribed with the use of Superscript II reverse transcriptase (Life Technologies). cDNA was amplified with 20 pmol of specific oligonucleotide primers (Bioneer, Daejeon, Korea) using *Pfu* polymerase (Stratagene, La Jolla, CA).

The primer sequences are 5′-GCCCTCGAGACCATGGCACCCAATGGCACAGC and 3′-GCCGGGCCCACCCCTGTCTGTGGCTCCCTCGG. The PCR products were separated by 1% agarose gel electrophoresis. The products were subcloned into a pGEM-T Easy Vector (Promega, Madison, WI) and sequenced with a dideoxynucleotide termination method.

$[Ca^{2+}]_i$ Measurement. Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined with the help of the fluorescent Ca^{2+} indicator fura-2/AM. In brief, the U937 cell suspension was incubated with fresh serum-free RPMI medium containing fura-2/AM (3 μM) for 40 min at 37°C with continuous stirring. The cells were then washed with Locke's solution and left at room temperature until use. Sulfinpyrazone (250 μM) was added to all solutions to prevent dye leakage. Fluorescence ratios were measured by an alternative wavelength time-scanning method (dual excitation at 340 and 380 nm; emission at 500 nm).

H_3 Histamine Receptor Expressing Human Embryonic Kidney 293 Cell. The full-length human histamine H_3 receptor gene was kindly provided by Dr. Liu Changlu (Johnson and Johnson Pharmaceutical Research and Development). H_3R genes were subcloned into the pCIneo expression vector. Four million cells detached with trypsin were mixed together with 10 μg of DNA in gene pulser cuvettes (0.4 cm; Bio-Rad, Hercules, CA) and electroporated at 260 V, $\infty\Omega$, 960 μFD using a Bio-Rad gene pulser. The cells from up to six electroporations were pooled subsequently on a cell culture dish (10 cm), trypsinized after 24-h incubation in normal culture medium, and seeded on 12-well plates for binding experiments performed the next day.

[^{3}H]Tiotidine Binding. The binding of [^{3}H]tiotidine to intact HL-60 cells was quantified by a method described previously (Monczor et al., 2003) with some modification. Triplicate assays were performed in polyethylene tubes in 50 mM Tris-HCl, pH 7.4. [^{3}H]Tiotidine were incubated with 10^6 cells/tube in the absence or presence of isoliquiritigenin in a total volume of 200 μl. After 40 min at 4°C, incubation was stopped by dilution with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4; rapid filtration onto GF/B glass-fiber filters (Whatman, Clifton, NJ) was performed under reduced pressure, followed by three washes with 3 ml of ice-cold buffer. Specific binding was defined as the difference in the amount of radioactivity bound in the absence and presence of 1 mM unlabeled tiotidine.

Three-Dimensional Model Building. All the simulations were performed on Linux workstations using Insight II 2005 software package (Accelrys, San Diego, CA). The primary sequence of the human H_2R (Swiss-Prot ID P25021) was used. Modeler program (Fiser et al., 2000), which is an implementation of an automated approach to comparative modeling by satisfaction of spatial restraints and derives final model by optimization process consisting of applying the variable target function method with conjugate gradients optimization as well as molecular dynamics refinement, was used to build the three-dimensional structure of human histamine H_2R by taking the structure of bovine rhodopsin (Protein Data Bank code 1U19) as template. The refined model thus obtained from Modeler was checked for the stereochemical parameters by the program PROCHECK.

Binding Site Analysis. Potential binding sites in the protein were identified with the help of Active_Site_Search program in Binding_Site module of Insight II 2005 software. Active_Site_Search characterizes protein active sites and binding sites by locating cavities in three-dimensional protein structures. The sites identified can be used to guide the protein-ligand docking experiments.

Ligand Preparation. Histamine and isoliquiritigenin were sketched with the help of Builder module in Insight II 2005. Then the charges and potentials were assigned using CHARMM forcefield and the minimization was done using the CHARMM module in Insight II 2005 with 1000 steps of steepest descents followed by 10,000 steps of conjugate gradients. To the minimized conformers, potentials and charges were once again assigned using CVFF forcefield and were saved in Sybylmol2 format.

Molecular Docking. The program GOLD (Genetic Optimization for Ligand Docking, Cambridge Crystallographic Data Centre, Cambridge, UK) was employed to dock the histamine and isoliquiritige-

nin into the histamine binding site of human histamine H_2 receptor. A homology model of human histamine H_2 receptor was used for molecular docking studies. Active site radius of 10.0 Å was defined from the binding site coordinates obtained from Active_Site_Search program in Insight II 2005 software. The root-mean-square deviation was considered within 1.5 Å, and the annealing parameter of van der Waals' interaction was 4.0; hydrogen bond interaction was 2.5.

Gastric Secretion in Pylorus-Ligated Rat. The pylorus-ligated rat model first described by Shay et al. (1954) was used with some modification. Male Sprague-Dawley rats weighing 200 to 250 g were used. Rats were deprived of food, but not water, for 18 to 24 h before each experiment. The test substances (vehicle, ranitidine, isoliquiritigenin) dissolved in saline were administered orally. After 1 h, rats were anesthetized by light ether, a small abdominal incision was made, the pylorus was ligated, and then indomethacin (40 mg/kg) was injected through the duodenum. The animals were sacrificed 8 h after ligation of the pylorus, the stomach was clamped at the esophageal and duodenal junctions, filled with 15 ml of 4% formalin, and then rapidly removed. After 24 h, the fixed stomach was opened along the greater curvature, gently rinsed in saline, and then pinned open to expose the gastric mucosa. The hemorrhagic and ulcerative lesions were counted and measured with a light microscope by an observer who was blinded to the treatment. The ulcer index was then calculated as the sum of the diameter of all lesions. The ulcer index was determined in six animals in each animal group.

Histological Examination. The histological study of the stomach was performed after evaluation of the ulcer index. Samples of the corpus were excised and transferred to fresh formalin and later processed by routine techniques before embedding in paraffin. Sections (5 μm thick) were mounted on glass slides and stained with hematoxylin and eosin (HE). Coded slides were examined by an experienced pathologist blinded to the treatment.

Statistical Analysis. All Quantitative data are expressed as mean ± S.E.M. Comparisons between two groups were performed using Student's unpaired t test. Differences were considered to be significant when the degree of confidence in the significance was 95% or better ($P < 0.05$).

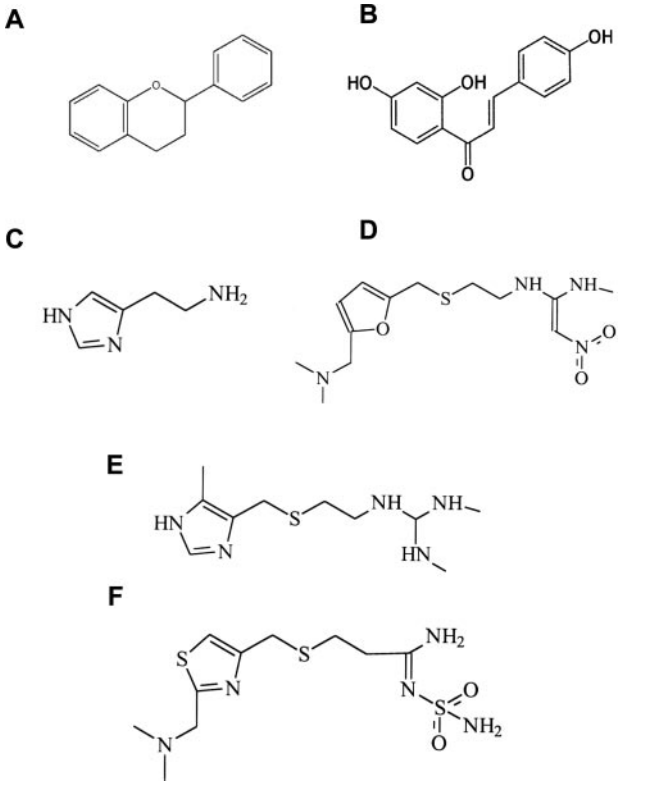

Fig. 1. Basic chemical structure of a flavonoid (A). Chemical structure of isoliquiritigenin (B), histamine (C), ranitidine (D), cimetidine (E), and famotidine (F).

Results

cAMP Production by H_2R Agonist in U937 and HL-60 Cells. Histamine and dimaprit are known as highly selective histamine H_2R agonists (Bakker et al., 2002). U937 and HL-60 cells express H_2R (Fig. 2A, inset), which is coupled to G_s protein and mediates the cAMP pathway (Shayo et al., 1997). As shown in Fig. 2A, histamine and dimaprit induced cAMP increase in a concentration-dependent manner, and half-maximally effective concentrations (EC_{50}) are 1.2 ± 1.3 and 4.7 ± 0.2 μM, respectively.

Effect of Isoliquiritigenin on H_2R-Mediated cAMP Production. We preliminarily investigated the inhibitory effect of licorice constituents (10 μM each) on cAMP production induced by histamine and dimaprit (10 μM) induced in U937 cell line (data not shown). Among the components, isoliquiritigenin (Fig. 1B) exhibited the strongest inhibitory effect. Figure 2B shows that isoliquiritigenin inhibited histamine and dimaprit-induced cAMP production in a concen-

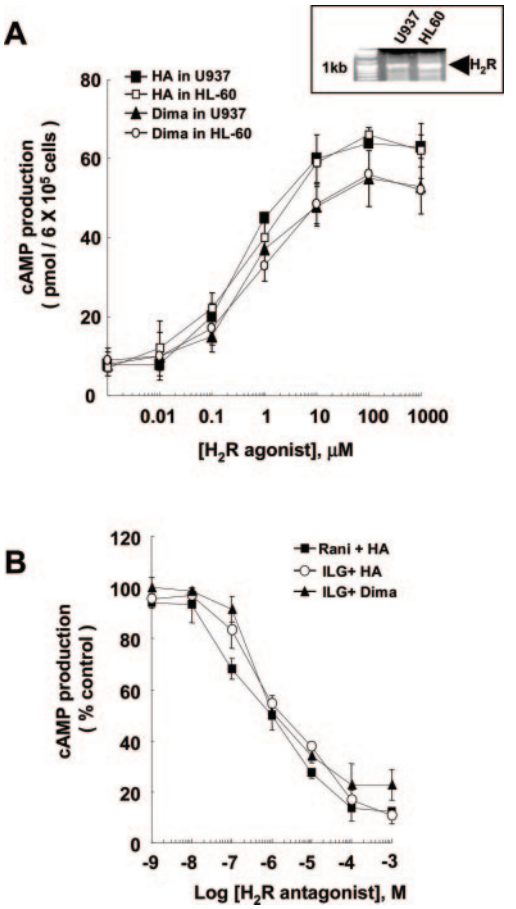

Fig. 2. Isoliquiritigenin attenuates H_2R signaling. A, H_2R agonists (histamine and dimaprit) induced cAMP production in U937 and HL-60 promyelocytes (HA, histamine; Dima, dimaprit). H_2R expression in U937 and HL-60 cells was confirmed by RT-PCR, inset. cAMP generation and RT-PCR were measured as described under *Materials and Methods*. B, U937 Cells were preincubated with indicated concentration of ranitidine (Rani) or ILG for 5 min, then cells were stimulated with 10 μM HA or Dima for 20 min. Rani or ILG was not removed during the H_2R agonist stimulation. The results are the mean ± S.E.M. of assay triplicates.

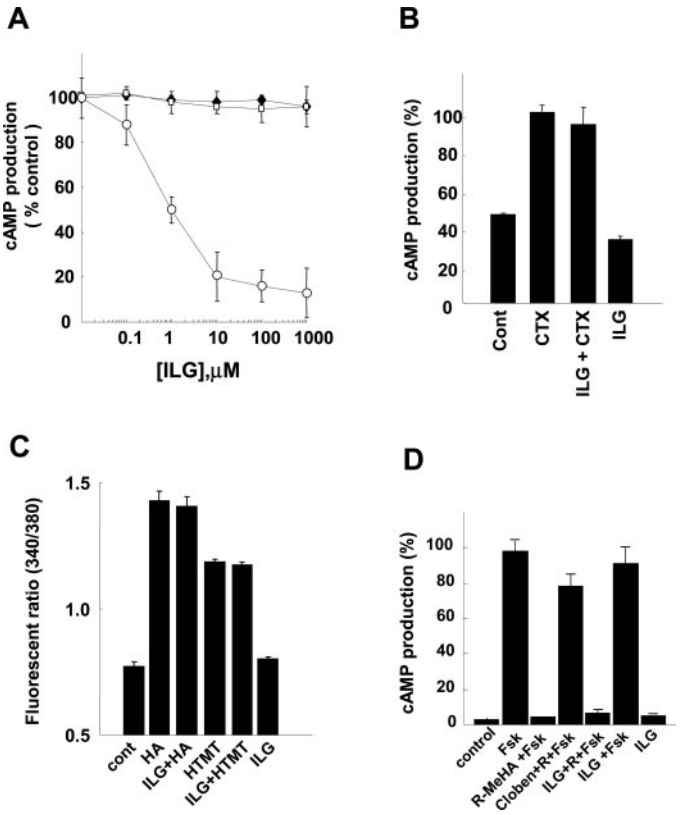

Fig. 3. Selective effect of isoliquiritigenin on H_2R. A, U937 cells were preincubated with indicated concentration of ILG for 5 min, then cells were stimulated with 10 μM dimaprit (○), 1 μM isoproterenol (□), or 1 μM forskolin (♦), respectively. B, bovine adrenal cortex cells were preincubated with 10 μM ILG, then cells were stimulated with 100 ng/ml cholera toxin (CTX). The results are the mean ± S.E.M. of assay triplicates. C, the intracellular $[Ca^{2+}]_i$ rise induced by 100 μM histamine (HA) or HTMT (H_1R-selective agonist) was measured in the absence or presence of 10 μM ILG in U937. Data are the means ± S.E.M. (bars) values of triplicate measurements. D, H_3R-transfected human embryonic kidney 293 cell were stimulated with 500 nM forskolin (Fsk) in the absence or presence of (*R*)-α-methylhistamine (R, H_3R-selective agonist), clobenpropit (Cloben, H_3R-antagonist) + R, ILG + R, or ILG, respectively. The results are the mean ± S.E.M. of assay triplicates.

tration-dependent manner similar to that of ranitidine [IC_{50} values of ranitidine = 1.0 ± 0.7 μM and isoliquiritigenin (ILG) = 2.3 ± 0.1 μM, respectively], suggesting that isoliquiritigenin inhibits H_2R-mediated signals with similar effect of ranitidine.

Lack of Direct Effects of Isoliquiritigenin on Signaling Mediated by G_s Protein, Adenylyl Cyclase, and β_2-Adrenoceptor. As we mentioned above, H_2R-mediated signaling activates G_s protein/adenylyl cyclase pathway (Klinker et al., 1996). To clarify whether the isoliquiritigenin affects only the H_2R, we treated the cells with forskolin (which directly activates adenylyl cyclase), isoproterenol (which activates G_s protein coupled β_2-adrenoceptor), and cholera toxin (which evokes G_s protein activity). Figure 3 shows that the addition of forskolin, isoproterenol, and cholera toxin caused a significant increase of cAMP level, and their cAMP generation were not attenuated by isoliquiritigenin pretreatment, suggesting that the site of the inhibition by isoliquiritigenin is neither the adenylyl cyclases, G_s protein, or G_s protein-coupled β_2-adrenoceptor. The result indicates that isoliquiritigenin selectively inhibits H_2R activation.

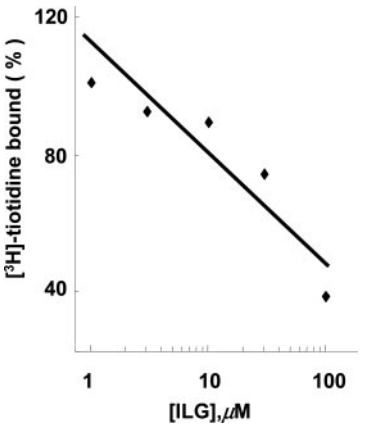

Fig. 4. Isoliquiritigenin inhibits binding of [^{3}H]tiotidine to H_2R. HL-60 cells were preincubated with indicated concentration of ILG for 10 min; then cells were treated with [^{3}H]tiotidine at 4°C for 80 min. Data were calculated as the mean ± S.E.M. of assay triplicates. Similar results were obtained in at least three independent experiments.

Selective Effect of Isoliquiritigenin on H_2R Subtype. As mentioned above, histamine exerts its effect not only through H_2Rs but also H_1Rs and H_3Rs. The H_1Rs are coupled to G_q proteins, which increase intracellular Ca^{2+} concentration, and H_3Rs are linked to G_i proteins, which down-regulate the activity of adenylyl cyclase. Thus, we checked whether isoliquiritigenin affects H_1R- and H_3R-mediated signaling. HTMT and (*R*)-α-methylhistamine are used as H_1R- and H_3R-selective agonists, respectively. Clobenpropit is used as H_3R antagonist. As shown in Fig. 3, C and D, isoliquiritigenin affect neither H_1R-mediated $[Ca^{2+}]_i$ nor H_3R-mediated signaling, suggesting that isoliquiritigenin is a selective H_2R antagonist.

[^{3}H]Tiotidine Binding Assay with Isoliquiritigenin. To determine whether the effects of ILG were due to its ability to block histamine binding to H_2R, we tested the effects of ILG on [^{3}H]tiotidine binding to H_2R in undifferentiated HL-60 cells (Monczor et al., 2003). Through RT-PCR and calcium measurements, we could not find H_1 receptor expression in our HL-60 system. In addition, [^{3}H]tiotidine is highly H_2R selective, suggesting that we can exclude the involvement of H_1 receptors with [^{3}H]tiotidine binding analysis. We found that isoliquiritigenin significantly blocked [^{3}H]tiotidine binding to undifferentiated HL-60 cells in a concentration-dependent manner (Fig. 4), indicating that isoliquiritigenin selectively and competitively inhibited histamine binding to the H_2R.

Three-Dimensional Model Building. The refined model obtained from the Modeler program was then checked for the stereochemical parameters by the program PROCHECK at 2.2 Å. For the modeled receptor, 87.3% of the backbone conformations fall within the most favored region of the Ramachandran plot, whereas the value for the A chain of bovine rhodopsin was only 79.9%. In addition, when analyzing the structural alignment of the C_α-trace between the human H_2R model and the A-chain of bovine rhodopsin, the root-mean-square deviation of the C_α-trace was 0.672 Å. This suggests that the homology model we obtained is reliable. To the human H_2R model thus obtained were added hydrogens at pH 7 to mimic the biological fluid environment. Then the potentials and charges were assigned using the CVFF forcefield and the receptor model was saved in Sybylmol2 format.

Binding Site Analysis and Docking. Then docking was performed with histamine and isoliquiritigenin. Goldscores of 39.08 and 48.91 were observed for histamine and isoliquiritigenin, respectively. The docked poses of both the ligands in the human H_2R model can be seen in Fig. 5, A and B, respectively. From our docking results, we demonstrated that histamine can form three hydrogen bonds with the receptor, which involve those from ethylamino group of histamine to side-chain carboxyl group of Asp 98, from ethylamino group of histamine to main chain carbonyl group of Lys 166, and from N1 proton of histamine to main chain carbonyl group of Leu 274, respectively (Fig. 6A), whereas isoliquiritigenin can also forms three hydrogen bonds with the receptor, which involve those from 4-OH-benzoyl group of isoliquiritigenin to the main chain carbonyl of Leu 274, from the 2-OH-benzoyl group of isoliquiritigenin to the main chain carbonyl of Lys 166, and from the carbonyl group of isoliquiritigenin to the main chain amino group of Asn 168.

Effect of isoLiquiritigenin on H_2R in MKN-45 Gastric Cancer Cell Line. MKN-45 gastric cancer cells express H_2Rs and increase H_2R expression upon retinoic acid treatment (Nakata et al., 1996). We treated MKN-45 with 5 μM retinoic acid for 72 h and tested the effect of isoliquiritigenin on dimaprit-induced cAMP generation. Similar to U937 cells, isoliquiritigenin also prominently inhibited dimaprit-induced cAMP production in MKN-45 gastric cancer cells. Isoliquiritigenin, however, did not affect forskolin-induced adenylyl cyclase activation (Fig. 7). These data indicate that isoliquiritigenin has an inhibitory effect on H_2Rs expressed not only in immune cell systems but also in gastric cells. For this reason, we extended our investigation whether

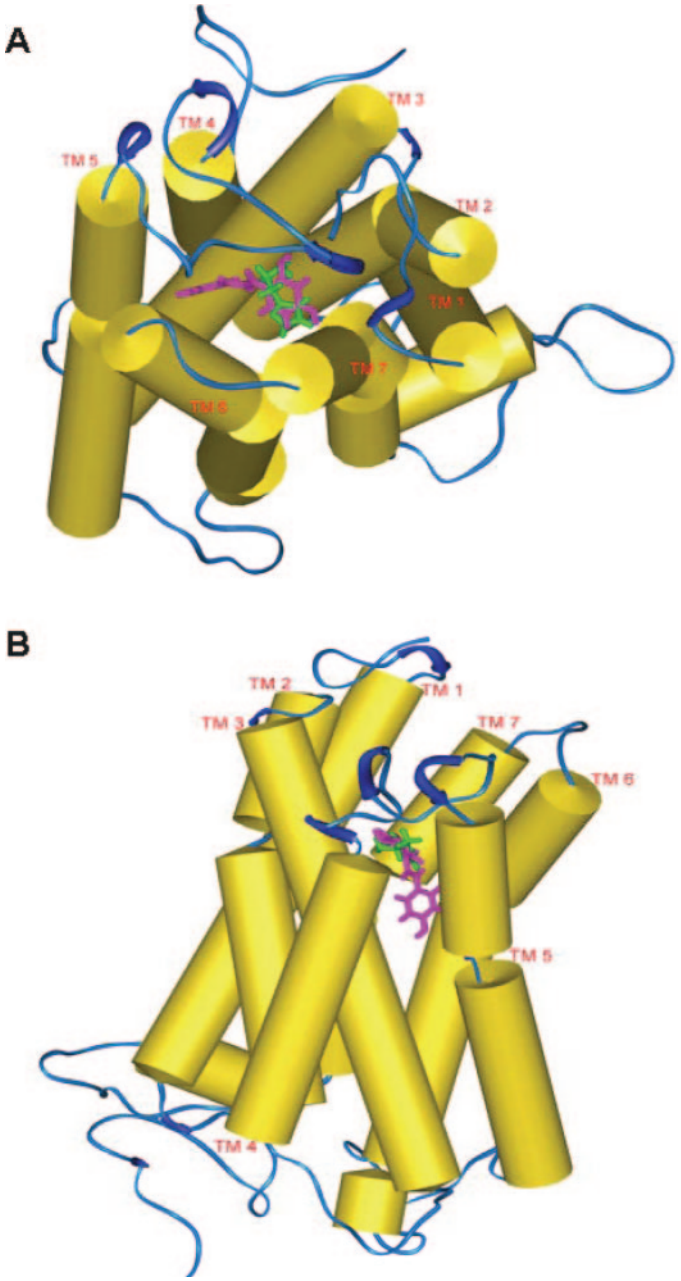

Fig. 5. Model of human H_2R complexed with histamine (green) and isoliquiritigenin (pink). The model was created by Insight II 2005 (Accelrys). A, top view of the docked poses of histamine and isoliquiritigenin. B, side view of the docked poses of histamine and isoliquiritigenin.

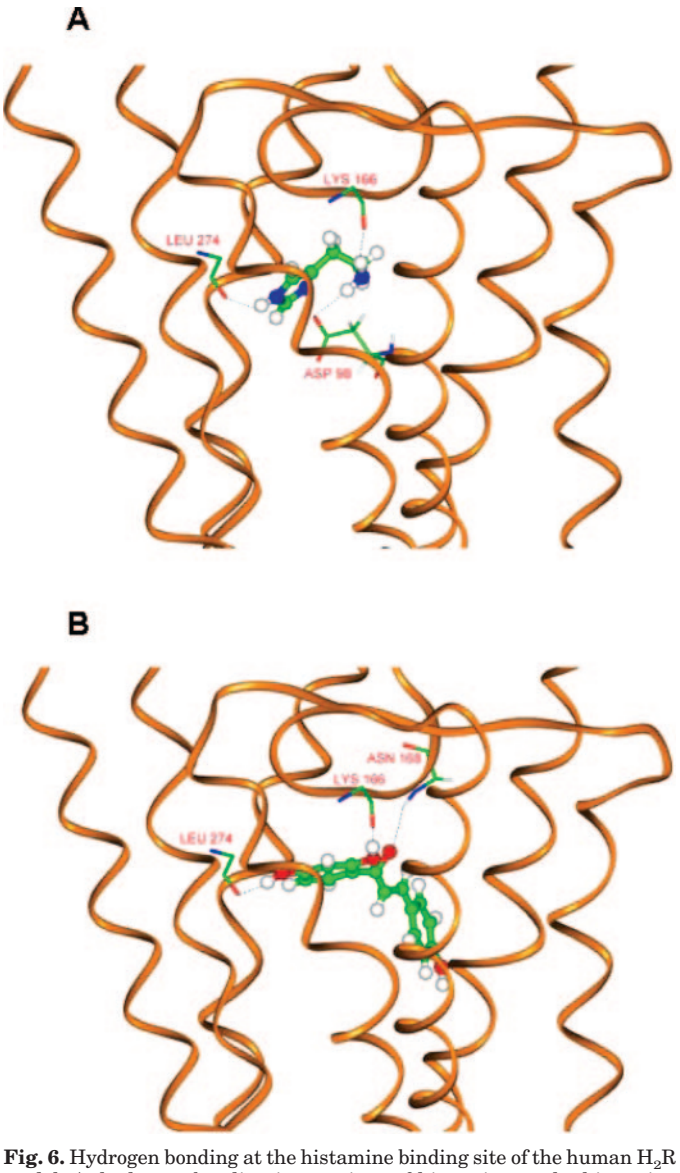

Fig. 6. Hydrogen bonding at the histamine binding site of the human H_2R model. A, hydrogen bonding interactions of histamine at the histamine binding site of human H_2R model. B, hydrogen bonding interactions of isoliquiritigenin at the histamine binding site of human H_2R mode. Hydrogen bonding is indicated by the dashed lines.

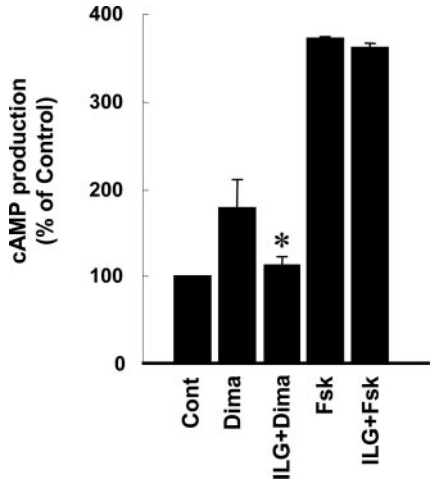

Fig. 7. Effect of isoliquiritigenin on H_2R-mediated signaling in MKN-45 gastric cancer cells. MKN-45 cells were preincubated with 10 μM ILG for 5 min; then cells were stimulated with 10 μM dimaprit (Dima) or 1 μM forskolin (Fsk) for 20 min. ILG was not removed during the H_2R agonist stimulation. The results are the mean ± S.E.M. of assay triplicates. *, $P < 0.05$ compared with Dima alone.

isoliquiritigenin can reduce the gastric ulceration in the stomach.

Effect of Isoliquiritigenin on Gastric Acid Secretion and Gastric Mucosal Lesion Formation. There are many reports about the inhibitory effect of H_2R antagonists on gastric acid secretion in animal model systems (Lamers, 1999). H_2R antagonists reduced volume of gastric acid production in pylori-ligated animal stomach and also significantly protected gastric mucosal lesion formation. In line with the above, we tested the effect of isoliquiritigenin on gastric acid-induced ulcer formation. In our experiment, isoliquiritigenin significantly prevented the gastric acid secretion (Fig. 8A) and gastric-ulcer formation (Fig. 8, B–D), similar to the ranitidine effect. Our results demonstrate that isoliquiritigenin effectively inhibits gastric acid secretion and protects gastric mucosal layer in a manner similar to that of other H_2R antagonists.

Discussion

Antagonism of histamine's action at H_2R has been the cornerstone of an immense market for pharmacological treatment of acid-peptic disorders of the gastrointestinal tract. Through this study, we provide evidence that isoliquiritigenin directly inhibited the H_2R activity and acted as an anti-peptic-ulcer drug. First, we confirmed the existence of H_2R in U937 and HL-60 cells through RT-PCR analysis, and isoliquiritigenin significantly blocked H_2R-mediated cAMP production. In addition, in MKN-45 gastric cells, isoliquiritigenin also inhibited H_2R-mediated signaling, indicating that isoliquiritigenin does not depend on the cell type. Second, isoliquiritigenin inhibits dimaprit-induced cAMP production via blocking of histamine binding to H_2R, rather than acceleration of degradation, because the inhibition was observed in the presence of the phosphodiesterase inhibitor, Ro 20-1724. Third, isoliquiritigenin did not affect the activity of other histamine subtype receptors (H_1R or H_3R), cholera toxin, the adenylyl cyclase- or β_2 adrenoceptor-mediated signaling pathway, suggesting that isoliquiritigenin is highly H_2R-selective. Fourth, isoliquiritigenin reduced [^{3}H]tiotidine binding on the H_2R, strongly suggesting that isoliquiritigenin inhibits only the binding affinity between H_2R and its corresponding ligand.

H_3 receptor agonists and antagonists, such as clobenpropit, imetit, (*R*)-α-methylhistamine, and thioperamide, show various degrees of cross-reactivity with the H_4 receptor. The H_4 receptor is a recently identified member of the histamine receptor family. Its closest relative in the histamine receptor family is the H_3 receptor; the similarity in the transmembrane region is even higher (68% sequence identity). Antagonists specific for the H_3 or H_4 receptor have been generated, and they are valuable tools for dissecting the biological roles of H_3 and H_4 receptors. As shown in Fig. 3D, isoliquiritigenin did not inhibit clobenpropit's antagonistic action on H_3 receptors, indicating that isoliquiritigenin does not interfere between the H_3/H_4 receptor ligand and receptor.

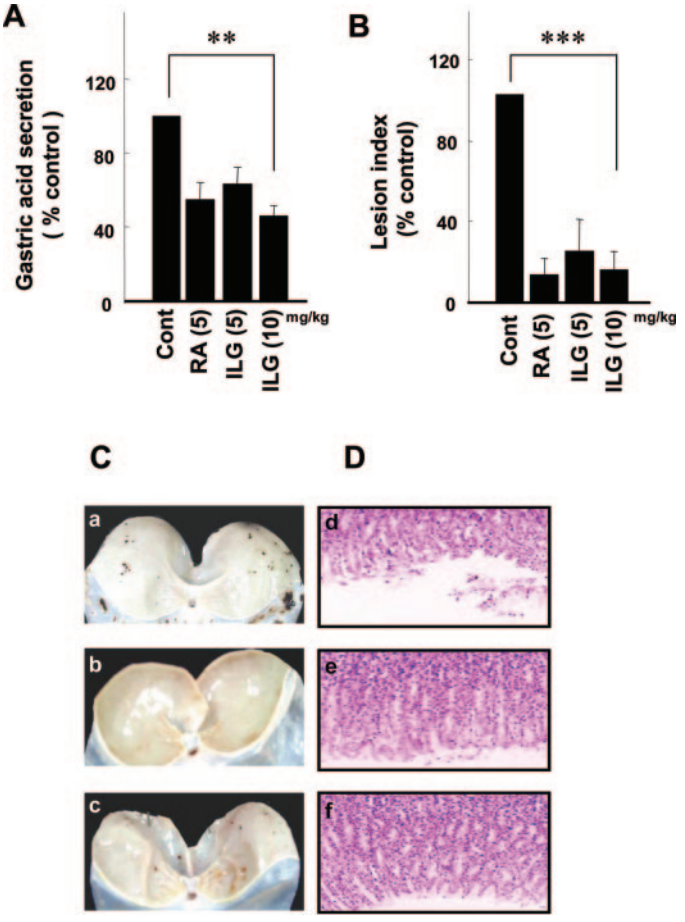

Fig. 8. Protection effect of isoliquiritigenin on the formation of gastric mucosal lesion. Test substances [vehicle (Cont), ranitidine (RA), and ILG] were administered orally, and then rats were killed 8 h after the surgery as described under *Materials and Methods*. A, effects of ILG and of ranitidine (RA) on the gastric acid secretion after pyloric ligature in SD rats. Each column represents the mean ± S.E.M. (n = 4–9 per group), and the difference between groups was determined by t test. **, $P < 0.01$ compared with control. B, each bar represents lesion score of Shay model-induced gastric damage. The hemorrhagic and ulcerative lesions were counted and measured with a light microscope by an observer who was blinded to the treatment. The ulcer index was then calculated as the sum of the length of all lesions. The ulcer index was determined in six animals in each animal group. ***, $P < 0.001$ compared with control. Representative pictures of 24 h pylorus-ligated Shay-model gastric walls (C) and HE-stained gastric mucosal layer (D). Vehicle-treated control group (a and d), ranitidine-treated group (b and e), and isoliquiritigenin-treated group (c and f).

The major physiological roles of gastrin include stimulation of acid secretion and the enterochromaffin-like cell is the principal cellular transducer of the gastrin-acid signal. Activation of its gastrin receptors results in synthesis and release of histamine with consequent activation of the parietal cell H_2Rs (Schubert, 2004). Histamine stimulates the parietal cells to secrete HCl (Lindstrom et al., 2001). Gastric acid secretion is under nervous and hormonal control (Lindstrom et al., 2001). The H_2 subclass of histamine receptors mediates gastric acid secretion, and antagonists for this receptor have proven to be effective therapy for acid peptic disorders of the gastrointestinal tract. It has been shown that H_2R activation mediates the gastric acid secretion via a G protein linked to adenylate cyclase activation and cellular cAMP generation. In the present study, 1-h pretreatment with isoliquiritigenin and ranitidine significantly inhibited the gastric acid secretion and gastric mucosal lesions in pylorus-ligated rat model (Fig. 8). Previous reports demonstrated that drugs that are unable to inhibit acid secretion cannot suppress the lesion formation in this model, because these lesions are related mainly to the significant increase in acidity of gastric secretions, and this correlates well with the severity of erosions (Shibata et al., 1998), showing that isoliquiritigenin can be very useful for peptic ulcer treatment.

Even though both histamine and isoliquiritigenin have three hydrogen bonds to the human histamine H_2R model, the higher goldscore for the latter arises from the more favorable hydrophobic interactions within the receptor. In addition, the isoliquiritigenin does not seem to have a hydrogen bond with Asp 98, which is crucial for histamine binding. From our studies, we can speculate that isoliquiritigenin has more favorable interactions with histamine H_2R because of the more hydrophobic interaction compared with histamine. In addition, suitable substitutions on the isoliquiritigenin that facilitates hydrogen bonding with the side-chain carboxyl group of Asp 98 may contribute to the antagonistic activity of isoliquiritigenin on the human H_2R.

As shown in Fig. 1, most H_2R antagonists [ranitidine (Fig. 1D), cimetidine (Fig. 1E), and famotidine (Fig. 1F)] are optimized through histamine-structure lead (Fig. 1C). However, the chemical structure of isoliquiritigenin is not optimized from histamine, suggesting that isoliquiritigenin can be used as novel H_2R antagonist. Mild diarrhea, neuropsychiatric disorders, gynecomastia, impotence, dizziness, rashes, confusion, headaches, and reversible liver damage have been reported by some people using histamine-derived H_2R antagonists as antiulcer treatments (Zimmerman, 1984). Because isoliquiritigenin is purified from a natural product, licorice (Ma et al., 2005), it may be considered a safe antiulcer treatment.

In previous reports, activation of H_2R has led to cell proliferation and has also activated transcription of the gene encoding c-fos in a protein kinase C-dependent manner (Wang et al., 1997). Cell proliferation often requires a series of signaling steps that act in a coordinated manner to regulate nuclear events responsible for controlling cell division. Previous reports proved that H_2R regulates c-Fos and c-Jun mRNA and protein level in numerous systems (Shayo et al., 1997). As in the previous reports, dimaprit transiently elevated c-Fos levels (Supplemental Data). In addition, we observed that the levels of c-Jun protein treated with H_2 agonist showed a similar pattern (Supplemental Data). However, in the presence of 10 μM isoliquiritigenin, dimaprit-induced elevation of c-Fos and c-Jun protein level was completely inhibited (Supplemental Data). These data demonstrate that isoliquiritigenin negatively regulates H_2R mediated c-Fos/c-Jun protein expression.

In conclusion, our results show that isoliquiritigenin is a novel, specific, and competitive histamine H_2R antagonist with antisecretory and antiulcer effects of similar potency to those of ranitidine. It also induces gastroprotective effects and might provide the basis for designing novel H_2R antagonists.

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