Isoliquiritigenin selectively inhibits $H_2$ histamine receptor signaling.

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Abbreviations

H₂R, histamine-2-receptor; cAMP, cyclic adenosine monophosphate
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 H₂ histamine receptor (H₂R) antagonistic effect and remarkably reduced several H₂R-mediated physiological responses. Preincubation of U937 and HL60 hematopoietic cells with isoliquiritigenin significantly inhibited H₂R agonist-induced cAMP response in a concentration-dependent manner without affecting the viability of cells. Isoliquiritigenin also blocked the binding affinity of [³H]-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 Gs protein, effector enzyme, adenylyl cyclase, or β₂-adrenoceptor. Isoliquiritigenin neither affect H₁R- nor H₃R-mediated signaling. In molecular docking studies, isoliquiritigenin exhibited more favorable interactions with H₂R than histamine. Isoliquiritigenin prominently inhibited H₂R 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 pylori ligated rat model. Taken together, the results demonstrate that isoliquiritigenin is an effective H₂R antagonist and provides the basis for designing novel H₂R antagonist.
Introduction

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 play 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 H₁, H₂, H₃, and H₄ receptors (Gutzmer et al., 2005). Specially, the H₂ histamine receptor (H₂R) is coupled to the Gs-protein/adenylyl cyclase system in a variety of tissues (e.g. brain, stomach, heart, gastric mucosa, lung) and produces intracellular cyclic adenosine monophosphate (cAMP) (Alewijnse et al., 1998). Gantz et al were the first to clone a cDNA encoding a 359 amino acid H₂R. Using degenerate primers based on the known sequence similarity of various GPCRs, the H₂R sequence was obtained from canine gastric parietal cDNA by PCR (Gantz et al., 1991). Soon thereafter, the intronless genes encoding the rat, human, guinea-pig and mouse H₂R were cloned by means of homology screening (Gantz et al., 1991). Many reports demonstrated the cellular function of H₂R and its importance. In immune system, the responses of T helper cell(Th1 and Th2) are negatively regulated by H₂R through the activation of different biochemical intracellular signals (Jutel et al., 2001). The presence of H₂R 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 (G. 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. Though it is considered to be the quintessential “servant” herb, it is often refered to as the King of Herbs. Although the flavonoid-rich fraction from the extract of licorice has been used as a gastrointestinal disorder and an anti-ulcer medicine (Fukai et al., 2002), the mechanism is still
elusive.

Since Black et al. first defined the H₂R and its involvement in gastric acid secretion, H₂R antagonists, for example, cimetidine, ranitidine, famotidine have been developed and used clinically as anti-acid secretagogues (Black et al., 1972). These histamine H₂R antagonists have revolutionized the treatment of peptic ulcers with their prominent therapeutic effects. Shibata reviewed that the components of *G. uralensis* have strong potency of anti-peptic ulcer effect (Shibata, 2000). Constituents of *G. uralensis* include glycyrrhizic acid, glycyrrhetic acid, isoliquiritigenin (Shibata, 2000). Since Fukai reported that flavonoid-rich fraction of licorice extract showed anti-ulcer effect (Fukai et al., 2002), 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 H₂R antagonist and examined its effect on H₂R activity. Isoliquiritigenin has been reported as a potent antioxidant, cancer-preventing properties, and anti-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 rat pylori-ligated model (Shay et al., 1954), to evaluate the potential activity of isoliquiritigenin as H₂R antagonist and anti-peptic ulcer agent against gastric ulcer. Through this study, we provide evidences that isoliquiritigenin negatively regulates most of the H₂R’s activity and clearly acts as a specific, competitive H₂R 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). [3H]-cAMP was obtained from NEN Life Science Products (Boston, MA) and [3H]-tiotidine was purchased from Perkin Elmer Life Sci. (Wellesley, MA). RPMI-1640 and penicillin-streptomycin were purchased from Gibco (Life Technologies, Gaithersburg, MD). Bovine calf serum was obtained from HyClone Laboratories (Logan, UT, USA)

Cell culture

U937, HL-60 cell line (American Type Culture Collection, Rockville, MD) and MKN-45 cell (Korean Cell Line Bank, Korea) was cultured in suspension at 37°C in RPMI 1640 medium supplemented with 10%(v/v) of heat-inactivated bovine calf serum and 1%(v/v) penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO2. Primary cultures of bovine adrenal cortex cells were prepared according to previous report (Reichenstein et al., 2004).

Measurement of [3H] cAMP

Intracellular cAMP generation was determined by [3H]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 H2R mRNA expression

Total RNA was isolated from the U937 and HL-60 cells using the CENTRIZOL (NEURONEX, Korea). Ten micrograms of total RNA were reverse-transcribed with the use of Superscript II reverse transcriptase (GIBCO BRL, Life Technologies). cDNA was amplified with 20pmol of specific oligonucleotide primers (Bioneer, Korea) using Pfu polymerase (Stratagene, La Jolla, CA).

The primer sequences are 5' -GCCCTCGAGACCA TGGCACCCAA TGGCACAGC and 3' -GCCGGGCCACCCCTGTCTGTGGCTCCCTCGG. 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.

[Ca2+]i measurement

Cytosolic free Ca2+ concentration ([Ca2+]i) was determined with the help of the fluorescent Ca2+ indicator fura-2/AM. Briefly, the U937 cell suspension was incubated with fresh serum-free RPMI medium containing fura-2/AM (3µM) 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 (250µM) 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).

H3 histamine receptor expressing HEK293 cell.

The full-length human histamine H3 receptor gene was kindly provided by Dr. Liu Changlu (Johnson & Johnson Pharmaceutical Research and Development). H3R genes were subcloned into the pCIneo expression vector. Four million cells detached with trypsin were
mixed together with 10 µg DNA in Bio-Rad gene pulser cuvettes (0.4 cm) and electroporated at 260 V, 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.

[3H]-tiotidine binding

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

Three-dimensional model building

All the simulations were performed on Linux workstations using InsightII 2005 software package (Accelrys, CA, USA). The primary sequence of the human H2R (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 (VTF) method with conjugate gradients optimization as well as molecular dynamics refinement, was used to build the 3D structure of human histamine H2R by taking the structure of bovine rhodopsin (PDB: 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 InsightII 2005 software. Active_Site_Search characterizes protein active sites and binding sites by locating cavities in 3D 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 InsightII 2005. Then the charges & potentials were assigned using CHARMM forcefield and the minimization was done using the CHARMM module in InsightII 2005 with 1000 steps of steepest descents followed by 10000 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 Optimisation for Ligand Docking, Cambridge Crystallographic Data Centre, UK) was employed to dock the histamine and isoliquiritigenin into the histamine binding site of human histamine H2 receptor. A homology model of human histamine H2 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 InsightII 2005 software. The RMS 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. (Shay et al., 1954) was used with some modification. Male Sprague-Dawley(SD) rats weighing 200-250g were used. Rats were deprived of food, but not water, for 18-24h prior to each experiment. The test substances (vehicle, ranitidine, isoliquiritigenin) dissolved in saline were administered orally (per os). After 1hr, rats were anesthetized by light ether, a small abdominal incision was made, the pylorus was ligated then indomethacine (40mg/kg) was injected through the duodenum. The animals were sacrificed 8hr after ligation of the pylorus, the stomach was clamped at the oesophageal and duodenal junctions, filled with 15ml of 4% formalin, and then rapidly removed. After 24hr, the fixed stomach was opened along the greater curvature, gently rinsed in saline, and then pinned open to expose the gastric mucosa. The haemorrhagic 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 following evaluation of the ulcer index. Samples of the corpus were excised and transferred to fresh formalin and later processed by routine techniques prior to embedding in paraffin. Sections (5µm thick) were mounted on glass slides and stained with haematoxylin 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% of better (P < 0.05).
Results

cAMP production by H2R agonist in U937 and HL-60 cells.

Histamine and dimaprit are known as highly selective histamine H2R agonists (Bakker et al., 2002). U937 and HL-60 cells express H2R (Fig. 2A, inset), which is coupled to Gs-protein and mediates cAMP pathway (Shayo et al., 1997). As shown in figure 2A, histamine and dimaprit induced cAMP increase in a concentration-dependent manner and half-maximal effective concentrations (EC50) are 1.2 ± 1.3 µM and 4.7 ± 0.2 µM, respectively.

Effect of isoliquiritigenin on H2R-mediated cAMP production.

We preliminarily investigated the inhibitory effect of licorice constituents (10 µM each) on histamine and dimaprit (10 µM each)-induced cAMP production in U937 cell line (data not shown). Among the components, isoliquiritigenin (Fig. 1B) exhibited the strongest inhibitory effect. Fig. 2B represents that isoliquiritigenin inhibited histamine and dimaprit-induced cAMP production in a concentration-dependent manner similar with ranitidine (IC50 values of ranitidine = 1.0 ± 0.7 µM and ILG = 2.3 ± 0.1 µM, respectively), suggesting that isoliquiritigenin inhibits H2R-mediated signals with similar effect of ranitidine.

Lack of direct effects of isoliquiritigenin on Gs-protein, adenylyl cyclase- and β2-adrenoceptor-mediated signaling.

As we mentioned above, H2R-mediated signaling posseses Gs-protein / adenylyl cyclase pathway (Klinker et al., 1996). To clarify whether the isoliquiritigenin only effects on the H2R, we treated the cells with forskolin, which directly activates adenylyl cyclase, isoproterenol, which activates Gs-protein coupled β2-adrenoceptor, and cholera toxin, which evokes Gs-protein activity. Fig. 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, Gs-protein, or Gs-protein coupled β2-adrenoceptor. The result indicates that isoliquiritigenin selectively inhibits H2R activation.

Selective effect of Isoliquiritigenin on H2R subtype.

As mentioned above, histamine exerts its effect not only through H2Rs but also H1Rs and H3Rs. The H1Rs are coupled to Gq-proteins which increase intracellular Ca2+ concentration and H3Rs are linked to Gi-proteins which down-regulate the activity of adenylyl cyclase. Thus, we checked whether isoliquiritigenin affect the H1R- and H3R-mediated signaling. HTMT and R-α-methylhistamine are used as H1R- and H3R-selective agonist, respectively. Clobenpropit is used as H3R antagonist. As shown in Fig. 3C and 3D, isoliquiritigenin neither affect H1R-mediated [Ca2+]i nor H3R-mediated signaling, suggesting that isoliquiritigenin is selective H2R antagonist.

[3H]-tiotidine binding assay with isoliquiritigenin.

To determine whether the effects of ILG were due to its ability to block histamine binding to H2R, we tested the effects of ILG on [3H]-tiotidine binding to H2R in undifferentiated HL-60 cells (Monczor et al., 2003). In our HL-60 cell system, we could not find H1 receptors but H2 receptors. In addition, [3H]-tiotidine is highly H2R selective, suggesting that we can exclude the involvement of H1 receptors with [3H]-tiotidine binding analysis. We found that isoliquiritigenin significantly blocked [3H]-tiotidine binding to undifferentiated HL-60 cells in concentration-dependent manner (Fig. 4), indicating that isoliquiritigenin selectively and competitively inhibited histamine binding to the H2R.
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 favoured region of the Ramachandran plot while for the A chain of bovine rhodopsin was only 79.9%. Also, when analyzing the structural alignment of the Cα-trace between the human H2R model and the A-chain of bovine rhodopsin, the root mean square deviation (RMSD) of the Cα-trace was 0.672 Å. This suggests that the homology model we obtained is reliable. To the human H2R model thus obtained was 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. A goldscore of 39.08 and 48.91 was observed for the histamine and isoliquiritigenin, respectively. The docked poses of both the ligands in the human H2R model can be seen in Fig 5A & 5B, 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 main chain carbonyl of Leu 274, from 2-OH-benzoyl group of isoliquiritigenin to main chain carbonyl of Lys 166, and from carbonyl group of isoliquiritigenin to main chain amino group of Asn 168.
Effect of isoliquiritigenin on H₂R in MKN-45 gastric cancer cell line.

MKN-45 gastric cancer cells express which mediate H₂R cAMP signaling and increase H₂R expression upon retinoic acid treatment (Nakata et al., 1996). We treated MKN-45 with 5µM of retinoic acid for 72hr 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 inhibitory effect on H₂Rs expressed not only in immune cell systems but also in gastric cells. For this reason, we extended our investigation whether 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 H₂R antagonists on gastric acid secretion in animal model systems (Lamers, 1999). H₂R 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. 8B, Fig. 8C, and Fig. 8D) similar to the ranitidine effect. Our results demonstrate that isoliquiritigenin effectively inhibits gastric acid secretion and protects gastric mucosal layer similar to other H₂R antagonists.
Antagonism of histamine’s action at H2R has been the cornerstone of an immense market for pharmacological treatment of acid-peptic disorders of the gastrointestinal tract. Through this study, we provided evidences that isoliquiritigenin directly inhibits the H2R activity and acts as an anti-peptic ulcer drug. First, we confirmed the existence of H2R in U937 and HL-60 cells through RT-PCR analysis and isoliquiritigenin significantly blocked H2R-mediated cAMP production. In addition, MKN-45 gastric cells, isoliquiritigenin also inhibited H3R-mediated signaling, indicating that isoliquiritigenin does not depend on the cell type species. Second, isoliquiritigenin inhibits dimaprit-induced cAMP production via bloking of histamine binding to H2R, rather than acceleration of degradation, since the inhibition was observed in the presence of the phosphodiesterase inhibitor, Ro20-1724. Third, isoliquiritigenin did not affect the activity of other histamine subtype receptors (H1R or H3R), cholera toxin, adenylyl cyclase- or β2 adrenoceptor-mediated signaling pathway, suggesting that isoliquiritigenin is highly H2R-selective. Fourth, isoliquiritigenin reduced [3H] tiotidine binding on the H2R, strongly suggest that isoliquiritigenin only inhibits the binding affinity between H2R and its corresponding ligand.

H3 receptor agonists and antagonists, such as clobenpropit, imetit, R-α-methylhistamine and thioperamide, show various degrees of crossreactivity with the H4 receptor. The H4 receptor is a new member of the histamine receptor family identified recently. Its closest member in the histamine receptor family is the H3 receptor, the similarity in the transmembrane region is even higher (68% sequence identity). Recently, antagonists specific for the H3 or H4 receptor have been generated and they are valuable tools for dissecting the biological roles of H3 and H4 receptors. As shown in Fig. 3D, isoliquiritigenin did not inhibit clobenpropit’s antagonistic action on H3 receptors, indicating that isoliquiritigenin does not interfere between the H3/H4.
The major physiological roles of gastrin include stimulation of acid secretion and the enterochromaffin-like (ECL) 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 H2Rs (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 H2 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 H2R activation mediate the gastric acid secretion via a guanine nucleotide-binding protein linked to adenylate cyclase activation and cellular cAMP generation. In the present study, one hour pretestment with isoliquiritigenin and ranitidine significantly inhibited the gastric acid secretion and gastric mucosal lesions in pylori-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 juice 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 bondings to the human histamine H2R model, the higher goldscore for the latter arises from the more favorable hydrophobic interactions within the receptor. Also the isoliquiritigenin does not appear 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 H2R due to more hydrophobic interaction compared to histamine. Also suitable substitutions on the isoliquiritigenin that facilitates hydrogen bonding with side chain carboxyl group of Asp 98 may
contribute to isoliquiritigenin’s antagonistic activity on the human \( H_2 \)R.

As shown in Fig.1, most \( H_2 \)R antagonists (ranitidine (Fig. 1D), cimetidine (Fig. 1E), and famotidine (Fig. 1F)) are optimized through histamine-structure lead (Fig. 1C). Isoliquiritigenin, however, neither have any structural similarity with these compounds nor have histamine moiety, suggesting that isoliquiritigenin can be used as novel \( H_2 \)R antagonist. Mild diarrhoea, neuropsychiatric disorders, gynecomastia, impotence, dizziness, rashes, confusion, headaches and reversible liver damage have been reported by some people using histamine-derived \( H_2 \)R antagonists as an anti-ulcer treatment (Zimmerman, 1984). Since isoliquiritigenin is purified from natural product, licorice (Ma et al., 2005), it may be considered as safe anti-ulcer treatment.

In previous reports, activation of \( H_2 \)R can lead cell proliferation and also activate transcription of the gene encoding c-fos in a protein kinase C (PKC)-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 \( H_2 \)R regulates c-Fos and c-Jun mRNA and protein level in numerous system (Shayo et al., 1997). Like as previous reports, dimaprit transiently elevated c-Fos levels (supplement data). In addition, we observed that the levels of c-Jun protein treated with \( H_2 \) agonist showed similar pattern (supplement data). However, in the presence of 10\( \mu \)M of isoliquiritigenin, dimaprit-induced elevation of c-Fos and c-Jun protein level were completely inhibited (supplement data). These data demonstrate here that isoliquiritigenin negatively regulates \( H_2 \)R mediated c-Fos / c-Jun protein expression.

In conclusion, our results show that isoliquiritigenin is a novel, specific, and competitive histamine \( H_2 \)R antagonist with antisecretory and antiulcer effects similar potent with those of ranitidine. It also induces gastroprotective effects and might provide the basis for designing novel \( H_2 \)R antagonists.
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References


Footnotes

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Legends for Figures

Figure 1.

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

Figure 2.

Isoliquiritigenin attenuates H₂R signaling.

(A) H₂R agonists (histamine and dimaprit)-induced cAMP production in U937 and HL-60 promyelocytes (HA: histamine, Dima: dimaprit). H₂R expression in U937 and HL-60 cells was confirmed by RT-PCR, inset. cAMP generation and RT-PCR were measured as described in Materials and Methods. (B) U937 Cells were preincubated with indicated concentration of ranitidine (Rani) or isoliquiritigenin (ILG) for 5min, then cells were stimulated with 10µM HA or Dima for 20min. Rani or ILG was not removed during the H₂R agonist stimulation. The results are the mean ± SEM of assay triplicates.

Figure 3.

Selective effect of isoliquiritigenin on H₂R.

(A) U937 cells were preincubated with indicated concentration of isoliquiritigenin (ILG) for 5min, then cells were stimulated with 10µM of dimaprit (open circles), 1µM of isoproterenol (open squares), or 1µM of forskolin (closed diamonds), respectively. (B) Bovine adrenal cortex cells preincubated with 10µM of ILG, then cells were stimulated with 100ng/ml of cholera toxin (CTX). The results are the mean ± SEM of assay triplicates. (C) The intracellular [Ca²⁺] rise induced by 100µM histamine (HA) or HTMT (H₁R-selective agonist).
was measured in the absence or presence of 10μM \textbf{ILG} in U937. Data are the means±SEM (bars) values of triplicate measurements. (D) H₃R-transfected HEK293 cell were stimulated with 500nM forskolin (\textbf{Fsk}) in the absence or presence of R-α-methylhistamine (\textbf{R} : H₃R-selective agonist), clobenpropit (\textbf{Cloben} : H₃R-antagonist) + \textbf{R}, \textbf{ILG} + \textbf{R}, or \textbf{ILG} respectively. The results are the mean ± SEM of assay triplicates.

**Figure 4.**

\textbf{Isoliquiritigenin inhibits binding of [³H]-tiotidine to H₂R.}

HL-60 Cells preincubated with indicated concentration of isoliquiritigenin (\textbf{ILG}) for 10min, then cells were treated with [³H]-tiotidine at 4°C for 80min. Data were calculated as the mean ± SEM of assay triplicates. Similar results were obtained in at least three independent experiments.

**Figure 5.**

Model of human H₂R complexed with histamine (green) and isoliquiritigenin (pink). The model was created by InsightII 2005 (Accelrys). (A) Top view of the docked poses of histamine and isoliquiritigenin is shown. (B) Side view of the docked poses of histamine and isoliquiritigenin is shown.

**Figure 6.**

Hydrogen bonding at the histamine binding site of the human H₂R model. (A) Hydrogen bonding interactions of histamine at the histamine binding site of human H₂R model is shown. (B) Hydrogen bonding interactions of isoliquiritigenin at the histamine binding site of human
H₂R model is shown. Hydrogen bonding is indicated by the dashed lines.

**Figure 7.**

**Effect of Isoliquiritigenin on H₂R-mediated signaling in MKN-45 gastric cancer cells.**

MKN-45 cells were preincubated with 10µM isoliquiritigenin (ILG) for 5min, then cells were stimulated with 10µM dimaprit (Dima) or 1µM forskolin (Fsk) for 20min. ILG was not removed during the H₂R agonist stimulation. The results are the mean ± SEM of assay triplicates. *P < 0.05 when compared with Dima alone.

**Figure 8.**

**Protection effect of isoliquiritigenin on the formation of gastric mucosal lesion.**

Test substances (vehicle (Cont), ranitidine (RA), isoliquiritigenin (ILG) were injected through per os and then rats were killed 8hr after the surgery as described in Materials and Methods. (A) Effects of isoliquiritigenin (ILG) and of ranitidine (RA) on the gastric acid secretion after pylorus ligature in SD rats. Each column represents the mean±SEM (N=4-9 per group) and the difference between groups was determined by t-test. **P<0.01 when compared with control. (B) Each bar represent lesion score of shay-model induced gastric damage. The haemorrhagic 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 when compared with control. Reresentative pictures of 24hr pylori-ligated Shay-model gastric walls (C) and Hematoxylin-Eosin (HE) stained gastric mucosal layer (D). Vehicle treated control group (a,d), Ranitidine treated group (b,e), and isoliquiritigenin treated group (c, f).
Fig. 1

A

B

C

D

E

F
Fig. 2

A

- HA in U937
- HA in HL-60
- Dima in U937
- Dima in HL-60

B

- Rani + HA
- ILG+ HA
- ILG+ Dima

H₂R production (pmol/6 X 10⁵ cells)

[H₂R agonist], µM

0.01 0.1 1 10 100 1000

Log [H₂R antagonist], M

CAMP production (% control)

-9 -8 -7 -6 -5 -4 -3

[Log H₂R antagonist], M

0 20 40 60 80

0.01 0.1 1 10 100 1000

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**Fig. 3**

(A) 

![Graph showing cAMP production (% control) vs. [ILG] in μM. The y-axis ranges from 0 to 100, and the x-axis ranges from 0.1 to 1000. Data points are marked with error bars.](image)

(B) 

![Bar chart showing cAMP production (%). The bars represent control (Cont), CTX, ILG + CTX, and ILG.](image)

(C) 

![Bar chart showing fluorescent ratio (340/380). The bars represent control (cont), HA, ILG+HA, HTMT, ILG+HTMT, and ILG.](image)

(D) 

![Bar chart showing cAMP production (%). The bars represent control, Fsk, R-MeHA + Fsk, Cloben + R + Fsk, ILG + R + Fsk, ILG + Fsk, and ILG.](image)
Fig. 4

![Graph showing the relationship between [ILG] in µM and [%] H]-tiotidine bound.](image-url)
Fig. 6

A

B
Fig. 7

![Graph showing cAMP production (% of Control)]
Fig. 8

**A**

Gastric acid secretion (% control)

- **Cont**
- RA (5) mg/kg
- ILG (5) mg/kg
- ILG (10) mg/kg

Note: Significant difference indicated by ***(p < 0.001)***

**B**

Lesion index (% control)

- **Cont**
- RA (5) mg/kg
- ILG (5) mg/kg
- ILG (10) mg/kg

Note: Significant difference indicated by ***(p < 0.001)***

**C**

- Image a
- Image b
- Image c

**D**

- Image d
- Image e
- Image f