Identification of Escherichia coli enterotoxin inhibitors from traditional medicinal herbs by in silico, in vitro, and in vivo analyses

Jaw-Chyun Chen, Tin-Yun Ho, Yuan-Shiun Chang, Shih-Lu Wu, Chia-Cheng Li, Chien-Yun Hsiang

Aim of the study: Heat-labile enterotoxin (LT), the virulence factor of enterotoxigenic Escherichia coli, induces diarrhea by initially binding to the GM1 on the surfaces of intestinal epithelial cells and consequently leading to the massive loss of fluid and ions from cells. Therefore, we evaluated the inhibitory effects of traditional medicinal herbs (TMH) on the B subunit of LT (LTB) and GM1 interaction.

Materials and methods: The inhibitory effects of TMH on LTB-GM1 interaction were evaluated by GM1-enzyme-linked immunosorbent assay (ELISA). The likely active phytochemicals of these TMH were then predicted by in silico model (docking) and analyzed by in vitro (GM1-ELISA) and in vivo (patent mouse gut assay) models.

Results: We found that various TMH, which have been ethnomedically used for the treatment of diarrhea, inhibited the LTB-GM1 interaction. Docking data showed that triterpenoids were the most active phytochemicals and the oleanane-type triterpenoids presented better LTB-binding abilities than other types of triterpenoids. Moreover, by in vitro and in vivo models, we demonstrated that glycyrrhizin was the most effective oleanane-type triterpenoid that significantly suppressed both the LTB-binding ability (IC50 = 3.26 ± 0.17 mM) and the LT-induced fluid accumulation in mice.

Conclusions: We found an LT inhibitor, glycyrrhizin, from TMH by in silico, in vitro, and in vivo analyses.

© 2008 Elsevier Ireland Ltd. All rights reserved.
phyllatics for the treatment and prevention of LT-induced diarrhea (Minke et al., 1999a; Pickens et al., 2002; Mitchell et al., 2004).

Many traditional medicines and their components have been used to treat LT-induced diarrhea. Some herbal extracts, such as the fruit of *Chaenomeles speciosa* (Chen et al., 2007a), the root of *Zingiber officinale* (Chen et al., 2007b), the leaf of *Camellia japonica* (Bruins et al., 2006) and the gall on *Rhus chinensis* (Chen et al., 2006), exhibit anti-LT-induced diarrheal abilities via several mechanisms. Traditional medicinal herbs (TMH) have been used in the treatment of gastrointestinal diseases in China for millennia. Therefore, we collected TMH, which have been ethnomedically used for gastrointestinal disorders, in this study. By GM1-enzyme-linked immunosorbent assay (ELISA), we found that some of TMH extracts were capable of inhibiting the binding of LT to GM1. The likely active phytochemicals of these TMH were then predicted by in silico model (docking) and analyzed by both in vitro (GM1-ELISA) and in vivo (patent mouse gut assay) models. Our data showed that glycyrrhizin exhibited anti-diarrheal effects in mice via the inhibition of GM1 and LT interaction. These findings suggested that glycyrrhizin might be a potent candidate for the treatment of LT-induced diarrhea.

2. Materials and methods

2.1. Chemicals and herbal materials

TMH were gifts from Sun Ten Pharmaceutical Co., Ltd. (Taipei, Taiwan), a renowned GMP manufacturer of concentrated herbal extracts by both Taiwanese and Australian authorities. The qualities of TMH samples were verified by authors (Prof. Yuan-Shiun Chang and Dr. Jaw-Chyun Chen) according to the Pharmacopoeia Commission of People’s Republic of China (2005). The voucher specimens were deposited in the Molecular Biology Laboratory, Graduate Institute of Chinese Medical Science, China Medical University. Specimen number was listed in supplemental table. TMH sample was ground with the homogenizer to a fine powder and extracted by mixing 100 g of herb powder with methanol at 4 °C overnight. The supernatant was then collected and stored at −30 °C in small aliquots. Glycyrrhizin was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide at 100 mM.

2.2. Expression and purification of *Escherichia coli* (E. coli) LT and LTB

Recombinant LT and LTB were expressed in *E. coli* BL21(DE3)pLysS strain and purified by affinity chromatography as described previously (Chen et al., 2006). Briefly, cells were induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside and collected 3 h after induction. The cell pellet was resuspended in 1× TEAN buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, 3 mM NaN3), lysed by sonication, and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was collected and mixed with d-galactose resin (Pierce, Rockford, IL, USA), and the recombinant LT and LTB were then eluted by 1× TEAN buffer containing 1 M galactose. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and quantified with a Bradford assay (Bio-Rad, Hercules, CA, USA).

2.3. Competitive G_{M1}-ELISA

LTB was biotinylated as described previously (Chen et al., 2006). Biotinylated LTB (16 ng) was mixed with various amounts of compounds and incubated at 4 °C for 3 h with shaking. Microtiter plates (MaxiSorp Nunc-Immum™ plates, Nunc, Denmark) were coated with 100 μl of 2 ng/μl G_{M1} (Sigma, St. Louis, MO, USA), which was diluted in phosphate-buffered saline (PBS) (137 mM NaCl, 1.4 mM KH_{2}PO_{4}, 4.3 mM Na_{2}HPO_{4}, 2.7 mM KCl, pH 7.2), per well and incubated at 4 °C overnight. The wells were washed with 200 μl of washing buffer (0.5% Tween 20 in PBS), blocked with 200 μl of blocking buffer (1% bovine serum albumin in PBS) at 37 °C for 1 h, and then incubated with 100 μl of biotinylated LTB/compound mixture at 37 °C for 1 h. After three washes with washing buffer, 50 μl of diluted peroxidase-conjugated avidin (Pierce, Rockford, IL, USA) was added to each well and incubated at 37 °C for 1 h. Following

![Fig. 1. Inhibitory abilities of TMH on the interaction between LTB and G_{M1}. Numbers in the brackets are the sum of herb species in the family. *p < 0.05, ** p < 0.01, compared with LTB.](image-url)
three washes, 50 μL of chromogenic substrate, 2,2’-azinobis(3-ethylbenzthiazoline-sulfonic acid) (Sigma, St. Louis, MO, USA), was added to each well and incubated at 37 °C for 15 min. The absorbance was read at 405 nm in an ELISA plate reader. The inhibitory ability (%) was calculated by \[1 - \frac{(OD \text{ value of mixture containing LTB and compound/OD \text{ value of mixture containing LTB only})}}{100}.\]

2.4. Docking technology

The docking technology was performed as described previously (Chen et al., 2007). The MEDock (Maximum Entropy based Docking) web server (http://medock.csie.ntu.edu.tw/) was used for the prediction of ligand binding sites (Chang et al., 2005). The input file was in the PDBQ format, which is an extension of the PDB format. The PDBQ format of ligands (phytochemicals) was generated by Dundee’s PRODRG server (http://davapc1.bioch.dundee.ac.uk/programs/prodrg/) (Schüttelkopf and van Aalten, 2004). The PDB file (PDB code 1EFI) of LTB was taken from the Protein Data Bank (http://www.rcsb.org/pdb/) (Sixma et al., 1993; Fan et al., 2001). The PDBQ file of LTB was derived from the PDB2PQR server (http://agave.wustl.edu/pdb2pqr/) (Dolinsky et al., 2004).

2.5. Patent mouse gut assay

Female BALB/c mice (8 weeks old, 20 ± 1 g weight) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mouse experiments were conducted under ethics approval from the China Medical University Animal Ethics Committee (Ethics Approval Number 96-42-N).

In vivo LT-induced diarrheal ability was determined by the patent mouse gut assay as described previously (Baselski et al., 1997; Chen et al., 2006). Briefly, five mice per group were starved with water only for 16 h. Each mouse was inoculated intragastrically with 0.5 ml of 10 μg LT alone or in conjunction with various amounts of TMH extracts or compounds. Six hours latter, mice were sacrificed. The entire intestine from duodenum to rectum was carefully removed to retain any accumulated fluid, and the residual mesentery was removed prior to weigh. The carcass was weighed separately. LT-induced diarrheal ability was presented as a gut/carcass ratio as followed. The IC50 value of compound was
Table 2
Binding energy scores of oleanane type of triterpenoids.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Energy score (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycyrrhizin</td>
<td><img src="image" alt="Glycyrrhizin structure" /></td>
<td>−18.03</td>
</tr>
<tr>
<td>Saikosaponin A</td>
<td><img src="image" alt="Saikosaponin A structure" /></td>
<td>−14.12</td>
</tr>
<tr>
<td>Friedelin</td>
<td><img src="image" alt="Friedelin structure" /></td>
<td>−11.01</td>
</tr>
<tr>
<td>α-Hederin</td>
<td><img src="image" alt="α-Hederin structure" /></td>
<td>−10.41</td>
</tr>
<tr>
<td>β-Amyrin</td>
<td><img src="image" alt="β-Amyrin structure" /></td>
<td>−8.45</td>
</tr>
</tbody>
</table>

Determined as the quantity of compound required to inhibit the LT-induced gut/carcass ratio at 50%.

\[
\text{Gut/Carcass ratio} = \frac{\text{Gut weight}(g)}{\text{Carcass weight}(g)}
\]

2.6. Statistical analysis

Data were presented as mean ± S.E. Student’s t-test was used for a comparison between two experiments. A value of \( p < 0.05 \) was considered statistically significant.
3. Results

3.1. The inhibitory abilities of TMH on the LTB and GM1 interaction

By GM1-ELISA, we found that various TMH extracts, which have ethnomedically been used for diarrheal disease, blocked the binding of LTB to GM1 (Table 1). We further divided these TMH into different taxonomic families, and their inhibitory abilities were shown in Fig. 1. In a total of 28 families, 8 families, including Campanulaceae, Convolvulaceae, Labiatae, Oleaceae, Polygonaceae, Rosaceae, Solanaceae and Umbelliferae, exhibited approximately 60% inhibitions at 1 µg/µl.

3.2. Docking analysis of the interaction between phytochemicals and LTB

We further analyzed the representative phytochemicals of these eight families by docking technology. The 3D structures of about 600 phytochemicals, which belong to 12 different kinds of structures (including monoterpenes, sesquiterpenes, iridoids, diterpenes, triterpenoids, alkaloids, quinones, flavonoids, tannins, phenylpropanoids, sterols, and others), were constructed and the interactions between phytochemicals and LTB were evaluated by docking analysis. Fig. 2A shows that several kinds of phytochemicals directly docked into the LTB. Triterpenoids were the most active phytochemicals and its average binding energy score was −10.15 ± 1.74 kcal/mol. By further analyzing the phytochemicals belonging to triterpenes, we found that oleanane-type triterpenoids presented better binding abilities than other triterpenoids, and its average binding energy score was −11.14 ± 2.21 kcal/mol (Fig. 2b). The docking data from part of oleanane-type triterpenoids were shown in Table 2. As shown in Table 2, glycyrrhizin exhibited the lowest binding energy score among the oleanane-type triterpenoids and its binding energy score was −18.03 kcal/mol.

3.3. Glycyrrhizin exhibited the anti-LT-induced diarrheal ability by blocking the binding of LTB to GM1

We further analyzed the anti-diarrheal effects of glycyrrhizin by competitive GM1-ELISA and patent mouse gut assay. Glycyrrhizin significantly blocked the binding of LTB to GM1, with the IC50 value of 3.26 ± 0.17 mM (Fig. 3). It also significantly suppressed the LT-induced fluid accumulation at 10 mM (Fig. 4). Therefore, these findings indicated that the glycyrrhizin was the likely active component for the suppression of LT-induced diarrhea.

3.4. Docking analysis of the interaction between glycyrrhizin and LTB

We further interpreted the binding sites of glycyrrhizin in LTB by docking technology. Glycyrrhizin was capable of docking into the LTB (Fig. 5). Glycyrrhizin fitted LTB very well, with the predicted binding energy score of −18.03 kcal/mol. Hydrogen bonds were formed directly between the carboxyl ketone group of glycyrrhizin and Asn98 residue of LTB. Extra hydrogen bonds were formed between the hydroxyl group of glycyrrhizin and Thr82 residue of LTB.
formed between the hydroxyl groups of glucuronic acids and the Gln49, Glu51, Val52, Gin56, Thr92, and Pro93 residues of LTB. Moreover, skeleton of oleanane and the oxane of glucuronic acids exhibited hydrophobic contacts with the Asn14, Arg55, Pro53, Gly54, Ser55, His57, Trp88, and Lys91 residues as well as with the peptide backbone of LTB.

4. Discussion

Antibiotics and antimotility agents are used to control diarrheal symptom. Antibiotics kill bacteria; however, they cannot inhibit the toxicity of bacterial toxin. Moreover, antibiotic therapy is not a viable solution because of the rapid increase of antibiotic resistance, particularly in endemic areas (Garg et al., 2000). Antimotility agents like loperamide can lessen stool frequency and volume in mild diarrhea. However, such agents are not suitable in severe diarrhea because they may cause pooling of large amounts of fluid in paralysed bowel loops (Field, 2003). Additionally, when loperamide is administrated in patients with moderately or severely dehydrated illness or bloody diarrhea, and in patients younger than 3 years old, the adverse effects of loperamide would outweigh benefits (Li et al., 2007). The most common principle of management for serious diarrhea is the fluid replacement combined with pharmacologic therapy (Ahluquist and Camilleri, 2001). Fluid and electrolyte replacement is used to replace fluid losses by the administration of sugar-electrolyte solutions; however, it does not facilitate the re-adsorption of secreted fluid and therefore does not lessen diarrhea. Because there is currently no specific prophylaxis against diarrhea caused by bacterial toxin, the discovery of agents that can block the function of toxin is important for the prevention and treatment of bacterial toxin-induced diarrhea.

To develop the agents that inhibit the toxin-induced diarrhea with specificity, we set up the LT- instead of castor oil-induced diarrheal model. Castor oil is experimentally used for the induction of diarrhea in animal model (Mascolo et al., 1992, 1993). However, the mechanism of castor oil-induced diarrhea is totally different from LT-induced diarrhea. Castor oil administration stimulates the liberation of ricinoleic acid from castor oil, results in the irritation and inflammation of the intestinal mucosa, and consequently leads to the release of prostaglandin, which in turn stimulates the intestinal motility and secretion (Pierce et al., 1971; Chitme et al., 2004). The fluid accumulation assay and patent mouse gut assay are frequently used as the toxin-induced diarrhea models (Gorbach et al., 1971; Hitotsubashi et al., 1992; Guidry et al., 1997). We used the patent mouse gut assay as the diarrheal model in this study because the induction of diarrhea is through the oral instead of intraintestinal administration of LT.

The phytochemicals derived from medicinal plants are abundant sources of biological active compounds. Many phytochemicals have been used as the basis for the development of new lead chemicals for pharmaceuticals (van Der et al., 2004). For example, quinine and artemisinin, isolated from the Cinchona bark and Artemisia annua, respectively, are the most important lead compounds against malaria (Wright, 2005). Because of increasing reliance on newer technologies, such as in silico analysis models and high-throughput screening, and their associated approaches for drug discovery, natural-product-based drug discovery has successfully and rapidly integrated rational approaches that exploit and evolve the structural diversity provided by nature (Haustedt et al., 2006). Therefore, in silico screening has shown a great promise in drug discovery because it plays an important role in identifying out lead (active) compounds from traditional medicine (Shen et al., 2003). In this study, we set up a 3D structure library of 600 phytochemicals from effective TMH and predicted the anti-toxin-binding abilities of phytochemicals by docking analysis. The docking results indicated that several kinds of phytochemicals docked into the binding site of LTB. For examples, Bupleurum chinense, Alisma orientalis, and Panax ginseng extracts blocked the binding of LTB to GM1, with the inhibitory abilities of 71.1, 69.5, and 65.0%, respectively. Their major triterpenoids Saikosaponin A (oleanane type), Alisol A (prostane type), and ginsenoside Rg2 (dammarene type) also docked into the active site of LTB, with the binding energy scores of −14.12, −10.29, and −9.99 kcal/mol, respectively. Additionally, we found that oleanane-type triterpenoids were the most effective triterpenoids in these families, except Convulvulaceae and Solanaceae (Vincken et al., 2007). Oleanane-type triterpenoids are widely distributed in many TMH and exhibit biological and pharmacological effects (Sparg et al., 2004). Many oleanane-type triterpenoids, such as β-amyrin, α-hederin and friedelin, docked into the active site of LTB and presented different binding energy scores (−8.45, −10.41, and −11.01 kcal/mol, respectively). Our previous study also demonstrated that three kinds of triterpenoids, including oleanan acid, ursolic acid and betulinic acid, dock into the active site of LTB (Chen et al., 2007a). Their binding inhibitory abilities are concentration dependent, with the IC_{50} value of 202.8 ± 47.8, 493.6 ± 100.0, and 480.5 ± 56.9 μM, respectively. These results indicated that oleanan acid (oleanane type) was more active than ursolic acid (ursane type) and betulinic acid (lupane type) in the suppression of LT and GM1 interaction. Therefore, we suggested that oleanane-type triterpenoids presented better abilities than other types of triterpenoids in the inhibition of LT and GM1 interaction.

Liquorice (radix Glycyrrhiza) is the root of several Glycyrrhiza species. Glycyrrhiza uralensis is one of the major species used in China. Liquorice has been used in China and Europe for thousands of years. It has been used for healing respiratory, gastrentestinal, cardiovascular, and genital-urinary disorders, such as asthma, cough, gastroenteritis, diarrhea, angina, and kidney stones in several ancient cultures (ChPC, 2005; Fiore et al., 2005). Glycyrrhizin is the principal component of liquorice and lots of biological, pharmacological, and toxicity effects have been demonstrated (Blumenthal et al., 2000; Wang and Nixon, 2001). For examples, glycyrrhizin exhibit anti-ulcer, anti-viral, and hepatoprotective effects. In this study, we first demonstrated that Glycyrrhiza uralensis extract and glycyrrhizin reduced the binding of LT to GM1, with the inhibitory abilities of 73.3 and 97.5%, respectively. Moreover, glycyrrhizin was capable of suppressing the LT-induced diarrhea at 10 mM. Because the exposure to glycyrrhizin compounds can induce hypermineralocorticoid-like effects in animal and human, these side effects may assuage the dehydration caused by diarrhea (Ishbrucker and Burdock, 2006). In addition to glycyrrhizin, our previous study also demonstrated that oleanolic acid exhibits anti-diarrheal effects (Chen et al., 2007a). Therefore, these findings suggested that oleanane-type triterpenoids and glycyrrhizin might be considered as lead therapeutic agents for the treatment of ETEC-induced diarrhea.

5. Conclusions

In this study, we evaluated the inhibitory abilities of TMH on the LTB and GM1 interaction by GM1-ELISA. In silico analysis model (docking technique) was used to predict the effective phytochemicals from TMH extracts. The docking results indicated that triterpenoids, especially oleanane type, presented a better binding ability to LT. Glycyrrhizin exhibited the lowest binding energy score among the oleanane-type triterpenoids. In vitro and in vivo models further showed that glycyrrhizin inhibited LT-induced diarrhea via the inhibition of GM1 and LTB interaction. Therefore, these findings suggested that an LT inhibitor, glycyrrhizin, was found from TMH by in silico, in vitro, and in vivo analysis.
Acknowledgments

This work was supported by grants from National Research Program for Genomic Medicine, National Science and Technology Program for Agricultural Biotechnology, National Science Council, Committee on Chinese Medicine and Pharmacy, Department of Health (CMCP96-RD-201, CMCP97-RD-201), and China Medical University (CMU97-CMC-004 and CMU97-064), Taiwan.

Appendix A. Supplementary data


References


