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Anti-SARS coronavirus 3C-like protease effects of *Isatis indigotica* root and plant-derived phenolic compounds

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Abstract

The 3C-like protease (3CL\(_{\text{pro}}\)) of SARS-coronavirus mediates the proteolytic processing of replicase polypeptides 1a and 1ab into functional proteins, becoming an important target for the drug development. In this study, *Isatis indigotica* root extract, five major compounds of *I.* _indigotica_ root, and seven plant-derived phenolic compounds were tested for anti-SARS-CoV 3CL\(_{\text{pro}}\) effects using cell-free and cell-based cleavage assays. Cleavage assays with the 3CL\(_{\text{pro}}\) demonstrated that IC\(_{50}\) values were in micromolar ranges for *I.* _indigotica_ root extract, indigo, sinigrin, aloe emodin and hesperetin. Sinigrin (IC\(_{50}:\ 217\ pm\)M) was more efficient in blocking the cleavage processing of the 3CL\(_{\text{pro}}\) than indigo (IC\(_{50}:\ 752\ pm\)M) and beta-sitosterol (IC\(_{50}:\ 1210\ pm\)M) in the cell-based assay. Only two phenolic compounds aloe emodin and hesperetin dose-dependently inhibited cleavage activity of the 3CL\(_{\text{pro}}\), in which the IC\(_{50}\) was 366\ pmM for aloe emodin and 8.3\ pmM for hesperetin in the cell-based assay.

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Keywords: SARS-coronavirus; 3C-like protease; *Isatis indigotica* root; Phenolic compounds

Severe acute respiratory syndrome (SARS) was reported in 8447 cases with 811 deaths worldwide from February to June 2003 (Poutanen et al., 2003; Peiris et al., 2003; Drosten et al., 2003). A novel coronavirus, SARS-coronavirus (SARS-CoV) was identified as the etiological agent of the disease (Ksiazek et al., 2003; Lee et al., 2003; Tsang et al., 2003; Hsueh et al., 2003). SARS-CoV particle contains a single positive-stranded RNA genome encoding for replicase, spike, envelope, membrane, and nucleocapsid (Lai, 2003; Enjuanes et al., 2001; Holmes, 2003). The SARS-CoV 3CL\(_{\text{pro}}\) mediates the proteolytic processing of replicase polypeptides into functional proteins, playing an important role in viral replication. Therefore, the SARS-CoV 3CL\(_{\text{pro}}\) can be considered an attractive target for developing effective drugs against SARS. Several potential 3CL\(_{\text{pro}}\) inhibitors with a 50% inhibitory concentration (IC\(_{50}\)) below 10\ pmM were identified from the large number of the structurally diverse small molecules (Kao et al., 2004; Hsu et al., 2004). *Isatis indigotica* root and phenolic Chinese herbs were frequently used for the prevention of SARS during the SARS outbreaks in China, Hong Kong, and Taiwan. *I.* _indigotica_ root (Radix isatidis), belonging to the family Cruciferae, is native to China. Antiviral effects of *I.* _indigotica_ root were found against influenza, hepatitis A and Japanese encephalitis (Qin and Xu, 1998; Wu et al., 1997). *I.* _indigotica_ root contains indigo, indirubin, indican (indoxyl-d-glucoside), beta-sitosterol, gamma-sitosterol, sinigrin, etc. (Gilbert et al., 2004). Indigo and indirubin were identified as the promiscuous chymotrypsin inhibitors (McGovern and Shoichet, 2003).
Recently, an anti-influenza virus effect of indirubin has been demonstrated (Mak et al., 2004). In addition, several herb-derived phenolics, aloeemodin, hesperetin, quercetin, and naringenin have been accredited with antiviral effects against poliovirus, vesicular stomatitis virus, Sindbis virus, herpes simplex virus types 1 and 2, parainfluenza virus, and vaccinia virus (Semple et al., 2001; Andersen et al., 1991; Paredes et al., 2003; Kim et al., 2001).

In this study, we characterized the anti-SARS-CoV 3CL\textsuperscript{pro} effect of the water extract of \textit{I. indigotica} root, \textit{I. indigotica} root-derived compounds, and herb-derived phenolics using a cell-free cleavage and cell-based cleavage assay.

The root of \textit{I. indigotica} was purchased from Sun Ten Pharmaceutical Corporation (Taiwan). The plant root of \textit{Isatis indigotica} was extracted twice with 10 volumes of distilled boiling water for 1 h. The aqueous extract was concentrated under the reduced pressure at 50 °C, passed through 0.22-µm filters for sterilization, and diluted in culture medium to make a stock concentration of 10 mg/ml. Indigo and indirubin were kindly provided by Dr. Yuan-Shiun Chang, professor for Institute of Chinese Pharmaceutical Sciences, China Medical University. Indican (indoxyl-β-glucoside), β-sitosterol, sinigrin, aloe emodin, hesperetin, quercetin, naringenin, daidzein, emodin, and chrysophanol were purchased from Sigma Chemical.

To examine the \textit{trans}-cleavage of SARS-CoV 3CL\textsuperscript{pro} in the cell-free assay, recombinant 3CL\textsuperscript{pro} was expressed in \textit{E. coli} and purified using the HisTrap Kit (Amersham) as described in our previous report (Lin et al., 2004). Coomassie Blue-staining revealed that recombinant 3CL\textsuperscript{pro} contained a major 34-kDa band for the monomer and a minor 68-kDa band for the dimer (Fig. 1A, lane 2). The cleavage substrate (TVRLQAGNATE) was generated as the substrate fusion protein with the N-terminal S-Tag and the C-terminal HSV-Tag. In the cell-free cleavage assay, the substrate fusion protein that was captured by anti-HSV-Tag antibodies in wells incubated with soluble 3CL\textsuperscript{pro} for 3 h at 37 °C. The non-cleavage substrate protein was detected by an Enzyme Linked Immunosorbent Assay (ELISA) using peroxidase-conjugated S protein. ELISA showed that cell-free proteolytic activity correlated, in concentration-dependent manner, with the serial twofold dilution of recombinant 3CL\textsuperscript{pro} protein in the range from 15 µg/ml to 240 µg/ml (Fig. 1B). Subsequently, the anti-3CL\textsuperscript{pro} effect by the extract of \textit{I. indigotica} root was evaluated using the cell-free cleavage assay.

The cell-free cleavage assay indicated that the extract of \textit{I. indigotica} root had a dose-dependent anti-3CL\textsuperscript{pro} effect with an IC\textsubscript{50} of 53.8 ± 4.2 µg/ml (Fig. 2; Table 1).

The cell-based cleavage assay of 3CL\textsuperscript{pro} for screening inhibitors does not require purification of the active 3CL\textsuperscript{pro},
Table 1
The inhibitory effect on cell-free and cell-based cleavage activity of the SARS-CoV 3CLpro

| Compound         | Structure | IC₅₀a of cell-free cleavage (µg/ml) | IC₅₀a of cell-based cleavage (µg/ml) | CC₅₀b of cell death (µg/ml) |
|------------------|-----------|------------------------------------|-------------------------------------|-----------------------------|
| Isatis indigotica root | ![Structure](image1) | 53.8 ± 4.2                         | 191.6 ± 8.2                        | >5000                       |
| Indigo           | ![Structure](image2) | 37.3 ± 8.1 (500 µM)                | 190 ± 2.6 (732 µM)                 | 917 ± 18 (7375 µM)          |
| Indirubin        | ![Structure](image3) | 81.3 ± 5.2 (209 µM)                | NS                                 |
| Indican          | ![Structure](image4) | 33.1 ± 1.2 (112 µM)                | NS                                 |
| Sinigrin         | ![Structure](image5) | 50.3 ± 1.5 (121 µM)                | 90.1 ± 4.2 (217 µM)               | >5000 (>10,000 µM)          |
| Beta-sitosterol  | ![Structure](image6) | 47.8 ± 8.6 (115 µM)                | 502.1 ± 2.9 (1210 µM)              | 613 ± 9 (1475 µM)           |
| Aloeemodin       | ![Structure](image7) | 35.7 ± 1.5 (132 µM)                | 99.1 ± 2.1 (366 µM)               | 3135 ± 9 (11,592 µM)       |
| Hesperetin       | ![Structure](image8) | 18.1 ± 0.6 (60 µM)                 | 2.5 ± 0.8 (8.3 µM)                 | 820 ± 15 (2718 µM)          |
| Daidzein         | ![Structure](image9) | 26.8 ± 1.2 (105 µM)                | NS                                 |

a IC₅₀ (50% inhibitory concentration) was the concentration requiring for 50% inhibition on the cis-cleavage activity of 3CLpro.

b CC₅₀ (50% cytotoxic concentration) was the concentration giving half the OD₅₇₀–₆₃₀ of mock cells in MTT assay. IC₅₀ and CC₅₀ were determined using a computer program based on Fisher’s statistical model.

c Not significant.
Fig. 2. Inhibition of the cell-free cleavage of the 3CLpro by the *Isatis indigotica* root extract. The extract of the *I. indigotica* root was added into the mixture of the substrate fusion protein and the 3CLpro, and then incubated at room temperature for 3 h. The non-cleavage of substrate fusion protein was detected using the S protein-HRP conjugate and ABTS/H2O2 substrates. The ELISA product was measured at A405 nm. The relative inhibition of cell-free cleavage activity was calculated as 1 - (A405no 3CLpro - A4053CLpro with inhibitor) / (A405no 3CLpro - A4053CLpro).

and represents closely the natural physiological state. Therefore, we used the cell-based cleavage assay for examining the inhibitory efficacy of the 3CLpro inhibitors. For the cell-based cleavage assay, the in-frame construction of the 3CLpro, the substrate, and the luciferase, designed as the plasmid pcDNA3.1-3CLpro-S-Luc, was co-transfected with the indicated vector pEGFP-N1 into Vero cells. The stable cell clone for the expression of the 3CLpro-substrate-luciferase fusion protein was selected by Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 800 μg/ml of neomycin G418 (Fig. 3A). Since a more than 30 kDa protein fused at the N-terminus of the luciferase resulted in a dramatic decrease of luciferase activity (Joubert et al., 2000), the detection of luciferase activity could be considered as a measure for the cis-cleavage by the SARS-CoV 3CLpro. Western blotting with the anti-luciferase monoclonal antibody showed a 94-kDa band for the fusion protein 3CLpro-S-Luc and a 60-kDa band for the luciferase in Vero cells transfected with the plasmid pcDNA3.1-3CLpro-S-Luc (data not shown).

The relative luciferase activity in the transfected cells was subsequently measured using the dual Luciferase Reporter Assay System (Fig. 3B). In the cell-based cleavage assay, the extract of *I. indigotica* root significantly inhibited the cis-cleavage activity of the SARS-CoV 3CLpro with an IC50.
higher than the IC50 value from the cell-free assay. The reason may be the inhibition of the 3CLpro by the compounds of beta-sitosterol, 121 and indigo, indirubin, indican, sinigrin, and beta-sitosterol.

The in vitro cytotoxicity profile of the I. indigotica root extract was examined using Vero cells. Vero cells in MEM medium with 10% FBS were plated in 96-well plates at 5 mg/ml was added to each well and incubated at 37°C for 3 h. After a three-time washing of phosphate buffer saline, 100 μl of a MTT solution were also tested for their inhibitory effects on the SARS-CoV 3CLpro (Fig. 6; Table 1). In the cell-free assay, the IC50 values were 132 μM for aloe emodin and 60 μM for hesperetin. Quercetin has been reported to block the entry effects on quinine reductase and glutathione S-transferase, antiprofenerative effects against cancer cells, and antimicrobial activity against Bacillus subtilis and Saccharomyces cerevisiae (Brabban and Edwards, 1995; Munday and Munday, 2002; Smith et al., 2004). This study is the first report that sinigrin significantly blocks the cleavage processing of a viral protease.

Seven phenolic compounds, aloesinodin, hesperetin, quererin, naringenin, daidzein, emodin, and chrysophanol were also tested for their inhibitory effects on the SARS-CoV 3CLpro (Fig. 6; Table 1). Only two of the phenolic compounds, aloesinodin and hesperetin dose-dependently inhibited cleavage activity of the 3CLpro in cell-free and cell-based assays (Fig. 6; Table 1). In the cell-free assay, the IC50 values were 132 μM for aloesinodin and 60 μM for hesperetin. Quercetin has been reported to block the entry effects on quinine reductase and glutathione S-transferase, antiprofenerative effects against cancer cells, and antimicrobial activity against Bacillus subtilis and Saccharomyces cerevisiae (Brabban and Edwards, 1995; Munday and Munday, 2002; Smith et al., 2004). This study is the first report that sinigrin significantly blocks the cleavage processing of a viral protease.

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et al., 2003). Of the compounds tested, hesperetin was the most potent inhibitor of SARS-CoV 3CL\(^{pro}\) (Table 1).

Our results have demonstrated significantly inhibitory effects on SARS-CoV 3CL\(^{pro}\) by \(I\). indigotica root extract, indigo, sinigrin, aloeemodin and hesperetin in the micromolar range. Particularly, the cell-based assay demonstrated that hesperetin (IC\(_50\)) 8.3 \(\mu\)M and sinigrin (IC\(_50\)) 217 \(\mu\)M could be potential inhibitors of SARS-CoV 3CL\(^{pro}\). In addition, sinigrin and hesperetin with a CC\(_{50}\) of over 2 mM were considerably less cytotoxic to Vero cells (Table 1). Akin to other reported anti-SARS substances, such as glycyrrhizin (Cinatl et al., 2003a), nelfinavir (Yamamoto et al., 2004), interferon (Cinatl et al., 2003b), the compounds reported here may be considered as potential leads in the development of inhibitors of SARS-CoV 3CL\(^{pro}\).

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