Inhibition of enterovirus 71 replication by chrysosplenetin and penduletin

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ABSTRACT

In recent years, enterovirus 71 (EV71) infections have caused an increasing epidemic in young children, accompanying with more severe nervous system disease and more deaths. Unfortunately, there is no specific medication for it so far. Here we investigated the anti-EV71 activity of chrysosplenetin and penduletin, two O-methylated flavonols isolated from the leaves of Laggera pterodonta. These two compounds were found to have strong activity in vitro against EV71 with low cytotoxicity. In the cytopathic effect (CPE) inhibition assays, both plaque reduction assay and virus yield inhibition assay, the compounds were found to have strong activity in vitro against EV71 with low cytotoxicity. In the cytopathic effect (CPE) inhibition assays, both plaque reduction assay and virus yield inhibition assay, the compounds showed a similar 50% inhibitory concentration (IC50) value of about 0.20 μM. The selectivity indices (SI) of chrysosplenetin and penduletin were 107.5 and 655.6 in African green monkey kidney (Vero) cells, and 69.5 and 200.5 in human rhabdomyosarcoma (RD) cells, accordingly. The preliminary mechanism analysis indicates that they function not through blocking virus entry or inactivating virus directly but inhibiting viral RNA replication. In the time-of-addition assay, both compounds inhibited progeny virus production and RNA replication by nearly 100% when introduced within 4 h post infection. In addition to EV71, both compounds inhibited several other human enteroviruses with similar efficacy. These findings provide a significant lead for the discovery of anti-EV71 drug.

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1. Introduction

Human enteroviruses belong to the Enterovirus genus of the Picornaviridae family and comprise a major subgroup of small RNA(-) viruses (Palacios and Oberste, 2005), which includes polioviruses, coxsackieviruses, echoviruses, and enteroviruses. Most of these viruses establish infection via the intestinal epithelial tract and lymphoid tissues, and cause paralytic disease, aseptic meningitis, enanthems, myocarditis, pericarditis, and nonspecific febrile illness. Enterovirus 71 (EV71) infections frequently manifest as hand, foot, and mouth disease, as well as encephalitis in infants and young children. EV71 can even cause severe central nervous system disease, complications, and fatalities (Huang et al., 1999; Lee et al., 2009; Solomon et al., 2010). Since it was first reported in 1974 (Schmidt et al., 1974), EV71 has been implicated in several outbreaks worldwide, especially in the Asia-Pacific region (Ooi et al., 2010; Weng et al., 2010). According to the latest reports from the Chinese Ministry of Health, there were over 1 million reported cases of EV71 infection in China in 2010, including 15,000 severe cases and more than 600 fatalities (Ministry of Health of the People's Republic of China, 2010).

At present, there is no specific treatment available for EV71 (Ooi et al., 2010). In the face of frequent outbreaks and the fatal consequences of EV71 infection, antiviral drugs for EV71 are urgently needed. Previous studies indicate that ribavirin and Type I interferon reduces mortality in EV71-infected mice (Liu et al., 2005; Li et al., 2008). Pleconaril, a Sterling-Winthrop (WIN) compound targeting viral protein 1 (VP1), is a successful clinical candidate for most enteroviruses, but not EV71 (Pevear et al., 1999; Shia et al., 2002; Florea et al., 2003; De Palma et al., 2008). On the other hand, pyridyl imidazolidinones derived from WIN compound templates show promising anti-EV71 activity in tissue cultures (Shia et al., 2002; Chen et al., 2009).

Laggera pterodonta has been used as traditional medicine in China for several centuries for its anti-inflammatory and antibacterial properties (Jiangsu New Medical College, 1977). L. pterodonta belongs to Compositae and is widely distributed in Southwestern China, especially in Yunnan and Sichuan Provinces. A number of chemical constituents have been isolated from this plant, mainly flavones and triterpenes (Liu et al., 2010). Chrysosplenetin and penduletin are two known flavonols that have been isolated from the leaves of L. pterodonta (Li and Ding, 1994). Besides penduletin has been reported to have anti-poliovirus activity and anti-inflamm
matory activity (De Meyer et al., 1991; Moscatelli et al., 2006), very little is known of the medicinal properties of these two flavonols.

In this study, the anti-EV71 activity of chrysosplenetin and penduletin were determined in African green monkey kidney (Vero) cells and human rhabdomyosarcoma (RD) cells using an EV71 infection-induced cytopathic effect (CPE) assay and a plaque reduction assay. And their modes of action were also preliminarily determined. In addition to the anti-EV71 studies, the antiviral activity of these two compounds against other enteroviruses was investigated.

2. Materials and methods

2.1. Cells and viruses

Vero cells (ATCC, Accession No. CCL-81) and RD cells (China Center for Type Culture Collection, Accession No. TCHu45) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal calf serum (FCS). EV71 (GZ-08-02 strain, GenBank Accession No. FJ360545) (Ding et al., 2009), enterovirus 84 (EV84), enterovirus 11 (EV11), coxsackievirus B3 (CVB3), coxsackievirus A10 (CVA10), and coxsackievirus A16 (CVA16) were isolated and identified by the Guangzhou Children’s Hospital, Guangzhou, China. These viruses were propagated and titrated in both Vero and RD cells.

2.2. Extraction and chemicals

Chrysosplenetin and penduletin were isolated from L. pterodonta. Fresh leaves of L. pterodonta (150 g) were crushed and placed in Me₂CO at 4 °C for 48 h. The Me₂CO extracts were evaporated in vacuo to yield a viscous residue extract, which was then partitioned with petroleum ether into petroleum ether and H₂O layers. The petroleum ether layer (648 mg) was subjected to medium pressure liquid chromatography with RP-18 CC (MeOH–H₂O, 10:1, 8:1). Chrysosplenetin and penduletin were finally identified by comparison with previously reported spectroscopic and physical data (Shun-Lin and Jing-Kai, 1994). Chrysosplenetin and penduletin were dissolved in dimethyl sulfoxide (DMSO). Ribavirin (Sigma) was dissolved in distilled water.

2.3. Cytotoxicity assay

The cytotoxicity of the compounds were evaluated in Vero and RD cells as previously described (Nociari et al., 1998). Briefly, the Vero or RD cells (1.5 x 10⁴ cells/well) were seeded into a 96-well plate. After 24 h, the medium was replaced with fresh medium (2% FCS) and a twofold serial dilution of the compound. After another 72 h, the culture medium was replaced with fresh medium with 2% FCS and 10% alamarBlue (AbD Serotec). After incubation at 37 °C for 2–3 h, the fluorescence of reduced alamarBlue was measured at a 530 nm excitation wavelength and a 590 nm emission wavelength using a microtiter plate reader (Synergy HT, BioTek). The cytotoxicity was determined with the equation:

\[ \text{Cytotoxicity} \%(%) = \frac{FU_{\text{ST}} - FU_{\text{T}}}{FU_{\text{ST}}} \times 100\% \]

where FU represents the mean fluorescence units for the triplicate, subscript ST indicates solvent-treated cells, and T indicates the testing compound-treated cells. The concentration of 50% cellular cytotoxicity (CC₅₀) of each testing compound was calculated using the Forecast function of Microsoft Excel (Pirrone et al., 2010).

2.4. CPE inhibition assay

The CPE induced by EV71 infection was measured in Vero and RD cells with alamarBlue assay. Confluent Vero or RD cells in a 96-well plate were inoculated in triplicate with the mixture of 100 × the 50% tissue culture infectious dose (TCID₅₀) of EV71 and serially diluted compounds in DMEM supplemented with 2% FCS. The plate was incubated at 37 °C for 72–96 h, after which cell survival was quantified using alamarBlue. The degree of CPE inhibition was determined after compound cytotoxicity was normalized using the following formulae:

\[ nFU_{\text{I}/\text{T}} = \frac{FU_{\text{I}/\text{T}}}{FU_{\text{I}/\text{U}}} \times 100\% \]

where nFU and FU represent the normalized fluorescence units and mean fluorescence units for the triplicate, respectively; subscript T/I indicates the testing compound-treated infected cells; UT/UI indicates untreated and uninfected cells; and UI/UI indicates untreated but infected cells.

The CPE induced by other enteroviruses was measured in Vero cells with classical MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) assay (Takeuchi et al., 1991). Briefly, confluent Vero cells in a 96-well plate were inoculated in triplicate with 100 × TCID₅₀ of EV84, EV11, CVB3, CVA10, or CVA16 mixed with the serially diluted testing compounds. After incubation at 37 °C for 72–96 h when the viral control cells showed complete CPE, 20 μl of MTT (5 mg/ml) was added to each well. The plate was then incubated at 37 °C for 2 h. And the formazan crystals were finally solubilized with 100 μl of acidified isopropanol containing 10% (v/v) Triton X-100. The A490 of the well was measured with a microtiter plate reader (Synergy HT, BioTek). The degree of CPE inhibition was calculated with the formula similar to that described above.

The 50% inhibitory concentration (IC₅₀) of the testing compound was calculated using the Forecast function of Microsoft Excel (Pirrone et al., 2010).

2.5. Plaque reduction assay

Vero cells were seeded into 24-well plates at a density of 10⁵ cells/well and incubated for 24 h to form monolayers. The cell monolayers were then inoculated with EV71 at 40–80 plaque forming units per well (PFU/well) and mixed with or without the serially diluted testing compounds. After incubation at 37 °C for 2 h, the inoculum was replaced with medium containing testing compounds at the corresponding concentrations, with 2% FCS and 1% methycellulose. The plate was incubated at 37 °C for 96 h until plaques appeared. The cells were fixed for 5 min with 10% formaldehyde and stained for 30 min with 0.05% crystal violet. The IC₅₀ of the plaque was determined.

2.6. Progeny EV71 virus yield assay

Confluent Vero cells in a 96-well plate were inoculated in triplicate with 100 × TCID₅₀ of EV71 mixed with or without the serially diluted testing compounds. After incubation at 37 °C for 96 h when the peak titer was reached, the culture medium and cell lysate were collected following three freeze–thaw cycles and then subjected to virus titration through a plaque-forming assay.

2.7. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis

Vero cells were seeded into a 12-well plate at a density of 3 x 10⁵ cells/well and grown overnight. The cells were then
inoculated with EV71 at multiplicity of infection (MOI) of 1. After allowing for adsorption at 37 °C for 1 h, the infected cells were washed once, and the medium containing 2% FCS and the serially diluted testing compounds were added. After incubation for 24 h, the cells were harvested and lysed using 100 μl of RIPA Lysis Buffer (Beyotime, China). After centrifugation, a 15 μl aliquot of each lysate was subjected to SDS—PAGE. Finally, the proteins were probed with anti-VP1 monoclonal antibodies (5C3, this study) and anti-GAPDH antibodies (Kangchen, China), respectively. Goat anti-mouse immunoglobulin G-peroxidase (ThermoFisher, USA) was then added as the secondary antibodies, and bands were visualized on X-ray film after the addition of ECL chemiluminescent substrate (Millipore, USA).

2.8. Effective stage analysis

Vero cell monolayers in 96-well plates were precooled at 4 °C for 10 min, and then the assay was continued using one of the following procedures:

(a): The cells were infected with 500× TCID₅₀ of EV71 for 1.5 h at 4 °C, followed by washing and replacement with the testing compound for 1.5 h at 4 °C. The plate was then incubated at 37 °C for 120 h. The assay was completed by measuring cellular survival using alamarBlue method.

(b): The testing compound was added to cells for 1.5 h at 4 °C, followed by washing and replacement with 500× TCID₅₀ of EV71 for 1.5 h at 4 °C. The cells were then washed with the medium and transferred to 37 °C for 120 h. The assay was completed by measuring cellular survival using the alamarBlue method.

(c): The plate was pre-cooled to 4 °C. At the same time, 500× TCID₅₀ of EV71 and the testing compound were mixed and incubated at 4 °C for 1.5 h. The mixture was then added and incubated with the pre-cooled plate at 4 °C for another 1.5 h. Subsequently, the plate was washed with the medium and shifted to 37 °C for 120 h. The assay was completed by measuring cellular survival using the alamarBlue method (Zhu et al., 2010).

2.9. Time-of-addition assay

Confluent Vero cells in a 96-well plate were inoculated in triplicate with EV71 (MOI = 1) at 37 °C. After 1 h, the infected cells were washed and treated with testing compound at different time points. At 16 h post-infection (p.i.), the cells were washed with PBS and harvested into two cell parts. One part was subjected to three freeze—thaw cycles in 200 μl compound-free medium, after which the viruses in the frozen-thawed cells were titrated with plaque assay. The other cell part underwent RNA extraction using QIAamp viral RNA mini kit (Qiagen, Germany) according to the manufacturer’s instructions. RNA was diluted with 50 μl DEPC-H₂O and frozen at −80 °C until quantification by real-time reverse transcriptase polymerase chain reaction (RT-PCR).

2.10. RT-PCR quantification of EV71 RNA

RNA samples from time-of-addition experiment were first subjected to cDNA synthesis with AMV reverse transcriptase (Fermentas, USA). The quantity of EV71 cDNA for each sample was then determined through the VP1-specific paired primers, RT-EV71-s (5′-CACACAGGTAGAGCATGAC-3′) and RT-EV71-a (5′-GTCTCAA TCATGCTCTCTGACT-3′), which was done with the kit of SYBRs Premix Ex Taq™ (Takara Bio, Japan) in CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). β-Actin was used as the normalizing gene (β-actin-F 5′-CTCTCTCTGACGCAAGTACTC-3′ and β-actin-R 5′-TCTGTGCTGCTGATCCACATC-3′). The specificity of the RT-PCR was examined by generating a dissociation curve after amplification. The relative folds of EV71 RNA were calculated according to the normalized threshold cycle (Ct) value.

3. Results

3.1. Chrysospleninetin and penduletin inhibit CPE induced by EV71 infection

The structure of chrysospleninetin and penduletin are shown in Fig. 1. To evaluate the anti-EV71 activity of these compounds, the inhibitory effect on CPE induced by the viral infection was examined on both Vero and RD cells using a cell viability assessment-based method. The cells were infected with 100× TCID₅₀ of EV71 with the testing compounds. The degree of CPE was detected with an alamarBlue assay, which quantified the metabolically active cells. This assay was performed when the untreated and infected control cells were 100% cytopathic. To minimize the cytotoxicity of the testing compound, its cytotoxicity was normalized before the calculation of inhibitive activity. The cytotoxicity of these compounds on Vero and RD cells were tested using the standard alamarBlue method. As shown in Table 1, chrysospleninetin has a CC₅₀ of 18.27 μM on Vero cells and 13.9 μM on RD cells, whereas penduletin has much higher CC₅₀ values (111.46 μM on Vero and 74.18 μM on RD). Consistent with previous reports, ribavirin, a compound with broad-spectrum antiviral activity, showed very low cytotoxicity in the assays. Compared with ribavirin (IC₅₀ = 490.75 μM, SI = 8.7), both chrysospleninetin (IC₅₀ = 0.20 μM, SI = 69.5) and penduletin (IC₅₀ = 0.37 μM, SI = 200.5) showed more potent inhibitory activity against EV71 in RD cells. Based on SI values, penduletin (Vero: SI = 655.6; RD: SI = 200) has higher anti-EV71 activity than chrysospleninetin (Vero: SI = 107.5; RD: SI = 69.5).

Aside from the alamarBlue-based assay, a plaque reduction assay was also performed to measure the inhibitory effects of these compounds against specific EV71 infection-induced CPE. In general, viral infectivity and the rate of replication are reflected by both the number and the size of plaques in a plaque assay. As indicated in Fig. 2, the number of plaques of EV71-infected cells markedly decreased with increasing chrysospleninetin and penduletin concentrations, with IC₅₀ of 0.14 and 0.20 μM, respectively. Compared with the untreated control, there was significant reduction in the size of the plaques in all treated groups (Student’s t-test, p < 0.01, data not shown). These results suggest that both chrysospleninetin and penduletin could effectively inhibit EV71 infection and replication at non-cytotoxic concentrations.

3.2. Chrysospleninetin and penduletin inhibit the production of EV71 progeny virus

To evaluate the effect of chrysospleninetin and penduletin on EV71 multiplication, a progeny virus yield assay was performed. Vero cells were infected with 100× TCID₅₀ of EV71 in the presence of chrysospleninetin or penduletin. Total viral yields at 96 h p.i. were detected using a plaque assay. As shown in Fig. 3, the total viral yields were greatly reduced in a dose-dependent manner. Almost...
100% reduction was observed when the chrysosplenetin or penduletin concentration was higher than 1.25 μM. Similar inhibitory effects were also observed in VP1 production (Fig. 4), which further confirmed that both chrysosplenetin and penduletin strongly inhibit the production of EV71 progeny virus.

3.3. Chrysosplenetin and penduletin target the post-attachment stage

The results generated from the experiments show that chrysosplenetin and penduletin could efficiently inhibit EV71 replication. During the preliminary investigation of potential inhibitory mechanisms, a modified effective stage analysis was performed to determine the stages of EV71 replication cycle targeted by these compounds (Zhu et al., 2010). The assays were performed using three different procedures, and the effects were measured with an alamarBlue assay. In Procedure (a), the cells were first exposed to EV71 at 4°C to allow virus attachment to cells. The unattached viruses were then removed and the testing compound was added. A positive effect indicates that the compound is most likely targeting the post-attachment steps of the viral exposure. Fig. 5 demonstrates that both chrysosplenetin and penduletin have dose-dependent inhibitory effects against EV71 infection in Procedure (a). To further confirm that these two compounds were functioning at the post-attachment stage, two pre-attachment inhibition assays were performed. In Procedure (b), the cells were exposed to the testing compound before viral exposure. A positive effect suggests the compound is targeting the pre-attachment functions. On the other hand, Procedure (c) involved incubation with the compound prior to cell addition. A positive result in this procedure suggests that the compound could directly inactivate the virus. For chrysosplenetin (Fig. 5A) and penduletin (Fig. 5B), only negative results were observed in Procedures (b) and (c), suggesting that both chrysosplenetin and penduletin inhibit EV71 by targeting the post-attachment stage of the viral infection.

To further understand the mechanism of action of chrysosplenetin and penduletin against EV71, a time-of-addition experiment was performed. Chrysosplenetin or penduletin at an excess concentration was added to the Vero cells at different time points after EV71 infection (0, 2, 4, 6, 8, 12, and 14 h p.i.). At 16 h p.i., the total virus yield was determined with a plaque assay. Both chrysosplenetin and penduletin inhibited progeny virus production by nearly 100% when added within 4 h p.i. (Fig. 6A). A significant increase in virus yield (58% and 41%, respectively) was observed when chrysosplenetin and penduletin were added 8 h p.i. Similar virus yields were sustained when chrysosplenetin or penduletin was added from 8 to 14 h p.i.

The results imply that chrysosplenetin and penduletin mainly acts on the early post-attachment stage of EV71 replication, which occurs within 4 h p.i.

### Table 1

<table>
<thead>
<tr>
<th>Testing compound</th>
<th>Vero&lt;sup&gt;a&lt;/sup&gt; (μM)</th>
<th>RD&lt;sup&gt;a&lt;/sup&gt; (μM)</th>
<th>Vero&lt;sup&gt;b&lt;/sup&gt; (μM)</th>
<th>RD&lt;sup&gt;b&lt;/sup&gt; (μM)</th>
<th>SI&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysosplenetin</td>
<td>18.27 ± 2.4</td>
<td>13.90 ± 6.7</td>
<td>0.17 ± 0.13</td>
<td>0.20 ± 0.01</td>
<td>107.5</td>
</tr>
<tr>
<td>Penduletin</td>
<td>111.46 ± 9.9</td>
<td>74.18 ± 25.7</td>
<td>0.17 ± 0.12</td>
<td>0.37 ± 0.01</td>
<td>655.6</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>&gt;20,000</td>
<td>4304.83 ± 374.3</td>
<td>108.6 ± 25.50</td>
<td>490.75 ± 13.6</td>
<td>&gt;184</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cytotoxicity (CC<sub>50</sub>) and antiviral activity (IC<sub>50</sub>) were determined by alamarBlue method on Vero and RD cells.

<sup>b</sup> Values represent the mean ± SD of three independent experiments.

<sup>c</sup> Selectivity Index (SI) is the ratio of CC<sub>50</sub> to IC<sub>50</sub>

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**Fig. 3.** Effect of chrysosplenetin and penduletin on the production of EV71 progeny virus. Vero cells were infected with 100 TCID<sub>50</sub> of EV71 in the presence of chrysosplenetin or penduletin. The total viral yields at 96 h p.i. were then detected using a plaque assay. The ratio of total viral yield in the treated group to that in the untreated control was calculated. The results are from three independent experiments.

**Fig. 4.** Effect of chrysosplenetin and penduletin on VP1 expression. Vero cells in 12-well plates were infected with EV71 at a MOI of 1. After 24 h, the cells were harvested and lysed. A 15 μl aliquot of each lysate was subjected to VP1 expression analysis through SDS–PAGE and Western blotting. The bands were visualized under an X-ray.

**Fig. 5.** Effect of chrysosplenetin and penduletin on EV71 plaque formation. Vero cells in 24-well plates were inoculated with EV71 mixed with or without serially diluted chrysosplenetin or penduletin. Four days later, the cells were fixed and the plaques were counted. The ratio of the number of plaques in the treated group to that in the untreated control was calculated. Data are expressed as mean ± SD of results from three independent experiments.

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**Fig. 6.** Effect of chrysosplenetin and penduletin on EV71 plaque formation. Vero cells in 24-well plates were inoculated with EV71 mixed with or without serially diluted chrysosplenetin or penduletin. Four days later, the cells were fixed and the plaques were counted. The ratio of the number of plaques in the treated group to that in the untreated control was calculated. Data are expressed as mean ± SD of results from three independent experiments.
and penduletin on viral RNA replication, a time-of-addition experiment was performed and the EV71 RNA of infected cells was quantified using two-step RT-PCR. Similar to the inhibition of virus production, both chrysosplenetin and penduletin inhibited EV71 RNA replication by nearly 100% when added before 4 h p.i. (Fig. 6B). The inhibitory effect diminished when they were at 8–12 h p.i. These findings suggest that chrysosplenetin and penduletin could efficiently inhibit EV71 RNA replication during the early stages of the viral infection.

3.5. Chrysosplenetin and penduletin inhibit the infectivity of a broad spectrum of human enteroviruses

The human enteroviruses include a number of viruses with similar genome and viral structures. To determine whether chrysosplenetin and penduletin also inhibit other enteroviruses, their effects against EV84, EV11, CVB3, CVA10, and CVA16 were examined. As summarized in Table 2, both chrysosplenetin and penduletin effectively inhibited the CPE induced by infection of these viruses with similar IC50 to that observed for EV71. Therefore, chrysosplenetin and penduletin are effective inhibitors of enteroviruses, and have a broad spectrum of antiviral activity.

4. Discussion

Chrysosplenetin and penduletin are two 4-methylated flavonols. Aside from L. pterodonta, they are also found in other plants such as Artemisia rupestris (Song et al., 2006). Flavonols are widely present in nature and have various bioactivities. However, very little is known about the bioactivity of chrysosplenetin and penduletin. In the current study, chrysosplenetin and penduletin extracted from L. pterodonta were demonstrated to possess potent antiviral activity against human enteroviruses in cell cultures. Furthermore, the evidence indicates that these two compounds function in post virus attachment during the early stages of virus infection, and could efficiently inhibit viral RNA replication.

In the current study, two EV71 infection systems, Vero cell- and RD cell-based systems, were used to evaluate the anti-EV71 effect of chrysosplenetin and penduletin. Chrysosplenetin and penduletin showed effective inhibition against EV71 infection in both infection systems, suggest that such antiviral activities are not cell type dependent. The EV71 infection-induced CPE was measured through two different methods. The alamarBlue assay measures the levels of cellular metabolism after viral infection and compound introduction. The plaque reduction assay, on the other hand, allows specific measurement of virus infection-induced cell death.

Table 2

<table>
<thead>
<tr>
<th>Virus</th>
<th>IC50 (μM)</th>
<th>Chrysosplenetin</th>
<th>Penduletin</th>
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<tbody>
<tr>
<td>EV84</td>
<td>0.232 ± 0.119</td>
<td>0.129 ± 0.076</td>
<td></td>
</tr>
<tr>
<td>CVB3</td>
<td>0.253 ± 0.043</td>
<td>0.305 ± 0.091</td>
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</tr>
<tr>
<td>EV11</td>
<td>0.401 ± 0.192</td>
<td>0.348 ± 0.113</td>
<td></td>
</tr>
<tr>
<td>CVA10</td>
<td>0.276 ± 0.133</td>
<td>0.173 ± 0.055</td>
<td></td>
</tr>
<tr>
<td>CVA16</td>
<td>0.320 ± 0.237</td>
<td>0.474 ± 0.307</td>
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* Antiviral activity (IC50) was determined by MTT method on Vero cells. Values represent the mean ± SD of two independent experiments.
at the point of infection. The similarity of the IC50 obtained from these two different assays for both compounds suggests that the compounds by themselves do not inhibit cell growth, and that the alamarBlue-based quantitative assay is a good indicator of the level of EV71 infection.

Experience with other viruses suggests that when a virus is incubated with the target cell at 4°C, cell surface attachment of the virus could be achieved, whereas virus entry is prevented or slowed. Cells were infected with EV71 at 37° and 4°C. Compared with that at 37°C, the cell attachment of EV71 was slightly reduced (data not shown). For this reason, in the experiment shown in Fig. 5, five times more viruses were used (Section 2). In addition, compared with inoculation at 37°C, incubation at 4°C could significantly slow down virus entry and penetration. For practicality and to allow sufficient interactions between the compounds and their targets during viral attachment and entry, the experiment was performed at 4°C for virus attachment. In the current study, the results clearly indicate that the compounds do not function through interaction with a cellular component to interfere with EV71 attachment or entry (Fig. 5A-b and B-b), nor do they function through interaction with the virus before cellular attachment (Fig. 5A-c and B-c). The experiments also excluded the possibility that the compounds function at the pre-attachment stage. In addition to the entry assay described in Fig. 5A-a and B-a, the time-of-addition experiment confirmed that the compounds function after attachment and that both compounds act within 4 h p.i.

Although the details of the EV71 life cycle are unclear, it generally involves virus attachment, uncoating, polyprotein translation and cleavage, viral RNA replication, and virus assembly. These critical steps are currently considered targets for antiviral development. Lead compounds that target some of these processes are being actively pursued (Chen et al., 2008). Picornaviruses could complete their lifecycle in approximately 8 h (MicrobiologyBytes, 2007), at which point, host protein production is shut down and CPE is induced. At 3–4 h p.i., viral protein synthesis is predicted to reach high levels and virus assembly could even be initiated. For both chrysosplenetin and penduletin, the IC50 for VP1 production is similar to the IC50 for progeny virus production, which is consistent with the finding that these two compounds inhibit viral RNA replication within 4 h p.i. Both compounds also showed inhibitory activity against five other human enteroviruses (Table 2), whereas no effect was observed against Herpes Simplex Virus or influenza A (H1N1) virus at 2.5 μM (data not shown). These suggest that both compounds might target a common mechanism for enterovirus replication. To understand the mechanism of action and to identify the target of the compounds, we attempted to select chrysosplenetin- and penduletin-resistant EV71 through continuous passage in the presence of the compounds. However, after 13 passages, EV71 remained sensitive to the compounds with similar IC50 (data not shown). More detailed analyses of the exact mechanisms of action of these compounds are currently being conducted.

The chemical structures of chrysosplenetin and penduletin differ by a methoxy group (Fig. 1). These two structures have similar anti-enteroviral activity. Another flavonol, 4,5-dihydroxy-3,3'-7-trimethoxyflavone, which is only a methoxy group less than chrysosplenetin, reportedly has anti-coxsackievirus B1 activity (Ishitsuka et al., 1982). Thus, compounds with enhanced drug ability could most likely be identified through further structural modifications.

In conclusion, both chrysosplenetin and penduletin could effectively inhibit the infection of EV71 as well as other human enterovirus in cell culture. Thus, chemicals with similar structures could be pursued as candidates for the treatment of these viral infections. In addition, using these compounds as probes, a study of their mechanisms of action would enhance our understanding of the molecular machinery of enteroviral infection and transmission.

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References


