Kaempferol inhibits enterovirus 71 replication and internal ribosome entry site (IRES) activity through FUBP and HNRP proteins

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ABSTRACT

Flavonoids are associated with multiple biological and pharmacological activities, including anti-enterovirus activity. An internal ribosomal entry site (IRES) required for viral protein translation is a potential drug target for enterovirus 71 (EV71). Regulation translation initiation requires the interaction of IRES specific trans-acting host factors with viral IRES element. By evaluation of 12 flavonoids again EV71 infection, we found that (a) 7,8-dihydroxyflavone, kaempferol, quercetin, hesperetin and hesperidin exhibited more than 80% of cell survival and inhibition of EV71 infection; however, no anti-oxidative effects were noted from these flavonoids; (b) Among them, only 7,8-dihydroxyflavone, kaempferol and hesperetin showed 40% of viral IRES activity; (c) Moreover, kaempferol interfered with EV71 virus replication and pseudotyped virus production; (d) FUBP1, FUBP3, HNRPD, HNRH1 and HNRPF proteins are associated with EV71 5'-UTR using RNA affinity pull-down assay coupled with LC-MS/MS analysis. We firstly found that kaempferol may change the composition of these IRES associated trans-acting factors, affect IRES function and EV71 virus replication. These studies help not only to understand the IRES function but also the mechanism by which drug induced cellular proteins are against EV71 infection.

Keywords:

Enterovirus 71

Kaempferol

Internal ribosome entry site

Trans-acting factors

1. Introduction

Enterovirus 71 (EV71) is considered one of the most important pathogens in the *Picornaviridae* family. It is known to trigger encephalitis; pulmonary edema; hand, foot, and mouth disease; herpangina; aseptic meningitis; and poliomyelitis-like paralysis (Singh, Chow, Phoon, Chan, & Poh, 2002). EV71-induced brainstem encephalitis has a high fatality rate (Mizuta, Aoki, Suto, Ootani, Katsushima, Itagaki, et al., 2009). Several neurological disease outbreaks associated with EV71 have occurred in Asian countries in the past two decades (Herrero, Lee, Hurrelbrink, Chua, Chua, & McMinn, 2003; Mizuta, et al., 2009; Shimizu, Utama, Onnimala, Li, Li-Bi, Yu-Jie, et al., 2004; Yang, Ren, Xiong, Li, Xiao, Zhao, et al., 2009). In Taiwan, EV71 outbreaks caused 78 deaths in 1998, 25 deaths in 2000, and 26 deaths in 2001. Small-scale outbreaks were observed from 2002 to 2005 (Chen, S. P., Huang, Li, Chiu, Huang, Tsao, et al., 2010). Until recently, the reemergence of EV71 in 2008 has resulted in the largest outbreak in the past 11 years (Huang, Hsu, Smith, Kiang, Tsai, Lin, et al., 2009). Studies have focused on antiviral agents and vaccine developments against enteroviruses (Chen, T. C., Weng, Chang, Lin, Huang, & Shih, 2008; Lee & Chang, 2010). However, there are still no sufficient pharmacologic agents or vaccine available

for clinical use.

Naturally occurring polyphenolic compounds such as flavonoids are found in several Chinese herbs including citrus herbs, Chrysanthemum morifolium Ramat, Tussilago farfara L. and Viola yedoensis MAKINO (as well as in some fruits and vegetables) (Benavente-Garcia & Castillo, 2008; Kim, Lee, Lee, Aryal, Kim, Kim, et al., 2006; Liu, Shan, Zhang, Ning, Lu, & Cheng, 2008; Lu, Sun, Chen, Chen, Li, Xu, et al., 2010; Xie, Veitch, Houghton, & Simmonds, 2003). Flavonoid researchers have identified multiple anti-cancer, anti-microbial, and anti-inflammatory effects (Manthey, Grohmann, & Guthrie, 2001; Orhan, Ozcelik, Ozgen, & Ergun, 2009). Particularly, flavonoids have been reported as having multiple anti-enterovirus characteristics, including the inhibition of Sabin type 2 poliovirus, hepatitis A, Coxsackie virus B1, B3, B4, A9 and echovirus 30 infections (Conti, Genovese, Santoro, Stein, Orsi, & Fiore, 1990; Superti, Seganti, Orsi, Divizia, Gabrieli, Pana, et al., 1989; Tait, Salvati, Desideri, & Fiore, 2006).

Like other enteroviruses, the EV 71 genome, which consists of a 7.4 kb single strand of positive-sense RNA, contains a 5'-untranslated region (UTR) with an internal ribosome entry site (IRES) domain for viral translation and replication (Balvay, Soto Rifo, Ricci, Decimo, & Ohlmann, 2009; Jang, Krausslich, Nicklin,

Duke, Palmenberg, & Wimmer, 1988; Pelletier & Sonenberg, 1988; Thompson & Sarnow, 2003). The IRES structure is highly structured, containing multiple stem-loop elements and can be divided into three types based on sequence and structure homology (Jackson & Kaminski, 1995; Wimmer, Hellen, & Cao, 1993). The Enterovirus IRES stem-loop I has been shown to be important for negative-strand RNA sysnthesis (Bell, Semler, & Ehrenfeld, 1999). Stem-loops II through VI contains major cis-acting elements and required for cap-independent translation (Murray, Steil, Roberts, & Barton, 2004). At least one research team has attempted to identify inhibitors that can be targeted at the 5'-UTR IRES site (Yuan, Stein, Lim, Qiu, Coughlin, Liu, et al., 2006).

Studies of flavonoids on multiple anti-enterovirus activities and structure-based inhibitors targeted at the viral IRES elements have suggested that translation driven by viral IRES element might be affected by intercalating these flavonoids to the virus occupied translational machinery. Here we test this hypothesis by evaluating the anti-viral effects of 12 flavonoids on EV71 replication and its viral IRES activity. Our results indicate that (a) 7,8-dihydroxyflavone, kaempferol, quercetin, hesperetin and hesperidin exhibited more than 80% of cell survival and inhibition of EV71 infection; however, no anti-oxidative effects were

noted from these flavonoids; (b) Among them, only 7,8-dihydroxyflavone, kaempferol and hesperetin showed 40% of viral IRES activity; (c) kaempferol interfered with EV71 virus replication and pseudotyped virus production; (d) FUBP1, FUBP3, HNRPD, HNRH1 and HNRPF proteins are associated with EV71 5'-UTR using RNA affinity pull-down assay coupled with LC-MS/MS analysis. Characterization of the EV71 IRES activity by silencing of newly identified cellular factors was also examined. To our knowledge, this is the first description of kaempferol exerting an anti-EV71 effect via a mechanism that blocks viral protein translation.

2. Materials and methods

2.1. Viruses and cells

An EV71 isolate (GeneBank accession number HM807310) was obtained from the clinical virology laboratory of China Medical University Hospital in Taichung, Taiwan. RD cells (ATCC accession no. CCL-136) were grown in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (FBS) (both

145 from Gibco). All media were supplemented with 100 U/mL penicillin, 100 U/mL
146 streptomycin, and 2mM L-glutamine.
147
148 *2.2. EV71 pseudovirus production*
149 Two types of cDNA fragments—the EV71 replicon encoding firefly luciferase
150 and the P1 gene of the EV71 virus (GeneDireX, Inc., Las Vegas City,
151 Nevada)—were cloned in the pcDNA3.1 vector under the control of a CMV/T7
152 promoter. EV71 replicon trans-encapsidation was performed to prepare EV71
153 pseudovirus stock (Fig. 3C). Briefly, RD cells were co-transfected with a pEV71
154 replicon and pEGFP-P1 vectors, and incubated for 72 h at 37°C. Cell culture
155 supernatants were harvested until all cells expressed the cytopathic effects of the
156 EV71 pseudovirus stock. Kaempferol-treated (35 μM for 24 h) and untreated RD
157 cells were infected with the EV71 pseudovirus at 10 MOI and held for an
158 additional 72 h for viral adsorption. Cells were harvested and assayed for
159 luciferase activity according to the manufacturer's instructions (Promega
160 Luciferase Assay System).
161
162 *2.3. EV71 bicistronic vector construction*

163 The pcDNA3.1 (Invitrogen) plasmid for the EV71 bicistronic vector contains a
164 CMV promoter. A firefly luciferase (LUC) gene was cloned adjacent to this
165 promoter, the EV71 5'UTR gene was cloned behind the LUC gene, and the
166 secreted alkaline phosphatase (SEAP) gene was cloned following the EV71
167 5'UTR gene. The resulting plasmid was transfected into RD cells. Culture medium
168 and cell lysates were collected for SEAP and LUC activity analysis after 24 h of
169 incubation at 37°C. Kaempferol (35 μM) treatment was performed as described in
170 Section 2.2, after which culture medium and cell lysates were collected for SEAP
171 and LUC activity analyses.

172

173 *2.4. Viral growth assays*

174 Cell samples were infected with EV71 at 1 MOI, incubated for 24 h at 37°C,
175 rinsed with PBS, and placed in fresh medium. Culture medium samples were also
176 collected at 0, 6, 12, 24 and 48 h. Virus titers were determined by tissue culture
177 infectious dose ($TCID_{50}$) assays, using RD cells and a computer program
178 generously provided by John Spouge of the National Center for Biotechnology
179 Information, U.S. National Institutes of Health.

180 To analyze the effects of kaempferol treatment, treated (35 μM for 24 h) and

181 untreated RD cells were infected with EV71 at 1 MOI and incubated for an
182 additional 24 h at 37°C (Fig. 3A). Cells were placed in fresh medium and
183 harvested at 0, 6, 12, 24 and 48 hours. $TCID_{50}$ assays were used to assess virus
184 yields.
185
186 *2.5. Chemicals*
187 7,8-dihydroxyflavone, chrysin, luteolin, kaempferol, quercetin, myricetin,
188 naringin, naringenin, hesperetin, hesperidin, genistein and biochanin A were
189 purchased from Sigma Chemical (St. Louis, USA).
190
191 *2.6. Cell viability assays using WST-1 assay (Cytotoxicity assay)*
192 RD cells were cultured overnight in 96-well plates. Media containing individual
193 chemicals (50 μM) were added prior to incubation for 24 h at 37°C followed by the
194 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
195 (WST-1; Roche, Indianapolis, IN) assays (Zhang, Peairs, Yang, Tyrrell, Roberts,
196 Kole, et al., 2007). WST-1 cell proliferation reagent was used for the
197 spectrophotometric quantification of cell proliferation, viability, and
198 chemosensitivity according to the manufacturer's instructions

199 (http://www.roche-applied-science.com/pack-insert/1644807a.pdf).

200 Following treatment, 10 µL of WST-1 were added to each well, followed by
201 incubation at 37°C for 1 h. Absorbances at 450 nm were measured against
202 background controls using a 96-well plate reader. Cell survival rates were
203 calculated as a ratio of the optical density of treated cells at 450 nm (OD_{450}) to the
204 OD_{450} of untreated cells. Four wells were analyzed for each concentration. The
205 data shown in Fig. 1A represent mean ± SD for three independent experiments.

206

207 *2.7. Anti-EV71 assays using WST-1 assay (Inhibition of EV71 infection)*

208 RD cells were cultured overnight in 96-well plates. Media containing individual
209 chemicals (50 µM) were added prior to incubation for 24 h at 37°C. Next, RD cells
210 were infected with EV71 (100 $TCID_{50}$) and held for 72 h at 37°C prior to WST-1
211 assays (Fig. 1B)(Chang, Wang, & Chiang, 2008; Guo, Pang, Wang, Shen, Jin, &
212 Li, 2006). Four wells were analyzed for each concentration. Percentage inhibition
213 of EV71 infection was calculated as ($OD450_{cell+drug+virus}$ – $OD450_{cell+virus}$) /
214 ($OD450_{cell}$ – $OD450_{cell+virus}$) x 100%.

215

216 *2.8. Total anti-oxidant capacity assay using Trolox equivalent antioxidant capacity*

(TEAC) assay

All procedures were performed according to the manufacturer's protocol (http://www.abcam.com/index.html?pageconfig=protocols&pid=996&intAbID=65329&strTab=protocols&mode=prot)(Gil, Tomas-Barberan, Hess-Pierce, Holcroft, & Kader, 2000; Pellegrini, Serafini, Colombi, Del Rio, Salvatore, Bianchi, et al., 2003). All standards and individual chemicals (200 µM) were added with 100 µl Cu^{++} working solution. The reactions were at room temperature for 1.5 hrs and read the absorbance at 570 nm.

2.9. Confocal image analysis

Treated (35µM kaempferol) and untreated RD cells were infected with EV71 at 1 MOI, fixed with 4% PFA for 15 mins at room temperature, treated with 0.3% Triton X-100 for 5 mins, and blocked with 3% BSA (Patkar, Larsen, Owston, Smith, & Kuhn, 2009). Next, cells were incubated with mouse anti-VP1 monoclonal antibodies for 2 h at room temperature and reacted with FITC-conjugated anti-mouse IgG antibodies for another 2 h at room temperature. After washing, cells were counterstained with DAPI (Hoechst 33342, Molecular Probes), mounted with 50% glycerol, and observed with a TCS SP2 AOBS laser-scanning

235 microscope (Leica Microsystems, Heidelberg GmbH, Germany).

236

237 *2.10. In Vitro RNA Transcription*

238 The plasmid including EV71 5'-UTR were linearized by EcoRI to produce DNA
239 templates for T7 runoff transcription of the EV71 IRES RNA. *In vitro* RNA
240 transcription was performed using the RiboMax T7 Transcription kit (Ambion,
241 Austin, TX), and the RNA products were further purified with Rneasy columns
242 (Qiagen, city) and quantified using a UV spectrometer.

243

244 *2.11. Affinity Extractions Using Biotin-Tagged RNAs*

245 The RD cells were washed with cold PBS 4× and pelleted by centrifugation at
246 1000 × *g*. Cells were lysed in ice-cold hypotonic buffer (10 mM K−Hepes, pH 7.5,
247 10 mM KOAc, 1.5 mM MgOAc, and 2.5 mM dithiothreitol). Nuclei and other cell
248 debris were removed by centrifugation at 1000 × *g* for 5 min. The supernatant was
249 subject to further centrifugation at 10000 × *g* for 20 min. The supernatant was
250 stored in small aliquots at −80 °C. Each *in vitro* transcribed biotin-tagged EV71
251 IRES RNA were incubated with the RD cell extract in a buffer containing 1X PBS,
252 70 mM KOAc, 2.5 mM MgOAc, 2 mM DTT, 1 mM ATP, and 40 units of RNasin at

30 C for 20 min. RNA-protein complexes were affinity-purified employing immobilized streptavidin-agarose beads**.** The RNA/protein/beads complexes were washed 3 times with cold PBS containing 70 mM KOAc, 2.5 mM MgOAc, and 40 units of RNasin.

2.12. 2-dimensional gel electrophoresis (2-DE)

The dried pellet was then extracted with lysis buffer containing 8 M urea, 4% CHAPS, 2% pH 3–10 non-linear (NL) IPG buffer (GE Healthcare) and the Complete, Mini, EDTA-free protease inhibitor mixture (Roche). After a 3-h incubation at 47C, the cell lysates were centrifuged for 15 min at 16000*g*. The protein concentration of the resulting supernatants was measured using the BioRad Protein Assay (BioRad, Hercules, CA, USA). Protein sample (100 mg) was diluted with 350 mL of rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer pH 3–10 NL, 18 mM DTT, 0.002% bromophenol blue), and then applied to the nonlinear Immobiline DryStrips (17 cm, pH 3–10; GE Healthcare). After the run of 1-D IEF on a Multiphor II system (GE Healthcare), the gel strips were incubated for 30 min in the equilibration solution I (6 M urea, 2% SDS, 30% glycerol, 1% DTT, 0.002% bromophenol blue, 50 mM Tris-HCl, pH 8.8), and for

another 30 min in the equilibration solution II (6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide, 0.002% bromophenol blue, 50 mM Tris-HCl, pH 8.8). Subsequently, the IPG gels were transferred to the top of 12% polyacrylamide gels (20 x 20 cm x 1.0 mm) for the secondary dimensional run at 15 mA, 300 V for 14 h.

2.13. Protein spot analysis

Separated protein spots were fixed in the fixing solution (40% ethanol and 10% glacial acetic acid) for 30 min, stained on the gel with silver nitrate solution for 20 min, and then scanned by GS-800 imaging densitometer with PDQuest software version 7.1.1 (BioRad). Data from three independently stained gels of each sample were exported to Microsoft Excel for creation of the correction graphs, spot intensity graphs and statistical analysis.

2.14. Nanoelectrospray MS and bioinformatics

The proteins were identified using an Ultimate capillary LC system (LC Packings, Amsterdam, The Netherlands) coupled to a QSTARXL quadrupole-time of flight (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City,

289 CA, USA). The peptides were separated using an RP C18 capillary column (15
290 cm x 75 μm id) with a flow rate of 200 nL/min, and eluted with a linear ACN
291 gradient from 10–50% ACN in 0.1% formic acid for 60 min. The eluted peptides
292 from the capillary column were sprayed into the MS by a PicoTip electrospray tip
293 (FS360-20-10-D-20; New Objective, Cambridge, MA, USA). Data acquisition from
294 Q-TOF was performed using the automatic Information Dependent Acquisition
295 (IDA; Applied Biosystem/MDS Sciex). Proteins were identified by the
296 nanoLC-MS/MS spectra by searching against NCBI databases for exact matches
297 using the ProID program (Applied Biosystem/MDS Sciex) and the MASCOT
298 search program (http://www.matrixscience.com).
299
300 *2.15. EV71 dicistronic expression coupled with siRNA assay*
301 RD cells were firstly transfected with FUBP1, FUBP3, HNRPD, HNRH1 and
302 HNRPF siRNAs, respectively. After 48 h, EV71 dicistronic plasmid was
303 transfected into RD cells. After 48 h, cell culture supernatants and cell lysates
304 were prepared for SEAP and LUC reporter assays according to the
305 manufacturer's instructions. For western blotting experiment, cell lysates were
306 incubated at 95°C for 5 min and was resolved on 12% SDS-PAGE and was

307 western blotted for FUBP1, FUBP3, HNRPD, HNRH1, HNRPF and actin (Cell
308 Signaling Technology, Inc, MA, USA).
309
310 **3. Results and discussion**
311 *3.1. Cytotoxicity, anti-EV71 and anti-oxidative activities of flavonoids*
312 The initial goal of our study was to determine the anti-EV71 activity of
313 flavonoids previous described in multiple anti-enterovirus characteristics (Conti, et
314 al., 1990; Genovese, Conti, Tomao, Desideri, Stein, Catone, et al., 1995; Salvati,
315 De Dominicis, Tait, Canitano, Lahm, & Fiore, 2004). Firstly, cytotoxicity to RD cells
316 was measured based on cell proliferation and viability. Results from treating
317 individual cell batches with 50 μM of each flavonoid indicate that 11 of 12
318 flavonoids exhibited 50% of cell survival. Among them, 7,8-dihydroxyflavone,
319 kaempferol, quercetin, hesperetin and hesperidin exhibited more than 80% of cell
320 survival. These flavonoids which exhibiting 80% of cell survival also showed at
321 least 80% of inhibition of EV71 infection (Fig. 1B). The total anti-oxidant capacity of
322 12 flavonoids was also evaluated using the TEAC assay (Gil, et al., 2000;
323 Pellegrini, et al., 2003) (Fig. 1C). The equivalent Trolox with the indicated
324 concentrations was used for the anti-oxidant activity. As shown, chrysin,

naringenin, hesperidin and biochanin A exhibited 1-4 TEAC (chrysin: 3.80 TEAC; naringenin: 1.13; hesperidin: 1.00; biochanin A: 1.91). However, there was no significant anti-oxidant activity detected in 7,8-dihydroxyflavone, kaempferol, quercetin and hesperidin.

By evaluations of cytotoxicity, anti-EV71 and anti-oxidative activities, we observed that 7,8-dihydroxyflavone, kaempferol, quercetin, hesperetin and hesperidin showed the most efficient anti-EV71 activity without toxic effects. However, no anti-oxidative effects were noted from these flavonoids. Therefore, there were no significant correlations between anti-oxidant activity and inhibition of the virus.

3.2. Inhibitory effects of flavonoids on EV71 IRES activity

The enterovirus 5'-UTR contains highly structured secondary elements with IRES activity for translation initiation (Balvay, et al., 2009; Jang, et al., 1988; Pelletier, et al., 1988; Thompson, et al., 2003). And this highly structured 5'-UTR IRES site has been used for identify enterovirus inhibitors (Yuan, et al., 2006). To further evaluate the effects of flavonoids on EV71 IRES activity, we constructed an EV71 bicistronic vector (Fig. 2A) and transfected the plasmid into RD cells

343 treated with 50 μM individual flavonoids for 24 h. Culture medium and cell lysates
344 were collected for SEAP and LUC activity analyses. As shown, 6 of 12 flavonoids
345 exhibited 40% of viral IRES activity (Fig. 2B). Among them, only
346 7,8-dihydroxyflavone, kaempferol and hesperetin also showed the most effective
347 inhibition of EV71 IRES activity and the least cytotoxicity. The exact mechanism
348 of IRES-mediated translation initiation has not been elucidated; however, it has
349 been postulated that the interaction of trans-acting host factors with cis-acting
350 stem-loop structures acts to recruit several translation factors and/or stabilize the
351 RNA for translation (Costa-Mattioli, Svitkin, & Sonenberg, 2004; Lin, Li, Huang,
352 Chien, Horng, & Shih, 2008; Lin, Li, & Shih, 2009; Walter, Parsley, Ehrenfeld, &
353 Semler, 2002). In this study, we postulated that flavonoids that exhibited
354 anti-EV71 activity may be due to affect vial IRES activity. And the viral IRES
355 activity may be affected by trans-acting host factors or translation factors.
356 Therefore, flavonoids may affect the trans-acting host factors or translation factors
357 and lead to their interaction with IRES structure, leading reduced virus replication.
358
359 *3.3. Inhibitory effects of kaempferol on EV71 virus replication and pseudotyped*
360 *virus production*

Among 7,8-dihydroxyflavone, kaempferol and hesperetin, we chosen kaempferol for the evaluation of EV71 virus replication and pseudotyped virus production. To further evaluate the anti-viral effects of kaempferol on EV71 replication, we analyzed virus yields at 0, 6, 12, 24 and 48 h post-infection. In cells treated with 35 μM kaempferol, changes in virus yields were observed in all infection phases (Fig. 3A). Specifically, amounts of infectious EV71 released from kaempferol-treated cells were at least 1 log unit lower than those measured in untreated cells, regardless of collection time. We also observed that kaempferol reduced virus yields by approximately 6 log units at 24 h post-infection. We used confocal images to confirm this finding. Kaempferol-treated and untreated RD cells were infected with EV71, double-stained with anti-VP1 monoclonal and FITC-conjugated anti-mouse IgG antibodies, and counterstained with DAPI. As shown in Fig. 3B, elevated anti-VP1 fluorescence was observed in EV71-infected cells and significantly reduced by kaempferol treatment. We performed trans-encapsidation of the EV71 replicon in an effort to further evaluate the antiviral effects of kaempferol on EV71 pseudotyped virus production (Fig. 3C). RD cells were co-transfected with pEV71 replicon and pEGFP-P1 vectors. Cell culture supernatant was harvested and used as EV71 pseudovirus stock.

Kaempferol-treated (35 μM, 24 h) and untreated RD cells were infected with the EV71 pseudovirus. As shown in Fig. 3D, relative firefly luciferase activity in kaempferol-treated cells was reduced to an 80% decrease compared to activity in untreated cells.

Increasingly, besides providing the anti-cancer, anti-oxidative and anti-inflammatory effects, flavonoids have also been becoming the subject of anti-infective research, and many groups have isolated and identified the structures of flavonoids possessing antifungal, antiviral and antibacterial activity (Cushnie & Lamb, 2005). Flavonoids have been reported as having multiple anti-enterovirus characteristics, including the inhibition of Sabin type 2 poliovirus, hepatitis A, coxsackie virus B1, B3, B4, A9 and echovirus 30 infections (Conti, et al., 1990; Superti, et al., 1989; Tait, et al., 2006). Thus, these flavonoids could not be specific for only one virus inhibition. Enterovirus inhibition data of various studies are also not absolutely conclusive. In Sabin type 2 poliovirus studies, two isoflavenes exhibited a significant inhibitory activity on the virus-induced cytopathic effect and plaque formation via the shutoff of host translation and viral RNA and protein synthesis. In hepatitis A virus (HAV) studies, 6,4'-dichloroflavan and 6,4'-dichloroisoflavan showed the highest inhibition of HAV replication during

397 an early stage (penetration and/or uncoating) of HAV infection. In coxsackie virus
398 B1, B3, B4, A9 and echovirus 30, homoisoflavonoids showed a low cytotoxicity
399 and a marked antiviral activity. Kaempferol (one member of the flavonols) found in
400 fruits, vegetables, and teas, and has exhibited inhibitory effects against influenza
401 and herpes simplex types 1 (HSV-1) and 2 (HSV-2) viruses (Jeong, Ryu, Park,
402 Kim, Kwon, Park, et al., 2009; Lyu, Rhim, & Park, 2005). To our knowledge, this is
403 the first demonstration that kaempferol decreases EV71 activity, and that the
404 anti-EV71 effect occurs via a mechanism that abolishes viral protein translation.
405
406 *3.4. Identification of kaempferol-induced cellular factors associated with the*
407 *5'-untranslated region (5'-UTR) of the EV71 genome*
408 From our previous study, we observed that kaempferol exerts anti-EV71
409 activity via eliminating viral IRES activity. Therefore, we hypothesis that the global
410 patterns of host cellular proteins may be altered when cells are treated with
411 kaempferol, and induced or inhibited cellular proteins associated with EV71 IRES
412 may influence virus replication. In order to detect kaempferol-induced cellular
413 factors associated with the 5'-untranslated region (5'-UTR) of the EV71 genome,
414 which is important for virus translation and replication, streptavidin beads were

415 used to capture the full-length, biotin-labelled EV71 5‘-UTR and associated
416 proteins. Fig. 4A outlines the design of the pull-down assay. The 2-dimentional
417 SDS-PAGE and LC/MS/MS technology were also performed to obtain the
418 identified cellular proteins (Fig.4.B). The identified cellular proteins were listed in
419 Table 2 with their accession numbers obtained from the NCBI protein database.
420 As shown, many of these proteins belong to the hnRNP family, splicing regulatory
421 factors and translational machinery, such as hnRNP D, HNRP H1, HNRP F,
422 HNRPR, IF2G, IF35, FUBP1 and FUBP3. So far, host trans-acting proteins or
423 translation factors interacting with picornavirus 5’-UTR and regulating virus
424 translation and replication have been identified including poly(rC)-binding protein
425 (PCBP), heterogeneous nuclear ribonucleoprotein K (hnRNP K), far upstream
426 element binding protein 2 (FUSE-binding protein 2), and autoantigen La
427 (Costa-Mattioli, et al., 2004; Lin, et al., 2008; Lin, Li, et al., 2009; Walter, et al.,
428 2002). Among them, PCBP 1/2 and the viral polymerase precursor 3CD with
429 poliovirus IRES stem-loop I RNA forms a ternary complex, which is required for
430 negative strand RNA synthesis (Perera, Daijogo, Walter, Nguyen, & Semler, 2007).
431 Beyond its role in viral RNA replication, PCBP2 interacts with enterovirus IRES
432 stem-loop IV RNA to affect IRES-mediated translation (Sean, Nguyen, & Semler,

2008).

3.5.EV71 IRES-dependent translation was inhibited by newly identified cellular factors associated with the 5'-untranslated region (5'-UTR) of the EV71 genome

To investigate the roles of the newly identified cellular factors in EV71 virus translation, the siRNA knockdown assay of individual cellular proteins (Fig. 5) and the EV71 dicistronic reporter plasmid (Fig. 2 A) were used to evaluate EV71 IRES activity. As shown, knockdown of endogenous FUBP1, FUBP3, HNRPD, HNRH1 and HNRPF proteins, respectively resulted in the decreased EV71 IRES activities ranging from 50% to 80% of that of the negative control (NC). No significant difference was observed in the HNRPR protein. The lower panel of western blot presents the knockdown efficiency of individual proteins. These results together indicate that FUBP1, FUBP3, HNRPD, HNRH1 and HNRPF proteins may act as regulators of EV71 IRES function.

We firstly found that kaempferol may change the composition of these IRES associated trans-acting factors, affect IRES function and EV71 virus replication.

FUBP1, FUBP3, HNRPD, HNRH1 and HNRPF proteins induced by kaempferol binds to the highly structured EV71 5'-UTR and against virus infection. Studies in picornavirus have suggested that many proteins that are primarily localized in the nucleus also interact with picornaviral RNA in the cytoplasm. These nuclear localization cellular factors, such as FUBP2 (Lin, Li, et al., 2009), PTB (Song, Tzima, Ochs, Bassili, Trusheim, Linder, et al., 2005), hnRNP C (Brunner, Nguyen, Roehl, Ho, Swiderek, & Semler, 2005), hnRNP K (Lin, et al., 2008), hnRNP A1 (Cammas, Pileur, Bonnal, Lewis, Leveque, Holcik, et al., 2007; Lin, Shih, Pan, Li, Lue, Stollar, et al., 2009) and nuclear factors (NF45)(Merrill & Gromeier, 2006), interact with picornaviral RNA and affect its viral replication and/or translation. These interactions between host cellular factors and virus RNA provide the important information for virus-host interactions when virus infections. When virus infection, these infection may affect the cellular distributions of these nuclear factors. And these affected nuclear factors may be involved in the virus life cycle such as viral RNA synthesis or translations.

4. Conclusions

To our knowledge, this is the first demonstration that kaempferol decreases EV71 activity, and that the anti-EV71 effect occurs via a mechanism that abolishes viral protein translation. Futhermore, the identification of the trans-acting factors associated with EV71 5'-UTR includes FUBP1, FUBP3, HNRPD, HNRH1 and HNRPF proteins. These newly IRES-specific trans-acting factors are involved in EV71 IRES activity. We firstly found that kaempferol may change the composition of these IRES associated trans-acting factors, affect IRES function and EV71 virus replication. These studies help not only to understand the IRES function but also the mechanism by which drug induced cellular proteins are against EV71 infection.

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Figure Legends

Fig. 1. Cell survival rates, inhibition of EV71 infection and anti-oxidative activities for flavonoids. (A) cell survival rate; (B) EV71 infection inhibition; (C) anti-oxidative activities . For cell survival rates, RD cells were placed on 96-well plates. Medium containing 50 μM of each flavonoid was added for 24 h incubation at 37°C. Cell survivals were detected using WST-1 assays and calculated as the ratio of optical density of treated cells at 450 nm (OD_{450}) to the OD_{450} of untreated cells. For anti-EV71 activity, RD cells were cultured overnight in 96-well plates. Medium containing 50 μM of each flavonoid was added for 24 h incubation at 37°C. Cells were then infected with EV71 (100 $TCID_{50}$) and held for an additional 72 h and detected using WST-1 assays. Percentage inhibition of EV71 infection was calculated as ($OD450_{cell+drug+virus}$ – $OD450_{cell+virus}$) / ($OD450_{cell}$ – $OD450_{cell+virus}$)] x

100%. Four wells were analyzed for each concentration. Data represent mean ± SD for three independent experiments. For anti-oxidative activities, All standards and individual chemicals (200 μM) were added with 100 μl Cu^{++} working solution. The reactions were at room temperature for 1.5 hrs and read the absorbance at 570 nm.

Fig. 2. Inhibitory effects of flavonoids on EV71 IRES activity. (A) Diagram of the EV71 bicistronic vector used in this study. Boxes indicate LUC (firefly luciferase) and SEAP reporter genes flanking the EV71 5'UTR in a pcDNA3.1 plasmid (Invitrogen) containing the CMV promoter. The LUC gene was cloned adjacent to the CMV promoter, the EV71 5'UTR gene was cloned behind the LUC gene, and the SEAP gene was cloned following the EV71 5'UTR gene. (B) Effects of flavonoids on EV71 IRES activity. Plasmids were transfected into RD cells prior to treatment with flavonoids at 50 μM and for 24 h incubation at 37°C. Culture medium and cell lysates were collected and analyzed for SEAP and LUC activity. Data are expressed as SEAP/LUC ratios (Medium only = 100%) from three independent experiments, each performed in triplicate.

Fig. 3. Inhibitory effects of kaempferol on EV71 virus replication and pseudotyped virus production. (A) Viral growth assays. Treated (35 μM kaempferol for 24 h) and untreated RD cells were infected with EV71 and harvested at 6, 12, 24 and 48 h. Virus yields in culture supernatant were assessed by $TCID_{50}$ assays. (B) Confocal image analysis. Treated (35 μM kaempferol for 24 h) and untreated RD cells were infected with EV71 and immunostained first with mouse anti-VP1 monoclonal antibodies and later with FITC-conjugated anti-mouse IgG antibodies. Cells were counterstained with DAPI (Hoechst 33342, Molecular Probes), mounted and observed using a confocal microscope. (C) Preparation of EV71 pseudoviruses. RD cells were co-transfected with pEV71 replicons and pEGFP-P1 vectors. Cell culture supernatant was harvested until all cells expressed a cytopathic effect for EV71 pseudovirus stock. (D) Effects of kaempferol on EV71 pseudovirus infection. Treated (35 μM) and untreated RD cells were infected with an EV71 pseudovirus at 10 MOI and held for 72 h at 37°C. Cells were harvested and assayed for luciferase activity according to the manufacturer's instructions (Luciferase Assay System, Promega).

Fig. 4. Identification of kaempferol-induced cellular factors associated with the 5'-untranslated region (5'-UTR) of the EV71 genome. (A) Flow chart for RNA affinity capture of cellular proteins interacting with EV71 5'-UTR. (B) 2-dimensional SDS-polyacrylamide gel electrophoresis (2D SDS-PAGE) of cellular proteins interacting with EV71 5'-UTR.

Fig. 5. Characterization of the EV71 IRES activity by silencing (siRNA) of identified cellular factors. RD cells were firstly transfected with FUBP1, FUBP3, HNRPD, HNRH1 and HNRPF siRNAs, respectively. EV71 dicistronic plasmid was then transfected into RD cells. After 48 h, cell culture supernatants and cell lysates were prepared for SEAP and LUC reporter assays according to the manufacturer's instructions. For western blotting experiment, cell lysates were resolved on 12% SDS-PAGE and was western blotted for FUBP1, FUBP3, HNRPD, HNRH1, HNRPF and actin.

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