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Identification of myricetin and scutellarein as novel chemical inhibitors of the SARS coronavirus helicase, nsP13

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SARS is an atypical pneumonia, primarily transmitted by respiratory droplets or personal contacts. SARS was an epidemic illness that occurred between 2002 and 2003, and caused more than 700 deaths around the world (more information can be found at http://www.who.int/csr/sars/en). Since the first diagnosis in Guangdong province, China, successive outbreaks occurred in 29 countries and about 20% of the patients inflicted with the SARS coronavirus (SARS-CoV) was isolated and shown to be a class of coronavirus that is a single stranded RNA virus with a genome of 29,751 bases. Based on the genome sequence, the SARS-CoV was found to be only moderately related to other human coronaviruses, HCoV-OC43 and HCoV-229E, and did not resemble any of the three previously known groups of coronaviruses. Coronaviruses are members of a family of enveloped viruses that replicate in the cytoplasm of animal host cells. Upon infection of target cells, the genome of SARS-CoV is translated into two large replicative polyproteins that are subsequently processed into a number of non-structural proteins (nsPs) by the viral protease. These nsPs include the RNA-dependent RNA polymerase and the helicase. Since the viral helicase is essential to viral genome replication, it is currently considered a potential target for anti-viral drug development.

The present study was conducted to identify natural compounds that might inhibit SARS-CoV helicase activity in vitro. In order to accomplish this goal, we prepared natural chemical stocks (Table 1) and examined their effects on the activity of SARS-CoV helicase, nsP13. Although SARS-CoV contains a RNA-dependent RNA polymerase, nsP13 has been reported to possess dsDNA unwinding activity.
unwinding activity as well as the ability to translocate along the ssDNA unless the helicase separates from the DNA. ATP hydrolysis was assessed using a colorimetric assay by measuring the release of ATP.6 We first attempted to screen compounds that suppress the DNA unwinding activity of nsP13 and the ATP hydrolysis activity of nsP13 by more than 90% at a concentration of 0.86 ± 0.48 M, respectively (Fig. 3B). To determine whether the ATP hydrolysis activity of nsP13 was measured using a fluorescent intensity at a wavelength of 535 nm. In these experiments, none of the natural chemicals inhibited the dsDNA unwinding activity of SARS helicase, nsP13 (Fig. 1B). In an identical experimental setup, we attempted to identify chemical inhibitors of the HCV viral helicase in vitro (Fig. 1C). We then assessed whether any of these natural compounds could exhibit inhibitory effects on the growth of MCF10A cells.9 As a result of this analysis, IC50 values of 6 and 8 were determined to be 2.71 ± 0.19 µM and 0.86 ± 0.48 µM, respectively (Fig. 3B). To determine whether myricetin or scutellarein (2 µM) or scutellarein (2 µM) and taraxerol (No. 58) exhibited some degree of inhibition (around 20%), as shown in Figure 2B. Again, we were not able to detect any compounds in our natural compound library that suppressed the ATPase activity of the HCV viral helicase (Fig. 2C). In order to determine the IC50 value of 6 and 8 (Fig. 3A) in suppressing nsP13 ATPase activity, we serially diluted 6 and 8 and measured their inhibitory effects on the ATPase activity of nsP13 in vitro. As a result of this analysis, IC50 values of 6 and 8 were determined to be 2.71 ± 0.19 µM and 0.86 ± 0.48 µM, respectively (Fig. 3B). To determine whether myricetin or scutellarein possesses potential cytotoxicity in normal cells, we have exposed normal breast epithelial MCF10A cells to myricetin (2 µM) or scutellarein (2 µM) and observed whether they could exhibit inhibitory effects on the growth of MCF10A cells.9 As a result, we observed that either myricetin or scutellarein did not affect the growth of MCF10A cells at cellular concentrations close to the IC50 of myricetin or scutellarein (Fig. 3C), suggesting that both myricetin and scutellarein are safe compounds at pharmacologically-effective concentrations.

Table 1

| No | Compound       | Source          | No | Compound       | Source          | No | Compound       | Source          |
|----|----------------|-----------------|----|----------------|-----------------|----|----------------|-----------------|
| 1  | Daedzin        | Chromadex 22    | 24 | ß-Mangostin     | Garcinia mangostana | 43 | Ursolic acid   | Bridelia cambodiana |
| 2  | Isobespin      | Chromadex 23    | 25 | ß-Mangostin     | Garcinia mangostana | 44 | Oleolectic acid| Bridelia cambodiana  
| 3  | Galangin       | Chromadex 24    | 26 | Thujia orientalis | Thujia orientalis  | 46 | ß-Sitosterol   | Bridelia cambodiana  
| 4  | Sophoroside    | Chromadex 25    | 27 | Aglaia peruvridis | Aglaia peruvridis  | 48 | Gynenoside XVII| Panax ginseng   
| 5  | Isoquercetin   | Chromadex 26    | 28 | 4-Hydroxyxy ramadatine | Aglaia peruvridis | 49 | Gynenoside Rib | Panax ginseng   
| 6  | Myricetin      | Chromadex 27    | 29 | Pyramadatine    | 50 | Imperatorin    | Saposhnikovia divaricata |
| 7  | Myricitrin     | Chromadex 30    | 30 | Verproside      | Phseudolysimachion | 51 | Hamaudol       | Saposhnikovia divaricata |
| 8  | Scutellarein   | Scutellaria baicalensis | 31 | 8-ethylvisamminol | Cinnamomum | 52 | 3-O-Angeloyl amaudol | Saposhnikovia divaricata  
| 9  | Chrysin        | Chromadex 32    | 32 | 6-O-Veratroyl Catalpol | Phseudolysimachion | 53 | 5-O-Methylvisaminol | Saposhnikovia divaricata  
| 10 | Silymarin      | Chromadex 33    | 33 | Minecoside      | Phseudolysimachion | 54 | Ledebouriellol  | Saposhnikovia divaricata |
| 11 | Icariin        | Chromadex 34    | 34 | Diosmetin-7-O-Glc | Diosmetin-7-O-Glc | 55 | Gallic acid    | Chromadex |
| 12 | Curcumin       | Chromadex 35    | 35 | Diosmetin-7-O-Glc-Xyl | Phseudolysimachion | 56 | Methoxyugenol  | Cinnamomum cambodiamum |
| 13 | Scutellaria    | Scutellaria baicalensis | 36 | 3ß-Friedelanol  | Bredelid cambodiana | 57 | Sputalenuil    | Thrysanthera subarobicularis |
| 14 | Baicalein      | Scutellaria baicalensis | 37 | Friedelin       | Bredelid cambodiana | 58 | Taraxerol      | Thrysanthera subarobicularis |
| 15 | Hyperoside     | Chromadex 38    | 39 | 24-Methyllanosta-9(11),25-dien-3-one | Bredelid cambodiana | 59 | 19-Hydroxy-1(10),15-rosadiene | Thrysanthera subarobicularis |
| 16 | Naringenin     | Chromadex 40    | 40 | 24-Dimethyllylosta-9(11),25-dien-3-one | Bredelid cambodiana | 60 | Aleuritolic acid| Thrysanthera subarobicularis |
| 17 | Naringenin     | Chromadex 41    | 41 | 24-Methyl-S-lanosta-9(11),25-dien-3-one | Bredelid cambodiana | 61 | Marilolide      | Cinnamomum cambodiamum |
| 18 | Amentoflavone  | Chromadex 42    | 42 | Betulinic acid  | Bredelid cambodiana | 62 | Sec-O-Glucosylmaudol | Saposhnikovia divaricata |
| 19 | Populinetin    | Chromadex 43    | 43 | ß-Amyrin        | Bredelid cambodiana | 63 | 4-O-ß-Glucosyl-5-O-methylvisaminol | Saposhnikovia divaricata |
| 20 | Icarin         | Chromadex 44    | 44 | Oleanolic acid  | 64 | Prim-O-Glucosylcimifugin | Saposhnikovia divaricata |

The table lists natural compounds used in our study. Table 1 shows the list of natural compounds used in our study.
Naturally-occurring chemicals are regarded as a great source of potential medications against various diseases. In particular, they have gained great scientific interest due to their strong neuroprotective, cardioprotective and chemopreventive activities. In addition, previous studies have demonstrated that selected naturally-occurring flavonoids exhibit anti-viral activities. For example, administration of silymarin, which is rich in milk thistle, significantly suppressed hepatitis B virus (HBV)-related hepatocarcinoma in HBV transgenic mice. Quercetin also inhibited HCV production in an HCV cell culture system. Epigallocatechin-3-gallate (EGCG), the major active constituent of green tea, suppressed human immunodeficiency virus (HIV) replication by degrading a

Figure 1. (A) Schematic representation of FRET-based dsDNA unwinding assay. (B) Inhibition of the dsDNA unwinding activity of the SARS CoV helicase in the presence of 10 μM natural compounds. (C) Inhibition of the dsDNA unwinding activity of the HCV helicase in the presence of 10 μM natural compounds.
semen-derived enhancer of virus infection (SEVI), which is required for HIV virus infection. Glycyrrhizin, an active ingredient in liquorice root, inhibited a SARS-associated virus in vero cells, although its clinical efficacy against the SARS virus in patients requires further verifications. In the present study, we present the evidence for the first time that myricetin and scutellarein are strong chemical inhibitors of SARS-CoV helicase and this effect is mediated through inhibition of ATPase activity, but not inhibition of helicase activity. On the other hand, myricetin and scutellarein did not suppress the helicase activity of HCV virus in our experi-

Figure 2. (A) Schematic representation of the ATP hydrolysis assay. (B) Inhibition of the ATP hydrolysis activity of the SARS CoV helicase in the presence of 10 μM natural compounds. (C) Inhibition of the ATP hydrolysis activity of the HCV helicase in the presence of 10 μM natural compounds.
mental setup. The reason for this discrepancy is currently unknown, but this may be due to structural difference of the ATPase domain between SARS-CoV helicase and HCV helicase. This result also indicates that suppression of SARS-CoV helicase by myricetin and scutellarein might not be mediated by affecting the protein stability and/or integrity of SARS-CoV protein in vitro, since these compounds did not seem to suppress the ATPase activity of HCV helicase protein. Therefore, it would be very interesting to examine which amino acid residues myricetin and scutellarein directly bind to on the SARS-CoV helicase to inhibit ATPase activity. Our modeling analysis shows that myricetin or scutellarein could fit in and directly interact with ATP/ADP binding pocket of the SARS-CoV helicase protein, thereby excluding a direct binding of ATP/ADP (Supplementary Fig. A). In particular, myricetin is likely to interfere with ATPase activity of the SARS-CoV helicase protein, possibly by directly interacting with critical residues of the ATPase domain, such as N265, Y269, and R443 (Supplementary Fig. B). Nonetheless, this structural proposition requires further experimental verifications in the future. Collectively, we propose that myricetin and scutellarein hold a great promise for use in treating and controlling potential future SARS outbreaks; however, more preclinical/clinical studies are necessary to examine whether this effect occurs after in vivo treatment.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.04.081. These data include MOL files and InChiKeys of the most important compounds described in this article.

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5. Natural compounds used in our study were directly purified from various medicinal plants or purchased from commercial vendor (Chromadex Inc.) Table 1. The integrity of the individual natural compounds, directly purified from natural plants was confirmed by NMR spectroscopy (More specific information is available upon request.). All natural compounds were dissolved in DMSO at a concentration of 10 mM as a stock solution before experiments.

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7. SARS-CoV helicase, nsP13, was expressed in Escherichia coli Rosetta™ and purified. The helicase domain of HCV, NS3h, was overexpressed in E. coli BL21(DE3) and purified. TAMRA and fluorescein-labeled DNAs were purchased from Integrated DNA Technologies (Coralville, IA), and the concentrations were determined by absorbance at 260 nm and their extinction coefficients. The base sequences of TAMRA-labeled and fluorescein-labeled DNAs were as follows: 5'-20T25Tam (5'-TGGAGCGGATTACTATACTACATTAGA(TAMRA)-3'), 3'-0T25Flu (5'-Fluorescein-TCTAATGTAGTATAGTAATCCGCTC-3'), and 3'-15T25Flu (5'-Fluorescein-TCTAATGTAGTATAGTAATCCGCTCT15-3'), respectively. The SARS-CoV helicase substrate was prepared by annealing 5'-20T25Tam and 3'-15T25Flu. The 5'-20T25Tam and 3'-15T25Flu were designed to have a 20-base dT overhang at the 5'-terminus and a 15-base dT overhang at 3'-terminus to load the SARS-CoV helicase and HCV NS3h, respectively.

8. ATP hydrolysis by helicases was assayed by measuring the amount of released inorganic phosphate from ATP using a colorimetric assay. Colorimetric measurements of complex formation with malachite green and molybdate (AM/MG) were performed in the presence of various concentrations of natural compounds. All experiments were repeated three times and averaged.

9. Normal breast epithelial MCF10A cells were maintained in DMEM (Invitrogen, Carlsbad, CA) media, supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 0.02 μg/ml epidermal growth factor (EGF), 5 μg/ml insulin, 1.25 μg/ml hydrocortisone (Sigma, St. Louis, MO, USA) at 37 °C in 5% CO2. MCF10A cells were seeded in six well plates at the number of 2.0 × 10^5 per well and exposed to myricetin or scutellarein at the concentration. Cells were collected every 24 h for 3 days and the viable cell number was calculated, using hemacytometer counting. Data are shown in mean ± standard deviation and a statistical analysis was conducted with Student t-test (n = 6). However, we did not observe any statistical significance between control group versus myricetin group or scutellarein group.

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