**Virologica Sinica**

**Supplementary Data**

**Identification of fangchinoline as a broad-spectrum enterovirus inhibitor through reporter virus based high-content screening**

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**Supplementary Materials and Methods**

**Simplified time of addition assay**

To preliminarily determine which part of the viral life cycle the compound inhibits, simplified time-of-addition assay was conducted. Vero cells were seeded in 24-well plates (8×104 cells per well). After one day cultivation, the cells were treated with fangchinoline (FAN) (10 μmol/L) or DMSO during the following period: full-time-infection (0 to 12 h), during-time-infection (0–2 h), and post-time-infection (2–12 h). The DMSO-treatment group of the final selected studies was consistent with that of the “full-time” group. For all the experimental groups, the cells were infected with CV-A10, CV-B3, CV-A16-24 and CV-A16-119 (MOI = 1/0.1/1/1, respectively) at 37 ℃ for 12 h. Viral titer in cell supernatant was quantified by plaque assay. Inhibition rates were calculated as “1 − the percentage of viral titer in treatment group relatively to that of DMSO control”.

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**Figure S1.** Analysis of simplified time of addition assay. Vero cells were infected with CV-A10 (**A**), CV-B3 (**B**), CV-A16-199 (**C**) and CV-A16-24 (**D**) at an MOI of 1/0.1/1/1 at 37 °C for 2 h, respectively. The infected cells were washed three times with PBS. FAN (10 μmol/L) was added into Vero cells full-time of-, during- or post- virus infection. The supernatants were assayed for determination of viral titers at 12 hours post-infection. The inhibition rates were calculated as “1 − the percentage of viral titer in treatment group relatively to that of DMSO control”.

**Table S1. Details of primers used in the infectious clones’ construction and RT-PCR amplification.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Primer Name** | **Genome position** | **Utilities** | **Sequence** |
| EV71-XbaI-1300F | 1341 | Fusion PCR/RT-PCR/Sequencing | CTAG TCTAGA GTCATTGGGACAGTAGCAGGC |
| EV71-HindIII-3799R | 3821 | Fusion PCR/RT-PCR/Sequencing | CTAG AAGCTT CTGCGTCAGTGAAGCCTGTT |
| EV71-VP1-E145G-F | \_\_ | E145G construction | CCACCGGGGgAGTTGTCCCACAATTGCTCC |
| EV71-VP1-E145G-R | \_\_ | E145G construction | TGTGGGACAACTcCCCCGGTGGGTGTGCAC |
| EV71-VP1-V258I-F | \_\_ | V258I construction | ATGAAGCACaTCAGGGCGTGGATACCTC |
| EV71-VP1-V258I-R | \_\_ | V258I construction | TCCACGCCCTGAtGTGCTTCATTCTCATG |
| EV71-T7-5NTR-F | 1 | RT-PCR/Sequencing | ACGACTCACTATTAAG CTG TGG GTT GCA CCC A |
| EV71-HidIII-1508-R | 1489 | RT-PCR/Sequencing | CTAG AAGCTT CCTCAAATTAATCCACTGGT |
| EV71-XbaI-3641F | 3641 | RT-PCR/Sequencing | CTAG TCTAGA CTCAGAACCTGGTGATTGC |
| EV71-HindIII-6440R | 6420 | RT-PCR/Sequencing | CTAG AAGCTT AATTTTATCAATCGAGCGCAG |
| EV71-XbaI-5878F | 5878 | RT-PCR/Sequencing | CTAG TCTAGA GCAATGGCAGACAAGGTTTT |
| EV71-HidIII-poly(T) | 3’ tail | RT-PCR/Sequencing | CTAG AAGCTT TTT TTT TTT TTT TTT TTT TTT TTT T |
| EV71-45-F | 45 | Sequencing | CGCAGCACTCTGGTACCTC |
| EV71-878-F | 878 | Sequencing | TCTCAAGCAGGATCCAGACA |
| EV71-1878-F | 1878 | Sequencing | AATAATGTGCCCACGAATGC |
| EV71-2388-F | 2388 | Sequencing | ATGAAATTGTGCAAGGATGC |
| EV71-2897-F | 2897 | Sequencing | TATGTTTGTGCCACCTGGAG |
| EV71-4911-F | 4911 | Sequencing | AAACGTTGCAGCCCATTAGT |
| EV71-6401-F | 6401 | Sequencing | CACTTATGTCAAGGACGAGCTG |