Discovery of Itraconazole with Broad-Spectrum *In Vitro* Antienterovirus Activity That Targets Nonstructural Protein 3A

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There is currently no approved antiviral therapy for the prophylaxis or treatment of enterovirus infections, which remain a substantial threat to public health. To discover inhibitors that can be immediately repurposed for treatment of enterovirus infections, we developed a high-throughput screening assay that measures the cytopathic effect induced by enterovirus 71 (EV71) to screen an FDA-approved drug library. Itraconazole (ITZ), a triazole antifungal agent, was identified as an effective inhibitor of EV71 replication in the low-micromolar range (50% effective concentrations [EC₅₀s], 1.15 μ M). Besides EV71, the compound also inhibited other enteroviruses, including coxsackievirus A16, coxsackievirus B3, poliovirus 1, and enterovirus 68. Study of the mechanism of action by time-of-addition assay and transient-replicon assay revealed that ITZ targeted a step involved in RNA replication or polyprotein processing. We found that the mutations (G5213U and U5286C) conferring the resistance to the compound were in nonstructural protein 3A, and we confirmed the target amino acid substitutions (3A V51L and 3A V75A) using a reverse genetic approach. Interestingly, posaconazole, a new oral azole with a molecular structure similar to that of ITZ, also exhibited anti-EV71 activity. Moreover, ITZ-resistant viruses do not exhibit cross-resistance to posaconazole or the enviroxime-like compound GW5074, which also targets the 3A region, indicating that they may target a specific site(s) in viral genome. Although the protective activity of ITZ or posaconazole (alone or in combination with other antivirals) remains to be assessed in animal models, our findings may represent an opportunity to develop therapeutic interventions for enterovirus infection.

Enteroviruses, belonging to the *Picornaviridae* family, are non-enveloped icosahedral RNA viruses with a diameter of about 30 nm. The four species of human enteroviruses (formerly named human enteroviruses A to D [HEV-A to HEV-D]) comprise more than 100 serotypes. Most enterovirus infections do not cause significant disease, but infection can lead to serious illness, especially in infants and in those who are immunocompromised (1). HEV-A viruses are associated most commonly with hand, foot, and mouth disease (HFMD) outbreaks. Coxsackievirus A16 (CVA16) and enterovirus 71 (EV71) are the main causative agents responsible for these outbreaks. In particular, infection with EV71 is more often associated with neurological complications in children and is responsible for the majority of fatalities (2–5). Coxsackievirus B3 (CVB3), a member of the HEV-B species, is an important human pathogen that may cause acute and chronic viral myocarditis and pancreatitis in children and young adults (6–9). Poliovirus (PV), a well-known member of the HEV-C species, is the causative agent of paralytic poliomyelitis. Despite the fact that polio has been officially eradicated in Europe and the Americas, the virus is still endemic in several countries and regions (<http://www.polioeradication.org/Dataandmonitoring.aspx>). The HEV-D species contains five viruses, including enterovirus 68 (EV-D68) and enterovirus 70 (EV-D70), which can cause clinical symptoms ranging from respiratory tract infections to paralysis and/or cranial dysfunction (10–12). Recent outbreaks of EV71 in the Asia-Pacific region and EV-D68 in the United States (13) highlight the public health dangers posed by enteroviruses. Unfortunately, for enteroviruses, a vaccine is now available only for PV, and approval is still pending for three EV71 vaccine candidates which completed phase III clinical trials in 2013 (14); no approved antiviral therapeutics are currently available, and treatment

remains limited to supportive care. Several antienterovirus compounds have been progressed into clinical trials, including Rupintrivir, a viral 3C protease inhibitor, and several capsid-binding compounds (Pirodavir, Pleconaril, V-073, and BTA-798), but none of them has been formally approved by the FDA (15, 16). Therefore, there is an urgent need for the discovery and development of antiviral agents against enterovirus infection.

The enterovirus genome is a single-stranded, positive-sense RNA of approximately 7.5 kb, with a 22-amino-acid (aa) virus-encoded protein (VPg) covalently linked to the 5' end and polyadenylated at its 3' end. Flanked by 5' and 3' nontranslated regions (NTRs), the single long open reading frame (ORF) encodes a large polyprotein which is processed into three primary precursors: one structural region (P1) and two nonstructural regions (P2 and P3). The P1 region encodes four structural capsid proteins (VP4, VP2, VP3, and VP1) which form the icosahedral virion structure, with VP1, VP2, and VP3 exposed on the surface and

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VP4 arranged internally. P2 and P3 encode seven nonstructural proteins: 2A, 2B, 2C, 3A, 3B (VPg), 3C, and 3D (17). The viral replication cycle of enterovirus involves a number of critical steps, including virus adsorption, uncoating, protein translation, polyprotein processing, viral RNA replication, and virus assembly. Antiviral compounds which can affect different stages of the virus replication cycle could potentially be developed for antiviral therapy.

Seven nonstructural proteins involved in genome replication and processing are potential targets for antiviral drug development (18). Among them, the 3D protein possesses RNA-dependent RNA polymerase (RdRp) activity, which is essential for viral RNA synthesis (19); the 2A and 3C proteins have protease activities (20, 21), and a target-based strategy has been used to search for those specific enzyme inhibitors. The nonstructural protein 3A and its precursor 3AB are critical components of the replication complex, but their exact role in viral replication still remains elusive. The 3A protein is a small hydrophobic protein (86 to 89 amino acids) that contains a C-terminal hydrophobic anchor which is responsible for its membrane association (22). The soluble N terminus exists as a symmetric dimer, and each monomer consists of an α -helical hairpin with unstructured N and C termini. A benzimidazole derivative, enviroxime, was first reported to have effective activity against enteroviruses by targeting the 3A protein (23, 24). However, the results of subsequent clinical trials were not promising (25–27), and the development of this compound was halted. Nevertheless, the multifunctional feature of 3A makes it an attractive candidate as a target for inhibition of viral replication.

In an attempt to identify additional antiviral compounds against enteroviruses with broad target specificity, we developed an unbiased cell-based screening system involving multiple rounds of EV71 infection in a 96-well format. Screening of the FDA-approved compound library identified itraconazole (ITZ), a triazole antifungal agent, as a broad-spectrum inhibitor of enterovirus which acts at the step of viral RNA replication or polyprotein processing. Resistant EV71 mutants selected upon propagation in the presence of ITZ contained mutations in the viral 3A protein, which was also targeted by enviroxime-like compounds. However, ITZ-resistant viruses do not exhibit cross-resistance to the enviroxime-like compound GW5074, suggesting that they may target a specific site(s) in the viral genome. Future studies investigating its efficacy *in vivo* and clinical applications should be explored.

MATERIALS AND METHODS

Cell lines, viruses, and compounds. RD (human rhabdomyosarcoma) cells, Vero (African green monkey kidney) cells, and BHK-21 (baby hamster kidney) cells were cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen) with 10% fetal bovine serum (FBS) (Thermo Scientific HyClone) and 100 U/ml penicillin-streptomycin (PS) (Invitrogen) at 37°C with 5% CO₂. EV71 strain Fuyang573 (GenBank accession number [HM064456](#)) was used for assay development and the cell-based high-throughput primary screen. EV71 strain G082, derived from an infectious cDNA clone, was used for the virus yield reduction assay and time-of-addition assay (28). EV71 strain SH12-036 (GenBank accession number [KC570452](#)), CVA16 strain SHZH05-1 (GenBank accession number [EU262658](#)), coxsackievirus B3 (CVB3) (strain Nancy; ATCC VR-30), poliovirus 1 (PV1) (strain Sabin), and enterovirus 68 (EV-D68) (ATCC VR-1076) were also used to evaluate antiviral activity. The U.S. Drug Collection (1,040 compounds) and the International Drug Collection

(240 compounds) were purchased from MicroSource Discovery Systems Inc. (Gaylordville, CT). GW5074, ITZ, fluconazole (Sigma-Aldrich), voriconazole, and posaconazole (Selleck) were dissolved in dimethyl sulfoxide (DMSO) for antiviral experiments.

Primary screening assay. RD cells (10,000 cells in 50 μ l of DMEM) were seeded into each well of a white 96-well plate (Corning Costar) and incubated at 37°C with 5% CO₂ for 24 h prior to infection. Five microliters of each test compound at a final concentration of 10 μ M (diluted in assay medium with a final DMSO concentration of 0.25%) was added to the plates (one compound per well). In cell control and virus control wells, 0.25% DMSO alone was added. Within 10 min of compound addition, 45 μ l of diluted virus (50 PFU, which corresponds to a multiplicity of infection [MOI] of 0.005 based on initial cell plating density of 10,000 cells/well) was added to each well. In cell control wells, 45 μ l of assay medium was added. The final assay volume was 100 μ l/well. Plates were incubated at 37°C for 96 h and then allowed to equilibrate to room temperature for 30 min. Afterward, 50 μ l of CellTiter-Glo (Promega) reagent was added to each plate well, and the plates were incubated at room temperature for 10 to 30 min before being read with a Veritas microplate luminometer (Turner BioSystems). The Z factor, an assessment of the quality of screening assays, was monitored in each plate, and compounds with greater than 30% inhibition of cytopathic effect (CPE) were selected for secondary confirmation assays.

Secondary confirmation assay. ITZ was repurchased from Sigma-Aldrich and dissolved in DMSO to achieve an initial concentration of 10 mM. A dose-response study (with virus) was performed for confirmation of compound efficacy against EV71-induced CPE. The assay was done similarly to the primary assay as described above. The cytotoxicity of each compound was assessed in parallel using the same assay without the addition of virus in order to determine the concentration that resulted in 50% inhibition of cell viability (CC₅₀).

Plaque assay and TCID₅₀ assay. For EV71 strain SH12-036, approximately 3×10^5 RD cells per well were seeded in a 12-well plate (Corning Costar) 24 h in advance. A series of 1:10 dilutions were made by mixing 25 μ l of virus sample with 225 μ l of DMEM containing 2% FBS and 100 U/ml PS. Two hundred microliters of undiluted and 10-fold dilutions of viral supernatant was seeded to individual wells of 12-well plates. The plates were incubated at 37°C with 5% CO₂ for 1 h with shaking every 15 min, and then the virus inocula were replaced with 1 ml of DMEM plus 0.8% methylcellulose (Aquacide II; Calbiochem) and 2% FBS. After 6 days of incubation at 37°C with 5% CO₂, the cells were fixed with 3.7% formalin and stained with 1% crystal violet. For other viruses, the time interval between the addition of overlay and the fixation of plate was 1 day for CVB3, 2 days for PV1, and 3 days for CVA16. For EV71 strain G082, Vero cells instead of RD cells were used for plaque assay.

For EV-D68, the viral titer was determined by using a standard 50% tissue culture infective dose (TCID₅₀) protocol. Briefly, 20,000 RD cells were seeded into each well of a 96-well plate and infected 24 h later using 100 μ l of 10-fold dilutions (10^{-1} to 10^{-8}) of virus samples. Each virus dilution was applied into 10 replica wells. Virus was allowed to adsorb for 1 h and was then removed and replaced with DMEM containing 2% FBS. Plates were further incubated at 37°C with 5% CO₂ for 7 days, after which the cells were fixed with 3.7% formalin and stained with 1% crystal violet. Viral titers were expressed as TCID₅₀/ml using the method of Reed and Muench (29).

Virus yield reduction assay. RD cells were seeded in 12-well plates at 3×10^5 cells per well in 1 ml of DMEM supplemented with 10% FBS and 100 U/ml PS and incubated at 37°C with 5% CO₂. After 24 h, medium was removed and cells were infected with EV71 strain SH12-036 at an MOI of 0.1. Serially diluted ITZ was added to the cell culture media. Plates were incubated at 37°C. After 42 h, the culture media were collected and then subjected to virus titration by plaque assay as described above. For CVA16 and PV1 infection, an MOI of 0.01 was used and the culture media were collected at 42 h postinfection (p.i.). For CVB3 infection, a low MOI of 0.001 was used, and viruses in the culture media were collected at 24 h p.i.

For EV-D68 infection, an MOI of 0.1 was used, and the culture media were collected at 48 h p.i. For EV71 strain G082, Vero cells were used for infection. The titers of these viruses were determined by plaque assay or TCID₅₀ assay as described above.

Time-of-addition assay. A time-of-addition assay was performed to study the mechanism of ITZ. Vero cells were seeded at 3×10^5 cells/well in a 12-well plate 24 h before the experiment and infected with EV71 at an MOI of 5 by adsorbing the virus for 1 h at 4°C. The infected cells were then washed thrice with cold medium, and then 1 ml of medium was added to the cells. ITZ (5 μM) was added at time zero and at 1, 2, 3, 4, 5, 6, 8, and 10 h, and supernatants were collected at 12 h p.i. The titers of the virus were determined by plaque assay as described above.

Transient-replicon assay. A transient-replicon assay was used to quantify compound-mediated inhibition of viral translation and suppression of RNA replication. Replicon RNA (1 μg) was electroporated into 8×10^6 BHK-21 cells (25 μF and 0.85 kV with three pulses at 3-s intervals). The transfected cells were resuspended in 15 ml of DMEM with 10% FBS, and 1 ml of the cells was seeded in a 12-well plate and immediately treated with 10 μM ITZ or treated with 0.25% DMSO or 12.5 μM GW5074 as a control. The cells were assayed for luciferase activities at 1 and 16 h posttransfection (p.t.). Duplicate wells were seeded for each data point. For harvesting of lysates, 12-well plates were spun at $700 \times g$ for 5 min at 4°C to ensure adherence of the transfected cells. The medium was then removed, and the cells were washed with phosphate-buffered saline (PBS) and spun for an additional 5 min at $700 \times g$. After the PBS washing, 250 μl of 1× lysis buffer was added to each well (Promega). The plates containing the lysis buffer were sealed with Parafilm and stored at -80°C. Once all the samples had been collected, 20 μl of the cell lysates was transferred to a 96-well white plate and assayed for luciferase signals in a Veritas microplate luminometer (Turner BioSystems).

In vitro transcription and RNA transfection. Both genome-length and replicon RNAs of EV71 were transcribed *in vitro* from the corresponding cDNA plasmids that were linearized with NotI. A MEGAscript T7 transcription kit (Ambion) was used for RNA synthesis according to the instructions of the manufacturer. For each transfection, 10 μg of genome-length RNA was electroporated into 8×10^6 Vero cells in 0.8 ml of cold PBS (pH 7.5) in a 0.4-cm cuvette with the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV and 25 μF, pulsing three times at 3-s intervals. After a 10-min recovery at room temperature, the transfected cells were resuspended in 15 ml of prewarmed medium and incubated in T75 flask at 37°C with 5% CO₂, and the viruses in the culture fluids were collected every 24 h until an apparent cytopathic effect was observed from 24 h posttransfection (p.t.). Aliquots of the viruses were stored at -80°C.

Plasmid construction. A full-length EV71 infectious cDNA clone (designated pFLEV71) was constructed by insertion of a T7 promoter immediately upstream of the 5' NTR in a plasmid containing the genomic cDNA of EV71 strain G082 (kindly provided by Zhong Huang, Institut Pasteur of Shanghai). The firefly luciferase reporter (F-Luc) replicon of EV71 (designated pEV71-LucRep) was constructed by replacing the structural gene in pFLEV71 with the firefly luciferase gene and a 2A recognition sequence, "AITTL," immediately downstream of the F-Luc reporter for autocleavage. Specific mutations in the 3A region were constructed with pFLEV71 using a Fast site-directed mutagenesis kit (TransGen Biotech) according to the manufacturer's instructions. The DNA fragment containing the desired mutation was cut and pasted back into the original pFLEV71 or pEV71-LucRep at ClaI and SpeI sites (nucleoside positions 4161 and 6275 of the viral genome, respectively). All constructs were verified by DNA sequencing.

Generation and sequencing of EV71 resistant to ITZ. ITZ-resistant EV71 was generated by passing the EV71 strain G082 on Vero cells in the presence of ITZ. For each round of passaging, Vero cells (3×10^5 per well) in 12-well plates were infected with 50 μl of EV71 (derived from the previous passaging, with the first round of infection at an MOI of 0.1) in the presence of increasing concentrations of ITZ or 0.25% DMSO (negative control). Three independent selections were carried out in parallel.

For passage 1 (P1) to passage 14, the concentration of ITZ was doubled every two passages starting from 0.3 μM (approximately $0.5 \times EC_{50}$ in the virus yield reduction assay), and for P15 to P16, EV71 was selected with 25 μM ITZ. (See Fig. 5A for an outline of the strategy for this selection.) For each passage, viral supernatants were harvested at 48 h p.i. Resistance was determined by the fold change in viral titer between wild-type (WT) and ITZ-treated virus at 25 μM ITZ. The selections were terminated at passage 16, when no further improvement of the resistance was observed. Viral RNAs from passage 16 were extracted from the culture supernatants using a QIAamp viral RNA minikit (Qiagen). The RNAs were subjected to amplification using SuperScript III one-step reverse transcription-PCR (RT-PCR) kits (Invitrogen). The gel-purified RT-PCR products were subjected to DNA sequencing for whole genome. Primers for RT-PCR and sequencing are available upon request.

Data analysis. Raw data were imported to Microsoft Excel 2010 software for determination of signal-to-background ratio (S/B), signal-to-noise ratio (S/N), Z factor, and percent inhibition for assayed compounds. Statistical calculations were made as follows: $S/B = \mu_c/\mu_v$, where μ_c is the mean cell control signal and μ_v is the mean virus control signal; $S/N = (\mu_c - \mu_v)/(\sigma_c - \sigma_v)$, where σ_c is the standard deviation of the cell control signal and σ_v is the standard deviation of the virus control signal; and $Z = 1 - (3\sigma_c + 3\sigma_v)/(\mu_c - \mu_v)$, where a Z factor between 0.5 and 1 indicates an excellent assay with good separation between controls (30). Antiviral activity is described as percent CPE inhibition = $(\mu_{cpd} - \mu_v)/(\mu_c - \mu_v) \times 100\%$, where μ_{cpd} is the mean test compound well signal, and percent cell viability = $\mu_{cpd}/\mu_c \times 100\%$. The 50% effective concentration (EC_{50}) was defined as the compound concentration required to achieve 50% of maximal CPE inhibition or viral titer reduction, the 50% cytotoxic concentration (CC_{50}) was defined as 50% reduction in luminescence and compared to control wells. EC_{50} and CC_{50} values were calculated using the Prism software (GraphPad Prism 5; GraphPad, San Diego, CA). The selectivity index (SI) was calculated for each compound as $SI = CC_{50}/EC_{50}$.

RESULTS

Primary screening of clinical compound library. A total of 1,280 clinical compounds were screened according to the scheme shown in Fig. 1A. All compounds were screened in single dose at 10 μM. The Z factors of screening plates were in the range of 0.62 to 0.94 with an average of 0.85, indicative of robust assay performance. The average S/B and S/N were 20.77 and 69.37, respectively. A "hit" for this assay was defined as any compound exhibiting greater than 30% inhibition of virus-induced CPE. Three hits were identified in the primary screening (Fig. 1B), yielding a hit rate of 0.23%. These compounds were cycloheximide, sucralfate, and ITZ. Among them, cycloheximide, a known inhibitor of eukaryotic protein synthesis, was not selected for further study due to its significant toxic side effects. Sucralfate, a cytoprotective agent, is used clinically for the treatment of active duodenal ulcers. The structure of sucralfate is similar to those of other polysulfated compounds, such as heparan sulfate, which serve as receptors/coreceptors or attachment receptors of numerous viruses, such as Sindbis virus (31), herpes simplex virus 1 (HSV-1) (32), and EV71 (33). Therefore, sucralfate may act as receptor mimetic, competing for virion binding to cellular heparan sulfate, which results in the inhibition of membrane attachment of viruses. ITZ is an orally active triazole antifungal agent with a wide spectrum of activity, including activity against a wide variety of *Aspergillus* and *Candida albicans* strains (34, 35), and has also been reported to be effective for the treatment of mycotic infections in children (36, 37). The pharmacokinetics of ITZ in healthy adult volunteers are characterized by good oral absorption, an extensive tissue distribution, and a relatively long elimination half-life ($t_{1/2}$) (about 1 day) (38).

A HTS scheme (96-well format)

FIG 1 Primary screening of the FDA-approved compound library. (A) Flow-chart of the HTS assay. Briefly, 10,000 RD cells per well were seeded in 96-well plates, infected with 50 PFU of EV71, incubated with compounds for 96 h, and measured for luminescence activities. (B) Primary screening of 1,280 compounds from the FDA-approved compound library in single dose at 10 μ M. Inhibitory effects were calculated as percent CPE inhibition. Hits were selected with a cutoff of 30% CPE inhibition (dotted line).

Hence, ITZ was selected for further characterization based on its favorable pharmacokinetic and safety profiles.

ITZ inhibits EV71 replication at noncytotoxic concentrations. ITZ (Fig. 2A) was reordered and dissolved in DMSO, and then dose-response analysis was performed in two secondary assays, including the same CPE assay as for primary screening and a parallel cytotoxicity assay. As shown in Fig. 2B, ITZ inhibits the EV71-induced CPE in a dose-responsive manner without significant cytotoxicity. The EC_{50} of ITZ was 1.15 μ M in the CPE assay and the CC_{50} was greater than 25 μ M (the highest tested concentration), yielding an SI of greater than 21.7. We then used a virus yield reduction assay to further confirm the results. Conventional plaque assays were employed to quantify the amount of EV71 produced by Vero cells treated with 3-fold serial dilutions of ITZ. ITZ suppressed EV71 replication with an estimated EC_{50} of 0.53 μ M (Fig. 2C) and reduced viral titers by more than 1.7 $\log_{10}$ units at 25 μ M. Taken together, these data reconfirmed that the primary hit ITZ could inhibit EV71 infection.

ITZ is a broad-spectrum inhibitor of enterovirus replication. The antiviral activity of ITZ against a panel of enteroviruses was determined by virus yield reduction assay. We chose another recent EV71 clinical isolate (EV71 strain SH12-036) to confirm that the antiviral activity of ITZ is not limited to the EV71 strain used in our screening assay and CVA16 to confirm that ITZ could inhibit the other main causative agent of HFMD. We also selected CVB3 (a human enterovirus B species member), PV1 (a human enterovirus C species member), and EV-D68 (a human enterovirus D

A Itraconazole

FIG 2 Reconfirmation of ITZ in secondary assays. (A) Chemical structure of ITZ. (B) Validation of ITZ by CPE assay. Threefold serial dilutions of compounds were added to RD cells, and the inhibitory effects of the compounds were analyzed by CPE assay (see details in Materials and Methods). Cytotoxicity was also examined by incubation of RD cells with the indicated concentrations of compounds. Cell viability was measured with the CellTiter-Glo reagent and is presented as a percentage of luminescence derived from the compound-treated cells compared with that from the mock-treated cells (with 0.25% DMSO). Average results from three experiments are shown. Error bars represent the standard deviations of three independent measurements. (C) Validation of ITZ by virus yield reduction assay. Vero cells were infected with EV71 strain G082 at an MOI of 0.1 and treated with 3-fold serial dilutions of ITZ. Supernatants were collected at 42 h postinfection, and viral titers were determined by plaque assay. The data presented were obtained from three independent experiments. Error bars represent the standard deviations from three independent experiments.

species member) to evaluate the antienterovirus activity of ITZ. As shown in Fig. 3A and B, ITZ consistently inhibited all the tested enteroviruses, with EC_{50} ranging between 0.12 and 1.81 μ M (Fig. 3C), and CVA16 was the most sensitive to ITZ (EC_{50} of 0.12 μ M), while EV71 SH12-036 was the most resistant to ITZ treatment (EC_{50} of 1.81 μ M). Interestingly, ITZ could reduce the viral titer of PV1 by 18,275.9-fold at 25 μ M. Altogether, the results indicate that ITZ may represent a novel class of broad-spectrum antiviral drugs effective against enterovirus replication.

FIG 3 Broad-spectrum antienterovirus activity of ITZ. (A) RD cells were infected with EV71 SH12-036 (MOI = 0.1), PV1 (MOI = 0.01), CVA16 (MOI = 0.01), or CVB3 (MOI = 0.001) and treated with various concentrations of ITZ. Supernatants were collected at 42, 42, 42, and 24 h p.i., respectively, and viral titers were determined by plaque assay as described in Materials and Methods. The data shown were obtained from two independent replicates. Error bars indicate the standard deviations from two independent experiments. (B) RD cells were infected with EV-D68 at an MOI of 0.1 and treated with ITZ at the indicated concentrations. At 48 h p.i., supernatants were analyzed for infectious virus titers by TCID₅₀ assay as described in Materials and Methods. The TCID₅₀/ml was determined using the method of Reed and Muench (29). The data shown were obtained from two independent replicates. Error bars indicate the standard deviations from two independent experiments. (C) Mean EC₅₀s of ITZ against different enteroviruses were calculated using Prism's nonlinear regression (GraphPad Prism5). Fold reduction was determined by dividing the titer from 0.25% DMSO-treated virus by that from 25 μM ITZ-treated virus.

ITZ acts at the step of viral RNA replication or polyprotein processing. Inhibition of virus replication in a multicycle assay could be the result of interference in any steps of the viral replication cycle by the compound. To identify the step(s) at which ITZ suppresses enterovirus infection, we analyzed the inhibitor in a time-of-addition assay and a transient-replicon assay. A time-of-addition experiment was initially performed to elucidate the mechanism of action of ITZ. Vero cells were synchronously infected with EV71 at an MOI of 5. ITZ (5 μM) was added to the infected cells at various time points postinfection. Viral titers in the culture medium were determined at 12 h p.i. As controls, 0.25% DMSO was added to infected cells at 0, 4, and 10 h p.i. for estimation of its effect on viral yield. As shown in Fig. 4A, the inhibitory effect of ITZ on viral titer gradually diminished when the compound was added later than 4 h after infection, indicating that ITZ does not function at an early (attachment, entry, or uncoating) or late (assembly or release) stage.

To narrow the step in the viral replication cycle blocked by the compound, we then employed a subgenomic EV71 replicon in which the capsid-coding region was replaced by the firefly luciferase gene. Replication of the F-Luc EV71 replicon yields large amounts of firefly luciferase. Quantification of intracellular luciferase levels is a sensitive measure to distinguish between the steps of viral RNA translation and replication and eliminates possible effects of the compound on virion assembly and release in the viral replication cycle. In our transient-replicon assay system, we found that luciferase activity at 1 h p.t. could represent the viral translation, while signal at 16 h p.t. could represent the viral RNA replication, which is consistent with the virus replication cycle (data not shown), and we therefore measured the luciferase activities at 1 and 16 h p.t. to differentiate between inhibition of viral translation and inhibition of RNA replication. GW5074, an enviroxime-like 3A inhibitor of viral RNA replication, was used as a control to distinguish between viral translation and RNA replication. As shown in Fig. 4B, treatment with ITZ and GW5074 did not result in a reduction in the luciferase signal at 1 h p.t.; however, treatment with ITZ and GW5074 reduced luciferase signals at 16 h p.t. by 89.2% and 85.5%, respectively. Taken together, the transient-replicon result was consistent with the result from the time-of-addition assay which demonstrated that ITZ strongly suppresses viral RNA replication or polyprotein processing.

Selection of ITZ-resistant EV71. Because resistance mutations usually locate to the viral protein physically targeted by the compound, we then generated resistant virus to identify the precise viral target of ITZ. ITZ-resistant viruses were generated by culturing EV71 in the presence of increasing concentrations of compound, starting at ~0.5 × EC₅₀, for 16 rounds (Fig. 5A). Viruses from P16 were assayed for the resistance phenotype by comparison of the fold change with the virus passaged in 0.25% DMSO in parallel (for the DMSO control, fold change was set as 1.0). Compared with the DMSO control, the P16 viruses were partially resistant to ITZ (Fig. 5B, left panel). As summarized in Fig. 5B (right panel), at 25 μM, the selection I (Sel I) to Sel III virus generated fold changes of 5.5 to 9.0 compared to the DMSO control. Further passaging of the P16 viruses did not improve the resistance phenotype (data not shown). We then sequenced the complete genome of the P16 resistant virus. Seven nucleotide changes were identified in 5 regions (Fig. 5C). For Sel I, three mutations were found: one silent mutation in VP1 (a C-to-U substitution at nucleotide position 2962); one silent mutation in 3D (a U-to-C sub-

FIG 4 Mechanism of ITZ-mediated inhibition of EV71. (A) Time-of-addition analysis of ITZ in EV71 infection. Vero cells were infected with EV71 at an MOI of 5 at 4°C for 1 h. The infected cells were washed three times with cold medium. ITZ (5 μ M) was then added to the cells at the indicated time points postinfection. The supernatants were assayed for determination of viral titers at 12 h p.i. As controls, 0.25% DMSO was added to the infected cells at 0, 4, and 10 h p.i. for estimation of its effect on viral production. The results shown are representative of one of two independent experiments. (B) Transient-replicon assay. A firefly luciferase reporting replicon (1 μ g) was electroporated into BHK-21 cells. The transfected cells were immediately incubated with 10 μ M ITZ, 12.5 μ M GW5074, or 0.25% DMSO (as controls), and the luciferase activities were measured at the indicated time points. Average results and standard deviations ($n = 3$) are presented. Differences in luciferase activities between each treated group and the DMSO control group were compared by two-way analysis of variance (ANOVA) (***, $P < 0.001$).

stitution at nucleotide position 6433); and a G-to-U substitution at nucleotide position 5213, resulting in an amino acid substitution of Val to Leu at position 51 (V51L) in the 3A protein. For Sel II, three mutations were also found: one silent mutation in VP3 (a G-to-U substitution at nucleotide position 2365); a U-to-C substitution at nucleotide position 5286, resulting in an amino acid substitution of Val to Ala at position 75 (V75A) in the 3A protein; and a C-to-U substitution at nucleotide position 7008, resulting in an amino acid substitution of Ala to Val at position 358 (A358V) in 3D. For Sel III, only two mutations were found: a C-to-A substitution at nucleotide position 634 in the 5' NTR and a common substitution 3A V75A, which was also found in Sel II. As a negative control, viruses cultured in 0.25% DMSO for 16 passages had a mutation (a C-to-A substitution at nucleotide position 3147, resulting in an amino acid substitution from Thr to Asn at position 237 in the VP1 protein) but did not exhibit the mutations described above. In summary, three mutations resulting in amino acid changes were mapped to the 3A and 3D regions for all three selections. Each of selections carried a single amino acid substitution in the 3A protein, and Sel II and Sel III shared a common mutation (3A V75A), indicating that the 3A protein could be the potential target of ITZ.

Sequence alignment of the 3A protein showed that (i) V51 is conserved among EV-D68, EV71, and CVA16, whereas PV1 and CVB3 have a Thr residue and an Ile residue at this position, respectively, and (ii) V75 is conserved among various enteroviruses but is distinct in CVB3, which has a Ile residue at this position (Fig. 5D). On the putative structure model of the 58-residue N-terminal domain of the EV71 3A protein, residue V51 is located at unstructured C termini (Fig. 5E), while residue V75 is located at the hydrophobic domain of the 3A protein (39).

Substitutions in 3A render EV71 resistant to ITZ. To evaluate whether the identified 3A mutations confer resistance to ITZ, we introduced single nucleotide substitutions into both the replicon and full-length cDNA clone of EV71 by site-directed mutagenesis. A transient-replicon assay was initially performed to validate the contribution of 3A mutations to resistance. In the absence of ITZ, both 3A mutant replicons yielded slightly lower levels of luciferase activities than the WT at 1 and 16 h p.t.; in contrast, the 3A V51L

and 3A V75A replicons exhibited luciferase signals 3.7- and 1.7-fold higher than that of the WT replicon, respectively, in the presence of 10 μ M ITZ at 16 h p.t. (Fig. 6A). These results demonstrate that the substitutions 3A V51L and 3A V75A do not affect viral translation but that they enhanced the ability of EV71 to replicate in the presence of ITZ. We then measured the sensitivities of mutant viruses to ITZ using a modified CPE assay. Because our EV71 strain G082 is well adapted to Vero cells, the optimized CPE assay conditions have two modifications: (i) the cell number is 5,000, and (ii) the MOI is 0.05 (data not shown). The 3A V51L and 3A V75A mutant viruses exhibited 2.6- and 3.4-fold shifts in EC₅₀s, respectively, and showed >20% reduction in peak CPE inhibition (Fig. 6B), confirming that both 3A mutant viruses were less susceptible to the antiviral effect of ITZ. Finally, we tested the infectivities of 3A mutant viruses in the presence and absence of 25 μ M ITZ. Both mutant viruses grew normally in the absence of ITZ (Fig. 6C, left panel). Resistance analysis showed that the 3A V51L and 3A V75A mutant viruses generated changes of 5.0- and 8.2-fold compared to the WT, respectively. The resistance levels of mutant viruses were comparable to what was observed for the original selections (Fig. 6C, right panel, versus Fig. 5B, right panel), demonstrating that the single substitutions V51L and V75A in the 3A protein confer EV71 resistance to ITZ.

To further examine the effect of the 3A mutations on viral replication, we compared the growth kinetics of the WT and mutant viruses. Vero cells were infected with WT or mutant viruses (MOI, 0.1) and were then monitored for viral yields. The growth kinetics of mutant viruses were not significantly different from those of WT virus at all tested time points (Fig. 6D, left panel). Inspection of the plaque phenotypes produced by the recombinant viruses showed that both the 3A V51L and 3A V75A mutant viruses were able to produce plaques similar to those produced by WT virus (Fig. 6D, right panel). These results suggest that the 3A mutations do not compromise viral fitness in cell culture.

Posaconazole, a new triazole antifungal agent, also exhibits anti-EV71 activity. There are 4 triazole antifungal agents (fluconazole, itraconazole, voriconazole, and the newest agent, posaconazole) currently available for systemic use in the United States (40). Posaconazole differs from ITZ by the presence of a

FIG 5 Selection and sequencing of ITZ-resistant EV71. (A) Scheme for the selection of ITZ-resistant EV71. From P1 to P14, the concentration of ITZ was doubled every two passages starting from 0.3 μ M, and P15 to P16 were selected at 25 μ M. (B) Resistance analysis. Vero cells were infected with wild-type virus passed in 0.25% DMSO (DMSO control) or P16 selections (Sel I to III) at an MOI of 0.1 in the presence of 25 μ M ITZ or 0.25% DMSO (as a negative control). At 42 h p.i., the viral titers in culture fluids were quantified by plaque assays. Fold reduction in viral titer at 25 μ M ITZ was calculated as for Fig. 3C, and resistance was quantified by fold change compared to the wild-type virus passed in 0.25% DMSO (DMSO control), which was set as 1.0. (C) Summary of mutations identified from the three selections. Locations of the nucleotide and/or amino acid changes are indicated. (D) Sequence alignment of 3A proteins from EV71, CVA16, CVB3, PV1, and EV-D68 were generated using the ClustalW2 program. Sequences with the following GenBank accession numbers were used: [FJ713137](#) (EV71), [EU262658](#) (CVA16), [M33854](#) (CVB3), [AY297760](#) (PV1), and [KF726085](#) (EV-D68). The arrows indicate mutations in ITZ-resistant EV71 identified in the present study. Two α -helices that form an α -helical hairpin in PV 3A protein are indicated as α 1 and α 2, respectively. (E) Location of the V51 mutation involved in EV71 resistance to ITZ in a structural model of the EV71 3A protein. The structural model generated using SWISS-MODEL (<http://swissmodel.expasy.org/>) with a template of the PV 3A soluble domain (Protein Data Bank code 1NG7) shows a homodimer of the N-terminal 58 aa of the EV71 3A protein. Residue V51 is shown in red.

FIG 6 Analysis of resistance mutations. (A) Transient-replicon assay. WT or mutant replicon (1 μg) was electroporated into BHK-21 cells. The transfected cells were immediately incubated with 10 μM ITZ or 0.25% DMSO (as controls), and the luciferase activities were measured at the indicated time points. Average results from three independent experiments are presented. Differences in luciferase activities between the ITZ-treated group and the DMSO control group were compared by two-way ANOVA (***, $P < 0.001$). (B) Resistance analyses of mutant viruses in CPE assay. The CPE assay was performed as described in Materials and Methods with the following modifications: 5,000 Vero cells were infected with EV71 strain G082 (WT) or mutant viruses at an MOI of 0.05. Other conditions were unchanged. The data presented were obtained from two independent experiments. Error bars represent the standard deviations from two independent measurements. (C) Resistance analyses of mutant viruses in virus yield reduction assay. The resistance analyses were performed as described in the legend to Fig. 5B. Average results from three experiments are shown. Error bars represent the standard deviations from three independent experiments. (D) Phenotypic characterization of resistance mutants. Left panel, growth kinetics of wild-type EV71 and recombinant viruses containing the 3A mutation. Confluent Vero monolayers in 12-well plates were infected with the WT and mutant viruses at an MOI of 0.1. After 1 h of incubation, the cells were washed three times with medium and replenished with 1 ml of medium. Viral titers in culture fluids were quantified at the indicated time points using plaque assays. The data shown were obtained from two independent replicates. Error bars indicate the standard deviations from two independent experiments. Right panel, plaque phenotypes of wild-type EV71 and recombinant viruses.

FIG 7 Identification of posaconazole, a new-generation oral triazole antifungal agent with anti-EV71 activity. (A) Anti-EV71 activities of three triazole antifungal agents. Upper panels, chemical structures of posaconazole, fluconazole, and voriconazole. Lower panels, CPE assay and cell viability assay were performed as described in the legend to Fig. 2B. The data presented were obtained from two independent experiments. Error bars represent the standard deviations from two independent experiments. (B) Posaconazole and GW5074 resistance analyses of mutant viruses in CPE assay. The CPE assay was performed as described in the legend to Fig. 6B. The data presented were obtained from two independent experiments. Error bars represent the standard deviations from two independent experiments. (C) Posaconazole and GW5074 resistance analyses of mutant viruses in virus yield reduction assay. Ten micromolar posaconazole and 5 μ M GW5074 were used to compare the fold change between 3A mutants and WT. The resistance analyses were performed as described in the legend to Fig. 5B. The data shown were obtained from two independent replicates. Error bars indicate the standard deviations from two independent experiments.

furan ring and substitution of chlorine with fluorine, while fluconazole and voriconazole lack an extended side chain like ITZ (Fig. 7A, upper panel, versus Fig. 2A). We next assessed the anti-EV71 activities of these compounds in the CPE assay. As shown in Fig. 7A, posaconazole, which has a structure similar to that of ITZ showed anti-EV71 activity with an EC_{50} of 1.29 μ M and a CC_{50} of greater than 10 μ M, while fluconazole and voriconazole did not inhibit the EV71-induced CPE at 100 μ M (the highest tested concentration). These results indicate that the side chain of ITZ is important for antienterovirus activity.

ITZ-resistant viruses do not exhibit cross-resistance to posaconazole or the enviroxime-like compound GW5074. Enviroxime was first reported to have effective activity against enteroviruses, which targets the 3A protein (24), and later several so-called enviroxime-like compounds (e.g., GW5074, TTP-8307, and AN-12-H5) were discovered, and all of them were found to have similar mutations in the 3A protein region causing resistance to enviroxime (a G5318A, 3A A70T mutation in PV) (41). To determine whether the ITZ-resistant virus had decreased sensitivity to posaconazole and another 3A inhibitor GW5074, we measured the sensitivities of mutant viruses to posaconazole and GW5074 in the CPE assay. For posaconazole, only a slight reduction (about 10%) in peak CPE inhibition was observed for both 3A mutant viruses; however, for both posaconazole and GW5074, the EC_{50} s of 3A mutant viruses were comparable to that of the WT (Fig. 7B), suggesting that ITZ-resistant EV71 viruses are not cross-resistant to posaconazole and GW5074. We also tested the infectivities of 3A mutant viruses in the presence of posaconazole or GW5074. As shown in Fig. 7C, posaconazole (5 μ M) and GW5074 (10 μ M) reduced the WT virus titer by 12.4- and 1,382.0-fold, respectively. In parallel, both 3A mutant viruses showed fold changes with posaconazole (0.6- to 0.9-fold) and GW5074 (1.0- to 1.5-fold) that were similar to those for WT virus (Fig. 7B, right panel), which indicates that these compounds may target a specific site(s) in the viral genome.

DISCUSSION

The goal of this study was to screen libraries of approved compounds which may potentially be repurposed as enterovirus antivirals. Given that licensed inhibitors have previously defined pharmacokinetic and safety profiles, the repurposing approach could shorten the time to provide clinically useful inhibitors of enterovirus. Although Arita and coworkers previously identified metrifudil, N6-benzyladenosine, NF449, and GW5074 as EV71 inhibitors by screening the LOPAC¹²⁸⁰ drug library using EV71 pseudovirus (42), we rescreened the FDA-approved compound library using a cell-based CPE assay and identified 3 new hits. Compared to screening with pseudovirus, which lacks the step of virus assembly and release, the cell-based CPE assay allows screening for inhibitors targeting all steps of the virus replication cycle. Among three primary hits, sucralfate together with suramin were also recently identified by our group as EV71 inhibitors targeting virion attachment using a quantitative RT-PCR (qRT-PCR)-based assay (43), which suggests that these polysulfated/poly-sulfonated compounds may have potential as therapeutic compounds for the treatment of EV71 infection. Because enteroviruses cause diseases in all age groups, a drug with a proved safety profile in children will broaden usage in the treatment of enterovirus disease. ITZ is well tolerated and safe in infants and children (44) and therefore is worthy of being further investigated.

There are more than 100 distinct serotypes of enteroviruses, and an ideal antienterovirus drug should exhibit effective activity against a broad spectrum of enteroviruses. Here we showed that ITZ exhibited broad-spectrum antiviral activity against enteroviruses in cell culture, including CVA16, CVB3, PV1, and EV-D68. Although the *in vivo* protective activity of ITZ remains to be assessed, pharmacokinetic studies in healthy adult volunteers have shown that mean peak ITZ concentrations in plasma (C_{max}) following a dosage of 200 mg twice daily for 2 weeks were 553 ng/ml (equal to 0.78 μ M) and 1,980 ng/ml (equal to 2.81 μ M) on days 1 and 15, respectively (38); the C_{max} on day 1 was lower than the *in vitro* EC_{50} (listed in Fig. 3C) only for EV71 strain SH12-036, while the C_{max} on day 15 exceeded all the EC_{50} s, indicating its use in a clinical context.

Time-of-addition and transient-replicon assays clearly showed that the antiviral effect of ITZ involves inhibition of RNA replication or polyprotein processing. ITZ resistance mutations were localized in the unstructured C termini and hydrophobic domain of the 3A protein. However, neither mutation could confer resistance to another enviroxime-like compound, GW5074, suggesting different mechanisms of action for these two compounds. In agreement with our result, ITZ was recently identified as a new member of the minor enviroxime-like compounds, which inhibit phosphatidylinositol 4-phosphate (PI4P) production and/or accumulation at the Golgi apparatus by targeting oxysterol-binding protein (OSBP) family I, while GW5074 belongs to the major enviroxime-like compounds, which target host phosphatidylinositol 4-kinase III beta (PI4KB) (45). Thus, it is possible that 3A mutations could functionally compensate for the compound-mediated loss of PI4P production and/or accumulation, resulting in resistance. Interestingly, the 3A V75A mutation was also found to be able to confer EV71 resistance to an adenosine analog, NITD008, which blocks viral RNA synthesis (46), suggesting that it is a "hot spot" for accumulation of resistance mutations. However, the precise resistance mechanism of the 3A mutations has yet to be determined. Unraveling the detailed mechanism may shed light on the biology of enteroviruses.

In summary, we identified ITZ as a broad-spectrum inhibitor of enteroviruses that targets the 3A protein. During the review process, we became aware that Strating and coworkers addressed ITZ activity against enterovirus using CVB3 as a model (47). ITZ and posaconazole could serve as important tools for understanding the fundamental aspects of the formation and function of enterovirus replication complexes. Moreover, our findings may also offer a starting point which deserves further evaluation with respect to its clinical potential for the treatment of patients infected with enteroviruses. Current efforts are focused on the evaluation of combinations of ITZ with other known pharmacologically active compounds with antienterovirus activity.

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