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Discovery of Structurally Diverse Small-Molecule Compounds with Broad Antiviral Activity against Enteroviruses

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Antiviral drugs do not currently exist for the treatment of enterovirus infections, which are often severe and potentially life-threatening. We conducted high-throughput molecular screening and identified a structurally diverse set of compounds that inhibit the replication of coxsackievirus B3, a commonly encountered enterovirus. These compounds did not interfere with the function of the viral internal ribosome entry site or with the activity of the viral proteases, but they did drastically reduce the synthesis of viral RNA and viral proteins in infected cells. Sequence analysis of compound-resistant mutants suggests that the viral 2C protein is targeted by most of these compounds. These compounds demonstrated antiviral activity against a panel of the most commonly encountered enteroviruses and thus represent potential leads for the development of broad-spectrum anti-enteroviral drugs.

The human enteroviruses (EVs) are a genus of small nonenveloped RNA viruses that are typically encountered during the summer and fall (1). Circulation of the three polioviruses (PV 1 to 3) has nearly been eliminated by immunization (2), but more than 110 other genetically distinct EVs with enormous medical impact also exist. These nonpolio EVs most often produce mild respiratory tract and gastrointestinal diseases and rash, but they are also among the most common causes of aseptic meningitis, encephalitis, and myocarditis (1). Progression from mild to severe clinical illness is also unpredictable, as seen in outbreaks of hand, foot, and mouth disease (HFMD) caused by enterovirus A71; most cases resolve uneventfully, while other individuals may develop brainstem encephalitis, pulmonary edema, or other life-threatening manifestations (3, 4). Enteroviruses also represent a perennially epidemic public health threat due to their genetic diversity and to the regular emergence of new, more pathogenic variants of known serotypes. For example, a new variant of coxsackievirus B1 (CVB1) emerged in the United States in 2007 and was associated with reports of sepsis and myocarditis in newborns at more than 40 locations in the continental United States and Alaska (5, 6). Similarly, in 2014, an outbreak of enterovirus D68 (EV-D68), previously likened to rhinoviruses in pathogenicity (7), was responsible for more than 1,100 reports of severe respiratory disease and was linked to more than 100 cases of acute flaccid myelitis (8–12).

Although enteroviruses vary greatly in their specific disease manifestations, they share many common virological features (13). They are small (27 to 30 nm) nonenveloped viruses with a single-stranded plus-sense RNA genome of approximately 7,500 nucleotides, which encodes a single large (~240 kDa) polyprotein. The single open reading frame encoding the polyprotein is flanked by untranslated regions (UTRs; 5′ UTR and 3′ UTR) involved in directing genome replication and translation. Cellular adhesion molecules typically function as viral receptors, which promote attachment and receptor-mediated endocytosis of virions into target cells. Following its release from virions, the genomic RNA is translated into the viral polyprotein. An autoproteolytic cleavage of the polyprotein gives rise to three proteins (P1 to P3), which are cleaved further into structural and enzymatic proteins. A key early event is the cleavage of the polyprotein by the 2A protease, releasing P1 from the remainder of the nascent polyprotein. P1 subsequently undergoes cleavage by the 3C protease to yield four peptides (VP1 to VP4) that make up the capsid of mature virions. Cleavage products of P2 and P3 are involved in the synthesis of minus-strand RNA and of additional copies of plus-sense RNA, leading to amplification of infection. The enterovirus life cycle is typically completed in 6 to 12 h and rapidly produces cell lysis in susceptible cells.

Despite decades of research, antiviral medications do not currently exist for coxsackievirus infections or for other enterovirus infections. Antiviral agents have been identified that bind to the viral capsid, that inhibit the virus-encoded RNA polymerase or proteases, or that interfere with other viral replication processes. Unfortunately, none have moved beyond initial clinical studies due to limited efficacy (enviroxime) or safety concerns (pleconaril) (14, 15). Others, including the viral capsid inhibitor BTA-798 (vapendavir), the protease inhibitor AG7088 (rupintrivir), and the viral 3D polymerase inhibitor (DTriP-22), remain at preclinical or early clinical phases of evaluation (15–18). Consequently, treatment of serious enterovirus infections presently consists of supportive care, such as management of seizures, hemorrhage, cardiac arrhythmias, and respiratory failure (1). Identification of clinically useful antiviral agents would reduce morbidity and mortality due to enteroviruses and may potentially play a role in the final elimination of poliovirus circulation (16).

In search of useful anti-enteroviral agents, we screened various

TABLE 1 List of enteroviruses used

EV species	Serotype	Source	Strain	Host cell
EV-A	Enterovirus A-71 (EV-A71)	CDC (courtesy of S. Oberste)	NA[a]	LLC-MK2
EV-B	Coxsackievirus A9 (CV-A9)	ATCC VR-186	PB (1950)	LLC-MK2
	Coxsackievirus B1 (CV-B1)	(31)	CVB-Chi07 (2007)	HeLa-RW
	Coxsackievirus B2 (CV-B2)	ATCC VR-29	Ohio-1 (1947)	HeLa-RW
	Coxsackievirus B3 (CV-B3)	Cell transfection with pH 3 (21)	H3 (1991)	HeLa-RW
	Coxsackievirus B4 (CV-B4)	ATCC VR-184	JVB (1951)	HeLa-RW
	Coxsackievirus B5 (CV-B5)	ATCC VR-185	Faulkner (1952)	HeLa-RW
	Echovirus 6 (E-6)	ATCC VR-36	D'Amori (1955)	LLC-MK2
	Echovirus 7 (E-7)	ATCC VR-37	Wallace (1953)	LLC-MK2
	Echovirus 9 (E-9)	ATCC VR-39	Hill (1953)	LLC-MK2
	Echovirus 11 (E-11)	ATCC VR-41	Gregory (1953)	LLC-MK2
	Echovirus 25 (E-25)	ATCC VR-1066	JV-4 (1957)	LLC-MK2
	Echovirus 30 (E-30)	ATCC VR-1660	Bastianni (1963)	LLC-MK2
EV-C	Poliovirus 1 (PV-1)	ATCC VR-1562	Chat (1957)	HeLa-RW
	Poliovirus 3 (PV-3)	ATCC VR-193	Fox [Wy 3] (1957)	HeLa-RW

[a] NA, not available.

small-molecule libraries containing 85,585 compounds and identified previously unrecognized inhibitors of enterovirus replication. We previously reported that fluoxetine, a selective serotonin reuptake inhibitor, demonstrated potent antiviral activity against coxsackieviruses (19). In this report, we describe additional enterovirus inhibitors, including a wide variety of novel antiviral agents. Interestingly, these inhibitors did not interfere with viral internal ribosome entry site (IRES) function or with the activities of the viral proteases, but they did drastically reduce viral RNA and viral proteins in cell culture, suggesting that inhibition of viral RNA replication may be the common inhibition mechanism shared by these structurally diverse compounds. Sequence analysis of compound-resistant mutants pointed to the viral 2C protein as the target of most compounds. These compounds represent candidates for further development toward clinical applications against enteroviral infections.

MATERIALS AND METHODS

Cells and viruses. HeLa-RW cells (20) were generously provided by J. Lindsay Whitton (The Scripps Research Institute, La Jolla, CA) and were grown in Dulbecco modified Eagle medium (DMEM) supplemented with penicillin, streptomycin, glutamine, and 10% fetal bovine serum. The LLC-MK2 cell line was obtained from ATCC (Manassas, VA). Stocks of CVB-H3 (21) and CVB3-eGFP (20) were produced by cotransfecting HeLa-RW cells with a plasmid expressing the T7 polymerase (pAR3126) and plasmid clones of the viral genome (22). In addition, important pathogenic enteroviruses (Table 1), including the reference strains of 12 enteroviruses most commonly identified in the United States between 1975 and 2008 (23, 24), were obtained from ATCC or other sources and were propagated in either LLC-MK2 Original cells or in HeLa-RW cells.

Virus quantification. For group B coxsackieviruses (B1 to B5) and polioviruses 1 and 3, infectious virus titers were determined by plaque assays on monolayers of HeLa-RW cells (22, 25). Viral quantification of enterovirus A71 (EV-A71), coxsackievirus A9, and echoviruses 6, 7, 9, 11, 25, and 30 was accomplished using a 50% tissue culture infective dose (TCID$_{50}$) (26) assay modified from an EV-A71 protocol (27).

Primary screening assay. A high-throughput cell assay was used to screen for novel inhibitors of enterovirus replication as previously described (19). Briefly, 20 μl of culture medium per well was dispensed into 384-well microtiter plates (Greiner One) using a Multidrop 384 dispenser (Thermo LabSystems), followed by the addition of 0.5 μl of 1 mM library

compound solution in dimethyl sulfoxide (DMSO) using a 500 nl V&P custom pin tool (San Diego, CA). In negative-control wells, 0.5 μl DMSO was added. HeLa-RW cells and CVB3-H3 virus were mixed, and 30 μl was added to each well using a Multidrop 384 dispenser to reach 3,000 cells with 20 PFU CVB3-H3 per well and a multiplicity of infection (MOI) of 0.007. Guanidine, known to potently inhibit enteroviral RNA synthesis (15, 28), was used as a positive control at a final concentration of 10 mM and resulted in complete protection from the cytopathic effects of CVB3 infection. After 48 h of incubation, cytopathic effect induced by CVB3-H3 was quantified by adding 25 μl ATPLite 1-Step reagent (PerkinElmer) to each well. The luminescence signals on the plate were read immediately using a FLUOstar Optima reader (BMG Labtech Inc., Cary, NC). The Z' of the assay was >0.5. A compound was designated a hit if the luminescence of cells incubated with CVB3 in its presence was at least 50% guanidine-treated infected cells.

Small-molecule compound libraries. The compound libraries are as follows: (i) the BioMol library consisting of 204 bioactive lipids, 60 endocannabinoids, 72 ion channel compounds, 84 enzyme inhibitors, and 84 phosphatase and kinase inhibitors and orphan ligands; (ii) an FDA-approved drug library consisting of 1,120 high-purity chemical compounds carefully selected for their structural diversity and broad therapeutic spectrum, including agents used for neuropsychiatric, cardiovascular, and immunologic conditions, as well as compounds used for their anti-inflammatory and analgesic effects; (iii) the Microsource Spectrum Collection consisting of 2,000 biologically active and structurally diverse compounds, including known drugs, experimental bioactive compounds, and pure natural products; (iv) the Druggable Compound Set consisting of 8,000 compounds targeted at various kinases, proteases, ion channels, and G-protein coupled receptors (GPCRs); (v) the Lead-Like Compound Set consisting of 20,000 compounds custom tailored for lead likeness; and (vi) the UCLA Chemically Diverse Library consisting of a collection of 30,000 chemically diverse small molecules in ChemBridge DIVERSet and a custom set of 20,000 compounds selected for low cellular toxicity and excellent coverage of the chemical space. All library compounds were dissolved in DMSO, allocated onto library plates at a stock concentration of 1 mM.

Determination of EC$_{50}$ against CVB3 infection and CC$_{50}$ of hit compounds. After the primary and secondary screens, 12 compounds that represented the structural diversity of the active compounds were chosen for further antiviral testing. These compounds were dissolved in DMSO to an initial concentration of 20 mM, and 19 serial 2-fold dilutions were subsequently prepared; the lowest concentration was 38 nM. Serially di-

luted compounds were added into HeLa-RW cell cultures to achieve final concentrations ranging from 200 μM to 380 pM. The cells were either infected with CVB3-H3 for the measurement of antiviral activity or were cultured without infection to measure compound toxicity. The ATPLite-based assay in the primary screen was adapted to estimate the 50% effective concentration (EC_{50}: compound concentrations associated with retention of 50% luminescence from infected cells) and the 50% cytotoxic concentration (CC_{50}: compound concentrations associated with 50% reduction in luminescence compared to DMSO-only wells) of each of the compounds. A selectivity index (SI), the ratio of CC_{50}/EC_{50}, was then calculated for each compound.

Assay of IRES-mediated translation of viral RNA. A bicistronic expression vector assay was performed (19) in which a cytomegalovirus (CMV) promoter directs transcription of mRNA containing the open reading frame of Renilla luciferase, followed by a thermostable hairpin, the IRES found in the 5′ untranslated region (5′ UTR) of coxsackievirus B3, and then the firefly luciferase gene. HeLa-RW cells were seeded in a 96-well microtiter plate and were transfected the next day with this dual-luciferase reporter plasmid. At 48 h posttransfection, the compounds were added to the transfected cells at a concentration of 6.25 μM, an effective concentration selected based on EC_{50} and CC_{50} data. DMSO alone was added to the control wells. At 72 h after the transfection, the cells were washed three times with phosphate-buffered saline (PBS), and the activities of the two luciferases were quantified using Promega's Dual-Luciferase reporter assay system. A stable cell line was produced using 1 mg/ml G418 to select and maintain cells after stable transfection with the dual-luciferase reporter plasmid and was used for some assays.

Inhibition of eGFP expression by recombinant CVB3-eGFP and time-of-addition experiment. HeLa-RW cells were plated at 30,000 cells per well in a 96-well microtiter plate in 100 μl of medium and were cultured overnight. The cells were infected on the following day with enhanced green fluorescent protein (eGFP)-expressing recombinant CVB3 (MOI, ~1), and compounds were added to the cells at 1-h intervals from 0 to 5 h postinoculation to a final concentration of 10 μM. DMSO only was added to control wells. Six hours after inoculation with virus, the cells were fixed with 1% formaldehyde and were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. The cells were washed three times with PBS before being analyzed using an ImageXpress micro high-content microscope (Molecular Devices).

Direct virucidal effect test. DMSO only or 10 μM compounds were mixed with virus stocks of CVB3-H3 at 37°C for 1 h. These pretreated viruses were diluted serially, and the residual infectious virus titers were determined by plaque assay as described previously (22).

Analysis of viral RNA and protein synthesis. Cells were pretreated with compounds for 30 min at 37°C prior to addition of CVB-H3 virus stock at an MOI of 1. After 6 h of incubation at 37°C, medium was aspirated from the wells and cells were washed with PBS and then lifted with 100 μl trypsin (2.5%). The trypsin was inactivated with 400 μl DMEM-based medium, and the cells were transferred into screw-cap tubes. The cells were washed twice and were resuspended in PBS, and the aliquots were frozen at −80°C prior to virus titration or real-time reverse transcriptase PCR (RT-PCR) and immunoblot analyses. For RT-PCR quantification of viral RNA, total cellular RNA was extracted from cell pellets using the Qiagen RNeasy kit and was quantified using a NanoDrop device (Thermo Scientific). Viral RNA was quantified by RT-PCR as previously described (29). Cellular RNA was also quantified by real-time reverse transcriptase PCR using primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (GAPDH-2524S: 5′-CCATCACCATCTTCCAGG AG-3′ and GAPDH-3099A: 5′-CCTGCTTCACCACCTTCTTG-3′) using serially diluted RNA from uninfected cells to produce a set of quantitative standards ranging from 200 pg to 200 ng. Viral RNA quantities are expressed as $\log_{10}$ copies of viral RNA per microgram of cellular RNA. For immunoblot detection of viral protein, trypsinized cells were pelleted with low-speed centrifugation, resuspended in PBS, disrupted in protein gel loading buffer, and homogenized by passage through small-

gauge needles. Following SDS-PAGE using 12% Bis-Tris gels (Invitrogen NuPAGE), viral proteins and cellular proteins were detected using antiserum obtained from the ATCC (V030-501-560) and using antibody to the cellular housekeeping protein glycyl tRNA synthetase. Anti-rabbit antiserum was used as a secondary antibody, and visualization was performed using a chemiluminescent substrate (Thermo Scientific SuperSignal West Pico).

Virus yield reduction assays. For CVB1 to 5, poliovirus 1 (PV1), and poliovirus 3 (PV3), HeLa-RW cells were plated at 30,000 cells per well in a 96-well microtiter plate in 100 μl of medium and were cultured overnight. The cells were infected on the following day at an MOI of 1. The compounds were added at a concentration of 10 μM to triplicate wells at the time of infection. DMSO without compounds was added to control wells. Six to eight hours after inoculation with virus, the plate was sealed and frozen at −80°C. After 3 freeze-thaw cycles, infectious virus titers of the infected cell lysates were determined by plaque assays on HeLa-RW cell monolayers. For EV-A71, echoviruses 6, 7, 9, 11, 25, and 30, and coxsackievirus A9, LLC-MK2 cells were plated at 10,000 cells per well in a 96-well microtiter plate in 100 μl of medium and were cultured overnight. The next day, the cells were infected at doses of 100 times the $TCID_{50}$ of the virus stocks. Compounds or DMSO were added to triplicate wells at the time of infection. The plates were cultured for 2 to 3 days. When the cytopathic effect in control wells were 100%, the plate was sealed and frozen at −80°C. After 3 freeze-thaw cycles, infectious virus titers of the cell lysates were determined by $TCID_{50}$ assays and by the Reed-Muench method (26). The reduction in the production of infectious virus was determined by comparing the titer of virus in DMSO-only wells to the titer of cells treated with a compound.

Pulse-chase analysis of protein processing. HeLa-RW cells were seeded into 6-well plates the day before use (200,000 cells per well). On the day of the experiment, cells were infected with CVB3-H3 at a high MOI (~10), and viral replication was allowed to proceed for 3 h before the cells were washed and placed in a starvation medium (DMEM with glutamine, but deprived of cysteine and methionine; Sigma-Aldrich). After 30 min, the starvation medium was removed, and 0.5 ml of labeling medium was added that contained the compound to be tested as well as ^{35}S-cysteine and methionine (EasyTag Express ^{35}S protein labeling mix [Perkin-Elmer]; 1,000 Ci/mmol and 22 μCi/ml final concentration). At the end of the 5-min pulse, the cells were washed with prewarmed PBS, and complete growth medium was added. Compounds to be tested were added at the time of starvation and were maintained during the pulse and chase periods. At selected time points, 5 min and 60 min into the chase period, the medium was removed, and the cells were washed with ice cold PBS and scraped in 60 μl PBS, which was followed by the addition of concentrated protein gel loading buffer containing sodium dodecyl sulfate. Following SDS-PAGE separation of proteins, the gels were dried and examined by autoradiography.

***In vitro* selection and analysis of CVB3 mutants with resistance to antiviral compounds.** Resistant CVB3 strains were selected by serial passage of the CVB3-H3 strain in the presence of increasing concentrations of the compounds. The serial passage was initiated by passing CVB3-H3 in HeLa-RW cells (MOI. 0.01) in the presence of a compound at a concentration of 0.3 μM, 0.6 μM, or the EC_{50} concentration of the compound. The cell culture was incubated until the cytopathic effect was >70% (usually after 2 days). Cell cultures were harvested and underwent 3 freeze-thaw cycles to release virions. The subsequent rounds were done by passing 3 dilutions (10^{-4}, 10^{-5}, and 10^{-6}) of lysate from prior viral cultures in HeLa-RW cells in the presence of the compound (or in the absence of the compound for control wells). When the viruses under selection produced a cytopathic effect in the presence of the compound as quickly as in the absence of the compound, the concentration of the compound was increased by 2-fold in the next round of the passage. After more than 20 passages and after reaching the concentration of 10 μM (about 10-fold higher than the EC_{50} of the compounds), compound-resistant strains were tested for susceptibility using viral yield reduction assays. Viral RNA

TABLE 2 Anti-CVB3-H3 compound screening procedures

Screening step	Assay and hit criteria	No. of hits/no. of compounds tested	Hit rate (%)
1a. Primary screen	Inhibition of CVB3-H3 induced cytopathic effect by 50% or higher in a single well test	277/85,585	0.32
1b. Validation	Same as above but in triplicates	54/319[a]	16.93
2a. Secondary screen 1	Inhibition of eGFP-CVB3 infection; <5% of eGFP-positive cells in the culture	65/73[b]	89.04
2b. Secondary screen 2	Reduction of CVB3-H3 titer by ≥ 1 $\log_{10}$ after one replication cycle	61/69[c]	88.41

[a] These 319 compounds include 277 hits from step 1a and 42 compounds below the 50% criteria.

[b] These 73 compounds include 54 hits (positive on 2 out of 3 plates) and 19 hits (positive on 1 out of 3 plates) from step 1b.

[c] These 69 compounds were tested in both step 2a and step 2b except 4 compounds that were not available for step 2b.

was extracted from resistant viruses for sequencing. The coding domains for the P2 and P3 domains of the viral genomes were amplified and analyzed as described previously (30, 31). BioEdit and DNAStar Lasergene programs were used to assemble the sequences and compare them to the reference sequence of CVB3-H3 (21).

RESULTS

Screen for small-molecule inhibitors of CVB3 replication. Applying a cell-based assay that detects the cytotoxic effects of infection by CVB3-H3, we previously identified seven compounds in the Prestwick Chemical Library that inhibited CVB3 infection in cell culture (19). Here, we expanded the screen to include additional chemical libraries in a search for novel small-molecule inhibitors of enterovirus replication. As described previously, HeLa-RW cells were seeded into 384-well plates containing library compounds and subsequently infected with CVB3-H3 at a low MOI (~0.007). After 48 h of incubation, protection from virus-induced cytotoxicity was assessed using ATP-driven luciferase as an indicator of cell viability. The quality control of the screen test was monitored by maintaining the Z factor (32), which was in a range of 0.5 to 0.7 throughout the primary screens of 6 different small-molecule libraries. After the primary screen, 277 out of 85,585 compounds passed a selection threshold, which was 50% protection from cytopathic effect of CVB3-H3 infection in reference to the positive control, guanidine (Table 2). We then reexamined these 277 initial hits on a customized 384-well plate together with 42 of the most active compounds that protected cells from cytopathic effects to 30% to 50% of the guanidine-positive control. These 319 compounds were reassessed three times in 3 separate plates as in the primary screen. This validation step reduced the number of the hits to 73 compounds. Of note, none of the 42 compounds below 50% threshold passed the validation step; it is unlikely that we missed potential active compounds due to false negatives.

We further tested the above 73 compounds in two secondary/confirmatory antiviral tests. We first reassessed a compound's antiviral activity by measuring inhibition of eGFP expression in HeLa-RW cells after inoculation with recombinant coxsackievirus that expresses this protein, CVB3-eGFP (20). Overall, 64 of the 73 compounds reduced the number of eGFP-expressing cells in culture by at least 80% compared to infected cell culture without compounds. Next, a compound's antiviral activity was assessed by measuring virus yield reduction after one viral replication cycle. There were 69 compounds available for testing by this method, and 61 compounds reduced the progeny virus titers at least 1 $\log_{10}$ compared to the titers in the cultures without compounds. Among active compounds, 21 of them had 1H-pyrazolo[3,4-b]pyridine-based structures, 17 of them had quinoline-based

structures, and several had benzimidazole structures (see Table S1 in the supplemental material).

Evaluation of antiviral efficacies of representative compounds against CVB3 replication. We selected 12 compounds that represented the structural diversity of the compounds identified above for further studies (Table 3). Four compounds (4, 6, 7, and 11) were from a group of 1H-pyrazolo[3,4-b]pyridine-4-carboxamide (PPC) molecules. To further evaluate their antiviral potential, we determined the EC_{50} and CC_{50} values of these 12 compounds. The four PPC molecules showed desirable EC_{50} and CC_{50} profiles with EC_{50}s in the range of 0.6 to 1.2 µM and CC_{50} values of 50 to 200 µM (Fig. 1 and Table 3). Compound 4 was the most potent in this group with an EC_{50} of 0.6 µM. Most of the compounds had EC_{50}s in a similar concentration range. Compound 9 had the lowest EC_{50} at 200 nM. The anti-CVB3 activity of the antimalarial compound mefloquine was difficult to assess due to its high toxicity in HeLa-RW cell culture with a CC_{50} of 10 µM.

Analysis of the points of inhibition in the viral replication cycle. Before we explored which replication steps these compounds inhibited, we tested whether these compounds were directly virucidal. We treated CVB-H3 with 10 µM of each compound for 1 h at room temperature followed by plaque assay to examine changes in the virus titer after the treatment. Only compound 9 reduced the numbers of plaques by at least 1 $\log_{10}$, whereas the other 11 compounds had no effect. Compound 9 may be virucidal or may be a viral entry blocker interfering with viral capsid protein binding to cell receptors. Compound 9 was reserved for future investigations and was excluded from further study here.

To gauge which replication stages the compounds inhibited, we performed a time-of-addition experiment. HeLa-RW cells were infected with CVB3-eGFP (MOI of 1), and the compounds were added into the cultures at 1-h intervals from 0 to 5 h after the inoculation of the virus. Since CVB3 virus reaches the peak of virus production at 6 h and starts to produce cytopathic effect by 8 h of inoculation, we stopped the infection at 6 h by fixation with 1% paraformaldehyde (PFA). All 11 compounds inhibited eGFP expression at maximal levels when added to cells from 0 to 2 h after virus inoculation, indicating that these compounds interfered with viral replication after virus entry. When added at the third hour after inoculation, 11 compounds still inhibited eGFP expression at maximal levels, but mefloquine became less effective (Fig. 2; see also Table S2 in the supplemental material). The antiviral effectiveness of all 11 compounds was compromised when added at the fourth hour after the inoculation. Thus, mefloquine seemed to exert its antiviral activities at a step that took place between the second and the third hour of the inoculation, while

TABLE 3 List of 12 representative compounds that inhibit the replication of CVB3

Compound	Structure	Systematic name	Antiviral activity[a]			
			Log10 reduction of virus titer[b]	Inhibition of infected cells[c] (%)	EC50 (µM)[d]	CC50 (µM)[d]
2. $C_{19}H_{16}FNO_3$		N-(4-fluorobenzyl)-N-(4-methoxyphenyl)-2-furamide	4.3	99.7	2.9	45.5
3. $C_{15}H_{13}N_3OS$		2-(5-Methyl-2-thienyl)-4-quinolinecarbohydrazide	3.7	100	0.8	27.8
4. $C_{21}H_{20}N_4OS$		1-Isopropyl-N-(2-methylphenyl)-6-(2-thienyl)-1H-pyrazolo[3,4-b]pyridine-4-carboxamide	4.4	100	0.6	>25
5. $C_{22}H_{22}N_4O_2$		1-Methyl-2-[2-(4-morpholinyl)-3-quinolinyl]-2,3-dihydro-4(1H)-quinazolinone	4.4	100	1.0	49.2
6. $C_{23}H_{20}N_4O_3$		N-(1,3-benzodioxol-5-yl)-1-isopropyl-6-phenyl-1H-pyrazolo[3,4-b]pyridine-4-carboxamide	4.5	100	1.2	>50
7. $C_{22}H_{20}N_4O$		1-Isopropyl-N,6-diphenyl-1H-pyrazolo[3,4-b]pyridine-4-carboxamide	4.5	100	1.2	>50
8. $C_{18}H_{17}N_5$		4-[(1E)-1-Phenyl-1-propen-2-yl]-1,4-dihydro[1,3,5]triazino[1,2-a]benzimidazol-2-amine	3.7	100	1.2	32.1

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Compound (formula)	Name	Structure				
9. $C_{22}H_{22}FNO_5$	2,3-Dihydro-1,4-benzodioxin-6-yl[1-(3-fluoro-4-methoxybenzoyl)-4-piperidinyl]methanone		2.0	82.1	0.2	46.1
10. $C_{21}H_{15}N_3O$	4-[(1-Phenyl-1H-benzimidazol-2-yl)methoxy]benzonitrile		4.5	100	2.9	28.2
11. $C_{20}H_{17}FN_4OS$	N-(2-fluorophenyl)-1-isopropyl-6-(2-thienyl)-1H-pyrazolo[3,4-b]pyridine-4-carboxamide		4.5	100	1.1	>100
12. $C_{22}H_{20}N_4OS_2$	N-[4-(1-pyrrolidinylmethyl)-1,3-thiazol-2-yl]-2-(2-thienyl)-4-quinolinecarboxamide		4.5	100	0.6	26.3
14. (Mefloquine hydrochloride, $C_{17}H_{17}ClF_6N_2O$)	[2,8-Bis(trifluoromethyl)-4-quinolinyl](2-piperidinyl)methanol hydrochloride (1:1)		4.0	100	1.6	10.0

[a] Antiviral activity quantification of secondary screen assays.

[b] The results of virus yield reduction assays after a 6-h viral life cycle in HeLa-RW cells. A reduction of virus titer is expressed as PFU per milliliter. Log_{10} reduction, log_{10} (viral titer with DMSO only/viral titer with inhibitor).

[c] Replication of eGFP-CVB3 in HeLa-RW cells with a compound or DMSO only was quantified by the percentage of eGFP-positive cells/DAPI-positive cells. Inhibition of eGFP-CVB3 infecting cells was defined by the formula: 1 − (% of eGFP-positive cells with a compound/% of eGFP-expressing cells with DMSO only).

[d] EC_{50} of a compound against CVB3-H3 replication in HeLa-RW cells and CC_{50} of a compound in HeLa-RW cell culture were calculated from EC_{50} and CC_{50} plots of the compounds in Fig. 1.
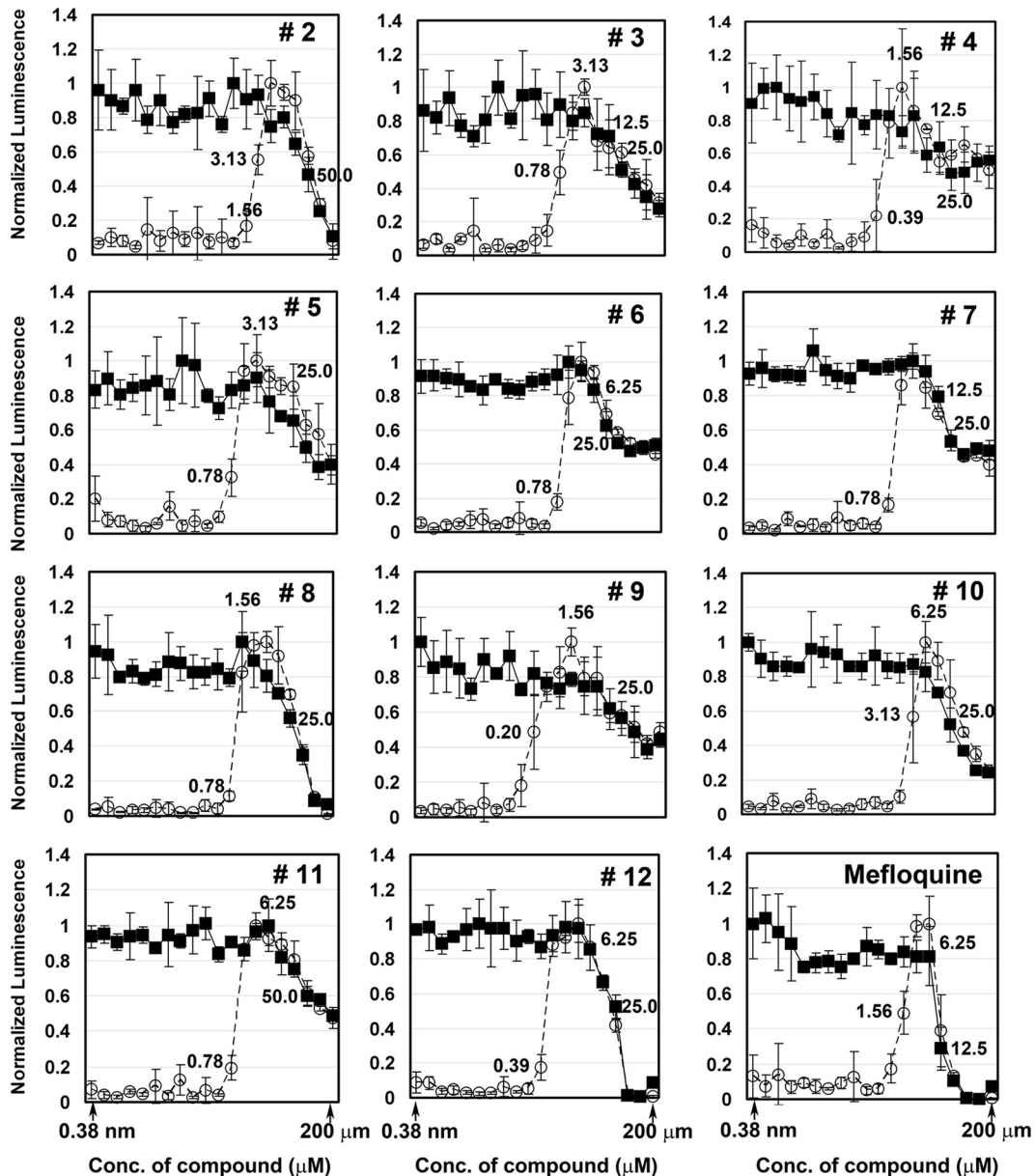
FIG 1 Evaluation of the potency of compounds against CVB3-H3. Dose-response plots were created from 2-fold serial dilutions of the compounds. The final concentration range was 0.38 nM to ~200 μM (numbers next to symbols represent concentrations in μM). Anti-CVB3 EC_{50} (open symbols) and CC_{50} (solid symbols) of each compound were determined by comparison of viability measured by ATPlite 1step reagent after 48 h of incubation, and the luminescence values were normalized to the wells of CVB3-H3-infected cells in the presence of guanidine, the positive control. Averages and standard deviations of triplicates are shown as symbols with error bars.

other compounds had their effect between the third and the fourth hour of the inoculation. Overall, all of these compounds appeared to interfere with early steps in the viral replication cycle, limiting the synthesis of viral RNA and the protein needed to produce progeny virions. The above time-of-addition results using the eGFP-CVB3 reporter virus were also corroborated by measuring viral titers of CVB3-H3 after adding compounds at different time points after inoculation (data not shown).

Inhibition of viral RNA and viral protein. To better understand the inhibition of viral replication by the compounds, we assessed the synthesis of viral proteins and RNA after the compound treatments. We infected cells at a high MOI (~10) with

CVB3-H3 and harvested total cellular RNA and protein after 6 h. Immunoblot revealed a total abrogation of detectable viral protein (Fig. 3A). RT-PCR analysis revealed that all of the compounds examined markedly reduced the amount of CVB3-H3 viral RNA at this time point, ranging from 100- to 10,000-fold compared to untreated cells (Fig. 3B). The suppression of viral RNA synthesis was comparable to the effects of guanidine, which interferes with the activity of viral protein 2C, thereby suppressing the formation of viral negative-strand RNA (28, 33).

We performed pulse-chase analysis to determine if either the synthesis or if the processing of the viral polyprotein is affected by these compounds. During a pulse step, newly synthesized viral
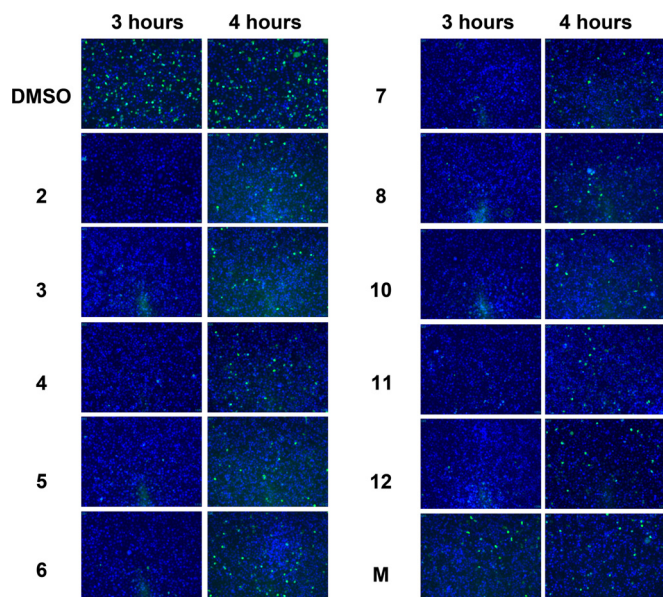
FIG 2 Time-of-addition experiment to identify the step of viral replication inhibited by compounds. Representative images of the wells in which compounds 2 to 12, mefloquine (M), and DMSO were added at 3 h and 4 h postinoculation are shown. The cells (blue fluorescence, DAPI stained) and the expression of eGFP (green fluorescence) in the infected cells were fixed and stained after 6 h of infection. The quantification of expression of eGFP in the infected cells in each well is also shown in Table S1 in the supplemental material.

proteins were labeled with ^{35}S and were subsequently cleaved into the expected viral proteins consistent with viral structural and enzymatic proteins during the chase period (Fig. 4). In control cells treated with rupintrivir, an inhibitor of the 3C protease of many enteroviruses and rhinoviruses (15), the labeled viral polyprotein was cleaved by the protease 2A into two expected fragments of approximately 90 kDa and 150 kDa, which corresponded to the nascent viral P1 and P2-P3 peptides (13). In contrast to this, in cell cultures treated with compounds, viral protein synthesis was unaltered during the pulse step, and the viral polyprotein was processed normally at 5-min and 60-min chase periods, which is comparable to the no-compound control cell culture (Fig. 4). These results indicate that the compounds in Table 3 do not interfere with viral protein synthesis from the viral IRES function and that they do not act as inhibitors of either the 2A or 3C viral proteases. Assays of IRES-mediated translation were performed and confirmed that the compounds in Table 1 did not reduce the IRES-mediated translation of a reporter gene in a bicistronic expression system (data not shown).

Broad anti-enteroviral spectrum of the compounds. A broad antiviral spectrum is a very desirable property of an antiviral agent. Among the more than 110 known enteroviruses, we tested the above compounds against 14 highly pathogenic enteroviruses that are commonly identified in the United States. We determined the magnitude of a compound's inhibition on virus production in cell cultures. The reduction in virus production of enteroviruses by exposure to 10 μM compounds compared to that of the DMSO control are shown in Table 4. These compounds demonstrated antiviral activities against almost all of the enteroviruses tested here except polioviruses 1 and 3, against which only the PPC compound 11 had significant antiviral activity.

Selection of compound-resistant mutants and mutation analysis of the mutants. In an effort to identify the target of the antiviral activity of these compounds, we selected resistant mutant viruses with serial passages of wild-type CVB3-H3 virus through gradually ascending concentrations of the compounds. After about 20 passages, the resulting viruses were resistant to the inhibitory effects of the compounds, inducing a 100% cytopathic effect that was indistinguishable in cells incubated with 10 μM of the compounds and in cells with DMSO only. The compound-resistant nature of the mutants was further confirmed using a one-step viral growth experiment followed by a plaque assay to quantify the viral yields. All of the mutants selected by PPC compounds exhibited cross-resistance against other PPC compounds. After plaque purification of the compound-resistant mutants, we sequenced the viral P2 and P3 domains of the virus and identified the mutations in Table 5. Interestingly, the 2C protein contains the most mutations, especially at amino acid positions 179 and 227. The C179F mutation, located near a conserved element of 2C, was inserted into a plasmid clone of CVB3-H3. Virus produced from this plasmid was resistant to all of the compounds tested (Fig. 5), suggesting that some function of 2C is targeted by these molecules.

DISCUSSION

Enteroviruses are commonly encountered pathogens that are known for their epidemic potential and diverse clinical presentations. No antiviral medications are available to treat meningitis, acute flaccid paralysis, hepatitis, myocarditis, or other serious complications of enterovirus infection. With this need in mind, we used a live virus assay to screen for antiviral activity in a set of chemical libraries that included a wide assortment of molecular structures (19). This cell-based screen approach had several advantages. First, it selected small molecules that have favorable cell permeability and toxicity. Second, the method searched through the entire viral replication cycle for inhibitors. Lastly, the screen assay itself was very simple and consistent, which is ideal for a high-throughput screen procedure. Using this approach, we discovered more than 60 chemical compounds that potently inhibited CVB3 replication. In a previous report, we described in detail our studies that demonstrated the antiviral activity of fluoxetine, best known for its use for the treatment of depression, against B group coxsackieviruses (19). Here, we have extended our studies to include additional medicinal agents and novel compounds, selecting 12 additional compounds for examination. The EC$_{50}$s for most compounds were generally <3 μM with CC$_{50}$ values in excess of 25 μM, yielding selectivity ratios of 15 or more. In addition to the high selectivity ratios, the obtained compounds are also very drug like. They have no obvious liabilities or reactive groups and do not belong to any class of known aggregators or nonspecific binders. Moreover, they obey Lipinski's rule of 5 (34), which makes them good starting points for becoming antiviral lead compounds. As these compounds have heterocycles such as quinoline and pharmacophore, modification and generation of the structure-activity relationship series around these compounds is facile.

To identify potential mechanisms of action, we performed additional experiments to determine if these compounds interfered with viral entry and to examine their impacts on the transcription and translation of the viral genome. Pretreatment of CVB3 with compound 9 interfered with the initiation of the viral life cycle, suggesting that it may bind to the surface of viral particles and block viral entry into target cells. Compound 9 achieved this ac-
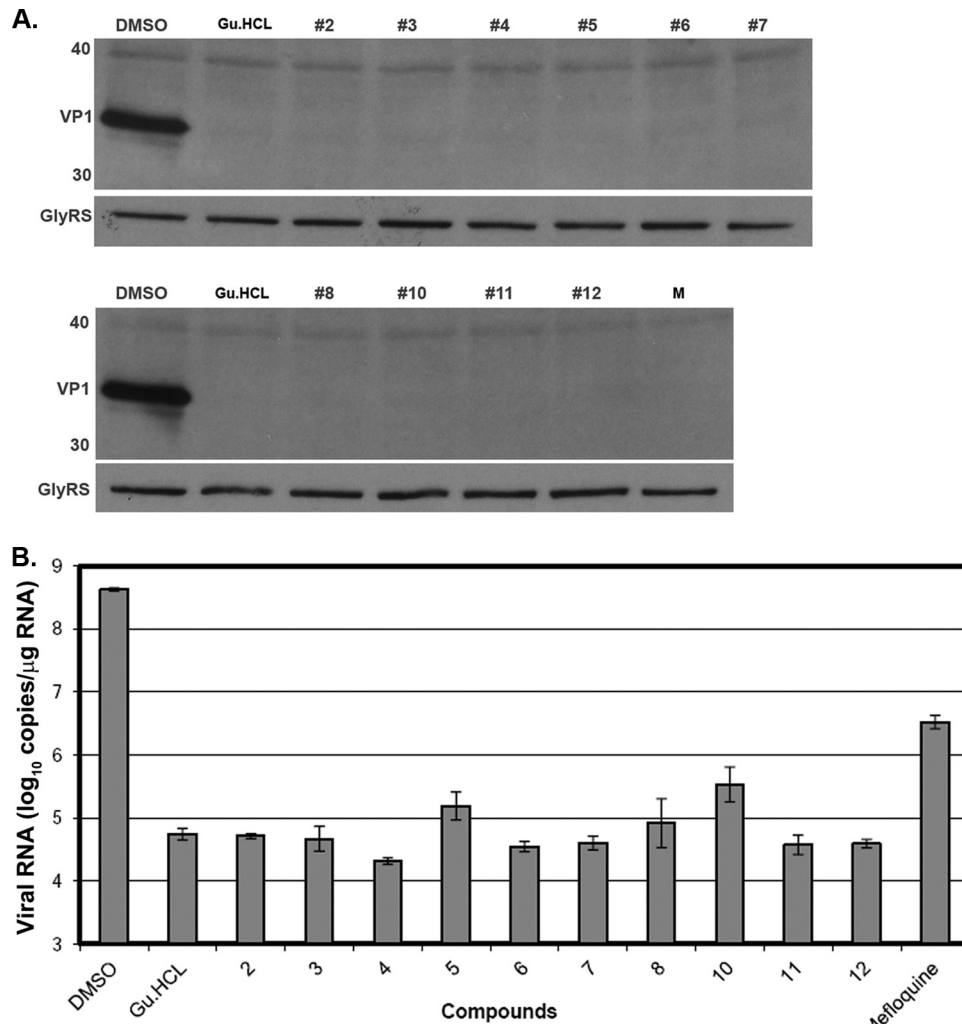
FIG 3 RT-PCR and immunoblot analysis of viral replication. (A) Immunoblot detection of the viral capsid protein 6 h after infection with CVB3-H3; no viral protein is detected in cells treated with identified compounds. Immunoblot detection of glycyl-tRNA synthetase protein (GlyRS) (a housekeeping protein) is shown as a loading control. (B) RT-PCR detection of CVB3 RNA in cells treated with identified compounds beginning 30 min before inoculation of cell cultures. Experiments were performed with triplicate wells in two separate infections. Symbols represent averages from triplicates, and results representative of two separate experiments are shown. Gu.HCL, guanidinium HCl.

tivity without apparent disruption of virions, as electron microscopy revealed that CVB3-H3 particles remained intact despite incubation with the compound (data not shown). We note that compound 9 is structurally distinct from pleconaril, pirodavir, and other previously reported capsid-binding agents (15).

We further demonstrated that the remaining 11 compounds possessed activity against a panel of 13 pathogenic nonpolio enteroviruses that include representatives of serotypes that commonly circulate in the United States (23, 24, 35). Interestingly, these studies also confirmed that fluoxetine, used here as a control, has broader antiviral activity than previously described by us and others (19, 36), although its clinical utility might be limited to infections of the central nervous system where it is known to concentrate (12). Detailed analysis of the antimalarial agent mefloquine was discouraging; its modest and somewhat narrow antiviral activity against enteroviruses was associated with a low CC_{50} value, yielding a low selectivity index of approximately 6 against CVB3. Among the nonpharmaceutical agents, compounds 4, 6, 7,

and 11 represented a group of 21 PPC molecules. All of them were active against representatives of the *Enterovirus A* and *B* species; compound 11 also exhibited activity against the two polioviruses tested (*Enterovirus C*). Since the completion of testing of these compounds against the viruses listed in Table 1, we were able to test the antiviral activity of compound 11 against enterovirus D-68 as well using two isolates recovered in 2014 (8–12). Compound 11 was highly potent in protecting cells from EV-D68 lysis, having an EC_{50} of a few hundred nanomolar and a selective index of >1,000 (data not shown). Additional studies to better define the antiviral activity of compound 11, and related PPCs, are underway.

In contrast to compound 9, time-of-addition experiments indicated that the remaining compounds exhibited antiviral activity subsequent to the entry of viral particles and in a fashion that drastically limited the amplification of viral RNA and the synthesis of viral protein. Pulse-chase experiments confirmed that none of the compounds interfered with the synthesis of the viral polyprotein or its cleavage by viral 2A or 3C proteases. Assays performed
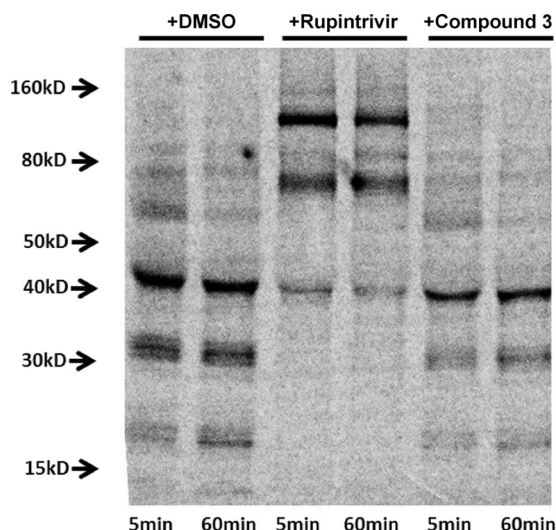
FIG 4 Pulse-chase experiment revealed no effects of the compounds on viral protein synthesis and processing. Cells were infected with CVB3 at a high multiplicity of infection. After 3 h of infection, the cells were washed, starved, and subjected to a 5-min pulse of ^{35}S-labeled cysteine and methionine. At 5 and 60 min into the chase period, proteins were harvested for analysis. Compounds to be tested were added at the time of starvation and were maintained during the pulse and chase periods. The third and fourth lanes demonstrate that treatment of cultures with rupintrivir, an inhibitor of the viral 3C protease, resulted in the formation of labeled proteins consistent with uncleaved viral P1 and P2-P3 proteins (~90 kDa and 150 kDa). In the example shown, compound 3 did not alter the protein cleavage products seen compared to those in the control conditions (DMSO).

TABLE 5 Mutations in the viral P2 and P3 domain selected by serial passage in the presence of antiviral compounds

Compound[a]	Amino acid position			
	2A	2C	3C	3D
2	S84G	S58N, C179F, I227V, N257D	T130A	
3	S84G	S58N, C179F, I227V, N257D	T130A	
4	S84G	S58N, C179F, I227V, N257D	T130A	
5	S64T	C179Y, F244L		T447A
6		A224V, I227V		Y385F
7		C179F, I227V	K156Q	
8	W25C	C179F, I227V		
10	N11S	A224V, I227L, L303I		
11		M193L, I227V		N67T
12		I227V, A239V		Y385F
F		I227V, N228S		T197A, Y385F
M		S58N, I227V		E429K

[a] F, fluoxetine; M, mefloquine.

mous substitutions occurred in the coding region for the viral 2C protein (Table 5).

The 2C protein is highly conserved among enteroviruses and other members of the picornavirus family, which likely explains the broad spectrum of activity demonstrated in our testing (Table 4). The 2C protein is a 329-amino-acid polypeptide that contains a nucleotide triphosphate (NTP)-binding domain containing three conserved sequences (boxes A, B, C) and that exhibits ATPase activity *in vitro* (28). Guanidine (37) inhibits the replication of most enteroviruses, and resistance to its antiviral activity maps to 2C, with resistance-associated amino acid changes flanking these boxes (38). Similarly, most of the mutations identified were in close proximity to the B and C boxes. These data may indicate that the compounds studied here (apart from compound 9) inhibit viral RNA synthesis in a fashion similar to guanidine. The idea that these compounds target 2C function was also supported by the fact that the introduction of the C179F coding mutation into CVB3-H3 conferred high-level resistance to most of the compounds evaluated (Fig. 5). We did not identify clear correlates between amino acid differences between polioviruses and the EV-A and EV-B viruses that predicted susceptibility

with a bicistronic expression vector (19) similarly showed no evidence of inhibition of IRES-mediated translation (data not shown). Similar results were found when we initially demonstrated inhibition of coxsackievirus B replication by fluoxetine, which was subsequently shown by others to target the viral 2C protein (36). In an effort to identify the target(s) of our compounds, we selected resistant mutants by serial passages of wild-type CVB3-H3 under the pressure of increasing concentrations of these inhibitors. Interestingly, most nonsynony-

TABLE 4 Antiviral spectrum of the compounds[a]

EV Species	Virus	Antiviral Compound											
		2	3	4	5	6	7	8	10	11	12	F	M
A	EV-A71	2.8	3.0	4.6	2.8	4.0	3.6	2.8	3.6	3.8	5.6	3.8	3.8
B	CV-A9	4.0	4.0	4.6	4.8	4.8	4.6	3.8	4.8	4.6	4.0	4.0	4.6
B	CV-B1	3.6	3.4	3.5	3.4	3.8	3.5	3.7	3.0	3.6	3.6	3.7	1.0
B	CV-B2	2.5	2.2	2.7	2.7	2.5	2.5	2.7	2.4	2.4	2.5	2.6	0.0
B	CV-B3	3.9	4.1	4.1	4.1	4.0	3.3	4.1	3.7	4.2	4.2	4.1	1.8
B	CV-B4	2.9	3.2	3.2	3.4	3.3	2.5	3.4	3.4	2.7	3.0	3.0	1.0
B	CV-B5	2.6	2.8	2.6	2.7	2.5	2.9	2.7	2.8	3.0	3.0	3.0	0.5
B	E-6	3.5	2.3	3.0	2.5	3.3	2.5	2.3	3.0	3.5	3.5	3.8	2.3
B	E-7	5.3	1.8	5.0	4.8	4.8	4.8	2.6	5.0	4.0	5.0	3.3	1.8
B	E-9	3.8	3.6	2.8	3.3	3.8	3.8	3.6	3.8	4.0	3.6	4.0	2.6
B	E-11	5.5	5.0	4.5	2.3	5.5	5.5	2.5	4.3	4.3	5.0	4.0	3.0
B	E-25	4.3	3.5	3.5	5.0	5.0	4.3	3.5	4.3	4.3	4.3	4.3	3.0
B	E-30	5.8	5.8	5.8	5.2	5.8	5.2	3.0	5.8	5.5	4.8	4.5	4.8
C	PV-1	0.1	-0.3	0.3	0.1	1.1	0.7	-0.2	1.3	3.3	0.2	0.6	-0.1
C	PV-3	-0.1	-0.1	-0.2	0.0	1.2	0.6	0.1	1.6	3.6	0.2	0.3	0.2

[a] The values represent the log$_{10}$ reductions of virus titers (PFU/ml: CV-B1, 2, 3, 4, and 5, PV-1 and PV-3; TCID$_{50}$ units/ml: E-6, E-7, E-9, E-11, E-25, E-30, EV-A71, and CV-A9) of compound-treated infected wells versus DMSO-treated wells. The magnitudes of the virus reductions are also demonstrated by the color shading, as the transition from red to blue indicates an increase of the virus titer reduction and antiviral potency of a compound. F, fluoxetine; M, mefloquine.
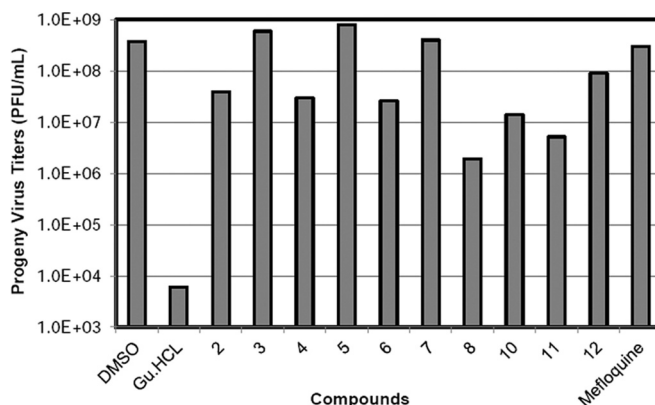

FIG 5 Compound resistance phenotype of the mutant virus with a single mutation C1279F in the CVB3-H3 2C protein. Bar graphs are viral yields (PFU/ml) of the mutant virus CVB3-H3-C179F in the presence of the compounds or DMSO only after one replication cycle (7 h). Averages of two tests are shown, and differences between the two tests are within 10% of each other. A separate experiment also showed similar results. Gu.HCL, guanidine HCl.

to the antiviral compounds studied here. This likely reflects the fact that amino acid sequences of the mature 2C proteins are highly similar within enterovirus species but differ markedly between species (39, 40).

However, these genetic data do not unambiguously identify a mechanism of action for these compounds. Genetic studies of 2C have indicated several potential functional roles for this polypeptide in picornavirus replication apart from the synthesis of viral RNA replication, including viral uncoating, alteration of cellular membranes, encapsidation of the viral genome, and assembly of infectious viral particles (37). The antiviral activities of these compounds might also involve the disruption of interactions between viral proteins or between 2C and cellular cofactors needed for viral replication.

For now, our results reveal new molecular structures as the basis of potential antiviral agents against enteroviruses, particularly the PPC compounds, which appear to target many of the most common and most widely distributed serotypes. Additional studies should include a larger number of EV-A and EV-C members, in view of their apparent recent increase in circulation in the United States (35).

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