Characterization of pharmacologically active compounds that inhibit poliovirus and enterovirus 71 infectivity

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Poliovirus (PV) and enterovirus 71 (EV71) cause severe neurological symptoms in their infections of the central nervous system. To identify compounds with anti-PV and anti-EV71 activities that would not allow the emergence of resistant mutants, we performed drug screening by utilizing a pharmacologically active compound library targeting cellular factors with PV and EV71 pseudoviruses that encapsidated luciferase-encoding replicons. We have found that metrifudil (N-[2-methylphenyl][methyl]-adenosine) (an A2 adenosine receptor agonist), N²-benzyladenosine (an A1 adenosine receptor agonist) and NF449 (4,4′,4″-[carbonylbis[mino-5,1,3-benzenetriyl bis(carbonyl-imino)]] tetrakis (benzene-1,3-disulfonic acid) octasodium salt) (a Gs-; inhibitor) have anti-EV71 activity, and that GW5074 (3-(3, 5-dibromo-4-hydroxybenzylidine-5-iodo-1,3-dihydro-indol-2-one)) (a Raf-1 inhibitor) has both anti-PV and anti-EV71 activities. EV71 mutants resistant to metrifudil, N²-benzyladenosine and NF449 were isolated after passages in the presence of these compounds, but mutants resistant to GW5074 were not isolated for both PV and EV71. The inhibitory effect of GW5074 was not observed in Sendai virus infection and the treatment did not induce the expression of OAS1 and STAT1 mRNA. Small interfering RNA treatment against putative cellular targets of GW5074, including Raf-1, B-Raf, Pim-1, -2, and -3, HIPK2, GAK, MST2 and ATF-3, did not consistently suppress PV replication. Moreover, downregulation of Raf-1 and B-Raf did not affect the sensitivity of RD cells to the inhibitory effect of GW5074. These results suggest that GW5074 has strong and selective inhibitory effect against the replication of PV and EV71 by inhibiting conserved targets in the infection independently of the interferon response.

INTRODUCTION

Poliovirus (PV) is a small, non-enveloped virus with a single-stranded positive genomic RNA of about 7500 nt, belonging to the genus Enterovirus of the family Picornaviridae. PV is the causative agent of poliomyelitis, where the motor neurons are the major target (Bodian, 1949). The tropism of PV to the motor neurons is attributable in part to the expression of the PV receptor (PVR) (Crotty et al., 2002; Ida-Hosonuma et al., 2002; Koike et al., 1994; Ren & Racaniello, 1992). Enterovirus 71 (EV71) belongs to the genus Enterovirus but was classified into a different species from PV, Human enterovirus species A. EV71 is a causative agent of hand, foot and mouth disease and herpangina, but sometimes causes severe neurological diseases, such as brainstem encephalitis and poliomyelitis-like paralysis (Chumakov et al., 1979; McMinn, 2002; Wang et al., 2003). The case-severity rate of EV71 in an outbreak in Taiwan was <0.3 % (Ho et al., 1999), suggesting a high neuropathogenicity of EV71 as well as PV, which causes poliomyelitis in 0.1–1.0 % of infected individuals (reviewed by Minor, 1992). EV71 causes fatal pulmonary oedema and/or pulmonary haemorrhage in young children by destruction of the vasomotor and respiratory centres in the brain stem (Chang et al., 1999; Ho et al., 1999; Huang et al., 1999; Komatsu et al., 1999; Lum et al., 1998; Wang et al., 1999).

The viral replication machinery of PV requires numerous cellular factors to hijack the cellular process. For viral protein synthesis, recruitment of ribosome to the internal ribosomal entry site (IRES) on the viral genome requires host factors polypyrimidine tract-binding protein (PTB), Unr, La, poly(rC)-binding protein 2 (PCBP2) and SRp20 that are not required for cap-dependent protein synthesis of cellular mRNA (Bedard et al., 2007; Hellen et al., 1993; Hunt et al., 1999; Meerovitch et al., 1993; Sanford et al., 2004; Walter et al., 1999), along with proteolytic modification of a translation initiation factor eIF4G by viral proteinase 2Apro to hijack the translation machinery from
cap-dependent protein synthesis to viral protein synthesis (Gradi et al., 1998; Krausslich et al., 1987). For the replication process, membrane rearrangement utilizing ADP ribosylation factors (Arfs) and guanine nucleotide exchange factors (GEFs) (GBF1, BIG1/2) are required via direct and indirect interaction with viral 3A and 3D proteins in coxsackievirus B3 (CVB3) and PV infections (Belov et al., 2007; Wessels et al., 2006). Cellular signalling via extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K) and stress-activated protein kinases (SAPKs) affected replication and/or the release of progeny virus in CVB3 infection by an unknown mechanism (Esfandiarei et al., 2004; Luo et al., 2002; Si et al., 2005).

These cellular processes are promising targets for the development of effective anti-PV drugs. PV mutants resistant to anti-PV inhibitors or anti-PV antibodies could emerge rapidly, because a mutant exists in a population of virus (10^3 to 10^6), if a single mutation was sufficient for resistance (Blondel et al., 1986; Crotty et al., 2001; de la Torre et al., 1992; Diamond et al., 1985; Pincus et al., 1986, 1987). Actually, PV isolates from an immunodeficient case chronically infected with PV showed resistance to pleconaril, which is an anti-PV drug that targets the viral capsid proteins to prevent uncoating (Abdel-Rahman & Kearns, 1998), despite the absence of pleconaril treatment (MacLennan et al., 2004). In contrast, emergence of virus mutant resistant to inhibitors against cellular factors was limited, depending in part on the capacity of viral protein activities in the target step. A PV mutant resistant to brefeldin A, which blocks membrane traffic between the cis- and trans-Golgi compartments and inhibits PV replication (Irurzun et al., 1992; Maynell et al., 1992), required two mutations and was isolated after five passages under a stepwise selection pressure by raising the concentration of the inhibitor (Crotty et al., 2004). The emergence of a PV mutant resistant to geldanamycin treatment, which targets Hsp90 and interferes with the folding of PV capsid probably in cooperation with Hsp70 (Maciejak & Sarnow, 1992), was not observed (Geller et al., 2007).

In this study, we performed drug screening by utilizing a pharmacologically active compound library with partially characterized targets and pseudoviruses, which have PV and EV71 replicons encoding firefly luciferase as a replication marker encapsidated in the PV and EV71 capsid proteins, respectively (Arita et al., 2006, 2008, Porter et al., 1998). We have identified four compounds and characterized their inhibitory effects on PV and EV71 infection.

**METHODS**

**Cells, viruses, reagents and drug library.** HEK293 cells (human embryonic kidney cell line) (Graham et al., 1977), RD cells (human rhabdomyosarcoma cell line), HeLa-2c (human larynx epidermoid carcinoma cell line) and L20B cells (mouse Ltk^- aprt^- fibroblast cell line expressing PV receptor) (Mendelsohn et al., 1989) were cultured as monolayers in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). HEK293 cells were used for preparation of pseudoviruses. RD cells were used for titration of pseudoviruses and for drug screening. PV and EV71 pseudoviruses, which encapsidated luciferase-encoding PV and EV71 replicons with capsid proteins derived from PV (Mahoney) and EV71 (Nagoya), respectively, were prepared as reported previously (Arita et al., 2006, 2008), except that the EcoRI site in the 5'NTR of PV replicon was removed by site-directed mutagenesis. A Sendai virus (SeV) mutant (SeV/luc), which encodes luciferase (Hasan et al., 1997), was a generous gift from Atsushi Kato, Department of Virology III, National Institute of Infectious Diseases, Tokyo, Japan. N^2^-Benzyladenosine (Acros Organics), SL 327 (Sigma-Aldrich) and U0126 (Sigma-Aldrich) were dissolved in DMSO to prepare a 10 mM solution, and this was diluted with 10% FCS/DMEM to prepare a 0.5 mM solution before use. LOPAC^{1280} drug library (Sigma-Aldrich) was used for drug screening. Each drug (10 mM solution in DMSO) was diluted with 10% FCS/DMEM to 0.5 mM before use.

**Virus titration.** The virus titre was determined by measuring 50% cell culture infectious dose (CCID_{50}) in a microtitration assay using RD cells, as described elsewhere (Nagata et al., 2002). Briefly, inoculated RD cells were cultured at 37 °C for 7 days, and were then observed for cytopathic effects (CPE). CCID_{50} was calculated using the Behrens–Kärber method (Kärber, 1931).

**Drug screening.** RD cells (1.0 × 10^4 cells per well in 100 μl medium) were cultured at 37 °C in 96-well plates (White Opaque Tissue Culture Plate; Becton Dickinson), followed by addition of 10 μl of 0.5 mM drug solution (final concentration of 45 μM). The cells were incubated at 37 °C for 0 or 48 h. The cells were inoculated with 100 infectious units (IU) of PV or EV71 pseudovirus in 50 μl of 10% FCS/DMEM, and then were incubated at 37 °C for 9 h (the final concentration of drugs was 31 μM). Luciferase activity of the cells was measured with Luciferase Assay System (Promega) using a TR717 Microplate luminometer (ABI) according to the manufacturer’s instructions. The inhibition index, which is the ratio of luciferase activity in the drug-treated cells to that in mock-treated cells (1.0 for mock-treated cells), was determined for each drug. 50% inhibitory concentration (IC_{50}), at which the drugs suppressed the infection of pseudovirus by 50% at 9 h post-inoculation (p.i.), was determined for the drugs that showed either anti-PV or anti-EV71 activity.

**Evaluation of cytotoxicity of drugs.** Cytotoxicity of drugs was evaluated by two methods: observation of cell viability at 3 days after drug treatment under the same condition as for the screening (described above) and determination of the 50% cytotoxic concentration (CC_{50}) of drugs by the measurement of ATP as a marker of metabolically active cells. For the measurement of ATP in cells, RD cells (3.8 × 10^4 cells per well in 100 μl medium) were cultured at 37 °C in 96-well plates (Becton Dickinson), followed by addition of 10 μl of drug solution. The ranges of the concentration of drugs examined were as follows: GW5074, 0.13–0.5 mM; metrifudil, 0.0063–0.05 mM; N^2^-benzyladenosine, 1.3–10 mM; NF449, 0.13–1.0 mM. The cells were incubated at 37 °C for 9 h, and were then subjected to the measurement of ATP by using a CellTitre-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer’s instructions.

**Measurement of anti-PV and anti-EV71 activities of inhibitors.** Virus solution (containing 10^4 CCID_{50} in 5 μl 10% FCS/DMEM) was added to RD cells (2.8 × 10^4 cells per well in 100 μl 10% FCS/DMEM) in 96-well plates (Becton Dickinson), and then the cells were incubated at 37 °C for 2 h. The cells were washed three times with 10% FCS/DMEM, followed by the addition of 100 μl of 10% FCS/DMEM containing each inhibitor or without the inhibitor as control.
The concentrations of inhibitors examined were: 50 μM GW5074, 25 μM metrifudil, 2.5 μM N6-benzyladenosine and 50 μM NF449. The cells were then incubated at 37 °C for 7 h and collected at 9 h p.i. and stored at −70 °C. For the analysis of the effect of NF449 on virus adsorption and/or uncoating, the inhibitor was added to the cells just before adding the virus solution, and the cells were then incubated at 37 °C for 2 h, followed by three washes with 10 % FCS/DMEM and incubated in the absence of NF449 at 37 °C for 7 h (0–2 h incubation, Table 2). The viral RNA was extracted from the collected cells using a High Pure Viral RNA purification kit (Roche), and the number of copies of viral RNA was quantified using a real-time TaqMan PCR system (described below). The inhibition index, which is the ratio of the luciferase activity or of the number of copies of viral RNA in inhibitor-treated cells to that in mock-treated cells (1.0 for mock-treated cells), was determined for each inhibitor (Fig. 2 and Table 2).

Quantification of viral RNA by real-time TaqMan PCR. Real-time TaqMan PCR was performed as previously described by Nijhuis et al. (2002). Isolated viral RNA was reverse transcribed using a Reverse Transcription System (Promega) with random hexamers according to the manufacturer’s instructions. The resultant cDNA was assayed in a 20 μl reaction mixture containing 2 μl cDNA solution, 10 μl TaqMan Fast Universal PCR Master Mix (Applied Biosystems) with primers (Forward primer and Reverse primer 1) and probe (Probe 1) (Nijhuis et al., 2002). Plasmid DNA of an EV71 infectious clone [pEV71(Nagoya)] was used as the control for quantification of the number of copies (Arita et al., 2007). The mixtures were subjected to real-time PCR; PCR conditions were: denaturation step at 95 °C for 20 s and 40 cycles of thermal cycling at 95 °C for 3 s and 60 °C for 30 s. The fluorescence emission of the probe was monitored and analysed by using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems).

Isolation of PV and EV71 mutants resistant to inhibitors. Virus mutants of PV1(Mahoney), EV71(BrCr-TR) and EV71(Nagoya) (containing 106 CCID50 in 5 μl 10 % FCS/DMEM) were added to RD cells (1.0 × 105 cells per well in 100 μl 10 % FCS/DMEM) in 96-well plates of Becton Dickinson in the presence of the inhibitors. The concentrations of inhibitors examined were: 25–100 μM GW5074, 10 μM metrifudil, 0.25–5 μM N6-benzyladenosine and 25–100 μM NF449. The cells were incubated at 37 °C and collected when all the cells exhibited CPE or at day 3 p.i. Collected cell lysates for each inhibitor were mixed and then used for the next passage in the presence of each inhibitor. The passage was repeated twelve times or until a resistant phenotype was observed for the isolates compared with the parental strains in the presence of each inhibitor. The viral RNAs of the isolates were extracted from the collected cell lysates by using a High Pure Viral RNA purification kit (Roche), and the sequences of the viral genomes of the isolates were analysed in the capsid protein coding region (for mutant resistant to NF449) or in the non-structural protein coding region (for mutants resistant to metrifudil and N6-benzyladenosine) to identify the mutations required for the resistance.

Knockdown analysis by RNA interference (RNAi). Knockdown analysis was performed by utilizing RNAi with small interfering RNA (siRNA; Elbashir et al., 2001). Three sets of siRNA against Raf-1, B-Raf, Pim-1, -2 and -3, HIPK2, GAK, MST2 and ATF-3 were designed in the conserved regions among the transcript variants and were prepared as 50 μM solutions of RNA duplex (Supplementary Table S1). For negative controls of each siRNA, RNA duplex with scramble sequences against each cellular-mRNA-specific RNA duplex, which did not show specificity to known cellular mRNAs but with the same nucleotide constitution as the cellular-mRNA-specific RNA duplex, was designed and prepared as 50 μM solutions of RNA duplex. RNA duplex solution (final concentration 0.1 μM) was transfected into RD cells (8.0 × 103 cells) in 96-well plates by using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer’s instructions. The cells were incubated at 37 °C for 48 h and the supernatant was removed and replaced with 100 μl per well of 10 % FCS/DMEM. At 72 h post-transfection, the cells were inoculated with 100 IU PV pseudovirus in 100 μl 10 % FCS/DMEM, and then were incubated at 37 °C for 8 h. Luciferase activity in the cells was measured with the Luciferase Assay System (Promega) using TR717 Microplate luminometer (ABI) according to the manufacturer’s instructions. For Western blot analysis of Raf-1 and B-Raf, RNA duplex solution (final concentration 0.1 μM) was transfected into RD cells (2.0 × 105 cells) in 6-well plates by using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer’s instructions. The cells were incubated at 37 °C for 48 h and the supernatant was removed and replaced with 2 ml 10 % FCS/DMEM per well. The cells were further incubated at 37 °C for 24 h, and were then subjected to Western blot analysis.

Western blot analysis. siRNA-treated RD cells (3.9 × 105 cells) were collected in 100 μl cell lysis buffer [21 mM HEPES buffer (pH 7.4), 1.8 mM disodium hydrogenophosphate, 137 mM NaCl, 4.8 mM KCl, 0.5 % Nonidet P-40 and 0.5 mM EDTA] at 72 h post-transfection, and then subjected to 5–20 % gradient PAGE (e-PAGE; Atto) in a Laemmli buffer system (Laemmli, 1970). The proteins in the gel were transferred to a polyvinylidene difluoride filter (Millipore) and blocked in phosphate-buffered saline (PBS) [10 mM phosphate buffer (pH 7.0), 135 mM NaCl and 2.6 mM KCl] containing 5 % non-fat dry milk. The filters were incubated with rabbit anti-Raf-1 antibody (Sigma) or with anti-B-Raf antibody (Santa Cruz Biotechnology) (1:1000 dilution in 10 % non-fat milk, 60 min) and then washed with PBS containing 0.1 % Tween 20 and 0.5 % non-fat dry milk, respectively, at room temperature for 1 h. The filters were washed with PBS containing 0.1 % Tween 20 and 0.5 % non-fat dry milk for 5 min each, and then incubated with donkey anti-rabbit IgG antibodies conjugated with horseradish peroxidase (Amersham Biosciences) (1:1000 dilution in PBS containing 0.1 % Tween 20 and 0.5 % non-fat dry milk) at room temperature for 1 h. The filters were washed with PBS containing 0.1 % Tween 20 three times for 5 min each, and then treated with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) for the detection of the signal.

Measurement of the interferon response. RD cells (2.8 × 105 cells) in 6-well plates were treated with siRNA (for Raf-1 and B-Raf, collected at 72 h post-transfection), GW5074 (6.25 μM or 25 μM at 37 °C, collected after 4 h incubation) or poly IC (collected at 24 h post-transfection), and the total RNA was extracted from the treated cells with a High Pure RNA Isolation kit (Roche). The relative expression levels of OAS1 and STAT1 mRNAs in the cells were determined by real-time PCR using primers in an IFN Response Watcher kit (TaKaRa) with a One Step SYBR PrimeScript RT-PCR kit II (Perfect Real Time) kit (TaKaRa). β-actin mRNA was used as the endogenous control, and the expression levels of OAS1 and STAT1 mRNAs in treated cells were normalized by the expression levels in the mock-treated cells.

RESULTS

Strategy of drug screening for anti-enterovirus drugs

To identify anti-PV and anti-EV71 drugs, we screened the LOPAC1280 drug library. We utilized PV and EV71 pseudoviruses that have PV and EV71 capsid protein-encapsidated luciferase-encoding PV and EV71 replicons.
respectively (Fig. 1a). Both the short and long-term effects of the compounds were examined by preincubating RD cells with compounds for 0 and 48 h before pseudovirus infection, respectively (Fig. 1c). We defined anti-enterovirus activity of the compounds as suppression of luciferase activity in the treated cells by less than 10\(^{-2}\) fold of that observed in mock-treated cells beyond the deviation of false-positive decline in luciferase signals caused by the drug treatment. The mean relative light units in mock-treated cells inoculated with PV and EV71 pseudoviruses (obtained from 16 wells of each plate) were 1.7 \(\times 10^7\) (with a standard deviation of 1.2 \(\times 10^6\)) and 4.5 \(\times 10^6\) (with a standard deviation of 7.2 \(\times 10^5\)), respectively.

Identification of anti-PV and anti-EV71 inhibitors

In the screening, we identified a total of 101 compounds that reduced either PV or EV71 pseudovirus infectivity. We examined the cytotoxicity of these compounds and found that most of them had strong cytotoxicity at the concentration examined, leaving substantially no viable cells after the treatment (Fig. 2b, toxic effect in the drug-treated cells). However, treatment with three compounds (GW5074, metrifudil and NF449) had apparently no effect on the viability of the cells (Fig. 2a and b). Among these compounds, metrifudil and NF449 specifically inhibited EV71 infection, with inhibition indices of 3.6 \(\times 10^{-7}\) and 2.6 \(\times 10^{-4}\), respectively. In contrast, GW5074 inhibited
both EV71 and PV infection, with inhibition indices of $2.2 \times 10^{-3}$ and $1.8 \times 10^{-4}$, respectively. Since metrifudil has just become commercially unavailable, we examined adenosine derivatives structurally related to metrifudil for their anti-EV71 activity, and identified $N^6$-benzyladenosine as a strong anti-EV71 inhibitor (inhibition index of $<1.4 \times 10^{-5}$). Cells treated with GW5074 and $N^6$-benzyladenosine showed characteristic morphological changes (flattened shape and enlargement of the cell size), in contrast to those treated with metrifudil or NF449. Treatment with $N^6$-benzyladenosine also affected the viability of the cells at 31 $\mu$M, but not at 1.6 $\mu$M, after incubation for 3 days (Fig. 2c).

Characterization of identified anti-PV and anti-EV71 inhibitors

We determined the IC$_{50}$ and CC$_{50}$ of identified inhibitors against pseudovirus infection (Fig. 3 and Table 1). The IC$_{50}$ values of the inhibitors against EV71 pseudovirus infection in RD cells were of the micromolar order, except $N^6$-benzyladenosine, whose IC$_{50}$ was 0.1 $\mu$M (Fig. 3a). IC$_{50}$ of GW5074 against PV pseudovirus infection was determined in RD cells and also in cell lines of different origins (Hep-2c and mouse L20B cells). GW5074 was effective in these cells, with an IC$_{50}$ of 2.3–6.0 $\mu$M (Fig. 3b). CC$_{50}$ of the inhibitors was determined by measuring ATP derived from
viable cells. The selectivity index (SI) of each inhibitor ranged from >38 to 33 000 (Table 1). To examine the specificity of the inhibitory effect of GW5074, we analysed the effect on the infection of SeV, which is a negative strand RNA virus belonging to the family Paramyxoviridae (Fig. 3c). GW5074 had no inhibitory effect on SeV infection (at most threefold reduction of luciferase activity in the treated cells), in contrast to its strong inhibitory effect on PV pseudovirus infection.

Next, we analysed the effects of identified inhibitors on the infectivity of PV and EV71 strains (Table 2). As observed for pseudovirus infection, they had strong inhibitory effect on the infectivity of virus strains examined (inhibition indices
of $10^{-1}$ to $10^{-5}$). The inhibitory effect of GW5074 was the highest against type 3 PV (Sabin) (inhibitory index of $7.0 \times 10^{-5}$) and lowest against EV71 (BrCr-TR) (inhibitory index of $1.5 \times 10^{-2}$). EV71 (BrCr-TR) also showed partial resistance to NF449 treatment. In this test, metrifudil, $N^6$-benzyladenosine and NF449 did not show any inhibitory effect on type 1 PV (Sabin) infection. Except for NF449, the inhibitors were effective when added to the inoculated cells after the uncoating step (2 h p.i.). NF449 was effective only when added to the cells during virus infection (0–2 h p.i.), suggesting that NF449 inhibits the adsorption and/or uncoating step of EV71, but not the replication step.

To characterize further the target of these inhibitors, we isolated virus mutants resistant to these inhibitors (Table 1). EV71 mutants resistant to metrifudil, $N^6$-benzyladenosine and NF449 treatments were isolated within five passages. Sequence analysis of the viral genomes showed mutations in protein 2C in mutants resistant to metrifudil (mutations at nt 4428 and 5048), and mutations in VP1 capsid protein at nt 5050 and 5815) and to $N^6$-benzyladenosine (mutations at nt 4428 and 5048), and mutations in VP1 capsid protein were observed in a mutant resistant to NF449 (mutations at nt 2734 and 3173). The critical mutations for the resistant phenotype against each inhibitor were determined by using pseudoviruses carrying corresponding mutations (Fig. 4). The determinants for the resistance to metrifudil, $N^6$-benzyladenosine and NF449 on the EV71 genome are a single mutation at nt 5050 (change of A to G) resulting in an amino acid change of glutamic acid to glycine at amino acid position 325 of protein 2C for metrifudil resistance, double mutations at nt 4428 and 5048 (changes of C to U and A to G, respectively) resulting in an amino acid changes of histidine to tyrosine and isoleucine to methionine at amino acid positions 118 and 324 of protein 2C for $N^6$-benzyladenosine resistance, and double mutations at nt 2734 and 3173 (changes of G to C and A to G, respectively) resulting in amino acid changes of glutamic acid to glutamine and lysine to arginine at amino acid positions 98 and 244 of protein VP1 for NF449 resistance. In contrast, PV and EV71 mutants resistant to GW5074 treatment were not isolated after 12 passages in the presence of GW5074. These results suggested that the target step of GW5074 was much more conserved in PV and EV71 infection than those of metrifudil, $N^6$-benzyladenosine and NF449, and that the inhibitory effect could not be overcome by the mutations of the viral proteins.

### Table 1. Properties of identified drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>$CC_{50}$ (μM)</th>
<th>$IC_{50}$ for EV71 (μM)</th>
<th>$IC_{50}$ for PV (μM)</th>
<th>SI for EV71</th>
<th>SI for PV</th>
<th>Reversion of EV71</th>
<th>Reversion of PV</th>
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<tr>
<td>Metrifudil</td>
<td>&gt;50</td>
<td>1.3</td>
<td>ND</td>
<td>&gt;38</td>
<td>ND</td>
<td>3 passages (2C)</td>
<td>ND</td>
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<td>$N^6$-benzyladenosine</td>
<td>3300</td>
<td>0.10</td>
<td>ND</td>
<td>33 000</td>
<td>ND</td>
<td>3 passages (2C)</td>
<td>ND</td>
</tr>
<tr>
<td>NF449</td>
<td>&gt;1000</td>
<td>6.7</td>
<td>ND</td>
<td>&gt;150</td>
<td>ND</td>
<td>5 passages (VP1)</td>
<td>ND</td>
</tr>
<tr>
<td>GW5074</td>
<td>170</td>
<td>2.0</td>
<td>2.7</td>
<td>85</td>
<td>63</td>
<td>No reversion</td>
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ND, Not determined.

Inhibitory effect of GW5074 is independent of Raf-1, B-Raf and of the interferon response

To examine the possible involvement of the putative cellular targets of GW5074 in its inhibitory effect, we analysed the effect of the MEK/ERK signalling pathway, which is the downstream signalling pathway of Raf-1, on PV infection by using MEK1/2 inhibitors (SL 327 and U0126) (Fig. 5a). After PV infection, clear CPE in the cells treated with MEK inhibitors appeared at 8 h p.i. The cells were completely destroyed at 32 h p.i., as observed for mock-treated cells infected with PV. However, treatment with GW5074 blocked the appearance of CPE in the cells from 8 h p.i. and at least until 32 h p.i. MEK inhibitors had no inhibitory effect on the pseudovirus infection (inhibition indices of 0.30 and 0.27 at 100 μM), in contrast to GW5074 (inhibition index of $6.3 \times 10^{-4}$ at 25 μM) (Fig. 5b). In addition, we did not observe an inhibitory effect from a MEK1 inhibitor, PD98059, which was included in the drug library (data not shown).

Next, we analysed the effect of putative targets of GW5074 on PV infection by using siRNAs specifically targeting Raf-1, B-Raf, Pim-1, -2, -3, GAK, HIPK2, MST2 and ATF-3 (Fig. 6a). Partial suppression of PV pseudovirus infection was observed for cells treated with B-Raf siRNA02 and with MST2 siRNA03, but not for other sets of siRNA targeting B-Raf and MST2. We analysed the induction of OAS1 and STAT1 mRNA expression in cells treated with siRNA or with GW5074 as an indicator of the interferon response, which could serve as a non-specific cellular response against PV infection (Yoshikawa et al., 2006). Induction of the interferon response was observed in cells treated with B-Raf siRNA02, but not in those treated with other siRNAs or with GW5074 (Fig. 6c). To examine further the role of Raf-1 and B-Raf, we analysed the effect of downregulation of Raf-1 and B-Raf on the sensitivity of RD cells to the inhibitory effect of GW5074 (Fig. 6a and b), and found that downregulation of Raf-1 and B-Raf did not affect the sensitivity of RD cells to GW5074. These results suggested that the inhibitory effect of GW5074 on PV infection did not depend on the MEK/ERK signalling pathway, expression of Raf-1 and B-Raf, or the interferon response, and that at least individual downregulation of previously known in vitro targets of GW5074 was not sufficient to show the inhibitory effect.
### Table 2. Effect of identified drugs on the infectivity of PV and EV71 strains

<table>
<thead>
<tr>
<th>Drug</th>
<th>Type 1 PV</th>
<th>Type 2 PV</th>
<th>Type 3 PV</th>
<th>Type 1 EV71</th>
<th>Type 2 EV71</th>
<th>Type 3 EV71</th>
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<td>BrCr-TR</td>
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<td>7.7 × 10^{-4}</td>
<td>7.0 × 10^{-7}</td>
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<td>1.9 × 10^{-3}</td>
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</tr>
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<td>9.1 × 10^{-4}</td>
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</tr>
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<td>9.2 × 10^{-4}</td>
<td>9.1 × 10^{-4}</td>
<td>9.1 × 10^{-4}</td>
<td>9.1 × 10^{-4}</td>
</tr>
<tr>
<td>Sabin</td>
<td>1.6 × 10^{-4}</td>
<td>7.2 × 10^{-4}</td>
<td>9.2 × 10^{-4}</td>
<td>9.2 × 10^{-4}</td>
<td>9.2 × 10^{-4}</td>
<td>9.2 × 10^{-4}</td>
</tr>
<tr>
<td>Mock-treated</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<td>1.0</td>
</tr>
</tbody>
</table>

*The genotype of each strain is shown in parentheses.

### DISCUSSION

By using a pharmacologically active drug library (LOPAC1280 library) with PV and EV71 pseudoviruses, we identified two compounds, metrifudil and NF449, with anti-EV71 activity and one compound, GW5074, with both anti-PV and anti-EV71 activities that suppressed the infection to less than 10^{-4} of that in mock-treated cells. We also found that N^6-benzyladenosine, which is structurally related to metrifudil, efficiently blocked the replication of EV71. For metrifudil and N^6-benzyladenosine, the spacer between the phenyl group and adenosine moiety of this compound seemed critical for the inhibitory effect, because no inhibitory effect was observed for the treatment with N^6-2-phenylethyladenosine (ethyl spacer) and N^6-phenyladenosine (no spacer) (data not shown). The determinants of EV71 mutants resistant to these compounds were identified in the viral protein 2C. Identified mutations were located in the C terminus portion of protein 2C (amino acid positions 325 and 324 for metrifudil and N^6-benzyladenosine resistance, respectively), which is responsible for the appearance of altered membranes in the PV-infected cells by unknown mechanism (Teterina et al., 1997). Mutations in this region were not observed in PV mutants resistant to guanidine hydrochloride, MRL-1237 and hydantoin, which suppress PV replication by inhibiting ATPase activity and/or encapsidation activity of protein 2C (Pfister & Wimmer, 1999; Shimizu et al., 2000; Vance et al., 1997). Therefore, the mechanisms of inhibition by metrifudil and N^6-benzyladenosine via EV71 protein 2C remain to be further studied.

NF449 blocked EV71 infection at the steps of binding and/or uncoating, and the determinants of the resistance to NF449 were identified in the viral capsid protein VP1. We observed a partial resistance for BrCr-TR strain against NF449 treatment, which had a positively charged amino acid residue at position 98 in protein VP1 (lysine in BrCr-TR strain and glutamic acid in other strains examined). Amino acid changes responsible for the resistance were located at the BC loop and near the fivefold axis of the virion (Ranganathan et al., 2002). In PV infection, the receptor binding brings a fivefold axis close to the membrane, suggesting a direct function of this site in the uncoating process (Bubeck et al., 2005). Interestingly, a mutation near the fivefold axis has been identified in a mouse-adapted EV71 strain, which affected the binding to the cells (Arita et al., 2008). A polyanionic property of NF449 might suggest an ionic interaction of EV71 with its receptor at the site near the fivefold axis. The direct interaction of these compounds with viral or cellular proteins remains to be further tested.

In contrast to metrifudil, N^6-benzyladenosine and NF449, the inhibitory effect of GW5074 did not allow the emergence of revertants under the conditions examined. GW5074 was originally identified as a Raf-1 inhibitor (Lackey et al., 2000), but also inhibits various kinases in vitro, including Pim isoforms (Pim-1, -2 and -3), HIPK2, RIP2, GAK and MST2, and those are considered as more potent targets of GW5074 than Raf-1 (Bain et al., 2007). RIP2 could be strongly inhibited by a p38 inhibitor,
Fig. 4. Characterization of EV71 mutants resistant to identified anti-EV71 inhibitors. Percentage of the pseudovirus replication relative to that in mock-treated cells, which was taken as 100%, is shown. (a, b) EV71 pseudovirus with a mutation in viral protein 2C was resistant to metrifudil treatment. RD cells were inoculated with 100 IU EV71 pseudovirus in the presence of metrifudil (16 μM) or of N6-benzyladenosine. (c) EV71 pseudovirus with mutations in viral capsid protein VP1 was resistant to NF449 treatment. RD cells were inoculated with 100 IU EV71 pseudovirus in the presence of NF449 (31 μM).

Fig. 5. GW5074 inhibits PV replication independently of the MEK/ERK signalling pathway. (a) Effect of MEK inhibitors (SL327 and U0126) and GW5074 on PV infection. RD cells were inoculated with PV (Mahoney) (at a m.o.i. of 10) in the presence of each drug (25 μM). (b) Effect of MEK inhibitors and GW5074 on PV pseudovirus infection. RD cells were inoculated with 100 IU PV pseudovirus in the presence of each drug. The cells were collected at 8 h p.i. and the luciferase activity in the cells was measured. Bars, 100 μM.
Fig. 6. Effect of siRNA treatment against putative cellular targets of GW5074 on PV replication. (a) Effect of siRNA treatment against putative targets of GW5074 on PV pseudovirus infection. Left panel: RD cells were treated with siRNAs against putative targets of GW5074; the cells were then inoculated with 100 IU PV pseudovirus at 72 h post-transfection. Percentage of the pseudovirus replication relative to that in mock-treated cells at 8 h p.i., which was taken as 100%, is shown. Right panel: Western blot analysis of Raf-1 and B-Raf in siRNA-treated cells. (b) Effect of downregulation of Raf-1 and B-Raf on the inhibitory effect of GW5074. RD cells treated with siRNAs against Raf-1 (left panel), B-Raf (right panel) and control siRNAs with scramble sequences were inoculated with 100 IU PV pseudovirus at 72 h post-transfection in the presence of GW5074. Percentage of the pseudovirus replication relative to that in mock-treated cells is shown. (c) Interferon response of siRNA- or GW5074-treated cells. The relative expression levels of OAS1 and STAT1 mRNAs in the cells were determined by real-time PCR using β-actin mRNA as the endogenous control normalized by the OAS1 and STAT1 mRNA expression in mock-treated cells. Poly IC-treated cells were taken as positive control of the interferon response.
SB202190, which showed no inhibitory effect on PV and EV71 infections (data not shown). Instead, the in vivo effect of GW5074 is enigmatic: GW5074 has a neuroprotective action in neurones by activating, but not by inhibiting, B-Raf (Chen et al., 2008). This action was independent of the MEK/ERK signalling pathway, but depended on down-regulation of ATF-3 mRNA (Chen et al., 2008). However, siRNA treatments against these putative targets did not consistently suppress PV replication (Fig. 6a). Interestingly, one set of siRNA against MST2 (siRNA03) showed a partial inhibitory effect without inducing an apparent interferon response. However, other sets of siRNA against MST2 failed to show the inhibitory effect, and one set (siRNA01) rather stimulated the infection (Fig. 6a). Therefore, the target of GW5074 for its inhibitory effect seemed different from those of in vivo neuroprotective action (B-Raf and ATF-3), but should be conserved among the enterovirus infections. It is also possible that GW5074 reacted with highly conserved region of the viral proteins for its inhibitory effect. The identity of the target of GW5074 in the inhibitory effect of PV replication remained to be further identified.

In summary, we have characterized four compounds with high anti-EV71 and/or anti-PV activity. Live and inactivated vaccines have been established for PV and used for global eradication of poliomyelitis (Sabin, 1965; Salk 1954). However, there is no effective therapy for vaccine-associated paralytic poliomyelitis, which is caused by virulent revertants of vaccine strains at a rate of one case per 520,000 doses associated with the first dose of the vaccine (Nkowane et al., 1987). For EV71, various vaccine candidates and therapies are being developed, but no vaccine has been established (Chen et al., 2006; Chiu et al., 2006; Liu et al., 2005, 2007; Shih et al., 2004; Tan & Cardosa, 2007; Tung et al., 2007; Wu et al., 2007; Yu et al., 2000). The results obtained in this study would be useful for the identification of novel targets to develop anti-enterovirus drugs that could serve as therapeutic and/or prophylactic agents against acute neurological diseases caused by enterovirus infection.

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