A bifunctional anti-enterovirus compound that inhibits replication and the early stage of enterovirus 71 infection

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Enviroxime is an anti-enterovirus compound that targets viral protein 3A and/or 3AB and suppresses a replication step of enterovirus by an unknown mechanism. To date, a number of anti-enterovirus compounds that have little structural similarity to enviroxime but induce common resistance mutations in the 3A-encoding region have been identified. The present study identified a novel type of functionally enviroxime-like compound, AN-12-H5. This compound had no structural similarity to enviroxime or to known enviroxime-like compounds, including TTP-8307, GW5074 and Flt3 Inhibitor II. A resistance phenotype of poliovirus (PV) to these compounds was conferred by a major enviroxime-resistance mutation of PV (G5318A, 3A-Ala70Thr), but not by resistance mutations to guanidine hydrochloride and brefeldin A. AN-12-H5 had a common structure with the anti-enterovirus 71 (EV71) compound AN-23-F6. AN-12-H5 and AN-23-F6 inhibited an early stage of EV71 infection after virus binding to the cells. Mutations in capsid proteins (G3112A, VP1-Ala224Thr, and G2396A, VP3-Arg227Lys mutations) were determined as resistant mutations to AN-12-H5 and AN-23-F6 in the early stage of EV71 infection. These results suggest that AN-12-H5 is a bifunctional anti-enterovirus compound that belongs to a novel class of enviroxime-like compounds and targets both a replication step and an early stage of EV71 infection.

INTRODUCTION

The genus Enterovirus, family Picornaviridae, consists of at least ten species of non-enveloped virus with a single-stranded, positive-sense genomic RNA. Enterovirus infection is mostly asymptomatic, but sometimes causes severe neurological symptoms, as exemplified by poliomyelitis. Among the enteroviruses, poliovirus (PV) and enterovirus 71 (EV71) are known to be highly neurotropic enteroviruses. Infection by PV, which is the causative agent of poliomyelitis and belongs to Human enterovirus species C, the motor neurons are the major target for neurovirulence (Bodian, 1949). EV71, which belongs to Human enterovirus species A, 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). Live attenuated oral PV vaccine and inactivated PV vaccine have been established for PV and used for global eradication of poliomyelitis (Sabin, 1965; Salk et al., 1954); however, currently no vaccine is available for EV71.

With available effective vaccines for PV, the role of antivirals for PV infection is limited in the current situation. In this post-eradication era of PV, anti-PV compounds are instead anticipated to have a role in the control of a circulating vaccine-derived PV along with inactivated PV vaccine, and for the treatment of patients chronically infected with PV and for persons exposed to PV (Collett et al., 2008; Committee on Development of a Polio Antiviral and Its Potential Role in Global Poliomyelitis Eradication, N. R. C., 2006). For EV71 infection, antivirals could be an effective therapeutic means during the course of the rapid progression of symptoms in fatal cases (≤ 12 h after hospitalization) (Chang et al., 1999).

To date, several anti-enterovirus compounds that target viral or cellular factors have been identified (reviewed by Barnard, 2006; Chen et al., 2008; Rotbart, 2002). Among the compounds with anti-PV activity, guanidine hydrochloride (GuHCl), brefeldin A and enviroxime inhibit replication steps with known targets. GuHCl belongs to a group of 2C inhibitors that inhibit initiation of negative-strand RNA synthesis by targeting viral proteins 2C and/or 2BC (Barton & Flanagan, 1997; Bienz et al., 1990; Caligiuri & Tamm, 1968). Some benzimidazole derivatives, including...
Identification of anti-enterovirus compounds

To identify anti-enterovirus compounds that have conserved targets in enterovirus infection, we performed a screening with a chemical library of 3000 compounds using a high-throughput screening system with a PV and EV71 pseudovirus infection system (Arita et al., 2008b). We identified 145 compounds that showed an apparent inhibitory effect on PV or EV71 pseudovirus infection. Among these candidate compounds, 11 had no detectable cytotoxicity towards human rhabdomyosarcoma (RD) cells. Finally, we identified three compounds that inhibited PV1 (Mahoney) and EV71 (Nagoya) infections (AN-22-A6, AN-11-E6 and AN-12-H5) and one compound that inhibited EV71 (Nagoya) infection but not PV1 (Mahoney) infection (AN-23-F6) (Fig. 1). These compounds were classified into two groups based on their structures: one group comprised AN-11-E6, AN-12-H5 and AN-23-F6 and the other AN-22-A6 and the other AN-22-A6. The EC_{50} values of AN-11-E6, AN-12-H5 and AN-22-A6 for PV pseudovirus were 4.5, 1.1 and 12 μM, respectively (Fig. 1b and Table 1). The EC_{50} value of AN-12-H5 (1.1 μM) was lower than that of GW5074 (4.6 μM) measured under the same conditions. The EC_{50} values of AN-11-E6, AN-12-H5, AN-22-A6 and AN-23-F6 for EV71 pseudovirus were 5.0, 0.55, 13 and 0.15 μM, respectively (Fig. 1b). The inhibitory effects of AN-22-A6 and AN-12-H5 on multistep infection of PV1 (Mahoney) were slightly weaker, but comparable or stronger on EV71 (Nagoya) infection compared with that of GW5074 (Fig. 1c). AN-23-F6 inhibited multistep infection of EV71 (Nagoya), but not that of PV1 (Mahoney).

Characterization of the target steps of the identified anti-enterovirus compounds

To determine the target step of the inhibitory effect of the identified compounds on PV and EV71 infection, we performed a time-of-addition experiment of the compounds (Fig. 2). The inhibitory effects of AN-22-A6, AN-11-E6 and AN-12-H5 after the uncoating step of PV infection [1 h post-infection (p.i.)] were comparable to those before the uncoating step, as observed for GW5074, a replication inhibitor of enteroviruses (Arita et al., 2008b). AN-22-A6 showed a similar inhibitory effect on EV71 infection before and after the uncoating step. In contrast, the inhibitory effects of AN-11-E6, AN-12-H5 and AN-23-F6 on EV71 infection were weaker after the uncoating step than before it (Fig. 2). These results suggested that the target steps of AN-11-E6 and AN-12-H5 were in a replication step of PV and EV71 infection, and also in an early stage of EV71 infection.

Characterization of mutants resistant to anti-enterovirus compounds

To characterize the target of the inhibitory effect of the identified compounds, we isolated mutants resistant to these compounds. After passages of PV1 (Mahoney) or EV71 (Nagoya) in the presence of AN-22-A6, AN-12-H5 or AN-23-F6, the resistance phenotypes of viruses were observed by the appearance of cytopathic effect (CPE) compared with that of parental virus infection in the presence of the compounds. Viral genomes of mutants resistant to AN-22-A6 and AN-12-H5 contained mutations in the 3A protein, including a G5318A mutation for AN-12-H5 (Table 2). Mutants resistant to AN-12-H5 contained mutations in the structural protein-coding regions. These results suggested that AN-12-H5 is an enviroxime-like compound but also belongs to a class of capsid-binding inhibitor.

To evaluate the importance of the identified mutations, the resistance phenotypes of pseudovirus mutants with identified mutations were analysed (Fig. 3). A resistance phenotype to AN-12-H5 of a PV pseudovirus mutant
was observed with a G5318A mutation in the 3A protein, but not with a G2957A mutation in the VP1 protein (Fig. 3a, d). EV71 pseudovirus mutants with a G5259A mutation (3A-Ala66Thr in strain BrCr-TR) and a G3112A mutation (VP1-Ala224Thr) showed a resistance phenotype to AN-22-A6 and AN-23-F6, respectively (Fig. 3b). A G2396A mutation (VP3-Arg227Lys) and a G3112A mutation, but not a A2797U mutation (VP1-Met119Leu), conferred partial resistance to AN-12-H5 and AN-23-F6 (Fig. 3c and Supplementary Fig. S1, available in JGV).
Online). A A5179G mutation (3A-Glu39Gly in strain BrCr-TR) conferred partial resistance to AN-12-H5 after the uncoating step (Fig. 3c, e). These results suggested that AN-12-H5 inhibits a replication step of PV and EV71 and also an early stage of EV71 infection by targeting the VP1 and VP3 proteins, similar to AN-23-F6.

**Specificity of resistance mutations to anti-enterovirus compounds**

To analyse the specificity of the resistance phenotype conferred by the G5318A mutation to AN-12-H5, we analysed the effect of known resistance mutations of PV, including a GuHCl resistance mutation (U4614A, 2C-Phe164Tyr) and brefeldin A resistance mutations (G4361A, 2C-Val192Met; C5190U, 3A-Ala27Val) (Baltera & Tershak, 1989; Crotty et al., 2004), to AN-12-H5. Each resistance mutation conferred a specific resistance phenotype to the corresponding inhibitors (Fig. 4). Among these mutations, only the G5318A mutation conferred a resistance phenotype to AN-22-A6, AN-12-H5 and AN-11-E6. The resistance phenotypes with the G5318A mutation to these compounds were weak compared with those observed to GW5074 and Flt3 Inhibitor II. These results suggested that AN-22-A6, AN-12-H5 and AN-11-E6 belong to a group of enviroxime-like compounds.

**Characterization of the inhibitory effects of AN-12-H5 and AN-23-F6 on the early stage of EV71 infection**

To characterize the effect of AN-12-H5 and AN-23-F6 on the early stage of EV71 infection, we analysed the binding of EV71 strains to cells in the presence of these compounds (Fig. 5). Mean copy numbers of viral genomes associated with the cells were 0.082, 1.9, 5.7, 4.2 and 1.5 % of those of input viruses for strains BrCr-TR, Nagoya, C7-Osaka, 1095 and 75-Yamagata-2003, respectively. The copy numbers of viral genomes associated with the cells in the presence of AN-12-H5 and AN-23-F6 were comparable to those in the presence of GuHCl (Fig. 5a). AN-12-H5 inhibited infection of EV71 strains with a 0–9 h p.i. treatment, but not with a 0–2 h p.i. treatment (Fig. 5b). These results suggested that AN-12-H5 acts as a bifunctional inhibitor for EV71 by targeting a replication step and an early stage of infection after the binding step.

Next, we examined the effect of a resistance mutation (Val192Met) to a capsid-binding inhibitor for EV71 (BPR0Z-194) on the inhibitory effect of AN-23-F6 (Shih et al., 2004). The mutation conferred only partial resistance to AN-23-F6, in contrast to the effect of the G3112A mutation (Fig. 5c). This suggested that AN-12-H5 and AN-23-F6 inhibited an early stage of EV71 infection by a mode that is probably different from that of the capsid-binding inhibitor BPR0Z-194.

**DISCUSSION**

In this study, we identified a novel type of enviroxime-like compound, AN-12-H5. AN-12-H5 suppressed PV and EV71 infection, as observed for other enviroxime-like compounds.

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**Table 1. Property of identified anti-enterovirus compounds**

Results are shown as means with SD in parentheses. SI, Selectivity index [ratio of 50 % cytotoxic concentration (CC50:EC50)]; ND, not determined.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC50 (2 days) (µM)</th>
<th>EC50 (PV) (µM)</th>
<th>EC50 (EV71) (µM)</th>
<th>SI (PV)</th>
<th>SI (EV71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW5074</td>
<td>96</td>
<td>4.6 (0.85)</td>
<td>6.4 (2.5)</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>AN-11-E6</td>
<td>120</td>
<td>4.5 (2.8)</td>
<td>5.0 (1.7)</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>AN-12-H5</td>
<td>78</td>
<td>1.1 (0.18)</td>
<td>0.55 (0.13)</td>
<td>74</td>
<td>141</td>
</tr>
<tr>
<td>AN-22-A6</td>
<td>290</td>
<td>12 (2.8)</td>
<td>13 (4.1)</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>AN-23-F6</td>
<td>84</td>
<td>ND</td>
<td>0.15 (0.047)</td>
<td>ND</td>
<td>550</td>
</tr>
</tbody>
</table>

Fig. 2. Determination of the target step of the inhibitory effect of anti-enterovirus compounds. RD cells were infected with PV or EV71 pseudovirus. Compounds were added to the infected cells at 0 or 1 h.p.i. for PV pseudovirus infection or 0 or 2 h.p.i. for EV71 pseudovirus infection at a concentration of 25 µM. The luciferase activities of the infected cells at 7 h.p.i. (for PV pseudovirus infection) or 9 h.p.i. (for EV71 pseudovirus infection) are shown as percentage pseudo virus infection. Pseudovirus infection in the absence of compounds was taken as 100 %. *, P<0.05.
Table 2. Mutations in the viral genomes of resistant mutants

Nucleotide sequences analysed for resistant mutants to AN-22-A6, AN-12-H5 and AN-23-F6 were the non-structural protein-coding region, structural and non-structural protein-coding regions and structural protein-coding region, respectively. ND, Not determined.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PV (Mahoney)</th>
<th>EV71 (Nagoya)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Passage no.*</td>
<td>Mutation</td>
</tr>
<tr>
<td>AN-22-A6</td>
<td>4</td>
<td>A4013G (2B-Thr61Ala)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C5151U (3A-Thr14Met)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C5366U (3A-His86Tyr)</td>
</tr>
<tr>
<td>AN-12-H5</td>
<td>12</td>
<td>G2957A (VP1-Val160Ile)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C4499U</td>
</tr>
<tr>
<td></td>
<td></td>
<td>U4765C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G5318A (3A-Ala70Thr)</td>
</tr>
<tr>
<td>AN-23-F6</td>
<td>–</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Numbers of passages when some resistance phenotype was observed.

compounds, but with little structural similarity to known enviroxime-like compounds, supporting the possibility of a large structural diversity of enviroxime-like compounds (De Palma et al., 2009) (Fig. 1). Interestingly, AN-12-H5 and an anti-EV71 compound, AN-23-F6, had a common structure, and inhibited the early stage of EV71 infection with a common target in capsid proteins (Figs 2 and 3, and Supplementary Fig. S1). The inhibitory effect of AN-12-H5 in the early stage of EV71 infection was weaker than that of AN-23-F6 (overall EC50 value of 0.55 vs 0.15 μM), but its contribution to the inhibitory effect on EV71 infection was comparable to that of a replication step. Therefore, a bifunctional inhibitory effect of AN-12-H5 is essential for its anti-EV71 activity.

Resistance mutations to the inhibitory effect of AN-12-H5 and AN-23-F6 on the early stage of EV71 infection were identified in the VP1 protein (Ala224Thr) and VP3 protein (Arg227Lys). These mutations have not been identified as resistance mutations to the capsid-binding inhibitor BPR0Z-194 (Shih et al., 2004), which is a pyridyl imidazolidinone derivative designed based on WIN compounds (Abdel-Rahman & Kearns, 1998; Otto et al., 1985), and are not known as corresponding sites of PV3 (Sabin) resistant mutants to the WIN compound disoxaril (WIN 51711; Mosser et al., 1994). A resistance mutation to BPR0Z-194 was identified in the VP1 protein of EV71 (Val192Met; Shih et al., 2004), which is located at the corresponding site of a resistance mutation of PV3 (Sabin) to disoxaril (Val194Leu; Mosser et al., 1994). Interestingly, the corresponding site of an Ala224Thr mutation in the VP1 protein of EV71 is aa 231 in the VP1 protein of PV1 (Mahoney), which is known as the site for a soluble receptor resistance mutation (Ala231Val; Colston & Racaniello, 1994). This mutation is located on the floor of the canyon structure at the interface between the protomers of the virion, and confers a resistance phenotype in the transition of 160S particles to 135S particles induced by the cell-surface PV receptor. Two mutations in the structural protein-coding region of a resistant mutant to AN-12-H5 (VP3-Arg227Lys and VP1-Met119Leu) are located at the interface between protomers, although their roles in the early stage of infection or in a resistance phenotype to WIN compounds are not known. A mutation in the VP1 protein of a PV mutant resistant to AN-12-H5 (VP1-Val160Ile) is known to be associated with in vitro persistence of PV1 (Sabin) and host-range expansion (Colston & Racaniello, 1995; Pelletier et al., 1998), but a resistance phenotype of the PV mutants to AN-12-H5 was not observed with this mutation under the conditions examined (Fig. 3). This mutation facilitates the uncoating step of PV1 (Sabin) (Pelletier et al., 1998) and thus might be introduced during selection with AN-12-H5 under conditions that restrict the infection of PV, possibly related to conditions resembling in vitro persistent infection (Benton et al., 1996; Carp, 1981; Colbere-Garapin et al., 1989). A resistance mutation to BPR0Z-194 (Val192Met) conferred only partial resistance to the mutant against AN-23-F6 (Fig. 5c), suggesting that AN-12-H5 and AN-23-F6 inhibit the early stage of EV71 infection by a mode that is probably different from that of capsid-binding inhibitors targeting hydrophobic pockets on the virion.

In the present study, we identified two independent enviroxime-like compounds in a screening of 3000 compounds. In our previous screening for pharmacologically active compounds, we identified one enviroxime-like compound (GW5074) out of 1268 compounds (Arita et al., 2008b). Some tyrosine kinase inhibitors could act as enviroxime-like compounds (Arita et al., 2009). This suggests that enviroxime-like compounds form a major group of anti-enterovirus compounds that suppress a replication step for a broad range of enteroviruses. The effect of a major resistance mutation of PV to enviroxime,
Fig. 3. Characterization of resistance mutations to anti-enterovirus compounds. RD cells were infected with PV or EV71 pseudovirus mutants with resistance mutations. Compounds were added to the infected cells at 0 or 1 h p.i. for PV pseudovirus infection or at 0 or 2 h p.i. for EV71 pseudovirus infection. The luciferase activities of the infected cells at 7 h p.i. (for PV pseudovirus infection) or 9 h p.i. (for EV71 pseudovirus infection) are shown as percentage pseudovirus infection. Pseudovirus infection in the absence of compounds was taken as 100 %. (a) Inhibitory effect of AN-12-H5 on PV pseudovirus mutant infection. PV pseudovirus mutants with mutations of an AN-12-H5-resistant mutant (G2957A, VP1-Val160Ile; G5318A, 3A-Ala70Thr) were examined. AN-12-H5 was added to the cells at a concentration of 25 μM. (b) Inhibitory effect of the identified compounds on EV71 pseudovirus mutant infection. EV71 pseudovirus mutants with mutations of an AN-22-A6-resistant mutant (G2957A, VP1-Val160Ile; G5318A, 3A-Ala70Thr) and an AN-23-F6-resistant mutant (G3112A, VP1-Ala224Thr) were examined. (c) Inhibitory effect of AN-12-H5 on EV71 pseudovirus mutant infection. EV71 pseudovirus mutants with resistance mutations of an AN-12-H5-resistant mutant (A5179G, 3A-Glu39Gly) and an AN-23-F6-resistant mutant (G3112A, VP1-Ala224Thr) were examined. For samples treated with 6.3 μM AN-12-H5 (drug treatment of 2–9 h p.i.), individual inhibitory effects on the mutants were compared with that on parental EV71 pseudovirus infection using Student’s t-test. (d) Dose-dependent inhibitory effect of AN-12-H5 on PV pseudovirus mutant infection. (e) Dose-dependent inhibitory effect of AN-12-H5 on EV71 pseudovirus mutant infection. *, P<0.05.
the G5318A mutation, was specific to these enviroxime-like compounds, but the extent of resistance varied among the compounds (Fig. 4). The resistance conferred by this mutation to AN-22-A6, AN-12-H5 and AN-11-E6 was weak compared with those to GW5074 and Flt3 Inhibitor II, although the mutation should be the critical resistant determinant to AN-12-H5 obtained after a number of passages (Table 2). Interestingly, the partial resistance conferred by the G5318A mutation was only clear in the treatment with AN-12-H5 at high concentration (Fig. 3d). This suggests that there is a threshold of resistance of PV to some enviroxime-like compounds conferred by the G5318A mutation.

AN-12-H5 and AN-23-F6 did not affect the binding step of EV71 to the cells, suggesting that the target in the early stages of infection is after the binding step, possibly at the uncoating stage (Fig. 5a). The inhibitory effect of AN-12-H5 in the early stages of infection was clearly observed for the BrCr-TR and Nagoya strains but not for other strains because of its specific inhibitory effect on the replication step of these strains. The early stage of infection was the major target of the inhibitory effect of AN-23-F6 on EV71 infection; however, an inhibitory effect was also observed in a replication step of some strains (data not shown).

These observations suggested that AN-12-H5 and AN-23-F6 are potent anti-EV71 compounds with multiple targets in EV71 infection, although the targets differ among the strains.

The bifunctional inhibitory effect of AN-12-H5 is reminiscent of betulinic acid derivatives that act as bifunctional inhibitors of human immunodeficiency virus (HIV) infection (Huang et al., 2004). The target specificity of the derivative is determined by the side-chain structure as the entry step and the maturation step of HIV infection. These bifunctional inhibitors were at least 1 log more potent in terms of EC₅₀ value than monofunctional inhibitors (Huang et al., 2004). Actually, the appearance of resistant mutants for AN-12-H5 was slower than those for AN-23-F6 (seven vs three passages), possibly because of its multiple targets in infection (Table 2). Nevertheless, the emergence of resistant mutants was inevitable under treatment with AN-12-H5, suggesting that combined treatment with other potent antivirals with different targets, including some potent capsid-binding inhibitors (Abdel-Rahman & Kearns, 1998; Oberste et al., 2009), will be essential to suppress the emergence of resistant mutants (Committee on Development of a Polio Antiviral and Its Potential Role in Global Poliomyelitis Eradication, N. R. C., 2006). The in vivo anti-enterovirus activities of the identified compounds are currently being evaluated by using a mouse EV71 infection model (Arita et al., 2008a).

In summary, we identified a novel type of enviroxime-like compound, AN-12-H5, that suppressed EV71 infection with a bifunctional inhibitory effect on a replication step and an early stage of the infection. These results suggested that the group of enviroxime-like compounds is a promising resource to explore bifunctional anti-enterovirus compounds due to its large structural diversity.

**METHODS**

**Cells, viruses and chemical library.** RD cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and used for the titration of viruses and pseudoviruses and for screening. PV and EV71 pseudoviruses (TE-PV-Fluc mc and TE-EV71-Fluc mc), which encapsidated luciferase-encoding PV and EV71 replicons with capsid proteins derived from PV1 (Mahoney) and EV71 (Nagoya), respectively, were prepared as reported previously (Arita et al., 2006, 2008a, b). Resistance mutations for PV, including G5318A (enviroxime and GW5074 resistance, 3A-Ala70Thr) (Arita et al., 2009; Heinz & Vance, 1995), G2957A (VP1-Val160Ile), U4614A (GuHCl resistance, 2C-Phe164Tyr) (Balter & Tershak, 1989), G4361A and C5190U (brefeldin A resistance, 2C-Val80Ile and 3A-Ala72Val) (Croft et al., 2004), were introduced into the cDNA of a luciferase-encoding PV replicon based on PV1 (Mahoney) or into a PV1 (Mahoney) capsid protein expression vector (Arita et al., 2006). Resistance mutations for EV71, including G3112A (VP1-Ala224Thr), A5179G (3A-Glu39Gly mutation of the BrCr-TR strain), G5259A (3A-Ala66Thr mutation of the BrCr-TR strain), G3016A and T3018G (BPKOZ-194 resistance, VP1-Val192Met mutation), were introduced into the cDNA of a luciferase-encoding EV71 replicon based on EV71 (BrCr-TR) or into an EV1 (Nagoya) capsid protein expression vector (Arita et al., 2008a). These mutations were introduced into the cDNAs by
site-directed mutagenesis as described previously (Arita et al., 2006, 2008a). EV71 strains BrCr-TR (genotype A), Nagoya (genotype B1), C7-Osaka (genotype B4), 1095 (genotype C2) and 75-Yamagata-2003 (genotype C4) (Mizuta et al., 2005) were used for analysis of the inhibitory effects of compounds. GuHCl (Sigma-Aldrich), brefeldin A (Sigma-Aldrich), GW5074 (Sigma-Aldrich) and Flt3 Inhibitor II (Calbiochem) were used for analysis of resistant mutants. A chemical library of the National Institute of Infectious Diseases (Tokyo, Japan), which consists of 3000 compounds based on natural product derivatives, was used for screening.

**Screening for anti-enterovirus compounds.** RD cells (1.0 x 10^4 cells per well in 100 μl medium) were cultured at 37 °C in 96-well plates (White Opaque Tissue Culture Plates; Becton Dickinson), followed by addition of 8.0 μl 0.24 mM compound solution (final concentration 18 μM). The cells were incubated at 37 °C for 24 h and then inoculated with 200 infectious units (IU) of PV or EV71 pseudovirus in 50 μl DMEM/10% FCS (the final concentration of the compounds was 12 μM). The cells were incubated at 37 °C. The luciferase activity of the infected cells was measured with a Luciferase Assay System (Promega) using a TR717 Microplate luminometer.
(ABI) at 7 h p.i. (for cells inoculated with PV pseudovirus) or 10 h p.i. (for cells inoculated with EV71 pseudovirus). Cut-off values for the screening were set to select at least one compound that showed anti-enterovirus activity without apparent cytotoxicity. For this criterion, we set the cut-off value at <5% of pseudovirus infection in mock-treated cells, as measured by luciferase activity.

The inhibitory effect of compounds on PV and EV71 infection was evaluated by the appearance of CPE of the infected cells and by measurement of the number of copies of viral genome in the infected cells in the presence or absence of the compounds. RD cells (1.4 × 10^6 cells per well in 100 μl medium) in a 96-well plate were infected with PV1 (Mahoney) or EV71 (Nagoya) at an m.o.i. of 0.1. Observation of CPE of the infected cells was performed at day 1 p.i. for cells infected with PV or at days 1–3 p.i. for cells infected with EV71. Cells infected with PV and EV71 were collected at days 1 and 3 p.i., respectively. Viral RNA was extracted from the cells using a High Pure Viral RNA Purification kit (Roche) and the number of copies of the viral genome was quantified using a real-time PCR system (described below).

**Quantification of viral RNA by real-time PCR.** Real-time PCR was performed as described previously by Dierssen et al. (2008) with modifications. The viral RNA was assayed in a 20 μl reaction mixture containing 5 μl viral RNA using a One Step SYBR PrimeScript PLUS RT-PCR kit (TaKaRa) with primers EQ-1 and EQ-2 (Dierssen et al., 2008). Viral RNA of PV1 (Sabin) was used to control the quantification of the number of copies. The mixtures were subjected to real-time PCR, consisting of a reverse transcription step at 42 °C for 30 min followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The fluorescence emission of SYBR Green 1 was monitored and analysed using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems).

**Measurement of cytotoxicity.** The cytotoxicity of compounds was evaluated by observation of cell viability at 7 h after treatment with the compounds under the same conditions as used for the screening, and by determination of the 50% cytotoxic concentration (CC_{50}) of compounds at 2 days after treatment by measurement of ATP as a marker of metabolically active cells. For the measurement of CC_{50}, RD cells (1.4 × 10^6 cells per well in 100 μl medium) were cultured at 37 °C in 96-well plates (Becton Dickinson), followed by the addition of 100 μl medium (containing 5 × 10^4 cells) were infected with PV1 (Mahoney) at an m.o.i. of 1 and incubated at 37 °C for 2 h in the presence of 2 mM GuHCl and 25 μM AN-12-H5 or AN-22-A6. Cells were incubated at 37 °C for 2 days and then subjected to measurement of ATP using a Cell Titre-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer’s instructions. The CC_{50} value of each compound was obtained by a non-linear regression analysis of the dose–response curve.

**Measurement of the inhibitory effect of anti-enterovirus compounds on PV and EV71 pseudovirus infection.** RD cells (1.4 × 10^6 cells per well in 100 μl medium) in a 96-well plate were inoculated with 400 IU PV or EV71 pseudovirus in the presence of the compounds as indicated above. Luciferase activity in the infected cells was measured at 7 h p.i. (for PV pseudovirus infection) or 9 h p.i. (for EV71 pseudovirus infection). The EC_{50} value of each compound was obtained by a non-linear regression analysis of the dose–response curve.

**Measurement of the inhibitory effect of anti-enterovirus compounds on PV and EV71 infection.** The inhibitory effect of the compounds on PV and EV71 replication was evaluated by measurement of the number of copies of viral genome in infected cells. RD cells (1.4 × 10^6 cells per well in 100 μl medium) in a 96-well plate were infected with PV1 (Mahoney) or EV71 (Nagoya) at an m.o.i. of 1 at 37 °C for 1 h (for PV infection) or 2 h (for EV71 infection). The cells were washed three times with DMEM/10% FCS, followed by the addition of 100 μl DMEM/10% FCS containing each compound, or without compound as a control. Cells infected with PV and EV71 were collected for measurement of the number of copies of viral genome in the cells as described above at 8 and 9 h p.i., respectively.

**Isolation of PV and EV71 mutants resistant to anti-enterovirus compounds.** For isolation of resistant PV mutants, RD cells (1.4 × 10^6 cells) were infected with PV1 (Mahoney) at an m.o.i. of 10, 1 and 0.1 in the presence of AN-12-H5 at 20 μM and AN-22-A6 at 20 and 40 μM. For isolation of resistant EV71 mutants, RD cells (1.4 × 10^6 cells) were infected with EV71 (Nagoya) at an m.o.i. of 10 in the presence of AN-12-H5 at 3.1–25 μM, AN-22-A6 at 25 μM and AN-23-F6 at 6.3–25 μM. The cells were incubated at 37 °C until all cells exhibited CPE or until day 3 p.i. Collected cell lysates for each compound were mixed and then used for the next passage. Passaging was repeated 12 times or until a resistance phenotype was observed by the appearance of CPE. Resistant mutants were isolated by limiting dilution. Non-structural protein-coding regions of the viral genomes were analysed for resistant mutants to AN-12-H5 and AN-22-A6, and structural protein-encoding regions of the viral genomes were analysed for resistant mutants to AN-12-H5 and AN-23-F6, as described previously (Arita et al., 2005, 2008a).

**Binding assay.** RD cells (2.0 × 10^4) in 96-well plates (Falcon) were infected with EV71 strains at an m.o.i. of 1 and incubated at 37 °C for 2 h in the presence of 2 mM GuHCl and 25 μM AN-12-H5 or AN-23-F6. The cells were washed three times with DMEM/10% FCS containing 2 mM GuHCl and harvested by adding 50 μl DMEM/10% FCS containing 2 mM GuHCl. Viral RNA was extracted from the cells and the number of copies of the viral genome was determined by real-time PCR.

**Statistical analysis.** The results of at least three experiments are shown as the mean ± SD. For comparison of the inhibitory effects between two samples, Student’s t-test was performed as appropriate. P values of less than 0.05 were considered significant.

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A bifunctional anti-enterovirus compound


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