Cellular kinase inhibitors that suppress enterovirus replication have a conserved target in viral protein 3A similar to that of enviroxime

Minetaro Arita, Takaji Wakita and Hiroyuki Shimizu

Department of Virology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan

Previously, we identified a cellular kinase inhibitor, GW5074, that inhibits poliovirus (PV) and enterovirus 71 replication strongly, although its target has remained unknown. To identify the target of GW5074, we searched for cellular kinase inhibitors that have anti-enterovirus activity similar or related to that of GW5074. With this aim, we performed screenings to identify cellular kinase inhibitors that could inhibit PV replication cooperatively with GW5074 or synthetically in the absence of GW5074. We identified MEK1/2 inhibitors (SL327 and U0126), an EGFR inhibitor (AG1478) and a phosphatidylinositol 3-kinase inhibitor (wortmannin) as compounds with a cooperative inhibitory effect with GW5074, and an Akt1/2 inhibitor (Akt inhibitor VIII) as a compound with a synthetic inhibitory effect with MEK1/2 inhibitors and AG1478. Individual treatment with the identified kinase inhibitors did not affect PV replication significantly, but combined treatment with MEK1/2 inhibitor, AG1478 and Akt1/2 inhibitor suppressed the replication synthetically. The effect of AG1478 in this synthetic inhibition was compensated by other receptor tyrosine kinase inhibitors (IGF-1R inhibitor II and Flt3 inhibitor II). We isolated mutants resistant to Flt3 inhibitor II and GW5074 and found that these mutants had cross-resistance to each treatment. These mutants had a common mutation in viral protein 3A that results in an amino acid change at position 70 (Ala to Thr), a mutation that was previously identified in mutants resistant to a potent anti-enterovirus compound, enviroxime. These results suggest that cellular kinase inhibitors and enviroxime have a conserved target in viral protein 3A to suppress enterovirus replication.

INTRODUCTION

The genus Enterovirus consists of at least ten species of non-enveloped viruses with a single-stranded, positive-sense genomic RNA that belong to the family Picornaviridae. Enterovirus infection is mostly asymptomatic, but sometimes causes severe neurological symptoms exemplified by poliomyelitis. In infection by poliovirus (PV), which is the causative agent of poliomyelitis and belongs to the species Human enterovirus C, motor neurons are the major target for neurovirulence (Bodian, 1949). Enterovirus 71 (EV71), another neurotropic enterovirus belonging to the species Human enterovirus 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). EV71 causes fatal pulmonary oedema and pulmonary haemorrhage in young children by destruction of the vasomotor and respiratory centres in the brainstem (Chang et al., 1999; Ho et al., 1999; Huang et al., 1999; Komatsu et al., 1999; Lum et al., 1998; Wang et al., 1999). For PV, live-attenuated and inactivated vaccines have been established and are currently being used for global eradication of poliomyelitis (Sabin, 1965; Salk et al., 1954). However, there is no effective therapeutic means for vaccine-associated paralytic poliomyelitis caused by virulent revertants of vaccine strains, which occurred at a rate of one case per 520 000 vaccinations with the first dose of the live-attenuated vaccine (Nkowane et al., 1987). For EV71, various vaccine candidates and therapeutic means are being developed, but no vaccine has been established (Arita et al., 2005a, 2007; 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).

To date, several anti-enterovirus compounds that target cellular factors and inhibit various stages of virus replication have been identified. Brefeldin A blocks membrane traffic between the cis- and trans-Golgi compartments by targeting a cellular guanine nucleotide-
exchange factor, GBF1, and inhibits the replication of PV, but not encephalomyocarditis virus (EMCV) (Belov et al., 2008; Cuconati et al., 1998; Iruzun et al., 1992; Maynell et al., 1992). Geldanamycin targets Hsp90 and interferes with the folding of PV capsid, but not with RNA replication, probably in cooperation with Hsp70 (Geller et al., 2007; Macejak & Sarnow, 1992). Hippuristanol, a natural product of the coral *Isis hippuis*, inhibits RNA binding of eIF4A and delays the appearance of virus proteins in PV replication for 2 h (Bordeleau et al., 2006). Calpain inhibitors inhibit the replication of echovirus 1, possibly by targeting the activities of calpain 1 and 2 (Upla et al., 2008). Pyrrolidine dithiocarbamate (PDTC) inhibits polypeptide processing of entero- and cyroviruses by transporting zinc ions into the cells with different mechanisms depending on the virus species (Krenn et al., 2005; Lanke et al., 2007). For PDTC, a mechanism via the ubiquitin–proteasome pathway is also proposed for the inhibitory effect on coxsackievirus B3 (CVB3) infection (Si et al., 2005). The advantage of using antiviral compounds that target cellular factors is the limited emergence of resistant mutants. Mutants resistant to geldanamycin and PDTC were not isolated despite attempts (Geller et al., 2007; Krenn et al., 2009), although a mutant resistant to brefeldin A was isolated after five passages in cultured cells (Crotty et al., 2004).

In our previous attempt to identify potent anti-enterovirus compounds that target cellular factors, we identified a Raf-1 inhibitor, GW5074, by screening pharmacologically active compounds (Arita et al., 2008b). However, inhibitors for the downstream signalling pathway of Raf-1 [MEK/extracellular signal-regulated kinase (ERK) signalling] did not affect PV replication. Actually, we did not identify any potent anti-enterovirus compounds other than GW5074 in the kinase inhibitor library examined in this screening. This suggested that GW5074 has several targets for its inhibitory effect, as observed in an in vitro kinase assay (Bain et al., 2007). Therefore, in the present study, we attempted to identify the target of GW5074 by searching for cellular kinase inhibitors that have anti-enterovirus activity similar or related to that of GW5074. We have found that combined treatment with inhibitors for MEK1/2 or Akt1/2 and a receptor tyrosine kinase inhibitor, AG1478, effectively suppresses PV and EV71 replication in the absence of GW5074. We have also found that some receptor tyrosine kinase inhibitors have anti-enterovirus activity by themselves. A single mutation in viral protein 3A is required for partial resistance to a receptor tyrosine kinase inhibitor, Flt3 inhibitor II, and to GW5074. The mutation was known previously as a determinant of resistance to envoixime, which is a potent anti-enterovirus inhibitor that suppresses the initiation of positive-strand RNA synthesis (Heinz & Vance, 1995; Wikel et al., 1980). These observations suggested that some cellular kinase inhibitors and envoixime have a conserved target in viral protein 3A to suppress enterovirus replication.

**METHODS**

**Cells, viruses, reagents and chemical library.** RD cells (a human rhabdomyosarcoma cell line) were cultured as monolayers in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and used for titration of pseudoviruses and screening of anti-PV and anti-EV71 compounds. 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 PV (Mahoney) and EV71 (Nagoya), respectively, were prepared as reported previously (Arita et al., 2006, 2008a, b). A PV pseudovirus mutant [TE-PV-Fluc mc (5318A)] that has adenine at nt 5318 was prepared with cDNA of a luciferase-encoding PV replicon by site-directed mutagenesis as described previously (Arita et al., 2006, 2008a). Kinase inhibitors included in the LOPAC1280 drug library (Sigma-Aldrich), ZM336372 (Calbiochem), Akt inhibitor VIII (Sigma-Aldrich), STO-609 (Calbiochem), FK506 (Fermentek), SL327 (Sigma-Aldrich) and U0126 (Sigma-Aldrich) were used for screening of kinase inhibitors (see Supplementary Table S1, available in JGV Online). For screening of receptor tyrosine kinase inhibitors that can substitute for the effect of AG1478, cFMS receptor tyrosine kinase inhibitor, Flt3 inhibitor II, IGF-1R inhibitor II, TGF-β RI inhibitor III and VEGF receptor tyrosine kinase inhibitor III were purchased from Calbiochem. Each compound (10 μM) was prepared in DMSO and then used for screening.

**Screening of kinase inhibitors.** For the screening of kinase inhibitors that have a cooperative inhibitory effect with GW5074, RD cells (1.0 x 10⁵ cells per well in 100 μl medium) were cultured at 37 °C in 96-well plates (Becton Dickinson), followed by addition of 1.6 μl kinase inhibitor solution (10, 5.0, 2.5 and 0 μM), 10 μl GW5074 (120 and 0 μM) and 200 IU PV pseudovirus in 50 μl DMEM/10% FCS. Cells were incubated at 37 °C for 7 h (final concentrations of kinase inhibitors: 100, 50, 25 and 0 μM; final concentrations of GW5074, 7.5 and 0 μM) (Fig. 1a) and the luciferase activity of the cells was measured with the Luciferase Assay system (Promega), using a TR717 Microplate luminometer (Applied Biosystems) according to the manufacturer’s instructions. For the screening of kinase inhibitors that have a synthetic inhibitory effect with SL327, U0126, AG1478 and wortmannin, RD cells (1.0 x 10⁶ cells per well in 50 μl medium) were cultured at 37 °C in 96-well plates, followed by addition of 1.6 μl kinase inhibitor solution (5.0, 2.5 and 0 mM), 50 μl SL327 (150 μM), U0126 (150 μM), AG1478 (150 μM) and wortmannin (6 μM) solution. The cells were inoculated with 200 IU PV pseudovirus and the luciferase activity of the cells at 7 h post-infection (p.i.) was measured (Fig. 1b). The inhibition index, which is the ratio of luciferase activity of the drug-treated cells to that of mock-treated cells (1.0 for mock-treated cells in the absence of GW5074 and for GW5074-treated cells in the presence of GW5074), was determined for each drug in the presence or absence of GW5074. Cutoff values of the screening were set to select at least one compound that showed an inhibitory effect without apparent cytotoxicity in the screening. For these criteria, we set the cutoff at below the mean –2SD, or at <10% of the mean in the case that the SD was larger than the mean. Accordingly, the cooperative inhibitory effect of the compounds was defined as a reduction of the inhibition index in the presence of GW5074 (7.5 μM) by <10-fold of that in the absence of GW5074. The synthetic inhibitory effect of the compounds was defined as reduction of the inhibition index in the presence of SL327 (25 μM), U0126 (25 μM), AG1478 (25 μM) or wortmannin (2 μM) by <10^2-fold of that in the absence of these kinase inhibitors.

**Evaluation of the cytotoxicity of compounds.** Cytotoxicity of the compounds was evaluated by two methods: one was observation of cell viability at 7 h under the same conditions as used for the screening and the other was the determination of 50% cytotoxic concentration (CC₅₀) of drugs by the measurement of ATP.
**RESULTS**

**Screening of kinase inhibitors that have a cooperative inhibitory effect with GW5074 on PV infection**

To identify cellular kinase inhibitors that have antienterovirus activity similar or related to that of GW5074, we performed two rounds of screening with a library of cellular kinase inhibitors. In the first screening, we aimed to identify cellular kinase inhibitors that have a cooperative inhibitory effect with GW5074 (see below for definition of the inhibitory effect). In the second screening, we aimed to identify cellular kinase inhibitors that have a synthetic inhibitory effect with the kinase inhibitors identified in the first screening in the absence of GW5074. Cooperative inhibitory effect was defined as an inhibitory effect that enhances the inhibitory effect of GW5074 on PV replication. Synthetic inhibitory effect was defined as an inhibitory effect on PV replication observed in a combined treatment of compounds, each of which showed no inhibitory effect in individual treatment.

To identify cellular kinase inhibitors that have a cooperative inhibitory effect with GW5074, we performed screening with a kinase inhibitor library in the presence or absence of a suboptimal concentration of GW5074 (7.5 µM) in RD cells (Fig. 1a; Supplementary Table S1). The inhibitory effect of GW5074 has been observed in L20B (murine), HEP-2c and RD (human) cells and thus is not species-specific (Arita et al., 2008b). Therefore, we used RD cells for the present screenings because this cell line is not species-specific (Arita et al., 2008b). Therefore, we used RD cells for the present screenings because this cell line is not species-specific (Arita et al., 2008b).

**Measurement of the interferon (IFN) response.** RD cells (4.2 × 10⁴) in 24-well plates were incubated at 37 °C for 7 h in the presence of the following compounds: GW5074, 50 µM; U0126, 25 µM; Akt inhibitor VIII, 2 µM; AG1478, 25 µM; IGF-1R inhibitor II, 25 µM; Flt3 inhibitor II, 25 µM. The relative expression levels of OAS1 and STAT1 mRNAs in the cells were determined by real-time PCR using an IFN Response Watcher kit (TaKaRa) as indicators of the IFN response. β-Actin mRNA was used as the endogenous control, and the expression level of OAS1 and STAT1 mRNAs in the treated cells was normalized by that in mock-treated cells.

Concentration as a marker of metabolically active cells, as described previously (Arita et al., 2008b). RD cells (1.4 × 10⁴ cells per well in 100 µl medium) were cultured at 37 °C in 96-well plates (Becton Dickinson), followed by addition of 100 µl compound solution in a range of concentrations (7.8–500 µM) for GW5074, AG1478, IGF-1R inhibitor II and Flt3 inhibitor II. The CC<sub>50</sub> value of each drug was also measured in the presence of U0126 (25 µM) and Akt inhibitor VIII (2 µM). Cells were incubated at 37 °C for 7 h and then subjected to measurement of ATP by using a Cell Titre-Glo Luminescent Cell Viability Assay kit (Promega) according to the manufacturer’s instructions.

**Quantification of virus RNA by real-time TaqMan PCR.** Real-time TaqMan PCR was performed as described by Nijhuis et al. (2002). Virus RNA was reverse-transcribed by using the Reverse Transcription system (Promega) and then subjected to real-time PCR by using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) with primers (forward primer and reverse primer 2) and a probe (probe 1) (Nijhuis et al., 2002). The fluorescence emission of the probe was monitored and analysed by using the Applied Biosystems 7500 Fast Real-Time PCR system, as described previously (Arita et al., 2008b).

**Isolation of PV mutants resistant to inhibitors.** RD cells (1.4 × 10⁴) were infected with PV (Mahoney) at an m.o.i. of 10 in the presence of inhibitors as follows: guanidine hydrochloride (GuaHCl), 0.5, 1.0 and 2.0 mM; GW5074, 12.5, 25 and 50 µM; Flt3 inhibitor II, 3.1, 6.3 and 12.5 µM. Cells were incubated at 37 °C until all cells exhibited cytopathic effect (CPE) or at day 3 p.i. Collected cell lysates for each inhibitor were mixed and then used for the next passage. Passage was repeated 12 times or until a resistant phenotype was observed by the appearance of CPE. Resistant mutants were isolated by limiting dilution, and then the non-structural protein-encoding regions of the viral genome were analysed as described previously (Arita et al., 2005b).

**RESULTS**

Screening of kinase inhibitors that have a cooperative inhibitory effect with GW5074 on PV infection

To identify cellular kinase inhibitors that have anti-entervirus activity similar or related to that of GW5074, we performed two rounds of screening with a library of cellular kinase inhibitors. In the first screening, we aimed to identify cellular kinase inhibitors that have a cooperative inhibitory effect with GW5074 (see below for definition of the inhibitory effect). In the second screening, we aimed to identify cellular kinase inhibitors that have a synthetic inhibitory effect with the kinase inhibitors identified in the first screening in the absence of GW5074. Cooperative inhibitory effect was defined as an inhibitory effect that enhances the inhibitory effect of GW5074 on PV replication. Synthetic inhibitory effect was defined as an inhibitory effect on PV replication observed in a combined treatment of compounds, each of which showed no inhibitory effect in individual treatment.

To identify cellular kinase inhibitors that have a cooperative inhibitory effect with GW5074, we performed screening with a kinase inhibitor library in the presence or absence of a suboptimal concentration of GW5074 (7.5 µM) in RD cells (Fig. 1a; Supplementary Table S1). The inhibitory effect of GW5074 has been observed in L20B (murine), HEP-2c and RD (human) cells and thus is not species-specific (Arita et al., 2008b). Therefore, we used RD cells for the present screenings because this cell line is susceptible to both PV and EV71. Cells were inoculated with PV pseudoviruses that have a luciferase-encoding PV
replicon genome encapsidated in a PV capsid, as described previously (Arita et al., 2006, 2008b), and then the luciferase activity in the cells was measured at 7 h p.i. The mean luciferase activity in the mock-treated cells infected with PV pseudoviruses was $1.3 \times 10^6$ relative light units (RLU), and that in GW5074-treated cells was $2.4 \times 10^5$ RLU. The mean inhibition index of kinase inhibitors (100 µM) in the presence or absence of GW5074 was 0.64 (with an SD of 0.33) for kinase inhibitors that did not cause apparent cytotoxicity. We identified MEK1/2 inhibitors (SL327 and U0126), an EGFR inhibitor (AG1478) and a phosphatidylinositol 3-kinase (PI3K) inhibitor (wortmannin) as compounds with a cooperative inhibitory effect with GW5074 (Fig. 2). SL327, U0126 and AG1478 showed the cooperative inhibitory effect with GW5074 at concentrations of ≥50 µM. Effective concentrations of wortmannin in the cells as a PI3K inhibitor or as an enhancer of EMCV mutant viral protein synthesis were 0.1–1.0 µM (Okada et al., 1994; Svitkin et al., 1998), thus far lower than that examined in the screening (25–100 µM). Therefore, we also examined lower concentrations (0.78–6.3 µM) and found that wortmannin showed a cooperative inhibitory effect in this range of concentrations.

**Screening of kinase inhibitors that have a synthetic inhibitory effect with MEK1/2 inhibitors, AG1478 and wortmannin on PV infection**

Next, to identify cellular kinase inhibitors that have a synthetic inhibitory effect in the absence of GW5074, we performed screening with the identified kinase inhibitors (SL327, U0126, AG1478 and wortmannin) that showed a cooperative inhibitory effect with GW5074 in the first screening (Fig. 1b). The mean inhibition index of kinase inhibitors in the presence or absence of SL327, U0126, AG1478 and wortmannin was 1.5–2.7 (with an SD of 1.5–3.8) for kinase inhibitors that did not cause apparent cytotoxicity. For SL327, U0126 and AG1478, but not for wortmannin, an Akt1/2 inhibitor (Akt inhibitor VIII) was identified as a compound that showed a synthetic inhibitory effect on PV pseudovirus infection. GW2974 (an EGFR/ERBB2 inhibitor) and diacylglycerol kinase inhibitor I showed a synthetic inhibitory effect with AG1478, but not with other inhibitors (Fig. 3a). The inhibitory effect of individual compounds was not significant compared with the synthetic inhibitory effect of these compounds (<10-fold reduction of replication); they caused at most a 50-fold reduction of replication [in the presence of 50 µM AG1478 (Fig. 3b)]. Combined treatment with U0126 (25 µM), Akt inhibitor VIII (2 µM), AG1478 (50 µM) and wortmannin (2 µM) showed a significant inhibitory effect (inhibition index of 2.1 × 10⁻⁴). In this combination, U0126, Akt inhibitor VIII and AG1478 were essential. The effect of wortmannin was weaker than that of Akt inhibitor VIII, and compensated only partially for the effect of Akt inhibitor VIII.

**Screening of receptor tyrosine kinase inhibitors that can substitute for the synthetic inhibitory effect of AG1478 on PV infection**

The effective concentration of AG1478 (12.5–50 µM) in the synthetic inhibitory effect was far higher than that reported for the inhibition of EGFR phosphorylation in cells (0.5–1.0 µM) (Isaacson et al., 2007; Soeda et al., 2008). Therefore, we analysed other receptor tyrosine kinase inhibitors that could substitute for the effect of AG1478 with U0126 (25 µM) and Akt inhibitor VIII (2 µM). We found that IGF-1R inhibitor II and Flt3 inhibitor II, but not GuaHCl, GW5074 or other receptor tyrosine kinase inhibitors, showed a cooperative inhibitory effect with U0126 and Akt inhibitor VIII, although these receptor tyrosine kinase inhibitors have a strong inhibitory effect by themselves, in contrast to AG1478 (Fig. 4a). CC₅₀ values of these receptor tyrosine kinase inhibitors and GW5074 were determined to be reduced by 3- to 10-fold in the presence of U0126 and Akt inhibitor VIII (Fig. 4b; see Discussion).

The inhibitory effect of these kinase inhibitors was also observed for EV71 pseudovirus and PV (Mahoney) infection (Fig. 5a, b). Treatment with kinase inhibitors was effective after the uncoating process of PV (Mahoney) (Fig. 5b), suggesting that the target step of the inhibitory
effect was the replication process after uncoating. Treatment with kinase inhibitors did not induce the expression of OAS1 or STAT1 mRNA (Fig. 5c), suggesting that the inhibitory effect of these kinase inhibitors was independent of the IFN response.

Isolation of mutants resistant to receptor tyrosine kinase inhibitors

We attempted to isolate mutants of PV (Mahoney) resistant to receptor tyrosine kinase inhibitors, in order to identify their targets in the viral proteins. We examined Flt3 inhibitor II because the inhibitory effect of AG1478 was weak and IGF-1R inhibitor II showed relatively high cytotoxicity (Fig. 3b). We also examined GuaHCl (0.5–2.0 mM) treatment as a positive control for the isolation of...

Fig. 3. Kinase inhibitors that showed a synthetic inhibitory effect on PV pseudovirus infection. (a) Identified kinase inhibitors that showed a synthetic inhibitory effect with SL327, U0126, AG1478 and wortmannin. Inhibition index of each compound in the synthetic inhibition is shown in parentheses. (b) Combined treatment with kinase inhibitors. RD cells (0.7×10⁴) in a 96-well plate were inoculated with 200 IU PV pseudovirus in the presence of AG1478 (12.5, 25 and 50 μM), U0216 (25 μM), Akt inhibitor VIII (2 μM) and wortmannin (2 μM). Luciferase activity at 7 h p.i. is shown. Pseudovirus replication in mock-treated cells was taken as 100 %.

Fig. 4. Synthetic inhibitory effect of receptor tyrosine kinase inhibitors on PV pseudovirus infection. (a) RD cells (1.4×10⁴) in a 96-well plate were inoculated with 200 IU PV pseudovirus in the presence of tyrosine kinase inhibitors, GuaHCl and GW5074 in mock-treated cells (without U0126 and Akt inhibitor VIII, ●) and in U0126 (25 μM)- and Akt inhibitor VIII (2 μM)-treated cells (○). Luciferase activity at 7 h p.i. is shown. Pseudovirus replication in mock-treated cells and in U0126- and Akt inhibitor VIII-treated cells was taken as 100 %. (b) CC₅₀ values of kinase inhibitors in the absence or presence of U0126 (25 μM) and Akt inhibitor VIII (2 μM).
resistant mutants, and GW5074 (12.5–50 μM) treatment as a negative control for the isolation (Arita et al., 2008b). After four passages of PV (Mahoney) in the presence of GuaHCl, clear CPE was observed in infected cells in the presence of GuaHCl (Fig. 6a). In contrast, CPE was not observed in infected cells during 12 passages in the presence of GW5074, and only weak CPE was observed in the presence of Flt3 inhibitor II (Fig. 6a; data not shown).

To confirm the inhibitory effect on replication, we analysed the copy number of the virus genome in infected cells. Surprisingly, substantial amounts of virus RNA were detected in infected cells inoculated with viruses after just four passages in the presence of GW5074 or Flt3 inhibitor II, as well as in those inoculated with a mutant resistant to GuaHCl (Fig. 6b). Moreover, these resistant mutants showed cross-resistance to each other, but not to GuaHCl. RD cells infected with the mutant resistant to GW5074 at an m.o.i. of 10–0.1 showed complete CPE at 24 h p.i. in the absence of GW5074 (Fig. 6c). However, the appearance of CPE in the infected cells was suppressed in the presence of GW5074 (at most 50 % of cells showed CPE when infected at an m.o.i. of 10, and no CPE was observed at m.o.i.s of 1.0 or 0.1), as observed in parental PV (Mahoney) infection. The genomic sequences of resistant mutants showed a common mutation in viral protein 3A [G (parental) to A (resistant mutants) at nt 5318] that caused an amino acid change at position 70 (Ala to Thr) (Fig. 6d). A PV pseudovirus mutant with this mutation [TE-PV-Fluc mc (5318A)] showed a phenotype resistant to GW5074 and Flt3 inhibitor II, as observed for the isolated resistant mutants, in contrast to its parental pseudovirus (TE-PV-Fluc mc) (Fig. 6c). However, TE-PV-Fluc mc (5318A) was still sensitive to GuaHCl and IGF-1R inhibitor II and to combined treatment with MEK1/2 and Akt1/2 inhibitors.

Fig. 5. Synthetic inhibitory effect of kinase inhibitors on EV71 pseudovirus and PV (Mahoney) infection. (a) Synthetic inhibitory effect of kinase inhibitors on PV and EV71 pseudovirus infection. RD cells (1.4×10⁴) in a 96-well plate were inoculated with 200 IU PV and EV71 pseudoviruses in the presence of the indicated inhibitors: GuaHCl (2 mM), GW5074 (50 μM), U0126 (25 μM), Akt inhibitor VIII (2 μM), AG1478 (25 μM), IGF-1R inhibitor II (25 μM) and Flt3 inhibitor II (25 μM). Luciferase activity at 7 h p.i. (for PV pseudovirus infection, filled bars) and 10 h p.i. (for EV71 pseudovirus infection, empty bars) is shown. Pseudovirus replication in mock-treated cells was taken as 100 %. (b) Synthetic inhibitory effect of kinase inhibitors on PV (Mahoney) replication. RD cells (4.2×10⁴) in a 24-well plate were infected with PV (Mahoney) at an m.o.i. of 5 and then incubated at 37 °C for 1 h in the absence of inhibitors. After washing, the cells were incubated with GuaHCl (2 mM), GW5074 (50 μM), U0126 (25 μM), Akt inhibitor VIII (2 μM), AG1478 (25 μM), IGF-1R inhibitor II (25 μM) and Flt3 inhibitor II (25 μM) at 37 °C for 7 h. Number of copies of the virus genome in infected cells at 8 h p.i. is shown. The number of copies of the virus genome in mock-treated cells (1.4×10⁹ copies) was taken as 100 %. (c) IFN response in cells treated with kinase inhibitors. Relative expression levels of OAS1 (filled bars) and STAT1 (empty bars) mRNAs in the treated cells are shown. Poly(IC)-treated cells were taken as a positive control for the IFN response.
DISCUSSION

In this study, we identified kinase inhibitors that could suppress enterovirus replication either cooperatively with GW5074 or synthetically with other kinase inhibitors that did not show the inhibitory effect by individual treatment. We identified MEK1/2 inhibitors (SL327 and U0126), an EGFR inhibitor (AG1478) and a PI3K inhibitor (wortmannin) that showed a cooperative inhibitory effect with GW5074, and an Akt1/2 inhibitor (Akt inhibitor VIII) that showed a synthetic inhibitory effect with MEK1/2 inhibitors and AG1478, but not with wortmannin (Fig. 3a). We also identified GW2974, an inhibitor for EGFR and ERBB2 [which both belong to the EGFR family of receptor tyrosine kinases (Rusnak et al., 2001)], and diacylglycerol kinase inhibitor I that showed a synthetic inhibitory effect with AG1478. GW2974 targets the same signalling pathway as AG1478, and thus would have an effect similar to that of AG1478. For diacylglycerol kinase inhibitors, a more active inhibitor (diacylglycerol kinase inhibitor II) did not show a synthetic inhibitory effect with AG1478 (data not shown). Therefore, we characterized the inhibitory effect of MEK1/2 inhibitors, AG1478, wortmannin and Akt1/2 inhibitor on enterovirus infection.

Combined treatment with MEK1/2 inhibitor, AG1478, wortmannin and Akt1/2 inhibitor showed a strong synthetic inhibitory effect (Fig. 3b). Individual treatment with these kinase inhibitors did not affect PV or EV71 infection, thus a synergy of these kinase inhibitors was essential for the inhibitory effect. This is consistent with a recent observation by Autret et al. (2008) that PI3K/Akt signalling does not affect the growth of PV. In EMCV infection, wortmannin treatment even increased viral protein synthesis and the yield of virus (Svitkin et al., 1998). In contrast, treatment with an inhibitor for PI3K significantly reduced viral RNA synthesis and release of progeny viruses in CVB3 infection (Esfandiarei et al., 2004). In CVB3 infection, a MEK1/2 inhibitor reduced viral protein synthesis and progeny virus release (Luo et al., 2002). Therefore, the MEK/ERK and PI3K/Akt signalling pathways might affect enterovirus infection in different ways depending on the virus species, but seemed to have little effect on PV and EV71 replication, at least by themselves. The effect of wortmannin in the synthetic inhibitory effect was weaker than that of Akt inhibitor VIII, but partially compensated for the effect of Akt1/2 inhibitor (Fig. 3b), suggesting that the target of the synthetic inhibitory effect was Akt1/2. The concentrations of MEK1/2 inhibitor (25 μM for U0126) and Akt1/2 inhibitor (2 μM for Akt inhibitor VIII) tested are in physiologically relevant orders of magnitude: 20 μM for U0126 (Favata et al., 1998) and 1–10 μM for Akt inhibitor VIII for its full inhibitory effect in cells (Logie et al., 2007). In contrast, the specificity of AG1478 was not supported by the effective concentration needed for its synthetic inhibitory effect (25–50 μM), which is far higher than those reported for its full activity in cells (0.5–1.0 μM) (Isaacson et al., 2007; Soeda et al., 2008). The inhibitory effect of other receptor tyrosine kinase inhibitors, an IGF1R inhibitor (IGF-1R inhibitor II) and an Flt3 inhibitor (Flt3 inhibitor II), was enhanced in the presence of MEK1/2 inhibitor and Akt1/2 inhibitor (Fig. 4a). Therefore, MEK1/2 and Akt1/2 seemed to be involved as the targets of the synthetic inhibitory effect, but the identity of the target(s) of these receptor tyrosine kinase inhibitors remains unknown. Combined treatment with MEK1/2 inhibitor and Akt1/2 inhibitor reduced the CC50 of kinase inhibitors by about 3- to 10-fold (Fig. 4b), suggesting that induced cytotoxicity, as measured by the amount of ATP of metabolically active cells, might have a role in the synthetic inhibitory effect. However, the apparent viability of the cells was not impaired severely by combined treatment under the conditions tested, except for those treated with IGF-1R inhibitor II, which showed relatively high toxicity by itself (11–24% of mock-treated cells; see Supplementary Fig. S1, available in JGV Online). Inhibition of Akt could sensitize tumour cells to apoptotic stimuli (DeFeo-Jones et al., 2005) and enhance cytotoxicity with MEK inhibitor in lymphoma cells (Leleu et al., 2007). Treatment with inhibitors for MEK1/2 (25 μM of U0126) and Akt1/2 (2 μM of Akt inhibitor VIII) did not affect the inhibitory effect of GuAHI and GW5074, although the CC50 value of GW5074 was also reduced with these inhibitors (Fig. 4a). These observations suggested that combined treatment with inhibitors for MEK1/2 and Akt1/2 could enhance the inhibitory effect with some receptor tyrosine kinase inhibitors, but would not generally affect PV and EV71 replication. The property of individual kinase inhibitors might affect the synthetic inhibitory effect with inhibitors for MEK1/2 and Akt1/2.

We isolated mutants resistant to Flt3 inhibitor II and GW5074 (Fig. 6). Interestingly, these resistant mutants showed cross-resistance to each other, with a common resistant determinant in viral protein 3A at position 70 [Ala (parental strain) to Thr (resistant mutants)] (Fig. 6d, e). This mutation has been observed previously in a PV mutant resistant to enviroxime, which inhibits the initiation of positive-strand RNA synthesis of rhinoviruses and enteroviruses, although a direct interaction of enviroxime with the 3A protein has yet to be detected (Brown-Augusburger et al., 1999; Heinz & Vance, 1995; Wikel et al., 1980). A compound called TTP-8307, which has a property similar to that of enviroxime in terms of the resistance determinant in resistant mutants, was recently described (De Palma et al., 2009). Interestingly, these ‘enviroxime-like’ compounds (enviroxime, TTP-8307, GW5074 and Flt3 inhibitor II) have little in common in their chemical structures. Our results suggest that a property of cellular kinase inhibitors would be an essential factor for an inhibitory effect similar to that of enviroxime.

Heinz & Vance (1995) isolated mutants resistant to enviroxime by a four-step enrichment procedure with enviroxime, because resistant mutants were not obtained by a conventional method that involved picking the mutant from plaques formed in the presence of the compound. Actually, all of the resistant mutants showed impaired growth in the presence of enviroxime, despite their resistant phenotype (Heinz & Vance, 1995). During
the passage of PV in the presence of GW5074 and Flt3 inhibitor II, we did not observe marked CPE in infected cells, in contrast to those in the selection with GuaHCl (Fig. 6a) (Arita et al., 2008b). Nevertheless, a substantial population of resistant mutants was detected within four passages in the presence of GW5074 or Flt3 inhibitor II, suggesting that the fitness of these resistant mutants was not completely recovered in the presence of these inhibitors to cause complete CPE in infected cells.

In summary, we identified cellular kinase inhibitors that suppress enterovirus replication. We found that MEK1/2 and Akt1/2 inhibitors showed an inhibitory effect on PV and EV71 replication when used in a combined treatment
Fig. 6. Characterization of mutants resistant to a receptor tyrosine kinase inhibitor. (a) Appearance of CPE in cells infected with resistant mutants. RD cells (1.4×10^5) in a 96-well plate were infected with mutants resistant to GuaHCl, GW5074 and Flt3 inhibitor II, obtained after four passages at an m.o.i. of 10 in the absence (mock treatment) and the presence of each inhibitor: GuaHCl (2 mM), GW5074 (50 μM) and Flt3 inhibitor II (25 μM). Phase-contrast views of the cells at 24 h p.i. are shown. Bar, 100 μm. (b) Replication of resistant mutants in the presence of inhibitors. RD cells (1.4×10^5) in a 96-well plate were infected with PV (Mahoney) or mutants resistant to GuaHCl, GW5074 and Flt3 inhibitor II obtained after four passages at an m.o.i. of 10, and then incubated at 37 °C for 1 h in the absence of inhibitors. The cells were washed three times and then incubated in the absence (mock-treated, filled bars) or the presence of inhibitors: GuaHCl (2 mM, empty bars), GW5074 (50 μM, light-shaded bars) and Flt3 inhibitor II (25 μM, dark-shaded bars). Number of copies of the virus genome at 8 h p.i. is shown. The number of copies of the virus in mock-treated cells infected with each virus was taken as 100 %. (c) Appearance of CPE in cells infected with a mutant resistant to GW5074 at different m.o.i.s. RD cells were infected with PV (Mahoney) and a mutant resistant to GW5074, obtained after four passages, at m.o.i.s of 10, 1.0 and 0.1 in the absence (mock-treated) and the presence of GW5074 (50 μM). Phase-contrast views of the cells at 24 h p.i. are shown. Bar, 100 μm. (d) Mutations observed in the non-structural protein-encoding region of the virus genome of resistant mutants. (e) Effect of combined treatment with kinase inhibitors on the replication of a PV pseudovirus mutant with the resistance mutation at nt 5318 [TE-PV-Fluc mc (5318A); empty bars] compared with its parental pseudovirus (TE-PV-Fluc mc; filled bars). RD cells (1.4×10^5) in a 96-well plate were inoculated with 200 IU PV pseudovirus in the presence of inhibitors: GuaHCl (2 mM), GW5074 (50 μM), U0126 (25 μM), Akt inhibitor VIII (2 μM), AG1478 (50 μM), IGF-1R inhibitor II (25 μM) and Flt3 inhibitor II (25 μM). Luciferase activity at 8 h p.i. is shown. Pseudovirus replication in mock-treated cells was taken as 100 %.

with receptor tyrosine kinase inhibitors. We also found that GW5074, receptor tyrosine kinase inhibitors and enviroxime have a conserved target and/or mechanism of the inhibitory effect. Our results suggest that cellular kinase inhibitors could serve as a promising resource for the identification of anti-enterovirus compounds.

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