

BPR2-D2 targeting viral ribonucleoprotein complex-associated function inhibits oseltamivir-resistant influenza viruses

Shin-Ru Shih^{1-4*}†, Jim-Tong Horng^{2,5}†, Leo L. M. Poon⁶, Tzu-Chun Chen², Jiann-Yih Yeh¹,
Hsing-Pang Hsieh¹, Sung-Nain Tseng¹, Chiayn Chiang², Wan-Ling Li³, Yu-Sheng Chao¹
and John T.-A. Hsu^{1,7}

¹Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Miaoli, Taiwan; ²Research Center for Emerging Viral Infections, Tao-Yuan, Taiwan; ³Department of Medical Biotechnology & Laboratory Science, Chang Gung University, Tao-Yuan, Taiwan; ⁴Clinical Virology Laboratory, Chang-Gung Memorial Hospital, Tao-Yuan, Taiwan; ⁵Department of Biochemistry and Molecular Biology, Chang Gung University, Tao-Yuan, Taiwan; ⁶Department of Microbiology, The University of Hong Kong, Hong Kong SAR, China; ⁷Department of Biological Science and Technology, National Chiao Tung University, Hsinchu, Taiwan

*Corresponding author. Department of Medical Biotechnology and Laboratory Science, Chang Gung University, 259 Wen-Hua 1st Rd, Kwei-Shan, Taoyuan, 333, Taiwan. Tel: +886-3-2118800, ext. 5497; Fax: +886-3-2118174; E-mail: srshih@mail.cgu.edu.tw

†The first and second authors contributed equally to this work.

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Objectives: The emergence of oseltamivir-resistant viruses raised the global threat with regard to influenza virus infection. To develop alternative antiviral agents against influenza virus infection is significant and urgent.

Methods: A neutralization test was applied as a screening assay and a plaque reduction assay was used for confirmation. Expression plasmids for viral ribonucleoproteins (RNPs) and a plasmid that allowed expression of a pseudoviral reporter RNA were transfected into cells to investigate the effects of a novel antiviral compound on viral RNA synthesis.

Results: BPR2-D2 was identified as a novel inhibitor against influenza virus from a hit obtained from high throughput screening of 20000 or more compounds. BPR2-D2 exhibited an excellent antiviral efficacy for the oseltamivir-resistant virus (EC₅₀ ranging from 0.021 to 0.040 μ M). No resistant virus was produced throughout 20 passages in the presence of BPR2-D2, whereas oseltamivir-resistant virus was generated at passage 8 using the same experimental system. A molecular target other than neuraminidase (NA) was found because BPR2-D2 inhibited the synthesis of viral RNA that was driven by influenza viral RNP in a transfection assay. BPR2-D2 also exhibited a broad antiviral spectrum against various strains of influenza A and influenza B viruses.

Conclusions: BPR2-D2 was identified as a novel inhibitor of influenza virus. It may target viral RNPs that are responsible for viral RNA synthesis. Targeting different molecules compared with NA allows BPR2-D2 to inhibit oseltamivir-resistant viruses.

Keywords: antiviral agent, viral RNA, influenza A virus

Introduction

Influenza is a highly contagious, acute, febrile, respiratory illness. The native hosts of influenza virus are wild water fowl and ducks; however, the virus can infect various host species, including humans, whales, swine, horses, cattle, chickens and even cats and tigers.¹ Influenza virus is an enveloped RNA virus, belonging to the family Orthomyxoviridae.² Its viral characteristics include a segmented genome structure that is attributed to the mechanism of reassortment and an RNA polymerase complex that contributes to a higher mutation rate; influenza virus has a very diverse genomic background. Because of this genomic

diversity and the wide variety of its hosts, influenza virus causes not only annual epidemics but also occasional pandemics. The 1918 influenza pandemic killed ~50 million people in 1 year.³ The other two pandemics caused by the H2N2 and H3N2 subtypes of influenza A viruses in 1957 and 1968, respectively, also killed millions of people.^{4,5} In recent years, the continuous outbreaks of the H5N1 subtype of influenza A virus in poultry in many Asian countries have been posing a threat to humans because of the high risk of another pandemic.⁶⁻¹⁰ More recently, a swine-origin influenza A (H1N1) virus that is being spread via human to human transmission has become a serious public

concern around the world, highlighting again that influenza virus is a very significant pathogen with the unique characteristic of causing pandemics.^{11–14}

Although vaccination is a good strategy for preventing and controlling seasonal influenza virus infections, antigenic drift may cause viruses to escape antibodies in vaccinated humans. Antiviral therapy is an effective means of prevention, control and treatment of influenza virus infection. However, an increasing number of amantadine- or oseltamivir-resistant viruses have emerged.^{15–22} For example, most human influenza A H1N1 (seasonal flu) viruses were found to be resistant to oseltamivir, and many human influenza A H3N2 viruses were resistant to amantadine.^{19,21–26} Hence, the development of more anti-influenza viral agents with various molecular targets is both urgent and important. Numerous efforts are under way to develop new antiviral agents for influenza treatment.²⁷ For example, DAS181 (Fludase; NexBio), which can be mass-produced in *Escherichia coli*, is a fusion construct that incorporates the sialidase from *Actinomyces viscosus* that is linked to human epithelium-anchoring domains.²⁸ This agent targets the viral attachment process. T705 (Toyama Chemical), another potential anti-influenza virus agent, targets viral RNA polymerase through a nucleoside analogue competition mechanism.^{29,30} While there are several potential anti-influenza viral agents being developed, the need to find more antivirals remains because combination therapy is a promising strategy for the treatment of RNA viruses, including influenza virus.

In this study we conducted a cell-based screening assay of at least 20 000 compounds and identified several promising compounds that inhibit replication of influenza virus. BPR2-D2 was obtained from one of the active compounds. The inhibition of oseltamivir-resistant viruses was examined and potential molecular targets and the corresponding mechanism were also elucidated.

Materials and methods

Cells, viruses and plasmids

293T cells were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Madin–Darby canine kidney (MDCK) cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% FBS. Influenza virus A/WSN/33 (H1N1) (WSN) was purchased from ATCC. Trypsin (2.5 µg/mL) was added to MEM for virus amplification, neutralization test and plaque reduction assay for influenza virus.

The four plasmids amplified using pcDNA plasmids encoding the sequences corresponding to the PB2, PB1, PA and NP genomic segments of WSN virus were kindly provided by George G. Brownlee, University of Oxford, UK. Plasmid pPolI-CAT-RT expressing the influenza virus-like RNA driven by a truncated human RNA Pol I promoter was also kindly provided by George G. Brownlee, University of Oxford, UK.³¹ It contains the chloramphenicol acetyltransferase (CAT) gene open reading frame in negative orientation, flanked by the 3'- and 5'-non-coding region of the NS segment of WSN virus.

Neutralization test

Confluent MDCK cells in 96-well tissue culture plates were added to influenza virus. The cells were then mixed with various concentrations of test compounds before incubation was performed at 37°C for 1 h. Following adsorption, the infected cell plates were overlaid with 50 µL of DMEM plus 2% DMSO. After they had been incubated at 37°C for 48 h, the

plates were fixed by adding 100 µL of 0.5% formaldehyde for 1 h at room temperature. Following removal of the formaldehyde, the plates were stained with 0.1% Crystal Violet for 15 min at room temperature. The plates were then washed and dried, and the density of the well was measured at 570 nm by a VERSAmax microplate reader. The concentration required for a test compound to reduce the virus-induced cytopathic effect (CPE) by 50% relative to the virus control was expressed as the 50% effective dose (EC₅₀). All assays were performed three times.

For viruses other than influenza viruses, Vero cells (African green monkey kidney cells; ATCC accession no. CCL-81) were maintained in MEM (Gibco) supplemented with 10% FBS. RD cells (rhabdomyosarcoma cells; ATCC accession no. CCL-136) were cultured in DMEM (Gibco) supplemented with 10% FBS. Coxsackievirus B3, human rhinovirus 2 (HRV-2) and herpes simplex virus type 2 (HSV-2) were isolated from clinical specimens in the Clinical Virology Laboratory of Chang Gung Memorial Hospital (Linkou, Taiwan). Enterovirus 71 (EV71/Tainan/4643/98) was obtained from Dr Jen-Ren Wang of the National Cheng-Kung University, Taiwan. HSV-2 was propagated in Vero cells. Enterovirus 71, coxsackievirus B3 and HRV-2 were cultivated in RD cells. For the neutralization test, 96-well plates were seeded with 200 µL of RD cells (for picornaviruses) at a concentration of 3×10^5 cells/mL in DMEM with 10% FBS. The plates were further incubated for 24 h at 37°C. Virus (100 TCID₅₀, 50% tissue culture infective doses) was added to the cells and incubated at 35°C for 1 h. Following adsorption, the infected cells were overlaid with 50 µL of DMEM plus 2% FBS and 0.5% DMSO or BPR2-D2. The plates were incubated at 37°C for 64 h. At the end of the incubation, the plates were fixed by adding 100 µL of 0.5% formaldehyde for 1 h and then staining with 0.1% Crystal Violet. The well density was determined at 570 nm by a VERSAmax microplate reader. The concentration of BPR2-D2 required to reduce the virus-induced CPE by 50% in relation to the virus control was expressed as the EC₅₀. Each experiment was performed in triplicate and repeated at least twice. The neutralization test for HSV-2 was performed in the same manner as that for enteroviruses and rhinovirus except that Vero cells were used.

Cytotoxicity test

Serial dilutions of BPR2-D2 were incubated with MDCK cells. Following incubation for 72 h, cells were fixed with paraformaldehyde and stained with 0.1% Crystal Violet; the optical density (OD) at 570 nm was measured in a microtitre plate reader. The percentage viability is expressed in relation to the vehicle control.

Plaque reduction assay

Different concentrations of compounds in a maintenance medium with trypsin were added to monolayers of MDCK cells in 6-well plates. Virus (250 pfu) was then added to each well that contained 5×10^5 cells [multiplicity of infection (MOI) = 5×10^{-4}]. The plates were then incubated for 1 h at 37°C for viral adsorption. After that, 3 mL of overlay medium was supplemented with 3% agarose and the compound was added. After they had been incubated for 48 h at 37°C, the cells were fixed with 10% formalin for 1 h. After the formalin had been removed, the cells were stained with Crystal Violet and plaques were visualized. Visible plaques were counted, and the number of pfu/mL was determined. The numbers and sizes of plaques were obtained from at least three independent experiments.

Primer extension assay

Primer extension reactions were performed using a primer extension system—the AMV Reverse Transcriptase kit (Promega)—following the manufacturer's instructions. Briefly, 5 µg of total RNA was mixed with CAT vRNA-specific ³²P-labelled primer and positive-sense RNA-specific

³²P-labelled primer, ATGTTCTTTACGATGCGATTGGG and negative-sense RNA-specific ³²P-labelled primer, CGCAAGGCGACAAGGTGCTGA. Primer extensions were performed at 42°C for 90 min. Transcription products were denatured at 90°C for 10 min, separated on 6% polyacrylamide gels that contained 7 M urea in Tris/borate/EDTA (TBE) buffer and detected by autoradiography.

Quantitative real-time RT-PCR

Total RNA from transfected 293T cells was collected using an RNeasy Mini kit (Qiagen) following the manufacturer's instructions. The first-strand poly(A) cDNA was made using a SuperScript II reverse transcription kit (Invitrogen) following the manufacturer's instructions. The specific primers used for the green fluorescent protein (GFP) gene were as follows: sense, 5'-AGAACGGCATCAAGGTGAAC-3'; and antisense, 5'-TGCTCAGGTAGTGGTTGTGCG-3'. Values were normalized using the β -actin mRNA level. The primers for β -actin were as follows: sense, 5'-GCTCGTCGTCGACAACGGCTC-3'; and antisense 5'-CAAACATGATCCTGGGTCATCTTTC-3'. The PCR amplification yielded a product of 135 bp in length. The cDNA was amplified using SYBR green PCR mastermix (ABI), 5 mM of each primer and 0.5 μ L of the cDNA product in a total volume of 20 μ L. Forty cycles of PCR (one cycle consists of 10 min at 95°C, 15 s at 95°C and 1 min at 60°C) were performed using ABI PRISM 7000 Sequence Detection systems. Melting curve analysis was performed to verify the specificity of the products; the relative values were calculated using the $\Delta\Delta C_t$ method. Each experiment was performed in triplicate.

CAT ELISA assay

293T cells were post-transfected for 48 h at 37°C; the cell extract was then lysed by 500 μ L of lysis buffer and tested for CAT levels using the CAT ELISA kit (Roche) following the manufacturer's instructions.

Selection of BPR2-D2-resistant and GS4071-resistant viruses

GS4071 is oseltamivir carboxylate and the active metabolite of oseltamivir. MDCK cells (4×10^5 cells/well) were seeded in a 6-well plate and incubated for 24 h. The cells were washed in PBS and infected with A/WSN/33 at an MOI of 0.0005 pfu/cell. After 1 h of virus adsorption the cells were washed twice and incubated in 2 mL of DMEM that contained 0.05 μ M BPR2-D2 or 0.3 μ M GS4071 (the EC_{50} value of the compound) for 3 days. The EC_{50} value of the compound was determined by counting the plaque number using the plaque reduction assay. The clear supernatant was collected and denoted passage 1. The passage 1 virus was used to infect a new cell monolayer (MOI of 0.0005 pfu/cell), which was further incubated in the presence of increasing concentrations of compound. The selection was performed using a total of 20 and 8 passages with BPR2-D2 and GS4071, respectively. Drug concentrations of BPR2-D2 were determined by doubling the concentrations from one passage to the next, from passage 1 to 11 (ranging from 0.05 to 51.2 μ M), and then maintaining a constant concentration from passage 12 to 20 (51.2 μ M). Drug concentrations of GS4071 ranged from 0.3 to 38.4 μ M, doubling from one passage to the next over eight passages. Drug susceptibilities were determined by performing a neutralization test. GS4071 (oseltamivir carboxylate) was synthesized by Dr K.-S. Shia at the National Health Research Institutes (NHRI), Taiwan.

Haemagglutination (HA) assay

Serial 2-fold dilutions of virus samples were mixed with an equal volume of 0.5% chicken red blood cells (RBCs) in PBS and incubated on ice for 1 h in round-bottom 96-well plates. The titre was determined by noting the highest dilution of the virus that caused an HA reaction. Wells containing

an adherent, homogeneous layer of erythrocytes were labelled as positive. A plaque assay was performed to estimate the reduced viral titre (pfu/mL) corresponding to the inhibition of HA. A series of 10-fold dilutions of samples was added to a monolayer of MDCK cells for 1 h at 37°C. Overlay medium (3 mL) was then supplemented with 1% agarose, and the compound was added. Following incubation for 48 h at 37°C, the cells were fixed with 10% formalin for 1 h. Following removal of the formalin, the cells were stained with Crystal Violet and plaques were visualized. Visible plaques were counted and then the concentration of pfu/mL was determined. The number of plaques was obtained from at least three independent experiments.

Results

Antiviral activity of BPR2-D2

Figure 1 displays the chemical structure of BPR2-D2, 8-benzoyl-4-methyl-9-phenyl-furo[2,3-h]chromen-2-one. BPR2-D2 was obtained from the hit identified from screening 20000 compounds by neutralization assay. A strain that is widely used in laboratories, influenza A/WSN/33 at an MOI of 0.001, was employed to infect MDCK cells and induce a CPE. Compounds that inhibited at least 50% of the CPE at a concentration of 1 μ M were selected for further investigation of their antiviral activities. A plaque reduction assay was performed to verify the antiviral activity (Figure 2). An MOI 5×10^{-4} of viruses was used to infect cells and a different concentration of compounds was present in the medium and the agarose throughout the viral adsorption (1 h) and 48 h post-infection. GS4071, an active form of oseltamivir, was used as a positive control in the plaque inhibition assay, and its EC_{50} was 0.091 ± 0.006 μ M. BPR2-D2 was found to inhibit 100% of the virus-induced CPE at a concentration of 0.05 μ M. The EC_{50} of BPR2-D2 was 0.043 ± 0.001 μ M. There is no cytotoxicity observed by MTT assay with a concentration of BPR2-D2 of up to 25 μ M. The cytotoxicity of BPR2-D2 was also examined by Crystal Violet staining of the attached live cells. Results of the analysis indicated that the CC_{50} (i.e. the concentration of the compound that causes 50% cytotoxicity) exceeded 25 μ M. The selective index (CC_{50}/EC_{50}) is therefore >581 ($25/0.043$).

Inhibition of oseltamivir-resistant viruses

The antiviral efficacy of BPR2-D2 was then tested against oseltamivir-resistant viruses. The recent seasonal influenza A H1N1 viruses were found to be resistant to oseltamivir.^{18,25,26} Accordingly, several clinical isolates of the H1N1 subtypes were tested to determine their susceptibilities to GS4071.

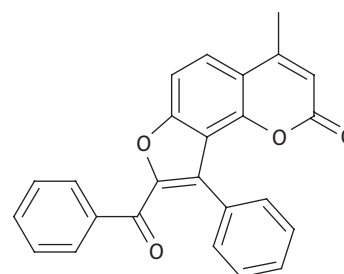


Figure 1. Chemical structure of BPR2-D2.

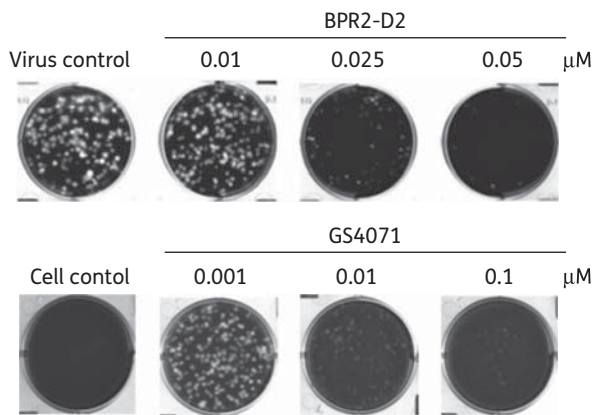


Figure 2. Effect of BPR2-D2 on influenza virus replication. MDCK cells were infected by influenza A/WSN/33 (H1N1) at an MOI of 5×10^{-4} . Various concentrations of BPR2-D2 (top panel) or GS4071 (bottom panel) were added to each well. At 48 h post-infection, cells were fixed and stained with Crystal Violet. Three independent experiments were performed and the results are presented here.

Table 1. Drug susceptibility of recent influenza H1N1 isolates to BPR2-D2

Virus	EC ₅₀ (μM) ^a	
	BPR2-D2	GS4071
Influenza A/TW/058/09 (H1N1)	0.022 ± 0.000	>25
Influenza A/TW/066/09 (H1N1)	0.040 ± 0.005	>25
Influenza A/TW/147/09 (H1N1)	0.021 ± 0.001	>25

Data are presented as means ± SD from at least two independent experiments.

^aNeutralization test was performed.

Influenza A/058/09, A/066/09 and A/147/09 with resistant marker Y at position 274 of the neuraminidase (NA) protein were all resistant to GS4071; however, BPR2-D2 inhibited those strains with EC₅₀ values from 0.021 to 0.040 μM (Table 1).

Time-of-addition experiments in influenza A virus-infected cells

To elucidate further the antiviral mechanism of BPR2-D2, a time-of-addition assay was conducted in influenza A/WSN/33-infected MDCK cells. Figure 3(a) plots the time of the addition of BPR2-D2 during the course of the viral infection. BPR2-D2 inhibited 98%–100% of the production of the progeny virus when it was added before 4 h post-infection (Figure 3b). A significant drop in the antiviral activity of the compound to 82% efficacy was observed when the compound was added 4 h post-infection, and the efficacy dropped further to 55% at 6 h and to 38% at 8 h post-infection. The results of the time-of-addition experiment in influenza A virus-infected cells suggest the potential involvement of BPR2-D2 in the early hours of viral replication.

Effects of BPR2-D2 on viral RNA synthesis that is driven by influenza A viral ribonucleoprotein (RNP) complex in transfected cells

Expression plasmids for PB2, PB1, PA and NP were transfected together with a plasmid that allowed expression of a pseudoviral reporter RNA (pPolI-CAT-RT plasmid) into 293 cells.³¹ The levels of CAT protein were measured by ELISA. The results in Figure 4(a) demonstrate that BPR2-D2 reduced the levels of CAT protein in a dosage-dependent manner, but had no significant effect when 25 μM (GS4071) or the solvent DMSO (0.25%) was present in the cell medium. The inhibition effect was unlikely to have been caused by an effect of the transfection efficiency because NP expression levels in all reactions were almost the same. To measure the levels of the transcription/replication of viral RNA, primer extension assays were performed using isotope-labelled primers that can differentiate among pseudoviral vRNA, cRNA and mRNA. The synthesis of viral mRNA and cRNA was strongly inhibited by BPR2-D2 but not by GS4071 or DMSO (Figure 4b). This study also attempted to determine whether BPR2-D2 affects any other expressed mRNA; a polymerase II-driven gene (GFP) was also co-transfected with influenza viral RNP genes into cells. In Figure 4(c), it can be seen that BPR2-D2 reduced the levels of CAT mRNA that was driven by viral RNP more significantly than those of GFP mRNA that was driven by cellular polymerase II. These results suggest that BPR2-D2 may target the viral RNP-associated function because it reduces the reporter gene expression when cells are transfected with viral RNP genes.

The effects of BPR2-D2 on viral RNA synthesis in influenza A virus-infected cells were also evaluated. MDCK cells were infected with A/WSN/33 at different viral titres (0.1–1 MOI) and treated or not with 0.025 μM BPR2-D2 (Figure 4d). At 8 h post-infection, RNAs were extracted from infected cells and a primer extension assay was conducted. BPR2-D2 was found to inhibit viral RNA synthesis (lanes 7, 10 and 13), whereas the control solvent (DMSO) did not (lanes 6, 9 and 12). Taken together, the results in Figure 4 suggest that BPR2-D2 reduced viral RNA levels, possibly by interfering with the influenza viral RNP function.

Selection of BPR2-D2-resistant viruses

Monitoring the generation of resistant viruses was performed in cell cultures. The amount of BPR2-D2 increased in each successive passage of the virus-infected cells. The susceptibility to BPR2-D2 in every passage was evaluated by performing a neutralization test. The EC₅₀ from passage 1 to 20 ranged from 0.019 to 0.46 μM, indicating that all of the progeny viruses were susceptible to BPR2-D2 (Table 2). However, the viruses that were resistant to GS4071 were produced in passage 8 when the culture medium contained GS4071. The results suggest that BPR2-D2-resistant viruses emerge less easily than do GS4071-resistant viruses.

Antiviral spectrum of BPR2-D2

The antiviral spectrum of BPR2-D2 was examined. Besides its activity against A/WSN/33, BPR2-D2 also exhibits antiviral activity against other strains of influenza A viruses and influenza B

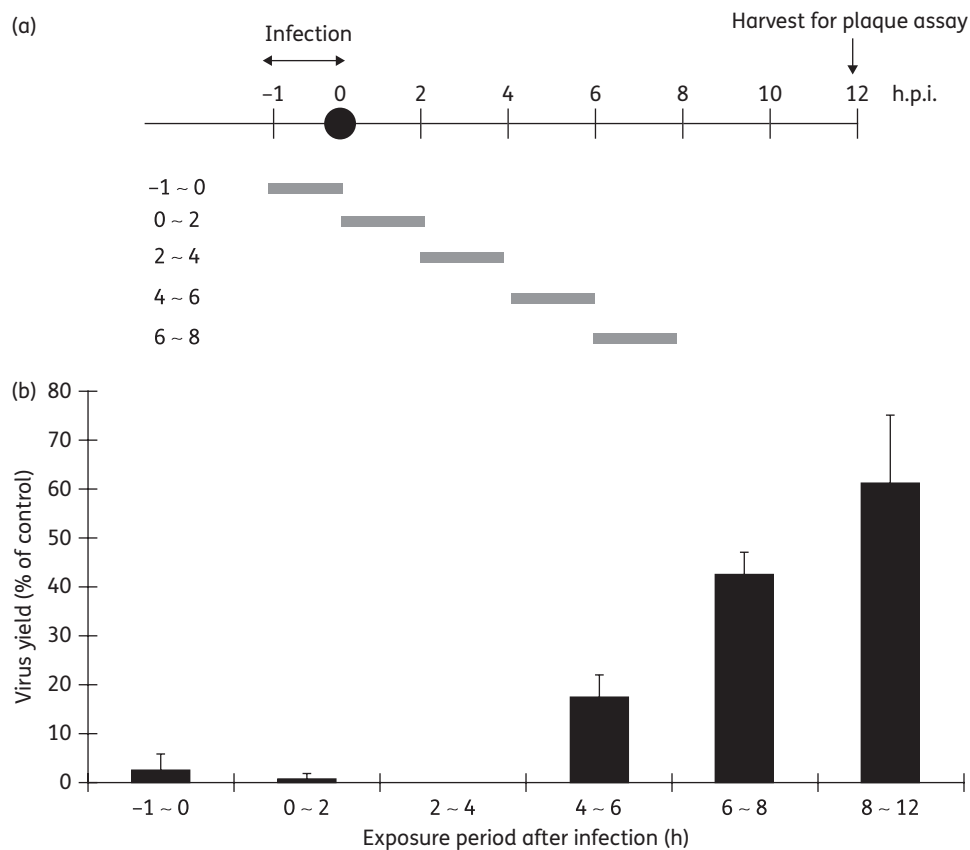


Figure 3. Time-of-addition assay. (a) Period of the presence of BPR2-D2 in the infection course and collection of supernatants for plaque assay to detect viral titres. (b) Viral yields after different intervals. MDCK cells were infected with influenza A/WSN/33 at an MOI of 1. After 1 h of adsorption, the infected cells were treated with 0.5 μ M BPR2-D2 at the indicated times. At 12 h post-infection (h.p.i.), the culture supernatant was collected and the viral yield was measured by plaque assay. Data are presented as means \pm SEM of results from triplicate experiments.

viruses. Several clinical isolates that are resistant to another antiviral, amantadine, are susceptible to BPR2-D2 (Table 3). Interestingly, BPR2-D2 also inhibited other RNA viruses, such as enterovirus 71, coxsackievirus B3 and HRV-2. No antiviral effect was found against HSV-2, a DNA virus.

The drug susceptibility of influenza A H5N1 to BPR2-D2 was also evaluated. Various concentrations of BPR2-D2 were added to the culture medium in which MDCK cells were infected by H5N1 viruses. Viral yields were measured by an HA test. The results in Figure 5 demonstrate that BPR2-D2 reduced the viral yield by >70% at a concentration of 0.02 μ M and by almost 100% at 0.1 μ M.

Discussion

Although two classes of antivirals are used in the clinical treatment of influenza viral infection, several works have demonstrated the emergence of viruses that are resistant to M2 ion channel inhibitors (amantadine and rimantadine) or NA inhibitors (oseltamivir and zanamivir).^{15,17,19–22,25,26} Accordingly, the urgency and significance of the development of novel antiviral agents that can inhibit such drug-resistant viruses are obvious. This study identified a compound, BPR2-D2, that inhibited oseltamivir-resistant influenza A viruses. Its chemical structure

(Figure 1) appears to differ markedly from that of oseltamivir. The inhibition by BPR2-D2 of the oseltamivir-resistant virus is understood by considering the difference between its antiviral mechanism and that of oseltamivir. BPR2-D2 inhibited reporter gene expression that was driven by influenza viral RNPs. This compound reduced viral-like cRNA and mRNA levels in cells that had been co-transfected with a set of expression vectors that encoded PB1, PB2, PA, NP and a pseudoviral reporter RNA. The possibility that BPR2-D2 targets viral RNP-associated function is further supported by the observation herein that viral RNA levels declined in virus-infected cells when BPR2-D2 was added. The vRNA level in infected cells also decreased upon the addition of BPR2-D2, but was unaffected in transfected cells. This discrepancy may be caused by the following. First, vRNA was overexpressed by cellular polymerase I through the polI promoter in the plasmid. BPR2-D2 seemed not to inhibit cellular polymerase I activity. Secondly, the viral RNP protein expression levels in infected cells fell because the viral mRNA levels were affected by BPR2-D2. In transfected cells, viral RNPs were overexpressed from cellular polymerase II-driven plasmids and were not affected by BPR2-D2.

BPR2-D2 targets the viral RNP-associated function, and viral RNP has been found to be highly conserved among various strains of influenza virus. Hence, this compound is expected to

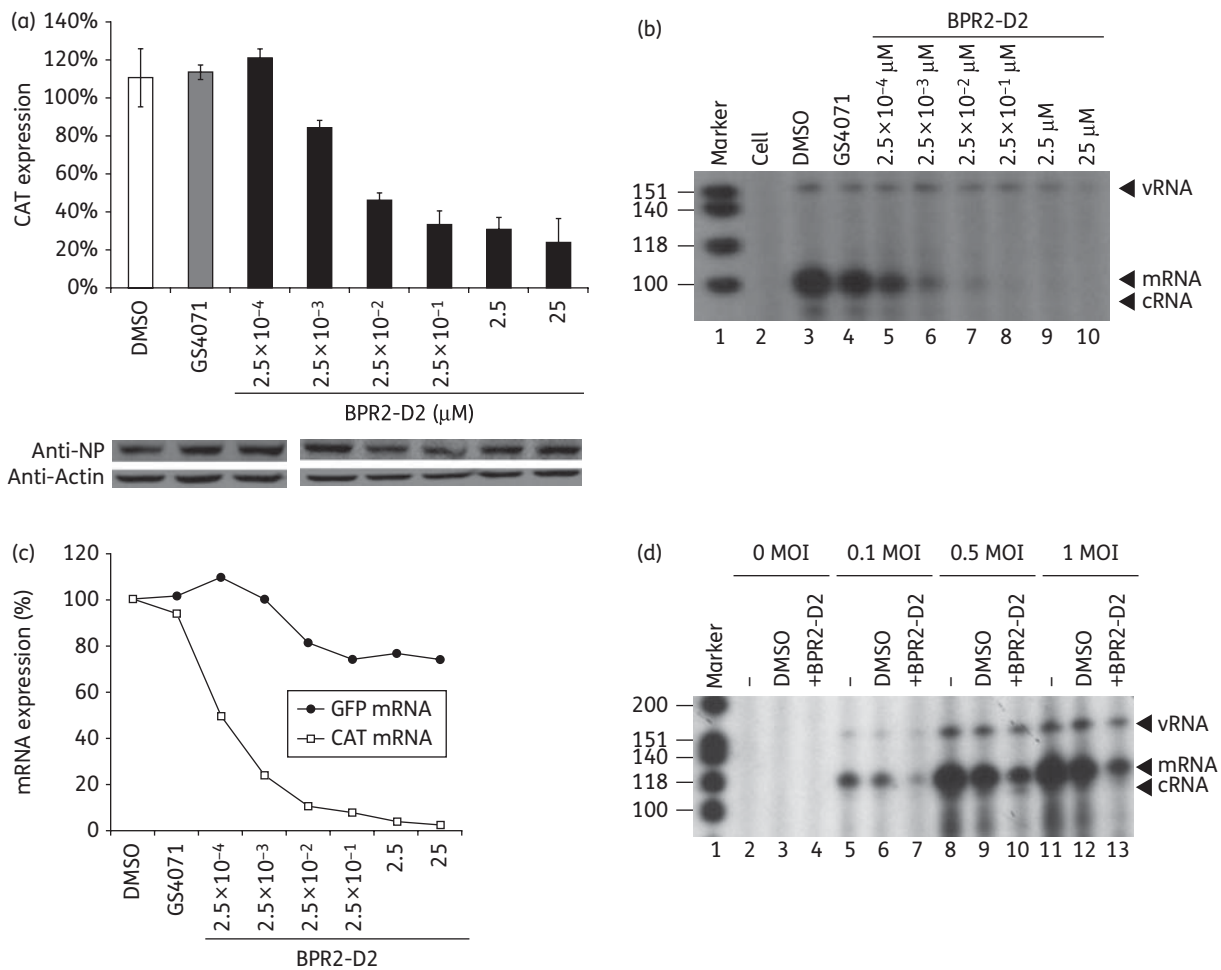


Figure 4. BPR2-D2 inhibited influenza A viral RNP activity. (a) 293T cells were transfected with expression plasmids for PB2, PB1, PA and NP together with a plasmid that allowed the expression of a pseudoviral reporter RNA (pPolI-CAT-RT plasmid). At 48 h post-transfection at 37°C, cell extracts were lysed and tested to determine CAT levels using a CAT ELISA kit. Various concentrations of BPR2-D2 were added to the cells during transfection. The levels of viral NP protein and cellular actin were monitored by western blot. (b) RNAs were extracted at 48 h post-transfection and subjected to primer extension assay with primers that can differentiate among vRNA, cRNA and mRNA. The reactions were then separated on 6% polyacrylamide gels that contained 7 M urea. (c) A polymerase II-driven gene (GFP) was co-transfected with influenza viral RNP genes into cells. GFP mRNA levels were detected by real-time RT-PCR. CAT mRNA levels were detected by primer extension assay and the band intensity was measured by Multi Gauge. The percentage mRNA expression was calculated in relation to the DMSO control. (d) MDCK cells were infected with various titres of influenza A/WSN/33 virus and viral RNAs were extracted at 8 h post-infection. A primer extension assay was performed to detect the levels of vRNA, cRNA and mRNA in virus-infected cells. BPR2-D2 (lanes 4, 7, 10 and 13) or DMSO (lanes 3, 6, 9 and 12) was added during viral adsorption. In lanes 2, 5, 8 and 11, nothing was added during viral adsorption.

exhibit a wide spectrum of antiviral activity. BPR2-D2 was demonstrated to inhibit various strains of influenza viruses effectively, including types A and B influenza viruses. Interestingly, BPR2-D2 also exhibited good antiviral activity against enterovirus 71, coxsackievirus B3 and HRV-2, which are three picornaviruses (Table 3).

BPR2-D2 displayed the ability to inhibit growth of both influenza viruses and picornaviruses, which exhibit various molecular mechanisms of viral RNA synthesis. Replication of picornaviral RNA that occurs via the RNA replication complex is associated with the membrane of virus-induced vesicles in the cytoplasm of infected cells.³² A virus-encoded RNA polymerase 3D is the major component of the RNA replication complex, which contains other viral and host proteins.³³⁻³⁶

The purified 3D protein from the infected lysate exhibits the polymerase activity.³⁷ For viral RNA synthesis of negative-strand influenza virus, RNA polymerases are composed of three subunits, PB1, PB2 and PA. The RNA polymerases (PB1, PB2 and PA) and NP form a viral RNP with genomic RNA. Although polymerase components of picornaviruses and influenza viruses differ significantly from each other, the picornavirus 3D and influenza virus PB1 subunit share typical common motifs, as has been shown by specific sequence alignment.³⁸ BPR2-D2 may inhibit the virus by interacting with the conserved structures in these two viral polymerases. No crystal structures of the full-length PB1 have yet been obtained, although some studies have demonstrated that a portion of the crystal structure of the PB1 region interacting with PB2 or PA was

resolved.^{39,40} According to the results of amino acid sequence alignments, there are four conserved motifs (motifs A, B, C and D) among RNA-dependent RNA polymerases (RdRps).³⁸ All motifs are located in the palm domain, based on the crystal structures of poliovirus, encephalomyocarditis virus and hepatitis C virus polymerases.⁴¹ Motif A is involved in magnesium coordination and possibly NTP selection. Although its function is unknown, motif B forms an α -helix that may be responsible for positioning the other motifs correctly. Motif C contains the highly conserved GDD motif found in RdRps. This GDD motif for poliovirus (SDD for influenza virus) is involved in magnesium coordination. Notably, motif D is the core palm structure.

Another possibility for its broad antiviral spectrum is that BPR2-D2 may target cellular proteins, which are involved in replicating both picornavirus and influenza viruses. A previous study identified heat shock protein 90 (Hsp90) as interacting with influenza virus PB2 protein and of being capable of stimulating vRNA synthesis.^{42,43} However, inhibition of Hsp90 functions by geldanamycin, a specific Hsp90 inhibitor, reduced poliovirus and coxsackievirus B and the replication ability of rhinoviruses.⁴⁴ BPR2-D2 may target such cellular proteins, which have an

inhibitory effect on the replication of both viruses. However, the antiviral mechanism of BPR2-D2 against these viruses must be further clarified.

In the present study, the exact molecular target of BPR2-D2 is unclear. An attempt was made to identify the genetic markers that are associated with the drug-targeted molecule by selecting resistant viruses. However, we failed to select a BPR2-D2-resistant virus after 20 passages. An oseltamivir-resistant virus appeared at passage 8 in the same culture system. Others have also reported the generation of oseltamivir resistance at early passages.⁴⁵ Although the failure of selection of resistant viruses impeded the investigation of the mechanism, it is a positive factor for BPR2-D2 as an antiviral agent because of the low probability of emergence of resistant viruses. The following may explain the difficulty in generation of BPR2-D2-resistant viruses. First, BPR2-D2 may target the highly conserved region of viral RNPs that is functionally essential for virus survival, such that its mutation is lethal or detrimentally affects the growth of the virus. Secondly, BPR2-D2 may target a cellular component that is functionally linked to

Table 2. Selection and drug susceptibility of passaged virus to BPR2-D2 and GS4071

Virus	EC ₅₀ (μ M)	
	BPR2-D2 ^a	GS4071 ^b
Wild-type	0.019 \pm 0.000	0.024 \pm 0.002
P3	0.037 \pm 0.004	0.391 \pm 0.301
P6	0.047 \pm 0.004	0.506 \pm 0.290
P8	0.037 \pm 0.004	>25
P20	0.460 \pm 0.110	—

Data are presented as means \pm SD from at least two independent experiments.

^aA/WSN/33 was serially passaged in the presence of BPR2-D2.

^bA/WSN/33 was serially passaged in the presence of GS4071.

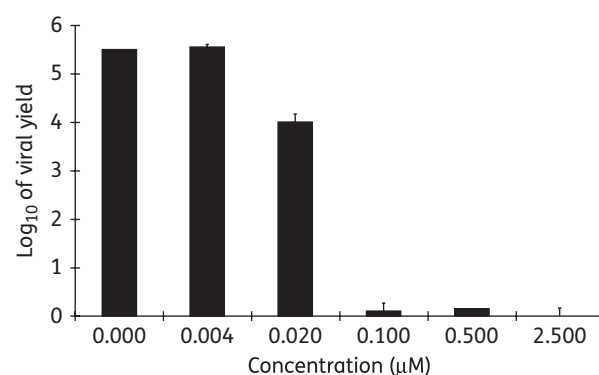


Figure 5. Reduction of influenza A H5N1 viral yields by BPR2-D2. MDCK cells were infected by a 0.001 MOI of H5N1 virus and the culture supernatants were collected at 48 h post-infection. Various concentrations of BPR2-D2 were added to cells when viral adsorption occurred. Viral yields were measured by HA assay.

Table 3. Antiviral activities of BPR2-D2, amantadine and zanamivir against various viruses

Virus	EC ₅₀ (μ M)			Assay ^a
	BPR2-D2	amantadine	zanamivir	
Influenza A/Udorn/72 (H3N2)	0.347 \pm 0.188	0.980 \pm 0.147	0.670 \pm 0.232	P
Influenza A/TW/83/05 (H3N2)	0.057 \pm 0.008	>25	0.918 \pm 0.026	P
Influenza A/TW/785/05 (H1N1)	0.052 \pm 0.001	0.382 \pm 0.238	1.056 \pm 0.982	P
Influenza B/TW/710/05	0.102 \pm 0.020	>25	0.030 \pm 0.019	N
Influenza B/TW/70325/05	0.067 \pm 0.002	>25	0.020 \pm 0.010	N
Influenza B/TW/99/07	0.089 \pm 0.014	>25	0.099 \pm 0.009	N
Enterovirus 71 (EV71/Tainan/4643/98)	0.002 \pm 0.000	>25	>25	N
Coxsackievirus B3	0.005 \pm 0.001	>25	>25	N
HRV-2	0.012 \pm 0.001	>25	>25	N
HSV-2	>25	>25	>25	N

Data are presented as means \pm SD from at least two independent experiments.

^aP, plaque reduction assay; N, neutralization test.

several regions of viral RNPs such that a single or a few mutations in RNPs do not render this virus resistant. Moreover, BPR2-D2 inhibited RNA viruses other than orthomyxoviruses, which may hint that BPR2-D2 targets a viral/cellular factor that is common in these RNA viruses.

In summary, a novel compound, BPR2-D2 was identified as a potent inhibitor against influenza virus using *in vitro* assay. It exhibited a broad range of antiviral activity against various strains of influenza viruses, including the H5N1 influenza A virus. This compound may target viral RNPs that are responsible for viral RNA synthesis. Targeting molecules other than NA and M2 allows BPR2-D2 to inhibit oseltamivir- and amantadine-resistant viruses efficiently.

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Transparency declarations

None to declare.

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