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Suramin inhibits EV71 infection

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ABSTRACT

Enterovirus-71 (EV71) is one of the major causative reagents for hand-foot-and-mouth disease. In particular, EV71 causes severe central nervous system infections and leads to numerous dead cases. Although several inactivated whole-virus vaccines have entered in clinical trials, no antiviral agent has been provided for clinical therapy. In the present work, we screened our compound library and identified that suramin, which has been clinically used to treat variable diseases, could inhibit EV71 proliferation with an IC_{50} value of 40 μM. We further revealed that suramin could block the attachment of EV71 to host cells to regulate the early stage of EV71 infection, as well as affected other steps of EV71 life cycle. Our results are helpful to understand the mechanism for EV71 life cycle and provide a potential for the usage of an approved drug, suramin, as the antiviral against EV71 infection.

1. Introduction

Enterovirus and coxsackievirus (CV) are the major causative agents of hand-foot-and-mouth disease (HFMD) in Pacific-Asia region. Among those, Enterovirus 71 (EV71) and coxsackeivirus A16 (CVA16) are the most prominent at present (Gong et al., 2011; Zhang et al., 2010). These agents have caused over 1,000,000 infections and 900 deaths in 2010 (Chen et al., 2013a). In particular, young children are more susceptible to EV71 infection (Chen et al., 2001; Wang et al., 2010), which causes severe aseptic meningitis, encephalitis, myocarditis, acute flaccid paralysis, and pulmonary edema, which lead to high fatality rates (Cui et al., 2010; Gautam, 2011; Yang et al., 2009; Zhang et al., 2010).

EV71 is a member of genus *Enterovirus* within family *Picornaviridae*. Its genome contains a single-stranded positive-sense polyadenylated RNA (King et al., 2000; McMinn, 2002). The single open-reading-frame translation is initiated by ribosomes that use an internal ribosomal-entry site located in the 5′-untranslated region (5′-UTR) region of the viral genome, which gives rise to a polyprotein of approximately 250 kDa (Gamarnik and Andino, 1998; Hillman et al., 2000; Pelletier and Sonenberg, 1988). The polyprotein encoded by the viral genome is initially processed into one structural (P1) and two non-structural (P2 and P3) regions. P1 region is further proteolytically processed into VP1–VP4 to form the viral capsid, while P2 and P3 are processed into replicase proteins. The most striking variation is that EV71 can infect central nervous system and causes severe infection and dead cases, but CV cannot. This clinical variation is believed to be related to the different functional receptors mediating the infections of EV71 and CV. Up to date, two extracellular membrane proteins, human P-selectin glycoprotein ligand-1 (PSGL-1) and scavenger receptor B2 (SCARB2), as well as heparan sulfate on the cell surface, are identified to be the functional receptors for EV71 infection (Nishimura et al., 2009; Tan et al., 2013; Yamayoshi et al., 2009). And another result suggests that binding of EV71 to human annexin II on the cell surface enhanced viral entry and infectivity, especially at a low infective dose (Yang et al., 2011).

Although vaccines have entered in clinical trials and the vaccines showed high efficacy and sustained immunogenicity (Zhu et al., 2013), no drug has been provided for clinical treatment of EV71 infection. Several lines of preclinical research identified a number of reagents can inhibit EV71 proliferation (Shang et al., 2013). For example, pleconaril and BPR0Z can block the multiplication of EV71 targeting virus entry (Shia et al., 2002; Zhang et al., 2012); Rupintrivir inhibits the function of EV71 proteases $3C^{pro}$ (Dragovich et al., 1999; Zhang et al., 2010); the peptide LVLQTM is working at EV71 $2A^{pro}$ (Falah et al., 2012); DTriP-22 and aurintricarboxylic acid targets at polymerase $3D^{pol}$ (Chen et al., 2009; Urbinati et al., 2008). However, none of them has been able to be advanced to clinical therapeutics. In the present work, we screened our compound library containing a number of proved drugs and identified that suramin, which is clinically used to treat variable diseases, can inhibit EV71 proliferation. We further revealed that suramin directly blocked the attachment of EV71 to host cells and mainly regulated the entry step of EV71 life cycle.

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2. Materials and methods

2.1. Viruses, cell lines, antibodies and inhibitor

Human rhabdomyosarcoma (RD) cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO) at 37 °C in 5% CO_2 humidified incubator.

EV71 virus strain SK-EV006 with GFP (EV71-GFP) was provided by Prof. Satoshi Koike as a kind gift for the initial phenotype screening. EV71 virus strain BrCr was kindly provided by Prof. Zheng Yin from Nankai University.

A single round pseudotype EV71 reporter virus system (EV71(FY)-Luc pseudotype virus system) containing plasmids of pcDNA6-FY-capsid and pEV71-Luc-replicon was kindly supplied by Wenhui Li from National Institute of Biological Sciences, Beijing (Chen et al., 2013b).

Antibodies used for Western blot (WB) analysis were purchased from Abcam (Mouse mAb to Enterovirus 71 VP1, 10F0), ProMab (GADPH), Southern Biotech (HRP-Conjugated Goat Anti-Mouse IgG(H + L)), and CoWin Bioscience (HRP-Conjugated Goat Anti-Rabbit IgG).

Suramin was purchased from Sigma–Aldrich and was freshly dissolved in a concentration of 100 mM in dimethyl sulfoxide (DMSO) as a stock, and added to culture medium at final concentrations at a series of dilution to virus infection and kept in the medium throughout the experiment, or added for different intervals as described in the text or figure legends.

2.2. Virus titration

Virus titers were determined by endpoint dilution assays (EPDA) using focus-forming units (ffu) as the read-out (Zhong et al., 2006). Briefly, the measurement was performed by seeding 1×10^4 RD cells per well in 96-well microtiter plates. After overnight culture, EV71 was serially diluted in 10-fold with DMEM containing 10% FBS (10^{-1} to 10^{-8} fold dilutions) and added to RD cell. The plates were then incubated at 37 °C in 5% CO_2. CPE or GFP expression level were observed under the microscope or monitored using an epifluorescence microscope to visualize the expression of GFP after 3–4 days. Determination of virus titer, the 50% tissue culture infectious dose ($TCID_{50}$) was performed using by EPDA.

2.3. Preparation of EV71 pseudotype virus

The EV71 pseudotype virus (EV71(FY)-Luc) was produced by using the method developed as previously described (Chen et al., 2013b). Briefly, the plasmid pEV71-Luc-replicon was linearized through digestion with SalI restriction enzyme, and was used as a template for RNA transcription. The EV71 replicon RNA transcripts were prepared in vitro by using the Ambion MEGAscript Kits. The pcDNA6-FY-capsid plasmid was transfected into HEK-293T cells at 60–80% confluence. After 24 hpi, EV71 replicon RNA was then transfected using Lipofectamine 2000 (Invitrogen). EV71 pseudotype virus was harvested at 24 h post-RNA transfection with two rounds of freeze–thaw cycle. To quantify the EV71 pseudotype virus, the virus stocks were diluted at a gradient of 1:10, and incubated with RD cells for 24 h at 37 °C. The cells were then harvested and the luminescence was detected as manufacturer's protocol of Bright-Glo Luciferase Assay System (Promega). For the inhibition assay, the final concentration of EV71 pseudotype virus was diluted to the numbers of relative luminescence units (RLU) as 1,000,000 RLU per well in a 96-well plate.

2.4. Western blot analysis

Cells were lysed in a buffer containing (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM $NaVO_4$, 10 mM NaF, and protease inhibitors), and the protein concentration in the lysates was determined by a spectrophotometer. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to NC membrane (Millipore). NC Membranes were blocked for 4 h with 5% nonfat dry milk solution in Tris-buffered saline, then blotted with specific primary antibodies, and followed by incubation with secondary antibodies conjugated with horseradish peroxidase. Proteins were visualized by chemiluminescence and clarity Western ECL substrate (BIO-RAD).

2.5. Quantitative RT-PCR (qRT-PCR) based infection assay

The antiviral activities of compounds were determined by using EV71 virus and RD cell in a qRT-PCR based assay. Briefly, 100,000 RD cells were seeded in each well of 24-well tissue culture plates and were allowed to attach in complete culture medium overnight. The culture medium was then replaced with medium containing EV71 virus at a MOI of 1 and serially diluted compounds in the presence of 10% FBS and 0.5% DMSO. After the cells were treated for 24 h, total cellular RNA was isolated by TRIZOL reagent by using standard protocols. Quantitative RT-PCR assay (for primer sequences, GADPH, forward primer 5′-CCC ACT CCT CCA CCT TTG ACG-3′ and reverse primer 5′-CAC CAC CCT GTT GCT GTA GCCA-3′, EV71 5′UTR forward primer 5′-TGA ATG CGG CTA ATC CCA ACT-3′ and reverse primer 5′-AAG AAA CAC GGA CAC CCA AAG-3′) was performed by QuantiTect SYBR Green RT-PCR kit (QIAGEN, Valencia, CA) as manufacturer protocol. EV71 and GAPDH transcript levels were determined by ΔΔCT method (Schmittgen and Livak, 2008). The percentage of inhibition was calculated as follows: % inhibition = (1 − (average of compound-treated cells)/(average of control cells)) * 100. IC_{50} is the concentration of compound at which the EV71 RNA level in the RD cells is reduced by 50%. To monitor the cytotoxic effect of the compounds, the viability of RD cells following 24 h of compound treatment was determined in 96-well tissue culture plates using cell proliferation reagent WST-1 (Roche). Each data point represents the average of three replicates in cell culture. The percentage of cytotoxicity was calculated as follows: % cytotoxicity = (1 − (average of compound-treated cells)/(average of control cells)) * 100. Each data point represents the average of three replicates in cell culture. The values of IC_{50} and cytotoxicity is plotted by the GraphPad Prism 5 software.

2.6. Virus binding assay

Virus binding assay was performed as a previously reported protocol with little modification (Cordey et al., 2012). Briefly, RD were seeded at 1×10^5 RD cells/well, respectively, in 24-well plates. The following day, culture medium was removed and cells were washed once with cold phosphate buffer saline (PBS). 500 μl of binding buffer (PBS containing 1% BSA and 0.1% sodium azide) were added to cell on ice for 10 min and the supernatant was subsequently removed from cells. The 10^8 $TCID_{50}$/ml of EV71 stocks (BrCr) were prepared as mentioned. 500 μl of EV71 virus diluted by DMEM complete medium (dilution fold = 1:10, or 1:20) with 0.5% DMSO or additionally suramin were added. After 1 h of incubation on ice, unbound virus was removed by three wash steps with 500 μl PBS and then cells were lysed in the wells with 500 μl TRIZOL reagent. Viral RNA was extracted and detected by qRT-PCR. The virus binding assays were performed systematically in duplicate in two individual experiments for each condition.

3. Results

3.1. Suramin suppress the multiplication of EV71

Phenotype screening of our compound collection by using rhabdomyosarcoma (RD) cells infected with EV71 strain SK-EV006 with GFP (Yamayoshi et al., 2009), which expressed GFP upon infection. We tested 450 compounds in our library and found 13 compounds can inhibit EV71 proliferation by checking the expression level of GFP in our first round screening. Among them, we found that suramin can clearly inhibit the proliferation of EV71 (Fig. 1A and B). This inhibition was dose-dependent and the expression of EV71 VP1, which is the major component of EV71 capsid, was obviously attenuated by the treatment of suramin. In a contrast, the expression of endogenous glyceraldehyde-3-phosphate dehydrogenase (GADPH) was not affected, being indicative that the inhibition of suramin to EV71 replication was specific (Fig. 1C).

By using a qRT-PCR assay, we further determined that suramin inhibited EV71 RNA replication with an IC_{50} value of approximately 40 μM (Fig. 1D). After treated RD cells by suramin at variable concentrations with 24 h, no significant cytotoxicity was observed with suramin at concentrations below 100 μM as demonstrated by WST-1 based assay, indicating again that the inhibition of EV71 multiplication was specific (Fig. 1E).

Because suramin is known to be used to treat several diseases, including human sleeping sickness caused by trypanosomes, onchocerciasis, and prostate cancer (Ahles et al., 2004; Anderson and Fuglsang, 1978; Darsaud et al., 2004), our interest in identifying anti-EV71 agents, prompted us to initiate further investigations to study the inhibitory mechanism of suramin in EV71 life cycle.

3.2. Suramin affected both the early and the late stage of EV71 proliferation

Suramin was reported to inhibit different viruses at variable stage of viral life cycle (Jiao et al., 2013; Mastrangelo et al., 2012; Rusnati and Urbinati, 2009; Tsiquaye and Zuckerman, 1985; Zhou et al., 2013b). We next checked which step in EV71 lifecycle was inhibited by suramin by analyzing growth curves of EV71 during its infection.

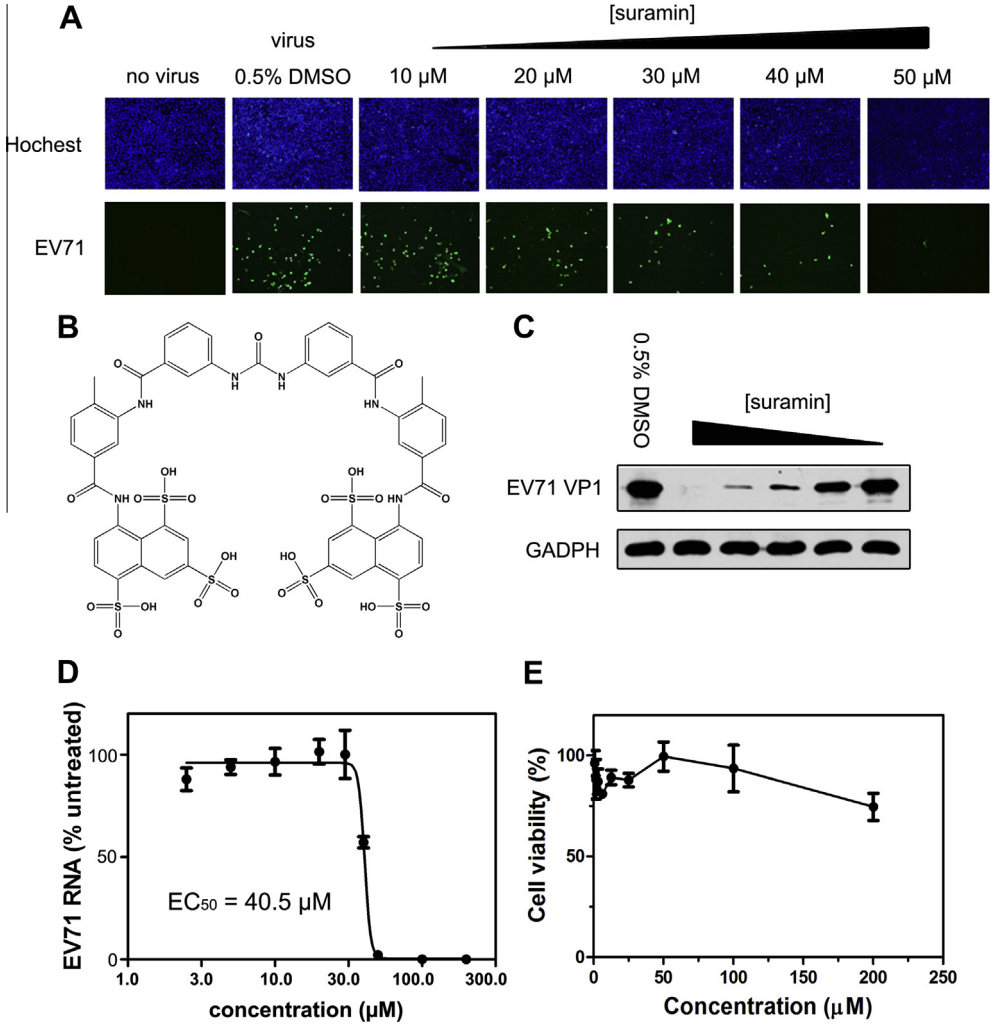

Fig. 1. Concentration-dependent reduction of EV71 proliferation following treatment with suramin. (A) Phenotype screening identified that suramin suppressed EV71 proliferation. RD cells were infected by EV71-GFP virus at a MOI of 0.5, with or without treatment by various concentrations of suramin (10–50 μM) for 24 h. Hochest was used to visualize the nuclei (top panel), marking up the host cells, and EGFP was used to monitor the virus growth (bottom panel). (B) Chemical structures of suramin. (C) Dose-dependent reduction of EV71 VP1 expression. The concentration of suramin in lane 2–6 were 50, 40, 30, 20, and 10 μM, respectively. (D) The inhibitory effect of suramin to EV71 RNA measured by qRT-PCR. RD cells were infected by EV71 virus at a MOI of 1, with or without treatment at various concentrations of suramin (2.5–200 μM) for 24 h. The levels of EV71 RNA were quantified by qRT-PCR and the data was expressed as the percentage of the level of EV71 RNA for the untreated control cells. Each data point represents the average of three replicates. (E) To monitor cytotoxic effect, the viability of RD following compound treatment (0.78–200 μM) of 24 h was determined using WST-1 based assay and compared to that of untreated control cells. Each data point represents the average for three replicates in cell culture.

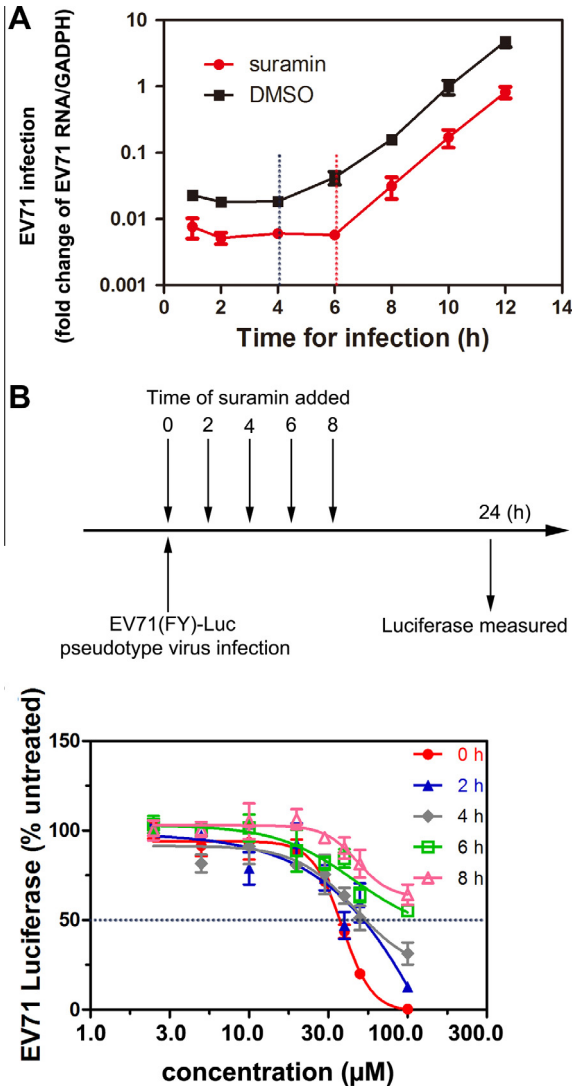

Fig. 2. The growth curves of EV71 in RD cells in absence or presence of suramin. (A) EV71 was incubated with RD cells in the absence or presence of 50 mM suramin. The cellular EV71 RNA was quantified by qRT-PCR at a MOI of 10 at 1, 2, 4, 6, 8,10, 12 hpi. The data represented the fold change of EV71 RNA relative to the GADPH endogenous gene, and each data was the means of three independent experiments. Error bars represent the SEM. (B) Inhibition of EV71(FY)-Luc pseudotype viral infection of RD cells by various concentrations of suramin (2.5 μM–100 μM) added at different time. EV71 pseudotype virus infected RD cells in a 96-well plate in a total volume of 100 μl in triplicates, and the suramin was added meanwhile (0 hpi) or after viral infection as indicated on the figure (2, 4, 6, and 8 hpi). The luciferase levels were quantified by measuring the firefly luciferase activity in relative luminescence units (RLU) at 24 hpi.

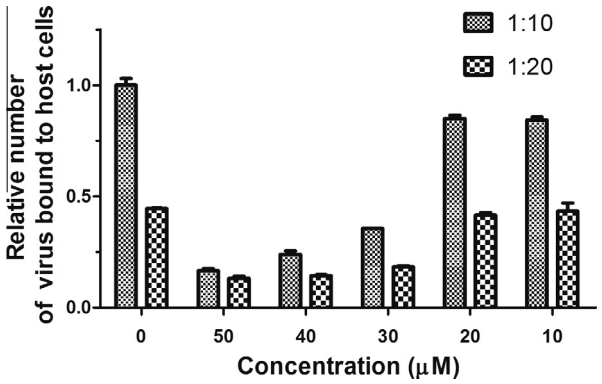

Fig. 3. Suramin blocked the attachment of EV71 virion to host cell. RD cells were used to compare the cell-binding capacity of EV71 under the treatment with suramin at different concentration (50 μM, 40 μM, 30 μM, 20 μM and 10 μM, respectively). Two conditions were assessed: 10-fold diluted (1:10) and 20-fold diluted (1:20) of standardized viral stocks (10^8 $TCID_{50}$/ml). Quantification of bound virus was measured by qRT-PCR and expressed relative to 0.5% DMSO (1:10 condition). Each data was the average of three replicates. Error bars represent the SEM.

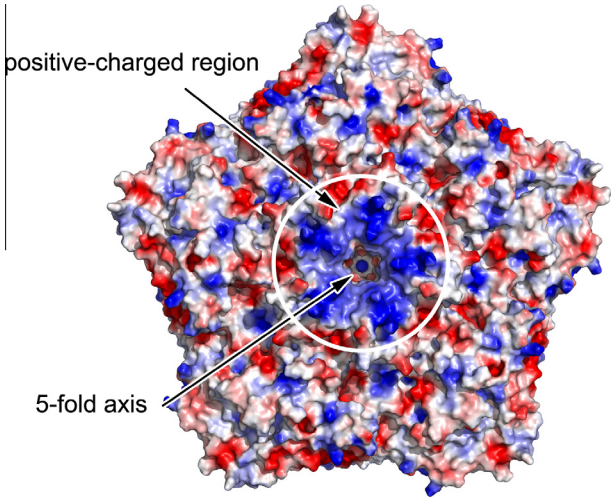

Fig. 4. The potential acting site of suramin on the molecular surface of EV71 virion. The electrostatic potential surface of five icosahedral asymmetric units (PDB code: 4AED). Negatively charged surfaces are shown in red, whereas positively charged areas are shown in blue. Fivefold axis and the positive-charged region are indicated by a black arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We infected RD cells with EV71 at a MOI of 10 in the absence or presence of suramin at a concentration of 50 μM, and detected the growth curves of EV71 virus by quantifying the viral RNA through qRT-PCR method at 1, 2, 4, 6, 8, 10, and 12 h post infection (hpi) (Fig. 2A). The result showed that the amount of cellular EV71 RNA after 1 hpi in the absence of suramin was reduced over 70% than that in the presence of suramin at a concentration of 50 μM, and the equal change was detected at 2 or 4 hpi. This result indicated that treatment of suramin impacted the early stage of EV71 life cycle.

We also found that the replication of viral RNA in the absence of suramin began at 4 hpi, but it was delayed in the presence of suramin. However, the slopes of the growth curves either in the absence or in the presence of suramin were the same. This result suggested that except the effect on early stage of EV71 life cycle, suramin might have an additional effect on the uncoating or protein translation process of EV71 lifecycle before genome replication.

To further dissect the inhibition mechanism of suramin, we used a single round pseudotype EV71 reporter virus system that allowed us to study EV71 infection at the entry or replication stages, which excluded the effect of virus reinfection. The single round entry virus was produced by sequential transfections of the pcDNA6-FY-capsid and EV71 replicon RNA that was transcribed from the plasmid pEV71-Luc replicon containing a firefly luciferase reporter gene. RD cells were infected with EV71(FY)-Luc pseudotype virus and treated with suramin at various concentrations of suramin (2.5–100 μM) at 0, 2, 4, 6, 8 hpi. The results showed that the inhibition of EV71 by suramin presented a clear dependence to the providing time (Fig. 2B). The treatment by suramin at the time of infection (0 hpi) displayed the best antiviral effects, and the intracellular luciferase level was reduced by above 99% and 80% when

treated by 100 and 50 μM suramin, respectively. The inhibition was clearly attenuated by the delay of the addition of suramin to 2 and 4 hpi. Moreover, the adding of suramin at 6 and 8 hpi showed no significant reduction of intracellular luciferase level at 50 μM.

Taken together, the growth curves indicated that the impact of suramin began at the early stage of EV71 life cycle, but had additional effect on the later stage.

3.3. Suramin blocked the attachment of EV71 virion to host cell

The entry stage of EV71 infection can be further subdivided into two steps: (1) the attachment of EV71 virion to host cell mediated by functional receptors and (2) uncoating to release viral genome into host cell. To further define the inhibitory mechanism of suramin on EV71 entry, we analyzed the binding affinity of EV71 virion to host cell with the treatment of suramin.

By using a previously reported virus binding assay (Cordey et al., 2012), we demonstrated that suramin clearly blocked the attachment of EV71 virion to RD cell (Fig. 3). We incubated RD cells with EV71 virus with the titer of 10-fold diluted (1:10) and 20-fold diluted (1:20) standardized viral stocks in the absence or presence of suramin. In the presence of 50 μM suramin, the virus bound to host cell in two conditions decreased to less than 20% of that seen in the absence of suramin. When the concentration of suramin dropped to 20 μM or 10 μM, the bindings to host cell were both rescued. These results revealed that suramin directly blocked the attachment of EV71 virion to host cell.

4. Discussion

Suramin is a symmetrical polysulfonated naphthylamine derivative of urea and has been widely used since the 1920s for the prophylactic treatment of human trypanosomiasis in Africa and for the treatment of the early stages of the disease before the nervous system is involved (Voogd et al., 1993). A variety of trypanosome enzymes have been reported to be inhibited by suramin in the range of 1–100 μM. These include *Trypanosoma cruzi* thymidilate kinase (Al Chalabi and Gutteridge, 1977), *T.b.* brucei glycerol-3-phosphate oxidase (Fairlamb and Bowman, 1977), and a number of enzymes associated with the trypanosome's plasma membrane (3′-nucleotidase and protein kinase), its flagellar pocket membrane (acid phosphatase, and acid pyrophosphatase), and its digestive apparatus such as the lysosomes (phospholipase Al) (Voogd et al., 1993). In the clinical usage of suramin, more results revealed that suramin could inhibit HIV-1 by functioning as a reverse transcriptase inhibitor and suramin was subsequently entered into clinical trials as a treatment for AIDS (Yarchoan and Broder, 1987). Moreover, suramin was also reported to inhibit hepatitis B virus targeting at its polymerase or be used in anticancer therapeutics (La Rocca et al., 1990). Very interestingly, suramin was also reported to impair the formation of ribonucleoprotein complex of Severe Fever with Thrombocytopenia Syndrome virus (SFTSV) by blocking the interaction of viral genome with the positively charged region of viral nucleocapsid protein (Jiao et al., 2013; Zhou et al., 2013a,b).

Although the working targets of suramin in different diseases are not consistent, the mechanism of action of suramin is believed to be binding with the positively charged regions on the molecular surface of target proteins or target cells through its negatively charged group, most likely the naphthalene trisulfonic acid groups. The crystal structure of matured EV71 particles (Plevka et al., 2012; Wang et al., 2012) revealed that a fully positively charged and most solvent-exposed region is located at the surface of virus particle close to the vertex of fivefold axis (Fig. 4). Although the precise positions for EV71 receptor binding is still not clear, several lines of evidence revealed that this positive-charged region plays important role in receptor binding. Nishimur et al. reported that H-I loop of VP1 in this region plays an essential role for EV71 recognition to one of its functional receptor, PSGL-1, and demonstrated that the substitution on VP1-K242 and K244, which are located at VP1 H-I loop, significantly attenuated virus binding to PSGL-1 (Nishimura et al., 2013). They also indicated that the residue VP1 E145 modulates the orientation of VP1 K244 and thus regulates exposure of the positively charged lysine side chain for receptor binding (Nishimura et al., 2013). Moreover, Tan et al. showed that EV71 binds to heparan sulfate on the cell surface, and suggested that heparan sulfate may bind to positively charged amino acids (including VP1-K242 and K244, as well as VP1-R161), that form a cluster around the fivefold symmetry axis (Tan et al., 2013). These findings suggested that the lysine residues at VP1-242 and 244 positions play essentials role for EV71 virus to bind with variable receptors. In a very recent result, Lee et al. reported an anti-EV71 neutralizing antibody, MA28-7, epitopes at the fivefold vertex covering VP1 H-I loop (Lee et al., 2013), agreeing with the critical role of the positive-charged region at the surface of EV71 virion in the entry step. These molecular features suggest that suramin may bind to the receptor-binding site located the molecular surface of EV71 virion and block the interaction of EV71 with its functional receptors. Further biophysical or crystallographic investigations are necessary to identify the precise action site and clarify the molecular mechanism of suramin to inhibit the proliferation of EV71. Moreover, the growth curves indicated that suramin can also block the late stage of EV71 infection in host cell, e.g. uncoating, protein translation, and replication and transcription. It is necessary to further clarify the inhibition mechanism of suramin impact on other stage of EV71 infection.

The work we described here highlights a new function of suramin as an anti-EV71 reagent through inhibiting multiple stages of EV71 lifecycle. Our results are significant for understanding of virus–host interactions and provide an potential for the usage of suramin as a clinical therapeutics against EV71 infection.

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