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Rupintrivir is a promising candidate for treating severe cases of enterovirus-71 infection: Evaluation of antiviral efficacy in a murine infection model

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ABSTRACT

Enterovirus-71 (EV71) infections can cause life-threatening diseases with neurological symptoms. Currently, no direct targeting antivirals are available to combat severe EV71 infection. Rupintrivir (AG7088) is a compound originally designed for Rhinovirus 3C protease. Previous computational analyses by us and crystallography studies by others suggested that rupintrivir is also a high affinity inhibitor to EV71 3C. Thus, we aimed to further evaluate its anti-EV71 activity in vivo at clinically acceptable doses. It was observed that administration of rupintrivir in suckling mice largely protected them from limb paralysis and dramatically improved survival (38.5% DMSO vs. 90.9% at 0.1 mg/kg, $p = 0.006$). Histological, immunohistochemical and quantitative RT-PCR analyses confirmed that rupintrivir profoundly alleviated virus induced necrotizing myositis, suppressed viral RNA and blocked EV71 VP1 expression in various tissues. In conclusion, we established that rupintrivir can strongly contain the spread of EV71 infection in vivo at a clinically acceptable dose (as low as 0.1 mg/kg). As its safety has been fully tested in previous clinical trials, rupintrivir is suitable for immediate evaluation of potential benefits in EV71-infected individuals with life-threatening neurological symptoms.

1. Introduction

Enterovirus 71 (EV71), a member of picornaviridae, is the etiology that has caused several epidemics of hand-foot-and-mouse disease (HFMD) in recent years. Most EV71 infections are self-limited, only causing mild symptoms such as rashes in children. However, severe cases with neurological manifestations that can rapidly deteriorate to cardiopulmonary failure have been frequently observed in recent outbreaks (Ho et al., 1999).

Currently, no direct acting antiviral drugs are available in the clinic to combat severe EV71 infection. Symptoms such as fever, encephalitis and meningitis are eased by supportive medication and intravenous immunoglobulin. Although ribavirin has been reported to decrease virus production in vivo, a very high dose is used (100 mg/kg) for treatment, which may raise safety concerns (Li et al., 2008). Other compounds that have shown promise in the research pipeline include viral capsid binding molecules such as the BPROZ series (Chen et al., 2008), inhibitors of protein 2C (metrifudil, etc.) (Arita et al., 2008), protein 3A inhibitors (enviroxime, etc.) (De Palma et al., 2009), 3D inhibitors (DTrip-22 and aurintricarbosylic acid) (Chen et al., 2009; Hung et al., 2010), and other natural products (raoulic acid, ursolic acid, etc.) (Chiang et al., 2005). Nevertheless, none of these molecules have been fully tested in animal models or clinical trials to establish their efficacy and safety profiles. Rupintrivir (AG7088), an irreversible inhibitor of HRV 3C protease originally developed by Agouron Pharmaceuticals, is by far the only 3C inhibitor that has entered clinical trial (Binford et al., 2005, 2007; Patick, 2006; Patick et al., 2005). The Phase I and Phase II results, according the company's press release, showed good safety and pharmacokinetic profiles. Furthermore,

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reduction in respiratory symptom scores and HRV titer in the upper respiratory tract of experimentally induced HRV infection was also documented (Witherell, 2000). In our previous study, the structure of EV71 3C protease was predicted by homology modeling. By molecular dynamics simulation, a favorable binding free energy between rupintrivir and EV71 3C was found (Zhang, 2010). This finding was further confirmed by crystallization of EV71 3C-rupintrivir complex (Lu et al., 2011; Wang et al., 2011). Based on these in vitro data, we aimed to evaluate the efficacy of rupintrivir as an anti-EV71 agent in cell culture and in an animal model.

2. Material and methods

2.1. Viruses, drugs, cell lines and mice

Human muscular rhabdomyosarcoma (RD) cells were maintained in Minimum Essential Medium Eagle (MEM) containing 10% FBS supplemented with L-glutamine, penicillin, and streptomycin (Gibco BRL, Grand Island, NY, USA). Rupintrivir (SC-208317) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enterovirus 71 strain SHAPHC695F/SH/CHN/10 (695F) was isolated from a 1.8-year-old patient in Shanghai public health clinical center (SHPHC) in 2010. Coxsackie virus 16 (CA16, strain 860F) and 10 (CA10, strain 798F) were also isolated from patients' oral swabs. Specific-pathogen-free ICR mice (Charles River Laboratories, Wilmington, MA, USA) were maintained in the animal facility of SHPHC. The animals were cared in accordance with the guidelines of the animal center of SHPHC.

2.2. EV71 infection in RD cells

RD cells in 96-well plates were infected with EV71, CA16, or CA10 at an MOI of 0.1. The infected cells were cultured with DMSO or rupintrivir at various concentrations for 3 days. Cell viability was measured using a cell counting kit (CCK-8; Dojindo Laboratories, Kumamoto, Japan), and a dose dependent response curved was plotted. TCID50 (50% tissue culture infectious dose) assays were performed and calculated as described (Tian et al., 2012). The plaque reduction assay was performed according to a simplified method (McKimm-Breschkin, 2004) with some modifications. Briefly, over 80% confluent RD cells in 6-well plate were incubated with serially diluted viral supernatant which was replaced with fresh medium containing 0.4% agarose 4 h later. After 2–3 days of culture, PBS containing 3.7% formaldehyde was added to each well and fixed overnight. After flicking off the agarose overlay, staining was performed with 0.05% crystal violet (20 min at room temperature). The plates were finally washed gently with deionized water, air dried and photographed.

2.3. EV71 infection in mice

Two-day-old suckling mice (2.0–2.3 g) were injected with 10^6 pfu EV71 (strain 695F) and subsequently underwent intraperitoneal (i.p.) injection with 5–15 μl of rupintrivir or ribavirin dissolved in DMSO to achieve a dosage of 0.1 mg/kg ($N = 13$), 1 mg/kg ($N = 13$) for rupintrivir or 100 mg/kg ($N = 10$) for ribavirin; an equivalent volume of DMSO was injected in the control group ($N = 14$). The drug was injected every day for 10 days. Infected mice were monitored daily for signs of morbidity and mortality. The sickness of mice was evaluated using a graded score (0, healthy; 1, slow movement; 2, weakness in hind limbs; 3, paralysis in single limb; 4, paralysis in two limbs; and 5, death). To better illustrate the recovery of the survived mice, deaths were calculated into the average score only once at the first observed date.

2.4. Histology and immunohistochemistry analysis

Routine hematoxylin and eosin (H&E) staining of various mouse tissues was performed using 3.7% formaldehyde-fixed and paraffin-embedded sections (4 μm). For immunohistochemistry of EV71 antigen, skeletal muscle, lung, and intestine tissues were embedded in OCT compound and frozen in liquid nitrogen. Cryosections were prepared using a CM 1800 cryostat (Leica, Wetzlar, Germany) onto poly-L-lysine-coated glass slides, which were further fixed with 3.7% paraformaldehyde in PBS and blocked with 1.5% horse serum in PBS followed by incubation with an anti-EV71 VP1 monoclonal antibody (Millipore, Billerica, MA, USA) at 4 °C overnight. The slides were serially incubated with biotinylated anti-mouse antibody supplemented with Vector M.O.M (mouse on mouse) agent and ABC reagent from a Vectastain ABC-peroxidase kit (Vector Laboratories, Burlingame, CA, USA). Slides were developed with DAB substrate and counterstained with Mayer's hematoxylin. Rigorous controls (matched tissue sections negative for EV71) were included in parallel to ensure the signals obtained were specific to viral antigen.

2.5. Real-time RT-PCR of viral RNA

Muscle, intestine, heart, lung, and brain-stem samples from various treatment groups were collected on day 6 post infection. Tissues were homogenized in 2% FBS culture medium using 1-mm glass beads (Biospec, Bartlesville, OK, USA), and RNA was subsequently extracted using QIAamp QIAXtractor Virus reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The VP1 fragment was amplified by real-time PCR using a one-step RT-PCR kit (TaKaRa Bio, Shiga, Japan). Mouse β-actin was amplified in parallel as an internal control. The viral load was expressed as $-(Ct_{EV71} - Ct_{actin}) + 10$.

2.6. Statistical analysis

Data are presented as mean ± SEM. Comparison of survival was performed with Mantel-Cox test. EC50 value was estimated using non-linear regression (variable slope) analysis employing Prims software (GraphPad Software, La Jolla, CA, USA). One-way ANOVA test was used for comparison of viral RNA between more than two groups. Mann–Whitney *U* test was used to compare viral RNA between two groups.

3. Results

3.1. Rupintrivir effectively eliminates EV71 replication in cell culture

We first evaluated the antiviral activity of rupintrivir in a cell culture model in which infection of EV71 strain 695F at an MOI of 0.1 leads to massive cytopathic effect. After inoculation, rupintrivir (8 nM–0.5 μM) was added and CPE was observed at 72 h post-infection. Indeed, complete protection against EV71-induced cell death was observed at low nanomolar concentrations (EC50 = 14 nM, 95% CI 12.16–16.33 nM; Fig. 1A). We also evaluated its activity against CA16 and CA10, two widely circulating agents also causing HFMD. It was observed that rupintrivir also blocked CA16 and CA10 proliferation with EC50 of 15 and 17 nM, respectively (Fig. 1B). Rupintrivir itself has minimal cell toxicity (Fig. 1A and B, see mock group) with CC50 over 50 μM (data not shown). For comparison, we also evaluated the antiviral effect of ribavirin (60 μM–4 mM). We found that ribavirin has a weak inhibitory effect against EV71 induced CPE (Supplementary Fig. 1) at concentrations over 1 mM. However, the cytotoxicity of ribavirin became apparent at the same dose range (Supplementary Fig. 1,

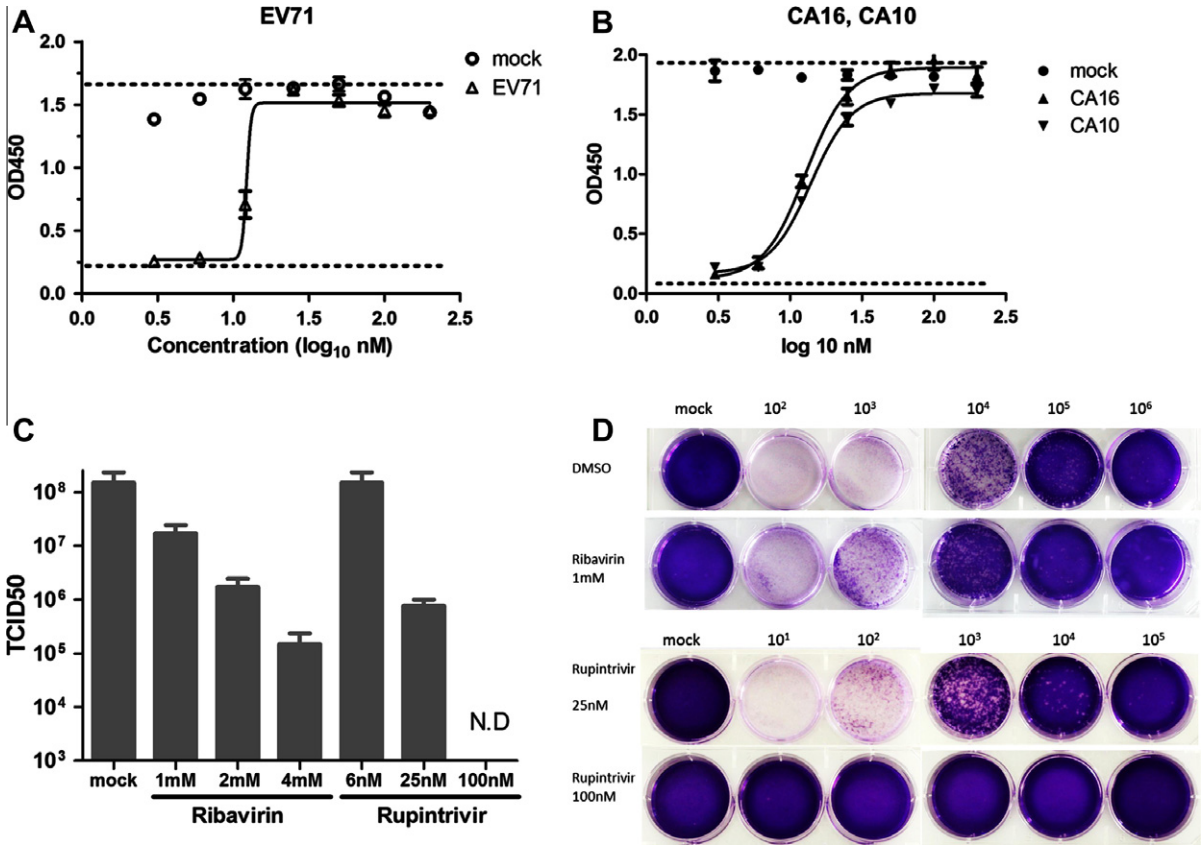

Fig. 1. The in vitro activity of rupintrivir. (A) and (B) Dose response curve of rupintrivir on cytopathic effects induced by EV71 (A), CA16, and CA10 (B). RD cells were mock infected or infected with EV71, CA16 or CA10 (MOI = 0.1) in the presence of rupintrivir (8–500 nM, $N = 4$) and cultured for 3 days. OD450 values obtained from CCK-8 assays were plotted against the concentration of rupintrivir in $\log_{10}$ scale. EC50 value was calculated as described in the materials and methods. The average OD values of infected cells and uninfected cell (DMSO treated) were plotted as dotted lines. These experiments were performed three times independently. (C) TCID50 assays of culture supernatant in various treatment group were performed and viral titers were calculated with Reed and Muench method. ND, not detected. (D) The DMSO, ribavirin or rupintrivir treated viral supernatant were serially diluted as indicated and subject to plaque forming assay, crystal violet staining was performed 3 day after inoculation.

see mock group). In addition, the infectivity of treated supernatant was measured by TCID50 and plaque assay. High dose of Ribavirin (1–4 mM) treatment gradually suppressed viral production up to 3 $\log_{10}$ (Fig. 1C), this result could be the combined effects of viral inhibition and cell toxicity as over 50% of RD cells died at 4 mM (Supplementary Fig. 1, see mock group). In contrast, only 25 nM of rupintrivir was able to generate a 2 $\log_{10}$ decline of virus production and 100 nM is sufficient to eradicate any sign of viral replication (Fig. 1C). This phenomenon was confirmed by viral plaque assay, in which no plaques were observed in 100 nM rupintrivir treated supernatant (Fig. 1D).

3.2. Rupintrivir effectively rescues EV71-induced paralysis and death in a suckling mice model

Having established the potency of rupintrivir in vitro, we next examined the efficacy of rupintrivir in a suckling mice model. After i.p. inoculation of EV71 strain 695F (10^6 pfu/mouse), rupintrivir (0.1 or 1 mg/kg) or ribavirin (100 mg/kg) was injected daily for 10 days; the same volume of DMSO was injected in the control group. In this model, without drug intervention, observable symptoms appeared on day 4 post infection, and this was characterized mostly by weakness in the hind limbs. At later days, the condition of infected mice further deteriorated into hind limb or front limb paralysis (single or both sided; Fig. 2C, upper panel, see black arrows) and reached a peak at 6–10 days post infection (Fig. 2B). At both dosages, rupintrivir significantly alleviated these symptoms (Fig. 2C, lower panel), as clinical score was markedly lower than that in DMSO group (Fig. 2B, $p < 0.01$ Wilcoxon rank test). As for the mortality of challenged mice, rupintrivir injection at 0.1 mg/kg was already sufficient for nearly complete protection (90.9% survival, Fig. 2A) whereas DMSO group yielded a 38.5% survival at the end of the observation ($p = 0.0063$, Mantel–Cox test). Administration of rupintrivir at 1 mg/kg did not further improve the overall survival rate (83.3% vs. 38.5% DMSO group, $p = 0.012$). In contrast, no significant improvement of mortality was observed in Ribavirin group (10% survival). Furthermore, ribavirin treated mice exhibited obvious morbidity at day 2–3 post infection, which is significantly earlier than DMSO or rupintrivir group (see Fig. 2B). We postulate that toxicity caused by high dose ribavirin may accelerate the morbidity and mortality after EV71 challenge.

In order to evaluate the extent of viral inhibition caused by rupintrivir, we further analyzed the viral RNA in various tissues at day 6 after infection. It was observed that skeletal muscle showed the highest viral load in the selected tissues (2000- to 4000-fold compared with other tissues in DMSO group), which is in accordance with a previous publication (Wang et al., 2004). Consistent with the symptoms, a significant decline in viral RNA was witnessed in intestine ($p < 0.0001$), lung ($p < 0.01$), muscle ($p < 0.05$), brain stem ($p < 0.01$), and cardiac muscle ($p < 0.0001$) when rupintrivir was administered (one-way ANOVA). Furthermore, 1 mg/kg of rupintrivir significantly strengthened this inhibition, as EV71 RNA level further declined compared with 0.1 mg/kg group (Fig. 2D, $p < 0.01$ in intestine, lung, and cardiac muscle; $p < 0.05$ in brain stem, Mann–Whitney U test). The decrease of viral RNA in muscle was evident but not as significant as the other tissues.

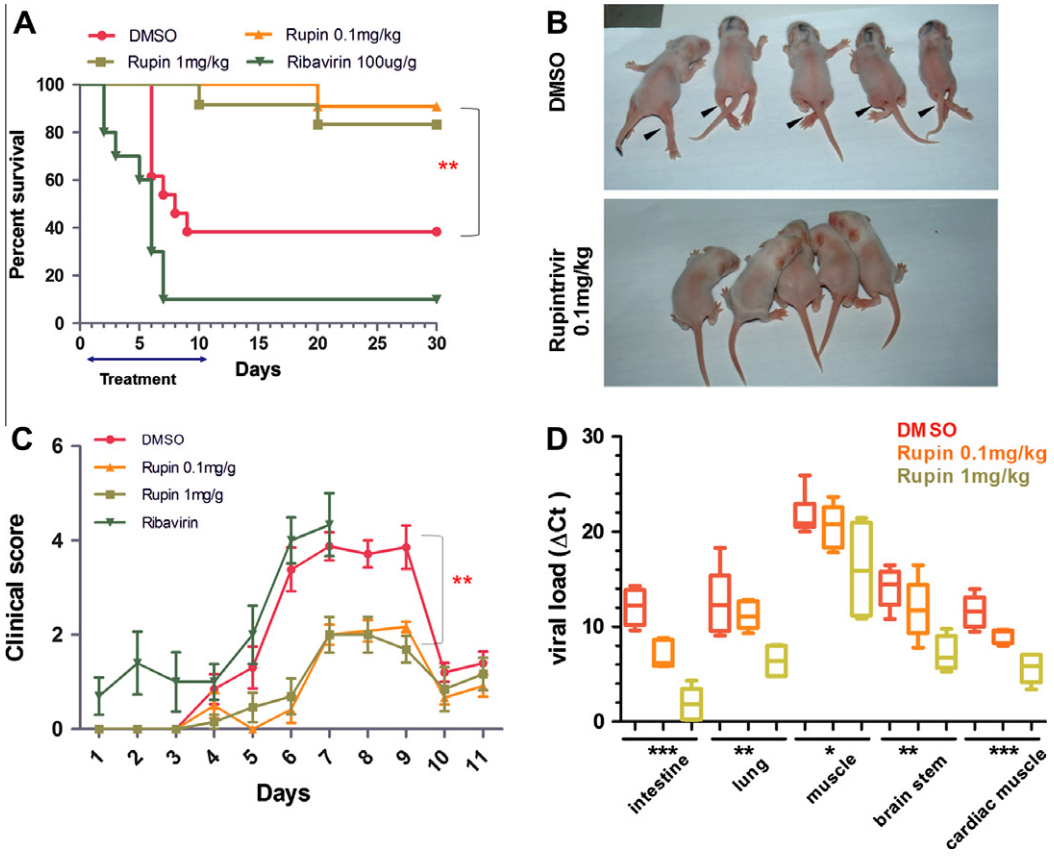

Fig. 2. The antiviral activity of rupintrivir in vivo. Kaplan–Meier curve (A) and clinical scores (B) of 2-day-old ICR mice i.p. inoculated with EV71 and treated with DMSO ($N = 13$) rupintrivir (0.1 mg/kg, $N = 13$, 1 mg/kg, $N = 14$) or ribavirin (100 mg/kg $N = 10$). Deaths were calculated into an average score only once at the first observed date. The scoring for ribavirin group was halted after 90% of the mouse died. Representative photographs were taken on day 6 post infection (C) showing apparent limb paralysis in the DMSO-treated infected mice (black arrow). (D) In separate experiments, the intestines, lungs, limb muscles, brain stems, and cardiac muscles ($N = 4$–6) from various groups (DMSO, rupintrivir 0.1 mg/kg and 1 mg/kg) were collected on day 6 post infection, Tissues were homogenized using 1-mm glass beads and RNA was extracted using QIAamp QIAXtractor Virus reagent. The VP1 fragment was amplified by real-time PCR using a one-step RT-PCR kit. Mouse β-actin was amplified in parallel as an internal control. The viral loads were expressed as $-(Ct_{EV71} - Ct_{actin}) + 10$ and presented as a box plot. These experiments were performed twice independently.

In particular, no significant difference was observed between DMSO and 0.1 mg/kg group. However, immunohistochemical analysis suggested that viral VP1 expression was significantly suppressed (see below).

3.3. Histological observations

We next examined the histological manifestations of the infected mice in various tissues. No obvious lesions and/or inflammatory signs were found in the heart or spinal cord when infected mice became morbid (data not shown). In contrast, massive necrotizing myositis with inflammatory infiltrates was observed in the limb muscles (Fig. 3B), and the administration of rupintrivir (0.1 mg/kg) significantly improved the integrity of limb muscle structure, although slight damages were still present (Fig. 3C). Immunohistochemistry staining of VP1 confirmed the RT-PCR results, viral replication was most active in skeletal muscle as intensive and widespread signal was detected (Fig. 3E 400×, see black arrows) in DMSO group but not in un-infected mice (Fig. 3D 400×). VP1 expression was largely suppressed by rupintrivir (0.1 mg/kg) in the muscle (Fig. 3F 200× and Fig. 3I 400×). Moderate inflammatory infiltration and thickening of alveolar septa in the lungs of DMSO-treated mice was observed, which was alleviated after rupintrivir treatment (0.1 mg/kg). In addition, significant staining of EV71 VP1 was detected in pulmonary alveoli (Fig. 3K) and in mucous and basement membrane of intestine (Fig. 3N, see black arrows). Accordingly, treatment with rupintrivir substantially decreased viral antigen expression in these tissues (Fig. 3L and O).

4. Discussion

Since the report of the first case of infection in California in 1969, EV71 has been identified in several small-scale outbreaks (Blomberg et al., 1974; da Silva et al., 1996; Gilbert et al., 1988; Ishimaru et al., 1980; Khetsuriani et al., 2006; Schmidt et al., 1974). In recent years, major EV71 outbreaks in Taiwan, Republic of China (Ho et al., 1999) in 1998 and in Anhui province (PR China) in 2008 resulted in substantial mortalities.

Considering the serious clinical manifestations associated with EV71, there is a pressing need to develop effective antivirals to prevent EV71-induced morbidity and mortality. Indeed, a growing body of literature on anti-EV71 drug development has been published in recent years (Wu et al., 2010). However, most of these drugs are still in the early phase of development and need further optimization of their pharmacokinetics and ADMET (absorption, distribution, metabolism, excretion, and toxicity) profiles. Although ribavirin, a wide spectrum antiviral, is reported to reduce mortality caused by EV71 in 12 to 14-day-old ICR mice (Li et al., 2008), a high dosage was used (100 mg/kg), which is approaching the LD50 value reported in adult mice (220 mg/kg/d) (Sidwell et al., 2005) and much higher than the clinical recommended dosage (10–16 mg/kg/d for adults with hepatitis C infection). Given that 95% of EV71 infections affect children younger than 4 years old (Chen et al., 2007), high dose of ribavirin may raise serious safety concerns. Indeed, we have observed very limited in vitro antiviral activity and accelerated death with i.p. injection of ribavirin in EV71 infected 2-day-old ICR mice. This result is at odds with Li

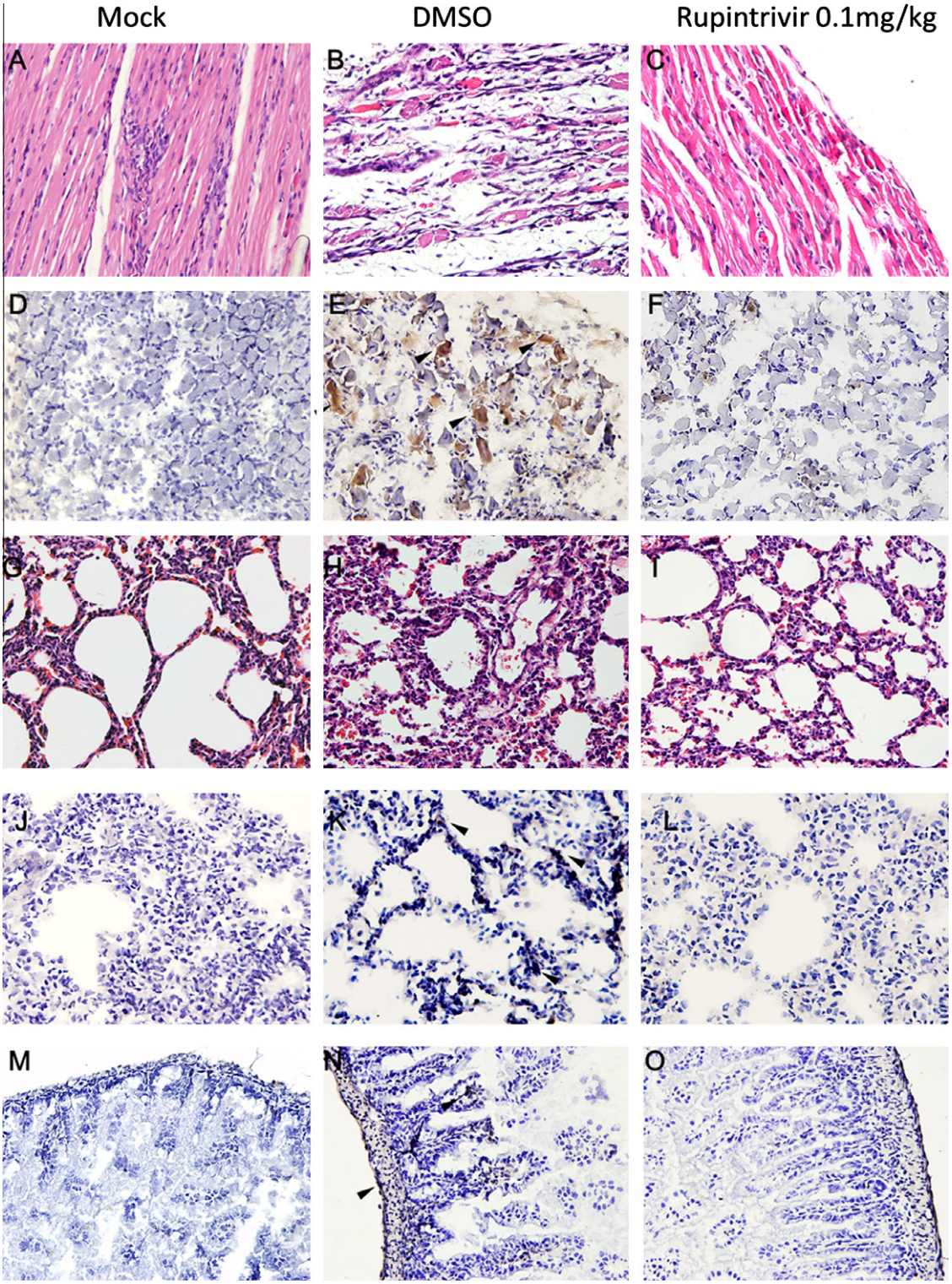

Fig. 3. Histological and immunohistochemical analysis of various tissues. Skeletal muscle (A–F), lung (G–L), and intestine (M–O) samples were collected on day 6 post infection and subjected to H&E staining (A–C, G–I) and immunohistochemistry (D–F, J–O) as described in the material and method section. The representative images in each group ($N = 6$) are shown (G–I 200× magnification, all others 400×).

et al's report (Li et al., 2008). We believe that the age of the mice used for protection assay is responsible for the discrepancy. In Li's report, 12–14 day old ICR mice were used, whereas 2 day old mice were used in our study. The younger age may render the mice more susceptible to drug induced toxicity.

Rupintrivir, also known as AG-7088, has long been established as an active agent against human Rhinoviruses, coxsackie viruses (CVB2 EC50 = 22 nM, CVB5 EC50 = 7 nM), and enteroviruses (EV6 EC50 = 51 nM, CVB5 EC50 = 11 nM) (Binford et al., 2005). Its activity against EV71, in particular, has also been documented using in silico

(computational simulation (Zhang, 2010)) and in vitro methods such as fluorescent resonance energy transfer (Tsai et al., 2009), 3C-rupintrivir complex structure, and in vitro protection assays (Wang et al., 2011). In addition, rupintrivir has gone through several large-scale clinical trials without obvious toxicity or adverse effects, which is a unique advantage over other anti-EV71 candidates. However, to the best of our knowledge, there is a dearth of reports describing the effects of rupintrivir against EV71-induced morbidity and mortality in experimentally infected animals. Therefore, in this study, we assessed its in vivo antiviral activity at doses that are comparable to clinical settings in a suckling mice model. We first confirmed the in vitro activity of rupintrivir with an EC50 of 14 nM, which is on par with a previous report (Wang et al., 2011). More importantly, the beneficial effects of rupintrivir were fully demonstrated by the nearly complete protection from EV71-induced death at a low dosage scheme (0.1 mg/kg). This feature is particularly desirable, as EV71-infected children are more vulnerable to drug-induced toxicity than adults. Immunohistochemical and real-time PCR analysis indicated that EV71 infection was most active in skeletal muscle where severe necrotizing myositis was observed. The administration of rupintrivir strongly suppressed viral replication and resulted in improved muscle histology and limb activity. Although viral RNA was not significantly suppressed in 0.1 mg/kg group, viral antigen expression was found to be largely inhibited. As EV71 exhibited the most active replication in skeletal muscle (2000- to 4000-fold viral RNA compared with other tissues in DMSO group), we postulate that the high level of viral RNA might mask the effects of rupintrivir on EV71 replication.

Antivirals that directly target viral enzymes would usually cause drug resistance. Indeed, in vitro selection of rupintrivir resistance in HRV 2 and 14 has been reported (Binford et al., 2007). It was found that single amino acid mutation only caused moderate reduction in susceptibility and that a significant resistance phenotype would require mutations on over three key residues. We also attempted to evaluate resistant mutants of EV71, however, selection by twelve serial passages with ascending concentrations of rupintrivir did not generate viable mutant virus. This might suggest that rupintrivir poses a high barrier for emergence of drug resistant virus, which further highlights its clinical advantage.

In conclusion, based on our in vitro and in vivo observations, together with its safety profile in previous Rhinovirus-related clinical trials, we propose that rupintrivir is a promising candidate for treating severe cases of EV71 infection. Further case-control clinical trials are needed to fully establish its activity in relieving EV71-induced symptoms and ultimately reducing mortality.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.12.029.

References

Arita, M., Wakita, T., Shimizu, H., 2008. Characterization of pharmacologically active compounds that inhibit poliovirus and enterovirus 71 infectivity. J. Gen. Virol. 89, 2518–2530.

Binford, S.L., Maldonado, F., Brothers, M.A., Weady, P.T., Zalman, L.S., Meador III, J.W., Matthews, D.A., Patick, A.K., 2005. Conservation of amino acids in human rhinovirus 3C protease correlates with broad-spectrum antiviral activity of rupintrivir, a novel human rhinovirus 3C protease inhibitor. Antimicrob. Agents Chemother. 49, 619–626.

Binford, S.L., Weady, P.T., Maldonado, F., Brothers, M.A., Matthews, D.A., Patick, A.K., 2007. In vitro resistance study of rupintrivir, a novel inhibitor of human rhinovirus 3C protease. Antimicrob. Agents Chemother. 51, 4366–4373.

Blomberg, J., Lycke, E., Ahlfors, K., Johnsson, T., Wolontis, S., von Zeipel, G., 1974. Letter: new enterovirus type associated with epidemic of aseptic meningitis and-or hand, foot, and mouth disease. Lancet 2, 112.

Chen, K.T., Chang, H.L., Wang, S.T., Cheng, Y.T., Yang, J.Y., 2007. Epidemiologic features of hand-foot-mouth disease and herpangina caused by enterovirus 71 in Taiwan, 1998–2005. Pediatrics 120, e244–e252.

Chen, T.C., Liu, S.C., Huang, P.N., Chang, H.Y., Chern, J.H., Shih, S.R., 2008. Antiviral activity of pyridyl imidazolidinones against enterovirus 71 variants. J. Biomed. Sci. 15, 291–300.

Chen, T.C., Chang, H.Y., Lin, P.F., Chern, J.H., Hsu, J.T., Chang, C.Y., Shih, S.R., 2009. Novel antiviral agent DTriP-22 targets RNA-dependent RNA polymerase of enterovirus 71. Antimicrob. Agents Chemother. 53, 2740–2747.

Chiang, L.C., Ng, L.T., Cheng, P.W., Chiang, W., Lin, C.C., 2005. Antiviral activities of extracts and selected pure constituents of *Ocimum basilicum*. Clin. Exp. Pharmacol. Physiol. 32, 811–816.

da Silva, E.E., Winkler, M.T., Pallansch, M.A., 1996. Role of enterovirus 71 in acute flaccid paralysis after the eradication of poliovirus in Brazil. Emerg. Infect. Dis. 2, 231–233.

De Palma, A.M., Thibaut, H.J., van der Linden, L., Lanke, K., Heggermont, W., Ireland, S., Andrews, R., Arimilli, M., Al-Tel, T.H., De Clercq, E., van Kuppeveld, F., Neyts, J., 2009. Mutations in the nonstructural protein 3A confer resistance to the novel enterovirus replication inhibitor TTP-8307. Antimicrob. Agents Chemother. 53, 1850–1857.

Gilbert, G.L., Dickson, K.E., Waters, M.J., Kennett, M.L., Land, S.A., Sneddon, M., 1988. Outbreak of enterovirus 71 infection in Victoria, Australia, with a high incidence of neurologic involvement. Pediatr. Infect. Dis. J. 7, 484–488.

Ho, M., Chen, E.R., Hsu, K.H., Twu, S.J., Chen, K.T., Tsai, S.F., Wang, J.R., Shih, S.R., 1999. An epidemic of enterovirus 71 infection in Taiwan. Taiwan Enterovirus Epidemic Working Group. N. Engl. J. Med. 341, 929–935.

Hung, H.C., Chen, T.C., Fang, M.Y., Yen, K.J., Shih, S.R., Hsu, J.T., Tseng, C.P., 2010. Inhibition of enterovirus 71 replication and the viral 3D polymerase by aurintricarboxylic acid. J. Antimicrob. Chemother. 65, 676–683.

Ishimaru, Y., Nakano, S., Yamaoka, K., Takami, S., 1980. Outbreaks of hand, foot, and mouth disease by enterovirus 71. High incidence of complication disorders of central nervous system. Arch. Dis. Child. 55, 583–588.

Khetsuriani, N., Lamonte-Fowlkes, A., Oberst, S., Pallansch, M.A., 2006. Enterovirus surveillance – United States, 1970–2005. MMWR Surveill. Summ. 55, 1–20.

Li, Z.H., Li, C.M., Ling, P., Shen, F.H., Chen, S.H., Liu, C.C., Yu, C.K., Chen, S.H., 2008. Ribavirin reduces mortality in enterovirus 71-infected mice by decreasing viral replication. J. Infect. Dis. 197, 854–857.

Lu, G., Qi, J., Chen, Z., Xu, X., Gao, F., Lin, D., Qian, W., Liu, H., Jiang, H., Yan, J., Gao, G.F., 2011. Enterovirus 71 and coxsackievirus A16 3C proteases: binding to rupintrivir and their substrates and anti-hand, foot, and mouth disease virus drug design. J. Virol. 85, 10319–10331.

McKimm-Breschkin, J.L., 2004. A simplified plaque assay for respiratory syncytial virus – direct visualization of plaques without immunostaining. J. Virol. Methods 120, 113–117.

Patick, A.K., 2006. Rhinovirus chemotherapy. Antiviral Res. 71, 391–396.

Patick, A.K., Brothers, M.A., Maldonado, F., Binford, S., Maldonado, O., Fuhrman, S., Petersen, A., Smith III, G.J., Zalman, L.S., Burns-Naas, L.A., Tran, J.Q., 2005. In vitro antiviral activity and single-dose pharmacokinetics in humans of a novel, orally bioavailable inhibitor of human rhinovirus 3C protease. Antimicrob. Agents Chemother. 49, 2267–2275.

Schmidt, N.J., Lennette, E.H., Ho, H.H., 1974. An apparently new enterovirus isolated from patients with disease of the central nervous system. J. Infect. Dis. 129, 304–309.

Sidwell, R.W., Bailey, K.W., Wong, M.H., Barnard, D.L., Smee, D.F., 2005. In vitro and in vivo influenza virus-inhibitory effects of viramidine. Antiviral Res. 68, 10–17.

Tian, D., Zhang, W., He, J., Liu, Y., Song, Z., Zhou, Z., Zheng, M., Hu, Y., 2012. Novel, real-time cell analysis for measuring viral cytopathogenesis and the efficacy of neutralizing antibodies to the 2009 influenza A (H1N1) virus. PLoS One 7, e31965.

Tsai, M.T., Cheng, Y.H., Liu, Y.N., Liao, N.C., Lu, W.W., Kung, S.H., 2009. Real-time monitoring of human enterovirus (HEV)-infected cells and anti-HEV 3C protease potency by fluorescence resonance energy transfer. Antimicrob. Agents Chemother. 53, 748–755.

Wang, Y.F., Chou, C.T., Lei, H.Y., Liu, C.C., Wang, S.M., Yan, J.J., Su, I.J., Wang, J.R., Yeh, T.M., Chen, S.H., Yu, C.K., 2004. A mouse-adapted enterovirus 71 strain causes neurological disease in mice after oral infection. J. Virol. 78, 7916–7924.

Wang, J., Fan, T., Yao, X., Wu, Z., Guo, L., Lei, X., Wang, M., Jin, Q., Cui, S., 2011. Crystal structures of enterovirus 71 3C protease complexed with rupintrivir reveal the roles of catalytically important residues. J. Virol. 85, 10021–10030.

Witherell, G., 2000. AG-7088 Pfizer. Curr. Opin. Investig. Drugs 1, 297–302.

Wu, K.X., Ng, M.M., Chu, J.J., 2010. Developments towards antiviral therapies against enterovirus 71. Drug Discov. Today 15, 1041–1051.

Zhang, X.-N., 2010. Rupintrivir is a promising candidate for treating severe cases of enterovirus-71 infection. World J. Gastroenterol. 16, 201.