

Synergistic Inhibition of Enterovirus 71 Replication by Interferon and Rupintrivir

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Background. Enterovirus 71 (EV71) can cause severe diseases and even lead to death in children. There is no vaccine or specific antiviral therapy to prevent or cure EV71 infection. Although interferon (IFN)- α has been used in the treatment of several viral infections, we found that IFN- α alone was ineffective in restricting EV71 replication in Vero cells.

Methods. Through a bioinformatics analysis, several cellular proteins in the IFN response pathway were identified as susceptible substrates that might be degraded by the EV71-encoded 3C protease (3C^{pro}).

Results. Indeed, IRF9 was shown to be vulnerable to 3C^{pro} cleavage, as revealed by enzyme-based and cell-based assays. Thus, the IFN-mediated antiviral mechanism compromised by the viral 3C^{pro} in EV71-infected cells may be accountable, at least partially, for that IFN- α cannot inhibit EV71 replication. Because rupintrivir (AG7088) is known to be an effective EV71 inhibitor, we investigated the effects of the combination of rupintrivir and IFN- α on EV71 replication and found that they strongly synergized with each other in inhibiting EV71 replication.

Conclusions. Because rupintrivir was shown to be generally tolerable in earlier clinical investigations, it is worth evaluating whether a combination of rupintrivir and IFN- α could be an effective treatment for EV71.

Host innate antiviral defense systems can be disrupted by viruses through a variety of mechanisms [1]. For instance, cleavage of host proteins by viral proteases has been shown to abrogate signaling pathways involving interferon regulatory factor 3 (IRF-3) and the synthesis of type 1 interferon (IFN- α/β) [2, 3]. Furthermore, the viral RNA recognition sensor RIG-I in the host cells is degraded by viral 3C protease (3C^{pro}) encoded by a number of different viruses in the picornaviridae [4].

Enteroviruses belong to the family *Picornaviridae* [5]. Among the enterovirus strains, Enterovirus 71 (EV71) infection may cause severe symptoms in young children

[6]. EV71 encodes 2 proteases, 2A and 3C, that are responsible for the processing of the viral polyprotein. These viral proteases also regulate host responses by mediating the cleavage of cellular proteins involved in the transcription and/or translation machinery and those involved in the linkage between the cytoskeleton and the extracellular matrix [7–9]. Currently, no antiviral drug is available for treating EV71 infection. Although IFN- α has been used to treat several viral infections, it is not recommended for EV71. Many studies have addressed the underlying mechanism for the interference of the IFN immune response by enteroviruses [10–12]. We previously identified the genes involved in the host's response to EV71 infection and found no significant increases in the expression levels of IFN- α , IFN- β , IFN- γ , IFN-induced PKR, or 2', 5'-oligoadenylate synthetase in the EV71-infected cells [10]. Recently, enteroviral 2A protease was reported to be essential for viral replication in cells treated with IFN [11]. In the present study, we showed that the 3C^{pro} of EV71 is able to cleave IRF9, a host protein involved in the signaling cascade triggered by type I IFN. We found that EV71 could be effectively inhibited by

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a combination of IFN- α and a 3C^{PRO} inhibitor [13]. One potential clinical ramification based on the results from this study will be discussed.

MATERIALS AND METHODS

Cells, Viruses, and Reagents

Human rhabdomyosarcoma cells (RD cells: ATCC-CCL-136) and African green monkey kidney cells (Vero cells: ATCC-CCL-81) were purchased from the American Type Culture Collection (ATCC); only cell passage numbers ≤ 20 were used to propagate viruses. EV71 (TW/2231/98) was isolated in the 1998 EV71 outbreak and supplied by the Clinical Virology Laboratory of Chang Gung Memorial Hospital, Taiwan [14]. Encephalomyocarditis virus (EMCV: VR-129B) was purchased from ATCC. Virus titer was measured by a plaque assay using Vero cells. Leukocyte IFN was obtained from PBL Biochemical Labs. Rupintrivir (AG7088) was a gift from JM Fang (National Taiwan University, Taipei, Taiwan) [15].

Expression and Purification of IRF9

The human total RNA was isolated from the RD cells using Trizol reagent (Invitrogen) and then subjected to reverse-transcription polymerase chain reaction (RT-PCR) according to the instructions from the manufacturer. Human cDNA was synthesized from the isolated total RNA and subjected to amplification using the Perkin Elmer Cetus DNA thermal cycler. The IRF9 cDNA was amplified by PCR from the human cDNA with primers encoding sites for *EcoRI* and *BamHI*. The sequence of the forward and reverse primers for amplifying IRF9 are 5' CGAATTCTATGGCATCAG GCAG GG 3' and 5'GCGGATCCCTCTACACCAGGGA 3', respectively. The thermal cycling profile was 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, with a final extension at 72°C for 10 min. After PCR amplification, the purified PCR products were digested with *EcoRI* and *BamHI* for subsequent cloning. The restriction enzyme digested-IRF9 PCR products were inserted into pET-23a plasmid (Novagen) at the corresponding sites. The correct construct was then transformed into *Escherichia coli* BL21 (DE3) pLysS competent cells, and expression of recombinant IRF9 protein was induced by the addition of isopropyl-D-thiogalacto-pyranoside. The histidine-tagged IRF9 was purified using a nickel-chelating column. The purified protein was dialyzed against storage buffer (25 mM Tris [pH, 7.0], 50 mM NaCl, 0.1% Triton X-100, 50% glycerol, 1 mM EDTA, and 1 mM DTT), and protein concentration was determined by the Bradford protein assay (BioRad).

In Silico Analysis of 3C^{PRO} Cleavage Target

The NetPicoRNA 1.0 server (<http://www.cbs.dtu.dk/services/NetPicoRNA/>) was used for identification of putative protease cleavage sites on type I IFN signaling pathway-associated

proteins, with scores $>.5$ considered to be potential cleavage sites.

Enzyme-Based Cleavage Assay

The expression and purification of recombinant wild-type EV71 3C^{PRO} and mutant EV71 3C^{PRO} (with a single amino acid change C147S [3CM^{PRO}]) were prepared as described elsewhere [7, 15, 16]. In these previous studies, a [³⁵S]-labeled protein substrate harboring a 3C^{PRO} cleavage site was generated for analyzing the catalytic activity of wild-type 3C^{PRO} or mutant 3C^{PRO} in the enzyme-based cleavage assay. In contrast to wild-type 3C^{PRO}, the 3CM^{PRO} could not cleave the 3C^{PRO} substrate, indicating the loss of catalytic activity. Recombinant IRF9 protein was diluted with cleavage buffer (2.5 M NaCl, 250 mM HEPES - KOH, pH 7.4, and 12.5 mM MgCl₂) and incubated for 2 h at 37°C with 2 μ g of purified recombinant 3C^{PRO} or 3CM^{PRO} in a total volume of 25 μ L. Cleavage of IRF9 was confirmed by Western blot analysis.

Cell-Based Assay

RD cells (6×10^5 cells per well) were plated onto 6-well plates and incubated overnight. Cells were pretreated with IFN (2,000 IU/mL) for 6 h and then infected with EV71 at a multiplicity of infection (MOI) of 10 in E2 medium (Dulbecco's modified Eagle's medium [Invitrogen] containing 2% fetal calf serum) at 37°C. At the times indicated, the medium was removed and the cells were centrifuged, washed with phosphate-buffered saline, and lysed with lysis buffer (1% IGEPAL CA-630, 50 mM Tris-HCl [pH, 8.0], and 150 mM NaCl) at 4°C for 1 h. The cell extracts were then centrifuged at 12,000 rpm for 10 min, and supernatants were stored at -80°C . Cleavage of IRF9 was assessed using Western blot analysis.

Cytopathic Effect Inhibition Test

Vero cells were seeded in 96-well tissue culture plates at a density of 2×10^4 cells/well in DMEM with 10% FBS. After cells were incubated for 18–24 h at 37°C, cells were pretreated with IFN at various concentrations for 6 h and then infected with EV71 at a multiplicity of infection (MOI) of 0.2., as described by Hung et al. [24].

Western Blot Analysis

Proteins with sample buffer were subjected to SDS-PAGE and were electroblotted onto Hybond ECL membranes (GE Healthcare Bio-Sciences). The membrane blocking and incubation buffers consisted of 5% nonfat dry milk in TBST (0.01M Tris HCl, 0.15M NaCl, and 0.05% Tween-20). The membrane was incubated with 1:2000 dilution of anti-IRF9 rabbit polyclonal antibody (Santa Cruz Biotechnology), either at 4°C overnight or at room temperature for 2 h. The membrane was then washed with TBST for 1 h, followed by incubation with 1:10,000 dilution of the HRP-conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). The enhanced chemiluminescence detection system was used for the detection of target proteins.

Plasmid Transfection and Luciferase Assay

The IFN-stimulated response element (ISRE) reporter plasmid (pISRE-Luc) was obtained from Clontech and the internal control plasmid pRL-SV40, with the SV40 early enhancer/promoter region upstream of *Renilla* luciferase, was purchased from Promega. The plasmids pISRE-Luc and pRL-SV40 were cotransfected into Vero cells (1×10^5 cells/well in 24-well plates) in Opti-MEM I (Invitrogen) medium using lipofectamine 2000 reagent (Invitrogen). At 5 h after transfection, the Opti-MEM I medium was replaced with E2 medium. After transfection for 24 h, cells were treated with or without IFN for 6 h, followed by EV71 infection at various MOIs. At 48 h after infection, cell lysates were collected to determine both *Renilla* and firefly luciferases activity with use of the Dual-Luciferase reporter assay system (Promega), according to manufacturer's instructions.

Analysis of the Combination Effects

CalcuSyn software (Biosoft) was used to evaluate the effects of combined drug treatments with use of the method described

by Chou and Talalay [17]. The synergism between 2 drugs was quantified by combination indices with use of CalcuSyn for automated analysis. Combination indices <1 indicate a synergistic effect, whereas combination indices of 1 indicate an additive effect and those >1 reflect an antagonistic effect.

RESULTS

EV71 Replication Reduces IFN-Activated Signaling

Type I IFN signaling plays an important role in modulating host cell responses to virus infection. In this study, we found that leukocyte IFN at concentrations >2000 IU/mL was ineffective in inhibiting EV71 replication in Vero cells (Figure 1A). In the same experiment, EMCV could be effectively inhibited by the leukocyte IFN, indicating that the IFN was active (Figure 1B). These results were reminiscent of several previous reports that examined the interplay between viral proteases and host responses to the treatment of infected cells with IFN [11, 18]. A critical mediator along the IFN signaling pathway is the IFN-

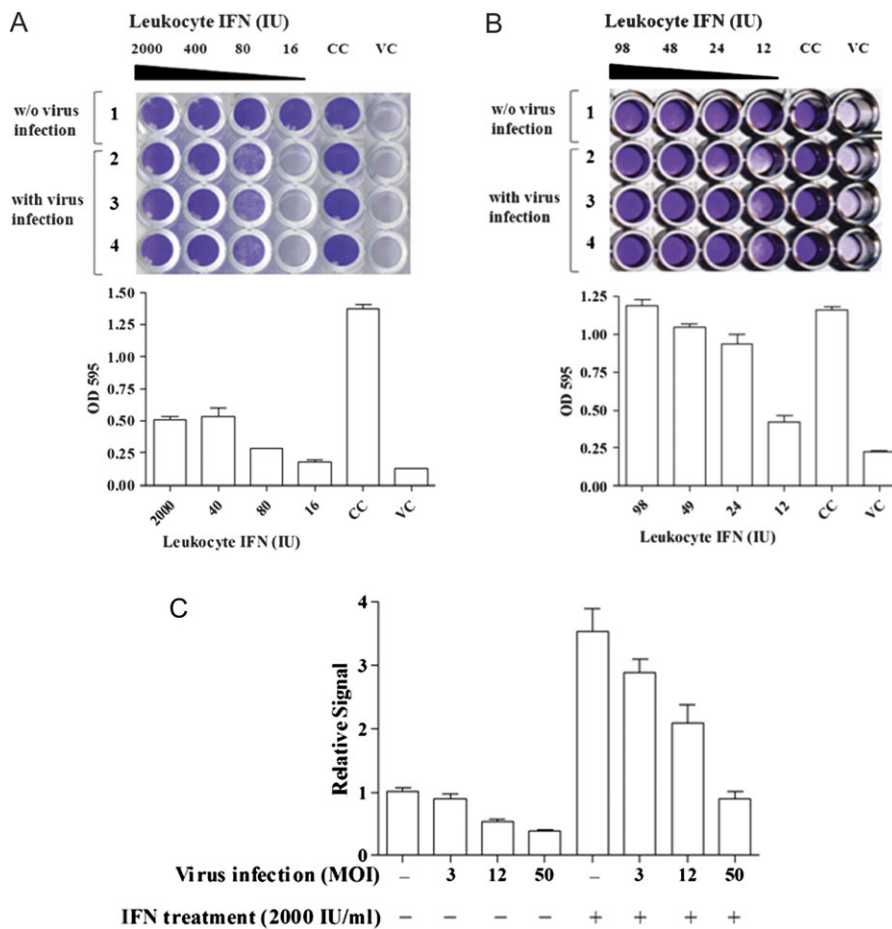


Figure 1. EV71 replication destroyed IFN-activated ISRE signaling. *A*, The effect of IFN in neutralizing the EV71-induced cytopathic effect (CPE). *B*, The effect of IFN in neutralizing the EMCV-induced CPE. *C*, The effect of EV71 infection on the ISRE-driven gene expression. Vero cells were cotransfected with pISRE-luc reporter plasmid and internal control pRL-SV40 plasmid, followed by treatment with IFN- α (2000 IU/mL), and then infected with various doses of EV71 for 48 h. Means \pm standard error of the mean for 3 experiments.

stimulated gene factor 3 (ISGF3) complex, which binds to the ISREs after IFN stimulation [18]. To examine whether EV71 replication interferes with the IFN signaling pathway in Vero cells, we used a transient transfection system using a plasmid, pISRE-Luc, consisting of the firefly luciferase reporter gene under the control of ISRE-binding sequence, located upstream of the herpes simplex virus thymidine kinase promoter.

Vero cells were cotransfected with pISRE-luciferase and pRL-SV40 (*Renilla* luciferase as an internal control) plasmids. At 24 h after transfection, cells in distinct plates were infected or not infected with EV71. The ISRE-driven firefly luciferase reporter gene activity was normalized to the *Renilla* luciferase activity. Infection of Vero cells with EV71 at various MOIs significantly decreased the ISRE-driven expression of the reporter gene (Figure 1C). However, in the IFN treatment experiment, cells were treated with leukocyte IFN (2000 IU/mL) 6 h before viral infection. IFN was present in culture media throughout the experiment. The maximal level of the normalized luciferase signal in cells was increased 5-fold after IFN stimulation. However, when cells were infected with EV71 at an MOI of 50, there was a pronounced decrease in the IRSE-driven luciferase signal after IFN stimulation (Figure 1C). Thus, IFN could no longer enhance the expression of the ISRE-mediated gene in EV71-infected cells. The results indicate that EV71 replication would lead to the disruption of IFN signaling in host cells.

IRF9 Was Degraded in EV71-Infected Cells and Enzyme-Based Assays

To evaluate how the IFN signaling pathway may be compromised after EV71 infection, a neural network approach, the *NetPicoRNA*, version 1.0, algorithm was used to predict whether

there are potential cellular proteins along the type I IFN signaling pathway that are substrates of EV71 3C^{pro} [19]. As shown in Table 1, JAK1, SATA2, IRF9, IRF3, and PRK ranked among the top 5 cellular proteins that are likely to be the substrates of viral 3C^{pro}. PKR was reported to be downregulated in poliovirus-infected cells [20]. Subsequent Western blot analyses revealed that IRF9 was indeed downregulated in EV71-infected cells. The cleavage product from IRF9 became evident at 5 h after infection, and the cleavage product became more prominent as the postinfection time progressed (Figure 2A). Thus, results from the cell-based assay were consistent with the bioinformatics prediction, suggesting that IRF9 is a substrate of EV71 3C^{pro}. To further confirm this observation, we examined whether purified recombinant IRF9 can also be cleaved by EV71 3C^{pro} in an enzyme-based assay.

The *E. coli* expressed recombinant IRF9 protein was treated with EV71 3C^{pro} or an inactive form of EV71 3C^{pro} with a C147S point mutation (3CM^{pro}) for 2 h at 37°C in an enzyme-based assay. Two likely cleavage fragments appeared after 3C^{pro} treatment (Figure 2B). In contrast, no cleavage fragment of IRF9 was detected after treatment with the 3CM^{pro} under the same conditions. These results further confirmed that IRF9 is a substrate of the viral 3C^{pro}. [15]

Synergistic Anti-EV71 Effects Were Exerted by the Combination of Rupintrivir and IFN

We have previously shown that rupintrivir (AG7088) is also a potent inhibitor of EV71, although it was originally developed to combat human rhinovirus, which causes the common cold [13]. Therefore, we evaluated the effects of the EV71-induced CPE on Vero cells treated with rupintrivir in the presence or absence of IFN.

Table 1. Identification of Potential EV71 3C^{pro} Substrates by a Bioinformatics Approach

No.	NCBI		Predicted cleavage				
	Accession No.	Name	Residue	Pos	Clv	Surf	Sequence
1	NP_002218	Janus kinase 1 (JAK1)	Q	149	0.914	0.698	YLFAQGQYD
2	NP_005410	Signal transducer and activator of transcription 2 (STAT2)	Q	129	0.846	0.665	AQLEQGEPV
			Q	394	0.824	0.618	KGQSQGLIW
			Q	779	0.71	0.672	PEPDQGPVS
			Q	649	0.691	0.532	IRHYQLLTE
3	NP_006075	Interferon regulatory factor 9 (IRF 9)	E	545	0.609	0.666	FTKRESPPG
			Q	202	0.922	0.679	EAPFQGDQR
			Q	381	0.86	0.605	YLLEQTPEQ
			E	168	0.738	0.725	NNEEGASG
4	Q14653	Interferon regulatory factor 3 (IRF3)	Q	337	0.65	0.619	VRYFQGLGP
			Q	237	0.513	0.523	EAQVQSLDC
5	NP_004696	Interferon-inducible double stranded RNA dependent inhibitor (PKR)	Q	422	0.886	0.697	GMDFQGPGE
			E	146	0.589	0.76	EEEGEGQDE
			Q	501	0.541	0.603	GKNLQGQTS

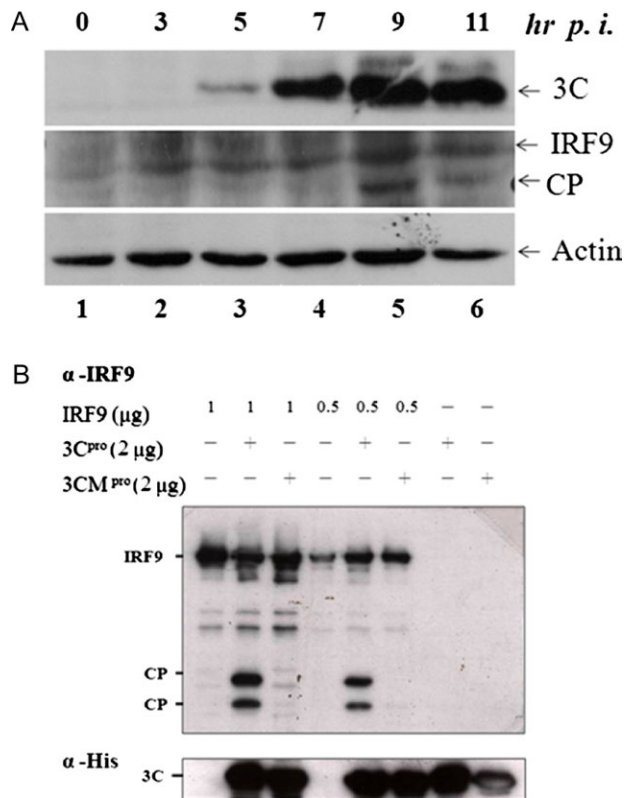


Figure 2. IRF9 was cleaved in *cell-based* and *enzyme-based* assays. *A*, IRF9 was cleaved in a *cell-based* assay system. Protein samples from EV71 (MOI 10) infected RD cells were separated on 10% SDS-PAGE, blotted, and stained using an anti-IRF9 antibody. *B*, Recombinant IRF9 was cleaved by 3C^{pro} in an *enzyme-based* assay system. Recombination IRF9 was incubated with 2 μ g of either EV71 3C^{pro} or 3CM^{pro} at 37°C for 2 h.

Dose-dependent inhibition was observed after treatment of the infected cells with the combination of rupintrivir and IFN. In fact, 40%–63% inhibition was observed when Vero cells were treated with IFN at 2000 IU/mL and rupintrivir at 10 μ M. The

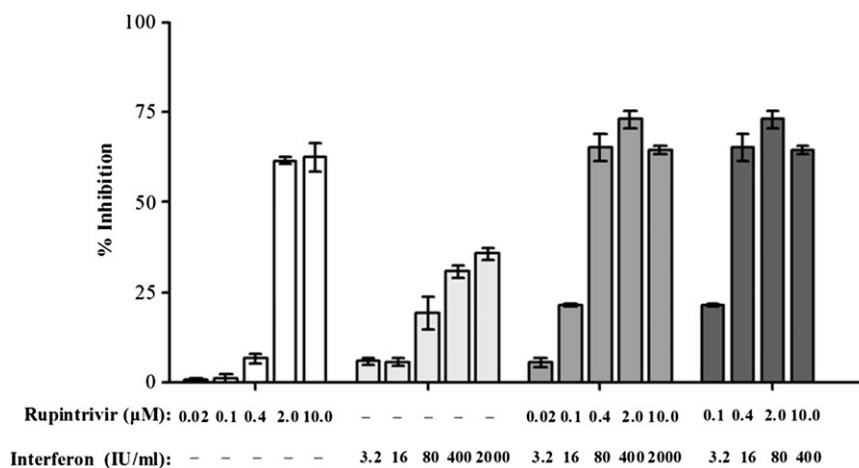


Figure 3. Inhibition of EV71 replication by combination of rupintrivir and IFN. The neutralization tests were designed for evaluations of the anti-EV71 activity of rupintrivir and IFN either alone or in combination. Vero cells were treated with rupintrivir and infected with EV71 (100 TCID₅₀) for 64 h. In the IFN treatment experiment, cells were treated IFN at various concentrations 6 h prior to viral infection. Means \pm standard error of the mean for 3 experiments.

individual 50% inhibitory concentrations of rupintrivir and IFN were 1.1 μ M and >2000 IU/mL, respectively (Figure 3). For more quantitative analysis of drug interactions, we then used the method developed by Chou and Talalay [21] for assessing drug-drug interactions to estimate whether a combination of rupintrivir and IFN exerts synergistic, additive, or antagonistic anti-EV71 effects. Inhibition of EV71 replication was evaluated in a neutralization test involving treatment with serial dilutions of rupintrivir (0, 0.016, 0.08, 0.4, 2, and 10 μ M) or IFN (0, 16, 80, 400, and 2000 IU/mL), or with a combination of the 2 agents at fixed 1:40 or 1:200 ratios (Figure 3 and Table 2). Results revealed statistically synergistic anti-EV71 effects at 50% inhibition levels. Treatment of EV71-infected cells with the combination of rupintrivir and IFN resulted in a more significant inhibition of CPE than did treatment with either agent alone, as revealed by the combination indices of < 1 (range, .14–.27) (Table 2). These data showed that rupintrivir and IFN exert strong synergistic anti-EV71 activities.

DISCUSSION

There is an urgent medical need for an effective treatment for EV71 infection. Understanding the interplay between virus and host is important for the identification of effective targets for the development of therapeutic strategies. Although IFN is an effective host defense mechanism to restrict the replication of many viruses, some viruses could escape from the IFN defense mechanism through inactivation of the essential signaling pathway in host cells [22]. In the present study, we first used a bioinformatics approach to examine whether there are potential viral 3C^{pro} cleavage sites residing in host proteins along the IFN signaling pathway. IRF9 was thus identified and subsequently confirmed to be degraded by 3C^{pro} in an enzyme-based assay. Destruction of IRF9 function is one way that a virus

Table 2. Effects of Rupintrivir in Combination With IFN on EV71-Induced Cytopathic Effect

IC	CI with the following rupintrivir/IFN ratio	
	1:40	1:200
50%	0.17	0.16
75%	0.15	0.20
90%	0.14	0.27

NOTE. CalcuSyn analysis provides combination index (CI) values to determine potential drug additivity. The CI determines the degree of the interaction of drugs, e.g., CI less than one, CI of one, and CI less than one are indicative of synergistic, additive, and antagonistic effects, respectively. The results show that rupintrivir combined with IFN (leukocyte) is synergistic, with CI values ranging from 0.14 to 0.27.

can abrogate the IFN signaling pathway [23]. The evidence presented in the present study suggests that EV71 replication would lead to the disruption of IFN-activated signaling. Overall, the 3C^{pro} encoded by EV71 was found to be a factor to disable the host IFN's antiviral defense mechanism through the cleavage of IRF9, an important transcription factor along the IFN signaling pathway.

We have previously demonstrated that EV71 3C^{pro} cleaves a number of host proteins to inhibit polyadenylation and induce apoptosis in infected cells [7, 16, 25]. To our knowledge, IRF9 is the first IFN-responding protein that can be degraded by EV71 3C^{pro}. Lei et al [12] showed that RIG-I, MDA-5, OPS-1, TBK1, and IKKi were not cleavable by EV71 3C^{pro}, although it was shown that the host innate immunity can be attenuated by the cleavage of viral RNA reorganization sensor RIG-I by 3C^{pro} in cells infected with other picornaviruses, such as poliovirus, rhinovirus, echovirus, and EMCV [4, 26].

Many viruses, such as HIV or hepatitis C virus, also rely on their own proteases to replicate in host cells, and a combination of viral protease inhibitors with IFN was shown to be effective against infections with these viruses [27, 28]. In view of the finding that 3C^{pro} cleaves the IRF9, it is important to examine whether treating EV71-infected cells with 3C^{pro} inhibitors would help to restore the undermined IFN signaling system. Rupintrivir was developed as a viral 3C^{pro} to combat human rhinovirus [29]. We have previously shown that rupintrivir was able to inhibit EV71 3C^{pro} activity and to neutralize the EV71-induced CPE [13, 15]. Therefore, we examined whether the combination of rupintrivir and IFN can act synergistically against the replication of EV71. Results showed that strong synergistic anti-EV71 activity was exerted by the combination of IFN and rupintrivir. The combination of IFN and rupintrivir may be considered for prospective trials in human subjects for evaluations of whether such a strategy would be effective in treating EV71 infection.

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