Idarubicin is a broad-spectrum enterovirus replication inhibitor that selectively targets the virus internal ribosomal entry site

Hsin-Yu Hou,1† Wen-Wen Lu,2† Kuan-Yin Wu,1 Cheng-Wen Lin3 and Szu-Hao Kung1

1Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Taipei, Taiwan, ROC
2Department of Clinical Pathology, Cheng Hsin General Hospital, Taiwan, ROC
3Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung, Taiwan, ROC

Enterovirus 71 (EV71) causes life-threatening diseases with neurological manifestations in young children. However, the treatment of EV71 infections remains an unmet medical need. Idarubicin (IDR) is an anthracycline compound that is used therapeutically for certain types of tumour. In this study, we identified IDR as an EV71 inhibitor, which displayed antiviral potency in the submicromolar range and substantially protected cells from the cytopathic effects and cell death caused by EV71 infections. The antiviral effects extended to several other enterovirus (EV) species, and these effects were independent of cytotoxicity or topoisomerase inhibition. Structure–activity relationship studies indicated the importance of the anthracycline scaffold for anti-EV potency. IDR effectively blocked the synthesis of viral protein and RNA, but not the viral proteolysis processes. Moreover, anthracyclines were demonstrated to suppress EV internal ribosomal entry site (IRES)-mediated translation; conversely, the cellular p53 IRES activity was not sensitive to IDR action. Inhibition of IRES-mediated translation by IDR correlated with the affinity of binding between IDR and the particular IRES. Moreover, IDR impaired binding between the EV71 IRES RNA and hnRNP A1, a known host IRES trans-acting factor. In sum, we have identified a USA FDA-approved anticancer drug with the new indication as a selective EV IRES binder and inhibitor. The finding may also provide leads for the development of novel antiviral therapies directed at the EV IRES RNA.

INTRODUCTION

Enteroviruses (EVs) include coxsackievirus, echovirus, poliovirus and rhinovirus species and are members of the family Picornavirus. EV infections have been associated with a broad array of clinical manifestations ranging from mild illnesses to more severe and even life-threatening diseases, such as myocarditis, pancreatitis, meningitis, encephalitis and acute paralysis (Rhoades et al., 2011). In recent years, EV71 infections have caused several outbreaks of hand, foot and mouth disease, with a notable proportion of infected young children manifesting severe neurological syndromes and even consequent death worldwide, particularly in the Asia–Pacific region (Bible et al., 2007; Ho et al., 1999; Liu et al., 2015; Solomon et al., 2010).

After viral entry, the EV71 positive-stranded genome acts directly as an mRNA and is translated into a large polyprotein. This viral translation is mediated at an internal ribosome entry site (IRES) with multiple stem–loop structures in the 5′ untranslated region (5′ UTR) of the viral genome. The IRES-driven translation is governed by functional interactions between the viral IRES and a number of cellular IRES trans-acting factors (ITAFs) that are not required for the canonical cap-dependent cellular mRNA translation (Niepmann, 2009; Shih et al., 2011). The polyprotein is cleaved post-translationally by virus-encoded proteases 2A (2APro) and 3C (3CPro) to yield the structural capsid proteins, termed VP1–4, and non-structural proteins, including proteases and polymerase. Viral genome replication is executed by the viral-encoded RNA-dependent RNA polymerase 3D. Positive-sense progeny viral RNAs are packaged into capsids to form virions, which the host cell releases through lysis (Bedard & Semler, 2004).

†These authors contributed equally to this study.

Five supplementary figures, one supplementary table and supplementary methods are available with the online Supplementary Material.
Despite the rapid expansion of EV infections, no effective clinical measures have been developed to treat the associated diseases. A broad-spectrum drug is warranted for treating EV infections because no single EV species is associated exclusively with any particular disease, and the treatment is primarily symptomatic (Norder et al., 2011; Thibaut et al., 2012). A number of compounds have demonstrated in vitro potency against several EV species by targeting various viral life cycle stages including the adsorption/uncoating, proteolytic process and genome replication (Norder et al., 2011; Thibaut et al., 2012). However, they either have remained largely unexplored in animal studies or have been dropped from further clinical trials (Norder et al., 2011; Solomon et al., 2010; Thibaut et al., 2012).

In the present study, we set out to identify novel inhibitors of EV71 replication by screening the Library of Pharmacologically Active Compounds (LOPAC), consisting of 1280 small molecular mass molecules (small molecules) with known bioactivities against diverse cell functions. We identified idarubicin (IDR) as a broad-spectrum inhibitor of EV replication. IDR is an anthracycline compound used clinically for cancer treatment (Kizek et al., 2012; Martincic & Hande, 2005). We demonstrated that IDR and its structural analogues potently suppressed the EV IRES activities, likely by preferentially binding to the EV IRESs but less so to the cellular IRESs, thereby impairing functional interactions between the EV IRESs and the ITAFs.

RESULTS

Anti-EV effects of IDR

We performed a screen by using a LOPAC drug library that consists of 1280 bioactive compounds. A cell-based biosensor, HeLa-G2AwtR cells, was used as a screening platform based on monitoring the conversion of fluorescence resonance energy transfer (FRET) following an EV71 infection. It was developed by stable expression of a fusion substrate composed of GFP linked to the red fluorescent protein by a cleavage motif of EV71 2APro (Hsu et al., 2007). Due to the close proximity of the two fluorescent proteins, pronounced FRET from the biosensor cells occurs until disruption by viral 2APro-mediated cleavage in the context of an EV infection. The level of the FRET can be measured quantitatively, and the repressed FRET is proportional to EV replication (Hsu et al., 2007). In this screening campaign, a hit was defined as a compound that significantly reversed the reduction in FRET levels caused by EV71 infections. IDR emerged as one of the hits, with no prior knowledge of its activity against EVs.

We first investigated if IDR treatments protected cells against EV71-induced cytopathic effects (CPEs), which are characterized by rounded, refractile and shrunken cells. Rhabdomyosarcoma (RD) cells were pre-incubated with IDR at 1–4 μM, or left untreated, and subjected to EV71 infections in parallel with uninfected control cells. Morphological analyses showed that the CPE levels declined in a dose-dependent manner (Fig. 1a). Quantitative analyses of the cell viability for all conditions were consistent with the morphological observations (Fig. 1b). Anti-EV71 potency was then measured using an immunofluorescence assay (IFA) on the infected cells treated with IDR at twofold serial dilutions (0.25–4 μM) 12 h after infection. A dose-dependent reduction was detected in the fluorescence foci representing the EV71 antigen-expressing cells (Fig. 1c), and the 50 % effective concentration (EC50) was 0.493 μM (Fig. 1c). Because IDR was selected and assessed using a single-cycle assay, we then proceeded to determine the antiviral activity of IDR by conducting a plaque reduction assay (PRA), which is a multicycle assay. The EC50 of IDR measured using a PRA was 0.538 μM (Fig. 1d). In addition, a cell viability assay to measure the 50 % cytotoxicity concentration (CC50) of IDR was conducted, and the CC50 was 192.1 μM (Fig. 1e). Based on these data, a selectivity index (SI = CC50/EC50) of approximately 389.7 (by IFA) or 357.1 (by PRA) was obtained. It was noted that only minimal cytoxicity was observed in the cells treated with IDR at 4 μM, the highest IDR concentration used to evaluate the antiviral potency in this study (Fig. 1). These data suggested that IDR exerts the antiviral effects independent of cytotoxicity. The antiviral effects of IDR were further investigated on a panel of representative members of EV species, including coxsackievirus A16 (CVA16), CVB1, CVB2, echovirus serotype 9 (Echo9) and Echo30. In all cases, IDR effectively inhibited EV replication, with EC50 and SI values ranging from 0.720 μM to 1.056 μM, and 181.9 μM to 266.8 μM, respectively (Table 1).

Structure–activity relationship

IDR falls into the class of anthracycline unrelated to any of the clinically used antivirals. To validate the anti-EV effects of IDR, two other anthracyclines with highly similar chemical structures, daunorubicin (DNR) and epirubicin (EPI), were assessed (Fig. S1, available in the online Supplementary Material). Both anthracycline analogues displayed inhibitory effects against EV71 and several other EV species, with EC50 values in the ranges of 0.516–1.613 μM for DNR and 0.546–1.825 μM for EPI (Table 1). Anthracyclines are well-known antineoplastic agents that act through DNA intercalation and topoisomerase II (topo2) inhibition (Kizek et al., 2012; Martincic & Hande, 2005). To determine whether the anti-EV effects were associated with topo2 inhibition, two other topo2 inhibitors with chemical structures unrelated to anthracyclines, etoposide and ellipticine, were assessed (Fig. S1). In contrast to the anthracyclines, both the topo2 inhibitors exhibited undetectable anti-EV71 effects even at higher concentrations (8 μM). Taken together, these findings indicate that a common chemical structure shared by these anthracyclines is associated with the anti-EV potency, which is independent of topo2 inhibition.
Fig. 1. IDR treatment inhibited EV71 replication, virus-induced CPE and cell death. Twofold serially diluted IDR at the concentrations indicated was added to RD cell cultures 1 h prior to inoculation of EV71 stock at an m.o.i. of 1.5 (a, b) or 0.1 (c) for 12 h. Compound-free wells contained 0.05 % DMSO. Cell morphology was observed by using a phase-contrast microscope (a). Cell viability was measured by using a CellTiter 96 AQuescent Cell Proliferation Assay and expressed as the percentage of the mock-infected cells (b). An IFA using an anti-EV71 Ab was carried out, and cells were counterstained with Hoechst 33258 dye for the nuclei. EV71 antigen-positive cells and the nuclei were quantified using Metamorph software, and
the antigen-positive cell numbers were normalized by the corresponding nucleus numbers. Data are expressed as EV71 antigen-positive cells (%) obtained in IDR-treated cells relative to the infected, IDR-free cells (c). A PRA was performed, with EC50 value presented (d). CC50 was determined by treating RD cells with twofold serially diluted IDR from 2 to 256 μM for 12 h. Cell viability was determined by the aforementioned method in (b), and expressed as the percentage of the mock-infected cells (e). EC50 (c, d) and CC50 (e) values were calculated using the GraphPad Prism5 software. Data represent means of three samples with SD (*P<0.05, **P<0.01 and *** P<0.001). Bar, 10 μm.

**Mechanism-of-action of IDR**

To identify which stage of virus replication was inhibited by IDR, a time-of-addition experiment was performed. RD cells were incubated with IDR 2 h prior to the end of virus adsorption, during virus adsorption (for 1 h), at the end of viral adsorption, and at 2 h intervals between 1 and 9 h post-adsorption. Infected cells devoid of the IDR treatment were also included (Fig. 2). In all cases, an IFA was conducted at 12 h post-adsorption, and the proportion of EV71 antigen-positive cells relative to that of the infected, untreated cells was determined. Virus antigen expression was noticeably inhibited by IDR even when added at 1 h post-infection (p.i.), and the effect was nearly as strong as when the compound was added during infection (0.86 ± 0.25 % compared with 0.51 ± 0.21 %), indicating that IDR blocked EV71 replication after viral entry. Additionally, virus antigen expression remained low when IDR was added at 3 h and 5 h p.i. (11.53 ± 2.65 % and 19.39 ± 4.23 %, respectively), whereas viral antigen expression rose drastically to a high level when added at 7 h p.i. (69.63 ± 6.11 %). These data suggested that IDR exerts its strong anti-EV71 effects prior to 5 h p.i. following viral entry, and that the inhibitory effect declined drastically after 7 h p.i.

Because the screen with the FRET-biosensor cells measured the virus proteolytic process, we investigated if IDR acts against EV71 2Apro or 3Cpro activity by an enteroviral protease assay (Hsu et al., 2007; Tsai et al., 2009). To this end, we used HeLa-G3CwtR cells, another FRET-biosensor cell line that responds specifically to EV71 3C activity (Tsai et al., 2009), in addition to HeLa-G2AwtR cells (Hsu et al., 2007). A 2Apro or 3Cpro expression plasmid was used to transfect HeLa-G2AwtR cells (Fig. 3a) or HeLa-G3CwtR cells (Fig. 3b), respectively, and cells were treated with IDR at various concentrations or left untreated. The FRET-biosensor cells were also infected with EV71 in the absence or presence of IDR to serve as a negative and positive control, respectively. While IDR dose-dependently reversed the FRET levels reduced by EV71 infection, the reduced FRET levels were not elevated by the IDR treatments, regardless of the concentrations, for the

<table>
<thead>
<tr>
<th>EV species</th>
<th>Drug</th>
<th>EC50 (μM)*</th>
<th>SI†</th>
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<tr>
<td>EV71</td>
<td>IDR</td>
<td>0.493 ± 0.133</td>
<td>389.7</td>
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<td></td>
<td>DNR</td>
<td>1.132 ± 0.286</td>
<td>&gt;226.1</td>
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<tr>
<td></td>
<td>EPI</td>
<td>1.415 ± 0.347</td>
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<td>IDR</td>
<td>1.041 ± 0.322</td>
<td>184.5</td>
</tr>
<tr>
<td></td>
<td>DNR</td>
<td>1.378 ± 0.402</td>
<td>&gt;185.7</td>
</tr>
<tr>
<td></td>
<td>EPI</td>
<td>1.825 ± 0.424</td>
<td>&gt;140.3</td>
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<tr>
<td>CVB1</td>
<td>IDR</td>
<td>0.720 ± 0.203</td>
<td>266.8</td>
</tr>
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<td></td>
<td>DNR</td>
<td>0.589 ± 0.185</td>
<td>&gt;434.6</td>
</tr>
<tr>
<td></td>
<td>EPI</td>
<td>0.546 ± 0.168</td>
<td>&gt;468.9</td>
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<tr>
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<tr>
<td></td>
<td>DNR</td>
<td>0.698 ± 0.236</td>
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<td></td>
<td>EPI</td>
<td>0.864 ± 0.219</td>
<td>&gt;296.3</td>
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<td>Echo9</td>
<td>IDR</td>
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<td>EPI</td>
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<tr>
<td></td>
<td>EPI</td>
<td>1.065 ± 0.283</td>
<td>&gt;240.4</td>
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</table>

*Data represent mean values ± SD from triplicate experiments by an IFA on RD cells.
†The CC50 values based on RD cells were 192.1 μM for IDR, and >256 μM for DNR and EPI.

**Table 1. Broad-spectrum anti-EV activity of anthracyclines**

**Fig. 2.** Timing of EV71 inhibition by IDR. IDR at 4 μM was added to RD cells at the times indicated relative to viral infection at an m.o.i. of 0.1, with the initiation (−1 h) and completion (0 h) of viral adsorption indicated. Cells were maintained in the compound throughout the viral infections. RD cells were also infected with EV71 without IDR treatment as a no-drug control (containing 0.05 % DMSO). In all cases, cells were subjected to an IFA at 12 h p.i., and the data expressed using the protocol in Fig. 1c. Data are means from three experiments with SD.
2A<sub>pro</sub>-expressing HeLa-G2AwtR and 3C<sub>pro</sub>-expressing HeLa-G3CwtR cells. The IDR treatments for the 3C<sub>pro</sub>-expressing HeLa-G2AwtR and 2A<sub>pro</sub>-expressing HeLa-G3CwtR cells did not significantly alter the FRET levels (Fig. 3). The data supported the notion that viral 2A<sub>pro</sub> and 3C<sub>pro</sub> exhibited the specific protease activities in the transfected cells and that the activities were not blocked by IDR.

To gain insight into the action mechanism of IDR, we assessed the effects of IDR on the synthesis of viral protein (Fig. 4a) and RNA (Fig. 4b). RD cells were incubated with 0.5–4 μM IDR or left untreated for 1 h as indicated or left untreated. IDR-free wells contained 0.05 % DMSO. Cell lysates were prepared for Western blot analysis by using anti-EV71 VP1 (VP1; 32 kDa) antibody and anti-β-actin (Actin; 43 kDa) antibody (a). Total RNA was prepared and subjected to reverse transcription (RT)-PCR, and results are shown as mean values ± SD (*$P<0.05$, **$P<0.01$ and ***$P<0.001$). In all cases, triplicate cultures were assayed (b).
IDR selectively targets EV IRES

(a) IDR selectively targets EV IRES

(b) IDR

(c) DNR

(d) EPI

(e) Quinacrine

(f) EV71 IRES

** Refer to the figure for detailed analysis.

* http://jgv.microbiologyresearch.org 1127
Fig. 5. Anthracyclines selectively inhibited the EV IRES activities but not the cellular IRES activities. Schematic diagram of dicistronic reporter plasmid (a). Expression of the dicistronic mRNA composed of RLuc, IRES from various sources (EV71, CVA16, Echo9 and p53) and FLuc gene is driven by the T7 promoter (Pt7) for in vitro transcription. RD cells grown in a 96-well Petri dish were transfected with 0.2 μg of in vitro-transcribed dicistronic mRNAs. At 5 h post-transfection, cells were treated with anthracycline derivatives IDR (b), DNR (c), EPI (d) or quinacrine (e), as well as non-anthracyclines etoposide or ellipticine (f) at the concentrations indicated, or left untreated (containing 0.05 % DMSO). Cell lysates were prepared at 20 h post-transfection, then analysed for RLuc and FLuc activity. IRES activity was calculated as FLuc divided by RLuc then plotted relative to the compound-free control (100 %). Each transfection was performed in triplicate with error bars representing sd (*P<0.05, **P<0.01 and *** P<0.001). p53 IRES (1+2) is p53 IRES (1 and 2).

IRI displays stronger affinities to the EV 5′ UTR RNAs

Anthracyclines are known to bind with and intercalate into the DNA double helix (Charak & Mehrotra, 2013; Ozluer & Kara, 2014), in addition to topo2 inhibition, which cannot explain the antiviral effects as demonstrated previously in this study. We hypothesized that the anthracyclines could have exerted varied effects on the EV and p53 IRESs because of their varying binding affinities. Fluorescence-quenching assays effectively measure the structural selectivity of ligand–nucleic acid interaction; this is based on the principle that anthracyclines (the ‘ligand’) are excellent fluorophores, and the intercalation causes a strong fluorescence quenching (Charak & Mehrotra, 2013; Ozluer & Kara, 2014). A fixed concentration of IDR (4 μM) was titrated with the EV 5′ UTR RNAs and cellular p53 IRES RNA samples at 0.25–1 μM, and the fluorescence intensities were measured. Poly(rU) was used as a negative control because it is known to be unstructured with limited binding to the known nucleic acid ligands (Chaires, 2005; Gasparian et al., 2010), whereas a highly polymerized, long-stretch calf thymus DNA that efficiently binds to IDR was used as a positive control (Chaires, 2005; Charak & Mehrotra, 2013; Ozluer & Kara, 2014). EMCV 5′ UTR RNA was also included for comparison. Whereas the fluorescence intensities of IDR titrated with the cellular p53 IRESs were decreased only slightly (<20 %), those of IDR treated with the EV IRES RNAs were decreased markedly (>70 %) in a dose-dependent manner (Fig. 6a). Titration with EMCV IRES RNA resulted in moderate decreases (approx. 50–60 %) in the fluorescence intensities (Fig. S5). These data indicate that IDR displays much stronger binding affinities to the EV IRESs than to the cellular p53 IRESs. The IDR binding efficiencies to the EV IRESs were even greater than those to calf thymus DNA, a reported IDR binder, whereas IDR binding to the poly(rU) was minimal (Fig. 6a). Taken together, these findings are consistent with the proposal that inhibition of IRES-mediated translation by IDR depends on the affinity of binding between IDR and the respective IRES element (Figs 5b, 6a). Moreover, IDR can bind to a specific site or multiple sites in EV71 5′ UTR RNA. To distinguish between these two possibilities, we generated overlapping subfragments A, B and C, ranging from nt 1 to 563, nt 242 to 445 and nt 242 to 743 of the EV71 5′ UTR RNA, respectively. In the fluorescence-quenching assay, titrations with the subfragments of EV71 5′ UTR RNA all resulted in marked decreases in the fluorescence intensity of IDR (Fig. 6b), indicating the possibility that IDR can bind to multiple sites in EV71 5′ UTR RNA.

IDR impairs IRES–ITAF interaction

The translation of the EV71 polyprotein is mediated by a mechanism that relies on interaction between the highly
drug-free control, respectively. Notably, treatment with reduced the binding efficiencies to 63 % and 5 % of the m not markedly impair the binding, IDR at 2
the IRES RNA and hnRNA A1. Although 1
host IRES ITAF), indicating the specific binding between biotin-labelled free RNA, captured hnRNP A1 (a known EV71 ITAF), implying the specific binding of biotinylated RNA to EV71 IRES RNA.

We showed that the biotinylated RNA, but not the unlabelled RNA, captured hnRNP A1 (a known EV71 ITAF), indicating the specific binding between the EV IRES RNA and hnRNP A1. Although 1 μM IDR did not markedly impair the binding, IDR at 2 μM and 4 μM reduced the binding efficiencies to 63 % and 5 % of the drug-free control, respectively. Notably, treatment with quinacrine resulted in a considerable level of reduction in hnRNA A1, whereas etoposide action caused a marked reduction in hnRNA A1 (Fig. 7a), consistent with their respective effects on the EV IRES activity (Fig. 5).

Functional interactions between IRES stem–loop structures with ITAFs are considered critical in promoting IRES activity (Niepmann, 2009; Shih et al., 2011). Six stem–loop subdomains were predicted in EV71 5′ UTR RNA, and the stem–loops II (nt 129 to 167) and VI (nt 566 to 636) displayed strong binding affinities to hnRNAP A1 (Lin et al., 2009a, b). Therefore, a pull-down assay was performed with EV71 5′ UTR RNA subfragments A (covering the stem–loop II), B and C (covering the stem–loop VI) in the presence or absence of IDR at 4 μM. Both subfragments A and C captured hnRNAP A1, and the pull-down activities were severely compromised by IDR action (Fig. 7b). Subfragment B showed minimal binding to hnRNAP A1 with or without IDR, indicating the high specificity of the assay. Together, these data indicate that the selective, multiple binding of IDR to the EV 5′ UTR RNAs (Fig. 6) can result in impairment of EV IRES RNA–ITAF interactions (Fig. 7), and thus the marked decreases in the EV IRES activities (Fig. 5).

**DISCUSSION**

In this study, we identified IDR as a novel, broad-spectrum anti-EV agent. We first validated that IDR exerted anti-EV71 effects at non-cytotoxic concentrations (Fig. 1).
IDR is a known antineoplastic drug, the mechanism of which is primarily attributed to its topo2 inhibition and its actions as a DNA intercalating agent that impairs cellular DNA replication and the subsequent RNA synthesis (Charak & Mehrotra, 2013; Kizek et al., 2012; Martincic & Hande, 2005). By using topo2 inhibitors that are either anthracycline analogues (DNR and EPI) or structurally unrelated (etoposide and ellipticine), we determined that the mode-of-action of the anti-EV potency of IDR is distinct from its topo2 inhibition activity. Time-course studies performed to further elucidate the action mechanism of IDR demonstrated that IDR exerted the maximal effect (>99% inhibition) against EV71 replication prior to 3 h p.i. and continued the inhibitory effect (>80% inhibition) until 5 h p.i. (Fig. 2). These data were consistent with the findings that viral replication at early stages, including the viral translation initiation, protein synthesis, and genome replication, markedly declined following IDR treatment (Figs 4 and 5). The translation of the EV71 RNA genome was mediated by a mechanism that relies on the highly structured IRES element and uses the host ITAFs, which is distinct from the canonical eukaryotic cap-dependent translation (Niepmann, 2009; Shih et al., 2011). Studies have proposed that ITAFs stabilize the functional IRES conformation and/or serve as additional bridges between the IRESs and the ribosomes (Komar & Hatzoglou, 2011). Accordingly, the preferential binding of IDR to EV IRESs and EMCV IRES (albeit to a lesser extent) compared with the cellular IRESs and the long stretch of chromosomal DNA (calf thymus DNA) (Figs 6 and S5) represented a plausible mechanism through which IDR selectively disrupts the assembly of the viral IRES-host protein–ribosome complex, thereby resulting in markedly reduced IRES activity (Figs 5 and S2). Because the viral translation initiation occurs prior to the viral protein synthesis, the observed decreases in viral protein/RNA accumulation caused by IDR (Fig. 4) could be a consequence of the reduced IRES activity. Nevertheless, IDR might exert pleiotropic effects targeting the viral and cellular factors associated with viral protein/RNA accumulation, in addition to the effect that selectively reduced the EV IRES activity. This could include a role that IDR plays as an enhancer in interferon signalling (Patel et al., 2012).

Translation initiation of EVs is mediated by viral IRESs with a highly ordered structure, a unique mechanism distinct from canonical cap-dependent translation initiation exploited by eukaryotic cells. Translation of cellular mRNA can likewise be mediated via initiation at cellular IRESs during many physiological and pathological stress conditions, as exemplified by p53 IRESs (Graber & Holcik, 2007; Komar & Hatzoglou, 2011; Sharathchandra et al., 2014) used in this study (Figs 5 and 6). RNA structures and canonical initiation factors/ITAFs involved in IRES-driven translation initiation appear to vary for IRESs in cellular and viral mRNA (Komar & Hatzoglou, 2011; Niepmann, 2009). The viral IRES region thus represents an ideal target for antiviral therapy. However, only few small molecules are proven to bind the EV IRES directly and impair its activity, including quinacrine (Gasparian et al., 2010; Wang et al., 2013) used here as a control (Figs 5 and 7). This is likely due to the complex, dynamic architecture of EV IRES RNA, rendering this type of nucleic acid entity especially difficult to target with small molecules (Chaires, 2005; Ren & Chaires, 2001). Herein, we add the anthracycline group of drugs to a short list of selective EV IRES antagonists, in that they are at least as potent as quinacrine is (Fig. 5). Structurally, all anthracyclines consist of a planar aglycone moiety and amino sugar ring. These well-known DNA intercalators can insert between adjacent DNA base pairs, using their planar aglycone moiety (Kizek et al., 2012; Martincic & Hande, 2005). The EV IRES element binding moiety of anthracyclines merits further probing. Picornavirus IRESs can be divided into four groups based on secondary structure and requirement for translation initiation factors or ITAFs. EV IRESs and the EMCV IRES are classified into the type I and type II IRESs, respectively (Niepmann, 2009). Further dissection of IDR-binding motifs in the EV IRESs and EMCV IRES might reveal the mechanism underlying the differential IDR binding affinities of the two IRES groups (Fig. S5). Moreover, we showed that IDR binds directly to multiple sites in the viral IRES RNA (Fig. 6b), thereby compromising the two known EV71 IRES-hnRNP A1 interactions (Fig. 7b). Indeed, attempts to select for drug-resistant mutants failed to yield any isolate with this phenotype on repeated passaging in the presence of the drug (data not shown). This could be at least partly explained by the finding that IDR targets multiple sites in the viral 5′ UTR, rendering it unlikely for a single viral genome to mutate simultaneously to confer drug resistance.

Clinically, IDR in conjunction with an anticancer drug is primarily used to treat patients with acute myeloid leukemia in all age groups, including young children who are the main patient population infected by EV71 (Kizek et al., 2012; Martincic & Hande, 2005; Teuffel et al., 2013). Anthracycline therapy poses a complication of adverse myocardial damage from an accumulated anthracycline dose. Nevertheless, several recently developed therapies, including use of an iron-chelating agent, liposomal encapsulation and alternative delivery approach, have substantially reduced the risk of anthracycline cardiotoxicity (Harake et al., 2012; Lipshultz et al., 2004). Moreover, treatment of EV71 acute infection would presumably require a much shorter drug course than cancer treatment does, thereby reducing the drug loading.

In sum, the agent identified, IDR, displays a wide spectrum of anti-EV potency, at least partly by suppressing viral IRES activity, most likely through selective, high-affinity binding with the viral IRES element. Discovery of this approved drug with anti-EV potency raises a possibility of repositioning or repurposing to treat EVs in a rapid, cost-effective manner (Ashburn & Thor, 2004). Well-known dosing and pharmacokinetic profiles of this drug should be useful in investigating anti-EV effects in animal models. Finally, as an alternative...
but not mutually exclusive development, IDR could serve as a lead compound for further optimization.

**METHODS**

**Cells, viruses and reagents.** RD (ATCC, CCL-136), Vero (ATCC, CCL-81), HeLa-G2AwtR and HeLa-G3CwtR cells (Hsu et al., 2007; Tsai et al., 2009) were cultured in minimum essential medium (MEM) (Gibco-BRL) supplemented with 10 % FBS. EV stocks including EV71 (strain BrCr), CVA16, CVB1 and -2, Echo9 and -30 (Tsai et al., 2009) were propagated in RD or Vero cell culture with MEM supplemented with 2% FBS, and titrated on a Vero cell monolayer by using a plaque assay. IDR (11656), DNR (D8809), EPI (E9406) and guanidium hydrochloride (GuHCl) were purchased from Sigma-Aldrich. Etoposide (120227) and quinacrine (120749) were obtained from Abcam, and ellipticine (3357) was from Tocris bioscience. A total of 1280 LOPAC (Sigma-Aldrich) compounds were provided by the Drug Screen Center, National Yang-Ming University, Taiwan. All compounds except the GuHCl (dissolved in a culture medium) were dissolved in DMSO, and the final concentration of DMSO in the culture medium did not exceed 0.05 %, a concentration tolerated by all cell lines tested.

**Plasmids.** The pCMV-FLAG-2A and pCMV-FLAG-3C plasmids, which encode the EV71 2A and 3C protease, respectively, have been described previously (Hsu et al., 2007; Tsai et al., 2009). The dicistronic reporter constructs used in this study were based on the pCRES plasmid, a dicistronic plasmid that encodes Renilla luciferase (RLuc) and firefly luciferase (FLuc), with the EMCV IRES in between (Créancier et al., 2000). The T7 promoter segment was cloned upstream to the dicistronic region. A pair of complementary oligonucleotides, Mull-T7 and T7-Xbal, that harbour the sense and antisense sequences of the T7 promoter with the engineered Mull and XbaI restriction sites at either end were synthesized (Table S1). The Mull-T7 and T7-Xbal oligonucleotides were annealed and subcloned into the corresponding sites in the pCRES plasmid and referred to as pT7-REL. Viral RNA was extracted from the EV71 (BrCr strain), CVA16 and Echo9 stocks, as described previously (Lu et al., 2004). The 5’ UTRs containing the IRES elements of the three EV species were amplified by RT-PCR, using the primer pairs EV71-IF/EV71-IR, CA16-IF/CA16-IR and E9-IF/E9-IR with the engineered restriction sites at either end (Table S1). The p53 IRES (1 and 2) was amplified from the p53 cDNA-containing plasmid pCMV-Neo-Bam p53wt (Baker et al., 1990) using the primer pair p53-IR5RI/p53-IR5R2 (each with the engineered restriction site at either end) (Table S1). The PCR products were restricted and cloned into the BamHI/BgIII sites in the intercistronic region of the pT7-REL plasmid as substitutes for the EMCV IRES. The resulting plasmids were designated pT7-R-EV71-F, pT7-R-E9-F, pT7-R-CA16-F and pT7-R-p53 (IR5RI and -2)-F for the dicistronic reporter constructs with the EV71, Echo9, CVA16 5’ UTR and p53 IRES (1 and 2), respectively.

**PRA.** Twofold serial dilutions of IDR ranging from 0.25 μM to 4 μM were added to confluent Vero cells grown in six-well tissue culture plates for 1 h, followed by inoculation with a viral stock at 80–100 p.f.u. Cells were then overlaid with a medium containing 0.8 % agar and 2 % FBS. The assay cultures were incubated for 4 days, and the plaques were counted after fixation and staining with a crystal violet formalin solution (Tsai et al., 2009). The EC50 value was calculated using GraphPad Prism5 (GraphPad Software).

**Cell viability assay.** Drug concentrations were a twofold serial dilution ranging from 2 μM to 256 μM. Cell viability was determined after 12 h of treatment using the CellTitre 96 AQueous Cell Proliferation Assay (Promega) as described by Lu et al. (2015). The CC50 value was calculated using GraphPad Prism5.

**IFA.** RD cells grown in 24-well dishes were fixed with 4 % paraformaldehyde and penetrated using 0.2 % Triton X-100. The following antibodies (Abs) were used to probe EV species: for EV71 and CVA16, mouse anti-EV71 Ab (1 : 1000; MAB979 Chemicon); for CVB1 and -2, mouse anti-coxsackie virus B blend Ab (1 : 1000; MAB9410 Chemicon); for Echo9 and -30, mouse anti-echovirus blend Ab (1 : 1000; MAB9670 Chemicon). A FITC-conjugated goat anti-mouse Ab (1 : 100; Jackson) served as a secondary Ab. The nuclei were counterstained with Hoechst 33258 (0.5 mg ml–1; Sigma). The cells were viewed using a fluorescence microscope (DM6000B, Leica) equipped with both FITC and UV filters (Lu et al., 2004). EV antigen-positive cells and Hoechst-positive cells from each field were counted and analysed using the associated MetaMorph software.

**In vivo viral protease assay.** The in vivo viral protease assay followed a protocol described previously (Tsai et al., 2009) but with certain modifications. An approximately 70 % confluent monolayer of the HeLa-G2AwtR and HeLa-G3CwtR cells grown on a black 96-well dish (SPL Life Sciences) was transfected with pCMV-FLAG-2A plasmid (2A pro expression plasmid), or pCMV-FLAG-3C (3C pro expression plasmid) at an amount of 0.2 μg each, using an X-tremeGENE HP DNA transfection kit (Roche), following the manufacturer’s instructions. After 18 h, the culture medium was replaced with MEM-2 with or without IDR. At 36 h post-transfection, fluorescence was measured by the aforementioned protocol to determine the FRET ratios.

**Western blot.** Western blots were conducted following the procedure used by Hsu et al. (2009). Primary Abs used were mouse anti-EV71 VP1 Ab (1 : 1000; MAB1255-M05, Abnova), rabbit anti-icmRNAP1 Ab (1 : 1000; AB17752, Abcam), and anti-beta-actin monoclonal Ab (1 : 1000; MAB1501, Chemicon) as a loading control. Horseradish peroxidase (HRP)-conjugated goat anti-mouse Ab (1 : 1000; sc-2060, Santa Cruz) or HRP-conjugated goat anti-rabbit Ab (1 : 1000; sc-2004, Santa Cruz) was used as a secondary Ab. Proteins were detected using an enhanced chemiluminescence (ECL) Western blot kit (Amersham).

**RNA extraction and RT-PCR.** Total cellular RNA was extracted using TRizol reagent (Invitrogen). An RT reaction was carried out using AMV Reverse Transcriptase XL (Takara) with 1 μg of total RNA, and real-time PCR was conducted with a FastStart Universal SYBR Green Master kit (Roche Applied Science), according to the manufacturer’s instructions. PCR primer pairs used were VP1-F and VP-R, which target the VP1 region of the EV71 (BrCr strain) genome (Lu et al., 2004), as well as Actin-F and Actin-R (Table S1), which detect human beta-actin as an internal control. The reaction parameters followed the protocol described by Lu et al. (2004).

**IRES activity.** The IRES activity was measured by a dual luminescence reporter system. To generate the in vitro transcript, the capped dicistronic mRNA was synthesized from the dicistronic reporter plasmids (linearized with a XbaI restriction enzyme) using an AmpliCap-Max T7 High Yield Message Maker kit (CellScript) and purified using a MEGAclear kit (Ambion). The integrity of the dicistronic mRNA was validated using gel fractionation. The in vitro-synthesized RNAs at 0.2 μg were transfected into RD cells grown in a 96-well plate dish by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The culture medium was replaced with MEM-2 with or without the test drugs 5 h post-transfection. Cell lysates were prepared at 20 h after the transfections, and RLuc and FLuc activities were measured by a dual luciferase reporter assay kit (Promega) using a luminometer (1420 Victor2, Wallac).

**Fluorescence-quenching assay.** Direct IDR-IRES RNA interaction was analysed based on the principle of fluorescence quenching referred to as a decrease in fluorescence intensity of a fluorophore, in...
this case IDR, when it interacts with the base pairs of nucleic acids (Charak & Mehrotra, 2013; Ozuer & Kara, 2014). The RNA fragments corresponding to various IRES elements were generated using in vitro transcription from corresponding PCR fragments carrying the T7 promoter. The aforementioned IRES-containing dicistronic reporter plasmids were used as templates. The following primer pairs were used to obtain the relevant PCR fragments: for the EV71 5′ UTR, T7-EV71-IF and EV71-IR; for the CVA16 5′ UTR, T7-CVA16-IF and CA16 IR; for the Echo9 5′ UTR, T7-E9-IF and E9-IR; for the p53 IRES (1 and 2), T7-p53-IR1F and p53-IR2R; for the p53 IRES (2), T7-p53-IR2F and p53-IR2R; for the EV71 5′ UTR subfragment A (nt 1 to 563), T7-EV71-IF and EV71-IR (563); for the EV71 5′ UTR subfragment B (nt 242 to 445), T7-EV71-IF (242) and EV71-IR (445); for EV71 5′ UTR subfragment C (nt 242 to 743), T7-EV71-IF (242) and EV71-IR. In vitro transcription was performed as described in the aforementioned protocol. In addition, poly(rU) (mean length, 250–300 nt; Sigma-Aldrich) and calf thymus DNA (polymerized, long-stretch DNA; Sigma-Aldrich) were included as a negative and a positive control, respectively. All RNA samples and the controls were dissolved in 90 μl distilled H2O, heated to 90 °C for 2 min and then gradually (15–20 min) cooled to room temperature to allow appropriate folding of the secondary structures. For a fluorescence-quenching assay, 10 μl of IDR was mixed with the RNA samples to reach a final concentration of IDR at 4 μM and RNA samples at 0.25–1 μM, and incubated for 1 h at room temperature. The fluorescence intensity of each reaction was determined using a Multimode Microplate Reader (Tecan) at 571 nm after excitation at 482 nm.

### RNA–protein pull-down assay

The IRES RNA–ITAF interaction in the presence of IDR action was studied in a pull-down assay conducted as described by Lin et al. (2009) and Shih et al. (2011) with some modifications. Streptavidin beads were used to capture biotinylated EV71 5′ UTR RNA bound to ITAFs in HeLa cell lysates, followed by protein elution and probing by Western blot.

Biotinylated RNA was synthesized by using an AmpliCap-Max T7 High Yield Message Maker Kit (CellScript) with the addition of bicinchoninic acid (BIA) and streptavidin (Ambion).

HeLa cell monolayer (1 × 10^7 cells) was harvested and washed with 50 mM HEPES/KOH (pH 7.5), 146 mM NaCl, 11 mM glucose three times. The cells were suspended in 90 μl hypotonic buffer [10 mM HEPES/KOH (pH 7.5), 15 mM KCl, 1.5 mM magnesium acetate, 6 mM 2-mercaptoethanol] and kept on ice for 10 min before sonication for 30 s. After sonication, cell suspension was centrifuged at 14,000 g for 10 min. The supernatant was designated a HeLa cell lysate.

To conduct a binding reaction, 300 μg HeLa cell lysate, 12.5 pmol of biotinylated RNA and IDR (0–4 μM) in 100 μl binding buffer [5 mM HEPES (pH 7.1), 40 mM KCl, 0.1 mM EDTA, 2 mM MgCl2, 2 mM DTT and 1 unit RNasin] was prepared, followed by a 30 min incubation at 30 °C. A total of 300 μl of streptavidin beads (ZS481, Promega) was then added to each reaction mixture for a 10 min incubation at room temperature. Beads were washed five times with the binding buffer. The RNA–protein complex was captured by the magnetic stand (Z5332, Promega), and the supernatant carefully removed. Bound proteins were eluted with SDS sample buffer and analysed using Western blots with anti-hnRNP A1 Ab.

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