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Short communication

A novel nucleoside analog, 1-β-D-ribofuranosyl-3-ethynyl-[1,2,4]triazole (ETAR), exhibits efficacy against a broad range of flaviviruses *in vitro*

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ABSTRACT

Antiviral therapies are urgently needed to control emerging flaviviruses such as dengue, West Nile, and yellow fever. Ribavirin (RBV) has shown activity against flaviviruses in cultured cells, but efficacy in animal models has generally been poor. In a preliminary screen of novel, synthetic 1-β-D-ribofuranosyl-azole analogs, two compounds, 1-β-D-ribofuranosyl-3-ethynyl-[1,2,4]triazole (ETAR) and 1-β-D-ribofuranosyl-4-ethynyl-[1,3]imidazole (IM18), significantly reduced the replication of dengue virus serotype 2 (DENV-2) in cultured Vero cells. In the current study we demonstrated that the effective concentration 50 (EC_{50}) of ETAR for DENV-2 is substantially lower than both IM18 and RBV. Moreover, ETAR reduced the replication of five additional flaviviruses, including DENV serotypes 1, 3 and 4, Langat virus and Modoc virus, ≥1000-fold relative to untreated controls. Addition of exogenous guanosine to DENV-2 infected cells negated the antiviral effects of both RBV and ETAR, indicating that GTP depletion is a major mechanism of action for both drugs. ETAR represents a promising drug candidate for the treatment of flavivirus infections.

The genus *Flavivirus* (family *Flaviviridae*) consists of more than 80 species of single-stranded, positive-sense RNA viruses (Cook and Holmes, 2006), including a number of globally significant emerging pathogens such as dengue virus (DENV) (Kyle and Harris, 2008), West Nile virus (WNV) (Kramer et al., 2008), and tick-borne encephalitis virus (Randolph, 2009). Effective antiviral therapies, currently unavailable for any flavivirus, are urgently needed to ameliorate the disease burden imposed by flaviviruses (Ghosh and Basu, 2008; Sampath and Padmanabhan, 2009; Stein and Shi, 2008). Ribavirin (RBV) has shown activity against all flaviviruses tested in a broad array of cell types *in vitro* but efficacy *in vivo* has generally been poor (Monath, 2008; Sampath and Padmanabhan, 2009). Additionally, RBV can be toxic *in vivo* (Bodenheimer et al., 1997; Russmann et al., 2006). A compound that exhibited a lower effective dose and toxicity than RBV while retaining its broad spectrum of activity would be particularly desirable as a candidate flavivirus therapy (Sampath and Padmanabhan, 2009).

We have previously synthesized a panel of 21 novel nucleoside analogs, some based on the structure of RBV (Chung et al., 2008; Kumarapperuma et al., 2007). One of these compounds, 1-β-D-ribofuranosyl-3-ethynyl-[1,2,4]triazole (ETAR) inhibited the replication of Hantaan and Andes virus with effective concentration 50 (EC_{50}) values of 10 and 4.4 μM, respectively (Chung et al., 2008). A previous screen at 50 μM showed that two of the 21 compounds, ETAR and 1-β-D-ribofuranosyl-4-ethynyl-[1,3]imidazole (IM18), inhibited DENV serotype 2 (DENV-2) replication in Vero cells by ≥tenfold. Both compounds possess an ethynyl group and isostructural relationship to RBV through replacement of the 3-carboxamide group of the parent scaffold (Fig. 1A).

Efficacy of both compounds was compared to RBV to assess the relative effect of the alkyne-substituents and the effect of replacing a nitrogen atom with CH at the 2-position of the heterocycle. Each compound was diluted in water to create a 10 mM stock and subsequently diluted in cell culture media. Vero cells were grown to confluency in 24-well plates as previously described (Hanley et al., 2003), media was removed, DENV-2 was added at a multiplicity of infection (MOI) of 0.1 in 100 μL of media and allowed to adsorb for 2 h, and then 900 μL of each compound was added to quadruplicate wells in serial twofold dilutions, giving final concentrations ranging from 400 to 1.6 μM. Control cells were infected and mock-treated with media. Cells were incubated for 5 days and supernatants were harvested and titered via serial dilution followed by immunostaining as previously described (Hanley et al., 2003). The EC_{50} and EC_{90} of each compound were determined using a 4 parameter, nonlinear regression of dose response inhibition by plotting log (inhibitor (concentration)) vs. viral titer (variable slope) using GraphPad Prism (GraphPad Software, San Diego, CA).

Abbreviations: ETAR, 1-β-D-ribofuranosyl-3-ethynyl-[1,2,4]triazole; IM18, 1-β-D-ribofuranosyl-4-ethynyl-[1,3]imidazole; DENV, dengue virus; LGTV, Langat virus; MODV, Modoc virus; WNV, West Nile virus; RBV, ribavirin.

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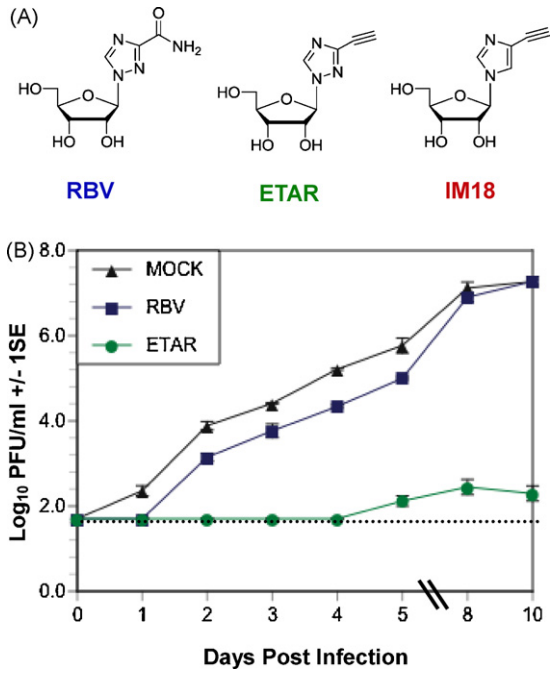

Fig. 1. (A) Structure of RBV, ETAR, and IM18. (B) Replication kinetics of DENV-2 in Vero cells infected at MOI 0.1 and treated with 50 μM ETAR, RBV, or media (mock) 2 h post-infection. Dashed line indicates limit of detection of the assay.

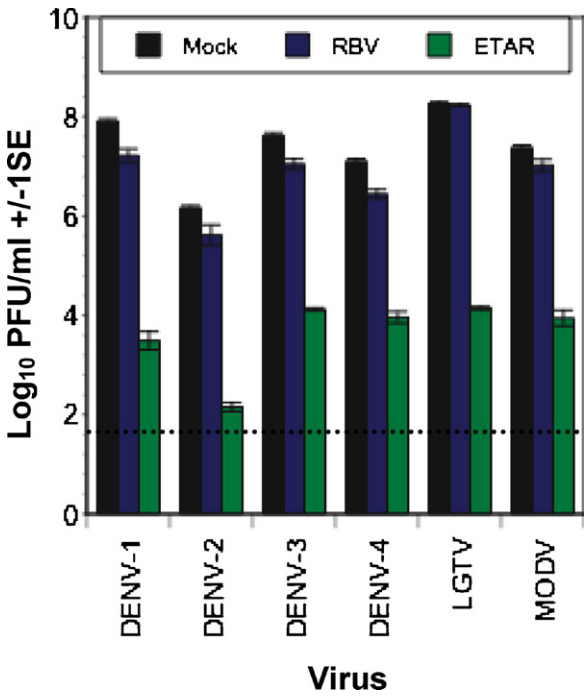

Fig. 2. Efficacy of 50 μM ETAR against six flaviviruses in Vero cells. Dashed line indicates limit of detection of the assay.

The EC_{50} of ETAR was 9.5 μM, an order of magnitude lower that that of RBV, which was 73.2 μM, a typical value for the efficacy of RBV against DENV infecting this cell type (Buckwold et al., 2007; Crance et al., 2003; Day et al., 2005; Huggins et al., 1984; Julander et al., 2007; Kirsi et al., 1983; Leyssen et al., 2000; Van Aerschot et al., 2003). The EC_{50} of IM18, 106.1 μM, was similar to that of RBV and thus IM18 was not characterized further. The EC_{90} values were 176.9, 259.7, and 402.9 μM for ETAR, RBV, and IM18, respectively.

To measure the effect of ETAR on virus replication kinetics, replicate wells of Vero cell monolayers were infected with DENV-2 and treated with ETAR or RBV or mock-treated with media as described above, and cell supernatants were harvested from quadruplicate wells from each treatment on days 0-5, 8 and 10 post-infection. Treatment with 50 μM ETAR delayed the onset of detectable replication by four days and suppressed titer at day 5 post-infection 100,000-fold relative to the control (Fig. 1B). In contrast, treatment with 50 μM RBV delayed the onset of detectable virus replication by only one day and no difference in virus titer between the RBV treatment and the control treatment was evident by day 5 post-infection (Fig. 1B). The data in Fig. 1B are representative of multiple, similar experiments that we have conducted, in which treatment with 50 μM ETAR delayed viral replication by 2–4 days and suppressed peak titer by 1000- to 100,000-fold. To determine the breadth of efficacy of ETAR against flaviviruses, Vero cell monolayers were infected as described above with DENV-1, 2, 3 or 4, Langat virus (LGTV) or Modoc virus (MODV) at MOI 0.1 in triplicate and treated with 50 μM ETAR or RBV or mock-treated with media. ETAR inhibited the replication of all six viruses by ≥1000-fold (Fig. 2) relative to mock-treated cells while RBV caused only about a fivefold suppression. In this experiment ETAR suppressed the replication of DENV-2 by 10,000-fold, slightly less than its effect in Fig. 1B. In many similar experiments we have conducted, the minimum suppression of any of the designated flaviviruses treated with 50 μM ETAR was 100-fold. In combination with the finding that ETAR inhibits the replication of bunyaviruses (Chung et al., 2008), these data suggest that ETAR may possess the same broad spectrum of activity as RBV.

To assess toxicity of ETAR, Vero cells were propagated to confluency in 96-well tissue culture treated plates and media was removed. RBV or ETAR was added to wells in triplicate to give twofold increasing concentrations from 25 to 1000 μM in a total volume of 100 μL. Mock-treated cells received 100 μL of media. Plates were incubated as described above for 5 days, after which 10 μL of resazurin (In Vitro Toxicology Assay Kit, Sigma–Aldrich, St. Louis, MO) was added to all wells. Plates were incubated 2 h and absorbance was read on a plate reader (TiterTek, Huntsville, AL, USA) at 600 nm. Cytotoxicity for Vero cells was not detected at 1000 μM, the highest concentration used, for ETAR or RBV. Thus the cytotoxic concentration 50 (CC_{50}) of ETAR greatly exceeded its EC_{50}. Chung et al. (2008) also found that ETAR was not toxic to Vero cells at concentrations below 880 μM. However the CC_{50} of ETAR for proliferating CEM cells was 7 μM. ETAR caused no clinical symptoms in suckling mice treated with 12.5 mg/kg, but treatment with 50 mg/kg caused 20% mortality (Chung et al., 2008).

Five different mechanisms for the antiviral action of RBV have been proposed (Graci and Cameron, 2006): (1) inhibition of the enzyme inosine monophosphate dehydrogenase (IMPDH), (2) inhibition of viral RNA capping, (3) inhibition of the viral RNA-dependent RNA polymerase (RdRp), (4) mutagenesis, leading to error catastrophe, and (5) immuno-modulation promoting a Th1 type immune response. Although RBV has been shown to cause error catastrophe and inhibition of methyltransferase in WNV (Day et al., 2005) and DENV-1 (Benarroch et al., 2004), respectively, the major mode of action of RBV against most flaviviruses appears to be inhibition of IMPDH, as demonstrated by the ability of supplementary guanosine to reverse the antiviral effect of RBV (Leyssen et al., 2005; Takhampunya et al., 2006)

To assess the importance of IMPDH inhibition for ETAR activity, Vero cell monolayers were infected with DENV-2 as described above. Triplicate wells were treated with either ETAR or RBV at final concentration of 12.5 or 200 μM, respectively, as described above, to reduce viral titer by at least 50% relative to mock-treated virus controls. Triplicate wells were also treated concurrently with one of the two drugs at the specified concentrations along with either

12.5 μM guanosine (Sigma-Aldrich), adenosine (Sigma-Aldrich) or cytidine (Sigma-Aldrich) Additionally, triplicate wells of DENV-2 infected cells were treated with each nucleoside alone at 12.5 μM, or mock-treated with media. Cell supernatants were harvested 5 days post-infection, stored and titered as described above. Treatment with RBV reduced DENV-2 replication by approximately 100-fold; addition of guanosine but not adenosine or cytidine reversed this effect. Similarly, guanosine but not adenosine or cytidine reversed the 50-fold reduction in titer caused by ETAR. None of the three nucleosides affected the level of viral replication in the absence of an antiviral drug treatment.

Chung et al. (2008) reported that treatment of Vero cells with 42 μM ETAR and 42 μM RBV for 4 h resulted in reduction of GTP levels by 79% and 42%, respectively. In contrast with the findings of the current study, they also found that supplementation with guanosine had little effect on the efficacy of RBV against Hantaan virus (Sun et al., 2007) and curtailed but did not abolish the antiviral effect of ETAR (Chung et al., 2008). In combination, these data suggest that ETAR enacts both direct antiviral effects on bunyaviruses as well as indirect effects mediated by GTP depletion, while its antiviral effects on flaviviruses are wholly attributable to GTP depletion

In conclusion, ETAR is a promising, broad-spectrum antiviral drug with substantially higher efficacy than RBV against flaviviruses and comparable toxicity *in vitro*. Moreover, the findings in the current study suggest that ETAR, like RBV, exerts its antiflaviviral effects via inhibition of IMPDH. The greater efficacy of ETAR *in vitro* offers hope that the drug may be efficacious *in vivo* at significantly lower concentrations than RBV, thereby mitigating its potential toxicity.

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