JVI Journals.ASM.org

Activation of Peripheral Blood Mononuclear Cells by Dengue Virus Infection Depotentiates Balapiravir

Yen-Liang Chen,[a] Nahdiyah Abdul Ghafar,[a] Ratna Karuna,[a] Yilong Fu,[a] Siew Pheng Lim,[a] Wouter Schul,[a] Feng Gu,[a] Maxime Herve,[a] Fumiaki Yokohama,[a] Gang Wang,[a] Daniela Cerny,[b] Katja Fink,[b] Francesca Blasco,[a] Pei-Yong Shi[a]

Novartis Institute for Tropical Diseases, Singapore[a]; Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Singapore[b]

In a recent clinical trial, balapiravir, a prodrug of a cytidine analog (R1479), failed to achieve efficacy (reducing viremia after treatment) in dengue patients, although the plasma trough concentration of R1479 remained above the 50% effective concentration (EC_{50}). Here, we report experimental evidence to explain the discrepancy between the *in vitro* and *in vivo* results and its implication for drug development. R1479 lost its potency by 125-fold when balapiravir was used to treat primary human peripheral blood mononuclear cells (PBMCs; one of the major cells targeted for viral replication) that were preinfected with dengue virus. The elevated EC_{50} was greater than the plasma trough concentration of R1479 observed in dengue patients treated with balapiravir and could possibly explain the efficacy failure. Mechanistically, dengue virus infection triggered PBMCs to generate cytokines, which decreased their efficiency of conversion of R1479 to its triphosphate form (the active antiviral ingredient), resulting in decreased antiviral potency. In contrast to the cytidine-based compound R1479, the potency of an adenosine-based inhibitor of dengue virus (NITD008) was much less affected. Taken together, our results demonstrate that viral infection in patients before treatment could significantly affect the conversion of the prodrug to its active form; such an effect should be calculated when estimating the dose efficacious for humans.

Dengue virus (DENV) is the most prevalent mosquito-borne virus that causes human disease. A recent study estimated that 390 million humans are infected and that 96 million infected humans exhibit disease symptoms annually (1). No licensed vaccine or antiviral for the prevention and treatment of DENV is currently available. Upon transmission by infected mosquitoes, the virus first infects dendritic cells, spreads to lymph nodes, and disseminates to various tissues and organs. Although the sites of DENV replication in natural human infections remain to be conclusively defined, monocytes and macrophages in peripheral blood mononuclear cells (PBMCs) were reported to be major replication sites in patients (2, 3).

Nucleoside analogs represent the major class of antiviral drugs in clinical use (4). To exert antiviral effects, nucleoside analogs must be converted to the triphosphate form (by host and/or viral kinases) before being incorporated into the viral DNA/RNA chain by viral polymerase. Balapiravir is an ester prodrug of the cytidine analog 4′-azidocytidine, also known as R1479 (Fig. 1A). It was originally developed as treatment against hepatitis C virus (HCV) infection (5, 6). Although balapiravir exhibited potency in HCV-infected patients, its clinical development was discontinued due to unacceptable toxicity (7). Since R1479 has anti-DENV activity *in vitro*, balapiravir was repurposed for a phase II trial for treatment of DENV infection. Surprisingly, no viremia reduction was observed in balapiravir-treated dengue patients, even though the maximum concentration in plasma (C_{max}) of R1479 surpassed the compound concentration required to reduce virus production by 50% (the 50% effective concentration [EC_{50}]) (8). Here we provide data showing that the discrepancy between the *in vitro* and *in vivo* efficacy outcomes is likely due to impaired compound metabolism in DENV-infected cells. The lesson learned from the balapiravir study in dengue patients has general implications for the development of prodrugs as therapeutics.

MATERIALS AND METHODS

Antiviral assays. Cryopreserved human PBMCs were purchased from AllCells, LLC (Alameda, California). Written consent from the donors was available for all samples. All experiments involving human PBMCs were approved by the Institutional Review Board at the Novartis Institute for Tropical Diseases prior to the start of the experiments. All compounds were dissolved in 100% dimethyl sulfoxide (DMSO). The final concentration of DMSO was 1% in all assays. For each experiment, cells were resuspended to 1×10^7 cells/ml in RPMI medium supplemented with 1% penicillin-streptomycin solution. Approximately 5×10^5 cells were seeded into each well in a 96-well plate. DENV serotype 2 (DENV-2; strain MY-10340) was preincubated with 0.38 μg/ml chimeric 4G2 antibody (in which the mouse Fc region is replaced with that from human; provided by Paul MacAry, National University of Singapore) for 30 min at 4°C to form a virus-antibody complex before it was added to the PBMCs at a multiplicity of infection (MOI) of 1. The plate was further incubated at 37°C for 1 h before addition of serially diluted compounds. Finally, RPMI medium was added such that the final concentration of the medium contained 2% fetal calf serum. At 24 or 48 h postinfection (p.i.), the viral titers in the culture fluids were quantified using a plaque assay. EC_{50}s were calculated by GraphPad Prism (version 5.02) software (GraphPad Prism, Inc.) using the equation for a sigmoidal dose-response (variable slope).

For the delayed-treatment experiment, human PBMCs were infected with DENV-2 without compound for the first 24 h, as described above. At 24 h p.i., compounds were added to the infected cells at the indicated concentrations. At 48 h p.i., culture fluids were subjected to plaque assay to estimate viral titers. EC_{50}s were calculated as described above.

For activation of PBMCs, PBMCs were pretreated with 5 ng/ml of phorbol myristate acetate (PMA) for 16 h in RPMI medium supple-

Received 27 September 2013 Accepted 14 November 2013
Published ahead of print 20 November 2013
Address correspondence to Pei-Yong Shi, pei_yong.shi@novartis.com.

doi:10.1128/JVI.02841-13

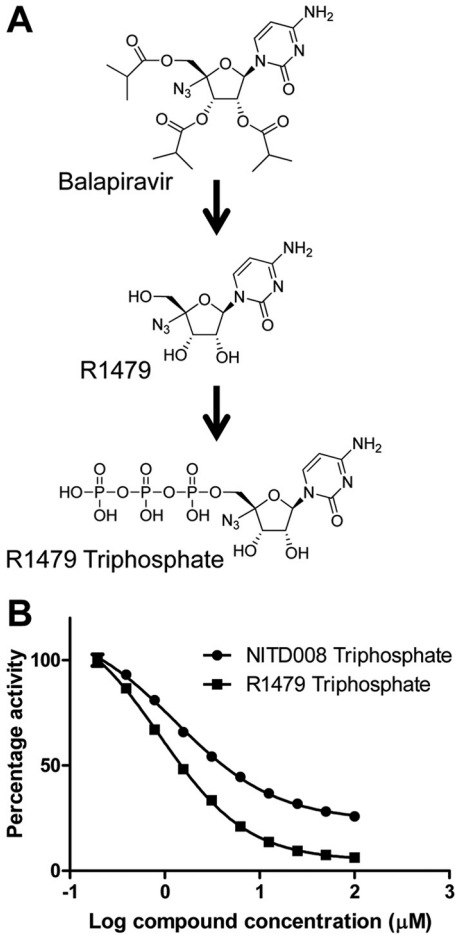


FIG 1 Balapiravir-related structures and R1479 triphosphate-mediated inhibition of DENV polymerase activity. (A) Structures of balapiravir, R1479, and R1479 triphosphate. After oral doing of balapiravir, balapiravir is converted to R1479, followed by R1479 triphosphate. (B) Inhibition of DENV polymerase activity by R1479 triphosphate and NITD008 triphosphate. See Materials and Methods for details. Average results of six independent experiments are shown; error bars represent standard deviations.

mented with 1% penicillin-streptomycin solution and 10% fetal calf serum. On the next day, the cells were washed to remove PMA, infected with DENV-2 (MOI, 1), and immediately treated with compounds, as described above. At 48 h p.i., viral titers in the culture fluids were measured by plaque assay to estimate EC_{50}s.

To determine the effect of cytokines on the EC_{50}s of the compounds, we first treated PBMCs with various cytokines for 16 h prior to viral infection in RPMI medium supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS). The final concentrations for the selected cytokines were 200 U/ml beta interferon (IFN-β), 10 ng/ml gamma interferon (IFN-γ), 25 ng/ml interleukin-10 (IL-10), 100 ng/ml IFN-γ-induced protein 10 (IP-10), 10 ng/ml tumor necrosis factor alpha (TNF-α), or 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). The cells were then spun down and used for infection in the presence of various concentrations of compounds and cytokines at the concentrations stated above. The final medium contained RPMI supplemented with 1% penicillin-streptomycin and 2% FBS. At 48 h p.i., the viral titers in the culture fluids were quantified using a plaque assay.

***In vitro* RdRp assay.** R1479 triphosphate was synthesized in-house. The inhibitory activity of the compound against full-length NS5 of DENV-4 was measured using a modified elongation RNA-dependent RNA polymerase (RdRp) assay as previously described (9, 10). Briefly, a cDNA representing 244-nucleotide single-strand heteropolymeric RNA [sshRNA; containing the sequence 5′-$(TCAG)_{20}(TCCAAG)_{14}(TCAG)_{20}$-3′] was prepared using overlapping PCR, cloned into pUC57 under the control of the T7 polymerase promoter, and verified by sequencing. The sshRNA transcripts, predicted to be devoid of secondary structure by RNA structure (version 3.5) software (11), were transcribed *in vitro* using a RiboMAX large-scale RNA production system T7 kit (Promega). After purification, 2.5 μM sshRNA was annealed with individual primers at a ratio of 1:2 by heating at 95°C for 5 min, followed by cooling to room temperature. The primers used were C1 (3′-AGTCAGTCAGTCAGTGT-5′), A1 (3′-GTCAGTCAGTCAGTCTC-5′), G1 (3′-TCAGTCAGTCAGT CACA-5′), and T1 (3′-CAGTCAGTCAGTCAGAG-5′).

For determination of the 50% inhibitory concentration (IC_{50}) using an elongation assay, 5 μl of serially diluted compounds was mixed with 15 μl of DENV-4 full-length NS5 in 50 mM Tris HCl, pH 7.5, 10 mM KCl, 0.5 mM $MnCl_2$, and 0.01% Triton X-100 for 20 min at room temperature. Thereafter, 10 μl of the RNA and nucleoside triphosphate mixture was added to the wells to start the reaction. The final concentrations of RNA and protein were 100 and 200 nM, respectively. For testing NITD008 triphosphate (an ATP analog), 1.5 μM 2′-[2-benzothiazoyl]-6′-hydrobenzothiazole (BBT)-ATP together with 0.5 μM GTP, CTP, and UTP was used. For testing R1479 triphosphate, 0.5 μM BBT-CTP together with 0.5 μM ATP, GTP, and UTP was used. The plate was sealed, shaken, centrifuged, and incubated at room temperature for 90 min. For terminating the reaction, 20 μl of 2.5× Stop buffer (200 mM NaCl, 25 mM $MgCl_2$, 1.5 M deoxyethanolamine (DEA), pH 10; Promega) containing 25 nM calf intestinal alkaline phosphatase (CIP; NEB) was added to the reaction mixtures. After further incubation at room temperature for 60 min, the plate was read at excitation and emission wavelengths of 422 nm and 566 nm, respectively (10). The values for the IC_{50} were similarly calculated using GraphPad Prism (version 5.02) software as described above.

Cytokine release from DENV-infected human PBMCs. Human PBMCs were infected with DENV-2 as described above. At 24 and 48 h p.i., culture fluids were subjected to IL-10 (Biolegend), TNF-α (Biolegend), and IFN-β (PBL Interferon Source) measurements by enzyme-linked immunosorbent assays according to the manufacturers' instructions.

Analysis of intracellular R1479 triphosphate. PBMCs were incubated in RPMI medium containing 2% FBS, 1% penicillin-streptomycin, and various concentrations of R1479. After the intracellular conversion into the corresponding triphosphate reached steady state (24 h at 37°C), the cells were spun down, washed with cold 0.9% NaCl solution in 1 mM HEPES, pH 7.5, and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Igepal CA-630) containing protease and phosphatase inhibitors (Roche Diagnostics). After lysing the cells at room temperature for 10 min, the cell debris was spun down at 13,000 × *g* and 4°C for 20 min. For every million PBMCs, 100 μl of RIPA buffer was used for lysis. The lysates were transferred into new tubes, snap-frozen in liquid N_2, and stored at −80°C. For bioanalytical studies, 50 μl of the PBMC lysate (in RIPA buffer) was mixed with 150 μl acetonitrile containing 1% NH_4OH and 3 μg/ml Br ATP as an internal control. The amount of triphosphate nucleoside analog was measured by liquid chromatography-tandem mass spectrometry. Since the volume of individual PBMCs was previously reported to be about 300 fl (12), the intracellular concentration of the triphosphate nucleoside analog was calculated (amount of triphosphate nucleoside analog per cell volume). The intracellular concentration of the triphosphate nucleoside analog was then plotted against the concentrations of the test compound used in the incubations. Using Michaelis-Menten kinetics with the formula $Y = A[X]/B + [X]$, where Y is the concentration of triphosphate R1479, A is the calculated maximum concentration of triphosphate R1479 extrapolated from the graph, $[X]$ is the concentration of R1479, and B is the calculated concentration of R1479 at which its triphosphate R1479 reached half of its maximal value, the intracellular concentration of the

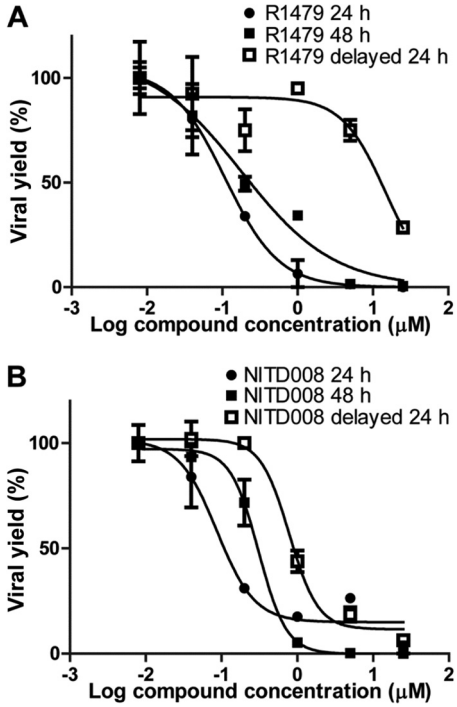


FIG 2 Effect of treatment procedure on the EC_{50}s of the compounds tested. PBMCs were infected with DENV-2 at an MOI of 1 and immediately treated with the indicated compounds; viral titers in the culture fluids were quantified at 24 h or 48 h postinfection. Alternatively, PBMCs were infected with DENV-2 for 24 h, after which the infected cells were treated with the indicated compounds for 24 h (labeled "delayed 24 h"). Viral titers in the culture fluids were measured by plaque assay. EC_{50} curves for R1479 (A) and NITD008 (B) are shown. Average results of five independent experiments are shown; error bars represent standard deviations.

triphosphate nucleoside analog at which 50% of the virus yield was inhibited (TP_{50}) was derived at the EC_{50}.

Pharmacological analysis and *in vivo* mouse efficacy. Pharmacological exposure of balapiravir was analyzed in CD-1 female mice. Briefly, CD-1 mice were dosed with 28.1 mg/ml of balapiravir dissolved in 5% (vol/vol) Solutol HS15 nonionic solubilizer and emulsifying agent in 50 mM citrate buffer, pH 3, via oral gavage. To calculate the bioavailability and half-life of balapiravir in plasma, mice were similarly dosed with 5.7 mg/kg of body weight of balapiravir via intravenous injection. For the efficacy study, AG129 mice deficient in IFN-α/β and IFN-γ receptors were used in a mouse DENV infection model (13) to test the antiviral activity of balapiravir. Briefly, DENV-2 strain TSV01 was injected intraperitoneally at 1.8×10^6 PFU per mouse. Immediately after viral inoculation, 0.2 ml of compound dissolved in the same vehicle used in the pharmacological experiment was given to AG129 mice via oral gavage twice per day for three consecutive days. On day 4, blood samples were taken and viral titers were determined using a plaque assay (13). Statistical analysis was performed by the log-rank test and Student's *t* test using SigmaPlot/SigmaStat software (Systat Software Inc.).

All experiments involving animals were approved by the institutional review board (IRB) of the Novartis Institute for Tropical Diseases prior to the start of the experiments.

RESULTS

R1479 triphosphate inhibits DENV polymerase-mediated RNA synthesis. Figure 1A shows the key molecules during conversion of balapiravir to its active triphosphate form. The ester moieties of balapiravir are removed after gut absorption, generating R1479 as the major circulating form in plasma. After uptake of R1479 into cells, cellular kinases convert R1479 to its triphosphate form, which is used by DENV polymerase and results in termination of viral RNA synthesis. Factors that change any step of the above-described process could affect the *in vivo* efficacy. To examine whether balapiravir has genuine anti-DENV activity, we chemically synthesized the triphosphate form of R1479 and tested its activity in a biochemical DENV polymerase assay. As a positive control, we included a triphosphate form of NITD008, a known adenosine analog inhibitor of DENV polymerase (14). The triphosphate molecules of both R1479 and NITD008 inhibited RNA synthesis, with IC_{50}s of 0.89 and 1.29 μM, respectively (Fig. 1B). The result demonstrates that R1479 triphosphate has intrinsic activity against DENV polymerase.

Decreased potency of R1479 in delayed treatment of DENV-infected PBMCs. We examined the activity of R1479 against DENV-2 in primary human PBMCs. Initially, we treated the PBMCs with R1479 immediately after infection. R1479 showed EC_{50}s of 0.103 and 0.249 μM when infected PBMCs were treated for 24 and 48 h, respectively (Fig. 2A). These EC_{50}s are more potent than the EC_{50}s previously reported for primary human macrophages (1.3 to 3.2 μM) (8). The difference in EC_{50}s between the two studies could be in part due to the different methods used to quantify virus: reverse transcription-PCR in the previous study (8) and plaque assay in the current study. As a control, NITD008 exhibited EC_{50}s of 0.179 and 0.275 μM when the infected PBMCs were treated for 24 and 48 h, respectively (Fig. 2B). The result indicates that R1479 has potent anti-DENV activity when cells are treated immediately after infection.

We reasoned that when patients received balapiravir, DENV infection had already been well established. Indeed, patients recruited in the clinical trial had a median interquartile range (IQR) of illness duration of 27.5 to 45.13 h (8). The EC_{50} derived from delayed treatment should be more reflective of the therapeutic condition. We therefore performed a delayed-treatment experiment. Specifically, R1479 was added to DENV-2-infected PBMCs at 24 h p.i.; viral titers were quantified at 48 h p.i. (giving a total treatment time of 24 h). Surprisingly, the delayed treatment resulted in an EC_{50} of 12.85 μM (Fig. 2A). Compared with the EC_{50} derived from the immediate 24-h treatment (0.103 μM), R1479 lost its potency by 125-fold in the delayed treatment. The results demonstrate that viral infection could change the potency of balapiravir in human PBMCs. As a control, the potency of NITD008 decreased by 7.2-fold, with a change in the EC_{50} from 0.179 μM in the immediate treatment to 1.288 μM in the delayed treatment (Fig. 2B; Table 1).

Intracellular conversion of R1479 to its triphosphate form. To exclude the possibility that the observed antiviral potency was due to an unknown off-target effect, we directly monitored the

TABLE 1 EC_{50} values of R1479 and NITD008 in DENV-infected PBMCs with different treatment procedures

Treatment	EC_{50} (μM)	
	R1479	NITD008
Immediate, 24 h	0.103 ± 0.013	0.179 ± 0.081
Immediate, 48 h	0.249 ± 0.016	0.275 ± 0.122
Delayed, 24 h	12.85 ± 4.00	1.288 ± 0.244
Immediate, 48 h, PMA-activated cells	4.86 ± 1.21	0.566 ± 0.039

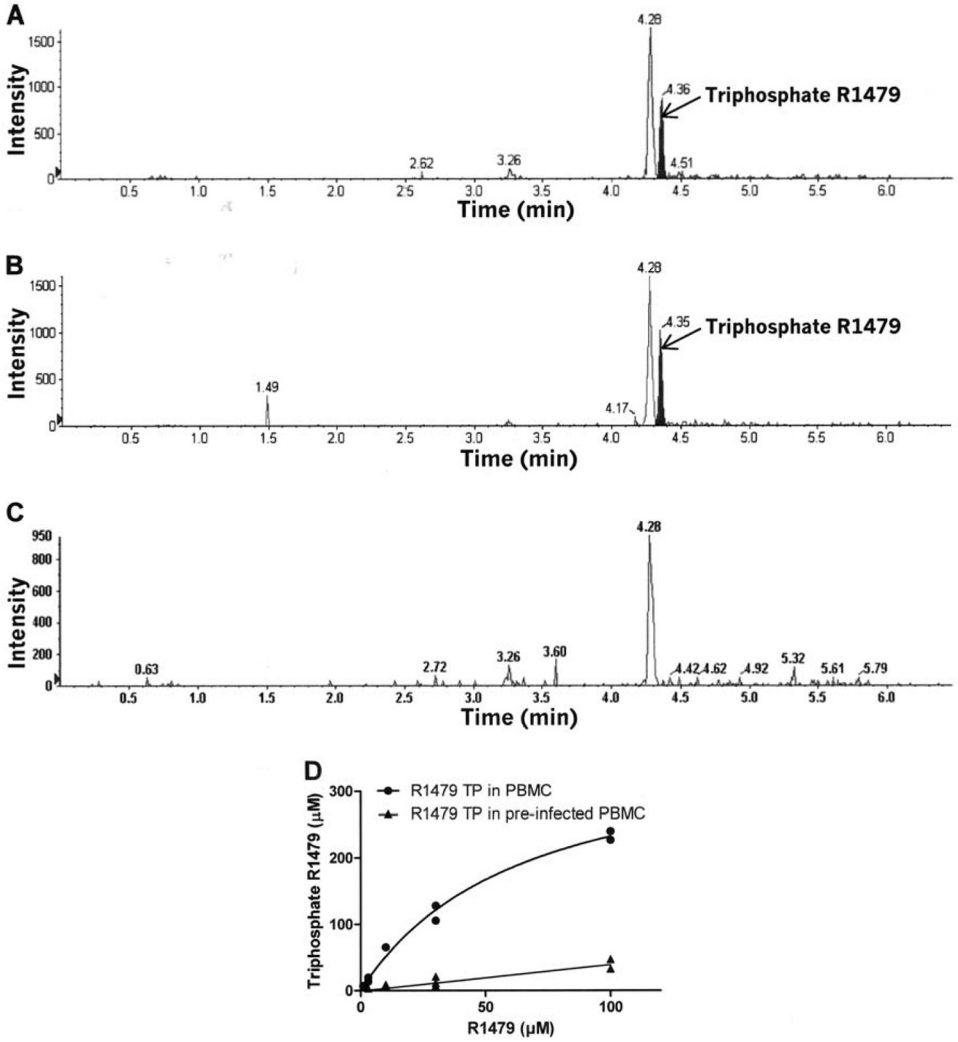


FIG 3 Measurement of intracellular R1479 triphosphate. (A to C) High-pressure liquid chromatography profiles of PBMC lysates spiked with 14.5 μM R1479 triphosphate (A), PBMC lysates after 24 h of incubation with 3 μM R1479 in the medium (B), and PBMC lysates without incubation with any compound (C). (D) Determination of the intracellular concentration of R1479 triphosphate at 24 h of incubation. Various concentrations of R1479 were incubated with PBMCs that were mock infected or preinfected with DENV-2 for 24 h. After 24 h of compound incubation, the cells were lysed and quantified for R1479 triphosphate. The best-fitting curves were generated using the Michaelis-Menten equation. One outlier data point at the 100 μM concentration under the preinfection condition was omitted in the plotting.

formation of R1479 triphosphate inside the cell. The identity of the intracellular derivative(s) of R1479 was monitored by mass spectroscopic analysis using a chemically synthesized R1479 triphosphate as a standard. The synthetic R1479 triphosphate appeared at an elution time of 4.36 min (Fig. 3A). After incubating PBMCs with 3 μM R1479 for 24 h, approximately 5.08 pmol of intracellular R1479 triphosphate was generated per million cells (Fig. 3B). No R1479 triphosphate was detected from cells without R1479 treatment (Fig. 3C). Since the average volume of individual PBMCs is about 300 fl (12), the intracellular concentration of R1479 triphosphate upon incubation with 3 μM R1479 was calculated to be 16.9 μM. Using the same method, we determined the intracellular concentrations of R1479 triphosphate after incubating PBMCs with various concentrations of R1479 (Fig. 3D); these data allowed us to estimate the intracellular concentration of R1479 triphosphate in PBMCs after 24 h of immediate treatment (when they were incubated with the EC_{50} of R1479 in the medium) to be 0.98 μM (TP_{50}). The TP_{50} value of R1479 in PBMCs is close to the IC_{50} (0.89 μM) derived from the polymerase enzyme assay (Fig. 1B).

As a positive control, we also quantified intracellular NITD008 triphosphate after incubating PBMCs with various concentrations of NITD008 under the same conditions (data not shown). The TP_{50} of NITD008 was estimated to be 1.89 μM, close to the IC_{50} (1.29 μM) of NITD008 triphosphate from the polymerase assay (Fig. 1B). Collectively, the results demonstrate that intracellular R1479 triphosphate is responsible for the antiviral activity observed in cell culture.

Next, we measured the intracellular concentration of R1479 triphosphate in PBMCs that were preinfected with DENV-2 (Fig. 3D). Specifically, PBMCs were first infected with DENV-2 for 24 h, the infected cells were incubated with various concentrations of R1479 for another 24 h, and the intracellular concentrations of R1479 triphosphate were measured. As shown in Fig. 3D, com-

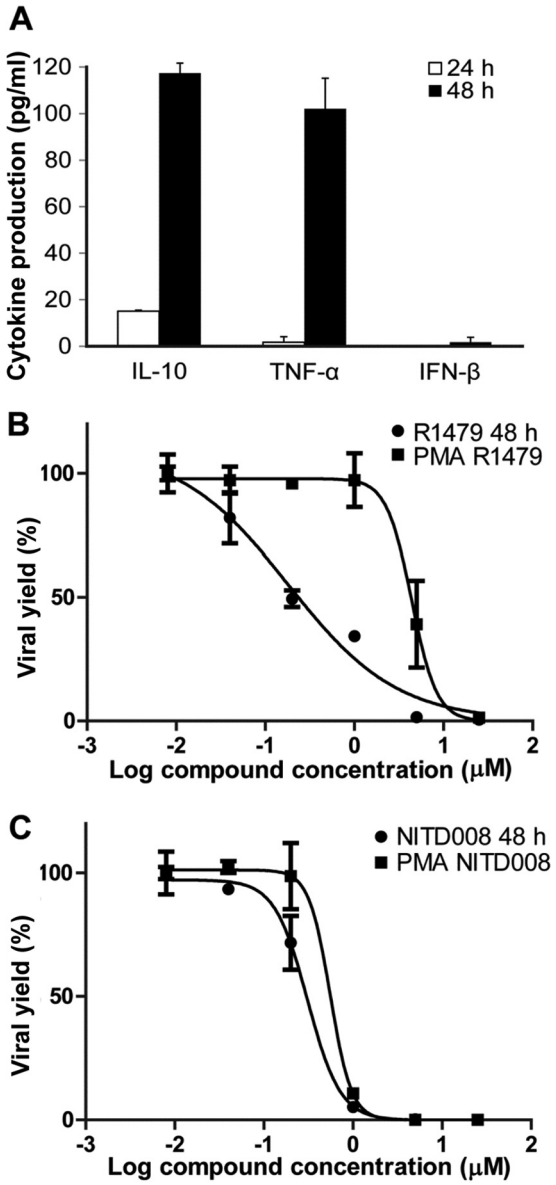
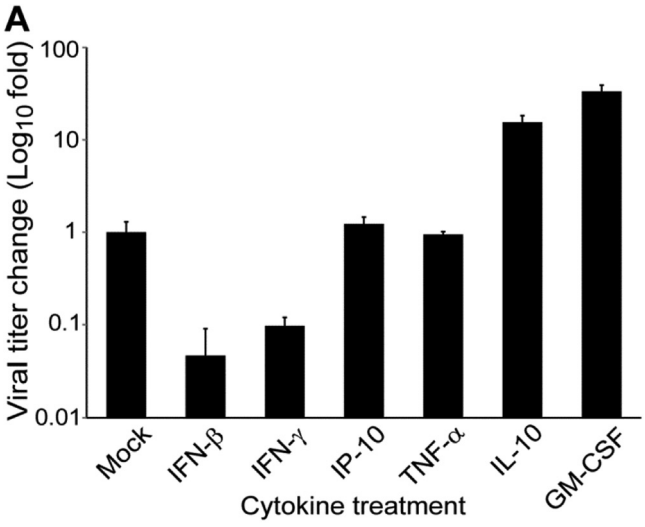


FIG 4 (A) Cytokine production after PBMCs were infected with DENV-2 for 24 h and 48 h. PBMCs were infected with DENV-2 at an MOI of 1; the indicated cytokines were measured at 24 and 48 h postinfection. (B and C) PBMCs were first activated with PMA for 24 h; the activated cells were infected with DENV-2 at an MOI of 1 and immediately treated with R1479 (B) or NITD008 (C); viral titers in the culture fluids were quantified by plaque assay at 48 h postinfection. Average results of four experiments are shown; error bars represent standard deviations.



B

		Mock	IP-10	TNF-α	IL-10	GM-CSF
R1479	EC_{50}	0.25	1.57	4.73	4.81	2.45
	Fold change	1	6.3	18.9	19.2	9.8
NITD008	EC_{50}	0.28	0.37	0.25	0.19	0.43
	Fold change	1	1.32	0.89	0.68	1.54

FIG 5 Effects of various cytokine treatments on viral yield and compound potency. (A) Measurement of viral yield under different cytokine treatments compared to that for a mock-treated sample; (B) EC_{50}s and fold change in R1479 and NITD008 potencies under various cytokine treatments.

pared with the intracellular concentrations of R1479 triphosphate in naive PBMCs, those in preinfected PBMCs were consistently lower. The results demonstrate that preinfection of PBMCs with DENV reduces the amount of R1479 converted to its triphosphate form.

Activation of PBMCs by DENV infection decreases the potency of R1479. We hypothesized that viral infection induced the production of cytokines and chemokines, which activated PBMCs to reduce the conversion of R1479 to its triphosphate form (15). To test this hypothesis, we measured the production of IL-10, TNF-α, and IFN-β in the culture fluids after PBMCs were infected with DENV-2 (Fig. 4A); these cytokines are typically associated with early DENV infection (3). Only IL-10 was detected at 24 p.i., whereas all three cytokines were observed at 48 postinfection. The results indicate that PBMCs produce cytokines upon DENV infection.

We examined whether stimulation of PBMCs by PMA affected the potency of R1479. After PBMCs were stimulated with PMA (5 ng/ml) overnight, the cells were infected with DENV-2 and immediately treated with R1479; the EC_{50} was determined to be 4.86 μM at 48 h p.i. (Fig. 4B). Compared with the EC_{50} (0.249 μM) derived from the inactivated PBMCs, R1479 lost its potency by almost 20-fold in the PMA-activated cells (Table 1). In contrast, PMA activation did not significantly affect the EC_{50} of NITD008 (Fig. 4C and Table 1). These results demonstrate that PBMC activation can affect the antiviral potency in a nucleoside-dependent manner.

We then assessed the impact of some major inflammatory mediators produced during DENV infection on the potency of R1479. PBMCs were pretreated with individual cytokines whose levels are reported to be elevated after DENV infection (IFN-β, IFN-γ, IP-10, TNF-α, IL-10, or GM-CSF) for 16 h, after which the cells were tested for the EC_{50}s of R1479. As previously reported (3), pretreatment of cells with IFN-β or IFN-γ suppressed virus production by >10-fold (Fig. 5A); consequently, the EC_{50}s could

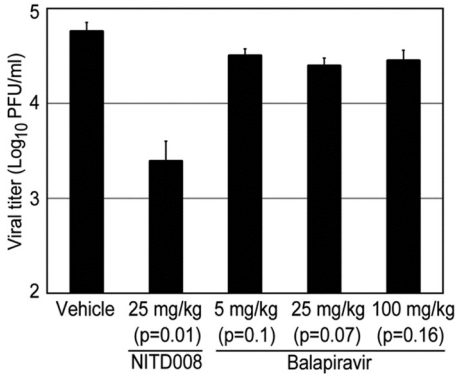


FIG 6 *In vivo* efficacy testing of balapiravir in a mouse model of DENV viremia. AG129 mice were intraperitoneally inoculated with DENV-2 and subsequently treated twice daily with vehicle fluid, positive-control compound NITD008, or a series of escalating doses of balapiravir of up to 100 mg/kg twice daily. The peak viremia levels on day 3 p.i. were quantified by plaque assay and are expressed as the number of PFU/ml of blood plasma, shown on a logarithmic scale. Whereas twice-daily dosing with NITD008 at 25 mg/kg resulted in a statistically significant 20-fold reduction of viremia compared to that in the vehicle-treated mice, twice-daily dosing with balapiravir at 5 mg/kg resulted in only a slight nonsignificant 2-fold reduction of viremia and no further reduction at higher doses.

not be accurately measured. Pretreatment with IP-10 or IFN-α did not affect the virus yield. In contrast, pretreatment of cells with IL-10 or GM-CSF increased the virus yield by 14-fold and 33-fold, respectively (Fig. 5A); it remains to be determined how IL-10 and GM-CSF stimulate DENV production. Remarkably, pretreatment of PBMCs with IP-10, TNF-α, IL-10, or GM-CSF decreased the potency of R1479 by 6.3-, 18.9-, 19.2-, or 9.8-fold, respectively (Fig. 5B). In contrast, pretreatment of PBMCs with the same panel of cytokines did not significantly affect the potency of NITD008 (Fig. 5B). The results demonstrate that distinct cytokines could activate PBMCs, leading to the reduced potency of R1479.

No efficacy of balapiravir in the DENV AG129 mouse model. A mouse model of DENV viremia was used to examine the *in vivo* efficacy of balapiravir (13). AG129 mice lacking IFN-α/β and IFN-γ receptors were infected with DENV-2 and treated with ascending doses of balapiravir by oral gavage twice daily at 5, 25, and 100 mg/kg per dose. Animals were sampled on day 3 p.i., at the peak of viremia, for analysis of viral titers in the blood. Balapiravir treatment produced a marginal reduction in viremia at all doses that did not reach statistical significance ($P > 0.05$) compared to the results for the vehicle-treated animals (Fig. 6), demonstrating no efficacy even when it was dosed at up to 100 mg/kg twice daily for 3 days. As a positive control, mice treated with NITD008 showed a robust 20-fold reduction of viremia when they were dosed at 25 mg/kg twice daily (Fig. 6).

Table 2 summarizes the pharmacokinetic parameters of balapiravir in mice. After oral dosing of the CD-1 mice with 28.1 mg/kg of balapiravir, R1479 reached a C_{max} and a minimum concentration in plasma (C_{min}) of 24.38 μM and 6.34 μM, respectively, at 2 h and 24 h postdosing. The results suggest that the lack of efficacy in mouse is not likely due to a low level of exposure to the compound, as the compound was dosed twice daily at up to 100 mg/kg. We currently do not know why balapiravir did not show protection in the DENV mouse model (see Discussion below).

DISCUSSION

The goal of this study was to understand why balapiravir failed to show efficacy in a recent clinical trial for the treatment of dengue virus infection. Human pharmacokinetic data showed that, after oral dosing at 1,500 and 3,000 mg of balapiravir, the median C_{min}s of R1497 reached 3.56 and 5.82 μM, respectively (8). Since the EC_{50}s of R1479 in primary human macrophages were estimated to be 1.3 to 3.2 μM in that study (8), balapiravir should have reduced the level of viremia in dengue patients. Here, we took two approaches to investigate the discrepancy between the *in vitro* and *in vivo* data. The first approach was to exclude the possibility that the antiviral activity observed in cell culture was caused by an off-target mechanism. To address this point, we showed that (i) R1479 triphosphate is a potent inhibitor of DENV polymerase in a biochemical RNA elongation assay and (ii) R1479 triphosphate is indeed generated inside cells after incubation with R1479. Our results clearly indicate that R1479 is a genuine inhibitor of DENV through blocking viral RNA synthesis.

The second approach was to examine the cell culture potency of R1479 in a delayed treatment. The rationale behind this approach is that, when patients receive treatment, viral replication has already been well established (8). Surprisingly, delayed treatment decreased the potency of R1479 by 125-fold compared with the potency of a treatment given concomitantly with the infection. The compromised EC_{50} is about 2- to 5-fold higher than the plasma C_{min} of R1497 in patients. Therefore, the decreased potency in delayed treatment could account for the lack of potency in patients. This information is critical for future antiviral development, as it is important to use the EC_{50} derived from the delayed treatment to estimate the minimum exposure required to achieve efficacy in humans.

Our data indicate that cytokines induced upon viral infection could affect the conversion of R1479 to its triphosphate form, leading to decreased potency in the delayed treatment. This conclusion was supported by three pieces of evidence. (i) Representative cytokines, TNF-α, IL-10, and IFN-β, were produced by human PBMCs upon DENV-2 infection. These data are in agreement with the observation that the levels of these cytokines were elevated in dengue patients (8). (ii) PMA stimulation of PBMCs decreased the potency of R1479 under a nondelayed treatment condition. In line with our results, activation of PBMCs was pre-

TABLE 2 Pharmacological parameters of balapiravir in mice[a]

Parameter[a]	Value
C_{max}[b]	24.38 μM
T_{max}[b]	2.00 h
AUC_{all}[b]	96.25 μg · h/ml
AUC_{inf}[b]	133.05 μg · h/ml
$t_{1/2}$[c]	14.15 h

[a] C_{max}, maximum concentration of balapiravir in plasma after dosing; T_{max}, the time at which C_{max} is reached; AUC_{all}, integral area under the plasma balapiravir concentration-versus-time curve for the time points at which they were measured (between 0 and 24 h); AUC_{inf}, integral area under the plasma balapiravir concentration-versus-time curve from 0 h and extrapolated to infinity; $t_{1/2}$, half-life of balapiravir, or the time required for the concentration of balapiravir to reach half of its original value.

[b] Parameters derived from CD-1 mice that were orally dosed with 28.1 mg/ml of balapiravir.

[c] Parameters derived from CD-1 mice that were dosed with 5.7 mg/kg of balapiravir via intravenous injection.

viously shown to change the activity of nucleosides in HIV infection (15). (iii) Pretreatment of PBMCs with individual cytokines significantly reduced the potency of balapiravir. Our results showed that the effect of preinfection on antiviral potency in PBMCs is nucleoside dependent. Compared with NITD008 (an adenosine analog), the potency of R1479 was more significantly affected by preinfection with DENV. Future studies are needed to determine whether the observed effect with R1479 is specific to this molecule or, rather, is pyrimidine specific.

Balapiravir has shown efficacy in a dose-dependent manner when treating HCV-infected patients. This is in contrast to the lack of efficacy in dengue patients. Although both HCV and DENV belong to the *Flaviviridae* family, the pathogenesis of the two viruses is different. HCV causes chronic liver diseases, whereas DENV causes systemic acute diseases. Besides the reduced potency of balapiravir in PBMCs that are preinfected with DENV, two other factors may contribute to the lack of efficacy in dengue patients. (i) The kinetics of conversion of R1479 to its triphosphate form could be too slow for the successful treatment of dengue patients. This is evidenced by the fact that when HCV patients were treated with balapiravir, there was a delay in the reduction in the level of viremia during the first 36 h after treatment (7). This is a critical time period for the treatment of dengue patients, as the viremia drops rapidly after fever development. Indeed, R1479 was shown to be a poor substrate for deoxycytidine kinase, the enzyme that converts R1479 to its monophosphate form; a closely related compound required more than 40 h to reach the maximum concentration when it was incubated with primary human hepatocytes at 2 μM (16). (ii) We found that R1479 had an EC_{50} of 22.85 μM in the Huh-7 DENV replicon assay (unpublished data). In contrast, R1479 showed an EC_{50} of 1.28 μM in the Huh-7 HCV replicon assay (6). Since liver hepatocytes were one of the major replication sites in dengue patients (17), the low efficacy of balapiravir in DENV-infected hepatocytes could also contribute to the lack of efficacy.

Nucleoside analogs are prone to cell type-specific activity (18, 19). This is reflected by the EC_{50} variation when R1479 was tested in PBMCs and various immortalized cell lines; the EC_{50}s were estimated to be 0.249, 3.57, 0.626, 14.25, and 22.85 μM in DENV-infected PBMCs, THP-1, KU812, A549, and Huh-7 cells, respectively (Table 1 and data not shown). This property underlines the importance of using the right cell type to determine antiviral activity. Although DENV replication can be detected in blood cells, other tissues and organs may also contribute to the overall viremia during infection. Therefore, for a potential anti-DENV drug, nucleoside analogs with consistent potency in various cell types are better candidates than compounds with potency limited to a few cell types.

In summary, this study has provided experimental evidence to explain why balapiravir failed to show efficacy in dengue patients. Viral infection-mediated cell activation can decrease the antiviral activity of a prodrug whose conversion to its active form requires a host enzyme(s). The magnitude of such an effect on efficacy is compound dependent (as exemplified by the cytidine-based compound R1479 and the adenosine-based compound NITD008) and should be factored to estimate the dose efficacious for humans.

ACKNOWLEDGMENTS

We thank Shamala Devi for providing DENV for the infection study. We thank Paul MacAry for the chimeric 4G2 antibody used in PBMC infection. We thank Suresh B. Lakshminarayana, Alex Chao, Ka Yan Chung, Anne Goh, Jofina Kim Cheow Lim, Boping Liu, Ghislain Bonamy, and Andy Yip for technical assistance. We thank Thierry Diagana for critical reading of the manuscript.

D.C. and K.F. were supported by the Agency for Science Technology and Research (A*STAR), Singapore.

REFERENCES

1. **Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI.** 2013. The global distribution and burden of dengue. Nature **496:**504–507. http://dx.doi.org/10.1038/nature12060.
2. **Durbin AP, Vargas MJ, Wanionek K, Hammond SN, Gordon A, Rocha C, Balmaseda A, Harris E.** 2008. Phenotyping of peripheral blood mononuclear cells during acute dengue illness demonstrates infection and increased activation of monocytes in severe cases compared to classic dengue fever. Virology **376:**429–435. http://dx.doi.org/10.1016/j.virol.2008.03.028.
3. **Wong KL, Chen W, Balakrishnan T, Toh YX, Fink K, Wong SC.** 2012. Susceptibility and response of human blood monocyte subsets to primary dengue virus infection. PLoS One **7:**e36435. http://dx.doi.org/10.1371/journal.pone.0036435.
4. **Cihlar T, Ray AS.** 2010. Nucleoside and nucleotide HIV reverse transcriptase inhibitors: 25 years after zidovudine. Antiviral Res. **85:**39–58. http://dx.doi.org/10.1016/j.antiviral.2009.09.014.
5. **Brandl M, Wu X, Holper M, Hong L, Jia Z, Birudaraj R, Reddy M, Alfredson T, Tran T, Larrabee S, Hadig X, Sarma K, Washington C, Hill G, Smith DB.** 2008. Physicochemical properties of the nucleoside prodrug R1626 leading to high oral bioavailability. Drug Dev. Ind. Pharm. **34:**683–691. http://dx.doi.org/10.1080/03639040701836636.
6. **Klumpp K, Leveque V, Le PS, Ma H, Jiang WR, Kang H, Granycome C, Singer M, Laxton C, Hang JQ, Sarma K, Smith DB, Heindl D, Hobbs CJ, Merrett JH, Symons J, Cammack N, Martin JA, Devos R, Najera I.** 2006. The novel nucleoside analog R1479 (4′-azidocytidine) is a potent inhibitor of NS5B-dependent RNA synthesis and hepatitis C virus replication in cell culture. J. Biol. Chem. **281:**3793–3799. http://dx.doi.org/10.1074/jbc.M510195200.
7. **Roberts SK, Cooksley G, Dore GJ, Robson R, Shaw D, Berns H, Hill G, Klumpp K, Najera I, Washington C.** 2008. Robust antiviral activity of R1626, a novel nucleoside analog: a randomized, placebo-controlled study in patients with chronic hepatitis C. Hepatology **48:**398–406. http://dx.doi.org/10.1002/hep.22321.
8. **Nguyen NM, Tran CN, Phung LK, Duong KT, Huynh HA, Farrar J, Nguyen QT, Tran HT, Nguyen CV, Merson L, Hoang LT, Hibberd ML, Aw PP, Wilm A, Nagarajan N, Nguyen DT, Pham MP, Nguyen TT, Javanbakht H, Klumpp K, Hammond J, Petric R, Wolbers M, Nguyen CT, Simmons CP.** 2013. A randomized, double-blind placebo controlled trial of balapiravir, a polymerase inhibitor, in adult dengue patients. J. Infect. Dis. **207:**1442–1450. http://dx.doi.org/10.1093/infdis/jis470.
9. **Hung M, Gibbs CS, Tsiang M.** 2002. Biochemical characterization of rhinovirus RNA-dependent RNA polymerase. Antiviral Res. **56:**99–114. http://dx.doi.org/10.1016/S0166-3542(02)00101-8.
10. **Niyomrattanakit P, Abas SN, Lim CC, Beer D, Shi PY, Chen YL.** 2011. A fluorescence-based alkaline phosphatase-coupled polymerase assay for identification of inhibitors of dengue virus RNA-dependent RNA polymerase. J. Biomol. Screen. **16:**201–210. http://dx.doi.org/10.1177/1087057110389323.
11. **Mathews DH, Sabina J, Zuker M, Turner DH.** 1999. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. **288:**911–940. http://dx.doi.org/10.1006/jmbi.1999.2700.
12. **Simiele M, D'Avolio A, Baietto L, Siccardi M, Sciandra M, Agati S, Cusato J, Bonora S, Di Perri G.** 2011. Evaluation of the mean corpuscular volume of peripheral blood mononuclear cells of HIV patients by a Coulter Counter to determine intracellular drug concentrations. Antimicrob. Agents Chemother. **55:**2976–2978. http://dx.doi.org/10.1128/AAC.01236-10.
13. **Schul W, Liu W, Xu HY, Flamand M, Vasudevan SG.** 2007. A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J. Infect. Dis. **195:**665–674. http://dx.doi.org/10.1086/511310.

14. **Yin Z, Chen YL, Schul W, Wang QY, Gu F, Duraiswamy J, Kondreddi RR, Niyomrattanakit P, Lakshminarayana SB, Goh A, Xu HY, Liu W, Liu B, Lim JY, Ng CY, Qing M, Lim CC, Yip A, Wang G, Chan WL, Tan HP, Lin K, Zhang B, Zou G, Bernard KA, Garrett C, Beltz K, Dong M, Weaver M, He H, Pichota A, Dartois V, Keller TH, Shi PY.** 2009. An adenosine nucleoside inhibitor of dengue virus. Proc. Natl. Acad. Sci. U. S. A. **106:**20435–20439. http://dx.doi.org/10.1073/pnas.0907010106.
15. **Gao WY, Shirasaka T, Johns DG, Broder S, Mitsuya H.** 1993. Differential phosphorylation of azidothymidine, dideoxycytidine, and dideoxyinosine in resting and activated peripheral blood mononuclear cells. J. Clin. Invest. **91:**2326–2333. http://dx.doi.org/10.1172/JCI116463.
16. **Klumpp K, Kalayanov G, Ma H, Le Pogam S, Leveque V, Jiang WR, Inocencio N, De Witte A, Rajyaguru S, Tai E, Chanda S, Irwin MR, Sund C, Winqist A, Maltseva T, Eriksson S, Usova E, Smith M, Alker A, Najera I, Cammack N, Martin JA, Johansson NG, Simth DB.** 2008. 2′-Deoxy-4′-azido nucleoside analogs are highly potent inhibitors of hepatitis C virus replication despite the lack of 2′-α-hydroxyl groups. J. Biol. Chem. **283:**2167–2175. http://dx.doi.org/10.1074/jbc.M708929200.
17. **Rosen L, Drouet MT, Deubel V.** 1999. Detection of dengue virus RNA by reverse transcription-polymerase chain reaction in the liver and lymphoid organs but not in the brain in fatal human infection. Metabolic activation of the anti-hepatitis C virus nucleotide prodrug PSI-352938. Am. J. Trop. Med. Hyg. **61:**720–724.
18. **Garcia-Lerma JG, Aung W, Cong ME, Zheng Q, Youngpairoj AS, Mitchell J, Holder A, Martin A, Kuklenyik S, Luo W, Lin CY, Hanson DL, Kersh E, Pau CP, Ray AS, Rooney JF, Lee WA, Heneine W.** 2011. Natural substrate concentrations can modulate the prophylactic efficacy of nucleotide HIV reverse transcriptase inhibitors. J. Virol. **85:**6610–6617. http://dx.doi.org/10.1128/JVI.00311-11.
19. **Niu C, Tolstykh T, Bao H, Park Y, Babusis D, Lam AM, Bansal S, Du J, Chang W, Reddy PG, Zhang HR, Woolley J, Wang LQ, Chao PB, Ray AS, Otto MJ, Sofia MJ, Furman PA, Murakami E.** 2012. Metabolic activation of the anti-hepatitis C virus nucleotide prodrug PSI-352938. Antimicrob. Agents Chemother. **56:**3767–3775. http://dx.doi.org/10.1128/AAC.00530-12.