# Lack of pharmacokinetic drug-drug interactions between bepirovirsen and nucleos(t)ide analogs

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# Supplementary Materials

## Supplementary Methods

### *1.1 Determination of cytochrome P450 (CYP) and uridine diphosphate (UDP)-glucuronosyltransferase (UGT) inhibition in suspended cryopreserved human hepatocytes*

To evaluate the direct inhibition of CYP and UGT enzymes, bepirovirsen was incubated in duplicate with appropriate probe substrate in suspended cryopreserved human hepatocytes at 1 million cells/mL. Bepirovirsen and hepatocytes suspended in Williams' E+ medium were added to 48-well plates and equilibrated for 5 minutes at ambient temperature prior to the addition of the probe substrate. Incubations were conducted at approximately 37°C in a mixture of 95% air and 5% carbon dioxide ($CO_2$) with 95% humidity. Reactions were terminated after approximately 10 minutes by the addition of acetonitrile containing an internal standard. The samples were centrifuged at 920 × g for 10 minutes at 10°C, and the supernatant fractions were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Positive control incubations, replacing bepirovirsen with a known direct inhibitor of each CYP enzyme or with a broad-spectrum inhibitor of UGT enzymes, were included.

To assess the time-dependent inhibition of CYP enzymes, bepirovirsen was preincubated in duplicate with hepatocytes in Williams' E+ medium at approximately 37°C for approximately 30 minutes. Incubations were initiated by adding an appropriate probe substrate and continued as described above. Incubations without bepirovirsen and those containing bepirovirsen but not preincubated, served as negative controls. Additionally, positive controls incubations, in which bepirovirsen was replaced with a known time-dependent inhibitor of each CYP enzyme, were included.

Supernatants were analyzed by LC-MS/MS for concentrations of probe substrate metabolite as described in section 1.1.1.

| Bepirovirsen concentration (μM) | Enzyme | Probe substrate | Probe substrate (μM)* |
|---|---|---|---|
| 0, 0.1, 0.3, 1, 3, 10, 30, and 100 | CYP1A2 | Phenacetin | 90 |
| | CYP2B6 | Bupropion | 90 |
| | CYP2C8 | Amodiaquine | 7 |
| | CYP2C9 | Diclofenac | 2 |
| | CYP2C19 | *S*-Mephenytoin | 60 |
| | CYP2D6 | Dextromethorphan | 10 |
| | CYP3A4/5 | Midazolam | 3 |
| | CYP3A4/5 | Testosterone | 60 |
| | UGT[†] | 4-Methylumbelliferone | 70 |

*Concentrations based on $K_m$ or $S_{50}$ data determined previously in incubations with human liver microsomes.
[†]UGT inhibition was measured using a probe substrate that is metabolized by multiple UGT isoforms.
$K_m$, substrate concentration supporting half the maximum rate of an enzyme-catalyzed reaction; $S_{50}$, substrate concentration for allosteric kinetics which supports half the maximum rate of an enzyme-catalyzed reaction

*1.1.1 Determination of metabolite concentrations by LC-MS/MS analysis*

Samples were analyzed by multiple reaction monitoring LC-MS/MS methods using an appropriate SCIEX (Framingham, MA, USA) mass spectrometer equipped with a Shimadzu Nexera (Shimadzu, Kyoto, Japan) or Shimadzu Prominence LC system interfaced by electrospray ionization. Methods were validated for absolute quantitation of probe substrate metabolite, using authentic metabolite reference standards and deuterated metabolites as internal standards, as detailed below.

| Enzyme | Metabolite monitored | Internal standard | Mode |
|---|---|---|---|
| CYP1A2 | Acetaminophen | Acetaminophen-d4 | Positive |
| CYP2B6 | Hydroxybupropion | Hydroxybupropion-d6 | |
| CYP2C8 | *N*-Desethyl Amodiaquine | *N*-Desethyl Amodiaquine-d5 | |
| CYP2C9 | 4′-Hydroxydiclofenac | 4′-Hydroxydiclofenac-d4 | |
| CYP2C19 | 4′-Hydroxymephenytoin | 4′-Hydroxymephenytoin-d3 | Negative |
| CYP2D6 | Dextrorphan | Dextrorphan-d3 | Positive |
| CYP3A4/5 | 1′-Hydroxymidazolam | 1′-Hydroxymidazolam-d4 | |
| CYP3A4/5 | 6β-Hydroxytestosterone | 6β-Hydroxytestosterone-d3 | |
| UGT | 4-Methylumbelliferone glucuronide | Hydroxycoumarin glucuronide-d5 | |

Metabolites were quantified by back calculation of concentrations against duplicate calibration curves using the simplest appropriate weighting and regression algorithm based on analyte/internal standard peak area ratios. Half maximal inhibitory concentration ($IC_{50}$) values were calculated using the following equation:

| Assessment | Equation |
|---|---|
| $IC_{50}$ determinations | $\mathrm{Y} = \mathrm{Min} + \frac{(\mathrm{Max} - \mathrm{Min})}{\left(1 + \left(\frac{\mathrm{Conc}}{\mathrm{IC}_{50}}\right)^{\mathrm{slope}}\right)}$ |

Average data (i.e., percent of control activity) obtained from duplicate samples for each bepirovirsen concentration were used to calculate $IC_{50}$ values.
Conc, concentration; $IC_{50}$, half maximal inhibitory concentration; Max, maximum; Min, minimum.

### *1.2 Determination of CYP induction in cryopreserved primary human hepatocytes*

To evaluate the induction of CYP1A2, CYP2B6, and CYP3A4, bepirovirsen was incubated with plateable human hepatocytes (BIOIVT, Baltimore, MD, USA) from 3 individual donors. Cells were thawed and plated as per manufacturer's instructions. Final hepatocyte density was 1.4 x 106 cell/mL. After 24 hours at approximately 37°C in 5% $CO_2$, cells were dosed in triplicate with bepirovirsen (0.01–200 µM) or the prototypical inducers rifampicin (0.01–30 µM), phenobarbital (1500 µM), and omeprazole (100 or 150 µM), or the negative control inducer flumazenil (50 µM) and incubated at approximately 37°C for 48 hours. Media was removed and replaced with fresh dosing medium 24 hours after the initial dose. After the addition of fresh dosing solution to the triplicate wells containing bepirovirsen, an aliquot of media was collected at 0, 4, and 24 hours and quantified for bepirovirsen concentration by LC-MS/MS, with actual measured concentrations used to calculate kinetic parameters.[1] At the end of the 48-hour incubation period, media was removed from all wells and an assessment of the effect of bepirovirsen or positive control on messenger RNA (mRNA) was evaluated by measuring mRNA levels of each CYP gene (CYP1A2, 2B6 and 3A4) via TaqMan real-time polymerase chain reaction analysis.

Maximum fold induction ($E_{max}$) and the unbound concentration eliciting half-maximal induction ($EC_{50,u}$) were determined for bepirovirsen using the following nonlinear regression model:[1]

| | |
|---|---|
| Sigmoidal 3-parameter nonlinear regression model | $y = bottom + \frac{E_{max} - bottom}{1 + e\left[\frac{\left(-[I] - EC_{50,u}\right)}{h}\right]}$ |

Where:

bottom is the lowest fold induction and is constrained to "1.0" to normalize to vehicle control
y is relative fold induction
h is the Hill slope
[I] is the test article (bepirovirsen) concentration
$EC_{50,u}$ is the unbound concentration causing half-maximal effect
$E_{max}$ is the maximum fold induction

***1.3 Assessment of bepirovirsen as an inhibitor or substrate of human drug transporters***

*1.3.1 Assessment of bepirovirsen as an inhibitor of MATE1, MATE2-K, OAT1, OAT3, OATP1B1, OATP1B3, OCT1, and OCT2*

The inhibitory potency of bepirovirsen against solute carrier (SLC) drug transporters was evaluated in human embryonic kidney mammalian (HEK293) cells overexpressing the respective transporter drug transporters organic anion-transporting polypeptide (OATP)1B1, OATP1B3, organic anion transporter (OAT)1, OAT3, organic cation transporter (OCT)1, OCT2, multidrug and toxin extrusion transporters (MATE)1 and MATE2-K. Cells were cultured at approximately 37°C in a humidified atmosphere containing 5% $CO_2$ and were plated onto standard 96-well tissue culture plates at an appropriate cell density.

Before the experiment, the culture medium was removed, and the cells were washed twice with 100 µL of pre-warmed (approximately 37°C) assay buffer (Krebs-Henseleit [pH 8.0] for MATE1, MATE2-K; Hank's balanced salt solution [pH 7.4] for OAT3, OATP1B1, OATP1B3, OCT2, OAT1, and OCT1). Thereafter, cells were preincubated at approximately 37°C for 30 minutes in assay buffer containing bepirovirsen at concentrations of 0.046–100 µM for MATE1, MATE2-K, OAT3, OATP1B1, OATP1B3, and OCT2, and 0.023–50 µM for OAT1 and OCT1, appropriate solvents for solvent controls and a known inhibitor for each transporter. In wells containing MATE1 and MATE2-K, buffer was supplemented with 20 mM NH4Cl during the 30 minutes preincubation. After the preincubation step, solutions were removed. Uptake experiments were performed at approximately 37°C in 50 µL of assay buffer containing the appropriate probe substrate (metformin [10 µM] for MATE1, MATE2-K, OCT2; tenofovir [5 µM] for OAT1; E3S [estrone-3-sulfate; 1 µM] for OAT3; $E_2$17βG [estradiol-17-β-

glucuronide; 1 µM] for OATP1B1; CCK8 [1 µM] for OATP1B3; sumatriptan [1 µM] for OCT1) and bepirovirsen or solvent or control inhibitor (incubation times: 1 minute for MATE2-K, OCT2 and OAT3; 2 minutes for OAT1 and OATP1B3; 3 mins for MATE1, OATP1B1, OCT1).

After the experiment, cells were washed twice with 100 µL of cold appropriate buffer and lysed with 50 µL of 0.1 M NaOH. Radiolabeled probe substrate transport was determined by measuring an aliquot (35 µL) from each well by liquid scintillation counting.

*1.3.2 Assessment of bepirovirsen as a substrate of MATE1, MATE2-K, OAT1, OAT3, OATP1B1, OATP1B3, OCT1, and OCT2*

For substrate assessment of SLC drug transporters by bepirovirsen, HEK293 cells overexpressing the respective transporter were incubated for 2 and 20 minutes in the appropriate assay buffer with bepirovirsen (0.1, 1, and 10 µM) in the presence and absence of transporter-specific inhibitor to determine whether bepirovirsen was actively taken up into the cells.

Cells were cultured at approximately 37°C in a humidified atmosphere containing 5% $CO_2$ and were plated onto standard 96-well tissue culture plates at an appropriate cell density.

Before the experiment, the medium was removed, and the cells were washed twice with 100 µL of the appropriate assay buffer. Cellular uptake of bepirovirsen into the cells was measured by adding 50 µL of the appropriate buffer containing bepirovirsen and incubating them at approximately 37°C. Recovery samples were collected from the buffer containing bepirovirsen at the end of the incubation. Reactions were quenched by removing the buffer containing bepirovirsen and the cells were washed twice with 100 µL of cold buffer. Cells were lysed by adding 150 µL of methanol:buffer mixture (2:1) and incubating for 20 minutes at 4 ± 1°C. The amount of bepirovirsen in the cell lysate was determined by LC-MS/MS. The amount of protein in each well was quantified using the bicinchoninic acid assay kit for protein determination (Sigma-Aldrich, St. Louis, MO, USA).

A positive control incubation was performed for each transporter using the appropriate probe substrate as described in section 1.3.1.

*1.3.3 Assessment of bepirovirsen as an inhibitor of BCRP, MRP2, and P-gp*

For the assessment of inhibition of human breast cancer resistance protein (BCRP), multidrug resistance-associated protein 2 (MRP2) and P-glycoprotein (P-gp), bepirovirsen was incubated with membrane vesicle preparations (total protein: 50 µg/well for MRP2 and P-gp; 12.5 µg/well for BCRP) and the probe substrate (E3S [1 µM] for BCRP; $E_2$17βG [100 µM] for MRP2; NMQ [N-methyl quinidine; 10 µM] for P-gp). Incubations were conducted in the presence of 4 mM adenosine triphosphate (ATP) or adenosine monophosphate (AMP) to distinguish between transporter-mediated uptake and passive diffusion into the vesicles. Bepirovirsen dissolved in 0.75 µL of solvent, was added across a concentration range of 0.046 to 100 µM into 50 µL of the reaction mixture. Then the reaction mixtures were preincubated for 15 minutes at 32°C for BCRP and P-gp and at approximately 37°C for MRP2. Reactions were initiated by the addition of 25 µL of pre-warmed 12 mM MgATP (or 12 mM AMP in assay buffer as a background control). Reactions were quenched by the addition of 200 µL of ice-cold washing buffer followed by immediate filtration via glass fiber filters mounted on a 96-well plate (filter plate). The filters were washed (5 × 200 µL of ice-cold washing buffer), dried and the amount of substrate inside the filtered vesicles was determined by liquid scintillation counting. Incubations were performed replacing bepirovirsen with known inhibitors of BCRP, MRP2, and P-gp.

*1.3.4 Assessment of bepirovirsen as a substrate of BCRP, MRP2, and P-gp*

The uptake of bepirovirsen into membrane vesicles was determined using inside-out membrane vesicles (total protein: 50 µg/well) prepared from cells overexpressing human ATP-Binding Cassette (ABC) transporters as well as from control cells. Vesicles were incubated for 10 minutes in the respective assay buffer containing bepirovirsen at two concentrations (1 and 10 µM) with or without ATP or AMP, and with or without a known inhibitor of the respective transporter to determine whether bepirovirsen is actively transported into the vesicles. Reactions were quenched by the

addition of 200 µL of ice-cold washing buffer and immediate filtration via glass fiber filters mounted to a 96-well plate (filter plate). The filters were washed with 5 × 200 µL of ice-cold washing buffer. The amount of accumulated bepirovirsen retained inside the vesicles was determined by LC-MS/MS after washing the vesicles 2 times with 100 µL methanol:water (2:1).

*1.3.5 Determination of bepirovirsen as a substrate of drug transporters by LC-MS/MS analysis*

Samples containing bepirovirsen and the analytical standard were analyzed by high-performance liquid chromatography (HPLC) MS/MS with methodology described below:

- HPLC was performed by using a Thermo Scientific Vanquish Flex UHPLC. Chromatographic separation was achieved using a gradient of 0.1% triethylamine (TEA) and 2% hexafluoroisopropanol (HFIP) in HPLC water and 0.1% TEA and 2% HFIP in Acetonitrile on an Agilent Poroshell 120 3.0x50 mm 2.7 µm column. Samples were analyzed by a Thermo Scientific Q-Exactive Focus Mass Spectrometer.
- HPLC was also conducted by using an Agilent 1260 HPLC. Chromatographic separation was achieved using a gradient of 0.1% TEA and 2% HFIP in HPLC water and 0.1% TEA and 2% HFIP in Acetonitrile on a Poroshell 120 EC-C18 50 × 3.0 mm, 2.7 µm column. Samples were analyzed by an Agilent 6460A Triple Quadrupole Mass Spectrometer.

Raw data was integrated using Analyst software (Sciex) to calculate peak area ratios to construct the calibration curves from which the concentration of bepirovirsen in the study samples was determined. The low concentration samples (e.g., 100 nM dosing samples for the substrate assays) were concentrated to ensure quantification of the compound in the samples.

In vesicular transport inhibition and SLC transporter inhibition assays, the $IC_{50}$ (µM) was calculated, where applicable. $IC_{50}$ was defined as the concentration of test article (TA) required to inhibit maximal activity by 50%. $IC_{50}$ values were derived from a four-parametric logistic equation (log(inhibitor) versus response – variable slope); the curve was fitted to the relative activity versus TA concentration plot using non-linear regression. Top (maximal response) and bottom (maximally

inhibited response) values were not constrained to constant values of 100 and 0, respectively, unless it is noted otherwise.

| Assessment | Equation |
|---|---|
| $IC_{50}$ determinations | $y = bottom + \frac{top - bottom}{(1 + 10^{((logIC_{50} - X) \times Hillslope)})}$ |

Where:

bottom is the maximally inhibited response
top is the maximal response
y is the response (decreases as X increases)
X is the log of concentration (TA media concentrations)
$IC_{50}$ is the half-maximal inhibition

***1.4 Static model equations and cut-off values for the potential of bepirovirsen to modulate CYP enzymes***

| Mechanism | Equation |
|---|---|
| Reversible inhibition of hepatic CYPs[2] | $1 + \left(\frac{I_{max,u}}{K_{i,u}}\right) \geq 1.02$ |
| Induction of CYPs[3] | $R_3 = \frac{1}{\left[1 + d \times \left(\frac{(E_{max} \times 10 \times I_{max,u})}{(EC_{50,u} + 10 \times I_{max,u})}\right)\right]} \leq 0.8$ |

Where:

$I_{max,u}$ is the maximal unbound plasma concentration of bepirovirsen.

$E_{max}$ is the maximum induction effect determined in vitro.

$EC_{50,u}$ is the unbound concentration causing half-maximal effect determined in vitro.

d is the scaling factor and is assumed to be 1.

$K_{i,u}$ is the unbound inhibition constant determined in vitro.

Model parameters are shown in **Supplementary Table 1**.

***1.5 Static model equations and cut-off values for the potential of bepirovirsen to modulate transporters***

| Mechanism | Equation |
|---|---|
| Hepatic efflux (after SC or IV administration) and renal uptake and efflux[4-6] | $\frac{Imax_u}{IC_{50}} \geq 0.1$ |

IV, intravenous; SC, subcutaneous.

Where:

$IC_{50}$ is the half-maximal inhibitory concentration.

$Imax_u$ is the maximal unbound plasma concentration of bepirovirsen.

## Supplementary Results

***Supplementary Table 1. Mathematical parameters used in the static model to evaluate bepirovirsen drug-drug interaction risk prediction***

| Parameter | Value | Definition |
|---|---|---|
| Molecular weight | 7344 | Molecular weight of bepirovirsen (free acid) |
| Dose (mg)<br>SC route of administration | 300 | Current therapeutic dose of bepirovirsen |
| Estimated human unbound $C_{max}$ (nM) | 57 | Systemic unbound $C_{max}$ (plasma) |
| CYP1A2 | No inhibition observed | Inhibition effect |
| CYP2C8 $IC_{50}$* (µM) | >100 | Inhibition constant |
| CYP2C9 | No inhibition observed | Inhibition effect |
| CYP2C19 | No inhibition observed | Inhibition effect |
| CYP2D6 | No inhibition observed | Inhibition effect |
| CYP3A4 | No inhibition observed | Inhibition effect |
| UGT | No inhibition observed | Inhibition effect |
| CYP1A2 | No induction observed | Induction effect |
| CYP2B6 | No induction observed | Induction effect |
| CYP3A4 $E_{max}$ | 2.28 | Maximum induction effect (Donor 2 only) |
| CYP3A4 $EC_{50,u}$ (µM) | 13.6 | Unbound concentration causing half-maximal induction effect<br>(Donor 2 only)<br>Bepirovirsen did not induce CYP3A4 in Donors 1 & 3 |
| P-gp | No inhibition observed | Inhibition effect |
| BCRP | No inhibition observed | Inhibition effect |
| OATP1B1 | No inhibition observed | Inhibition effect |
| OATP1B3 | No inhibition observed | Inhibition effect |
| MRP2 $IC_{50,u}$ (µM) | 8.49 | Unbound concentration causing half maximal inhibitory effect |
| MATE1 | No inhibition observed | Inhibition effect |

| Parameter | Value | Definition |
|---|---|---|
| MATE2-K | No inhibition observed | Inhibition effect |
| OAT1 | No inhibition observed | Inhibition effect |
| OAT3 | No inhibition observed | Inhibition effect |
| OCT1 | No inhibition observed | Inhibition effect |
| OCT2 | No inhibition observed | Inhibition effect |

*$IC_{50}$/2 was used as an estimate of Ki for CYP2C8 inhibition prediction. No induction observed (<2-old max response).

BCRP, human breast cancer resistance protein; $C_{max}$, maximum concentration; CYP, cytochrome P450; $E_{max}$, maximum induction effect; $IC_{50}$, half-maximal inhibitory concentration; MATE, multidrug and toxin extrusion transporter; MRP2, multidrug resistance-associated protein 2; OAT, organic anion transporter; OCT, organic cation transporter; OATP, organic anion transporting polypeptide; P-gp, P-glycoprotein; SC, subcutaneous; UGT, uridine diphospho-glucuronosyltransferase.

***Supplementary Table 2. Summary of bioanalytical assay performance in the B-Clear study***

| Analyte | Matrix | Validated Assay Range (LLOQ to ULOQ) | Inter-Assay Precision (% CV) | Accuracy (% Bias) |
|---|---|---|---|---|
| Bepirovirsen | Plasma | 1.00 to 1000 ng/mL | 5.0 to 7.0 | −3.0 to 4.0 |
| ETV | Plasma | 0.0400 to 16.0 ng/mL | 1.8 to 10.1 | −2.1 to 4.4 |
| TFV | Plasma | 1.00 to 500 ng/mL | 3.6 to 9.4 | −1.6 to 3.0 |

CV, coefficient of variation; ETV, entecavir; LLOQ, lower limit of quantification; TFV, tenofovir; ULOQ, upper limit of quantification.

***Supplementary Table 3. Summary of bioanalytical assay performance in the CS3 study***

| | **Bepirovirsen** | **ETV** | **TFV** |
|---|---|---|---|
| Analytical matrix | $K_2$EDTA Plasma | $K_2$EDTA Plasma | $K_2$EDTA Plasma |
| Internal standards | ISIS440762 | Entecavir -$^{13}C_2$,$^{15}N$ | Tenofovir –d7 |
| Validated range (LLOQ to ULOQ) | 1.00 to 1000 ng/mL | 0.04 to 16.0 ng/mL | 1.00 to 500 ng/mL |
| Quality control levels (ng/mL) | 3.0, 30.0, 480, 800 | 0.12, 0.96, 6.4, 12.8 | 3.0, 30.0, 250, 400 |
| Analytical technique/ method of detection | Liquid-liquid extraction | Solid-phase extraction | Protein precipitation |
| Sample aliquot volume | 100 µL | 100 µL | 50 µL |
| Calibration model | Linear regression | Linear regression | Linear regression |
| Weighting factor | $1/x^2$ | $1/x^2$ | $1/x^2$ |
| Total number of samples analyzed | 871 | 178 | 139 |
| Total number of samples reassayed | 315 Analytical (36.2% of total number of samples analyzed)<br>95 ISR (10.9% of total number of samples analyzed) | 25 ISR (14.0% of total number of samples analyzed) | 1 Analytical (0.719% of total number of samples analyzed)<br>24 ISR (17.3% of total number of samples analyzed) |
| Sample storage conditions | -60 to -80°C | -60 to -80°C | -60 to -80°C |
| ISR | Required/Pass | Required/Pass | Required/Pass |
| Samples analyzed within known stability | Yes | Yes | Yes |

CV, coefficient of variation; ETV, entecavir; ISR, incurred sample reanalysis; LLOQ, lower limit of quantification; TFV, tenofovir; ULOQ, upper limit of quantification.

## References


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