Intracellular islatravir pharmacology differs between species in an in vitro model: implications for preclinical study design

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Background: Islatravir (4′-ethynyl-2-fluoro-2′-deoxyadenosine; EFdA) is a first-in-class nucleoside reverse transcriptase translocation inhibitor (NRTTI) being investigated for HIV treatment and prevention. EFdA is intracellularly phosphorylated to EFdA-triphosphate (EFdA-tp), a competitive substrate of deoxyadenosine-triphosphate (dATP). Thus, translating safety and efficacy findings from preclinical studies relies on the assumption that EFdA’s intracellular pharmacology can be extrapolated across species.

Objectives: We investigated how EFdA is phosphorylated across animal species commonly used for preclinical models in drug development to identify those that most closely matched humans.

Methods: PBMCs were isolated from whole blood of six species (human, rhesus macaque non-human primate (rmNHP), rat, minipig, dog, and rabbit) using Ficoll separation and counted on a haemocytometer by Trypan blue staining. One million live cells were cultured in media supplemented with 10 U/mL human IL-2, 10% FBS and 1% antibiotics and treated with 0, 17, 170, and 1700 nM EFdA (n = 3 replicates per concentration). After 24 h, representative cell counts were derived from untreated control wells (as above), cells were washed in PBS, and lysed with 70:30 methanol:water. EFdA-tp and dATP concentrations were quantified by HPLC-MS/MS and normalized to the representative live cell counts for each species.

Results: When compared to human values, EFdA-tp concentrations for each EFdA treatment concentration were lower in all species (rmNHP 1.5–2.1-fold, rat 4.5–15-fold, minipig 37–71-fold, dog and rabbit >100-fold). Additionally, rmNHP and dog PBMCs exhibited significantly higher (7–10-fold; P < 0.001) dATP when compared with human PBMCs.

Conclusions: Given intracellular pharmacology differences, these preclinical models may be a conservative estimate of EFdA’s intracellular pharmacokinetics and efficacy in humans.

Introduction

Islatravir (4′-ethynyl-2-fluoro-2′-deoxyadenosine; EFdA) is a potent prodrug being investigated as the first-in-class nucleoside reverse transcriptase translocation inhibitor (NRTTI) for HIV treatment and prevention. EFdA exhibits favourable pharmacokinetic/pharmacodynamic (PK/PD) characteristics for a long-acting formulation, including an extremely long intracellular half-life of its active metabolite (120 hours) and effective concentrations in the sub-nanomolar range (EC50 = 0.05–0.1 nM).1 EFdA is intracellularly phosphorylated to EFdA-triphosphate (EFdA-tp), which competes against deoxyadenosine-triphosphate (dATP) for incorporation into the nascent HIV DNA strand to terminate reverse transcription. Additionally, EFdA’s intermediary compound, EFdA-monophosphate (EFdA-mp) inhibits reverse transcriptase translocation by preventing the enzyme from binding incoming deoxynucleotides.1

The validity of safety and efficacy findings from preclinical studies relies on the assumption that EFdA’s intracellular pharmacology can be extrapolated across species. We used an in vitro model to investigate whether EFdA is differentially phosphorylated across animal species commonly used for pre-clinical models in drug development with the goal of identifying which most closely match the intracellular pharmacology of humans.
Methods

Whole blood in K2EDTA was shipped overnight from BioIVT (Westbury, NY) at ambient temperature for six species: human, rhesus macaque non-human primate (rmNHP), Sprague Dawley rat, Gottingen minipig, Beagle dog, and New Zealand White (NZW) rabbit. PBMCs were isolated by Ficoll separation using lymphocyte separation medium (MP Biomedicals) and 50 mL leucosep centrifuge tubes (Greiner bio-one). Contaminating red blood cells were lysed (eBioscience 1× XBC Lysis Buffer, ThermoFisher Scientific) and resulting PBMCs were counted using Trypan blue staining and a haemocytometer. One million live PBMCs were cultured in 1 mL of selected media for each species based on previously published culture procedures as follows: RPMI for human, dog, and rabbit;1,2 AIM-V for rmNHP;3 and DMEM for rat and minipig.2,5 All culture media were prepared with 10 U/mL of recombinant human IL-2, 10% FBS, and 1% penicillin/streptomycin, then treated with 0, 17, 170, and 1700 nM EFdA in triplicate. 1 mg/mL EFdA in DMSO stock solution (Pharmaron, Beijing China) was further diluted in cell culture media to achieve these concentrations. PBMCs were incubated at standard culture conditions for 24 hours, then representative count and viability were assessed in an untreated control well for each species as above and PBMCs were collected from each well, washed with normal saline, lysed in 76:30 methanol:water and stored at −80°C.

Nucleotides (EFdA-tp, dATP and deoxycytidine triphosphate; dCTP) were quantified by LC-MS/MS. Briefly, 125 μL of PBMC lysate was mixed with 100 μL of 70-30 methanol:water containing stable isotopically labelled internal standards: [13C10,15N5]dATP (for dATP), [13C9,15N3]dCTP (for dCTP), and [13C10,15N5]dGTP (for EFdA-tp). Following vortexing and centrifugation, extracts were evaporated to dryness. Extracts were reconstituted in 75 μL of 1 mM ammonium phosphate (pH 7.4) prior to LC-MS/MS analysis. Chromatographic separation was achieved using a Thermo Biobasic AX (50 × 2.1 mm, 5 μm particle size) column operated under anion exchange conditions. Gradient elution was performed with the following mobile phases; 10 mM ammonium acetate and 75:25 5 mM ammonium acetate:acetonitrile (pH 10.1). Compounds were detected on an AB Sciex API-5000 triple quadrupole mass spectrometer using the following transition: 200 ng/mL (EFdA-tp) and 0.005–100 ng/mL (dATP and dCTP). Resulting concentrations were normalized to the representative live cell count for each species and reported in units of fmol/million live cells. Values below limits of quantification (BLQ) were imputed at a standard level of 10 fmol/million live cells. Linear regression was used to quantify the relationship between natural log (Ln) transformed EFdA treatment concentration and Ln EFdA-tp concentration (reported by the R² value) only when EFdA-tp was quantifiable at all three treatment concentrations. A Kruskal–Wallis one-way analysis of variance (ANOVA) with the Dunn’s pairwise multiple comparisons method was performed to determine whether dATP concentrations in PBMCs differed between species when compared with human PBMCs. Statistical analyses were conducted in SigmaStat v2.0 (Systat Software Inc).

Results

PBMC viability from each batch of blood immediately following separation was as follows: human 93%; rmNHP 99%; rat 75%; minipig 99%; dog 90%; and rabbit 95%. With the exception of rabbit, viability at 24 hours of culture was generally high, as follows: human 96%; rmNHP 92%; rat 87%; minipig 93%; dog 97%; and rabbit 34%. As expected, EFdA-tp was not detected in any of the untreated control samples (data not shown). EFdA-tp (Figure 1) was quantifiable across all three treatment concentrations only for human (9/9 samples quantifiable), rmNHP (9/9 samples quantifiable), and rat (7/9 samples quantifiable). Human PBMCs exhibited the highest EFdA-tp concentrations, which were 1.5–2.1-fold higher than in rmNHP and 4.5–15-fold higher than in rat. Ln-transformed EFdA-tp concentrations increased predictably with LnEFdA in these three species (human r² = 0.96, P < 0.001; rmNHP r² = 0.99, P < 0.001; and rat r² = 0.73, P = 0.003). rmNHP cells exhibited the closest phosphorylation capacity to that of human cells, with nearly parallel regression lines (regression line slope = 0.85 versus 0.89, respectively, compared with 0.73 in rat cells). EFdA-tp was not quantifiable at the 17 nM treatment concentration for minipig PBMCs and EFdA-tp concentrations at 170 and 1700 nM were 37–71-fold lower than those at 1700 nM EFdA for each of the six species.

![Figure 1](https://academic.oup.com/jac/article/77/4/1000/6519800)
than in human cells. EFdA-tp was not quantifiable at 17 and 170 nM treatment concentrations for dog and rabbit PBMCs and at 1700 nM EFdA the EFdA-tp concentrations were 100-fold lower than in human cells. Concentrations of EFdA’s competing endogenous nucleotide, deoxyadenosine triphosphate (dATP), in PBMCs isolated from the blood of six species are summarized by the box plots as follows: centre lines, median; box edges, 25th/75th percentiles; whiskers, 10th/90th percentile; and open symbols, outliers. Because no differences in dATP concentrations were observed between treatment concentrations, all replicates (treated and untreated control, n = 12 per species) were summarized together. Values below limits of quantification (BLQ) were omitted from analyses as they represented only a small proportion of the quantifiable replicates (16%) as follows: 1/12 for minipig, 3/12 for rat, and 2/12 for rabbit. Both rmNHP and dog exhibited significantly higher concentrations of dATP when compared with human (Dunn’s Method pairwise comparison ANOVA P values <0.001).

Figure 2. PBMC dATP concentrations are higher in rhesus macaque non-human primates (rmNHP) and dogs when compared with humans. Concentrations of EFdA’s competing endogenous nucleotide, deoxyadenosine triphosphate (dATP), in PBMCs isolated from the blood of six species are summarized by the box plots as follows: centre lines, median; box edges, 25th/75th percentiles; whiskers, 10th/90th percentile; and open symbols, outliers. Because no differences in dATP concentrations were observed between treatment concentrations, all replicates (treated and untreated control, n = 12 per species) were summarized together. Values below limits of quantification (BLQ) were omitted from analyses as they represented only a small proportion of the quantifiable replicates (16%) as follows: 1/12 for minipig, 3/12 for rat, and 2/12 for rabbit. Both rmNHP and dog exhibited significantly higher concentrations of dATP when compared with human (Dunn’s Method pairwise comparison ANOVA P values <0.001).

Discussion

This study examined differences in EFdA intracellular pharmacology across species. The purpose was to determine how EFdA PK/PD findings in preclinical animal models would translate to humans. Dose selection efforts for efficacy studies of oral EFdA HIV treatment and prevention have aimed to achieve a target of 50 fmol/million PBMCs. Based on these in vitro data, we project that achieving this EFdA-tp target is unlikely in minipigs, rabbits, and dogs within the clinical range of plasma concentrations observed for investigational long-acting formulations of EFdA (1–10 nM). While rmNHP PBMCs most closely matched humans in their phosphorylation capacity of EFdA, they exhibited significantly higher dATP concentrations, which could have implications for EFdA’s potency in this animal model. Interestingly, in two rmNHP challenge studies weekly oral EFdA dosing protected against an IV challenge of SIV and rectal challenges of SHIV at an inhibitory quotient (IQ; defined as the ratio of EFdA-tp trough concentration and in vitro IC$_{50}$) of 1.6 and 2.4, respectively, which is lower than that associated with the lowest effective dose in clinical studies (IQ = 5). This finding may indicate that EFdA’s potency is not diminished by high dATP concentrations, which contrasts with what has been reported for tenofovir diphosphate (TFV-dp), another dATP analogue with a similar mechanism of action and long intracellular half-life. The relationship between EFdA-tp and dATP in rat PBMCs was most similar to that observed in human PBMCs. While it is uncertain whether EFdA phosphorylation capacity is innate to the species or results from the cellular environment, these rat data warrant further characterization of EFdA’s intracellular pharmacology in humanized mice, which may be a useful model to study drug efficacy. Interestingly, significant kinetic differences have been previously described for mouse and human deoxycytidine kinase (the main phosphorylating enzyme of EFdA) and represent a potential mechanism of our findings.

Our study has some limitations. We only explored EFdA-tp and did not characterize intermediary compounds of the phosphorylation pathway such as EFdA-mp. Because structure–activity relationship studies indicate that EFdA-mp may also be pharmacologically active a more complete characterization of intracellular pharmacology is warranted. Additionally, we
used a representative cell count to normalize our nucleotide concentrations, which could confound these concentrations if PBMC counts and viability were highly heterogeneous between our treatment replicates within species. However, given that dATP and dCTP exhibited different between-species relationships (with no difference in dCTP concentrations when compared with human cells), it is unlikely that unrepresentative cell counts drive our conclusions. To ensure EFdA did not exert a cytopathic effect, we conducted a sub-study where PBMCs from each species were isolated and cultured for 24 hours in 0 or 1700 nM EFdA using identical procedures and conditions. The median (range) change in percentage viability relative to untreated control was 1.3% (−2.5% to 13.5%) demonstrating no cytotoxicity across our species. The low PBMC viability in rabbits (34%) after just 24 hours warrants caution in interpreting these rabbit findings. Given that non-viable cells are unable to contribute EFdA-tp, live cell count was used to normalize concentration. However, it is possible that unfavourable culture conditions prevented phosphorylation of EFdA even in live rabbit cells. These in vitro data corroborate findings from an in vivo PK study of an EFdA-eluting subdermal implant in rabbits where sustained EFdA plasma concentrations ranging from 2–93 nM resulted in no quantifiable EFdA-tp in matched PBMC samples for up to 48 days of study (data not shown). Finally, we measured EFdA-tp following 24 hours of incubation based on previous data exhibiting maximal TFV-dp concentrations in human PBMCs after 24 hours of ex vivo tenofovir and tenofovir alafenamide treatment. While TFV-dp has a similarly long half-life (112 hours), it is possible that the time to achieve maximal concentrations of these phosphorylated analogues differs.

To our knowledge, this is the first between-species comparison of EFdA-tp intracellular pharmacology. Herein, we report significant species differences in both EFdA-tp and its competing nucleotide, dATP. Our data demonstrate that, with the exception of rmNHP, the PK efficacy threshold of 50 fmol/million PBMCs should not be used to make ‘go’/‘no go’ decisions for advancing long-acting formulations of EFdA in preclinical PK studies using the animal species tested here. These preclinical models should be considered a conservative estimation of EFdA’s intracellular PK and efficacy in humans.

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Transparency declarations

None to declare.

Disclaimer

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