Development of an LC-MS/MS Assay for Measurement of Intracellular Concentrations of Cidofovir Prodrug USC-505 and its Metabolites

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Abstract

LC-MS/MS assays were developed to measure USC-505 (Figure 1A), a prodrug for the antiviral therapeutic Cidofovir (Figure 1B), and its intracellular metabolites in human foreskin fibroblast (HFF-1) cells.

Introduction

Cidofovir (CDV, Figure 1B) is a nucleoside phosphonate that exhibits broad spectrum therapeutic activity against numerous DNA viruses.[1] The mechanism of action of the drug is through inhibition of viral DNA polymerase upon intracellular conversion to Cidofovir Diphosphate (CDV-PP, Figure 1C). However, cellular uptake of CDV is limited due to the hydrophilic properties of the drug. USC-505 (Figure 1A), a CDV prodrug, has been designed to facilitate improved transmembrane transport of CDV for higher intracellular exposure and potency.[2] The specific mechanisms associated with the uptake of USC-505 and conversion to CDV-PP have yet to be determined. In principle, the hydrophobic tail of the pro-moiety (USC-575, Figure 1D) facilitates transport of the prodrug USC-505 into the cell through the membrane where activating enzymes (undetermined) release CDV (Figure 2). Figure 2 also illustrates the conversion of CDV to the viral DNA polymerase inhibitor CDV-PP.[3,4] To assess the effectiveness of USC-505 for introducing higher intracellular levels of CDV and CDV-PP and to further characterize the intracellular metabolism of the prodrug, LC-MS/MS assays were developed to measure the prodrug and its metabolites in human foreskin fibroblast (HFF-1) cells.

Methods

Cell Growth Conditions: HFF-1 cells were seeded at 6 x 10^6 concentration /T-75 flask in 15% DMEM and incubated for 24 hours at 37˚C incubator with 5% CO_2. The cells were treated with USC-505 at 1uM in 2% DMEM and incubated an additional 72 hours. As summarized in Figure 3, cells were harvested via suspension in chilled 50% acetonitrile. The suspensions were vortexed and frozen immediately at -80˚C to facilitate cell lysis. After the lysates were thawed and centrifuged, the supernatant was collected and analyzed via qualified LC-MS/MS assays for the prodrug USC-505 and its metabolites.

LC-MS/MS Assay Development: USC-505 and its metabolites were analyzed using a Shimadzu 8050 triple quadrupole mass spectrometer equipped with a Nexera UPLC system (Shimadzu). USC-505 and the pro-moiety metabolite were analyzed on a perfluorophenyl (PFP) LC column. The metabolites CDV and CDV-PP were analyzed on a C8 LC column. The compounds were measured in MRM mode using optimized settings. Drug and metabolite concentrations were determined via extrapolation of peak area responses onto external calibration curves prepared by spiking diluted amounts of stock, synthetic compounds into blank HFF-1 lysates.

Assay Qualification Acceptance Criteria:
Accuracy: ± 20% R.E.
Precision: < 20% C.V.
Linearity: r2 > 0.98
Range: 10 (LOQ) – 1,000 ng/mL lysate

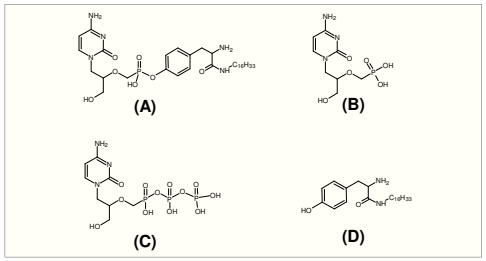

Figure 1: Chemical structures of USC-505 (A) and its intracellular metabolites CDV (B), CDV-PP (C) and the pro-moiety USC-575 (D).

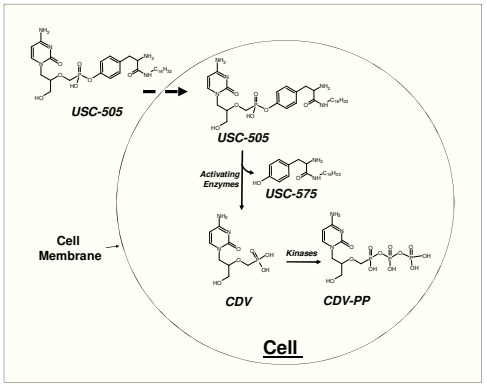

Figure 2: Proposed uptake and conversion of USC-505 to CDV-PP

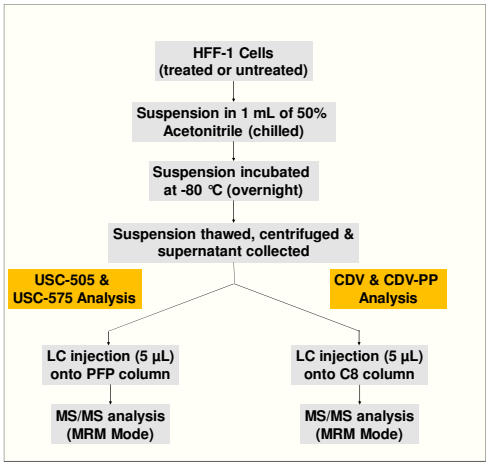

Figure 3: Workflow for extraction and measurement of USC-505 and its corresponding metabolites from HFF-1 cellular lysates.

Results

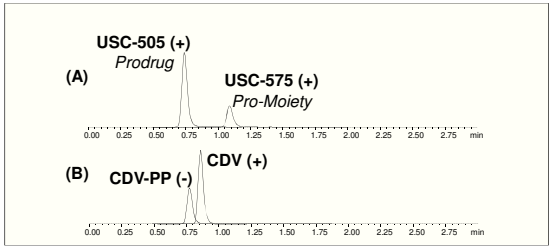

Figure 3: Representative MRM chromatograms of USC-505 and metabolites measured in HFF-1 cells after 72 hr incubation with USC-505. (A) MRM measurement of USC-505 and USC-575 using PFP chromatography. (B) MRM measurement of CDV and CDV-PP using C8 chromatography.

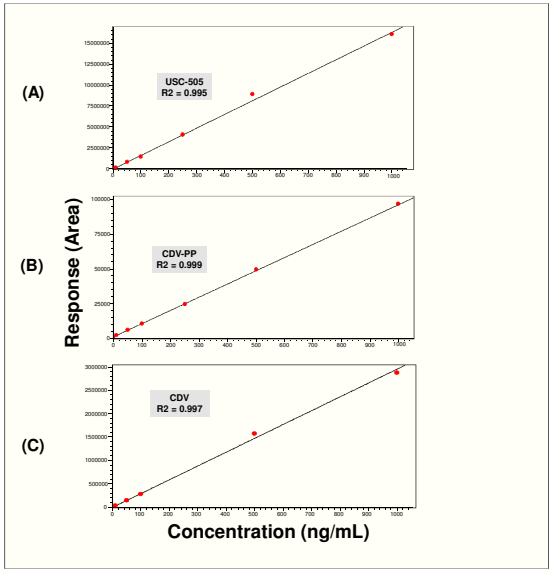

Figure 4: Calibration curves quantification of USC-505 (A), CDV-PP (B) and CDV in HFF-1 cellular lysate.

Table 1: Intracellular concentrations of USC-505 and its corresponding metabolites measured in 1 mL of HFF-1 cellular lysate after 72 hr incubation with the prodrug.
* Approx. 17 million cells per mL

Compound	Identity	Concentrations (nmol/mL lysate)*
USC-505	Prodrug	3.8
CDV	Parent drug	0.3
CDV-PP	Active metabolite	1.0
USC-575	Pro-moiety metabolite	Detected, not quantified

Discussion & Observations

- **The chromatograms displayed in Figure 3A and 3B indicate that the compounds are detected above the background noise with sufficient signal intensity and selectivity.**
- **The assays exhibit sufficient linearity and detection sensitivity to measure the prodrug and its metabolites in cellular lysates (Figure 4A-C).**
- **All three anticipated metabolites were detected intracellularly (as described in Figure 2) implying that HFF-1 cells contain the necessary enzymes to convert USC-505 to CDV and CDV-PP (Table 1). Note: USC-575 was not assayed. Optimized MRM settings for USC-575 were included in the method for USC-505 for qualitative detection of the metabolite.**
- **The performance of the qualified assays described here is sufficient to pursue full analytical validation in multiple cell lines.**
- **The analytical performance of these assays indicates they will be keys tools in establishing the uptake mechanism(s) of USC-505 as well as its intracellular conversion pathways to CDV-PP.**
- **Moreover, the elucidation of the intracellular conversion pathways of USC-505 will be essential in fully characterizing the pharmacokinetic, efficacy and toxicity profile of USC-505 in its treatment of viral infections.**

Future Work

- **Full analytical validation of the assays for the prodrug USC-505 and its metabolites will be performed for application in multiple cell lines, including HFF-1, MRC5, HepG2, and human liver microsomes.**
- **The activating enzymes associated with the intracellular conversion of USC-505 to CDV will be determined. The assays described in this work will be applied to assess USC-505 substrate activity against various enzymes (e.g. phosphatases, lipases, etc.).**

References

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Slide 1

EL4 add student who made compound

Elke Lipka, 9/2/2015