SUPPLEMENTAL DIGITAL CONTENT: DETAILED ASSAY METHODS FOR HUMAN OAT-MEDIATED CELLULAR UPTAKE OF BRINCIDOFOVIR AND MAJOR METABOLITES

Cell Culture and Uptake Assay

The OAT-mediated uptake of brincidofovir, cidofovir, CMX103 and CMX064 was conducted using an established protocol (Optivia Biotechnology, Menlo Park, CA)[1]. Briefly, epithelial Madin-Darby canine kidney type 2 (MDCK-II) cells were seeded on semipermeable filters (1 μm pore size; polyethylene terephthalate (PET) membrane) and cultured for 2 days in a 24-well culture plate supplemented with Dulbecco's Modified Eagle Medium containing 1 g/L glucose, 3.7g/L sodium bicarbonate, and 10% fetal bovine serum. Cells were maintained under 5% carbon dioxide atmosphere at a temperature of 37°C. Approximately 24 hours after seeding cells, media was removed and cells were transiently transfected with OAT1, OAT3, or a green fluorescent protein (GFP) control vector using a proprietary technique. Cells were cultured an additional 24 hours prior to the initiation of the uptake experiment. Transfection efficiency was assessed by inspection of the GFP control using a fluorescent microscope.

Immediately prior to initiation of the uptake assay, cell culture media was removed from the apical and basolateral compartments of the 24-well plate, and cells were rinsed with blank assay buffer (Hank's Balanced Salt Solution, HBSS). The cells were pre-incubated under assay conditions for 15 minutes. During the pre-incubation period, the basolateral compartment contained either blank HBSS or HBSS supplemented with the OAT reference inhibitor probenecid, 100 μM. The apical compartment contained mineral oil. The uptake study was initiated upon replacement of the basolateral pre-incubation assay buffer with fresh assay buffer containing test compounds or control substrates (with and without probenecid). Uptake was conducted for a period of 5 minutes at 37°C with orbital agitation. The duration of the incubation period used for brincidofovir, cidofovir, CMX103 and CMX064 was chosen based on previous studies with test compounds and control substrates for which linear conditions were maintained. At the end of the incubation period, drug solutions were removed from the basolateral compartment and cells were rinsed with cold phosphate buffered saline. Cells were lysed with 50% (v/v) acetonitrile/water.

The concentration used for brincidofovir uptake (5 μM) was based on concentrations used for previous compounds that did not appreciably saturate OAT-mediated uptake, and that allowed detection of compound in mock-transfected cells. Higher incubation concentrations of cidofovir, CMX103 and CMX064 (25 μM) were required to allow detection in mock-transfected cells. All experiments were performed using 3 – 4 wells per experimental condition. However, in instances in which cell lysate concentrations were below the limit of quantification (BLQ), the BLQ results were excluded from summary statistics, and the lower replicate number was noted. The uptake results were

reported as net OAT-mediated uptake, which was determined by subtracting permeation in mock-transfected cells from uptake in OAT-expressing cells.

The control substrate for OAT1 activity was [^{3}H]p-aminohippurate (2 μM). The control substrate for OAT3 activity was [^{3}H]estrone-3-sulfate (0.75 μM). The established assay acceptance criteria for OAT1 activity was >0.71 pmol/min/cm^2 for p-aminohippurate, with >70% inhibition in the presence of probenecid. The minimum established acceptance criteria for OAT3 activity was >0.62 pmol/min/cm^2 for estrone-3-sulfate, with >76% inhibition in the presence of probenecid. The performance of these controls were within assay acceptance criteria. Statistical significance ($p<0.05$) of the cellular uptake of test compounds in transfected versus control cells, or ± probenecid, was assessed using an unpaired t test. Statistical analysis of multiple parameters was performed using analysis of variance.

LC-MS/MS Analysis of Cell Extracts

Brincidofovir

Cell lysate concentrations of brincidofovir were determined using a non-validated assay employing a reverse-phase liquid chromatography and tandem mass spectrometry (LC-MS/MS) method (Integrated Analytical Solutions, Inc., Berkeley, CA). Chromatographic separation was performed using a Peeke Scientific Polymeric SDB (10 x 2 mm) column with an initial mobile phase of 90% water/8% acetonitrile/2 % tetrahydrofuran (containing 0.1% (v/v) formic acid), held constant for 0.25 min, and then changed to 80% acetonitrile/20% tetrahydrofuran (containing 0.1% formic acid, v/v) over a 1.25 min linear gradient. The flow rate was 0.8 mL/min. Calibration standards at 6 different concentrations over the range 0.3 to 100 ng/mL and quality control (QC) samples containing brincidofovir at two concentrations (3 and 30 ng/mL) were prepared in diluted (1:2 lysate:50% (v/v) acetonitrile/water) blank cell lysate. The internal standard was imipramine. The single bioanalytical run included duplicate calibration standards (singlet of each concentration at the beginning and end of run), triplicate QC samples, and matrix blanks. The accuracy (% bias) of calibration standards ranged from -13 to 15%. The %bias of QC samples was 42% and 35% (% relative standard deviation 8.5% and 6.0%) at the 3 and 30 ng/mL concentration, respectively. The QC results suggest a potential bias in sample concentrations. Overall, the potential bias in sample concentrations was judged not to affect the overall study results since all treatment conditions resulted in comparable brincidofovir concentrations (i.e., the net OAT-mediated brincidofovir uptake was approximately zero). Therefore, the assay results were accepted.

Cidofovir, CMX103, CMX064

Cell lysate concentrations of cidofovir, CMX103 and CMX064 were determined using a non-validated assay employing a mixed mode (ion exchange/reverse-phase) LC-MS/MS method (Tandem Labs, Durham, NC). Chromatographic separation was performed using an SIELC Primesep B4 column fit with an SIELC Primesep B4 guard column operated under mixed-mode anionic/reversed-phase conditions and a gradient mobile phase of 200 mM ammonium acetate in water (pH 4.5) and acetonitrile at a total flow rate of

0.8 mL/min. Calibration standards of each analyte at 9 different concentrations over the range 1 to 625 ng/mL were prepared in dilute rabbit plasma (25% v/v in 100 mM Ammonium Acetate, pH 4.5). The dilute rabbit plasma matrix was used to prepared calibration standards and QC samples due to limited availability of blank cell lysate. QC samples at three concentrations (6, 40 and 200 ng/mL) were prepared in 25% rabbit plasma. Additional QC samples (6 and 200 ng/mL) were prepared in blank cell lysate in order to assess the ability of the standards to quantify analyte concentrations in the sample matrix. Stable isotopically labeled internal standard solutions in acetonitrile were added to all samples in a 1:1 (v/v) sample:internal standard solution ratio. The bioanalytical run included duplicate calibration standards (singlet of each concentration at the beginning and end of run), duplicate QC samples and matrix blanks. The assay accuracy (%bias) of standards ranged from -6.0 to 4.0%, -9.0 to 6.2% and -3.6 to 4.2% for cidofovir, CMX103 and CMX064, respectively. The %bias of QC samples ranged from -7.5 to 1.0%, -5.5 to -4.7% and -7.5 to -4.2% for cidofovir, CMX103 and CMX064, respectively. The assay precision (% coefficient of variation) was not calculated because QCs were only prepared in duplicate. The QC samples prepared in blank cell lysate were ≤ 15% of nominal concentrations, with the exception of the low QC for CMX064 that had % bias of -37% due to a low measured concentration for one of the replicates. Overall, the cell lysate QC performance confirmed the ability of the diluted rabbit plasma standards to accurately quantify analyte concentrations in the cell lysate sample matrix.

Reference

1. Wang J, Lustig D, Huang Y. A Robust, Consistent and Scalable Functional Transporter Assay Method Based on Transient Expression of Specific Transporters in Polarized Cell Monolayers. Drug Metab Rev. 2008;40(Suppl 3):271