Simultaneous Delivery of Tenofovir and Acyclovir *via* an Intravaginal Ring

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SUPPLEMENTAL MATERIAL

Bioanalysis methods used in the rabbit study. 9-[(2-Hydroxyethoxy-*d4*)methyl]guanine (ACV-*d4*, Toronto Research Chemicals, Inc.), {[(2*R*)-1-(6-amino-9*H*-purin-9-yl)propan-2-yl]oxy}methyl)-*d6*-phosphonic acid (TFV-*d6*, Toronto Research Chemicals, Inc.), tenofovir diphosphate (TFV DP), and tenofovir diphosphate, [adenine-^{13}C(U)]- (IS for TFV DP, Moravek Biochemicals) were used as internal standards and reference compounds. "Total TFV" refers to phosphatase-treated samples as described below.

Weck-Cel sponges were extracted with internal standard solution (50 µL; for TFV, 200 ng mL^{-1} TFV-*d6*; for ACV, 50 ng mL^{-1} ACV-*d4*) and methanol-water (10:90, 1000 µL), followed by vortex agitation and centrifugation for 5 min at 4500 rpm and 5°C. A 150 µL aliquot of the supernatant was transferred to a 96-well plate for analysis of TFV. A 25 µL aliquot of the supernatant was transferred to a 96-well plate and diluted with 500 µL of acetonitrile for analysis of ACV. Sample analysis was performed by LC/MS using an Agilent Series 1200 HPLC system coupled to an Applied Biosystems/MDS SCIEX API 5000 MS/MS detector. For TFV analysis: Synergi Polar-RP column (2.0 × 75 mm, 4 µm, Phenomenex) and a SecurityGuard Polar-RP (2 × 4 mm, Phenomenex) guard column; injection volume 10 µL; mobile phase 3% acetonitrile in 0.1% formic acid in water run isocratically at a flow rate 0.3 mL min^{-1}; run time 4 min; single ion mode detection, *m/z* 288→176 (TFV), 294→182 (IS, TFV-*d6*). For ACV analysis: Atlantis HILIC Silica column (2.1 × 50 mm, 3 µm, Waters); injection volume 10 µL; gradient program, 1 min hold 10:90 A:B (A, 5 mM ammonium acetate; B, acetonitrile), 0.2 min ramp to 50:50 A:B; 1.1 min hold at 50:50 A:B; 0.2 min ramp to 2:98 A:B; 1.5 min hold at 2:98 A:B; 0.2 min ramp to 10:90 A:B; 0.8 min hold at 10:90 A:B at a flow rate 0.8 mL min^{-1}; run time 6 min; single ion mode detection, *m/z* 226→152 (ACV), 230→152 (IS, ACV-*d4*).

Plasma samples (100 μL) were transferred to a 96-well plate and treated with internal standard solution (50 μL; for TFV, 50 ng mL^{-1} TFV-*d6*; for ACV, 50 ng mL^{-1} ACV-*d4*) and 0.5% formic acid (500 μL), followed by purification by solid phase extraction, evaporation of the eluents under a stream of nitrogen at 50°C, and reconstitution of the residue with 200 μL of water. Sample analysis was performed by LC/MS using an Agilent Series 1100 HPLC system coupled to an Applied Biosystems/MDS SCIEX API 5000 MS/MS detector. For TFV analysis: Synergi Polar-RP column (2.0 × 75 mm, 4 μm, Phenomenex) and a SecurityGuard Polar-RP (2 × 4 mm, Phenomenex) guard column; injection volume 10 μL; mobile phase 2% acetonitrile in 0.1% acetic acid in water run isocratically at a flow rate 0.3 mL min^{-1}; run time 4 min; single ion mode detection, *m/z* 288→176 (TFV), 294→182 (IS, TFV-*d6*). For ACV analysis: Atlantis dC18 column (2.1 × 150 mm, 3 μm, Waters); injection volume 10 μL; mobile phase 97:3 mM ammonium formate in water with 0.1% formic acid:acetonitrile run isocratically at a flow rate 0.3 mL min^{-1}; run time 6 min; single ion mode detection, *m/z* 226→152 (ACV), 230→152 (IS, ACV-*d4*).

Tissue samples were homogenized mechanically with dry ice to produce a finely ground material. Following sublimation of the dry ice at -20°C, the ground tissue was diluted tenfold (100 mg tissue in 1 mL of solvent) with acetonitrile:water (1:1, v/v) and sonicated for 5 min in an ice-water bath. The resulting mixture was centrifuged at 4500 rpm for 10 min and a 50 μL aliquot (100 μL in the case of total TFV) of the supernatant was transferred to a 96-well plate. Internal standard solution (50 μL; for total TFV, 25 ng mL^{-1} TFV-*d6*; for TFV, 50 ng mL^{-1} TFV-*d6* and 100 ng mL^{-1} TFV-DP, adenine-$^{13}C_5$; for ACV, 100 ng mL^{-1} ACV-*d4*) and acetonitrile (250 μL) were added to each well, followed by vortex agitation and centrifugation for 5 min at 4000 rpm and 5°C. A 150 μL aliquot of the supernatant was transferred to a 96-well plate and the

solvent was evaporated under a stream of nitrogen at 50°C. The dried residue was reconstituted with 150 µL of water followed by vortex agitation and centrifugation for 5 min at 4500 rpm and 5°C. For total TFV, the sample and internal standard were treated with acid phosphatase (25 units mL^{-1} in 50 mM ammonium acetate, pH 4.0) at 40°C for 40 min, followed by filtration and processing as described above. Rabbit lymph node homogenate was prepared by freezing the samples at -78°C directly followed by pulverization. For each 20 mg of crushed lymph node, methanol:water (60:40 v/v, 1 mL) was added and the samples were sonicated, centrifuged at 4000 rpm, 5°C, for 10 min and a 200 µL aliquot of the supernatant was transferred to a 96-well plate. Internal standard solution (20 µL; 500 ng mL^{-1} TFV-*d6*) was added to each well, followed by vortex agitation. The samples then were membrane filtered at 14000 rpm for 20 min through Microcon sample reservoir (50K membrane) vial inserts. A 65 µL aliquot of the mixed, filtered solution was transferred to a 96-well plate and another 65 µL of 1% formic acid in water was added followed by mixing and centrifugation for 5 min at 4000 rpm and 4°C.

Sample analysis was performed by LC/MS using an Agilent Series 1100 HPLC system coupled to an Applied Biosystems/MDS SCIEX API 5000 MS/MS detector. For total TFV analysis, the method described above for plasma samples was used. For TFV and TFV-DP analysis: BioBasic AX column (3.0 × 50 mm, 5 µm, Thermo Scientific); injection volume 5 µL; gradient program, 0.5 min hold 10:90 A:B (A, acetonitrile:10 mM ammonium acetate in water, 30:70 v/v, pH 6; B, acetonitrile:1 mM ammonium acetate in water, 30:70 v/v, pH 10.5) at a flow rate of 0.4 mL min^{-1}, 0.5 min ramp to 50:50 A:B; 1.0 min hold at 50:50 A:B; 0.1 min ramp to 100% B; 5.9 min hold at 100% B; 0.1 min ramp to 95:5 A:B at 1.0 mL min^{-1}; 3.9 min hold at 95:5 A:B; run time 12 min; single ion mode detection, *m/z* 288→176 (TFV), 294→182 (IS, TFV-*d6*), 446→159 (TFV-DP), 451→159 (IS for TFV-DP). For TFV in lymph tissue: Zorbax

XDB-C8 column (4.6 × 75 mm, 3.5 μm, Agilent); injection volume 10 μL; gradient program, 0.5 min hold 80:20 A:B (A, 0.2% formic acid, 5 mM ammonium acetate in water; B, 0.2% formic acid in methanol) at a flow rate of 0.7 mL min^{-1}, 0.5 min ramp to 5:95 A:B; 1.0 min hold at 5:95 A:B; 0.1 min ramp to 80:20 A:B; 3.1 min hold at 80:20 A:B; run time 5.2 min; single ion mode detection, *m/z* 288→176 (TFV), 294→182 (IS, TFV-*d6*). For ACV, the analytical method described above for the plasma samples was employed.