Clinical Extrapolation of the Effects of Dolutegravir and Other HIV Integrase Inhibitors on Folate Transport Pathways

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Supplemental Methods 1: LC-MS/MS bioanalytical method summary.

Sample preparation

In PCFT assay, eight concentrations of the test article were applied to the apical side of columns 3 through 10 in a 96-well cell plate transfected with a control vector in Rows A to D, and PCFT in Rows E to H. At the end of 5 min incubation, 20 µL samples were taken from the apical side and transferred to the corresponding wells in a 96-well sample plate preloaded with 180 µL internal standard in 50% acetonitrile (acetonitrile:H_2O=1:1). Samples were frozen at -80ºC for later detection.

In RFC assay, eight concentrations of the test article were applied to the basal side of columns 3 through 10 in a 96-well cell plate transfected with a control vector in Rows A to D, and RFC in Rows E to H. At the end of 5 min incubation, 20 µL samples were taken from the basal side and transferred to the corresponding wells in a 96-well sample plate preloaded with 180 µL internal standard in 50% acetonitrile (acetonitrile:H_2O=1:1). Samples were frozen at -80ºC for later detection.

In FRα assay, eight concentrations of the test article were applied to both sides of columns 3 through 10 in a 96-well cell plate transfected with a control vector in Rows A to D, and FRα in Rows E to H. At the end of 120 min incubation, 20 µL samples were taken from the apical side and basal side of Rows E-H and transferred to Rows A to D, and E to H, respectively, in a 96-well sample plate preloaded with 180 µL internal standard in 50% acetonitrile (acetonitrile:H_2O=1:1). Samples were frozen at -80ºC for later detection. Rows A-D in the cell plate (cells transfected with a control vector) were not sampled.

LC-MS/MS analytical conditions for dolutegravir, cabotegravir, bictegravir, elvitegravir, raltegravir, methotrexate, and valproic acid

1. Equipment

LC model (Shimadzu)	Shimadzu LC-20AD, and Shimadzu SIL-20A Analyst 1.6.2. software for instrument control and data analysis
MS model (AB Sciex)	API4000 triple quadrupole MS Analyst 1.6.2. software for instrument control and data analysis

2. LC Conditions

Column type	Kinetex Polar-C18, 2.6 µm particle size, 3.0 x 50 mm
Eluent A	5% ACN in H_2O, w/ 0.1% formic acid and 5mM ammonium formate
Eluent B	95% ACN in H_2O, w/ 0.1% formic acid and 5mM ammonium formate
Flow rate	800 µL/min
Column temperature	Room temperature
Injection volume	2 µL

Gradient

time (min)	A (%)	B (%)
BIC, CAB, DOL, EVT, MTX		
0	98	2
0.2	60	40
0.4	20	80
0.8	0	100
1.2	0	100
1.25	98	2
2	98	2
RAL, VPA		
0	100	0
0.2	98	2
0.4	20	80
0.8	0	100
1.2	0	100
1.25	100	0
2	100	0

3. MS parameters

bictegravir, cabotegravir, dolutegravir, elvitegravir, and methotrexate:

Ionization:	ESI(+)
Scan type	MRM
Ionspray Voltage	4500 V
Ion Source gas 1	50 psi
Ion Source gas 2	50 psi
Curtain gas	20 psi
Temperature	500 °C
Resolution Q_1	Unit
Resolution Q_3	Unit
DP	50
EP	10

raltegravir and valproic acid:

Ionization:	ESI(-)
Scan type	MRM
Ionspray Voltage	4500 V
Ion Source gas 1	50 psi

Ion Source gas 2	50 psi
Curtain gas	20 psi
Temperature	500 °C
Resolution Q_1	Unit
Resolution Q_3	Unit
DP	50
EP	10

	Q1 mass (Da)	Q3 mass (Da)	Dwell time (msec)	DP	CE	CXP
bictegravir	450.2	289.1	150	116	39	18
cabotegravir	406.3	127.0	150	106	37	10
dolutegravir	420.2	277.2	150	111	35	16
elvitegravir	448.2	430.2	150	81	29	12
methotrexate	455.3	308.2	150	90	40	12
IS_Carbutamide(+)	270.0	156.2	150	50	25	12
raltegravir	443.2	315.9	150	-95	-24	-9
valproic Acid	142.9	142.9	150	-55	-8	-11
IS_Carbutamide(-)	269.9	171.0	150	-40	-20	-15

Internal standard (IS): Carbutamide

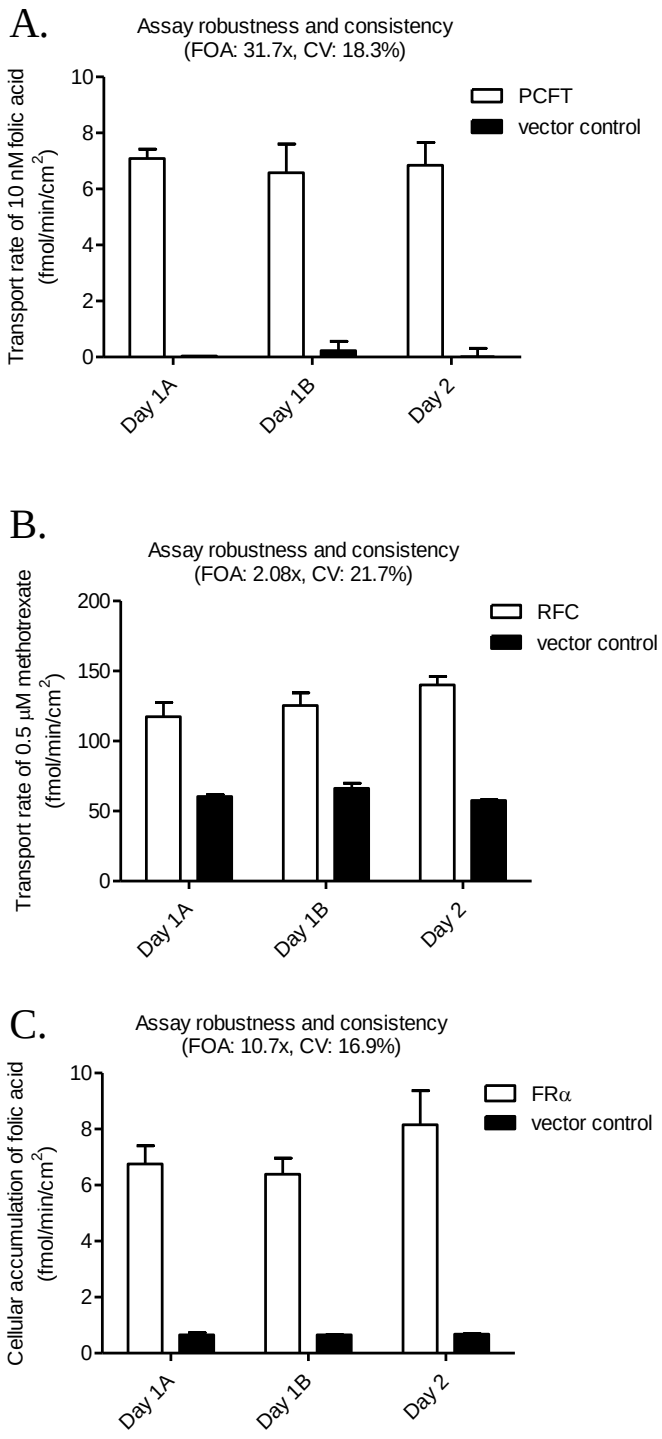
Retention time(min.):

bictegravir	1.32
cabotegravir	1.29
dolutegravir	1.30
elvitegravir	1.45
methotrexate	1.18
raltegravir	1.34
valproic acid	1.32
IS_Cabutamide (+)	1.26
IS_Cabutamide (-)	1.32/1.36

LOD for BIC, CAB, DUL, EVT, MTX, RAL	0.5 ~ 1 nM
LOD for valproic acid	100 nM

Matrix: HBSS+50% Acetonitrile

Supplemental Figure 2: Functional verification of optimized folate *in vitro* transport assays. PCFT transport of 10 nM [^{3}H]-folic acid (A). RFC transport of 0.5 μM [^{3}H]-methotrexate (B). FRα-mediated endocytosis of 50 nM [^{3}H]-folic acid (C). Note all inhibitor concentrations and IC_{50} values are nominal. Mean ± S.D., n = 4.



Supplemental Table 3: Evaluation of cytotoxicity by lactate dehydrogenase (LDH) leakage.

To evaluate the possibility that inhibition observed in the FRα endocytosis assays (longest incubation duration on both apical and basolateral sides) was an artifact of cytotoxicity, LDH release was investigated in FRα-expressing and control cells at the two highest test article concentrations. As this was the longest incubation of cells and from both sides, a negative cytotoxicity results in this assay was deemed adequate to address cytotoxicity in PFCT and RFC assays (35 min vs 2.5 h incubation and only from apical or basal side vs both). Only elvitegravir exhibited statistically significant >12.5% cytotoxicity in the FRα-expressing cells at the top two tested concentrations (Supplemental Table 3.1). However, elvitegravir cytotoxicity was not observed under the incubation conditions of the PCFT assay, where inhibition of this transporter was observed at the two highest elvitegravir concentrations, and which cannot be attributed to cytotoxicity (Supplemental Table 3.2).

Supplemental Table 3.1. LDH release in vector control and FRα-expressing cells following incubation with test articles.

Treatment	Concentration (μM)	Absorbance-Blank (FRα receptor)	Absorbance-Blank (vector control)	% Cytotoxicity (FRα receptor)	% Cytotoxicity (vector control)
Vehicle		641 ± 64	1,005 ± 187	0.0 ± 0.0	0.0 ± 0.0
dolutegravir	15.9	875 ± 128	1,350 ± 102	2.7 ± 1.5	4.5 ± 1.4
dolutegravir	37.3	924 ± 153	1,036 ± 99	3.3 ± 1.8	0.4 ± 1.3
cabotegravir	11.6	1,189 ± 204	1,047 ± 242	6.3 ± 2.4	0.6 ± 3.2
cabotegravir	25.8	1,033 ± 144	1,125 ± 398	4.5 ± 1.7	1.6 ± 5.2
bictegravir	103	1,691 ± 36	1,495 ± 462	12.1 ± 0.7	6.4 ± 6.1
bictegravir	442	1,479 ± 117	1,462 ± 1,020	9.7 ± 1.4	5.9 ± 13.4
elvitegravir	4.18	2,975 ± 474	809 ± 193	26.9 ± 5.6*	-2.6 ± 2.5
elvitegravir	27.6	1,817 ± 233	1,925 ± 682	13.6 ± 2.8*	12.1 ± 8.9
raltegravir	171	1,738 ± 645	916 ± 167	12.7 ± 7.5	-1.2 ± 2.1
raltegravir	472	1,134 ± 114	1,028 ± 126	5.7 ± 1.3	0.3 ± 1.6
valproic acid	352	1,150 ± 111	1,031 ± 79	5.9 ± 1.3	0.3 ± 1.0
valproic acid	1,380	1,192 ± 485	7,626 ± 4,240	6.5 ± 5.6	86.9 ± 55.7
methotrexate	271	717 ± 186	710 ± 250	0.9 ± 2.1	-3.9 ± 3.3
methotrexate	616	1,200 ± 80	1,177 ± 458	6.4 ± 1.0	2.3 ± 6.0
1% Triton-X 100		9,312 ± 393	8,627 ± 592	100 ± 6.4*	100 ± 8.9*

% of toxicity was calculated assuming vehicle was 0% and 1% Triton-X was 100%.
* >12.5% cytotoxicity, which is significantly different from vehicle by t-test with Bonferroni's correction. Mean ± S.D., n = 3.

Supplemental Table 3.2. LDH release in vector control and PCFT-expressing cells after incubation with elvitegravir.

Treatment	Concentration (μM)	Absorbance-Blank (PCFT)	Absorbance-Blank (vector control)	% Cytotoxicity (PCFT)	% Cytotoxicity (vector control)
Vehicle		1,547 ± 319	1,279 ± 160	0.0 ± 0.0	0.0 ± 0.0
elvitegravir	8.24	1,210 ± 217	1,968 ± 223	-3.9 ± 2.5	7.8 ± 2.5
elvitegravir	30.0	701 ± 168	1,088 ± 155	-9.8 ± 1.9	-2.2 ± 1.7
1% Triton-X100		10,140 ± 95	10,160 ± 125	100 ± 1.5*	100 ± 1.9*

% of toxicity was calculated assuming vehicle was 0% and 1% Triton-X was 100%.
* >12.5% cytotoxicity, which is significantly different from vehicle by t-test with Bonferroni's correction. Mean ± S.D., n = 3.