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BRINCIDOFOVIR (BCV) DELIVERS HIGH INTRACELLULAR CONCENTRATIONS OF CIDOFOVIR DIPHOSPHATE

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OBJECTIVE

To determine the intracellular concentration of cidofovir diphosphate in brincidofovir and cidofovir-treated cells *in vitro*

INTRODUCTION

Brincidofovir (BCV, CMX001) is a lipid conjugate nucleotide in Phase 3 development for the prevention of CMV infection in HCT recipients. BCV is administered orally, circulates as BCV, and is converted to the active antiviral cidofovir diphosphate (CDV-PP) within cells. BCV shares the broad-spectrum antiviral activity of cidofovir (CDV) against all five families of dsDNA viruses which cause disease in humans. The 50 to 500-fold improved *in vitro* activity of BCV versus CDV has been hypothesized to result from more efficient transport of circulating BCV across the cell membrane, resulting in higher intracellular concentrations of CDV-PP. The goal of this study was to determine if cells exposed to the same concentration of BCV and CDV would have similar or different concentrations of CDV-PP. Five cell types that have been historically used for generating antiviral activity of BCV and CVD were used in this study.

METHODS

Cells

Human foreskin fibroblast cells (HFF), MRC-5 cells, A549 cells, HepG2 cells and Vero cells were obtained from ATCC and grown in the recommended medium in the presence of 10% FBS. During treatment with BCV and CDV, the FBS concentration was 2% to reflect the FBS concentration used in the antiviral assays.

Treatment of cells with BCV and CDV

Cells were seeded in duplicate sets of T75 flasks at 6E+06 cells/flask. Compound (BCV or CDV) was added to a final concentration of 1µM. Flasks were incubated at 37^{0}C in a CO_2 incubator for 72 hrs. Cells were harvested as follows. Cells from one set of two flasks were rinsed with saline twice and treated with 2mL of 0.05% trypsin for 5 minutes at 37^{0}C to dislodge the cells. To the trypsinized cells, 2mL of medium containing FBS was added followed by 35 mL of chilled saline. Cells were then collected by centrifuging at 250xG for 5 minutes at 4^{0}C. Cells were washed with a second lot of 40mL of chilled saline and then resuspended in 10mL chilled saline. A 50µL aliquot of cells was used to determine the cell number, the rest was spun down and 1mL of chilled Methanol:DW mixture (70:30) added to the cell pellet. Cells were vortexed and cell suspension frozen immediately on dry ice. Cells from a second set of two flasks were harvested using accutase similar to the method used for cells treated with trypsin excepting no medium was added after cells were dislodged after accutase treatment. A third set of flasks was harvested as follows. After medium was aspirated, cells in the flasks were rinsed twice with 40mL of chilled saline. 1mL of chilled methanol:DW (70:30) was added to each flask and cells were scraped off using a cell scraper. The collected cell suspension was vortexed and frozen immediately on dry ice.

Human peripheral blood mononuclear cells (PBMC)

Buffy coats were prepared from human blood and PBMCs stimulated with 2µg/mL phytohemagglutinin (PHA, Sigma) for 48 hrs. After stimulation, cells were resuspended in fresh medium without PHA and treated with compound for 72 hrs. Cells were processed as mentioned above to determine CDV-PP concentration. Briefly cells were rinsed in chilled saline, counted and extracted by resuspending cell pellet in methanol:DW (70:30).

Analytical measurements of BCV, CDV and CDV-PP

Intracellular levels of BCV, CDV and CDV-PP were determined by LC/MS/MS by Pyxant Laboratories, Colorado Springs, CO using similarly prepared calibration standards.

RESULTS

Table 1 CDV-PP estimation in A549 cells treated with 1µM BCV and 1 & 5µM CDV for 3 days

Compd	Harvest	CDV-PP (pg/mL)	Total Cell#	CDV-PP[1]	CDV-PP (% of MeOH:DW)
BCV 1µM	Accutase	12300	1.2E+07	2.40	26.0%
	Trypsin	16800	1.1E+07	3.40	36.8%
	MeOH:DW	46500	1.2E+07*	9.24	100.0%
CDV 1µM	Accutase	258	1.6E+07	0.04	56.9%
	Trypsin	250	1.6E+07	0.04	54.3%
	MeOH:DW	457	1.6E+07*	0.07	100.0%
CDV 5µM	Accutase	914	1.6E+07	0.13	42.5%
	Trypsin	1110	1.7E+07	0.15	48.5%
	MeOH:DW	2220	1.6E+07*	0.31	100.0%
Control	MeOH:DW	BLOQ			

* Cell number taken as an average of accutase and trypsin treatments
[1] pmol/10^6 cells
BLOQ – below limit of quantitation

- Dislodging cells with trypsin and accutase from flasks prior to extracting gave similar yields of CDV-PP
- Extracting cells directly in methanol:DW (70:30) gave the highest yield of CDV-PP

Table 3. CDV-PP levels in stimulated human peripheral blood mononuclear cells treated with BCV or CDV for 3 days

Compd	Compd Conc. (µM)	CDV-PP*	CDV-PP (%)
BCV	3	10.49	100%
	10	15.27	
CDV	3.3	0.03	0.54%
	10	0.11	

* pmol/10^6 cells

Table 2. BCV, CDV and CDV-PP levels in cells treated with 1µM CMX001 or 1µM CDV for 3 days

Cell Type	Compd	pmol/1E+06 cells			CDV (%)	CDV-PP (%)
		CDV-PP	CDV	CMX001		
Vero	BCV	13.6	34.7	309.2	100%	100%
		12.5	31.4	307.4		
	CDV	0.08	0.25		0.8%	0.6%
		0.07	0.27			
HFF	BCV	57.6	69.6	113.1	100%	100%
		60.9	68.6	91.0		
	CDV	0.13	0.42		0.6%	0.22%
		0.13	0.46			
HepG2	BCV	6.4	19.1	161.7	100%	100%
		6.1	12.8	158.8		
	CDV	0.16	0.78		5%	3.0%
		0.21	0.74			
MRC5	BCV	25.9	147.7	123.5	100%	100%
		31.6	122.2	112.3		
	CDV	0.175	BLOQ		-	0.6%
		0.179	BLOQ			

BLOQ – below limit of quantitation

Table 4. Higher levels of CDV-PP in BCV-treated cells correspond to an increase in antiviral activity of BCV in cell-based assays[1]

Viral Family	Virus	Activity (EC_{50}, µM)		Enhanced Activity (CDV/BCV)	Cell Line	CDV-PP (pmol/10^6 cells)		Enhanced CDV-PP Level (BCV/CDV)
		BCV	CDV			BCV trreated cells	CDV trreated cells	
Herpes	CMV	0.001	0.4	400	MRC-5	29	0.177	162
	HSV 1	0.01	3	300	MRC-5	29	0.177	162
	HSV 2	0.02	6.5	325	MRC-5	29	0.177	162
	VZV	0.0004	0.5	1250	HFF	59	0.129	457
Adenovirus	AdV 5	0.02	1.3	65	HFF	59	0.129	457
Pox	Variola	0.1	27	270	Vero	13	0.073	178
	Vaccinia	0.8	46	58	HFF	59	0.129	457

[1]Antiviral data from Williams-Aziz (1) and Lanier (2)

SUMMARY

- The method used for harvesting cells affected the yield of CDV-PP in the extract
  - Comparable levels of CDV-PP were measured when trypsin or accutase were used to dislodge cells from tissue culture flasks
  - Harvesting cells directly in methanol:DW doubled the yield of CDV-PP detected
- BCV-treated cells had higher levels of CDV and CDV-PP compared to CDV-treated cells
  - Increase in intracellular CDV concentration ranged from 20 to over 100 fold
  - Increase in intracellular CDV-PP concentration ranged from 33 to 450 fold
- Concentrations of CDV-PP in stimulated human PBMCs were similar to those seen in other cell types

CONCLUSIONS

- Concentrations of CDV and CDV-PP in cells treated with BCV are orders of magnitude higher than those seen in CDV treated cells
- The increase in intracellular levels of CDV-PP corresponds well with an increase in potency of BCV over CDV in antiviral assays *in vitro*
- The enhanced level of CDV-PP in BCV-treated cells used in antiviral assays were comparable to those observed in human PBMCs

REFERENCES

1. Williams-Aziz. et al. (2005) Antimicrob. Agents Chemother. 49: 3724-3733
2. Lanier R. et al. (2010) Viruses. 2: 2740-2762.

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