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IN VITRO SELECTION OF BRINCIDOFOVIR-RESISTANT AND CIDOFOVIR-RESISTANT HUMAN ADENOVIRUS

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OBJECTIVE

To generate brincidofovir (BCV, CMX001) and cidofovir (CDV) resistant adenovirus (AdV) through *in vitro* selection and to evaluate sequence changes in the AdV DNA polymerase gene.

INTRODUCTION

Brincidofovir is an orally bioavailable lipid acyclic nucleoside phosphonate which is converted intracellularly to the active antiviral cidofovir diphosphate (CDV-PP). BCV shares the broad-spectrum antiviral activity of CDV against all five families of dsDNA viruses which cause disease in humans, including adenoviruses (AdV). The 50 to 500-fold improved *in vitro* activity of BCV vs CDV is likely due to efficient transport of BCV across the cell membrane, resulting in higher intracellular concentrations of CDV-PP (see poster ####). Mutations in the AdV DNA polymerase gene have been reported to impart resistance to CDV (1, 2). Since the active antiviral is qualitatively the same for BCV and CDV, sequence changes in BCV-resistant and CDV-resistant viruses selected under identical conditions were compared.

METHODS

Passaging AdV 5 in BCV and CDV
T75 flasks were seeded with 2E+06 A549 cells. After overnight growth at 37^{0}C, the cells were infected with AdV5 at an MOI of 0.01. At the end of the infection period, cells were rinsed with medium and 20mL of fresh medium containing the appropriate concentration of CDV or BCV was added and incubation continued at 37^{0}C. Flasks were harvested when distinct CPE was observed. Passaging in the presence of BCV or CDV was continued using harvested virus, free of cellular debris.

The concentration of BCV and CDV used over the 15 passages ranged from 1x EC_{50} to 21x EC_{50}. Passaged viruses were tested in a phenotyping assay to determine if there was an increase in EC_{50} over the wild-type (WT) strain. The DNA polymerase region was sequenced to determine if there were any changes in the DNA sequence.

Phenotyping assay
The AdV phenotyping assay was carried out in A549 cells with a qPCR readout to quantitate the amount of AdV DNA. The assay was used to report the EC_{50} and fold-change (FC) of the test specimen compared to the WT reference virus.

Cells were seeded in a 96-well plate at a density of 2.5E+04 cells/well along with virus at an MOI of 0.01. Appropriate compound dilutions were then added to the wells. After 3 days of incubation at 37^{0}C in a CO_2 incubator, the medium was aspirated and DNA harvested for qPCR analysis as described in (3). AdV DNA was quantified from 2.5µL of processed sample using the Life Technologies 7500 Real Time PCR System in a 25µL reaction mixture using TaqMan® Gene Expression Master Mix. The qPCR cycling parameters were 95^{0}C for 10 mins, followed by 45 cycles at 95^{0}C for 15 secs and 60^{0}C for 1 min. An AdV DNA quantitation standard with a dynamic range of 10^{10} to 10^3 DNA copies/mL was used.

DNA sequencing
DNA was extracted from virus grown in cell culture using the QIAamp MinElute Virus Spin Kit. The AdV DNA polymerase region was amplified, excepting for the first three amino acids, by PCR and sequenced. Sequencing was carried out at Eton Biosciences using an ABI 3730xl DNA Sequencer. The DNA sequence was assembled using Sequencher (v5.1) and subsequently translated to the amino acid sequence of the amplified AdV pol gene. BioEdit (v7.2.0) was used to align the nucleotide and amino acid sequence of the sample against the reference sequence to identify any alteration or discordant changes.

RESULTS

Table 1: Fold Change in EC_{50} against BCV and CDV with BCV and CDV-resistant isolates selected *in vitro*

Mutant / Source	AdVC5	Fold Change In EC_{50}	
		BCV	CDV
---	Wild-type	1.0	1.0
AdV-BCV p5 [a]	V303I	2.2	ND
AdV-BCV p15 [a]	T87I, V303I [b]	6.0	6.1
AdV-CDV p15[c]	T1151I	4.5	7.4
R1, Kinchington [d]	F882L/S1183R	5 - 10	7.2
R4, Kinchington [d]	V303I, F882I	8.1	5.1
R5 & R6, Kinchington [e]	A501E, L677F	8.7	5.6

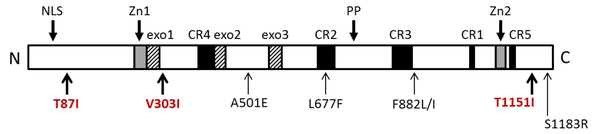
[a] Resistant mutant selected in the presence of BCV at Chimerix
[b] Plaque purified virus
[c] Resistant mutant selected in the presence of CDV at Chimerix
[d] Resistant virus obtained from Kinchington (2)
[e] Data from Kinchington mutant R5
ND – not determined

Table 2. Sequence changes identified in the DNA polymerase gene of BCV and CDV passaged virus

Virus	Amino Acid Positions						
	87	303	501	677	882	1151	1183
CR-P (WT)	T	V	A	L	F	T	S
	ACC	GTC	GCG	CTC	TTT	ACC	AGC
AdV_p5_BCV		V/I					
		RTC					
AdV_p10_BCV		I					
		ATC					
AdV_p12_BCV		I					
		ATC					
AdV_p14_BCV	I/T	I					
	AYC	ATC					
AdV_p15_BCV	I	I					
	ATC	ATC					
AdV5_p15_CDV						I	
						ATC	
CR-R1*					L		R
					CTT		AGA
CR-R4*		I			I		
		ATC			ATT		
CR-R5*			E	F			
			GAG	TTC			
CR-R6*			E	F			
			GAG	TTC			

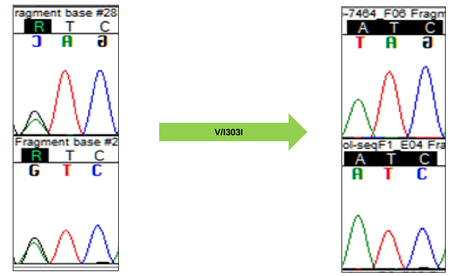
* CDV resistant mutants generated by Kinchington (1, 2)

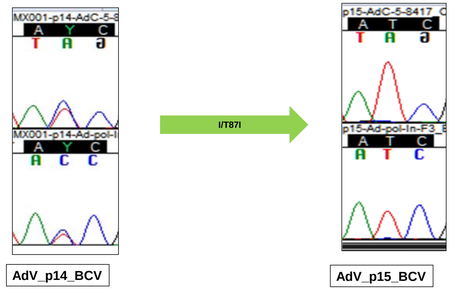
Figure 1. Schematic representation of amino acid changes in BCV and CDV-resistant AdV5 DNA polymerase



Changes in red are those identified in resistance passage studies conducted at Chimerix. Numbers in black are those identified by Kinchington (2). Conserved domains of the protein are indicated: Nuclear localization signal (NLS); putative Zinc finger DNA binding domains (Zn1-2); sequences conserved in exonucleases (exo1-3); DNA polymerase conserved regions (CR1-5); a conserved sequence in DNA polymerases which use protein priming to initiate DNA replication (PP). Figure adapted from Parker (4).

Figure 2. Sequence changes identified in the DNA polymerase gene of BCV passaged virus **(**AdV_p15_BCV)





SUMMARY

- BCV and CDV-resistant AdV were selected in cell culture
- Even though the active antiviral agent within cells is the same in BCV and CDV treated cells (CDV-PP), the genotyping profiles for the resistant viruses were different:
  - BCV resistant AdV had two changes in the DNA pol sequence T87I, V303I
  - Both changes (T87I, V303I) were necessary for the virus to exhibit a 5-fold increase in EC_{50} to BCV
  - CDV-resistant AdV had only one change in the DNA pol sequence T1151I
- One of the three changes in the DNA polymerase gene we observed was also seen earlier by Kinchington (2)

CONCLUSIONS

- This is the first report of selecting a BCV-resistant AdV in cell culture
- BCV and CDV-resistant AdV have a partial overlap in their resistance profile
- AdVs resistant to BCV and CDV displayed similar relative increases in EC_{50} as compared to wild type with BCV being about 100-fold more potent than CDV
- The observed difference in mutations selected could be stochastic or a real difference, possibly reflecting higher intracellular levels of CDV-PP in BCV-treated cells (see poster ###)

REFERENCES

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CHIMERIX, INC.