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FINAL TECHNICAL REPORT

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SELECTION AND CHARACTERIZATION OF VIRUS STRAINS RESISTANT TO MDH-1-38 AND PRODRUG NS104~~FULLERENE POLY-AMINO CAPROIC ACID~~

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Introduction

This final report summarizes the results of the *in vitro* selection of HIV-1 resistant to fullerene poly-amino caproic acid (FPACA) for six months. *In vitro* resistance selection assays were performed to evaluate the relative rate of appearance of drug-resistant virus strains when virus was cultured in the presence of increasing concentrations of FPACA and to define the resistance-conferring mutations which appear in the selected virus isolates. The virus strain (HIV-1_{IIIB}) employed for the assay was serially passaged in CEM-SS cells in the presence of FPACA for seventeen passages. RNA was extracted from cell culture supernatant at passage 14 and passage 17 for sequencing of the virus envelope and protease genes.

Materials and Methods

Compounds:
Fullerene poly-amino caproic acid (FPACA) provided by CJSC Intelpharm was solubilized at 40 mg/mL in DMSO and stored at -20°C.

Drug Resistant Virus Selection By Dose Escalation:

Acute virus infections were initiated with 8 x 10^5 CEM-SS cells in RPMI1640 tissue culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptamycin in T25 flasks. Inoculum consisted of HIV-1_{IIIB} virus at a multiplicity of infection (MOI) predetermined by virus titration to yield complete cell killing by 6 days post-infection in the absence of anti-HIV compound (virus control). FPACA was added at two-times the mean EC_{50} concentration determined in the anti-HIV cytoprotection assay. Infection was allowed to proceed for one hour at 37°C in 200 µL of tissue culture medium. The medium volume was then increased to 5 mL after the one hour incubation. The medium volume was increased to 10 mL on day 3.

Beginning on day 4, daily observations for syncytium formation were made and supernatant collected to determine the peak day of virus replication by RT assay. The recovered virus-containing supernatants were placed into aliquots and stored at -80°C until used. With each successive *de novo* infection of CEM-SS cells, the concentration of FPACA was increased two-fold up to passage 17. Changes in the concentration of compound employed at each passage (or a determination to continue to passage the virus several times at a fixed concentration) were made based on the ability of the virus to grow in the presence of FPACA at a given concentration. When virus production was not observed at any given passage, fine tuning of the selection process to reduce the incremental dose escalation and return to earlier passage number with smaller increments of dose increase were attempted. Upon completion of a given passage, the virus produced from the cell culture in the presence of FPACA was evaluated for sensitivity to both FPACA and AZT (drug control) in the microtiter anti-HIV cytoprotection assay in parallel with similarly passaged but untreated virus. Figure 1 in **Appendix I** summarizes the process of selecting for FPACA-resistant HIV.

Anti-HIV Cytoprotection Assay for Drug Susceptibility:

Cell Preparation

CEM-SS cells were passaged in RPMI1640 supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin in T-75 flasks prior to use in the antiviral assay. On the day preceding the assay, the cells were split 1:2 to assure they were in an exponential growth phase at the time of use. Total cell and viability quantification were performed using a hemocytometer and Trypan Blue dye exclusion, respectively. Cell viability was greater than 95% for the cells to be utilized in the assay. The cells were resuspended at 5 x 10^4 cells per mL in tissue culture medium and added to the drug-containing microtiter plates (FPACAconcentrations of 100 µg/mL to 0.32 µg/mLM in 100 µL per well) in a

volume of 50 μL. The control compound AZT (0.5, 0.16, 0.05, 0.016, 0.005, and 0.0016 μM) was evaluated as an internal assay standard.

Virus Preparation

The virus used for the cytoprotection assays was the lymphocyte-tropic strain HIV-1_{IIIB}. The virus was obtained from the NIH AIDS Research and Reference Reagent Program and stock virus pools were produced in CEM-SS cells. A pretitered aliquot of each untreated virus in parallel with each passage of virus treated with FPACA was removed from the freezer (-80°C) and allowed to thaw slowly to room temperature in a biological safety cabinet. Virus was resuspended and diluted into tissue culture medium such that the amount of virus added to each well in a volume of 50 μL was the amount determined to yield 85 to 95% cell killing at 6 days post-infection.

Plate Format

Each microtiter plate contained cell control wells (cells only), virus control wells (cells plus virus), drug toxicity wells (cells plus drug only), drug colorimetric control wells (drug only) as well as experimental wells (drug plus cells plus virus). Samples were tested in triplicate over a range of five half-log dilutions.

Efficacy and Toxicity Evaluation

Following incubation at 37°C in a 5% CO_2 incubator for 6 days, the test plates were stained with the tetrazolium dye XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide). XTT-tetrazolium is metabolized by the mitochondrial enzymes of metabolically active cells to a soluble formazan product. XTT solution was prepared daily as a stock of 1 mg/mL in RPMI1640. Phenazine methosulfate (PMS) solution was prepared at 0.15 mg/mL in PBS and stored in the dark at –20°C. XTT/PMS stock was prepared immediately before use by adding 40 μL of PMS per mL of XTT solution. Fifty microliters of XTT/PMS was added to each well of the plate prior to incubation for 4 hr at 37°C. Plates were sealed with adhesive plate sealers

and shaken gently or inverted several times to mix the soluble formazan product. The plate was read spectrophotometrically at 450/650 nm with a Molecular Devices Vmax plate reader.

Data Analysis

Raw data was collected from the Softmax Pro 4.6 software and imported into a Microsoft Excel XLfit4 spreadsheet for analysis by four parameter curve fit calculations. A summary of test compound antiviral activity and toxicity with a graphic representation of the data are provided in a Plate Analysis Report (PAR). The PAR for each compound consists of the following:

- the plate design demonstrating the microtiter plate assay format,
- the raw optical density data values imported from Softmax,
- the cell type and virus strain used in the assay,
- the technician responsible for performing the antiviral assay,
- the project number identification,
- the dates of assay initiation and completion,
- the mean values for the reagent, virus and cell controls and differential of virus to cell control,
- a summary table of calculated toxicity ($TC_{25, 50, 95}$) values, efficacy ($EC_{25, 50, 95}$) values, and therapeutic index ($TI_{25, 50, 95}$) values,
- a summary table of mean optical density values at each compound concentration in the efficacy, toxicity and colorimetric wells along with calculated percentage of reduction values for viral cytopathic effect (CPE) and cell viability;
- graphical presentation of the percentage of cell viability and percentage of reduced viral CPE at each test concentration.

Reverse Transcriptase Activity Assay:

Reverse transcriptase activity was measured in cell-free supernatants using a standard radioactive incorporation polymerization assay. Tritiated thymidine triphosphate (TTP; New England Nuclear) was purchased at 1 Ci/mL and 1 μL was used per enzyme reaction. A rAdT stock solution was prepared by mixing 0.5 mg/mL poly rA and 1.7 U/mL oligo dT in distilled water and was stored at -20°C. The RT reaction buffer was prepared fresh daily and consists of 125 μL of

1 mol/L EGTA, 125 μL of dH_2O, 125 μL of 20% Triton X-100, 50 μL of 1 mol/L Tris (pH 7.4), 50 μL of 1 mol/L DTT, and 40 μL of 1 mol/L $MgCl_2$. For each reaction, 1 μL of TTP, 4 μL of dH_2O, 2.5 μL of rAdT and 2.5 μL of reaction buffer were mixed. Ten microliters of this reaction mixture was introduced to each well of round bottom microtiter plate and 15 μL of virus-containing supernatant was added and mixed. The plate was incubated at 37°C in a humidified incubator for 90 min. Following incubation, 10 μL of the reaction volume was spotted onto a DEAE filter mat in the appropriate plate format, washed 5 times (5 min each) in a 5% sodium phosphate buffer, 2 times (1 min each) in distilled water, 2 times (1 min each) in 70% ethanol, and then air dried. The dried filtermat was placed in a plastic sleeve and 4 mL of Opti-Fluor O was added to the sleeve. Incorporated radioactivity was quantified utilizing a Wallac 1450 Microbeta Trilux liquid scintillation counter.

HIV-1 Genotype Analysis: Viral RNA was extracted from cell culture supernatants of CEM-SS cells infected with $HIV\text{-}1_{IIIB}$ and cultured in the presence or absence of escalating concentrations of FPACA using QIAamp Viral RNA Mini Kit spin columns and reagents (Qiagen, Valencia, CA) according to the manufacturer's recommended procedure. Viral RNA was subjected to either one-step or two step RT-PCR amplification using Superscript III reverse transcriptase and hi-fidelity Taq polymerase (Invitrogen, Carlsbad, CA). For two step amplification, 5 μl of extracted viral RNA was reverse transcribed using random hexamers primers and Superscript Reverse Transcriptase at 50°C for 30 minutes. The resulting cDNA was then amplified by PCR with hi-fidelity Taq Polymerase and HIV gene specific primers for gp120 and protease listed below.

Table 1: GENOTYPING PRIMERS

HIV_{IIIB}-Env Primer	Sequence (5'-3')
5'amp	GCAATAGTTGTGTGGTCCATAG
369F	AAAGCCATGTGTAAAATTAACC
679F	GCTGGTTTTGCGATTCTAA
779R	TGCTAGACTGCCATTTAACA

991F	GGAAATATGAGACAAGCACAT
1104R	CTGTGCGTTACAATTTCTGGG
1740R	AGGTATCTTTCCACAGCCAGG
1610F	CACTATGGGCGCAGCGTCAA
1934F	CACAAGCTTAATACACTCCTT
1997R	CCAATTCCACAAACTTGCCC
2249F	GATCCATTCGATTAGTGAA
8924R	GCTACTTGTGATTGCTCCATGTTT
HIV_{IIIB}-PR Primer	**Sequence (5'-3')**
Pol-F	TTCTTCAGAGCAGACCAG
747R	GTCATTGACAGTCCAGCTGTCC

Results

Selection of $HIV\text{-}1_{IIIB}$ Virus Resistant to FPACA: $HIV\text{-}1_{IIIB}$ virus was propagated in CEM-SS cells in the presence of increasing concentrations of FPACA. **Table 2** summarizes the following information from each passage: passage number, FPACA concentration, RT values for the peak day of virus replication, the FPACA EC_{50}, TC_{50} and TI values in parallel with AZT, and fold-increase compared to wild type from the compound susceptibility evaluation. The compound susceptibility assay results are presented in **Appendix I**.

Passage 1 was initiated with a FPACA concentration of 3.8 µg/mL and slight increases in compound concentration were utilized up to a concentration of 15 µg/mL at virus passage 17. Attempts to grow virus at passage 2 and passage 4 at higher concentrations of FPACA than used for passage 1 and passage 3 were unsuccessful, so it was decided that dose escalation would proceed in slight increments. EC_{50} values for FPACA increased 5.9-, 12.1-, 28.5- and 37.9-fold against FPACA-treated virus at passages 14 through 17, respectively, compared to wild type HIV. Upon review of the sensitivity data, resistance selection has been extended for an additional six months.

Table 2: HIV-1_{IIIB} FPACA RESISTANCE SELECTION IN CEM-SS CELLS

HIV-1 Virus	Passage Number	Peak RT Value (cpm)	Fullerene (µg/mL)			AZT (µM)		
			EC_{50}	TC_{50}	TI	EC_{50}	TC_{50}	TI
IIIB	1	9634	1.06	>100	>94.3	0.004	>0.10	>25.0
IIIB+ 3.8µg/mL FPACA		3086	0.62	>100	>161	0.003	>0.10	>33.3
IIIB	2	9298	1.81	>100	>55.2	0.005	>0.10	>20.0
IIIB+ 3.8µg/mL FPACA		4352	0.82	>100	>122	0.001	>0.10	>100
IIIB	3	26993	0.61	>100	>164	0.001	>0.10	>100
IIIB+ 4.5µg/mL FPACA		2671	1.25	78.5	62.8	0.004	>0.10	>25.0
IIIB	4	3949	0.62	>100	>161	0.002	>0.10	>50.0
IIIB+ 4.5µg/mL FPACA		7969	1.26	80.8	64.1	0.004	>0.10	>25.0
IIIB	5	11701	0.9	>100	>111	0.002	>0.10	>50.0
IIIB+ 5.0µg/mL FPACA		10536	1.13	>100	>88.5	0.01	>0.10	>10.0
IIIB	6	4204	1.93	>100	>51.8	0.002	>0.10	>50.0
IIIB+ 5.5µg/mL FPACA		4109	0.67	>100	>149	0.0005	>0.10	>200
IIIB	7	6032	1.89	>100	>52.9	0.002	>0.10	>50.0
IIIB+ 6.0µg/mL FPACA		10738	4.54	>100	>22.0	0.005	>0.10	>20.0
IIIB	8	9085	1.56	>100	>64.1	0.006	>0.10	>16.7
IIIB+ 6.5µg/mL FPACA		5721	1.09	35.6	32.7	0.001	>0.10	>100
IIIB	9	5654	1.19	>100	>84.0	0.007	>0.10	>14.3
IIIB+ 7.5µg/mL FPACA		10462	1.92	>100	>52.1	0.003	>0.10	>33.3
IIIB	10	5542	1.24	>100	>80.6	0.003	>0.10	>33.3
IIIB+ 8.5µg/mL FPACA		4863	1.76	>100	>56.8	0.002	>0.10	>50.0
IIIB	11	5209	1.45	>100	>69.0	0.003	>0.10	>33.3
IIIB+ 9.0µg/mL FPACA		5834	11.6	>100	>8.62	0.001	>0.10	>100
IIIB	12	5814	1.01	>100	>99.0	0.003	>0.10	>33.3
IIIB+ 10µg/mL FPACA		5922	3.92	>100	>25.5	0.002	>0.10	>50.0
IIIB	13	4746	0.60	>100	>167	0.002	>0.10	>50.0
IIIB+ 11µg/mL FPACA		6342	2.68	>100	>37.3	0.0007	>0.10	>143
IIIB	14	7452	2.73	>100	>36.6	0.01	>0.10	>10.0
IIIB+ 12µg/mL FPACA		5972	**16.1**	>100	>6.21	0.007	>0.10	>14.3
IIIB	15*	4684	1.51	>100	>66.2	0.01	>0.10	>10.0
IIIB+ 13µg/mL FPACA		4634	**18.2**	>100	>5.49	0.004	>0.10	>25.0
IIIB	16	8381	0.6	>100	>167	0.002	>0.10	>50.0
IIIB+ 14µg/mL FPACA		6080	**17.1**	>100	>5.85	0.01	>0.10	>10.0
IIIB	17	4742	0.68	>100	>147	0.002	>0.10	>50.0
IIIB+ 15µg/mL FPACA		4933	**25.8**	>100	>3.88	0.008	>0.10	>12.5

*Indicates FPACA powder batch change to that used in the pre-formulation studies

Genotypic Evaluation of FPACA-Resistant Virus: Virus passages 14 and 17 were chosen for genotypic analysis prior to continuing resistance selection. Sequence analysis of passage 14 and 17 viruses **(Table 3)** was compared to the wild-type gp120 and protease sequences from control viruses cultured in parallel. The same eleven mutations in the gp120 region of HIV envelope and two mutations in the gp41 region were found in passage 14 and 17 virus grown in the presence of FPACA (**Appendix II**). Virus resistant to the FDA-approved fusion inhibitor, T20, or the CCR5 coreceptor inhibitor, Maraviroc, contains amino acid changes in gp41 codons 36 to 45 or within the V3 loop of gp120 at codons 290 to 325, respectively. FPACA-resistant HIV contained S166N and I173L gp41 mutations. The eleven mutations within the gp120 sequence of FPACA-resistant HIV were primarily within codons 138 to 188 and codons 306 to 401. The impact of the individual gp120 and gp41 mutations on fold-resistance to FPACA could be determined in future studies. No significant mutations were identified in the protease gene of passage 14 and 17 virus grown in the presence of FPACA.

Table 3: GENOTYPIC ANALYSIS OF FPACA-RESISTANT HIV ENVELOPE

HIV-1_{IIIB}	Virus Passage	HIV_{IIIB} gp120 Amino Acid											HIV_{IIIB} gp41 Amino Acid	
		16	138	143	171	181	188	306	325	344	398	401	166	173
Wild Type	**14**	R	T	N	K	I	N	R	N	Q	E	N	S	I
	17	R	T	N	K	I	N	R	N	Q	E	N	S	I
FPACA	**14**	K	I	S	E	V	T	K	D	K	K	I	N	L
	17	K	I	S	E	V	T	K	D	K	K	I	N	L

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APPENDIX I

Compound Susceptibility Results

APPENDIX II

Genotype Results