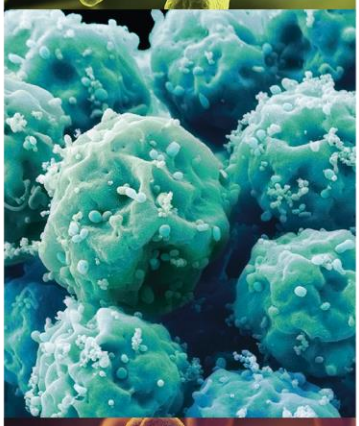
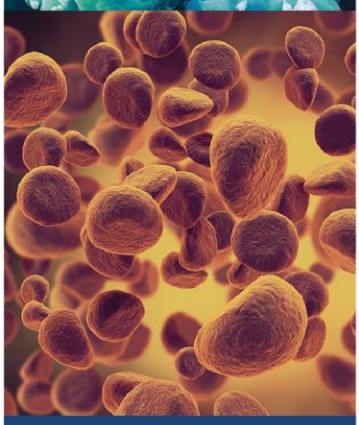
ImQuest BioSciences





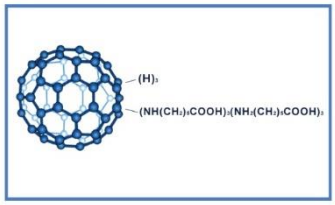
ImQuest BioSciences is a preclinical contract research and development company that evaluates the potential of new and novel pharmaceutical products. We specialize in the development of drugs, vaccines and biologic products for the treatment and prevention of infectious disease, cancer and inflammatory disease.

Robert W. Buckheit, Jr., Ph.D.
Chief Scientific Officer
rbuckheit@imquestbio.com

HIV Antiviral Drug Development
Evaluation of Fullerene Poly-Aminocaproic Acid

Introduction

The development of highly active anti-retroviral therapies (HAART) has greatly suppressed HIV replication and helped to prevent progression to AIDS in many infected patients. In the absence of a functional cure or effective vaccine, drug safety issues, and the ability of HIV to acquire resistance to drugs in current use, continued efforts to develop new anti-HIV compounds are warranted.



Fullerenes, especially C60 derivatives, have unique carbon cage structures reported to bind the hydrophobic cavity of HIV protease, inhibiting the access of substrates to the catalytic site of the enzyme.

ImQuest BioSciences, in collaboration with Intelpharm, has evaluated the anti-HIV activity of fullerene poly-aminocaproic acid (FPACA). The results reported herein demonstrate that FPACA inhibits virus entry as its primary mechanism of action and also inhibits the HIV-1 protease thereby supporting its potential as an anti-HIV drug.

Methodology

HIV Cytoprotection Assays: Following a six-day acute infection of CEM-SS or MT-4 cells with wild-type or drug-resistant HIV-1 in the presence of test compound, cell viability was measured spectrophotometrically using XTT dye reduction.

PBMC and Monocyte Macrophage Assays: Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll hypaque gradient centrifugation from whole blood and activated with PHA. Monocytes were further purified by adherence to plastic and washing to remove lymphocytes. Purified monocytes were infected with the clinical clade B strain HT/92/599 and incubated in the presence of compound. After seven days, supernatant reverse transcriptase (RT) activity or p24 antigen expression by ELISA was measured to quantify virus replication. Cell viability was measured in parallel using XTT dye reduction.

Attachment Inhibition Assay: Test compound and a pre-determined titer of virus were added to TZM-bl-FcRI cells that had been plated in a 96-well flat-bottomed plate 24 hours before assay initiation. Cells, test compound and virus were allowed to incubate for 48 hours at 37°C, 5% CO_2. Following the incubation, the cells were lysed and luciferase expression evaluated in the assay medium using a chemiluminescent endpoint (Gal-Screen, Tropix).

Fusion Inhibition Assay: Compound was incubated with HeLa-CD4-LTR-β-Gal cells in a 96-well flat-bottom plate for 1 hour at 37°C, 5% CO_2 before being co-cultured with HL2/3 cells for 48 hours. The cells were then lysed and β-galactosidase expression evaluated using Gal-Screen.

HIV Reverse Transcription (RT) Inhibition: Reaction mixtures containing recombinant, purified HIV-1 RT enzyme in 100 μL containing 25 mM Tris-HCl, pH 8.0, 75 mM KCl, 8 mM $MgCl_2$, 2 mM DTT, 10 μM dGTP, 0.01U rC:dG template

primer (Pharmacia), 10 μCi [^{32}P]-α-dGTP (800 Ci/mmol), and the test compound were prepared. After incubation for 30 min at 37°C, 10% TCA and 100 μg of heat-denatured, sonicated salmon sperm DNA were added to the mixtures. TCA-precipitated material was harvested onto glass-fiber filters, washed twice with ice-cold 10% TCA and subjected to liquid scintillation counting. Radioactivity was quantified using a MicroBeta scintillation counter.

HIV Integrase Inhibition: The HIV-1 Integrase Assay Kit (Bioproducts) was used to examine integrase inhibition. The plate was coated with double-stranded HIV-1 LTR U5 donor substrate (DS) oligonucleotide containing an end-labeled biotin. Full-length recombinant HIV-1 integrase protein was added, followed by test articles, then a different double-stranded target substrate (TS) oligonucleotide containing 3'-end modifications. Reaction products were detected colorimetrically using an HRP-labeled antibody against the TS 3'-end modification.

HIV Protease Inhibition: The SensoLyte 520 HIV-1 Protease Assay Kit (Anaspec Inc.) and recombinant HIV-1 protease were used to examine protease inhibition. The cleavage of the p17/p24 junction by HIV-1 protease and HIV-1 protease activity in the presence of test compound was monitored in a fluorescent plate reader using excitation and emission wavelengths of 490nm/520nm.

Cell-Cell Transmission Assay: Uninfected CEM-SS cells were cultured with chronically infected CEM_{IIIB} cells at various densities in the presence of compound for three days. Syncytia and RT activity were quantified at 48 hours.

Chronically Infected HIV-1 Cell Assay: CEM-SS cells chronically infected with and constitutively producing HIV-1_{IIIB} were incubated in the presence of the compound for 6 days. Efficacy was determined by evaluating RT activity in the supernatant and toxicity was evaluated by XTT staining.

Combination Drug Interaction Evaluation: The efficacy and toxicity of the compounds alone and in combination were analyzed using an in-house software program, which calculates %CPE reduction or reduction in virus replication, % cell viability, EC25, 50 & 95, TC25, 50 & 95 and other indices. Combination antiviral effects (efficacy and toxicity) were analyzed statistically by the Prichard and Shipman MacSynergy II methodology. Effects of drug combinations on efficacy and toxicity were calculated based on the activity or toxicity of the two compounds when tested alone. The expected additive inhibition of virus production or the expected additive cell viability were subtracted from the experimentally determined percent inhibition or cell viability at each combination concentration, resulting in a positive value (Synergy), negative value (Antagonism) or zero value (Additivity).

Results

Efficacy and Range of Anti-HIV Activity

The efficacy of FPACA was evaluated against a panel of 24 low passage clinical HIV-1 isolates in fresh human PBMCs. EC_{50} values ranged from 1.5 to 13 μg/mL with no observed toxicity up to a high test concentration of 100 μg/mL.

FPACA inhibited CXCR4-tropic, CCR5-tropic and dual-tropic virus strains with equal efficacy. FPACA also inhibited the replication of geographically diverse subtypes (clades) of HIV-1 at equivalent concentration levels.

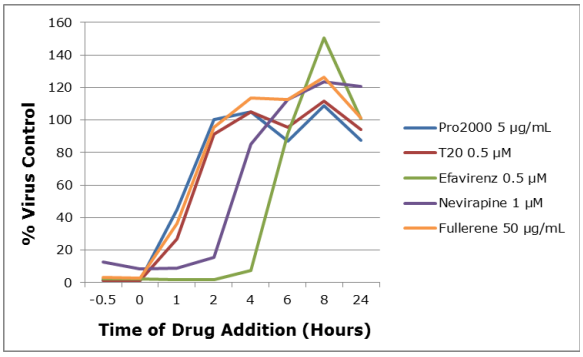
These data demonstrate that FPACA possesses a broad range of anti-HIV activity and would be expected to be active throughout the world as either a therapeutic or prevention product.

Clinical HIV-1 Isolate	Clade, Co-receptor Usage	AZT		FPACA	
		EC_{50} (μM)	TI	EC_{50} (μg/mL)	TI
92RW016	A, CCR5	0.004	>276	4.27	>23.4
92RW009	A, CCR5/CXCR4	0.006	>163	1.15	>87.0
92UG029	A, CXCR4	0.011	>94.3	1.50	>66.7
92US727	B, CCR5	<0.003	>333	2.49	>40.2
92HT594	B, CCR5/CXCR4	0.007	>152	2.45	>40.8
92HT599	B, CXCR4	0.01	>101	2.22	>45.1
93MW959	C, CCR5	0.007	>138	1.48	>67.6
97ZA009	C, CCR5	0.0005	>213	4.32	>23.2
98IN017	C, CXCR4	<0.0003	>333	1.80	>55.6
92UG005	D, CCR5	0.0005	>203	2.41	>41.5
92UG001	D, CCR5/CXCR4	0.005	>203	2.74	>36.5
92UG024	D, CXCR4	0.009	>109	3.13	>32.0
93TH060	E, CCR5	0.004	>225	1.51	>66.2
93TH051	E, CCR5/CXCR4	0.005	>218	2.13	>47.0
CMU02	E, CXCR4	0.0103	>97.1	3.32	>30.1
93BR029	F, CCR5	0.007	>138	2.09	>47.9
93BR020	F, CCR5/CXCR4	<0.003	>333	0.83	>120
93BR019	F, CXCR4	0.007	>135	2.34	>42.5
G3	G, CCR5	0.006	>173	2.18	>45.9
JV1083	G, CCR5	0.01	>97.1	1.64	>61.0
RU132	G, CCR5	0.01	>94.3	2.43	>41.2
BCF01	O, CCR5	0.007	>144	1.92	>52.1
BCF02	O, CCR5	<0.003	>333	1.92	>52.1
BCF03	O, CCR5	0.01	>105	3.99	>25.1

Mechanism of Action

In time-of-drug-addition assays, FPACA lost antiviral activity when compound addition was delayed by several hours similar to the activity observed with the fusion inhibitor Fuzeon (T20) and the CD4-targeted naphthalene sulfonate attachment inhibitor PRO2000.

The results indicate that FPACA inhibits entry of HIV-1 into target cells.



In cell-based entry inhibition assays using TZM-bl cells, FPACA inhibited virus entry with a mean EC_{50} value of 0.45 ug/mL. The compound inhibited the fusion of cells expressing cell surface CD4 with cells expressing viral envelope glycoproteins at 2.3 ug/mL. FPACA also reduced the cell-to-cell transmission of HIV-1 with EC_{50} values ranging from 0.5 to 7 µg/mL.

Assay	Control EC_{50} (µg/mL)	FPACA EC_{50} (µg/mL)
Attachment Inhibition	0.47	0.45
Fusion Inhibition	0.14	2.30
Reverse Transcriptase Inhibition	0.75	59.40
Integrase Inhibition	0.09	8.87
Protease Inhibition	0.02	0.71
Chronic Infection Inhibition	0.26	>100
Cell-Cell Transmission Inhibition	0.05	7.00

FPACA inhibited the HIV-1 protease activity (IC_{50} = 0.71 µg/mL) and also inhibited the activity of integrase but at concentrations one-log higher (IC_{50} = 8.9 µg/mL) with no activity against reverse transcriptase. FPACA was not able to inhibit virus production from cells which were chronically infected with HIV suggesting the compound had no effect on viral transcription, translation or assembly.

Evaluation of cross-resistance to FPACA with HIV-1 isolates resistant to reverse transcriptase or protease inhibitors yielded EC_{50} values similar to wild-type HIV-1. These results support those described above.

Virus	Positive Control EC50 (µM)	Negative Control EC50 (µM)	FPACA EC_{50} (µg/mL)
Wild Type	0.0010	---	0.51
K103N/Y181C	0.0005	0.07	0.48
M184V	0.0006	>10	0.63
4xAZT	0.0200	0.45	0.55
V82A/I84V	0.0009	>1	0.59

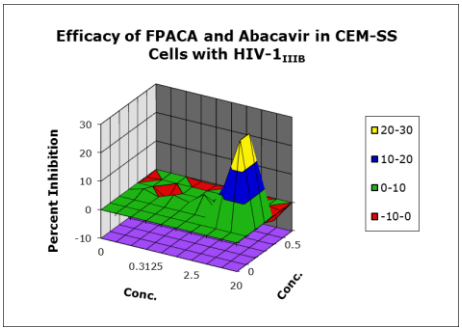
Taken together, our results indicate that the primary antiviral mechanism of FPACA is the inhibition of virus attachment, fusion, and entry with a second potential mechanism involving protease inhibition.

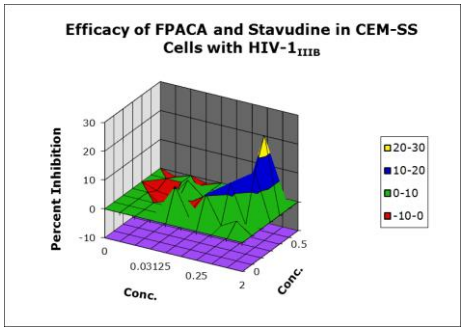
Resistance Selection and Characterization

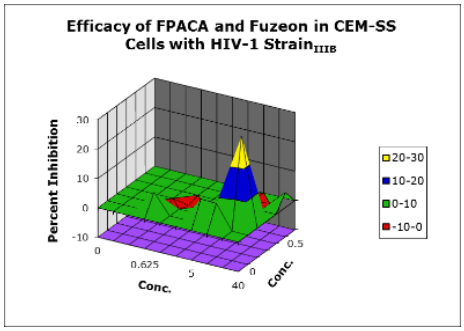
To date through 15 passages in a serial virus passage selection with dose-escalation, resistance to FPACA has not been achieved. Our resistance selections will continue for up to one year in efforts to generate and characterize viruses which are selected for resistance to FPACA.

Combination Interactions with Other Anti-retroviral Agents

The results of the evaluation of FPACA combinations with 23 FDA-approved HIV inhibitors yielded additive to synergistic antiviral interactions.







FPACA was most synergistic with abacavir and stavudine (mean synergy volumes of 187 and 128 µM2%, respectively) and marginally synergistic with Fuzeon (mean synergy volume of 73 µM2%). No evidence of synergistic toxicity was observed with the various combinations of products.

Toxicity Evaluation

FPACA was evaluated for *in vitro* cytotoxicity and mechanism of cytotoxicity using in ImQuest's ToxiSENS platform.

FPACA was not toxic up to a high test concentration of 100 µg/mL to a panel of primary human cells. However, FPACA de-creased PHA-stimulated PBMC proliferation when evaluated by BrdU ELISA, yielding a TC_{50} value of approximately 13.2 µg/mL. Synthesis of RNA and protein were slightly decreased in stimulated PBMCs treated with FPACA. FPACA did not induce apoptosis, oxidative stress or affect membrane integrity.

Cell Type	Control TC_{50} (µg/mL)	FPACA TC_{50} (µg/mL)
Unstimulated PBMC	0.070	> 100
PHA-Stimulated PBMC	0.280	> 100
Monocyte/Macrophage	0.050	> 100
Dendritic	0.250	> 100
Bone Marrow Progenitor	0.650	> 100
Hepatocyte	1.230	> 100
iPS Cardiomyocytes	64.300	> 100
iPS Neuron	0.640	> 100
RPTEC Kidney	0.001	> 100

Assay	Control ED_{50} (µg/mL)	FPACA ED_{50} (µg/mL)
Apoptosis	0.04	> 100
Membrane Integrity	0.07	> 100
Cellular Proliferation	7.76	13.20
Oxidative Stress	95.30	> 100

Conclusion

The ViroSens efficacy, range of action, mechanism of action, combination drug and toxicity studies indicate that FPACA represents a new potent anti-HIV drug candidate which could potentially be used in combination with approved anti-HIV drugs.

ImQuestSUCCESS

Select drug candidates with the highest probability of clinical success

The ImQuest*SUCCESS* preclinical services platform is used to critically evaluate the potential of a test compound and to assure that its efficacy, toxicity, and pharmaceutical properties are evaluated in a comprehensive and interactive way. Successful completion of platform objectives provides significant confidence in the potential of a test compound to transition to human clinical trials, enhances the robustness of drug development efforts and reduces the risk of expensive clinical development failures by the exclusion of candidates which are likely to fail during advanced preclinical and clinical development at early (and less expensive) time points.

7340 Executive Way, Suite R, Frederick, MD 21704
www.imquestbio.com | p: 301-696-0274 | f: 301-696-0381