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Small-Molecule Inhibits HIV-1 Replication by Interacting with HIV Capsid

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Introduction

Continuation of the success of HAART requires identification of novel compounds that target novel sites or proteins and have thus no cross-resistance with existing therapeutics. HIV-1 capsid (CA) is an essential viral protein for early and late events of the replication cycle and so far has been an untapped target, therefore providing excellent opportunities for the discovery of novel antiretrovirals that act by novel mechanisms of action.

Here, we identified a novel small molecule antiretroviral and study its mechanism of action using a combination of virological, biochemical and molecular modeling methods.

Methods

Protein Purification: CA proteins were expressed in *E. coli* BL21 and purified by ammonium sulfate precipitation and Mono-Q FPLC chromatography.

Capsid Multimerization Assay: Compound was added to 125 µl of 88 µM CA protein in 50 mM sodium phosphate buffer, pH 8.0. Capsid assembly was initiated by addition of 5 M NaCl. The increase in optical density at 350 nm was monitored every 10s (Lanman *et al*., *J Virol* 76: 6900-8, 2002).

Drug Susceptibility Assay: Susceptibilities to various compounds were determined using H9 and MAGI-CCR5 cells (Hachiya *et al., Antimicrob Agents Chemother* 45: 495-501, 2001).

Cell to Cell Fusion Assay: The HIV-envelope and HIV-1 tat expression vectors (NIH AIDS Research and Reference Reagent Program) were co-transfected into 293T cells. On the next day, the co-transfected cells were incubated with TZM-bl cells and the luciferase activity was detected using the Bright-Glo Luciferase Assay system.

Time-of-Addition Assay: MAGI-CCR5 cells were infected with HIV-1_{NL4-3} and test compounds (at EC_{90}) were added at different times after infection as described previously (Donzella *et al., Nat Med,* 4: 72-77, 1998). The number of blue cells was counted at 48 hours post-infection as a measure of HIV-1 infection.

Quantitative Real-Time PCR: Virus supernatants were treated with 20 U of DNase I. H9 cells were infected with 500 BFU virus or heat-inactivated virus as a control. Genomic DNA was isolated at appropriate time after infection. Products were quantified by real-time PCR analysis using the primers MH531 and MH532 and the probe LRT-P for late RT products, the primers MH535 and MH536 and the probe MH603 for 2-LTR circles, and the primer Alu1 and LM667 for 1st PCR of integrated DNA products and the primer λT and LR and the probe ZXF for integrated DNA products as described previously (Butler *et al., Nat Med,* 7: 631-634, 2001 and Tan *et al., J Virol,* 80: 1939-48, 2006).

Selection of Resistant Virus: MAGI-CCR5 cells infected with HIV-1_{NL4-3} were cultured in the presence of 0.1 µM 18E8. When cytopathic effects were observed, supernatants were harvested and transferred to new MAGI-CCR5 cells. Cells and supernatant samples were taken at regular time points and stored at -80°C until subsequent analysis.

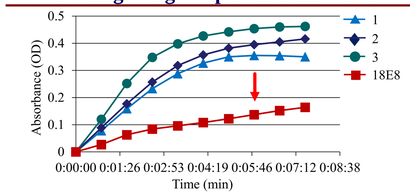
Docking Simulation: The molecular model of 18E8 and the crystal structure of HIV-1 CA were prepared in appropriate formats recognized by the Glide docking program (Schrödinger Inc., NY). The structure data file (sdf) of compound provided by manufacturer was subjected to LigPrep, a ligand preparation tool that is interfaced with Maestro (Schrödinger Inc., NY) to obtain a molecular model of the compound. The protonation state and tautomer search of the compound was carried out by Epik (Schrödinger Inc., NY). The crystal structure of the hexameric HIV-1 CA (PDB file 3H4E, Pornillos *et al., Cell,* 137: 1282-1292, 2009), suitable for Glide docking was prepared by 'Protein Preparation Wizard' of Schrödinger Suite. The molecular model was subjected to limited minimization (1000 iterations) using the OPLS_2005 force field in Maestro. The molecular model of 18E8 generated by 'LigPrep' was docked into a CA pocket within a cubic box (20Å x 20Å x 20Å) centered at N74. In order to adjust the conformation of side chains in the binding pocket, we used the induced-fit docking (IFD) of Schrödinger Suite. A total of 17 docked poses were obtained from this protocol. All poses were visually inspected.

Acknowledgements

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Results

1. Screening using a capsid multimerization assay



This assay measures the effect of compounds on the rate of formation of higher order tube-like CA multimers.

18E8 significantly decreased the rate of CA multimerization

2. *In vitro* antiretroviral activity in cell-based assays

Cell line	EC_{50} [µM]	CC_{50}	Therapeutic Index[a]
Multiple-round			
H9	4.9 ±0.1	31.5 ±4.5	6.4
Single-round			
MAGI-CCR5	1.8 ±0.3	28.9 ±8.5	16

[a] Therapeutic index is calculated as (mean CC_{50})/(mean EC_{50}).

EC_{50} of 18E8 showed 1.8 µM using MAGI-CCR5, suggesting that it targets a step prior to transcription in the HIV-1 replication cycle

3. Antiretroviral activity against clinical isolates

HIV-1 isolate	Clade	Fold Increase 18E8	AZT	EFV	LPV
ART-Naive					
189	B	0.5	0.9	0.5	0.5
210	B	1	0.9	2	2
215	B	1	0.2	2	0.2
123	**A**	1	0.4	0.2	0.2
4	**C**	1	1	0.7	0.2
214	**CRF01_AE**	0.5	2	4	0.5
68	**CRF01_AE**	2	0.6	0.9	<0.04
107	**CRF02_AG**	**5**	2	2	1
ART-Experienced					
56	B	2	**168**	1	1
130	B	2	**44**	**>588**	1
334	B	2	**9**	**>625**	0.2
423	B	3	**4**	**88**	1
126	**D**	1	**314**	1	**>33**
427	**CRF01_AE**	2	2	1	**109 (DRV)**

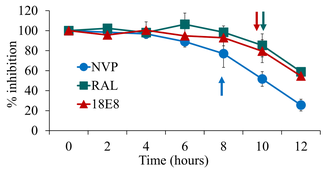
18E8 showed broad antiretroviral activity against drug-resistant and non-clade B viruses

4. Antiretroviral activity against pseudotyped viruses

	Pseudovirus IC_{50}[µM] (fold increase)					
	VSV-G			MuLV (Env)	HIV (Env)	Real HIV
(Core)	MuLV	XMRV	HIV	HIV	HIV	
AZT	-	-	0.1 (3.3)	0.01 (0.3)	0.1 (3.3)	0.03 (1)
18E8	>20 (>11)	>20 (>11)	**>20 (>11)**	**>20 (>11)**	**6.5 (3.6)**	1.8 (1)

18E8 showed no antiretroviral activity against an HIV-1 core pseudotyped with VSV-G envelope

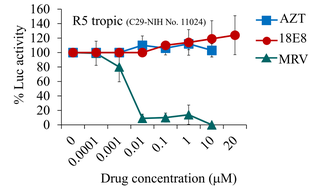
5. Synchronized time-of-addition assay



To determine the specific step of the viral replication cycle affected by 18E8, we performed time-of-addition assays that determine how long the addition of 18E8 could be postponed before losing its antiviral activity in cell culture.

18E8 targeted an early step in the HIV replication cycle, after reverse transcription

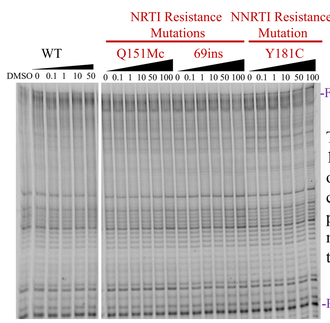
6. Cell to cell fusion assay / Entry step



To determined whether the 18E8 inhibits the entry step of the viral replication cycle, we performed cell to cell fusion assays in the presence 18E8.

18E8 did not affect viral entry

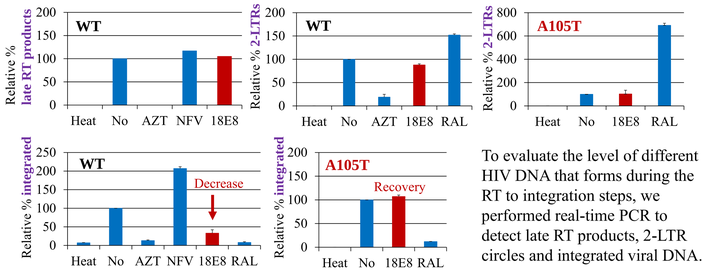
7. Primer extension assay / RT step



To determined whether 18E8 inhibits the RT step of the viral replication cycle, we performed primer extension assays to monitor DNA synthesis in the presence 18E8.

18E8 did not inhibit RT

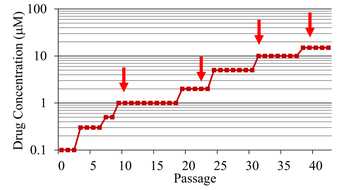
8. HIV DNA quantification / Late RT to integrate steps



To evaluate the level of different HIV DNA that forms during the RT to integration steps, we performed real-time PCR to detect late RT products, 2-LTR circles and integrated viral DNA.

Although 18E8 targeted CA, it decreased the amount of integrated viral DNA

9. Selection of 18E8-resistant HIV *in vitro*



To positively identify the antiviral target of the compound, 18E8-resistant viruses were selected in *in vitro* serial passage experiments using MAGI-CCR5 cells.

Passage	Mutation (population) Matrix	Capsid	Integrase
10	-	**A105T** (10%)	-
23	-	**A105T** (50%)	-
31	K103E (50%)	Q4K, **A105T** (50%) (70%)	-
40	K103E (50%)	**A105T** (80%)	-

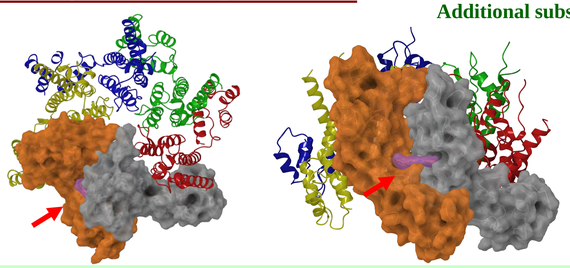
A105T in CA was selected in the presence of 18E8

10. Susceptibilities of HIV-1 CA recombinant molecular clones

Mutation	18E8 EC_{50} [µM]	Fold Increase	Reference
WT	1.6	1	
N74D	5.6	3.5	Resistance to CPSF6 (*Cell Host Microbe*, 2010, 7, 221-233)
A105T	11.3	**7.1**	Compensatory mutation confers CsA sensitivity (*J. Virol* 81, 3749)
T107N	11.3	**7.1**	Resistance to PF-3450074 (*PLoS Pathogens*, 2010, e1001220)
N74D/A105T	11	**6.9**	
A105T/T107N	10.4	**6.5**	

A105T in CA conferred 18E8 resistance
Additional substitutions did not enhance 18E8 resistance when combined with A105T

11. Docking simulation of capsid and 18E8



A105 is located in α-helix 5 of N-terminal domain (NTD) of CA.
18E8 binds to a preformed pocket (magenta) consisting of α-helix 3, 4 and 5 in the NTD of one CA molecule (gray) and α-helix 2 of another CA molecule (orange).

In the hexamer, 18E8 seems to affect interactions between the NTD of two capsid monomers

Conclusions

- **We identified a small compound, 18E8, that targets CA and shows no cross-resistance with existing therapeutics.**
- **18E8 acts after reverse transcription and at or before integration by interacting with the CA, which is an unexpected function of CA.**
- **A deeper knowledge of this mechanism of action might enable a more informed approach to improving the spectrum of CA inhibitors.**