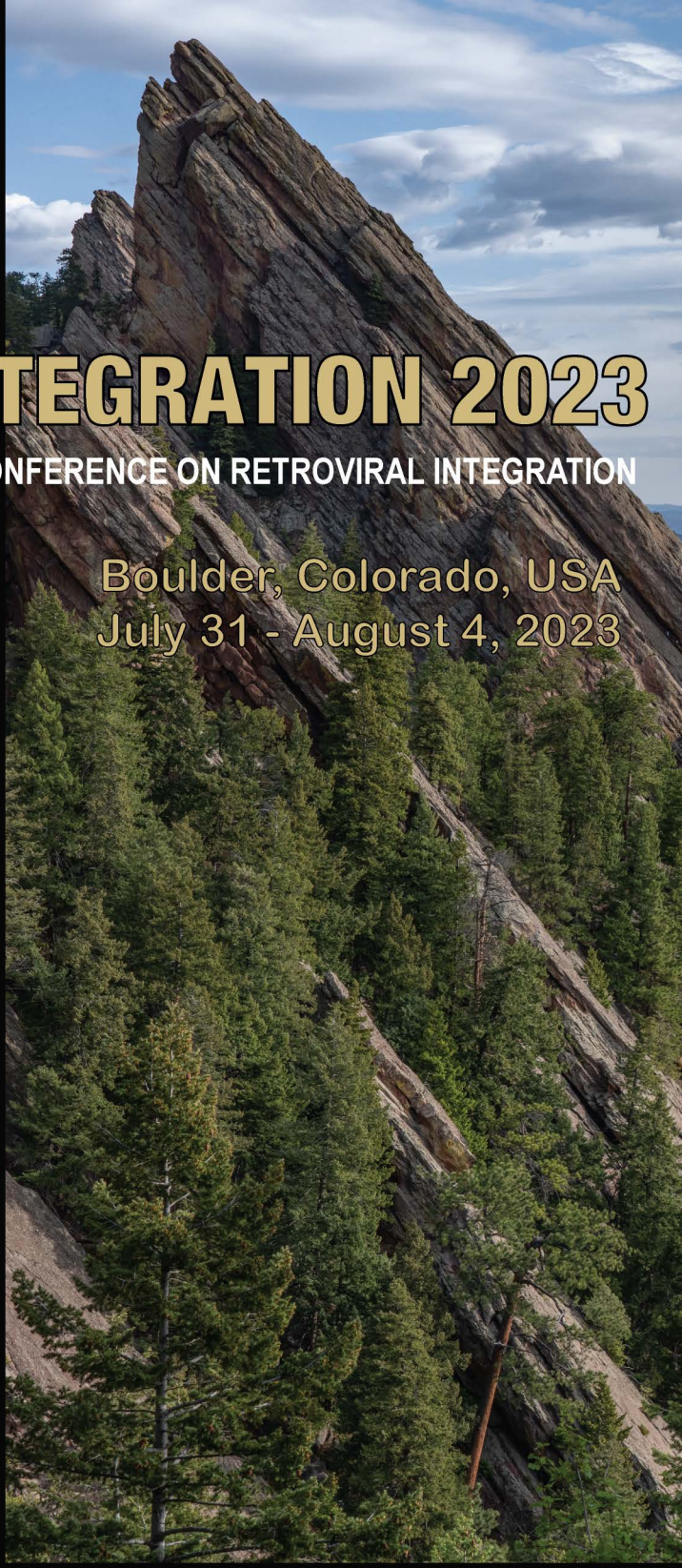
RETROINTEGRATION 2023

7th INTERNATIONAL CONFERENCE ON RETROVIRAL INTEGRATION

Boulder, Colorado, USA
July 31 - August 4, 2023



University of Colorado **Anschutz Medical Campus**

RETROINTEGRATION 2023

7th International Conference on Retroviral Integration

Embassy Suites, Boulder, Colorado, USA
July 31 – August 4, 2023

Main topics:
- Retroviral integrases: structure and function
- Integrase-host factor interactions
- Nuclear import of HIV-1 cores/preintegration complexes
- HIV-1 integrase inhibitors and novel antiretroviral compounds
- Retrotransposons and serine integrases
- Retroviral integration site selectivity and latency

Organizers:
Mamuka Kvaratskhelia, *University of Colorado AMC*
Alan Engelman, *Dana-Farber Cancer Institute*
Duane Grandgenett, *St. Louis University*
Goedele Maertens, *Imperial College London*
Kristine Yoder, *The Ohio State University*

Administrative Support:
David Brandon, *University of Colorado AMC*
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Division of Infectious Diseases

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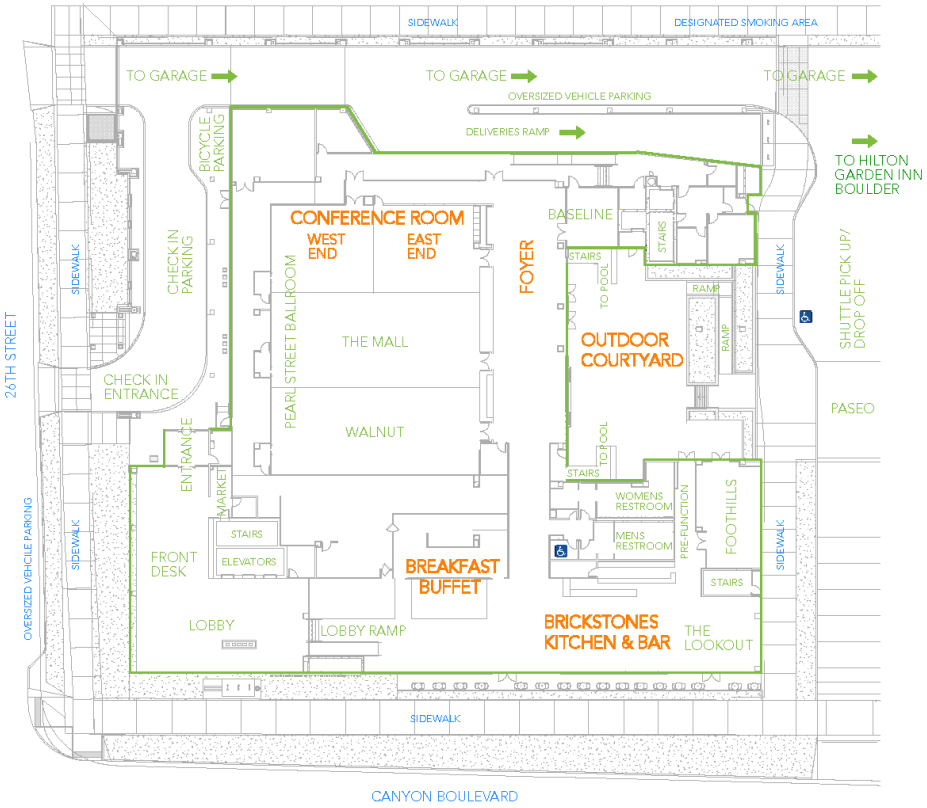

PROGRAM

Monday, July 31

Brickstones Kitchen and Bar

05:00 PM – 06:00 PM — Evening Reception (included with Embassy Suites Reservation)

Outdoor Courtyard

06:00 PM – 07:30 PM — Registration & Welcome Reception/Dinner Buffet

East End/West End Conference Room

07:30 PM – 07:45 PM — Welcome & Logistics
Mamuka Kvaratskhelia, *University of Colorado AMC*

07:45 PM – 07:55 PM — Introduction of the Keynote Speaker
Alan Engelman, *Dana-Farber Cancer Institute*

07:55 PM – 09:00 PM

KEYNOTE SPEAKER

Peter Cherepanov
Francis Crick Institute
Retroviral DNA integration through the lens of structural biology
14

Tuesday, August 1

Breakfast Buffet

06:30 AM – 08:00 AM — Breakfast (included with Embassy Suites Reservation)

East End/West End Conference Room

SESSION 1: **RETROVIRAL INTEGRASES: STRUCTURE AND FUNCTION**

Chairperson: **Monica Roth**, *Rutgers University*

08:00 AM – 10:00 AM

Robert Craigie
NIH, Bethesda, MD, USA.
15 HIV Intasomes: Where we are and where we are going.

Dmitry Lyumkis
The Salk Institute for Biological Studies, La Jolla, CA, USA.
16 Implications for Integrase Functional Plasticity from the Structure of the HIV-1 Integrase Tetramer.

Krishan Pandey
Saint Louis University, MO, USA.
17 Molecular determinants of the Rous sarcoma virus intasome assembly.

Kushol Gupta
University of Pennsylvania, Philadelphia, PA, USA.
Structural Consequences of Resistance Mutations on the Formation of ALLINI-Induced Branched Polymers of HIV-1
18 Integrase.

Foyer

10:00 AM – 10:20 AM Coffee Break

East End/West End Conference Room

10:20 AM – 12:20 PM **Kristine Yoder**
The Ohio State University, Columbus, OH, USA.
DNA Strand Breaks and Gaps Target PFV Intasome Binding
19 and Catalysis.

Chandravanu Dash
Meharry Medical College, Nashville, TN, USA.
An optimal substrate for HIV-1 preintegration complex
20 mediated viral DNA integration.

Stephen Goff
Columbia University Medical Center, NY, USA.
Working overtime: Jump-starting provirus transcription, redirecting sites of integration, and activating DNA damage
21 repair pathways.

Wesley Sundquist
University of Utah School of Medicine, Salt Lake City, UT, USA.
Reconstitution and characterization of a cell-free system for
22 HIV-1 capsid-dependent replication and integration.

Brickstones Kitchen and Bar

12:20 PM – 1:30 PM Lunch

East End/West End Conference Room

SESSION 2: **INTEGRASE-HOST FACTOR INTERACTIONS**
Chairperson: **Marina Lusic,** *University Hospital Heidelberg*

1:30 PM – 3:30 PM **Monica Roth**
Robert Wood Johnson Medical School, Rutgers, Piscataway, NJ, USA.
Studies of the common binding motif BRD3 ET domain: polymorphic structural interfaces with host/viral proteins and
23 small molecules.

Goedele Maertens
Imperial College London, United Kingdom.
Investigating the role of PP2A-B56 in establishing HTLV-1
24 infection.

Ganjam Kalpana
Albert Einstein College of Medicine, New York, NY, USA.
25 INI1/SMARCB1 IN binding domain mimicry to TAR RNA and its influence on viral late events and particle morphogenesis: Development of novel class of INI1-derived inhibitors.

Marina Lusic
University Hospital Heidelberg, Germany.
26 HIV-1 integration into R-loop enriched genomic regions is mediated by Aquarius helicase of the Intron Binding Complex.

Foyer

3:30 PM – 3:50 PM Coffee Break

East End/West End Conference Room

3:50 PM – 5:05 PM **Henry Levin**
Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, MD,
27 The role of LEDGF in transcription is intertwined with its function in HIV-1 integration.

Selected Short Talks

Joshua Hope
The Francis Crick Institute, London, UK.
28 The rules of engagement between lentiviral integration machinery and chromatin.

Ross Larue
The Ohio State University, Columbus, OH, USA.
29 Single molecule visualization of intasome assembly.

Arpa Hudait
University of Chicago, IL, USA.
30 Multiscale simulations of HIV-1 capsid nuclear entry and host factor interactions.

Brickstones Kitchen and Bar

5:05 PM – 6:30 PM Evening Reception (included with Embassy Suites Reservation)

6:30 PM – 9:00 PM **Networking Event at Avanti**: Outside Flatiron Rooftop 1401 Pearl Street, Boulder, CO 80302 (walk or Uber/Lyft)

Wednesday, August 2

Breakfast Buffet

6:30 AM – 8:15 AM Breakfast (Included with Embassy Suites Reservation)

8:30 AM – 1:00 PM **Excursion to the Rocky Mountain National Park.**
Busses depart from Embassy Suites at 8:30 AM.

East End/West End Conference Room

SESSION 3: **NUCLEAR IMPORT OF HIV-1 CORES/PREINTEGRATION COMPLEXES**

Chairperson: **Melissa Kane,** *University of Pittsburgh*

2:00 PM – 3:00 PM

Hans-Georg Kräusslich
Heidelberg University, Heidelberg, Germany.

31 Capsid as key orchestrator of early HIV-1 replication.

Vinay Pathak
HIV DRP, NCI-Frederick, MD, USA.

32 Mechanisms of HIV-1 Core Uncoating, Nuclear Import Kinetics, and Integration Site Selection.

Foyer

3:00 PM – 3:20 PM Coffee Break

East End/West End Conference Room

3:20 PM – 5:20 PM

Kate Bishop
The Francis Crick Institute, London, UK

33 HIV-1 requires capsid remodelling at the nuclear pore for nuclear entry and CPSF6 binding.

Edward Campbell
Loyola University Chicago, IL, USA.

34 Distinct utilization of nuclear import pathways allows HIV-1 integration into transcriptionally active regions of the chromatin.

Ashwanth Francis
Florida State University, Tallahassee FL, USA.

35 Live-cell imaging of HIV-1 nuclear transport and association with nuclear speckles.

Selected Short Talks

João Mamede
Rush University Medical Center, Chicago, IL, USA.

36 Fluorescent labeled CA correlates progressive uncoating from the cytoplasm to the nucleus to productive HIV infection in primary cells.

Melissa Kane
University of Pittsburgh, Pittsburgh, PA, USA.

37 Effects of the cyclophilin homology domain of RanBP2 on HIV-1 infection and Mx2 activity.

Brickstones Kitchen and Bar

5:20 PM – 6:30 PM Evening Reception (Included with Embassy Suites Reservation)

Outdoor Courtyard

6:30 PM – 8:00 PM **Gala Dinner at the Embassy Suites Courtyard**

East End/West End Conference Room

8:00 PM – 9:00 PM **"Where Do You See Your Future?"**
The discussion panel:
Goedele Maertens, Imperial College London (moderator)
Stephen Yant, Gilead Sciences
Suzanne Sandmeyer, University of California Irvine
Mark Underwood, ViiV Healthcare
Wesley Sundquist, University of Utah

Thursday, August 3

Breakfast Buffet

6:30 AM – 8:00 AM Breakfast (Included with Embassy Suites Reservation)

East End/West End Conference Room

SESSION 4: **HIV-1 INTEGRASE INHIBITORS AND NOVEL ANTIRETROVIRAL COMPOUNDS**

Chairperson: **Daniel Adu-Ampratwum**, *The Ohio State University*

8:00 AM – 10:00 AM

Kyungjin Peter Kim
ST PHARM, Seoul, Republic of Korea.
38 The Fellowship of the Ring: Quest to develop Pirmitegravir, a novel potent and safe HIV-1 allosteric integrase inhibitor (ALLINI).

Jacques Kessl
University of Southern Mississippi, Hattiesburg, MS, USA.
39 Optimizing the binding of substituted quinoline ALLINIs within the HIV-1 integrase oligomer.

Stephen Yant
Gilead Sciences, Inc., Foster City, CA, USA.
40 Lenacapavir: A First-in-Class, Long-Acting HIV Capsid Inhibitor for Treatment and Prevention.

Daniel Adu-Ampratwum
The Ohio State University, Columbus, OH, USA.
41 Developing novel small molecules as inhibitors targeting HIV-1 Integrase and capsid proteins.

Foyer

10:00 AM – 10:20 AM Coffee Break

East End/West End Conference Room

10:20 AM – 12:20 PM

Eric Gillis
Discovery Chemistry, ViiV Healthcare, Branford, CT, USA.
42 Potent long-acting inhibitors targeting HIV-1 capsid based on a versatile quinazolin-4-one scaffold.

Mark Underwood
ViiV Healthcare, Durham, North Carolina, USA.
43 Second Generation Integrase Inhibitor Resistance in the Clinic: Dolutegravir Resistance Mechanisms and Structural Underpinnings.

Selected Short Talks

Yuta Hikichi
HIV Dynamics and Replication Program, NCI-Frederick, MD, USA.
44 Mutations outside integrase lead to high-level resistance to integrase strand transfer inhibitors.

Jose Dekker
Amsterdam UMC – University of Amsterdam, Amsterdam, The Netherlands.
45 HIV-1 3'-polypurine tract mutations confer dolutegravir resistance by switching to an integration-independent replication mechanism via 1-LTR circles.

Roberto DiSanto
Sapienza University of Rome, Rome, Italy.
46 New small molecule derivatives as dual inhibitors of the HIV-1 integrase catalytic site and integrase-RNA interactions.

Szu-Wei Huang
University of Colorado AMC, Aurora, CO, USA.
47 Sub-stoichiometric drug to HIV-1 capsid ratio enables ultra-potent antiviral activity of lenacapavir.

Brickstones Kitchen and Bar

12:20 PM – 1:30 PM Lunch

Outdoor Courtyard

SESSION 5: **POSTER PRESENTATIONS**
1:30 PM – 3:30 PM **61-83**

East End/West End Conference Room

SESSION 6: **RETROTRANSPOSONS AND SERINE INTEGRASES**
Chairperson: **Kate Bishop,** *The Francis Crick Institute*

3:30 PM – 5:30 PM **Suzanne Sandmeyer**
University of California, Irvine, CA, USA.
48 Ty3: We should have known it wouldn't be random.

David Garfinkel
University of Georgia, Athens GA.
49 Ty1 Gag stories: mechanism of copy number control, domestication of a restriction factor, and an interchangeable prion-like domain.

Phoebe Rice
The University of Chicago, IL, USA.
50 Large serine integrases: how do they know which way to go?

Selected Short Talks

Eric Arts
Western University, London, Ontario, Canada.
51 Evidence of significantly reduced HIV proviral integrants within genes and increased integration into transcriptionally silent elements in HIV-1 infected individuals failing an INSTI treatment regimen with or without INSTI resistance mutations.

Ariberto Fassati
Institute of Immunity and Transplantation, London, UK.
52 Functional mapping of integration sites connected to latent HIV-1 infection.

Brickstones Kitchen and Bar

5:30 PM – 6:30 PM Evening Reception (included with Embassy Suites Reservation)

6:30 PM – **Special Dinner Reception at The Flagstaff House**
Busses depart from Embassy Suites at 6:30 PM.

Friday, August 4

Breakfast Buffet

6:30 AM – 8:00 AM Breakfast (Included with Embassy Suites Reservation)

East End/West End Conference Room

8:00 AM – 8:10 AM Awards for best poster presentations and short talks presented by **Kristine Yoder**

SESSION 7: **RETROVIRAL INTEGRATION SITE SELECTIVITY**
Chairperson: **Mary Kearney**, *HIV Dynamics & Replication Program*

8:10 AM – 10:10 AM **Frederic Bushman**
University of Pennsylvania, Philadelphia, PA, USA.
53 Retroviral DNA Integration: Target Site Selection and Genomic Consequences.

Charles Bangham
Imperial College London, London, United Kingdom.
54 HTLV-1 integration site: impact on viral persistence and host chromatin structure and expression.

Alan Engelman
Dana-Farber Cancer Institute, Boston, MA, USA.
55 CPSF6 Liquid-Liquid Phase Separation Determines Higher-Order Capsid Binding, Nuclear Core Incursion, and HIV Integration Targeting.

Zeger Debyser
KU Leuven, Flanders, Belgium.
56 The chromatin landscape of the HIV provirus determines its transcriptional state. Implications for a functional block-and lock cure strategy.

Foyer

10:10 AM – 10:30 AM Coffee Break

East End/West End Conference Room

SESSION 8: **LATENCY**
Chairperson: **Eric Arts**, *Western University*

10:30 AM – 12:30 PM **Mary Kearney**
HIV Dynamics & Replication Program, NCI-Frederick, MD, USA.
57 Divergent Populations of Infected Naïve and Memory CD4+ T Cell Clones in Children on ART.

Mathias Lichterfeld
Harvard Medical School, Boston, MA, USA.
58 Chromosomal Integration sites as biomarkers of HIV-1 reservoir cell selection.

Frank Maldarelli
HIV DRP, NCI-Frederick, MD, USA.
59 Anatomic Distribution of HIV-Infected Cells After Long Term Antiretroviral Therapy.

Duane Grandgenett
Saint Louis University, St. Louis, MO
60 Concluding remarks: retrovirus integrase, integration, HIV-1 integrase inhibitors.

Brickstones Kitchen and Bar

12:30 PM – 2:00 PM Lunch & Departure

Abstracts
of Oral Presentations

Retroviral DNA integration through the lens of structural biology

Peter Cherepanov

The Francis Crick Institute and Imperial College London, UK

A unique aspect of retroviral life cycle, integration has left a huge imprint in vertebrate genomes. Integration is the principal reason for the ability of HIV-1 and other members of this viral family to establish life-long infections, at the same time making them excellent vectors for gene therapy applications. Unlike reverse transcriptase and protease, both of which had well-studied functional homologs, integrase would claim life efforts of many before finally yielding to HIV drug developers. In the past 13 years, X-ray crystallography and cryo-EM studies of intasomes from a range of retroviral species revealed surprising structural diversity as well as shared structural features. The structures explained the mode of action of integrase inhibitors and explained the mechanisms of drug resistance. In this lecture, I will summarise the progress in the structural biology of retroviral DNA integration and the important outstanding questions.

HIV Intasomes: Where we are and where we are going

Robert Craigie

NIH, Bethesda, MD, USA

HIV DNA integration is essential for HIV replication and is mediated by a nucleoprotein complex comprising a pair of viral DNA ends and a multimer of integrase (intasome). In infected cells, intasomes are a subcomponent of a much larger nucleoprotein complex called the preintegration complex. Intasomes, rather than free integrase protein, are the target of a class front-line drugs used in the treatment of HIV-AIDS. High-resolution structures of HIV intasomes are therefore needed to understand their mechanism of action, mechanisms by which integrase can escape by acquiring resistance, and the development of improved drug candidates.

Structural studies of HIV intasomes have been frustrated by many obstacles. Integrase protein itself has a notorious propensity to aggregate. Intasomes also aggregate at the concentration required for structural studies; the vast majority of intasomes assembled with wild-type integrase are in the form of very large aggregates, even at high ionic strength. Several different strategies have contributed to overcoming these obstacles. The first is the N-terminal fusion of the non-specific DNA binding domain Sso7d to HIV integrase. This, together with advances in cryo-EM enabled the determination of the first high-resolution structures of HIV-1 intasomes and more recently structures of HIV intasomes bound to drugs and structures of HIV drug-bound intasomes assembled with integrase carrying drug resistance mutations. This work is part of a collaboration with Dmitry Lyumkis, and other groups with expertise in virology, molecular dynamics, and chemistry. We focused on the mechanism(s) of resistance caused by mutations at positions 138, 140, and 148. Our results elucidate the mechanisms by which dolutegravir loses potency in the background of these drug resistance mutations. Such data can provide more direct pathways to the design of drugs with improved resistance profiles.

Despite improvements in methods of intasome assembly, preparation of grids for cryo-EM is laborious due to the vast majority of intasomes being in the form of aggregate and the many steps of purification that are required to obtain samples suitable for single particle cryo-EM. The aggregates are stacks formed by domain swapping of intasomes and we find that stacks can be virtually eliminated from the intasome preparations by assembly in the presence of the isolated C-terminal domain of HIV integrase. This greatly simplifies the steps needed to obtain samples suitable for grid preparation, increases the abundance of single particles, reduces orientation bias, and has enabled us to obtain HIV intasome structures at 2Å resolution. These structures have revealed additional order in residues 271-279 of the C-terminal tail, which is essential for integration activity both in vitro and in vivo. Residues in this tail region make numerous inter-domain interactions within the intasome and we propose these interactions contribute to intasome stability.

Implications for Integrase Functional Plasticity from the Structure of the HIV-1 Integrase Tetramer

Tao Jing[1‡], Zelin Shan[1‡], Tung Dinh[4], Juliet Greenwood[5], Min Li[6], Terry Zhang[1], Bo Zhou[1], Sriram Aiyer[1], Robert Craigie[6], Alan N. Engelman[5,7], Mamuka Kvaratskhelia[4], Dmitry Lyumkis[1,2,3]

‡ These authors contributed equally to this work
[1]The Salk Institute for Biological Studies, La Jolla, CA, 92037, USA.[2]Department of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, CA, 92037, USA.[3]Graduate School of Biological Sciences, Section of Molecular Biology, University of California San Diego, La Jolla, CA 92093, USA.[4]Division of Infectious Diseases, Anschutz Medical Campus, University of Colorado School of Medicine, Aurora, CO 80045, USA.[5]Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, MA 02215, USA.[6]National Institutes of Health, National Institute of Diabetes and Digestive Diseases, Bethesda, MD, 20892, USA.[7]Department of Medicine, Harvard Medical School, Boston, MA 02115, USA

The primary function of HIV integrase is to insert a DNA copy of the viral RNA genome into host chromatin, a process that occurs in the early stages of the viral replication cycle. Recent evidence also revealed that HIV IN can bind viral RNA in the late stages of the replication cycle, facilitating the proper packaging of viral ribonucleoprotein complexes inside the capsid lattice during virion maturation. Whereas integration is mediated by the viral intasome – a large multimeric assembly of up to sixteen IN subunits bound to the linear ends of viral DNA – RNA binding is mediated by an IN tetramer in its apo form. How IN coordinates these two distinct events remains elusive. Here, we determined a cryo-EM structure of the full-length HIV IN tetramer bound to the IN-binding domain (IBD) of LEDGF, resolved to 3.5 Å. Structural comparisons to two-domain structures of HIV IN and to full-length IN protomers within lentiviral intasome assemblies reveal a remarkable structural plasticity inherent to IN that allows the protein to make diverse interactions with distinct interfaces. We also determined a structure of the HIV intasome assembled using WT IN and resolved the flanking IN regions that were absent in prior reconstructions. This allowed us to position individual domains from all sixteen IN protomers into the map and explain how tetrameric INs form the core building block of the fully formed hexadecameric HIV intasome. The tetrameric IN-IBD structure revealed a novel interface containing an inter-subunit salt bridge formed by E35 and K240. Whereas the single mutants E35K and K240E abrogated integration activity and RNA binding, remarkably, the charge swapped double mutant substitution E35K/K240E partially restored both distinct functions of IN. Finally, using molecular modeling, we suggest several scenarios for how viral RNA may bind to the IN tetramer. These data highlight how the plastic molecular architecture of IN can mediate its diverse functions and provide a molecular blueprint for the design of novel inhibitors that can block the function of IN in distinct stages of the viral replication cycle.

Molecular determinants of the Rous sarcoma virus intasome assembly

Krishan K. Pandey[1], Sibes Bera[1], Ke Shi[2], Hideki Aihara[2], Duane P. Grandgenett[1]

[1]Department of Molecular Microbiology and Immunology, Saint Louis University, MO USA. [2]Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN USA

Integration of retroviral DNA into the host genome involves the formation of integrase (IN)-DNA complexes termed intasomes. Detailed characterization of these complexes is needed to understand their assembly process. We used single-particle cryo-EM to determine the structures of Rous sarcoma virus (RSV) intasomes. Our structures demonstrated the flexibility of the distal IN subunits relative to the IN subunits in the conserved intasome core. An extensive analysis of these structures helped in identification of nucleoprotein interactions important for intasome assembly. Using structure-function studies, we determined the mechanisms of several IN-DNA interactions critical for the assembly of RSV intasomes. We demonstrated that assembly of the mature octameric intasome is mediated through a novel tetrameric intermediate in the RSV intasome assembly pathway. Taken together, these studies advance our understanding of different RSV intasome structures and molecular determinants involved in their assembly.

Structural Consequences of Resistance Mutations on the Formation of ALLINI-Induced Branched Polymers of HIV-1 Integrase

Saira Montermoso[1], Kushol Gupta[1], Grant Eilers[1, 2], Audrey Allen[1,2], Nathan Stewart[1], Amanda Gore[1], Nancy An[1], Young Hwang[2], Robert Sharp[1], Frederic D. Bushman[2*], Gregory Van Duyne[1*]

From the Perelman School of Medicine, University of Pennsylvania. [1]Department of Biochemistry and Biophysics 242 Anatomy-Chemistry Building Philadelphia, PA, 19104-6059 U.S.A. [2]Department of Microbiology 3610 Hamilton Walk Philadelphia, PA 19104-6076 U.S.A.

HIV-1 integrase (IN) can be targeted by inhibitors that act at the catalytic core domain (CCD) active site (INSTIs) and allosteric inhibitors that bind at a secondary location in the CCD dimer interface (ALLINIs). ALLINIs binding at the CCD dimer interface blocks the interaction of IN with its cellular cofactor LEDGF/p75 and promotes abnormal IN polymerization which consequently leads to aberrant viral maturation. Emergence of several ALLINI-resistant IN variants *ex vivo* revealed IN substitutions at or near the IN CCD dimer interface. We previously determined the structure of full-length HIV IN bound with the ALLINI GSK1264 at 4.4 Å resolution. To achieve crystallization, we employed the mutation Y15A, which provided desirable solution properties but does not support replication in HIV.

Here, we have extended these structural studies to include HIV IN variants that are replication-competent and contain amino acid substitutions conferring resistance to ALLINIs (IN^{W131C} and IN^{N222K}), at a similar crystallographic resolution of ~4.5 Å and with another prototype ALLINI, BI-D. These structures reveal long-range structural perturbations in the ALLINI-induced open-polymer of resistant INs that correlate with their resistance to formation of drug-induced branched polymers *in vitro,* suggesting a mechanism of ALLINI resistance. Supporting biophysical characterization of the two IN variants *in vitro* reveal their native structural and oligomeric properties. SEC-SAXS analyses of the N222K variant, which is a mutant not in direct contact with protein-protein or protein-drug contacts, reveals a perturbation in native IN dimer structure that increases protein flexibility and disorder and would be predicted to inhibit formation of ALLINI-induced branched polymers. Together, these results illuminate the structural mechanisms for drug resistance against the ALLINI class of antivirals and inform future drug-design efforts.

DNA Strand Breaks and Gaps Target PFV Intasome Binding and Catalysis

Gayan Senavirathne[1], Anne Gardner[1], James London[1], Richard Fishel[1,2,*] and Kristine E. Yoder[1,2,3]

[1]Cancer Biology and Genetics, The Ohio State University College of Medicine, Columbus, OH 43210. [2]Molecular Carcinogenesis and Chemoprevention Program, The James Comprehensive Cancer Center. [3]Center for Retrovirus Research, The Ohio State University

Real time single molecule imaging of prototype foamy virus (PFV) intasomes interacting with linear naked DNA indicated the complexes remain in continuous contact with the helix for an average of 2.1 seconds interrogating 1.6 kb. Although hundreds of interactions were visualized, strand transfer was rare. Using single molecule Förster resonance energy transfer (smFRET), we show PFV intasomes bind to DNA strand breaks and gaps. The increased DNA binding events targeted half site integration 4 bp from the break/gap site without inducing detectable intasome conformational changes. The BER substrate 8-oxo-guanine and a G/T mismatch or a +T nucleotide insertion that introduce a bend or localized flexibility into the DNA did not increase intasome binding or targeted integration. These results suggest that DNA breaks or gaps can modulate dynamic intasome-target DNA interactions enhancing site-specific integration.

An optimal substrate for HIV-1 preintegration complex-mediated viral DNA integration

Nicklas Sapp[1,2,3], Nathaniel Burge[4], Khan Cox[4], Prem Prakash[1,2], Muthukumar Balasubramaniam[1,2], Devin Christenson[5], Min Li[6], Jared Linderberger[7], Mamuka Kvaratskhelia[7], Jui Pandhare[1,3], Robert Craigie[6], Michael G Poirier[4], and Chandravanu Dash[1,2,8]

[1]Center for AIDS Health Disparities Research, Meharry Medical College, Nashville, TN, USA. [2]Department of Biochemistry and Cancer Biology, Meharry Medical College, Nashville, TN, USA. [3] School of Graduate Studies, Meharry Medical College, Nashville, TN, USA. [4] Department of Physics, Department of Chemistry & Biochemistry, Ohio State University, Columbus, OH, USA. [5]Department of Biochemistry, University of Utah, Salt Lake City, UT, USA. [6]Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA. [7]University of Colorado School of Medicine, Division of Infectious Diseases, Aurora, CO, USA. [8]Department of Microbiology, Immunology and Physiology, Meharry Medical College, Nashville, TN, USA.

HIV-1 infection is dependent on integration of the viral DNA into specific regions of the host chromosomes. The preintegration complex (PIC) containing the viral DNA, the virally-encoded integrase (IN) enzyme, and other viral/host factors, carries out HIV-1 DNA integration. Yet, the mechanisms and factors that guide the PIC and facilitate PIC-associated viral DNA into specific hot spots of the human chromosomes remains largely speculative. In this study, we describe an optimal substrate for the preference of PIC-mediated viral DNA integration. We used HIV-1 PICs extracted from acutely infected T-cell lines and measured the ability of these viral replication complexes to integrate the viral DNA into isolated chromatin, genomic DNA substrates, biochemically assembled nucleosomes, and analogous naked DNA. To study whether viral DNA integration by PICs is distinct, we carried out comparative analysis of integration with intasome (INS)-biochemically assembled PIC substructures. Our results demonstrated that PIC-mediated integration into biochemically assembled nucleosomes without histone tail modifications was lower compared the naked DNA substrates. Notably, the addition of a trimethylated histone tail modification H3K36me3 significantly enhanced PIC-mediated integration into nucleosomes. The addition of linker DNA to the modified nucleosomes optimally supported PIC-mediated integration but not INS-mediated integration. Surprisingly, the cellular cofactor LEDGF/p75 rendered distinct biochemical effects on integration by the PIC compared to the INS. Furthermore, chromatin compaction by the linker histone H1° negatively regulated HIV-1 integration. Finally, using sequencing analysis, we identified integration preferences within specific regions of the nucleosomal DNA. Overall, our study provides critical biochemical evidence for HIV-1 PIC- mediated integration preference into open chromatin and report a mechanistic link between H3K36me3 and integration preference.

Working overtime: Jump-starting provirus transcription, redirecting sites of integration, and activating DNA damage repair pathways

Winans, S.[1], Yu, H.J.[2], KewalRamani, V.N.[3], de Los Santos, K.[1], Wang, G.Z.[1], Fu, S.[4], Phan, A.T.[5], Gao, G.[4], Zhu, Y.[5], and Goff, S.P.[1]

[1]Columbia University Medical Center, NY. [2]Basic Science Program, Leidos Biomedical Research, Frederick National Laboratory, Frederick, MD. [3]Basic Research Laboratory, Center for Cancer Research, NCI-Frederick, MD. [4]CAS Key Laboratory of Infection and Immunity, Institute of Biophysics, University of Chinese Academy of Sciences, Beijing, China. [5]Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY.

We are interested in identifying additional roles for the HIV-1 integrase beyond its main functions of mediating integration of the viral DNA and assisting the assembly of properly formed virions. The HIV-1 IN is known to acquire several post-transcriptional modifications, including acetylation of four lysine residues near the C-terminal tail. A mutation changing all four to arginines, retaining the basic charge, had little or no impact on integration *per se*, but profoundly blocked expression of reporter genes on vectors delivered by the mutant integrase. Reporters driven by the viral LTR were poorly transcribed, as well as those expressed from heterologous cellular promoters. Expression was not permanently blocked but only delayed for 1-2 days and returned to normal levels after 2-4 days. The data suggest that the wild-type integrase normally "jump-starts" active transcription of the provirus after integration, and that the mutant fails to perform this task.

Analysis of mutants with individual, single mutations of each of the four lysines to arginine also had minimal effects on transduction, but one mutant exhibited a remarkable phenotype: K258R virus integrates at wildly elevated frequency into centromeric heterochromatin. Sequence analysis of integration site junction fragments, and PCR-based readouts, identified "alpha repeat" sequences as the major target. Probing the intranuclear localization of the mutant PICs by FISH confirmed the increased overlap with centromeric markers. This mutant would be selected out of a pool of replicating virus, but might be positively selected in the pool of silent virus in HIV-1 latency.

Proteomic screens by mass spectrometry have revealed that FANCI and FANCD2, two Fanconi Anemia gene products, interact with the C-terminal tail of HIV-1 IN, requiring sequences that include the acetylated lysine residues. HIV-1 infection led to FA pathway activation, marked by monoubiquitinylation of FANCI/D2, and formation of nuclear foci containing FANCD2 and gH2AX. Raltegravir, or KO of the ubiquitin ligase FANCA, blocked this activation. Further tests revealed that FANCI/D2, REV1, involved in translesion repair, four specific DNA polymerases (H, I, K, and REV3L), and the flap nuclease FAN1 are all important for the stable formation of integrated HIV-1, but not MLV DNA. The specific benefit of activating the FA pathway for HIV-1 remains to be understood, but perhaps inducing DNA repair helps virus persist or evade detection.

Reconstitution and characterization of a cell-free system for HIV-1 capsid-dependent replication and integration

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The conical core of the mature infectious HIV-1 virus is organized by an outer capsid shell comprising CA protein subunits. Upon infection, the core is released into the cytoplasm of the newly infected cell where it converts into a pre-integration complex that carries the reverse transcribed viral DNA into the nucleus to integrate into the host chromosome. In my talk, I will discuss the development of a cell-free system the reconstitutes HIV-1 replication and integration, the use of that system to demonstrate that the capsid plays an essential role in viral replication, recent improvements in the assay that increase integration efficiency, and our efforts to understand why the integration step requires cell extracts to proceed efficiently.

Studies of the common binding motif BRD3 ET domain: polymorphic structural interfaces with host /viral proteins and small molecules

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Gammaretroviruses bias integration at promoter/enhancer regions through the interaction the IN protein with the extraterminal (ET) domain of BET proteins (BRD2, BRD3, BRD4). For MLV IN, the ET binding motif is separated from the IN CTD SH3 fold through a partially flexible 10 amino-acid linker region affecting its potential range of orientations in the IN:nucleosome complex. Using solution NMR, the structures of both the 79-residue murine leukemia virus integrase (IN) C-terminal domain ($IN_{329-408}$) or its 22-residue IN tail peptide ($IN_{386-407}$) alone reveal similar intermolecular three-stranded β-sheet formations complexed with the Brd3 ET domain. The complex of the ET-binding peptide of the host Nuclear Receptor Binding SET Domain Protein 3 (NSD3) protein ($NSD3_{148-184}$) and the BRD3 ET domain includes a similar three-stranded β-sheet interaction, but the orientation of the β hairpin is flipped compared with the two IN:ET complexes.
The interaction domains of both the substrate and ET complex involve intrinsically disordered regions, undergoing a disorder-to-order transition upon binding. This limits the ability of modeling tools to predict the three-dimensional structures of such complexes. To address this problem, we have taken a tandem approach combining NMR chemical shift data and molecular simulations with the MELD (Modeling Employing Limited Data) technique, applied to polypeptide complexes formed with the extraterminal domain (ET). In a blind study, MELD successfully modeled bound-state conformations and binding poses, using only protein receptor backbone chemical shift data, in excellent agreement with experimentally determined structures for the IN:ET complex, a moderately tight (K_d ~100 nM) binder. The hybrid MELD + NMR approach required additional peptide ligand chemical shift data for NSD3, a weaker (K_d ~250 μM) peptide binding partner. MELD was also used to estimate relative binding affinities consistent with the experimental ITC data.
In parallel work, using computer-aided drug design (CADD) tools, we have identified small-molecule lead compounds showing interactions to BRD3 ET using NMR-based water-LOGSY and Saturation Transfer Difference (STD) NMR. Additionally, we are currently testing alternative peptides predicted to bind within the ET binding cleft.

Investigating the role of PP2A-B56 in establishing HTLV-1 infection

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Human T-cell lymphotropic virus type 1 (HTLV-1), a deltaretrovirus, causes debilitating and sometimes fatal disease in humans. To establish infection, a retrovirus needs to integrate a DNA copy of the viral RNA genome into host chromatin. This is not a random process but is instead regulated by interactions with host proteins. Our laboratory identified protein phosphatase 2A-B56 (PP2A-B56) as a functional binding partner and host factor for deltaretroviral integration. Using a combination of X-ray crystallography and cryo-EM we previously solved the structure of the deltaretroviral integration machinery (intasome) in complex with B56, the regulatory subunit of PP2A. Our (and others) structural work revealed that IN binds B56 by mimicking a short linear motif (SLiM) present on dozens of endogenous binding partners of PP2A-B56. HTLV-1 IN SLiM mutants abolished binding to B56 whilst retaining intrinsic integration activity. We generated mutant viral particles from a molecular clone and were able to detect virus-associated reverse transcriptase, p19/p24^{gag}, and cell-associated p55/p19/p14^{Gag} and GP46^{Env} from all HTLV-1 variants. Here, we show how these SLiM mutations affect HTLV-1 infectivity.

INI1/SMARCB1 IN binding domain mimicry to TAR RNA and its influence on viral late events and particle morphogenesis: Development of novel class of INI1-derived inhibitors

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INI1 (also known as hSNF5, SMARCB1, BAF47) is an HIV-1 IN-interacting host factor and is a component of the human SWI/SNF complex and a tumor suppressor. INI1 directly binds to IN within the context of Gag-Pol and is incorporated into HIV-1 virions. Lack of INI1 leads to inhibition of HIV-1 particle production and INI1-interaction-defective IN mutants impair virion morphogenesis. Recent NMR and modeling studies revealed that IN-binding-Rpt1 domain of INI1 and Trans Activating Response element (TAR) structurally mimic each other (Dixit et al Nat. Comm. 2021). Protein-nucleic acid mimicry exists in nature, but mimicry of Rpt1 to TAR is a novel and provides new insights into the possible mechanism by which INI1 facilitates HIV-1 late events.

Based on Rpt1 and TAR mimicry, and the functional overlap, we hypothesized that drugs that target IN-INI1 interaction will be “dual acting” inhibiting both IN-INI1 and IN-TAR interactions.

Structural overlap of Rpt1 with TAR identified a α1 helix in Rpt1 positioned at the IN-CTD/INI-Rpt1 interface. As a proof-of-concept, we generated a 19 aa long hydrocarbon staples stapled peptide derived from the Rpt1 α1 helix (termed SP-38). We found that SP-38 (but not its mutants) inhibited both IN-INI1 and IN-RNA interactions *in vitro* and in RNA-co-IP in cells, with an IC50 of 20 nM. SP-38 exhibited no cell toxicity but potently inhibited HIV-1 replication in CEM-GFP T-cells, in a multiday replication assay. Further analysis indicated that while SP-38 did not affect intracellular and virion associated p24, virions produced in the presence of SP38 were defective for infection. Transmission Electron Microscopy (TEM) analysis of the virions (~200 each) indicated that SP-38 (but not the controls) resulted in morphologically defective virions with “eccentric” electron dense material with empty capsids. Together, these studies indicated that SP-38 derived from α1 helix of INI1 potently inhibits particle morphogenesis.

Our studies provide a proof-of-concept for the hypothesis that INI1 Rpt1 mimics RNA in binding to IN and that INI1-derived drugs inhibit both IN/INI1 and IN/RNA interactions and inhibit particle morphogenesis. SP-38 represents a novel inhibitor derived from the host factor INI1.

HIV-1 integration into R-loop enriched genomic regions is mediated by Aquarius helicase of the Intron Binding Complex (IBC)

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HIV-1 Integration in the complex environment of the cell nucleus has been the matter of thorough investigation in the recent years. We now know that nuclear speckles (NS) represent a subnuclear environment where the virus completes retrotranscription, to then integrate within the adjacent chromatin regions assigned as speckle associated domains (SPADs). NS seem to be devoid of DNA and it is still unclear how does the Pre-Integration Complex (PIC) target the intronic regions of transcribed and spliced genes and what is the role of HIV-1 Integrase (IN) and its main cellular partner lens epithelium-derived growth factor LEDGF/p75 in this process.

We discover that HIV-1 IN, which binds DNA: RNA hybrids with high affinity through its C-terminal RNA- and DNA- binding moiety, mediates integration into *in vitro* formed hybrid structures or R-loops in the presence of a nuclease. We further show that HIV-1 integration requires a helicase which enables R-loop resolution, and that a splicing helicase Aquarius, member of the intron binding complex (IBC) is needed for efficient HIV-1 integration in cell lines and primary CD4 T cells. Helicase activity of AQR is required for efficient integration, that cannot be reconstituted in the presence of a helicase mutant form of the protein. AQR and the IBC are capable of resolving RNA:DNA hybrids and R-loops, they bind to HIV-1 intasome in vitro colocalize with incoming viral PIC/cDNA in cells. Interaction between HIV-1 IN and components of the IBC and their concomitant binding to R-loops reflect HIV-1 integration patterns into R-loops, which we mapped for the first time in primary CD4 T cells to show that HIV-1 Recurrent Integration Genes are highly enriched in these genomic features, together with H3K36me3 histone mark, a well defined chromatin signature of HIV-1 integration.

Hence, dynamic R-loops are sites of preferential HIV-1 integration, which is mediated by the IBC and its helicase AQR, that we identify as an important interacting partner of IN and LEDGF/p75. Our findings bring into focus the C-terminal domain of HIV-1 IN, and the significance of both DNA and RNA structures in integration site selection, opening new possibilities for novel therapeutic strategies.

The role of LEDGF in transcription is intertwined with its function in HIV-1 integration

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HIV-1 integration occurs across actively transcribed genes and this specificity is due to the interaction of host factor LEDGF with integrase. Our understanding of HIV-1 integration is incomplete in part because the cellular function of LEDGF is unclear. Although LEDGF was originally isolated as a co-activator of promoters in purified systems, this model is inconsistent with LEDGF-mediated integration across gene bodies and with data suggesting LEDGF can regulate alternative splicing. To clarify the roles of LEDGF in transcription we conducted RNA-seq. By knocking out LEDGF we found 516 genes were differentially expressed (>1.6-fold change), underscoring a significant role in gene regulation. To examine the role of LEDGF in splicing, we analyzed genes that produce differentially expressed mRNA isoforms as the result of knocking out LEDGF. The majority of these isoforms were expressed from different start sites, suggesting the dominant function of LEDGF is to regulate promoter activity, not splicing. To determine how LEDGF regulates transcription, we measured H3K4me3 enrichment, a mark of active promoters. Cells lacking LEDGF had reduced H3K4me3 at down-regulated genes. We asked whether such changes were the direct result of LEDGF association at promoters by performing ChIP-seq in LEDGF-3XFLAG knock-in cells. We observed sharp peaks of LEDGF at 5' ends of transcription units that matched the peaks of H3K4me3 of active promoters. Levels of H3K4me3 at LEDGF bound promoters were significantly reduced as the result of knocking out LEDGF, indicating LEDGF functions to stimulate promoter activity. We also observed by ChIP-seq that levels of RNA Pol II at promoters were reduced when LEDGF was knocked out. Efforts to understand how LEDGF is recruited to promoters tested the function of factors that bind LEDGF such as MLL1. ChIP-seq showed that MLL1 peaks match the positions of LEDGF at promoters and importantly, when levels of MLL1 were reduced with siRNA, the peaks of LEDGF at promoters were significantly reduced. Reduction of MLL1 also resulted in substantially lower levels of RNA Pol II Ser5 phosphorylation, a mark of active polymerase. Additional experiments with truncated alleles of LEDGF showed that the N-terminal PWWP chromatin reader domain does not contribute to promoter occupancy. These data support a model where LEDGF is recruited to active promoters by MLL1 and subsequently travels across transcription units to effect HIV-1 integration.

The rules of engagement between lentiviral integration machinery and chromatin

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Large-scale parallel integration site sequencing from infected cells revealed the role of LEDGF/p75 in fine-tuning HIV-1 and generally lentiviral integration site selection. However, such studies cannot tell us the exact status of the chromatin at the time integration took place. We have previously shown that the interactions of the spumaretroviral PFV intasome with the nucleosome core particle overwhelmingly direct integration into specific positions on the nucleosome, separated ~35 bp from the dyad axis. However, we find that this property is not conserved among the retroviral genera. Because retroviral integration must coordinate integration in the context of chromatin, it is likely different retroviruses have evolved alternative mechanisms to enable this direct interaction. LEDGF/p75 is the key candidate to allow direct interaction of the HIV-1 preintegration complex (PIC) with chromatin. Structural studies of both the LEDGF integrase binding domain complexed with lentiviral INs or intasomes, and the LEDGF PWWP domain complexed with histone H3 K36Me$_3$-modified nucleosomes have revealed the nature of the binary interactions. Yet, the structural details of LEDGF-mediated intasome-chromatin tethering remains to be clarified. To address this important question, we developed *in vitro* real-time quantitative PCR assays, which allow quantification of pre-integration complex (PIC) and intasome strand-transfer activity into defined poly-nucleosomal arrays. For both PICs and defined lentiviral intasomes, we found LEDGF/p75 is essential and minimally sufficient for increased integration into H3K36Me$_3$ nucleosomes. Finally, we developed a novel method using long-read Nanopore sequencing to quantify integration sites produced by strand transfer of HIV-1 intasomes into defined chromatin targets. This technique revealed that LEDGF/p75 is responsible for targeting HIV integration outside of the nucleosome body of H3K36Me$_3$ modified chromatin. In the presence of increasing LEDGF/p75 concentration, a dose-dependent inhibition of integration into unmodified chromatin leads the intasome to preferentially search for more exposed DNA targets.

Single molecule visualization of intasome assembly

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Retroviruses integrate their viral genome into the host cell's chromosome, which is essential for the virus to replicate and persist within the host. Integration is facilitated by a complex termed the intasome, which consists of the retrovirus-encoded integrase enzyme (IN), the long terminal repeat (LTR) ends of the viral DNA, and certain cellular factors. The intasome can vary in size and complexity depending on the retrovirus. For example, the intasome of the Prototype Foamy Virus (PFV) is a simple IN tetramer, the intasomes of Mouse Mammary Tumor Virus (MMTV) are IN octamers, and intasomes of HIV-1 and Maedi-Visna virus (MVV) have been reported to be higher order IN multimers including octamer, decamer, dodecamer (12-mer) and hexadecamer (16-mer). Despite these differences, all retroviral intasomes maintain a conserved intasome core (CIC) configuration that is similar to the PFV intasome. However, the exact mechanism by which the intasome assembles and forms these different multimeric structures is not yet fully understood.

To study the intasome assembly process, we utilized a technique called single molecule mass photometry (smMaPh) linked with state-of-the-art microfluidic delivery of complexes that are traditionally unstable in dilute concentrations. This label-free approach measures molecular mass by analyzing light scattering from individual biomolecules in solution. By using smMaPH, size exclusion chromatography and integration activity assays, we were able to determine assembly intermediates and endpoint complexes for different retroviral intasomes, including wild-type PFV, MMTV, and MVV IN. These studies showed that despite the differences in intasome size and complexity across different retroviruses, they all follow a similar IN multimer assembly and kinetics process. Understanding these dynamic and kinetic processes of intasome assembly is important for determining how and when IN subunits are appended to a developing intasome, which could potentially inform the development of antiviral therapies targeting this crucial step in the retroviral life cycle.

Multiscale simulations of HIV-1 capsid nuclear entry and host factor interactions

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Nuclear import and uncoating of the viral capsid are critical steps in the HIV-1 life cycle to transport and release genomic material into the nucleus. Viral core import involves docking and translocating HIV-1 capsid at the nuclear pore complex (NPC) embedded in the nuclear envelope. Notably, the central channel of NPC accommodates and allows passage of intact HIV-1 capsid, though mechanistic details of the process remain to be fully understood. Here, we investigate the molecular interactions that work in concert between HIV-1 capsid, capsid-binding factors, and NPC scaffold to promote capsid translocation through the NPC central channel. We report the development of a “bottom-up” coarse-grained (CG) model of the human NPC from recently released cryo-electron tomography structural data. These components are combined to generate the composite CG model of dilated and constricted membrane-embedded NPC. Applying the CG model of human NPC, we investigate the early stages of translocation dynamics of HIV-1 capsids into the central channel using CG molecular dynamics (MD) simulations. Our simulations demonstrate that dilated state of NPC allows translocation of cone-shaped capsid when approaching with the narrow end at the central channel and tubular pill-shaped capsid. Structural analysis of the capsid lattice bound to the central channel reveals correlated striated patterns of lattice disorder even though the capsids remain structurally intact when translocating into the central channel. We also find that uncondensed genomic material inside the docked capsid augments the overall lattice disorder of the capsid. Our results uncover key factors that regulate the viral nuclear import dynamics and provide molecular insight into the structural state of capsid during distinct stages of nuclear entry. We also simulate CPSF6 oligomerization templated by HIV-1 capsid. First, we use all-atom (AA) MD simulations to perform conformational analysis of GST-CPSF6$_{261\text{-}358}$ bound to CA-hexamer lattice. The AA MD simulations demonstrate that an ensemble of N-terminal LCR conformations mediates the formation of a highly interactive network of primarily unstructured CPSF6 chains. The conformational ensembles of the tri-CPSF6 complex observed in our AA MD simulations can be interpreted as the minimal LCR-mediated motifs for large-scale assembly of CPSF6 templated by a mature CA lattice. We then performed CG MD simulations CPSF6 oligomerization templated by full capsid. Our results show distinct patterns of CPSF6 oligomerization regulated by capsid curvature. In the pentamer-rich tips, typically, the zig-zag patterns are disrupted. Zig-zag patterns similar to cryo-EM maps of CPSF6 oligomer in capsid nanotube are observed in other regions away from the tip. We also uncover that LEN at comparable concentrations to CPSF6 impedes the CPSF6 nucleation of CPSF6 oligomers.

Capsid as key orchestrator of early HIV-1 replication

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After cell entry, HIV-1 replication involves reverse transcription of the RNA genome, nuclear import of the subviral complex without nuclear envelope breakdown, and integration of the viral cDNA into the host genome. Recent evidence indicated that completion of reverse transcription and viral genome uncoating occur in the nucleus rather than in the cytoplasm, as previously thought. Furthermore, multiple recent studies revealed that the cone-shaped HIV-1 capsid, which encases the genome and replication proteins, serves not only as reaction container for reverse transcription and as shield from innate immune sensors, but may also constitute the elusive HIV-1 nuclear import factor. Using high-precision on-section CLEM, we were able to correlate the signal of fluorescently-labeled HIV-1 complexes to positions in the cytosol, in the vicinity of the nucleus and inside the nucleoplasm in infected T-cell lines and primary monocyte-derived macrophages (MDM), and to acquire 3D electron tomograms from these regions. These tomograms revealed cone-shaped structures containing electron-dense nucleic acid complexes that appeared indistinguishable from intravirion capsids in the cytosol, adjacent to and inside nuclear pore complexes. Nuclear capsid-derived structures were mostly empty and broken and accumulated in large numbers in nuclear speckle domains. Super-resolution STED microscopy allowed further analysis of NPC-associated and nuclear capsid-related structures with respect to relevant cellular proteins and to study the influence of capsid-targeting drugs on their localization and accessibility of the reverse-transcribed HIV-1 genomic DNA.

Mechanisms of HIV-1 Core Uncoating, Nuclear Import Kinetics, and Integration Site Selection

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HIV-1 uncoating is essential for release of the viral DNA for integration into the host genome. Our recent live-cell imaging studies have indicated that intact viral cores enter the nucleus and uncoat near (<1.5 μm) the genomic integration site <1.5 hours before integration. However, the factors that trigger HIV-1 uncoating are unknown. We observed that inhibition of reverse transcription resulted in a 5-fold decrease in uncoating of nuclear viral cores. Uncoating was impaired even when reverse transcription was inhibited at a late stage, indicating that long double-stranded viral DNA must be synthesized to efficiently trigger uncoating. Viral genome size was directly correlated with uncoating efficiency, and genomes >3.5 kb were needed to trigger uncoating. A small (3.1 kb) GFP-reporter genome was efficiently reverse transcribed, but it was defective for uncoating, integration and GFP reporter gene expression. Integration and GFP reporter gene expression was rescued by inducing disassembly of the viral cores containing the small genomes with PF74 or Lenacapavir treatment, which also resulted in rapid degradation of most of the viral DNA, suggesting that the viral cores remain intact until just before uncoating to protect the viral DNA from degradation by the host. These results are consistent with a model in which HIV-1 uncoating is triggered upon the completion of long double-stranded viral DNAs in the viral cores.

HIV-1 core-nuclear pore complex interactions mediate nuclear import but the capsid protein (CA) determinants essential for these interactions are not well defined. We found that CA mutant M10I exhibits a longer NE residence time before nuclear entry compared to WT. Low expression of cleavage and polyadenylation specificity factor 6 (CPSF6) resulted in M10I cores having even longer NE residence times, a nuclear import defect, uncoating at the NE, and integration into nearby lamina-associated domains. Conversely, high expression of CPSF6 restored M10I nuclear import kinetics to WT. These studies identify a CA determinant that affects the nuclear import kinetics in a CPSF6-dependent manner and show that core-CPSF6 interactions regulate nuclear import of viral cores. Additionally, these studies reveal that the kinetics of viral core nuclear import can influence integration site selection.

HIV-1 requires capsid remodelling at the nuclear pore for nuclear entry and CPSF6 binding

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The HIV-1 capsid (CA) protein assembles into an organised lattice enclosing the viral RNA and enzymes to form the mature HIV-1 core. This lattice is crucial during the early stages of viral replication as it interacts with many host cell factors, but it must break down (uncoat) at the right time and place for successful integration. The spatio-temporal aspects of uncoating are currently hotly debated.
We have investigated the effects of increased core stability on the early stages of HIV-1 infection by introducing cysteine residues into CA in order to induce disulphide bond formation and obtain hyper-stable mutants that are slower or unable to uncoat. We found that the A14C/E45C and E180C mutants were able to reverse transcribe to approximately WT levels but were markedly impaired for integration. Intriguingly, these mutants only had a 5-fold reduction in 2-LTR circle production and we observed CA protein in both nuclear and chromatin-associated cellular fractions suggesting that the cores entered the nucleus. However, using a proximity ligation assay, we found that although the A14C/E45C CA reached the nuclear pore with the same kinetics as WT CA, it was retained at the pore in association with Nup153. This highlights the importance of CA lattice flexibility for nuclear entry and suggests some CA remodelling occurs at the nuclear pore. Furthermore, infection with the hyper-stable mutants did not promote CPSF6 puncta formation, despite the mutant capsid being competent for CPSF6 binding. We have further investigated how CA induces CPSF6 puncta formation at nuclear speckles in relation to reverse transcription, nuclear entry and integration and are currently assessing how this affects cellular processes.

Distinct utilization of nuclear import pathways allows HIV-1 integration into transcriptionally active regions of the chromatin

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Human immunodeficiency virus (HIV-1) nuclear import is mediated by a series of interactions between the viral capsid (CA) and host factors, including CypA, CPSF6, and nucleoporins (Nups). Interactions between CA and host factors determine downstream nuclear events of infection, including targeting HIV-1 integration into gene-rich and transcriptionally active chromatin regions. By altering the interaction of CA with CPSF6 and CypA, the Nup requirement of HIV-1 is altered, and integration occurs in gene-sparse rather than gene-rich regions. Using an inducible nuclear pore blockade, we observed that CA mutants N74D and P90A were relatively insensitive to Nup62 (central FG Nup) mediated nuclear pore blockade, while virus with WT CA was more potently inhibited. This suggests that Nup62 blockade does not block all populations of NPCs, which further suggests that heterogeneous populations of NPCs exist and that HIV-1 can utilize different populations of nuclear pores during infection, which is in turn dependent on its interaction with host factors such as CPSF6 and CypA. The components of the NPC machinery that facilitate viral nuclear import and how passage through different populations of nuclear pores potentially influence intranuclear trafficking and viral integration are unknown. Utilizing a panel of 25 Nups fused to the dimerization domain, we observe the inducible blockades differentially inhibit infection by HIV-1 with WT, N74D and P90A CA mutants, providing insight into the composition of NPCs utilized by HIV-1 during nuclear import. In addition, to determine the role of CPSF6 in driving utilization of these NPC populations, we generated chimeric CPSF6 constructs engineered to contain divergent NLS sequences to promote the utilization of specific nuclear import pathways. With these systems, we observe that certain Nup blockades and NLSs fused to CPSF6 showed differential effects on reporter gene expression and 2-LTR circle formation and localization to speckle associated domains (SPADS). These results indicate that passage into the nucleus via specific nuclear import pathways influences the intranuclear trafficking and likely integration into transcriptionally active regions of chromatin.

Live-cell imaging of HIV-1 nuclear transport and association with nuclear speckles

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Following its cellular entry, the HIV-1 RNA genome is reverse transcribed into a cDNA by the process of reverse transcription. The reverse transcribing viral genome is enclosed inside a protective capsid shell, which is transported into the nucleus of non-dividing cells through nucleopore complexes (NPCs) embedded on the nuclear membrane. The passage of the capsid and its contents through NPCs is facilitated by interactions between capsid and multiple nucleoporins, including NUP358 situated in the cytoplasmic side, and NUP153 situated at the nucleopore basket facing the nucleoplasm. At some point during its passage through the NPCs, the capsid interacts with the cellular polyadenylation specific factor (CPSF6), which is primarily located in the nucleus. The interactions between capsid and CPSF6 is critical to release HIV-1 from NPCs and for its penetration into the nucleoplasm. We have recently identified that capsid-CPSF6 interactions drives the transport and preferential accumulation of HIV-1 replication complexes in phase-separated membrane-less organelles called nuclear speckles. Notably, nuclear speckles sequester actively transcribing genes which are known targets for viral integration. Accordingly, our findings demonstrated that integration of the newly completed HIV-1 cDNA takes place in proximal nuclear speckle associated genomic domains (SPADs). The mechanisms of how the interactions between capsid and CPSF6 target HIV-1 integration into SPADs remains unclear. We will discuss recent live-cell imaging insights of HIV-1 transport in the nucleoplasm (from NPCs to speckles), and highlight the different components, involving the release of viral cores from the nuclear pore and association with nuclear speckles, which is orchestrated by mechanism involving distinct domains of the CPSF6 protein.

Fluorescent labeled CA correlates progressive uncoating from the cytoplasm to the nucleus to productive HIV infection in primary cells

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Imaging and analysis of individual viral particles has become a widely utilized tool to study HIV replication. Although this approach has advanced the field, conflicting results are obtained due to different viral behaviors that occur within cells throughout infection and detrimental effects of the labeling methods We propose that it is important to distinguish between viral behavior that leads to infection from the one that does not. We constructed a directly labeled CA by circularly permuting a fluorophore to a.a. residues facing the outside surface of the core while ensuring that all viral particles are labeled. The behavior of the labeled viruses was characterized and compared to wild-type NL4-3 in different steps of infection with biochemistry, fluorescence imaging, cryo-CLEM and functional assays. We show that the fluorophore is incorporated into the capsid lattice and is not segregated to the inside or excluded from the capsid, unlike N and C-term fusions to CA. Thus, our construct enables direct quantification of CA content in HIV cores. By combining variable time-lapse and light intensity imaging of multiple fields of cells over 48-hour periods with machine learning methods for segmentation and image restoration, we tracked hundreds of thousands of viral particles that distribute into different behaviors previously described by multiple research groups: **i**. falling apart after fusion, **ii.** completely losing CA signal in the cytoplasm, **iii.** initiating uncoating at the cytoplasm and further shedding at the nuclear pore, and **iv.** retrograde nuclear pore complex interaction. We challenged HOS cells and primary monocyte derived macrophages (MDM) with ultra-low MOI and tracked individual viral particles that achieved a productive infection. We identified 53 productively infected macrophages and 195 infected HOS cells out of a total of 19591 MDM and 64054 HOS cells, identified by nascent fluorescent virus particles. We find that the viral behavior linked to infection of wild-type CA in cell lines and macrophages was only observed in particles undergoing initiation of uncoating in the cytoplasm. In some particles we detect further CA shedding at the nuclear envelope before nuclear entry. We did not find any intact cores inside of the nucleus of non-dividing cells. These observations support that the model of cytoplasmic initiation and progressive uncoating with some CA retention until integration is associated with infection.

Effects of the cyclophilin homology domain of RanBP2 on HIV-1 infection and Mx2 activity

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Mx2 is an effector of the type I IFN-mediated inhibition of the early, preintegration phases of HIV-1 infection that is localized to the nuclear pore complex (NPC). The HIV-1 capsid (CA) is the major viral determinant for sensitivity to Mx2, and complex interactions between Mx2, CA, nucleoporins (Nups), and other cellular proteins influence the outcome of viral infection. In prior studies, we explored these interactions using a panel of siRNAs targeting Nups and nuclear import factors. While this method provided significant insight concerning the importance of Nups in HIV-1 infection, such depletions have significant pleiotropic effects and often dramatically alter the formation/structure of the NPC, confounding interpretation of results. Additionally, genetic studies have identified direct interactions between Nups and the viral CA, but do not demonstrate that such interactions have functional consequences in the context of infection. Furthermore, not all Nups that affect HIV-1 infection interact with the viral CA, indicating that their impact on infection may be indirect.

To specifically assess the role of individual Nups in HIV-1 infection and Mx2 activity, we have utilized a CRISPR/AAV approach to generate cells expressing mutant Nups from their endogenous loci. The precise construction of these mutants allows for the modification of the functional capabilities of the NPC without disrupting its assembly or altering overall expression levels.

We initially generated cells with a deletion of the cyclophilin (Cyp) homology domain of RanBP2 and found that both HIV-1 and HIV-2 infection were modestly reduced in cells endogenously expressing truncated RanBP2ΔCyp. Importantly, although Mx2 still localized to the NPC in RanBP2ΔCyp cells, antiviral activity against HIV-1 in these cells was decreased. However, inhibition of HIV-2 infection by Mx2 was unaffected, indicating that the Cyp domain of RanBP2 affects Mx2-CA interactions in a virus- specific manner. Additionally, both sensitivity of HIV-1 and HIV-2 to depletion of other nucleoporins, and the antiviral activity of Mx2 were dramatically altered in RanBP2ΔCyp cells. To address whether virus- specific differences are the result of altering RanBP2-CA and/or RanBP2-Mx2 interactions, we generated cells expressing specific point mutations in the RanBP2-Cyp domain that abrogate HIV-1 or HIV-2 CA binding but should not affect enzymatic activity or cellular function. We have also produced cells with further truncations in RanBP2 to address Cyp-domain-independent effects on HIV infection and Mx2 activity.

Further characterization of these RanBP2 mutant cells and the application of this approach to other Nups will now allow us to precisely define the role of individual Nups in HIV-1 infection, Mx2 activity, and cellular nucleocytoplasmic trafficking.

The Fellowship of the Ring: Quest to develop Pirmitegravir, a novel potent and safe HIV-1 allosteric integrase inhibitor (ALLINI)

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Over the past few decades, a number of ALLINIs were discovered and developed along with the progress of HIV-1 integrase research. However, numerous efforts failed to move forward to the clinical development stage due to various obstacles in the early discovery and development stage.

Pirmitegravir (PIR), a pyrrolopyridine derivative ALLINI, is a first-in-class HIV-1 allosteric integrase inhibitor with a novel mechanism of action. It binds to the LEDGF/p75 binding site of integrase (IN) and inhibits viral maturation.

PIR had demonstrated its moderate druggability possessing excellent physicochemical and ADME properties and confirmed its efficacy and safety profiles in nonclinical development. The safety, tolerability and pharmacokinetics of PIR was recently proved in the first-in-human phase 1 study and therefore it is into the clinical phase 2a to confirm its antiviral potency in clinical settings.

In this talk, we will introduce the overall development journey of this novel potent and safe HIV-1 ALLINI.

Optimizing the binding of substituted quinoline ALLINIs within the HIV-1 integrase oligomer

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Allosteric HIV-1 integrase (IN) inhibitors or ALLINIs bind at the dimer interface of the IN, away from the enzymatic catalytic site and block viral replication by triggering an aberrant multimerization of the viral enzyme. Interestingly, these inhibitors are capable to impact both early and late stages of the viral replication. To further our understanding of the important binding features of multi-substituted quinoline-based ALLINIs, we have examined the IN multimerization and antiviral properties of substitution patterns at the 4, 6 and 8 positions. We have explored the hypothesis that the early/late efficacy of these inhibitors can be modulated by the nature of the substitutions at those positions. We have found that the dual properties of ALLINIs could be decoupled toward the late-stage viral replication effects by generating additional contact points between the bound ALLINI and a third subunit of IN. These features not only improve the overall anti-viral potencies of these compounds but also significantly shift the selectivity toward the viral maturation stage. Thus, to fully maximize the potency of ALLINIs, the interactions between the inhibitor and all three IN subunits need to be simultaneously optimized.

Lenacapavir: A First-in-Class, Long-Acting HIV Capsid Inhibitor for Treatment and Prevention

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A research program building on prior extensive structural and functional characterization of the HIV capsid and spanning a decade of drug discovery work has yielded lenacapavir (LEN, formerly GS-6207), a first-in-class clinically active small molecule inhibitor targeting several critical functions of the HIV capsid. LEN binds at a conserved interface between adjacent capsid protein (CA) monomers and interferes with protein-protein interactions essential for multiple phases of the HIV replication cycle, including capsid nuclear import and disassembly, virion production from CA precursor polyproteins, and proper capsid formation. LEN exhibits antiviral activity at picomolar concentrations against all subtypes of HIV, including strains resistant to other antiretroviral classes, and is amenable to both oral and long-acting subcutaneous administration due, in large part, to its exceptional potency and metabolic stability. In a phase 1 study in treatment-naïve people with HIV (PWH), LEN (50 to 750 mg) showed a rapid and dose-dependent antiviral effect, with up to 2.3 mean $\log_{10}$ decrease in HIV-1 RNA at day 10 of monotherapy. In people with multidrug-resistant HIV, subcutaneous LEN administered every 6 months in combination with an optimized background regimen led to high rates of virologic suppression and was well tolerated. LEN is now approved as a twice-yearly subcutaneously dosed therapy for heavily treatment-experienced PWH with multidrug resistant virus and is now undergoing phase 2 clinical trials as a once-weekly oral partner agent for the reverse transcriptase inhibitor islatravir. Building on favorable emerging data from non-human primate studies investigating LEN as a long-acting agent for pre-exposure prophylaxis (PrEP), twice-yearly LEN is also being studied for HIV prevention in two phase 3 clinical trials (PURPOSE 1 and PURPOSE 2) to overcome some of the barriers that can limit the uptake of daily oral medications in people who could benefit from PrEP. The discovery, mechanism of action, and development of lenacapavir will be described.

Developing novel small molecules as inhibitors targeting HIV-1 Integrase and capsid proteins

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HIV-AIDS continues to be a global health issue with over 38 million people currently living with the virus. Although decades of intense research in the area of drug development have resulted in discovering critical life-saving drugs that prolong the lives of patients living with HIV, drug resistance remains a major concern. Therefore, there is a continuing need to develop novel drug molecules with possibly different target and/or mode of action against emerging drug-resistant variants. Our research group is interested in developing small molecules that target HIV-1 integrase (IN), and the capsid protein (CA) which encapsulates the viral genome.

Allosteric HIV-1 IN inhibitors (ALLINIs) are a promising, new class of antiretroviral agents that induces higher-order IN multimerization to disrupt proper viral maturation. We used several medicinal chemistry techniques such as high-throughput screening, scaffold-hopping and fragment-based approaches to design our ALLINIs. One of our promising lead compounds, KF116, is a pyridine-based ALLINI that exhibits remarkable selectivity for IN tetramers versus lower order protein oligomers. We have been able to show that cells treated with KF116 yield malformed viral particles. We have extended our studies towards the synthesis of the highly active (–)-KF116 enantiomer, which displays EC_{50} of ~7 nM against wild type HIV-1 and ~10-fold higher, sub-nM activity against clinically relevant dolutegravir resistant mutant virus suggesting potential clinical benefits for complementing dolutegravir therapy with pyridine-based ALLINIs. We have also identified two new quinoline-based ALLINIs KF-255 and KF-257, which show notable differential abilities to promote IN multimerization.

Lenacapavir (LEN; Sunlenca®) is an FDA approved first-in-class long-acting HIV-1 CA-targeting inhibitor that exhibits exceptional potency against all major HIV-1 subtypes including the viral variants resistant to current antiretroviral therapies. We have established the synthesis of LEN in our academic lab and elucidated the structural and mechanistic bases behind the exceptional antiviral potency of the inhibitor. Our high-resolution x-ray crystallography experiments with drug-resistant CA hexamers uncovered structural basis for viral resistance to LEN. We have used these findings to develop an improved analog KFA-012 which exhibits enhanced activity against the viral Q67H/N74D capsid phenotype compared with that of parental LEN. These findings provide a proof-of-concept for the rational development of second-generation HIV-1 CA-targeting inhibitors.

Potent long-acting inhibitors targeting HIV-1 capsid based on a versatile quinazolin-4-one scaffold

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Long-acting (LA) human immunodeficiency virus-1 (HIV-1) antiretroviral therapy characterized by a ≥1 month dosing interval offers significant advantages over daily oral therapy. These include increased convenience; reduced logistical and psychosocial demands; fewer opportunities for stigma, discrimination, or disclosure of HIV status; and improved adherence in certain situations. However, relative to once-daily oral therapy, injectable long-acting therapy imposes more stringent criteria on the profile of compounds that enter clinical development. In particular, exceptional potency and low plasma clearance are required to meet dose size requirements, and excellent chemical stability and/or crystalline form stability is required to meet formulation requirements. Additionally, new antivirals in HIV-1 therapy need to be largely free of side-effects and drug-drug interactions. In view of this high bar, a scaffold for capsid inhibitor design which could be efficiently assembled and offer a conformationally-constrained, single isomer was sought. Herein is reported the discovery that capsid inhibitors comprising a quinazolinone core tolerate a wide range of structural modifications while maintaining picomolar potency against HIV-1 infection in vitro, are assembled efficiently in a multi-component reaction, and can be isolated in a stereochemically pure form. Additionally, the feasibility of both an efficient library synthesis strategy and a more targeted scale-up synthesis strategy are demonstrated. Further, the detailed characterization of a prototypical compound, GSK878, is presented, including an X-ray co-crystal structure; subcutaneous and intramuscular pharmacokinetic (PK) data in rat and dog; and virology data including time of addition experiments and integration site analysis.

Second Generation Integrase Inhibitor Resistance in the Clinic: Dolutegravir Resistance Mechanisms and Structural Underpinnings

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The SAILING and DAWNING studies enrolled integrase strand transfer inhibitor (INSTI)-naïve adults with HIV who respectively had: failed virologically with ≥two-class resistant HIV-1 and randomized to receive dolutegravir (DTG) or raltegravir (RAL) based regimens that included ≥one fully active background agent or; virologically failed first-line therapy of non-nucleoside reverse transcriptase inhibitor (NNRTI)+2 nucleos(t)ide reverse transcriptase inhibitors (NRTI) with resistance, and randomized to either DTG or lopinavir/ritonavir plus 2 NRTIs, ≥one being fully active. The DTG arm had superior efficacy at Week 48 in both studies. We assessed HIV-1 resistance and replication capacity (RC%) using population samples and derived clones, DTG dissociation rates, and structural underpinnings focused on integrase resistance substitutions G118R and R263K.
Through Wk132 in SAILING, 5 participants receiving DTG had emergent IN substitutions: R263K,V260I; R263K; N155H; T97A,N155H; or A49G,S230R,R263K. Through Week 158 in DAWNING, 6 participants receiving DTG had on study IN substitutions G118R; H51H/Y, G118R, E138E/K, 263R/K; G118G/R, E138E/K, 148Q/R, R263R/K; E138K, G140S, Q148H, N155H; T66T/I, L74I, G118R, E138E/K; and G118R. DTG fold change IC50 and RC% for key on study isolate clones formatted as “IN substitutions (median DTG FC, RC%)” include: R263K (1.8; 27%); G118R (30.5, 9.6%); L74I, G118R (40, 14.5%); H51Y, G118R (11, 28%); G118R, E138K, R263K (13, 27%); L74I, E138K, Q148R (6.8, 41%); L74I, E138K, Q148R, R263K (17, 23.5%); L74I, G118R (10; 24%); T66I, L74I, G118R (31.5; 13%); T66I, L74I, G118R, E138K (35; 37%).
To rationalize these mutations, we compared HIV-1 integrase wildtype and mutant homology models. In our G118R model, R118 partially occludes the integrase catalytic binding site and forms a dual hydrogen bond with E92 and a hydrogen bond with the tDNA terminal thymine, interactions that are not observed for wildtype G118. In our R263K model, fewer hydrogen bond interactions to substrate and catalytic loop were observed with K263 relative to wild-type R263. DTG T1/2 (half-life of dissociation) from R263K or G118R integrase-DNA complexes remained prolonged at 52 and 10.7 hours, versus 9.6 and 2.7 hours for RAL.
Consistent with continued prolonged DTG binding in the presence of G118R or R263K substitutions, and with structural observations, DTG’s high resistance barrier appears to be the result of a challenging balance between impaired replication capacity and level of resistance.

Mutations outside integrase lead to high-level resistance to integrase strand transfer inhibitors

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Second-generation integrase (IN) strand transfer inhibitors (INSTIs) are highly potent antiretroviral compounds that exhibit a high genetic barrier to resistance. Recent clinical studies concluded that some INSTI-treated individuals experience virological failure in the absence of resistance mutations in IN. To elucidate INSTI resistance mechanisms and pathways, we performed long-term passaging of lab-adapted and primary viral isolates using human T-cell lines or primary PBMCs over nearly one year with an escalating concentration of the INSTI dolutegravir (DTG) or, in parallel, the RT inhibitor islatravir (EFdA). Independent of viral subtype and coreceptor usage, HIV-1 became resistant to DTG by sequentially acquiring mutations in Env and Gag-nucleocapsid (NC) in the absence of resistance mutations in IN. In contrast to the accumulation of mutations outside the drug-target gene observed with DTG, HIV-1 acquired high-level resistance to EFdA by accumulating multiple mutations in RT. The NC mutations selected with DTG clustered in the zinc-finger domains and conferred modest (3-5- fold) resistance to INSTIs but not the RT inhibitor. By cloning *env* from the DTG-treated viruses selected in the SupT1 T-cell line or PBMC, we obtained heavily mutated Env clones, 7XEnv and WD-3, respectively. Both Env mutants exhibit faster-than-WT replication in spreading infection. 7XEnv exhibits resistance to multiple classes of ARVs, with the fold resistance being ~2-logs higher for INSTIs than for other classes of drugs. WD-3 confers 5-fold resistance to DTG in PBMC. Viral transmission of 7XEnv through cell-cell contact is more efficient than that of WT at the cost of cell-free infection. In contrast, WD-3 exhibits more efficient cell-free infection than WT virus. These results suggest that the selected Env mutations confer resistance to INSTIs by increasing infection capacity through cell-cell transmission or cell-free viral infection. Interestingly, viral infection using VSV-G-pseudotyped viruses over a range of multiplicity of infections (MOIs) revealed that INSTIs are more readily overwhelmed by high MOI than RT inhibitors, leading to high-level resistance to INSTIs. Overall, these findings demonstrate that a combination of mutations in Env and NC can confer high-level resistance to INSTIs in the absence of IN mutations. These results advance the understanding of how HIV-1 can evolve resistance to ARVs in the absence of mutations in drug-target genes.

HIV-1 3'-polypurine tract mutations confer dolutegravir resistance by switching to an integration-independent replication mechanism via 1-LTR circles

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The integrase inhibitor dolutegravir (DTG) is a potent inhibitor of HIV replication and is currently recommended in drug regimens for people living with HIV. Whereas HIV normally escapes from antiviral drugs by the acquisition of specific mutations in the gene that encodes the targeted enzyme, recent in vitro studies indicate that mutations in the HIV 3'-polypurine tract (3'PPT) motif, an RNA element that has a crucial role in the viral reverse transcription process, allow HIV replication in the presence of DTG.

Using an in vivo SELEX approach, we discovered that different mutations in the 3'PPT can confer DTG resistance, suggesting that inactivation of this critical reverse transcription element causes resistance. Analysis of the viral DNA products formed upon infection of cells with these 3'PPT mutants revealed that they replicate without integration into the host cell genome, concomitant with an increase in the production of 1-LTR circles. Additionally, we demonstrate that replication of the 3'PPT-mutated virus variants is activated by the HTLV-1 Tax protein, a factor that reverses epigenetic silencing of episomal HIV DNA.

Together, our data indicate that the 3'PPT-mutated viruses escape from DTG by switching to a unique integration-independent replication mechanism. Whether this exotic escape route can also contribute to viral escape in HIV-infected persons remains to be determined, but our results indicate that screening for 3'PPT mutations in patients that fail on DTG therapy should be considered.

New small molecule derivatives as dual inhibitors of the HIV-1 integrase catalytic site and integrase-RNA interactions

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Over the past decades, the outcome of HIV infection has been revolutionized by undisputed progress resulting from the introduction of the antiretroviral regimen. However, multi-drug resistant strains can still be detected and long-term drug toxicities represent an unresolved concern.
HIV integrase (IN) is vital for viral replication and it is an important therapeutic target. IN strand transfer (ST) inhibitors (INSTIs) have proven to be highly effective, becoming a potent first-line therapy to treat infected patients. However, despite the high genetic barriers with the recently FDA-approved INSTIs, the drug therapy selects for mutations responsible for multiple INSTIs resistance, underscoring the need of more effective antiretroviral compounds. The development of small molecule protein-protein interaction inhibitors is an attractive strategy to discover anti-HIV agents. In this field of research, allosteric IN inhibitors (ALLINIs), are a promising new class of antiretroviral agents. They act differently in respect to INSTIs, causing aberrant IN multimerization, which was recently unraveled as cause of the inhibition of IN-vRNA interactions.[1] In doing so, ALLINIs indirectly disrupt the IN-vRNA binding, leading to the formation of defective viral particles with greatly reduced infectiveness.[2] While the indirect disruption of IN-vRNA binding has been described with ALLINIs, the direct disruption of this binding (without affecting IN multimerization) by small molecules has not been reported so far. We describe a series of compounds identified as inhibitors of the IN-vRNA binding *via* a direct mechanism deepening the mechanism of action of some compounds previously described as INSTIs by us.[3] Indeed, we speculated that these quinolinonyl derivatives, being endowed with two DKA chains, could also act as protein-nucleic acid interaction inhibitors. To verify our hypothesis, we tested a set of derivatives endowed with a variable "base-like" functional group. We assessed their capability of inhibiting both the IN 3'-processing and ST reactions in a LEDGF/p75 independent assay. In addition, we performed *in vitro* binding assays, and we found that they are able to disrupt the IN-vRNA interaction, vital for a correct generation of infective virions. The data coming from the biological assays will be shown and discussed.

Sub-stoichiometric drug to HIV-1 capsid ratio enables ultra-potent antiviral activity of lenacapavir

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Lenacapavir (LEN) is the most potent antiretroviral therapy. Yet, the underlying mechanism for such high potency is unknown. Here we have developed an LC-MS-based methodology to quantitate LEN concentrations in virions and found that sub-stoichiometric LEN to capsid protein (CA) ratios hyper-stabilize HIV-1 capsid and block infection. Furthermore, we investigated an additional antiviral activity of LEN to impair proper virion maturation. Strikingly, LEN binding to CA monomers specifically blocked the formation of CA pentamers, whereas the inhibitor promoted the assembly of hyper-stable hexametric CA lattices. Moreover, we have elucidated structural and mechanistic bases for the emergence of the major clinical drug-resistant HIV-1 (CA M66I) isolate. We show that Ile66 specifically introduces steric hindrance to LEN, whereas the M66I change does not affect binding to the key cellular cofactors CPSF6, NUP153, and SEC24C to the same hydrophobic CA pocket. Collectively, our findings elucidate previously undescribed mechanisms for ultra-potent antiviral activity of and drug resistance to LEN and provide means for developing improved antiretrovirals.

Ty3: We should have known it wouldn't be random

Suzanne Sandmeyer

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In the early 1980's Maynard Olson, a chemist newly arrived and on sabbatical in the Ben Hall laboratory at University of Washington showed that budding yeast $tRNA^{Tyr}$ genes, mapped as suppressors in different yeast strains to identical positions using classical genetics--for which UW faculty were rightly famous--disconcertingly could not be confirmed in his hands to be in the same physical positions using two new--and highly acclaimed--tools: restriction enzymes and Southern blots. Understanding the basis of those disparate observations triggered multiple rounds of research inquiries in our laboratory leading eventually to the discovery of the full-length Ty3 retroviruslike element, the observation that Ty3 targets RNA polymerase III transcription factor IIIB, and more generally the notion of retrointegration position specificity. We now know, thanks to the work of many attendees at this meeting, that retrointegration site choice can be influenced by multiple properties of the target site and in turn, much of the impact of elements on host genomes is a consequence of that target site choice. The story of course cannot end there. In an inversion of perception brought on by high throughput sequencing, retroelements are now considered not just punctuation, but rather a major, poorly understood, structural component of host genomes.

Ty1 Gag stories: mechanism of copy number control, domestication of a restriction factor, and an interchangeable prion-like domain

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Retrotransposons and endogenous retroviruses shape genome structure and function. *S. cerevisiae* and its close relatives harbor several families of LTR retrotransposons with different properties. In particular, Ty1 retrotransposition is restricted via a process termed Copy Number Control (CNC). We reported a novel self-encoded restriction factor called p22 that is necessary and sufficient for CNC. p22 is translated from internally initiated Ty1 RNA and is identical to the C-terminal region of Gag. Mutations in Gag that escape CNC exclude p22 from Ty1 VLPs, suggesting that p22 restricts Ty1 transposition by interfering with VLP assembly.

CNC. Genetic, biophysical, and structural analyses of the minimal restrictive segment within p22 that contains the capsid C-terminal domain (CTD) led to a model for CNC based on CTD interactions between Gag and p22 that preclude assembly of a spherical shell.

Domestication. We provide evidence that p22 was endogenized in the ancestral Ty1 subfamily Ty1'. Endogenization took place at the Relic 2 locus on chromosome IV between $tRNA_{Gly}$ and $tRNA_{Ser}$ genes, which are preferred targets for Ty integration. Relic 2 is structurally diverse and rapidly evolving in human-associated and natural isolates. *DRT2* (*D*omesticated *R*estriction *T*y1') maintains CTD coding potential in different contexts at Relic 2 and is present in many strains, including ancestral isolates from China. Retromobility and RNA analyses demonstrate *DRT2* restriction factor functionality and co-sedimentation profiles suggest an association between Drt2 and Ty1' assembly complexes/VLPs. Interestingly, Ty1' is not under a self-encoded form of CNC.

A prion-like domain (PrLD). Ty1 forms cytoplasmic foci called that contain Ty1 proteins and RNA, are sites where VLPs assemble, and resemble liquid-liquid phase separated (LLPS) compartments. We show that a predicted PrLD upstream of the capsid domain is required for foci formation, VLP assembly and retrotransposition. The Ty1 PrLD's amino-acid composition is similar to yeast prions and nucleates prionogenesis in a *SUP35* prion reporter system. Ty1 Gag chimeras containing PrLDs from yeast and mammalian prionogenic domains display a range of retrotransposition phenotypes. We examine the chimeras throughout the Ty1 replication cycle and find that Sup35 and mouse PrP support VLP assembly and retrotransposition. Our work invites study into phase separation as an organizing component in other retroelements and viruses.

Large serine integrases: how do they know which way to go?

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Site-specific DNA recombinases catalyze DNA insertions, inversions, and deletions in an extremely tidy fashion, leaving not a single broken phosphodiester bond. However, the mechanism by which they do so leaves them with an interesting thermodynamic problem: the net number of high-energy bonds in the product is the same as that in the substrate. How do these enzymes drive their reactions to near completion? Furthermore, how do they "decide" which pairs of sites to pair as substrates and in what relative orientation? I will describe our progress on answering these questions for the serine-family group of site-specific recombinases termed the large serine integrases.

Large serine integrases are encoded by temperate bacteriophages and have evolved to catalyze efficient insertion of large payloads. Furthermore, unlike tyrosine integrases, they do not require accessory DNA sites. Efficient insertion requires with only a tetramer of integrase and two DNA sites: an ~40bp attB and an ~50bp attP site. Expression of the phage-encoded RDF (recombination directionality factor) protein, which binds the integrase itself, triggers the reverse (excision) reaction and inhibits integration. Surprisingly, although the RDFs encoded by a wide variety of phages are all predicted to bind to the same region of their cognate integrase proteins, they share no sequence or structural homology with one another and are instead scavenged phage proteins. We are using cryoEM, modeling, and biochemistry to understand how these disparate RDFs control the directionality of large serine integrase-catalyzed reactions.

Evidence of significantly reduced HIV proviral integrants within genes and increased integration into transcriptionally silent elements in HIV-1 infected individuals failing an INSTI treatment regimen with or without INSTI resistance mutations

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Recent studies by our team have provided new evidence that divergent retroviruses exhibit distinct integration site profiles with strong preferences for integration near non-canonical B-form DNA (non-B DNA), analyses which included sequencing of DNA from primary T cells of individuals infected with HIV-1 group M subtypes A, C and D in the absence of treatment. Cells with proviral integrants at or near transcriptionally silent regions may be archived whereas cells with HIV-1 integrated in transcriptional active regions are in constant turnover, suggesting a reduction in their numbers. During effective combined antiretroviral treatment (cART), there is a further increase in at or near transcriptionally silent regions. In this study, we have explored the impact of treatment of integrase strand transfer inhibitiors (INSTIs) on sites of HIV-1 proviral integration. Individuals receiving cART (including Raltegravir or Dolutegravir) with undetectable viremia or with virologic failure (N = 52) provided whole blood samples for genotypic drug resistance testing and for proviral DNA integration site analyses using Illumina MiSeq. We mapped the HIV-1 proviral integration sites near or within (a) “longer, functional” regions which included genes/oncogenes, endogenous retroviruses, nuclear elements, etc and (b) shorter Non-B DNA motifs including A phased, cruciform, slipped, triplex and Z-DNA motifs as well as G-quadruplex, inverted, short tandem and mirror repeats. When compared to analyses on DNA from individuals on stable cART, already with enrichment of HIV-1 integrants at transcriptionally silent sites, individuals failing an INSTI-based cART had DNA with transcriptionally active sites (i.e. genes) almost devoid of HIV-1 DNA and with HIV-1 integration mostly appearing in the cellular DNA sites that are “dead” for transcription. In contrast, individuals on a cART including a protease inhibitor (PI), instead of INSTIs, had integration patterns more similar to infected individuals in the absence of cART. Interestingly, integration patterns biased to transcriptionally silent sites were observed in patients receiving INSTI-based cART whether or not they were failing the treatment and independent of the presence of INSTI resistance mutations. At the meeting, we will provide new evidence that HIV-1 integration in silent DNA (away from transcriptionally active sites) may be linked to the direct binding of the INSTI to HIV-1 integrase, impacting host protein and DNA binding, even in the presence of INSTI resistance mutations, and thereby, altering site selection by the integration complex.

Functional mapping of integration sites connected to latent HIV-1 infection

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Long-term persistence of HIV-1 in latent reservoirs hinders a cure for people living with HIV-1. Several approaches have been proposed, and some have been tested clinically to eradicate the latent reservoirs, but our partial understanding of how latency is regulated has limited their success. Here, we have investigated how the site of HIV-1 integration affects entry and exit from latency. We have used a comparative approach that takes advantage of the different integration profiles of wild type (WT) HIV-1 and a mutant virus bearing the N74D change in the capsid. We found that WT virus becomes latent more slowly and can be reactivated more efficiently than N74D virus by TCR stimulation or PMA.

To understand the mechanisms for this different phenotype, we mapped WT and N74D unique integration sites (UISs) onto HiC maps of 3D chromatin compartments and observed that WT HIV-1 integrated 8 times more frequently in A1 compartment than N74D HIV-1, whereas N74D integrated 7 times more frequently into lamina associated domains (LADs) and 2 times more frequently in A2 compartments than WT virus. Epigenetic profiling showed that the broad histone deacetylase inhibitor SAHA and the G9a inhibitor BIX-0124 reversed both WT and N74D latency with equal efficiency; however, selective inhibition of HDAC6, inhibition of FOXO-1 or AzadC and depletion of the HUSH complex component periphilin reversed WT more potently than N74D latency. These results indicated that latency reversal is position-dependent and can be selectively targeted by epigenetic modifiers.

To identify UISs functionally relevant for latency, we stimulated latently infected cells (WT and N74D) serially by TCR engagement followed by inhibition of HDAC6 and at each step we sorted cells into GFP+ (reversible latency) and GFP- (deep latency) cell populations. Integration site analysis on these cell populations showed that, in deeply latent cells, WT UISs were more frequent in B1 and B2 compartments, and were further away from genes and closer to LADs. Remarkably, in deeply latent cells, N74D UISs showed the opposite trend, such that the distribution of N74D and WT UIS became similar after serial sorting. We propose that deep latency may be linked to a particular integration site distribution, which eventually uncouples CD4 T cell activation from HIV-1 latency reversal.

Retroviral DNA Integration: Target Site Selection and Genomic Consequences

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DNA integration is a required step in HIV replication. Our previous work, together with collaborators, helped establish that HIV integrase protein catalyzes the initial steps in the integration reaction. In later studies, we showed that HIV favors integration in active transcription units. In subsequent collaborative studies, we reported that targeting is mediated in part by integrase binding to the cellular factor LEDGF p75, and further binding of additional factors to HIV CA protein. In recent years we have worked with many groups to analyze the consequences of integration of retrovirus-based vectors, which today are commonly used to introduce new genes into humans. Some of our recent work has focused on understanding the consequences of integration targeting in specific host genes, which in some cases has altered cell growth and affected health outcomes. In fact, today, there is evidence for four different mechanisms by which integration of retroviral DNA in humans can alter the activity of a gene at the integration acceptor site and promote cell proliferation: enhancer insertion, promoter insertion, gene inactivation, and 3' end substitution. Some of the data specify cellular factors that can be modulated to promote cell proliferation, which is commonly a limiting step in lentiviral-vector mediated CART therapy. Currently we are using studies of integration site distributions to identify genes and pathways modulating CART cell proliferation in treated subjects, with the long term goal of carrying out combinatorial screens for enhancing factors in CART-treated cancer patients. These and other results from recent studies will be discussed.

HTLV-1 integration site: impact on viral persistence and host chromatin structure and expression

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It is widely believed that human retroviruses persist in the reservoir in vivo largely by maintaining transcriptional latency. We compared >160,000 HTLV-1 integration sites (>40,000 HIV-1 sites) from T cells isolated ex vivo from naturally-infected individuals with >230,000 HTLV-1 integration sites (>65,000 HIV-1 sites) from in vitro infection, to identify genomic features that determine selective clonal survival (1). Remarkably, long-term survival of HTLV-1-infected clones in vivo was strongly correlated with the spatial position of the provirus: its radial position in the nucleus, and its genomic distance from the centromere; transcriptional latency was also correlated with survival, but was statistically independent of the spatial factors. These three factors combined explained over 40% of the observed selective clonal survival of HTLV-1 in vivo. Unexpectedly, in vivo clonal survival of HIV-1 was correlated with these spatial factors, but transcription intensity was not significantly correlated. We propose that the human retroviruses exploit some fundamental, unidentified features of the spatial distribution of the genome in the nucleus.

We previously showed that the HTLV-1 provirus causes aberrant transcription in the flanking host genome and, through binding the chromatin architectural protein CTCF, forms abnormal chromatin loops with the host genome. However, it remained unknown whether these effects were exerted simply by the presence of the provirus or were induced by its transcription. We show here (2) that proviral plus-strand transcription induces aberrant transcription and splicing in the flanking genome, but suppresses aberrant chromatin loop formation with the nearby host chromatin. Treatment with an inhibitor of transcriptional elongation allowed recovery of chromatin loops in the plus-strand-expressing population. We conclude that aberrant host transcription induced by proviral expression causes reversible disruption of chromatin looping in the vicinity of the provirus.

CPSF6 Liquid-Liquid Phase Separation Determines Higher-Order Capsid Binding, Nuclear Core Incursion, and HIV Integration Targeting

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HIV infection induces nuclear CPSF6 puncta that colocalize with nuclear speckles (NS) and integration occurs preferentially in speckle-associated domains (SPADs) of human DNA. Although these phenomena strictly depend on the known capsid–CPSF6 interaction, potential roles of additional CPSF6 effector functions are unknown. CPSF6 harbors an N-terminal RRM domain that mediates binding to its polyadenylation partner CPSF5, followed by two low complexity regions: a prion-like domain (PrLD) that mediates capsid binding and a C-terminal mixed-charge domain (MCD) that binds TNPO3. MCD deletion restricts HIV infection due to premature engagement of mis-localized CPSF6 in the cell cytosol. We have mutated the RRM and MCD (the PrLD was not targeted due to its role in capsid binding) and assessed HIV restriction, CPSF6 puncta formation, viral nuclear penetration distance, and integration site targeting. Due to the requirement of the MCD in CPSF6 nuclear import, a heterologous cMyc nuclear localization signal was also tested with these constructs. Unexpectedly, deletion of the MCD ablated SPAD-proximal integration, puncta formation, and nuclear HIV incursion, whereas RRM deletion partially reduced these metrics. Thus, MCD deletion seemingly phenocopied loss of capsid binding. To dissect MCD functionality, we tested missense mutants known to ablate TNPO3 binding and substituted heterologous TNPO3 (MCD or RS) binding domains. Missense mutants partially supported SPAD-proximal integration, puncta formation, and nuclear HIV incursion, while RS domains were non-functional. The synthetic RD50 MCD failed to support CPSF6 nuclear import yet promoted effective nuclear functionality in the presence of cMyc. Both the CPSF6 MCD and RD50 are known to display liquid-liquid phase separation (LLPS) activity in vitro, and we have developed a robust bacterial expression and purification system to assess full-length (FL) CPSF6 functionalities. FL CPSF6 displayed robust LLPS activity while delta-MCD CPSF6 displayed residual activity. Capsid binding was measured by nanotube spin-through and single viral particle staining assays. Whereas FL and delta-MCD CPSF6 displayed similar capsid binding affinities, FL displayed a second, unsaturable binding phase. Because this second phase was inhibited by 1,6-hexanediol, we conclude that MCD LLPS activity determines high avidity capsid binding critical for nuclear HIV incursion, CPSF6 puncta formation, and SPAD integration targeting.

The chromatin landscape of the HIV provirus determines its transcriptional state. Implications for a functional block-and lock cure strategy

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A permanent cure remains the greatest challenge in HIV research. In order to reach this goal a profound understanding of the molecular mechanisms controlling HIV integration and transcription is needed. We will review recent advances in the field. The transcriptional co-activator lens epithelium-derived growth factor p75 (LEDGF/p75), tethers and targets the HIV integrase to regions of active transcription in the chromatin via an interaction with the epigenetic mark H3K36me2/3. This finding prompted us to propose a “block-and-lock” strategy to retarget HIV integration into deep latency. A decade ago we pioneered protein-protein interaction inhibitors for HIV and discovered LEDGINs. LEDGINs are small molecule inhibiting the interaction between the integrase binding domain (IBD) of LEDGF/p75 and HIV integrase. They modify integration site selection and therefore might be molecules with a “block-and-lock” mechanism of action. We will describe how LEDGINs may become part of future functional cure strategies. We will also explain recent data from our lab on the mechanism underlying the observed block-and-lock phenotype. The data reveal how the chromatin landscape of the integrated provirus affects its transcriptional state.

Divergent Populations of Infected Naïve and Memory CD4+ T Cell Clones in Children on ART

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Pediatric HIV remains a public health issue with 1.7 million children living with HIV worldwide. Although HIV primarily infects memory $CD4^+$ T cells, recent studies suggest that naïve $CD4^+$ T cells are a significant contributor to the HIV reservoir. Here, we characterized HIV persistence in naïve and memory $CD4^+$ T-cells in children on long-term ART. The cohort consisted of 8 children who initiated ART at a median of 4 weeks of age and had viremia suppressed on ART for a median of 8.5 years. PBMC were sorted into naïve ($CD45RO^-CD28^+CD27^+CD95^-CCR7^+CD45RA^+$) and memory ($CD45RO^+CD95^+$) $CD4^+$ T cells. Multiple displacement amplification (MDA) was used to amplify endpoint diluted proviruses from the sorted cells, and probe-based PCR methods were used to estimate the frequency of infection and the proportion of proviruses that were predicted to be intact. Integration site analysis (ISA) was performed on LTR+ MDA wells to characterize the population of integration sites in naïve vs. memory T cells. HIV-infected naïve cells were detected in all 8 children at a median of 37.5 infected cells/million (range 6-231), a mean of 11-fold lower than the infected memory $CD4^+$ T cells. Of 200 proviruses in naïve cells that were analyzed with primers and probes from the Intact Proviral Detection Assay (IPDA), only 8 were predicted to be intact. ISA identified 8 clones of infected naïve T cells, none of which harbored intact HIV proviruses and none of which matched infected memory T cell clones. In fact, in one child with the greatest depth of sampling (85 integration sites obtained from naïve cells and 174 from memory cells), no matching integration sites were detected across the subsets, including from the largest infected clones in each subset. Consistent with a previous finding that proviruses in the largest infected memory CD4+ T cell clones consist of a single LTR (solo LTRs), a majority of the HIV+ MDA wells in these children appeared to lack internal HIV sequences. Our findings demonstrate that infected naïve $CD4^+$ T cells can proliferate into cell clones, can have a distinct population of integration sites from infected memory cells, and may be comprised mostly of highly defective proviruses, including solo LTRs.

Chromosomal Integration sites as biomarkers of HIV-1 reservoir cell selection

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With more than 38 million people living with HIV-1 worldwide, developing a cure for HIV-1 remains a major global health priority. Lifelong persistence of HIV-1 is frequently attributed to a pool of stable, transcriptionally silent HIV-1 proviruses, which are unaffected by currently available antiretroviral therapy (ART) or host immune activity. Our recent work suggests a more dynamic evolution of the viral reservoir, primarily characterized by a longitudinal selection of genome-intact proviruses integrated in heterochromatin locations. These proviruses are frequently transcriptionally repressed, which likely protects them from host immune recognition and seems to confer a longitudinal selection advantage. Such selection mechanisms appear to be most obvious in persons with natural (drug-free) control of HIV-1, but are also detectable in individuals undergoing suppressive antiretroviral therapy for very long periods of time (>20 years). While the immunological mechanisms underlying this selection process are not clear at this point, recent data from our lab demonstrate that selection of proviruses can be accelerated through the pharmacological administration of latency reversing agents; for instance, we noted in a randomized controlled human clinical trial that during treatment with the histone deacetylase inhibitor Panobinostat, proviruses in proximity to H3K27ac marks were preferentially eliminated, while proviruses in greater distance to acetylated histones persisted. Selection of proviruses in deeper latency over time may act in favor of the host and could be regarded as a therapeutic objective for clinical studies aiming for HIV-1 cure. Moreover, longitudinal monitoring of HIV-1 integration sites may allow to unravel immune selection processed that remain masked when viral reservoir analysis is solely based on quantitative evaluations of intact proviral frequencies.

Anatomic Distribution of HIV-Infected Cells After Long Term Antiretroviral Therapy

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HIV persistence during combination antiretroviral therapy (cART) is the principal obstacle to cure in persons living with HIV (PWH). Mechanisms contributing to persistence including clonal expansion of infected cells in anatomical compartments are not well understood. Here we characterized the persistence of HIV-infected cells from autopsy donations of people undergoing autopsy after long term ART.

PWH enrolled in HIV protocols at the NIH Clinical Center with suppressed HIV RNA on long term ART consented to research autopsy underwent post-mortem examination after expiration. Levels of HIV provirus in tissues obtained at autopsy were quantified using a single copy DNA PCR, Intact Proviral DNA Analysis (IPDA), and multiplexed HIV-LTR/*gag* digital droplet PCR assays. *Gag* proviral populations (1.1 kb) were obtained by single genome sequencing (SGS) and analyzed by average pairwise distance (APD) as a measure of genetic diversity and phylogenetics. Integration sites were identified as previously described.

PWH underwent autopsy within 3-48 hours. HIV-infected cells were widely distributed in tissues; highest HIV-DNA levels in lymph node (240-410 copies/million cells), and lowest in brain (1-9 copies/million cells). Multiplexed LTR/*gag* quantification revealed diverse proviral populations structure with LTR:*gag* ratio ranging from 2.09-8.27 across tissues. IPDA noted extensive DNA shearing, exceeding 60-70% of total DNA, precluding the quantification of intact proviruses. Analyses of unique HIV sequences without hypermutations showed a low level of genetic diversity (APD=0.19%-0.9%) and intermingled proviral populations across tissues. Individual clones of HIV infected cells were detected in lymphoid and non-lymphoid tissues.

These data demonstrate that HIV proviruses are widely distributed in anatomic compartments with significant differential levels of identical, defective and hypermutated proviruses, suggesting a role forf local effects in shaping the proviral landscape.

Retrovirus integrase/integration/HIV-1 integrase inhibitors

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I was asked to present a brief summary of the past research accomplishments of scientists in the retrovirus integrase (IN) and integration fields. The discovery of IN (alias p32 DNA endonuclease) in alpha-retroviruses started out in 1978 and was quick to gain tempo with the emergence of HIV-1. Because of basic research discoveries studying IN and integration from different retroviruses, virologists soon recognized that HIV-1 IN would be a highly desirable third target for inhibitors besides the reverse transcriptase and protease. Several early p32 DNA endonuclease studies followed by subsequent identification of IN from other retroviruses, with highlights of the previous six International Retrovirus Integration meetings, will be presented.

Abstracts
of Poster Presentations

Investigating the Chromatin Landscape Effects on HIV-1 Latency

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Persistence of HIV-1 in latent reservoirs is a major obstacle that prevent its eradication and cure. Understanding the mechanisms governing HIV-1 latency is crucial for eliminating this viral reservoir. Recent evidence highlights the role of chromatin spatial folding in gene expression regulation. Although HIV-1 prefers integrating into active genes, the impact of chromatin spatial folding on HIV-1 gene expression and latency is not well understood. We mapped unique integration sites (UISs) for HIV-1 WT and capsid mutant (N74D), that has a different integration preference relative to WT, to previously published DamID and HiC data. We found that WT virus preferentially integrates into active topologically associated domains (TADs) associated with T-cell activation and metabolism while avoiding lamina associated domains (LADs). N74D virus exhibits the opposite pattern.

To investigate the impact of chromatin spatial folding, we examined changes during the transition of CD4 T cells from activated to quiescent states and vice versa. Jurkat cells were transduced with either WT or N74D virus vectors expressing GFP. GFP+ cells were sorted after 48 hours, and latency was established. Subsequent sort was done to separate GFP+ (active) and GFP- (latent) cells. We observed that WT HIV-1 exhibited slower silencing compared to N74D and could be more efficiently reactivated using various stimuli. These results led to the hypothesis that different levels of latency (poised, deep, and deeper) may exist, and integration site selection (WT/N74D) could influence the probability of falling into these latency types.

To address this hypothesis, latently infected cells (WT and N74D) were stimulated and sorted into reversible (GFP+) and deep (GFP-) latency populations. We then applied Linker mediated PCR (LMPCR) to identify UISs that are enriched in the latent cells at each sort. Integration site analysis revealed WT UISs' initial presence in A1 and A2 compartments and infrequency in B compartments. N74D UISs were more frequent in A2 and B3 compartments. WT UISs were also closer than N74D UISs to transcription start sites and more frequently intragenic. However, in deeply latent cells, WT UISs became more frequent in B1 and B2 compartments, were more frequently intergenic and closer to LADs. N74D UISs in deeply latent cells resembled WT UISs, indicating convergence of integration profiles. An analysis of the UISs in poised and deeply latent cells will be presented.

Structural studies on the selectivity of quinoline-based ALLINIs as promoters of HIV-1 integrase multimerization

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Allosteric inhibitors of HIV-1 integrase (IN) that bind to the LEDGF/p75 site, also known as ALLINIs or LEDGINs, interfere with the replication cycle of HIV-1 by binding to the catalytic core domain (CCD) of viral encoded IN. Though originally designed to compete with LEDGF/p75, several ALLINIs have instead been reported to interfere with viral maturation during the later stages of the integration process. In addition to inhibition due to direct binding at the CCD dimer interface, many ALLINIs possess moieties that project away from the CCD and are thought to pick up additional interactions from the C-terminal domain (CTD) subunit of an adjacent integrase protein, thereby inhibiting the viral lifecycle through induction of aberrant multimerization. Many of the commonly employed groups projected towards the incoming CTD domain are neutral, planar substituents including aryl and heteroaryl moieties. Though these moieties are typically believed to promote interaction with the CTD and enhance multimerization, further structural exploration is necessary to characterize these interactions and understand what groups are necessary for either promoting or preventing IN multimerization. In this study, we have synthesized a series of ALLINIs to explore this relationship. Two of these compounds, KF-255 and KF-257, which are adorned with piperazine and morpholine rings, respectively, have shown dramatically different abilities to promote multimerization, suggesting that the mechanism of IN inhibition can be controlled through careful manipulation of chemical structure and electronic properties. Interestingly, the selectivity in this case is due to a single atom replacement, potentially leading to an alternative mechanism of inhibition.

Structural basis for viral resistance to allosteric HIV-1 integrase inhibitor Pirmitegravir and rational development of an improved analog

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Allosteric integrase inhibitors (ALLINIs) are a new class of antiretroviral compounds which bind to the noncatalytic site of HIV-1 integrase (IN) and potently impair correct virion maturation by inducing aberrant IN multimerization. Pirmitegravir (PIR), a highly potent and safe pyrrolopyridine-based ALLINI developed by ST Pharm, is currently in clinical trials. Previous cell culture based viral breakthrough assays identified emergence of the Y99H/A128T IN variant that confers substantial resistance to the inhibitor. Here, our efforts have focused on elucidating structural and mechanistic bases for viral resistance to PIR with an aim to rationally modify the inhibitor to enhance its barrier to resistance. In cell culture-based assays HIV-1 (IN Y99H/A128T) conferred >150-fold resistance to PIR compared to the WT virus. Biochemical assays revealed that Y99H/A128T substitutions substantially (>60-fold) reduced PIR IC_{50} values for inhibiting IN:RNA but not IN:LEDGF/p75 binding. In turn, these results suggest that inhibition of IN:RNA binding during virion maturation is the primary target for PIR. High resolution structures obtained by X-ray crystallography and complementary surface plasmon resonance assays revealed that the Y99H/A128T changes do not affect PIR binding to IN catalytic core domain (CCD); instead, these IN substitutions introduced steric hindrance at the inhibitor mediated interface between CCD and C-terminal domain. Based on these findings we have developed an analog EKC110, which exhibited ~15-fold improved potency against HIV-$1_{(Y99H/A128T\ IN)}$ compared to the parental PIR. These findings serve as a compelling example for rationally modifying the parental ALLINI, and provide means for future development of improved second generation ALLINIs with a higher barrier to resistance for their potential clinical use.

Designer nucleosome substrates for probing retroviral integration

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Integration of viral DNA into the host target DNA establishes a permanent infection in host cells and is a hallmark of all retroviruses. The host target DNA is compacted into chromatin, a mixture of nucleic acids and proteins found in the nucleus of eukaryotic cells that helps to condense and organize genetic information. The nucleosome represents the core repeating unit of chromatin and constitutes 147 bp of DNA wrapped around a histone octamer. Nucleosomes are dynamic structures that can be assembled and disassembled by chaperones or chromatin remodelers and modified through post-translational modifications. The local nucleosomal environment imparted by these factors plays an important role in establishing a successful integration event. We are interested in understanding the mechanistic bases for how retroviruses insert their viral DNA into diverse nucleosome substrates. To achieve this objective, we have adapted and developed protocols for generating and purifying designer nucleosomes. These nucleosomes can be varied by their DNA sequence, histone content and subtype, and the presence of post-translational modifications. The strategies used to generate various nucleosomal substrates will be presented and discussed. We are currently exploring how two distinct viruses – the prototype foamy virus (PFV) and HIV-1 – target actively transcribing regions of chromatin for integration. Preliminary results with these two systems will be presented. A longer-term goal for the customized workflows presented here is to clarify how local charge distributions, posttranslational modifications, transcription intermediates, and the proximity of neighboring nucleosomes influence PFV and HIV-1 integration. Using these systems, we aim to elucidate the mechanistic bases by which retroviruses select specific regions of chromatin for integration. Our findings will provide insights into this key step of the viral replication cycle and may assist with the development of novel therapies that inhibit integration into chromatin.

Optimization of affinity reagents for purification and detection of HIV1 pre-integration complexes

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High-resolution structures of *in vitro* reconstituted intasomes have been determined from five retroviral genera, including *Lentivirus*. Yet, the intasome represents only a small part of the PIC: while IN footprints just ~20 bp at each viral DNA end within an intasome, a PIC contains a much larger protein assembly that spans ~200 bp. The specific components and the structural organization of the PIC remain completely open questions. Because only one PIC is produced during a successful infection event, structural characterization of this critical nucleoprotein assembly presents formidable technical challenges. The PIC needs to be purified away from cellular components that are present in cell extracts at colossal excess or labeled for detection in its natural, highly packed environment. By revisiting studies of PICs generated using replication-competent HIV-1, we have been able to isolate and purify active PICs from the nuclei of cells transduced with single-cycle HIV-1 based vectors. To optimize purification of the PICs, it was critical to establish a real-time quantitative PCR assay to directly quantify integration junctions with minimal material, rather than using a nested PCR approach. Having screened an extensive panel of monoclonal antibodies and nanobodies against HIV-1 IN and matrix protein, we identified reagents that can pulldown PICs, while maintaining robust strand transfer activity. In addition, we found that the host cofactor LEDGF/p75 was very effective at pulling down active HIV-1 PICs, which can be subsequently eluted from the affinity beads. Further, we have synthesized integrase strand transfer inhibitors (INSTIs) with a biotin tether that maintains robust inhibitory potency in strand transfer reactions, similar to the parental compound. Because INSTIs recognize the IN active site only within an intasome, these reagents specifically pull-down functional HIV-1 PICs. To validate these reagents, the limits of single-molecule total internal reflection microscopy (TIRF) to detect molecules with minimal material have been determined – laying the foundation for biochemical and structural characterization of HIV-1 PICs.

Probing HIV-1 integration using designer nucleosomes

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The integration of HIV-1 viral DNA (vDNA) into host chromatin is essential for viral replication. This critical step is mediated by the intasome, a nucleoprotein assembly of Integrase (IN) multimers bound to the linear vDNA ends. In vivo, HIV integration is facilitated by the host factor protein Lens Epithelium Derived Growth Factor (LEDGF). LEDGF recognizes H3K36me3 posttranslational modifications on nucleosomes through its PWWP domain, and a structure containing LEDGF and a mononucleosome containing the methyl-lysine mimic (H3K36Cme3) has been previously reported. LEDGF also binds HIV IN through its Integrase Binding Domain (IBD). We accordingly hypothesize that LEDGF directs integration into chromosomal DNA through a tethering interaction that leads to the formation of a ternary complex composed of nucleosomes containing the H3K36me3 modification, LEDGF, and HIV-1 intasomes. In our lab, we have made different types of mononucleosomes with varying DNA linker lengths in order to identify an optimal substrate for HIV-1 integration. Our preliminary biochemical and sequencing data suggest that HIV integration may be directed toward a superhelical position on the nucleosome near the entry/exit site, whereas the bulk of the integration events are directed into free linker DNA. We are currently expanding our efforts in three directions: (1) we are developing a strategy to install the native H3K36me3 mark and incorporate these modified histones into nucleosomes; (2) we are developing a dinucleosome substrate to understand integration preferences into the minimal substrate for “beads-on-a-string” chromatin; (3) we are working to identify an optimal chromatin substrate to enable structural biology studies of the ternary assemblies. Our efforts are aimed at dissecting the molecular and structural bases of HIV integration into chromatin and should yield a mechanistic understanding of this key step in the viral replication cycle.

Nucleosome chaperone activity of LEDGF and HDGF2 characterized with single molecule force spectroscopy

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The nucleosome is the fundamental structure of DNA organization in eukaryotic cells. It consists of two loops of DNA wrapped around a histone octamer. Lens epithelium-derived growth factor (LEDGF) and hepatoma-derived growth factor 2 (HDGF2) are recently discovered proteins that facilitate transcription without ATP hydrolysis, suggesting roles as nucleosome chaperones during the integration of lentiviral DNA, including HIV-1 DNA, into infected cell genomes. Both proteins have methyl-lysine reading PWWP domains that can bind to biologically relevant histone H3 containing epigenetic H3K36me3 marks. To investigate the roles of LEDGF and HDGF2 in nucleosome engagement and structure modulation, we used optical tweezers to stretch reconstituted DNA nucleosome arrays formed on twelve Widom 601 positioning sequences with unmodified (WT) and methylated histones (mimicking H3K36me3 modifications) with of LEDGF and HDGF2. Furthermore, to test chaperone function during HIV-1 DNA integration, we exposed modified nucleosomes to HIV-1 integrase (IN) together with equal concentrations of LEDGF and HDGF2. Analysis of force-extension data showed that, while some destabilization of the inner wrapping of nucleosomes occurs due to histone methylation, exposure to chaperones results in further destabilization. LEDGF may destabilize the whole histone as seen by the lowered release forces for both outer and inner wrap. Interestingly, there may be a higher incidence of cooperative nucleosome openings in the presence of LEDGF. HDGF2 may be more specific to dimer sites, leading to more disruption to the outer wrap. This effect is more pronounced when HDGF2 acts together with IN. Kinetic analysis of histone-DNA interactions in the presence of these chaperones via survival probability and confocal fluorescence measurements indicates that chaperones not only facilitate nucleosome unwrapping but also moderately promote nucleosome reassembly. Though neither protein is as efficient as the well-known chaperone FACT (facilitates chromatin transcription), LEDGF is more effective at reassembly of wild type nucleosomes at low protein concentrations and HDGF2 is more effective at high concentrations. Confocal fluorescence image tracking reveals rapid release of dimers from nucleosomes in the presence of LEDGF and HDGF2 after disruption. These results offer insight into the functions of LEDGF and HDGF2 as nucleosome chaperone proteins and their effect on IN binding to the nucleosome array.

Cell culture evaluation hints first-line HIV medicines are primed for success if repurposed for HTLV-1 prevention

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With an estimated 10 million people infected, human T-cell lymphotropic virus type 1 (HTLV-1) is the second most prevalent pathogenic retrovirus in humans after HIV-1. Like HIV-1, HTLV-1 overwhelmingly persists in a host via a reservoir of latently infected $CD4^+$ T cells. Although most patients are asymptomatic, HTLV-1-associated pathologies are often debilitating and include childhood infective dermatitis and adult T-cell leukaemia/lymphoma (ATLL), which presents in mature adulthood and is associated with poor prognosis with short overall survival despite treatment. Curiously, the strongest indicator for the development of ATLL is the acquisition of HTLV-1 through breastfeeding. As it stands, there is no therapeutic or preventative regimen for HTLV-1. However, antiretrovirals (ARVs) have been developed for and revolutionised HIV treatment, and the conserved mechanistic properties of retroviral polynucleotide-processing enzymes mean some HIV-1 drugs have the potential to treat or prevent HTLV-1. We previously reported that both first- and second-generation integrase strand transfer inhibitors (INSTIs), including raltegravir and bictegravir, and the nucleoside reverse transcriptase inhibitor (NRTI) formulation tenofovir disoproxil fumarate (TDF), effectively blocked intercellular transmission of HTLV-1 in cell culture. In this study, we report that dolutegravir, the INSTI currently recommended as the foundation in all first-line combination therapy by the World Health Organisation, also potently inhibits HTLV-1 infection in cell culture. We also show that the latest formulation of tenofovir, tenofovir alafenamide (TAF), is as effective as TDF in blocking transmission. Our results, if replicated in a clinical setting, suggest vertical transmission rates of HTLV-1, and future caseloads of ATLL, could be dramatically cut by the appropriation of already widely available HIV pills. Considering this work with the old medical adage ‘it’s better to prevent than cure’, we are calling for the inclusion of INSTIs and NRTIs in upcoming HTLV-1 clinical trials.

Discovery and development of novel pyrrolopyridine derivatives as a highly potent and safe allosteric HIV-1 integrase inhibitor

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Integrase (IN) plays an essential role in HIV-1 replication and represented as an important and unique target for treatment of human immunodeficiency virus (HIV) infection since there is no human homolog of IN. In particular, allosteric HIV-1 integrase inhibitors (ALLINIs) are a new class of HIV-1 inhibitors that targets HIV-1 maturation process.
Herein, we introduce the pyrrolopyridine derivatives, which binds to the host LEDGF/p75 protein binding pocket of the IN dimer and showed high antiviral potency and safety in HIV-1. A novel pyrrolopyridine derivative, Pirmitegravir (PIR or STP0404), has demonstrated its druggability through physicochemical and its excellent ADME properties in both preclinical animal studies and recently completed phase 1 study. Phase 2a clinical study of PIR is now on-going and will be completed by Q1 2024.

ALLINI Sensitivity Genetically Maps to Residues within HIV-1 Integrase C-terminal Domain

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With its multifunctional roles in HIV-1 replication, HIV-1 Integrase (IN) is one of the most promising candidates for developing alternative anti-HIV drugs. Besides catalyzing the integration of the viral genomic RNA (gRNA) into host DNA, IN binds to the gRNA in mature virions to facilitate the proper encapsulation of the viral ribonucleoproteins (vRNPs) inside the mature CA lattice. Allosteric integrase inhibitors (ALLINIs) are an emerging class of drugs that inhibit this second function of IN. ALLINIs compete with LEDGF binding to IN by engaging the V-shaped binding pocket created by the catalytic core domain of two IN dimers. Based on ALLINI-IN structures, ALLINIs also engage the C-terminal domain (CTD) of a nearby dimer resulting in the formation of open IN oligomers. It is the formation of these oligomers that is thought to prevent IN binding to the gRNA resulting in the information of virions with eccentric morphology. In contrast, Class II mutations within IN-CTD (i.e., R269A/K273A) inhibit IN- gRNA binding without altering IN oligomerization in virions and in vitro. We have recently published that this defect can be reversed entirely upon charge reversal substitution at the nearby D278 residue.
Genetic evidence for the involvement of CTD of IN in the ALLINI mechanism of action is lacking. Based on structural studies, the amino acids residues Y226, W235, K266, and I268 within the CTD contact with ALLINI-bound CCD. However, we demonstrate that mutation of these residues results in class II phenotype in the absence of ALLINIs making it difficult to assign a role for CTD in the ALLINI mechanism of action genetically. Here we demonstrate that a class II IN mutant bearing a compensatory substitution (R269A/K273A/D278R) is significantly more sensitive to ALLINIs. This sensitivity maps to R269A/K273A substitutions as a similar IN mutant, R262A/R263A/D256R does not display increased sensitivity to ALLINIs. We will present these, and ongoing studies aimed at determining the role of aberrant IN multimerization in ALLINI sensitivity. Collectively, our studies present the first genetic evidence highlighting the role of IN-CTD in the ALLINI mechanism of action.

Intasomes assembled with full-length HIV-1 integrase and CTD facilitate structural studies by cryo-EM and reveal the role of the integrase C-terminal tail

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Retroviral DNA integration is mediated by nucleoprotein complexes referred to as intasomes, in which a pair of viral DNA ends are bridged by a multimer of integrase (IN). Atomic-resolution structures of HIV-1 intasomes provide detailed insights into the mechanism of integration and inhibition by integrase strand transfer inhibitors (INSTIs). Currently, structures of HIV-1 intasomes have been facilitated by HIV-1 IN with a Sso7d domain fused to the N-terminus. Sso7d-IN is much more soluble in solution than wild-type IN and has been critical for structural studies of HIV-1 intasomes. However, intasomes assembled with full-length Sso7d-IN are highly heterogeneous and have the tendency to form intasome stacks, which is one of the limiting factors in determining high-resolution cryo-EM maps. We have assembled HIV-1 intasomes by mixing of full-length Integrase with the isolated CTD domain. CTDs are readily incorporated into the intasomes and the purified intasomes are much less heterogenous and exhibit minimal tendency to form stacks. The intasome particles are well dispersed on EM grids during cryo plunge freezing, which improves the efficiency and accuracy of the data processing. The resolution of cryo-EM map was improved to 2.0 Å, and this approach will greatly facilitate structural studies of mechanisms of INSTIs resistance.

The C-terminal tail 18 residues (271-288) of HIV-1 integrase is critical for virus replication and integration *in vitro*, but is not resolved in any previous structures of HIV integrase or intasomes and its function has remained unclear. We show that the C-terminal tail participates in intasome assembly and resides within the intasome core. It forms numerous interactions with other integrase protomers and likely plays a key role in stabilizing the intasome structure.

The Nonclinical & Clinical Development of a Novel Potent HIV-1 Allosteric Integrase Inhibitor, Pirmitegravir

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Pirmitegravir (PIR, also known as STP0404) is a first-in-class HIV-1 allosteric integrase inhibitor (ALLINI) with a novel mechanism of action. It binds to the LEDGF/p75 binding site of integrase (IN) and inhibits viral maturation.
PIR has shown potent *in vitro* anti-HIV-1 activities, a favorable nonclinical pharmacokinetics (PK), safety pharmacology and toxicology profiles. It also demonstrated a distinctive *in vitro* resistance profile from other catalytic-site integrase inhibitors (CINIs, also known as integrase strand transfer inhibitor, INSTI) and it raised no concern in cross-resistance.
The first-in-human phase 1 study of PIR was a double-blinded, placebo-controlled, randomized phase 1 trial in healthy male adult volunteers with once daily oral dose. PIR was very well tolerated in the tested population and has presented a favorable PK profile over the tested dose range. The average C_{trough} for 200 mg dose group gave out a minimum 700-fold therapeutic range when compared to the *in vitro* efficacy concentration.
Based on our successful phase 1 study, PIR is now moving forward with its phase 2a proof-of-concept clinical study targeting HIV-treatment-naïve participants living with HIV-1. We received IND clearance for phase 2a trial by US FDA IND in 2022 and is currently in process of patient recruitment.

Beyond the Consensus: Unraveling Diverse DNA Motifs in Retroviral Integration Sites

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The targeting of retroviral integration is governed by a complex interplay between virus-encoded proteins, target DNA (tDNA), and cellular factors. The processes governing the integration targeting eventually manifest as detectable patterns in proviral genomic distribution. Genome-wide analysis of tDNA sequences has revealed a subtle yet consistent palindromic consensus, implying the existence of intrinsic integrase preferences for the tDNA nucleotide composition. The prevailing model posits that most tDNA sequences lack palindromic characteristics and that the palindromic motif emerges in sequence alignment as the overlap of non-palindromic reverse-complementary sub-motifs. While this model provides a plausible explanation for the formation of palindromic motifs from non-palindromic tDNA sequences, it may still oversimplify the complexity of the underlying selection processes.

In our work, we describe an in-depth investigation of tDNA sequence composition utilizing advanced statistical modeling. Employing multicomponent mixture models, we discerned several sub-groups of patterns including nucleotide-content-specific patterns and patterns specific for integration into deproteinized tDNA. Most importantly, we observed patterns consistent with the co-existence of palindromic and non-palindromic sub-motifs within tDNA datasets. These findings were corroborated by quantitative analyses, which confirmed contrasting selection processes acting on individual positions at tDNA. Moreover, the model identified a palindromic hotspot of HIV-1 integration that locates to *Alu* repeats, is present consistently once in 175 tDNA sequences, and its targeting is altered in integrase mutants. Consequently, we suggest that such a hotspot could serve as a distinctive marker for HIV-1 wild-type integrase target selection within *in cellulo* datasets.

Collectively, our findings challenge both a uniform palindromic consensus and the prevailing notion of a single non-palindromic motif as simplistic representations of forces governing the tDNA selection. We provide arguments for diverse sequence preferences, as reflected in the motif footprints, highlighting the nuanced interplay between retroviral integrase and tDNA. Our study underscores the power of advanced statistical approaches in deciphering complex biological processes, showcasing the significance of sequence composition in retroviral target site selection.

Suppression of HIV-1 reactivation from latently infected CD4+ T cells by pyrrolopyridine-based allosteric integrase inhibitors

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HIV-1 integrase (IN) dimers bind to host LEDGF/p75 protein during viral integration. Transcription activator LEDGF/p75 tethers the IN-proviral DNA complex (pre-integration complex) for integration specifically to transcriptionally active regions of the chromosomes. New Allosteric IN (ALLINI) class inhibitors target non-catalytic sites of HIV-1 IN; LEDGINs are ALLINI compounds that can also inhibit IN binding to LEDGF/p75 are reported to interrupt the integration site preference of HIV-1. The Debyser group reported LEDGINs CX014442 and GS-9822 retarget HIV-1 integration site profiles to transcriptionally non-active regions of the chromosomes which reduced viral transcription activation from latently infected HIV-1 CD4+ T cell reservoirs. LEDGINs indicate potential utility to reduce HIV-1 reactivation from latently infected T cell reservoirs as a “Block and Lock” strategy, yet no ALLINI /LEDGIN compounds have been clinically developed. Recently, we characterized antiviral efficacy and MOA of a pyrrolopyridine based ALLINI STP0404. Based on outstanding preclinical efficacy, animal safety, and phase 1 trial data STP0404 became the first-in-human ALLINI. We previously reported STP0404 binds the LEDGF/p75-binding site of IN dimer and blocks IN-LEDGF/p75 binding with ~190nM IC_{50}. Moreover, we developed the pyrrolopyridine ALLINI derivative, EKC110, which inhibits the Y99H/A128T STP0404-resistant mutant virus and possibly elevates its genetic barrier, compared to parental STP0404. Here, we report STP0404 suppresses HIV-1 reactivation from latently infected CD4+ T cells in three different reactivation conditions, 1) IL15, 2) PMA/Ionomycin, and 3) latency reversing agents. Next, we examined STP0404 anti-reactivation activity from CD4+ T cells latently infected with Y99H/A128T mutant HIV-1. Also, we tested whether the second-generation pyrrolopyridine, EKC110, suppresses HIV-1 reactivation from the CD4+ T cells latently infected with wild type virus or Y99H/128T STP0404 resistant virus. HIV-1 reactivation was quantified by p24 ELISA for produced viruses as well as vRNA/vDNA copy numbers in the reactivated cells. Collectively, this study supports the use of our pyrrolopyridine ALLINIs as “Block and Lock” agents to reduce HIV-1 reactivation from latently infected T cell reservoirs.

Data driven computational analysis of ALLINIs at the CCD-CTD interface

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Allosteric HIV-1 integrase inhibitors (ALLINIs) are small molecules that disrupt the viral lifecycle by inducing aberrant integrase (IN) multimerization and eliciting eccentric viral morphologies in which the viral genome is localized outside the capsid. Here, we perform molecular dynamics simulations of IN in complex with four ALLINIs: BID, Pirmitegravir (PIR), gs9822 and EKC110 to study the role of these molecules in IN multimerization. We find that the ALLINIs work as a “molecular glue” that induces and stabilizes an interface between the IN catalytic ore domain (CCD) and the carboxyl terminal domain (CTD) via electrostatic and hydrophobic interactions with conserved residues in the CTD and CCD domains of IN. All ALLINIs share the same chemical design principles: an aromatic scaffold with tertbutoxyl and carboxylate sidechains and a bulky hydrophobic group; however, small changes in these moieties lead to increased potency against HIV-1 mutants. Thus, we perform free energy perturbation (FEP) calculations in silico mutation simulations, to estimate the energetic favorability of interactions with residues in the CCD-CTD interface to different ALLINIs. Overall, our computational pipeline allows the study of ALLINI induced IN condensation and provides insights for the rational development of this series of antiretrovirals through the optimization of their key contacts with the viral target.

Characterization of HIV-1 Vpr interactions with double stranded DNA and nucleosome-DNA arrays by optical tweezers and atomic force microscopy

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In the attempt to understand physical nature of the multiple roles of the HIV-1 Vpr protein reported for the HIV-1 life cycle, including pro-viral DNA integration and chromatin formation, as well as its role in the viral and cellular gene expression, we study the direct interaction of purified Vpr with polymeric double stranded (ds) DNA and also with an array of twelve nucleosomes assembled on dsDNA with positioning sequences *in vitro*. We use a Lumicks correlated optical tweezers and fluorescence instrument and an atomic force microscope (AFM) to measure Vpr filament formation on dsDNA accompanied by dsDNA rigidification. Measurements on dsDNA allows us to characterize the Vpr/dsDNA complex persistence length, binding strength, and cooperativity. Measurements on nucleosome arrays show that Vpr is able to significantly destabilize the nucleosomes by unwinding their outer turn and strongly reducing histone octamer/dsDNA interactions within the nucleosome strong sites. In contrast to the nucleosome chaperone complexes, like FACT, which facilitate both nucleosome disassembly and re-assembly, thereby promoting the transcription through nucleosomes, Vpr leads to the nucleosome destabilization and displacement followed by irreversible histone loss upon mechanical, and likely transcriptional, nucleosome unwinding. Thus, Vpr activity is characterized primarily by nucleosome destabilization and removal rather than the nucleosome chaperone activity, which may contribute to pro-viral DNA transcription activity.

Two new structures of HIV Integrase assemblies solved using cryo-EM

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HIV Integrase (IN) plays multiple roles in the viral replication cycle. In the early stages, IN catalyzes the insertion of the viral DNA into host target DNA, a process that is mediated through the formation of a large nucleoprotein assembly called an intasome, which contains up to sixteen IN protomers bound to a pair of viral DNA ends. In the late stages, IN binds the viral RNA and facilitates the proper packaging of viral ribonucleoprotein complexes inside the capsid lattice, a process that is mediated by tetrameric IN. Here, we report two new structures of IN assemblies, solved using cryo-EM. The first structure is a tetramer of IN in its apo form, which is refined to a nominal resolution of ~3.5 Å. The second structure is the fully-formed intasome hexadecamer, which is refined to a resolution that ranges between ~4-10 Å for the different regions of the complex. We describe the methods employed to derive both structures. For the IN tetramer, we used the IN binding domain (IBD) of LEDGF to stabilize the complex and employed tilted data collection strategies to address the pathological orientation bias of the vitrified complex on cryo-EM grids. We also employed multiple different cryo-EM processing packages to iteratively improve the resolution for the reconstructed map, leading to an atomic model of full-length tetrameric IN. For the hexadecameric intasome, we used crosslinking to stabilize the intasome prior to data collection. For cryo-EM, we employed density subtraction, and iterative global and focused classification to improve the quality of the map in the most heterogeneous regions of the assembly. This allowed us to derive a complete atomic model of the intasome hexadecamer through rigid-body docking. Comparisons of the atomic models for the IN protomers within these assemblies, and across other INs and intasomes, highlight the structural plasticity of IN and how the protein's modular scaffold allows it to assemble into numerous oligomeric forms.

High-resolution co-crystal structures reveal ALLINI-induced interface responsible for HIV-1 IN hypermultimerization and loss of function

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Allosteric HIV-1 integrase (IN) inhibitors (ALLINIs) are an emerging class of small molecules that disrupt viral maturation. The compounds act as molecular glues to promote formation of an unnatural IN-IN interface involving the catalytic core domain (CCD) dimer of one IN multimer and the C-terminal domain of a second IN multimer. Their unusual mode of action results in hypermultimerization and aggregation of HIV-1 IN, making ALLINIs very challenging targets for co-crystallography.

To visualize the critical drug-induced protein-protein interface, we designed an HIV-1 IN construct harbouring a monomerized CTD flexibly linked to the CCD. The strategy allowed us to co-crystallize the protein construct with eight ALLINIs and refine X-ray structures to 1.7 - 2.4 Å resolution. The ligand series included the current clinical leads, Pirmitegravir (STP0404; developed by ST Pharm, South Korea) and BDM-2 (Biodim, France), as well as the benchmark ALLINI BI-D, which feature pyrrolopyridine, benzene, quinoline scaffolds, respectively.

The co-crystal structures revealed atomistic details of the ALLINI-induced interface involving the pair of HIV-1 IN domains. Protruding from their principal binding pocket on the IN CCD dimer, the compounds recruit a triad of HIV-1 IN CTD residues, Tyr226, Trp235, and Lys266, to nucleate the pathological CTD-CCD interaction. The three CTD residues are highly conserved within *Lentivirus* genus and, importantly, are invariant among circulating HIV-1 strains. The drug-induced interface involves the CTD SH3-like fold and extends to the beginning of the IN carboxyl-terminal tail region, burying a total surface area of ~1,200 Å. Comparison of the individual structures revealed that the binding pose of the CTD on the CCD dimer can adapt to accommodate diverse ALLINI molecules. We show that mutations of HIV-1 IN CTD residues that participate in the interface with the CCD greatly reduce the IN-aggregation properties of Pirmitegravir. Our results explain the mechanism of the ALLINI-induced condensation of HIV-1 IN and provide a reliable template for the rational development of this series of antiretrovirals through structure-based optimization of their key contacts with the viral target.

HIV-1 Preferentially Targets Genes Regulated by PAF-1 and U2 snRNP for Integration

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Pre-mRNA splicing is coupled with promoter-proximal Pol II pausing and alternative polyadenylation (APA). Splicing inhibitors increase pausing and the use of proximal polyadenylation sites (PAS) in intron rich genes by impairing P-TEFb recruitment, which is a core component of the super elongation complex (SEC). The CFIm complex consisting of CPSF6 and CPSF5 also regulates APA by promoting the use of distal PAS. CPSF6 binds viral capsid (CA) to license HIV-1 intranuclear trafficking and integration targeting. Previously, we showed that HIV-1 preferentially integrates into intron rich, Pol II-paused genes. Based on the interconnections between splicing, pausing, and APA, we hypothesized that APA might play a role in HIV-1 integration targeting. Indeed, in Jurat T cells, APA genes regulated by U2 snRNP contained 24% of integration sites (3x compared to RIC or random integration control; p<1E-5). In contrast, nonregulated genes were targeted similarly to all genes (p< 0.2). Further, paused genes regulated by PAF-1, which is also important for APA and for post-integration viral expression, were preferentially targeted (3.5x RIC; p<1E-5), whereas the reciprocal gene set was preferentially avoided (p<1E-5). To test the role of splicing, we infected Jurkat T cells in the presence of the U2 snRNP inhibitor Pladenolide B (Plad B) or the SEC inhibitor KL-2. Plad B significantly reduced genic integration in PAF-1 paused genes but not in unpaused genes. We defined chromosomes with reduced genic integrations (p<1E-04) as Plad B sensitive chromosomes (PBSC) and the remaining chromosomes as Plad B insensitive chromosomes (PBIC; p<0.02)). KL-2 reduced genic integration significantly for PBSC but not for PBIC, suggesting that splicing targets HIV-1 integration into genes regulated by P-TEFb/SEC. To test the roles of integration targeting cofactors, we mapped sites for CPSF6-defective CA mutant viruses or wild type (WT) HIV-1 in LEDGF/p75 knockout (LKO) cells. PBSC supported significantly less genic integration for CA mutants and for WT virus in LKO cells (p<1E-7). However, while PBIC were significantly less targeted by WT virus in LKO cells, these genes were significantly more targeted by CA mutants (p<1E-7 for both comparisons). Thus, the CPSF6-CA interaction is critical for preferential HIV-1 integration targeting of paused genes and APA genes regulated through P-TEFb/SEC.

Comparative analyses of the efficacies of the second-generation INSTIs and 4d against a panel of IN quadruple mutants with mutations at IN positions L74, E92, or T97 combined with E138A/K +G140S + Q148H

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Integrase strand transfer inhibitors (INSTIs) have emerged as leading therapeutics both to treat HIV-1 infections and to reduce HIV-1 transmission when used in pre-exposure prophylaxis (PrEP). The FDA-approved second-generation INSTIs, dolutegravir (DTG), bictegravir (BIC) and cabotegravir (CAB) are prescribed in combination with other classes of antiretroviral drugs (cART). The development of resistance to second generation INSTIs has been relatively infrequent, particularly if these drugs are used in combination therapies in people living with HIV-1 (PLWH) who are drug naive. Although the resistance pathways to second generation INSTIs are still being defined, it appears that resistance can develop along at least four distinct pathways: G118R, G140A/S + Q148H/K/R, N155H, and R263K. An important aspect of our research has been directed at overcoming resistance associated with the clinically important G140A/S + Q148H/K/R pathway. Additional mutations, described in previous clinical studies, can arise at IN positions L74, E92, T97, and E138. We have extended our previous studies by measuring, in a single round infection assay, the EC_{50} values of our leading compound, **4d**, and the second-generation INSTIs against a panel of eight IN quadruple mutants that have mutations at positions E138A/K + G140S/Q148H and fourth mutation either at IN positions L74, E92, or T97. All four INSTIs failed to retain potency against all the mutants in this panel of eight IN quadruple mutants. However, **4d** and **BIC** retained greater potency against the mutants we tested than either **DTG** or **CAB**. In particular **CAB**, which is important in long-acting formulations, was largely ineffective against this panel of IN quadruple mutants. When compared to **BIC, 4d** had an improved antiviral profile against the four IN quadruple mutants in the L74I/M + E138A/K +G140S + Q148H group. With one exception, the infectivity of the quadruple mutants was >50% of WT in our one-round assay. However, when we generated and tested a related set of quintuple mutants, none of the mutants we tested showed detectable infectivity. The ability of **4d** to retain considerable antiviral potency against complex IN mutants is encouraging and the compound is currently undergoing preclinical testing.

Comparative biochemical and single molecule imaging analysis of octameric MMTV and tetrameric PFV intasome dynamics

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A multimeric complex of retroviral integrase bound to cDNA ends mediates integration, an essential step in viral replication. Purified integrase combined with DNA oligomers mimicking the viral cDNA ends (vDNA) may form a multimeric complex called an intasome. Structural studies have revealed that integrases of different retroviruses form multimers from tetramer to hexadecamer. We have used bulk biochemical and single-molecule imaging techniques to study the dynamics of intasomes interacting with target DNA. We compared the tetrameric intasome of the non-pathogenic spuma retrovirus prototype foamy virus (PFV) to octameric intasomes of pathogenic beta-retrovirus mouse mammary tumor virus (MMTV). Recombinant integrases were assembled with fluorophore labeled or unlabeled vDNA and purified by size exclusion chromatography. The tetrameric and octameric multimers of PFV and MMTV intasomes were confirmed by single molecule mass photometry. Integration assays with supercoiled plasmid target DNA revealed that the MMTV intasomes are active for at least 80 minutes while PFV intasomes are active for only 2 minutes. The length of time between strand transfer events was measured by single molecule magnetic tweezers. MMTV intasomes displayed an average time of 1.3 seconds between strand transfers while this time for PFV intasomes was 0.47 seconds. Single molecule total internal reflection fluorescence microscopy (smTIRF) was used to visualize the fluorophore labeled intasomes interacting with 23 kb naked DNA attached at both ends to the surface. This visualization technique was used to determine values for both the lifetime of association with DNA (MMTV = 4.6 sec; PFV = 2.1 sec) and the diffusion coefficient (D_{MMTV} = 0.035 $\mu m^2/s$, 110 mM NaCl; D_{PFV} = 0.082 $\mu m^2/s$, 110 mM NaCl). The diffusion coefficient did not change over multiple salt concentrations suggesting that both MMTV and PFV intasomes remain in continuous contact with the DNA during a search. Intriguingly smTIRF also revealed MMTV intasomes forming stable immobile filaments on naked target DNA, a phenomenon that has not been observed with PFV intasomes. Our comprehensive analysis elucidates the contrasting dynamics of MMTV and PFV intasomes, showcasing the extended activity and formation of stable immobile filaments exhibited by MMTV, shedding light on the dynamics of variable multimeric retroviral intasomes.

Structure-guided Optimization of HIV Integrase Strand Transfer Inhibitors with Improved Efficacy Against a Broad Panel of Virus Having Resistant Mutant Forms of Integrase

Xue Zhi Zhao,[1] Steven J. Smith,[1,2] Dario O. Passos,[3] Min Li,[4] Mathieu Métifiot,[5] Daniel P. Maskell,[6] Valerie E. Pye,[6] Peter Cherepanov,[6] Yves Pommier,[5] Robert Craigie,[4] Dmitry Lyumkis,[3] Stephen H. Hughes,[2] and Terrence R. Burke, Jr.[1]

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FDA-approved integrase strand transfer inhibitors (INSTIs) include the first-generation agents raltegravir (RAL) and elvitegravir (EVG) and the second-generation drugs, dolutegravir (DTG), bictegravir (BIC), and cabotegravir (CAB). These INSTIs are widely used as first-line antiviral therapies. Although second-generation INSTIs have shown improved clinical success against integrase mutations compared with first-generation INSTIs, mutations that diminish the efficacy of the second-generation INSTIs have arisen and the mechanisms by which these mutations cause resistance are often poorly understood. We have developed noncytotoxic naphthyridine-based INSTIs that retain low-nanomolar antiviral potencies against HIV-1 variants harboring the major INSTI-resistant mutations. By analyzing crystal structures of these inhibitors bound to the prototype foamy virus (PFV) intasomes, we found that the most successful inhibitors exhibit striking mimicry of binding interactions shown by uncleaved viral DNA and target host DNA. Based on these observations, we proposed a concept of “bi-substrate mimicry,” as an extension of the "Substrate Envelope" principle, in which the efficacy of INSTIs against resistant mutants can be enhanced by mimicking aspects of both bound target DNA and viral DNA, and by filling the substrate-binding regions of the catalytic site. We also examined the effects of substituents at different positions on our naphthyridine-based INSTIs. Substituents at the 6-position are particularly efficacious, with the best compound **XZ426** retaining better potency against a broad panel of known INSTI-resistant mutants than other analogs that we have described. We analyzed the cryo-EM structures with **XZ426** or DTG bound to HIV-1 intasomes formed from IN wild-type and mutants. We showed that **XZ426** is more effective at inhibiting these mutant forms of IN than DTG. Several structural features contribute to the favorable interactions of **XZ426** with both with the mutant forms of IN and the DNA substrate. These interactions contribute to the improved profile of this INSTI against the mutants. Notable characteristics of **XZ426** include the fact that pyridine hydroxyamide promotes a coplanar metal-chelation heteroatom triad, the flexible halobenzylamide and naphthyridine can make a π-π stacking interaction with two of the 3’-end nucleobases of cleaved viral DNA substrate. Having a better understanding of the binding of **XZ426** to WT and drug resistant intasomes provides information that can be used to further the development of this important class of anti-HIV agents.

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