Allosteric integrase inhibitor potency is determined through the inhibition of HIV-1 particle maturation

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Edited by Alan R. Rein, National Cancer Institute, Frederick, MD, and accepted by the Editorial Board April 1, 2013 (received for review January 14, 2013)

Integration is essential for HIV-1 replication, and the viral integrase (IN) protein is an important therapeutic target. Allosteric IN inhibitors (ALLINIs) that engage the IN dimer interface at the binding site for the host protein lens epithelium-derived growth factor (LEDGF)/transcriptional coactivator p75 are an emerging class of small molecule antagonists. Consistent with the inhibition of a multivalent drug target, ALLINIs display steep antiviral dose–response curves ex vivo. ALLINIs multimerize IN protein and concordantly block its assembly with viral DNA in vitro, indicating that the disruption of two integration-associated functions, IN catalysis and the IN-LEDGF/p75 interaction, determines the multimode mechanism of ALLINI action. We now demonstrate that ALLINI potency is unexpectedly accounted for during the late phase of HIV-1 replication. The compounds promote virion IN multimerization and, reminiscent of class II IN mutations, block the formation of the electron-dense viral core and inhibit reverse transcription and integration in subsequently infected target cells. Mature virions are recalcitrant to ALLINI treatment, and compound potency during virus production is independent of the level of LEDGF/p75 expression. We conclude that cooperative multimerization of IN by ALLINIs together with the inability for LEDGF/p75 to effectively engage the virus during its egress from cells underscores the multimodal mechanism of ALLINI action. Our results highlight the versatile nature of allosteric inhibitors to primarily inhibit viral replication at a step that is distinct from the catalytic requirement for the target enzyme. The vulnerability of IN to small molecules during the late phase of HIV-1 replication unveils a pharmacological Achilles' heel for exploitation in clinical ALLINI development.

AIDS | antiretroviral therapy

Highly active antiretroviral therapy (HAART), which treats patients with combinations of antiviral drugs to suppress HIV-1 replication, is the standard of care in the AIDS clinic (1). HAART is most often formulated from compounds that inhibit the activities of the viral reverse transcriptase (RT) and protease (PR) enzymes. The activity of the viral integrase (IN) enzyme is also crucial to HIV-1 replication, and raltegravir (RAL), which was licensed by the US Food and Drug Administration in 2007, is the first-in-class IN strand transfer inhibitor (INSTI) (2). Although the INSTI elvitegratir (EVG) has since been licensed, IN mutations that confer resistance to RAL in large part convey EVG cross-resistance, thereby limiting clinical INSTI treatment options (3). Dolutegravir, an investigational INSTI that remains active in the face of most RAL resistance mutations, may help to circumvent the limitation of cross-resistance to the current clinical IN inhibitors (3). INSTIs inhibit DNA strand transfer activity by binding to the intasome nucleoprotein complex at the enzyme active site and displacing the terminal deoxyadenylate residue of retroviral DNA that would otherwise be used by IN to cut chromosomal DNA (4). Inhibitors that engage HIV-1 IN at sites separate from the active site, which should retain potency in the face of INSTI resistance mutations, are therefore an important class of investigational antiretroviral drug (5).

HIV-1 preferentially integrates along the bodies of active genes (6), a trait that is largely attributable to an interaction between IN and the host protein lens epithelium-derived growth factor (LEDGF)/transcriptional coactivator p75 (reviewed in refs. 7 and 8). LEDGF/p75 functions as a bimodal tether during integration: elements within its N-terminal region confer constitutive binding to chromatin, whereas a downstream IN-binding domain (IBD) binds lentiviral IN proteins (9, 10). HIV-1 IN is composed of three domains, the N-terminal domain, the catalytic core domain (CCD), and the C-terminal domain, and LEDGF/p75 engages IN at a cleft formed through the dimerization of the CCD (11). IN is a highly dynamic protein that functions as a tetramer, and its assembly in the presence of viral DNA is critical to enzyme function (12, 13). Small molecules that compete for LEDGF/p75 binding enhance IN multimerization in the absence of viral DNA and accordingly allosterically inhibit IN activity (14–16). Although various terms, including LEDGINs for LEDGF/p75-IN inhibitors, have been coined for these investigational compounds, we prefer "allosteric IN inhibitor" or ALLINI to highlight the mechanistic basis of compound action (14, 17).

Different classes of antiretroviral drugs display characteristic dose–response curve slopes in antiviral activity assays, which has important implications for the mechanism of drug action. IN is considered a monovalent INSTI target because these compounds display slope parameters of close to 1 (18). Because ALLINIs display significantly steeper slopes, IN by contrast behaves as a multivalent target for this drug class (14). The inhibition of two integration-related functions, IN catalysis and the IN-LEDGF/p75 interaction, are proposed to underlie the multimode mechanism of ALLINI action (14–16). Herein we show that ALLINI potency is accounted for during the late stage of HIV-1 replication. The ability for ALLINIs to engage and multimerize IN at a point in the viral life cycle when the virus is apparently unable to interact with the LEDGF/p75 host factor accounts for the unique pharmacology of this class of antiretroviral compounds.

Results

ALLINI Potency Is Accounted for During the Late Stage of HIV-1 Replication. The ALLINI BI-1001 (Fig. S1) displayed an effective concentration 50% (EC_{50}) of 5.8 μM in a spreading HIV-1 replication assay (14). IN catalysis is required during the early

Author contributions: K.A.J., J.J.K., M.K., and A.E. designed research; K.A.J., H.W., A.S., L.F., J.J.K., and Y.K. performed research; W.W., P.A.P., and J.R.F. contributed new reagents/analytic tools; K.A.J., H.W., A.S., L.F., J.J.K., Y.K., W.W., A.B.-C., M.K., and A.E. analyzed data; and K.A.J., L.F., M.K., and A.E. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. A.R.R. is a guest editor invited by the Editorial Board.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 4ID1).

See Commentary on page 8327.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1300703110/-/DCSupplemental.

phase of retroviral replication (1), where the infection of SupT1 cells by an HIV-luciferase (HIV-Luc) reporter virus was potently inhibited by the INSTI RAL (Table 1). BI-1001 concentrations of up to 50 μM, by contrast, failed to inhibit HIV-Luc. HIV-Luc producing HEK293T cells were therefore treated with BI-1001, which did not affect the extent of virus accumulation in the cell supernatant. Subsequent challenge of SupT1 cells revealed an EC_{50} of 1.9 ± 0.2 μM despite omitting additional BI-1001 from the target cell cultures (Table 1). These results are consistent with a recent report that virus produced in the presence of LEDGIN Cx05045 is noninfectious (15), yet extend it to show that HIV-1 is significantly more vulnerable when it is exposed to an ALLINI in virus producer compared with challenge target cells. Because target cells treated with BI-D (Fig. S1) are protected from infection by HIV-Luc (17), we next used the more potent compound to dissect the full spectrum of ALLINI action.

BI-D yielded an EC_{50} of 1.17 ± 0.1 μM with an accompanying slope of 1.3 ± 0.2 during the early or acute phase of infection (Fig. 1*A*, black line, and Table 1), indicating that IN in large part behaves as a monovalent target under these conditions (18). The potency of the inhibitor increased ∼13-fold, to 0.089 ± 0.023 μM, following titration on HEK293T producer cells. The slope of the dose–response curve, 2.4 ± 0.4, was moreover noticeably greater than 1 (Fig. 1*A*, solid gray line). Because an EC_{50} of 0.090 ± 0.031 μM with an accompanying slope of 2.8 ± 0.4 was determined in the spreading replication assay (Fig. 1*A*, dashed gray line), we conclude BI-D potency is principally accounted for during the late phase of HIV-1 replication. To address physiological relevance, peripheral blood mononuclear cell (PBMC) cultures were treated with 10 μM BI-D, a dose equivalent to ∼110 EC_{50} units, during the peak of HIV-1_{NL4-3} replication. Similar levels of HIV-1 were produced from BI-D and DMSO-treated PBMCs (Fig. 1*B*). Viruses were washed by ultrafiltration to remove excess drug before target cell infection. BI-D treatment rendered HIV-1_{NL4-3} noninfectious (Fig. 1*C*).

ALLINIs Inhibit the Formation of the Electron-Dense HIV-1 Core. Mutational studies provide precedence for the involvement of IN during the late stage of HIV-1 replication. IN mutant viruses are classified I or II based on the nature of associated replication block(s) (19). Class I mutants are specifically blocked for integration, whereas class II mutants are additionally defective for particle assembly/release and/or reverse transcription. The effects of ALLINI treatment were accordingly compared with two class II IN mutant viruses, V165A, which carries a missense mutation in the CCD (20), and ΔIN, which harbors a stop codon at the RT-IN boundary in the *pol* gene and hence does not express IN (21). Viral protein processing and virion incorporation were analyzed by metabolic labeling followed by immunoprecipitation. Consistent with prior observations (21), IN deletion reduced the level of incorporated RT p66/p51 heterodimer (Fig. S2*A*, lane 4). Neither BI-D treatment nor the V165A mutation reproducibly affected HIV-1 protein processing or virion incorporation (Fig. S2*A*, lanes 1–3 and 6–8). The incorporation of HIV-1 genomic RNA into virions was also unaffected by BI-D treatment (Fig. S2*B*).

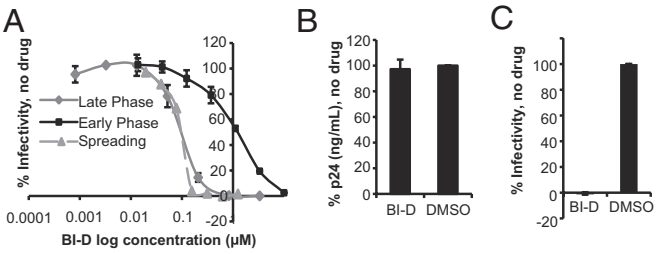

Fig. 1. BI-D potency is accounted for during the late phase of HIV-1 replication. (*A*) Dose–response curves under the indicated conditions of drug treatment. The similarity in late phase and spreading replication EC_{50} values is statistically significant (P = 0.94); error bars represent the variation obtained from two to three independent experiments. (*B*) Release of HIV-1_{NL4-3} from PBMC cultures following 24 h of BI-D (10 μM) or solvent control treatment at peak of virus replication. (*C*) Infectivity of ultrafiltered particles from *B* as assessed in CEMx174 5.25 M7 indicator cells. Results in *B* and *C* are averages ± SDs from three independent experiments.

Virion morphology was analyzed by thin-section electron microscopy. Particles purified and concentrated by ion exchange and size exclusion chromatography (22) were categorized as (*i*) mature, containing conical or round cores with associated electron density; (*ii*) eccentric, with electron dense material situated between translucent cores and the viral membrane; and (*iii*) immature. Most (93%) HIV-1_{NL4-3} particles were mature, whereas IN deletion yielded gross morphological defects (21): only ∼9% of particles were mature, with about 61% and 30% eccentric and immature, respectively (Fig. 2). The V165A mutation yielded somewhat less intense alterations, with ∼18% of particles mature, 70% eccentric, and 12% immature. Similar to the class II mutations, ALLINI treatment significantly enhanced the formation of eccentric HIV-1 cores. BI-D yielded ∼8%, 89%, and 3% mature, eccentric, and immature particles, respectively, whereas the values for BI-1001 were ∼17%, 76%, and 7% (Fig. 2).

ALLINI Treatment Renders HIV-1 Defective for Reverse Transcription and Integration. Quantitative PCR was used to assess the effects of ALLINI treatment on reverse transcription and integration. Primers and probes were designed to detect viral R and U5 (R-U5) sequences indicative of early reverse transcription (ERT) products, the late reverse transcription (LRT) product R-*gag*, integrated proviruses, and two-long terminal repeat (LTR)-containing circles that form at low levels in the nucleus through the action of cellular nonhomologous DNA end joining (23, 24). Integration-specific blocks yield transient increases in two-LTR circles due to the increased availability of nuclear viral DNA for cellular DNA metabolism (21, 25).

HIV-Luc supported similar levels of LRT product formation in SupT1 cells treated with DMSO, RAL, or BI-D (Fig. 3*A*). BI-D and RAL moreover both inhibited integration (Fig. 3 *B* and *C*); the lack of two-LTR circle increase in BI-D–treated cultures is likely attributable to relative drug dose (10 μM RAL = 3,333

Table 1. Antiviral EC_{50} values and slope parameters

	BI-1001		RAL		BI-D	
Treatment condition	EC_{50}, μM	Slope	EC_{50}, μM	Slope	EC_{50}, μM	Slope
Target cell	>50*	NA	0.003 ± 0.001†	1.2 ± 0.06†	1.17 ± 0.1†	1.3 ± 0.2†
Producer cell	1.9 ± 0.2†	2.2 ± 0.7†	ND	ND	0.089 ± 0.023†	2.4 ± 0.4†
Replicative spread	5.8 ± 0.1‡	3.7 ± 0.2‡	0.005 ± 0.002‡	1.1 ± 0.1‡	0.090 ± 0.031*	2.8 ± 0.4*

NA, not applicable; ND, not determined.
*Average ± SD of two independent experiments.
†Average ± SD of three independent experiments.
‡Data from ref. 14.

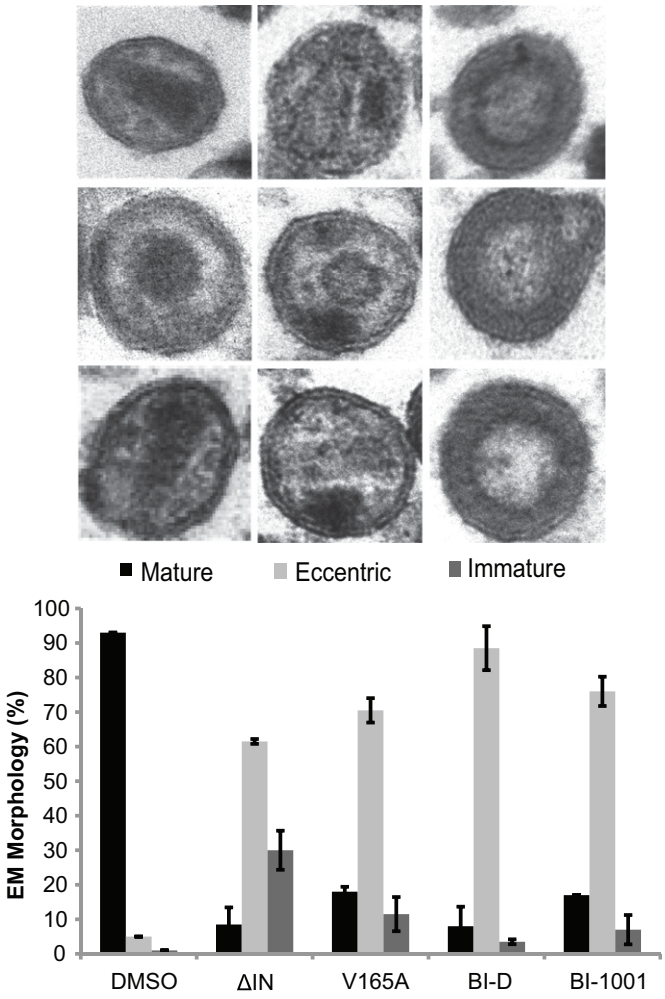

Fig. 2. ALLINIs inhibit the formation of the electron-dense HIV-1 core. *Upper*, representative images of mature, eccentric, and immature particle morphologies. *Lower*, quantitation of core morphology frequencies (average ± SD for $n = 2$ experiments) for ΔIN, V165A, and wild-type HIV-1_{NL4-3} made in the presence of BI-D (10 μM), BI-1001 (50 μM), or DMSO solvent control. Particles (100 in each experiment) were counted and typed as described in text.

EC_{50} units, where 10 μM BI-D = 8.5 EC_{50} units; Table 1). These results agree with prior reports that ALLINIs behave as integration inhibitors during the acute phase of HIV-1 replication (16, 26). Infection with virions produced in the presence of BI-D revealed strikingly different results. As expected (27), the V165A mutation reduced ERT and LRT product formation by ~70%–80% (Fig. 3 *D* and *E*). BI-D–treated virus was also defective for reverse transcription, yielding 11%–18% of the level of ERT and LRT products compared with controls (Fig. 3 *D* and *E*). The DNAs that did form were additionally defective for integration (Fig. 3 *F* and *G*). Both BI-D–treated and V165A mutant virions entered SupT1 cells normally as assessed by the Vpr–beta-lactamase (Vpr-BlaM) fusion protein assay (Fig. 3*H*).

IN Is the Target of ALLINI Action During Viral Egress. IN is processed from the Gag-Pol polyprotein precursor by the viral PR during HIV-1 maturation (28). To investigate the nature of the drug target, ΔIN virions were transcomplemented with Vpr-IN harboring wild-type IN or the H171T IN mutant that carries a substitution in the ALLINI binding pocket (Fig. S3*A*) and confers ~44-fold resistance to BI-D (29). A similar experimental design previously demonstrated that Gag-Pol, and not IN, was the target of a dominant-negative version of the IN-interacting INI1 host factor during HIV-1 egress (30). The ΔIN transcomplemented virions would accordingly resist BI-D action if the precursor Gag-Pol protein were the drug target. BI-D by contrast retained full potency, and the H171T mutation moreover conferred similar drug resistance profiles regardless of its expression *in cis* from Gag-Pol or *in trans* from Vpr-IN (Fig. S4). We therefore conclude that IN is the likely target of BI-D action during the late stage of HIV-1 replication.

Ultrafiltration was used to remove excess compound following incubation with cell-free HIV-Luc to assess virucidal activity. Despite testing concentrations of up to 100 μM, BI-D antiviral activity was not detected. Consistent with its low micromolar virucidal activity (31), the nonnucleoside RT inhibitor (NNRTI) efavirenz (EFV) yielded an EC_{50} of 4.2 ± 3.5 μM ($n = 3$).

ALLINI Potency Is Independent of LEDGF/p75 Expression Level During HIV-1 Production. LEDGF/p75 and ALLINIs compete for binding to a pocket formed through the dimerization of the HIV-1 IN CCD (11, 26) (Fig. S3*A*). To address if LEDGF/p75 affects BI-D potency, EC_{50} values were assessed in constitutively knocked down versus control HEK293T cells (32) (Fig. S5). Consistent with results using mouse knockout cells (17), LEDGF/p75 knockdown yielded a significant 29-fold increase in BI-D potency during the acute phase of HIV-1 infection, whereas RAL potency was unaffected (Table 2). By contrast, LEDGF/p75 depletion did not significantly alter BI-D potency during HIV-1 production. Potency during the acute phase of HIV-1 infection in LEDGF/p75-depleted cells was moreover similar to BI-D titer during virus production regardless of LEDGF/p75 expression level (Table 2).

Purified Protein and Virion-Associated IN Are Oligomerized by BI-D Treatment. Integration is catalyzed by an IN tetramer (4, 33); in the absence of viral DNA, recombinant HIV-1 IN assumes a variety of multimeric forms, from monomer to higher-order oligomers, depending on buffer conditions and protein concentration (34). Resonance transfer-based assays previously demonstrated that ALLINIs promote IN multimerization (14–16). Homogenous time resolved fluorescence (14) accordingly yielded an in vitro stimulatory concentration 50% of 0.027 ± 0.003 μM with an accompanying slope of 1.97 ± 0.36 for BI-D (Fig. S3*B*), and the compound expectedly cocrystallized in the LEDGF/p75 binding pocket at the CCD dimer interface (Fig. S3*A* and Table S1). Size exclusion chromatography was used to monitor distinct protein species, which revealed that BI-D effectively converted IN tetramers to higher-order oligomers (Fig. 4*A* and Table S2). To assess effects of ALLINI treatment on IN multimerization during virus production, reducing agent was omitted from virion lysates (35), which revealed ~3-fold enhancement of IN dimer formation by BI-D (Fig. 4*B*, lanes 1–6). The substitution of Asp at position 124 in the ALLINI binding pocket (Fig. S3*A*), which confers ~250-fold resistance to BI-D (29), importantly negated compound-induced IN multimerization in vitro and in virions (Fig. 4 *A* and *B*).

Discussion

Some antiretroviral drugs, like potent NNRTIs, display weak activities (<1% of full potency) against secondary steps in the viral lifecycle (36). Low micromolar styrylquinoline-based IN inhibitors can inhibit recombinant IN nuclear transport in vitro (37) and reverse transcription and integration during HIV-1 infection (38), although the contributions of these different activities to compound potency have remained unclear (39). The results reported here differ starkly from these prior reports: ALLINI potency is determined through the inhibition of viral core maturation, a step in the HIV-1 lifecycle that is clearly distinct from the catalytic requirement for the targeted IN enzyme (Figs. 1 and 2 and Table 1). We expect that future allosteric inhibitors of viral targets will similarly unveil the versatile nature of these compounds to primarily inhibit replication in unanticipated ways. Because styrylquinolines

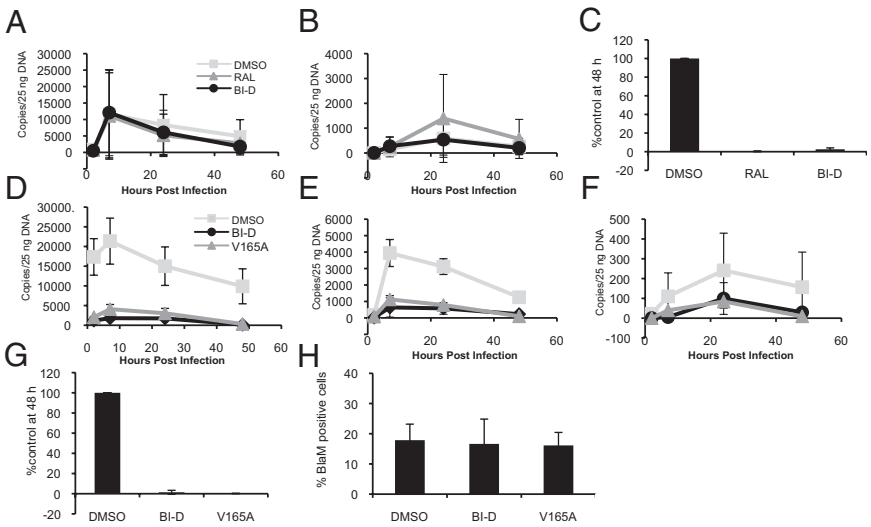

Fig. 3. BI-D–treated HIV-1 is defective for reverse transcription and integration. (*A*) DNAs (25 ng) isolated from SupT1 cells infected with HIV-Luc in the presence of DMSO (light gray squares), 10 μM BI-D (black circles), or 10 μM RAL (dark gray triangles) were assessed for LRT product formation by quantitative PCR. (*B*) Time course of two-LTR circle formation. (*C*) Alu-PCR values at 48 h postinfection (DMSO sample set at 100%) for extracts described in *A*. (*D*) DNAs isolated from SupT1 cells infected with IN mutant V165A (dark gray triangles), or wild-type HIV-Luc pretreated during virus production with DMSO (light gray squares) or 10 μM BI-D (black circles), were assessed for ERT product formation. (*E* and *F*) Levels of LRT and two-LTR circle product formation, respectively, as a function of time. (*G*) Alu-PCR values at 48 h postinfection for extracts described in *D*; values obtained from DMSO-treated HIV-Luc were set at 100%. (*H*) Percentage of cells positive for beta-lactamase; similar results were found for HIV-Luc carrying the HIV-1 envelope glycoprotein. Results in *A* and *C*–*G* are averages and SDs of two independent infection experiments, with DNA samples queried in duplicate by PCR for each infection; *B* and *H* values are averages and SDs from three independent experiments.

harbor the quinolone pharmacophore common to many ALLINIs (Fig. S1), it will be instructive to ascertain if they too impact late events in HIV-1 replication.

Although there is strong consensus for a role for LEDGF/p75 during the early phase of HIV-1 replication, a potential postintegration role for this protein is less clear. Expression of a LEDGF/p75-binding peptide in virus producer cells reduced HIV-1 infectivity (40). Overexpression of IBD-containing dominant interfering fragments by contrast potently restricted HIV-1 ingress, but not egress (41). Our finding that BI-D potency is unchanged by depleting LEDGF/p75 from virus producer cells is consistent with the fact that the IBD and ALLINIs engage the same CCD binding pocket on IN. We therefore conclude that ALLINI-mediated multimerization of IN through CCD dimer binding in the absence of competing LEDGF/p75 protein during HIV-1 egress underlies the multimode mechanism of ALLINI action. Although our work indicates that inhibition of the interaction between IN and LEDGF/p75 or its close relative hepatoma-derived growth factor (HDGF) related-protein (HRP) 2 does not significantly contribute to ALLINI potency (17) (Table 2), it nevertheless underscores the utility of searching for inhibitors of protein–protein interactions. ALLINIs can block the interaction between a physiologically relevant host factor and a viral protein dimerization interface, and we speculate that small molecules that block interactions between binding partners and target multimerization interfaces will allosterically alter target function even when the binding partner (e.g., recombinant antibody) plays a limited physiological role.

INSTIs display slope parameters of ∼1 because each infectious particle yields a single intasome (4, 18). The underlying theme for steeper slopes, participation of multiple copies of the drug target in the relevant step of the lifecycle (18), is consistent with the results reported here for ALLINIs. The cooperative behavior of ALLINIs in IN multimerization assays (14) (Fig. S3*B*), combined with the apparent inability of LEDGF/p75 to engage HIV-1 during virus egress, determines ALLINI potency (EC_{50} and slope parameter). Because LEDGF/p75 depletion from target cells sharply increases BI-D EC_{50} values, the host factor competes with the drug for binding to the CCD dimer interface during the acute phase of infection (17). The slope of the dose–response curve remains close to 1 under this condition (Table 2) because only a minor fraction of the IN during viral ingress is required for intasome formation. IN therefore is a monovalent target of INSTIs and ALLINIs during the early phase of HIV-1 replication, where both types of compounds specifically target integration. We

Table 2. LEDGF/p75 expression level does not influence BI-D titer during HIV-1 egress

	RAL		BI-D	
Treatment condition	EC_{50}, μM	Slope	EC_{50}, μM	Slope
293T-si1340/1428 target cell	0.009 ± 0.001*	0.86 ± 0.13	0.131 ± 0.003†,§,**	0.85 ± 0.05
293T-siScram target cell	0.01 ± 0.001*	0.94 ± 0.86	3.8 ± 1.1†	1.27 ± 0.67
293T-si1340/1428 producer cell	ND	NA	0.161 ± 0.05‡,§	2.40 ± 0.41
293T-siScram producer cell	ND	NA	0.144 ± 0.07‡,**	2.44 ± 0.12

NA, not applicable; ND, not determined.
*$P = 0.79$.
†$P = 0.016$.
‡$P = 0.56$.
§$P = 0.46$.
**$P = 0.84$.

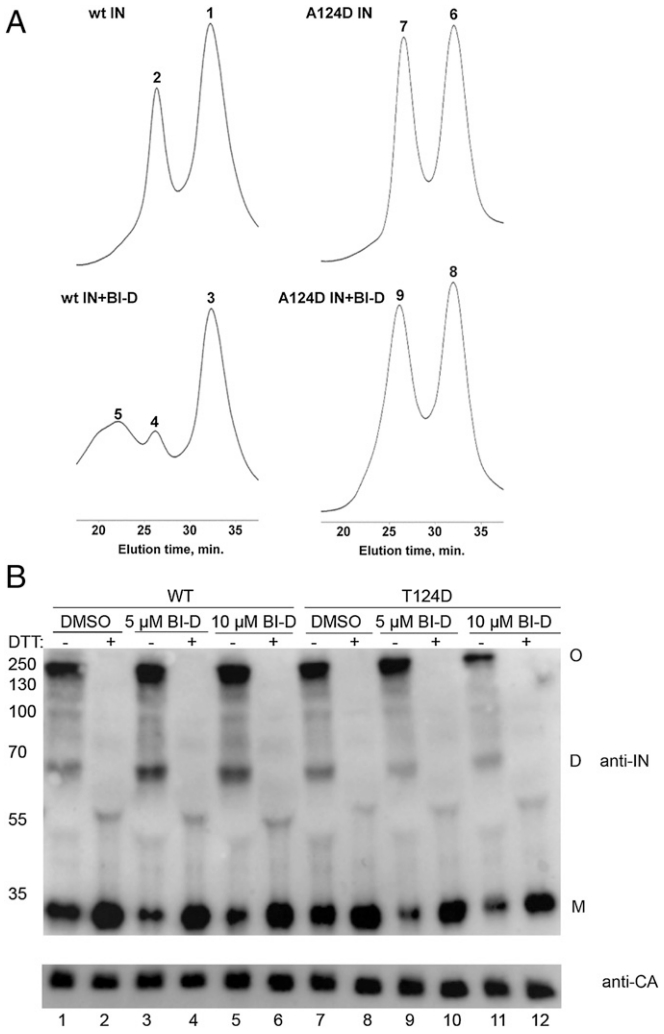

Fig. 4. BI-D enhances the formation of IN oligomers. (*A*) Elution profiles of purified wild-type (WT) and A124D mutant IN (20 μM) in the absence of drug (*Upper*) or in the presence of 10 μM BI-D. Elution times of indicated peak values, and associated estimates of oligomeric states, are summarized in Table S2. (*B*) Wild-type HIV-1_{NL4-3} and IN mutant T124D (position 124 is polymorphic; HIV-1_{HXB2} carries Ala where HIV-1_{NL4-3} harbors Thr) made in the presence of DMSO solvent control or 5 μM or 10 μM BI-D as indicated were prepared with and without DTT reducing agent in the SDS/PAGE sample buffer. *Upper*, blot was probed with anti-IN monoclonal antibody 8E5; *Lower*, blot probed with anti-p24 antibody. Results are representative of three independent experiments. D, IN dimer; M, IN monomer; O, IN oligomer.

consider that inhibition of multiple viral replication steps (core formation, reverse transcription, and integration) could also contribute to the multimode mechanism of ALLINI action.

Whereas INSTIs recapitulate the phenotype of class I IN mutant viruses, our findings highlight that ALLINI treatment during HIV-1 production induces the class II IN mutant viral phenotype (Figs. 2 and 3). The behavior of certain class II mutant viruses indicated a potential role for IN during the late stage of HIV-1 replication (19), although the underlying mechanism has remained elusive. The particle release defect of IN deletion mutant viruses is overridden by inhibiting PR activity (42), and suboptimal concentrations of PR inhibitors can yield the eccentric core morphology described here (43). One interpretation of our results is an active role for IN in the formation of the electron-dense HIV-1 core. However, structural rearrangements subsequent to Gag and Gag-Pol proteolysis that underlie HIV-1 core formation might by contrast be particularly sensitive to deregulated IN multimerization. Concordantly, a variety of mutations that either enhance or inhibit IN oligomerization confer the class II IN mutant phenotype (44–47). Although we cannot rule out that ALLINIs engage the IN domain of the Gag-Pol precursor protein before proteolysis, the results of Vpr-IN complementation assays indicate that the drug target during HIV-1 production is the post-cleavage IN protein (Fig. S4). ALLINIs lack virucidal activity because mature HIV-1 particles have proceeded past the point in the lifecycle where IN unveils a CCD–CCD Achilles' heel for engagement by small molecule inhibitors. Further evaluation of class II mutant viruses may shed additional light on the multimode mechanism of ALLINI action. Hypersensitivity to multimerization-inducing small molecules during viral egress highlights IN as an attractive target for clinical ALLINI development.

Materials and Methods

Cells, Viruses, and Antiviral Assays. HIV-$1_{NLX.Luc.R-}$ (HIV-Luc) was pseudotyped by cotransfecting HEK293T cells with pNLX.Luc.R- and an envelope expression vector (HIV-1_{NL4-3} or vesicular stomatitis virus G) using FuGENE 9 (Promega) (27). DMSO and ALLINIs were maintained in media throughout transfection procedures. Cell-free virus concentration, typically 500–800 ng/mL, was determined using a commercial p24 ELISA kit (Advanced Biosciences Laboratories).

SupT1 (2×10^5/mL), CEMx174 5.25 M7 (2×10^5/mL), and shRNA-expressing HEK293T (1.5×10^5/mL) cells were infected in triplicate with 5 ng/mL p24, unless specified otherwise. Where indicated, compound or DMSO was added to target cells at the time of infection. Luciferase values, expressed as relative light units, were determined 48 h postinfection. Virucidal activity was analyzed after 1 h incubation with drug at 37 °C. Drug was removed before infection via three rounds of successive ultrafiltration in 100 kDa concentrators (Amicon), theoretically yielding a 28,000-fold reduction in the concentration of flow-through molecules. Additional details are provided in *SI Materials and Methods*.

DNA Analysis. DNA was extracted from SupT1 cells infected with ultrafiltered, DNase-treated (1 h at 37 °C) HIV-Luc (100 ng p24/mL) using the DNeasy blood and tissue kit (Qiagen). Parallel infections were performed in the presence of 10 μM EFV to account for residual plasmid after DNase treatment, and values obtained from separate PCR assays were subtracted from experimental samples. The PCR conditions are elaborated in *SI Materials and Methods*.

Virion Analyses. HEK293T cells (9×10^5) were transfected with pNL4-3/XmaI-based plasmids (48) (4.4 μg) using FuGENE 9. See *SI Materials and Methods* for expanded radiolabeling, Western blotting, genomic RNA detection, and virion purification details.

IN Biochemistry. Recombinant HIV-1 IN proteins were purified following their expression in *Escherichia coli* as described previously (14, 49, 50). See *SI Materials and Methods* for details of IN multimerization assays, protein crystallization, and structure determination.

Statistical Analysis. Significant differences between data groups were determined by a paired-sample *t* test (two tailed).

ACKNOWLEDGMENTS. We thank J. Kappes (University of Alabama) for pRL2P-Vpr-IN, M. D. Miller (Merck Research Laboratories) for pMM310, E. Poeschla (Mayo Clinic) for 293T-si1340/1428 and 293T-siScram cells, N. Landau (New York University) for CEMx174 5.25 M7 cells, C. Tipper for advice on HIV-1 purification for microscopy, and P. Cherepanov for pCPH6P-HIV1-IN and for critical review of the manuscript. This work was funded in part by National Institutes of Health Grants AI039394 (to A.E.), AI062520 and AI081581 (to M.K.), GM103368 (to A.E. and M.K.), AI097044 (to J.J.K. and J.R.F.), and P30AI060354 (to the Harvard University Center for AIDS Research). K.A.J. is a recipient of a National Academies' Ford Foundation Predoctoral Fellowship.

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