Targeting of mTOR catalytic site inhibits multiple steps of the HIV-1 lifecycle and suppresses HIV-1 viremia in humanized mice

Alonso Herediaa,1, Nhung Lea, Ronald B. Gartenhausb, Edward Sausvilleb, Sandra Medina-Morenaa, Juan C. Zapataa, Charles Davisb, Robert C. Galloa,1,1, and Robert R. Redfielda

*Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD 21201; andbMarlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201

Contributed by Robert C. Gallo, June 19, 2015 (sent for review March 2, 2015)

HIV necessitates host factors for successful completion of its lifecycle. Mammalian target of rapamycin (mTOR) is a conserved serine/threonine kinase that forms two complexes, mTORC1 and mTORC2. Rapamycin is an allosteric inhibitor of mTOR that selectively inhibits mTORC1. Rapamycin interferes with viral entry of CCR5 (R5)-tropic HIV and with basal transcription of the HIV LTR, potently inhibiting replication of R5 HIV but not CXCR4 (X4)-tropic HIV in primary cells. The recently developed ATP-competitive mTOR kinase inhibitors (TOR-KIs) inhibit both mTORC1 and mTORC2. Using INK128 as a prototype TOR-KI, we demonstrate potent inhibition of both R5 and X4 HIV in primary lymphocytes (EC50 < 50 nM), in the absence of toxicity. INK128 inhibited R5 HIV entry by reducing CCR5 levels. INK128 also inhibited both basal and induced transcription of HIV genes, consistent with inhibition of mTORC2, whose activity is critical for phosphorylation of PKC isoforms and, in turn, induction of NF-κB. INK128 enhanced the antiviral potency of the CCR5 antagonist maraviroc, and had favorable antiviral interactions with HIV inhibitors of reverse transcriptase, integrase and protease. Targeting of cellular mTOR with INK128 (and perhaps others TOR-KIs) provides a potential strategy to inhibit HIV, especially in patients with drug resistant HIV strains.

HIV resistance | mTOR inhibitors | HIV transcription | CCR5 | humanized mice

Current antiretrovirals (ARTs) against HIV target several different steps in the viral lifecycle. However, there is a continuous need for novel classes of ARTs targeting additional stages of viral replication, primarily due to emergence of drug resistance. There are currently several ARTs classes against reverse transcription, integration and maturation. Within each class, the availability of several drugs with distinct resistance profiles make it possible to switch to an alternative drug from the same class in the event of resistance. In contrast, the HIV lifecycle steps of entry and transcription are underrepresented in current therapy. First, there are only two licensed entry inhibitors: the CCR5 antagonist Maraviroc (5) and the fusion inhibitor Enfuvirtide (6). However, the virus tropism specificity of Maraviroc (7) and the need for twice-daily s.c. injection of Enfuvirtide (8) limit their clinical potential. Second, there are no licensed inhibitors of HIV transcription. Therefore, new approaches for targeting entry and transcription may provide alternative treatment options for patients, especially those with drug-resistant HIV strains.

Targeting cellular proteins that HIV necessitates in its lifecycle is an attractive approach to overcome HIV drug resistance because cellular proteins have lower mutations rates than do HIV proteins under drug pressure, and because there are so many host proteins needed by HIV for its replication. A downside, of course, is the possibility of side effects from inhibition of a cellular protein. Cellular mammalian target of rapamycin (mTOR) is a serine/threonine kinase that forms two complexes, mTORC1 and mTORC2. mTORC1 promotes translation initiation and synthesis of host proteins, whereas mTORC2 regulates full activation of the protein kinase B (Akt) pathway and also regulates PKC signaling pathways (reviewed in ref. 9). Previous work has shown that rapamycin, which targets mTORC1 but not mTORC2 (10), interferes with the HIV steps of CCR5-mediated entry and with basal (but not induced) transcription of the HIV LTR (11–14). These activities of rapamycin effectively inhibit replication of CCR5 (R5)-tropic HIV, but not CXCR4 (X4)-tropic HIV, in primary lymphocytes (11, 13, 15).

The recently developed ATP-competitive mTOR kinase inhibitors (TOR-KIs) inhibit both mTORC1 and mTORC2 complexes (16–19). Because mTORC1 controls CCR5 expression and basal HIV transcription (11–13), and because mTORC2 controls phosphorylation of PKC (9, 20–22), required for NF-κB induction of HIV transcription (23, 24), we hypothesized that TOR-KIs could inhibit both R5 and X4 HIV. We have evaluated the anti-HIV potential of TOR-KIs using INK128 (25–27), currently in clinical trials of cancer.

Results
INK128 Inhibits R5 and X4 HIV Replication in Primary Cells. The chemical structure of INK128 is shown in Fig. 1A. We first evaluated the effect of INK128 on proliferation of peripheral blood lymphocytes (PBLs) from four different donors. For each donor, PBLs were activated by treatment with anti-CD3/CD28 antibodies for 3 d, cultured in the presence of IL-2 and various dilutions of INK128 for 5 d, followed by measurement of cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

Significance
Most HIV antiretrovirals target viral proteins. Unfortunately, HIV mutates under drug pressure, which can lead to drug resistance. Targeting cellular proteins that HIV necessitates in its lifecycle may help overcome HIV drug resistance because cellular proteins have lower mutations rates than do HIV proteins. Mammalian target of rapamycin (mTOR) is a cellular kinase that forms two complexes (mTORC1 and -2), regulating protein translation and transduction signaling. We demonstrate that dual targeting of mTORC-1/2 with the catalytic inhibitor INK128 blocks HIV by interfering with entry and with transcription (basal and induced). Importantly, INK128 suppressed HIV in a preclinical animal model, suggesting that mTORC-1/2 catalytic inhibitors may help control HIV in patients, particularly those with drug-resistant HIV.


The authors declare no conflict of interest.

1To whom correspondence may be addressed. Email: aheredia@ihv.umaryland.edu or rgallo@ihv.umaryland.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1511144112/-/DCSupplemental.
assays (Fig. 1B). INK128 did not inhibit cell proliferation at concentrations of up to 100 nM. We therefore selected 100 nM as the highest INK128 concentration in subsequent experiments evaluating antiviral activity.

We evaluated the antiviral activity of INK128 in PBLs infected with CCR5 (R5)-tropic and CXCR4 (X4)-tropic HIV reference strains BaL and HXB2, respectively. In experiments with PBLs from three donors, INK128 inhibited replication of both viruses, but it inhibited BaL more potently than HXB2 (EC$_{50}$s of 10.5 vs. 38 nM; $P = 0.007$ by two-tailed, unpaired $t$ test) (Fig. 1C). Similarly, in primary isolates evaluated in three donors, INK128 was more potent against R5 (EC$_{50}$s ranging 2.9–10.1 nM) than against X4 (EC$_{50}$s ranging 17.5–36.7 nM) (Table S1). The difference in INK128 potency against R5 vs. X4 primary isolates was again significant ($P = 0.01$ by two-tailed, unpaired $t$ test). In addition, INK128 inhibited a multidrug-resistant HIV molecular clone NL4329129–2, which carries the RT gene amplified from plasma of a patient with multidrug resistant HIV (28), with an EC$_{50}$ of 10.9 nM (Fig. S1). Together, these data show that INK128 inhibits replication of R5 and X4 strains of HIV, both laboratory adapted and primary isolates, in PBLs.

**INK128 Inhibits Entry of R5, but not X4, HIV in Primary Lymphocytes.** We next evaluated the mechanism of INK128 inhibition of HIV. Because INK128 activity was more potent against R5 than against X4 HIV, we hypothesized that INK128 affects entry of these viruses differently. To test this, we performed cell–cell fusion assays between 293T cells expressing R5 or X4 HIV envelopes (effectors) and INK128-treated primary CD4$^+$ T cells (targets). In this assay, targets are labeled with the fluorescent dye calcein (green) and effectors with CMTMR (red) before coculture. Fused cells score positive for both dyes. INK128 inhibited fusion of CD4$^+$ T target cells expressing R5 Env compared to X4 Env (Fig. 2A). Early products of reverse transcription (R/U5 region) in PBLs infected with R5 (JRFL) or X4 (HXB2) HIV and treated with INK128 for 16 h showed reduced levels of R/U5 DNA in R5-infected compared to X4-infected cells (Fig. 2B). Similarly, integrated HIV DNA in PBLs infected with R5 (JRFL) or X4 (HXB2) HIV and treated with INK128 for 72 h showed reduced levels of HIV integration in R5-infected compared to X4-infected cells (Fig. 2C)
cells with R5 HIV JRFL Env, but not with X4 HIV HXB2 Env (Fig. 2A). These data, obtained with CD4 targets from two different donors, suggested inhibition at an early step of the R5, but not X4, HIV lifecycle. We next evaluated downstream steps in HIV infection by measuring early products of reverse transcription and integrated provirus using real-time PCR in PBLs from two donors. As expected from the cell–cell fusion data, PBLs infected with R5 HIV (JRFL) and treated with INK128 had decreased levels of early products of reverse transcription (R/U5 transcripts) (Fig. 2B) and integrated provirus (Fig. 2C). In contrast, INK128 did not decrease R/U5 transcripts or integrated provirus on infection with X4 HIV (HXB2). Together these data demonstrate that INK128 inhibits entry of R5 HIV, but it does not inhibit X4 HIV infection before, or at the level of, integration.

To gain insight into the mechanism of INK128 inhibition of R5 HIV entry, we evaluated the effects of INK128 on the receptor CD4 and the coreceptors CCR5 and CXCR4 by flow cytometry analysis in PBLs from three donors. In these experiments PBLs were stimulated with IL-2 alone (without previous activation with anti-CD3/CD28), in the presence and absence of INK128, for 7 d. INK128 reduced percentages of CCR5 expressing cells in both the CD4+ and CD8+ subsets of T cells (Fig. 3A). INK128 also decreased CCR5 receptor density (molecules/cell). In contrast, INK128 did not change CXCR4 levels, either percentage or density (Fig. 3B). In addition, INK128 did not impact CD4 receptor levels (Fig. 3C). Together, these data suggest that INK128 inhibits R5 HIV entry by decreasing CCR5 levels, consistent with the observation that CCR5 levels are limiting for R5 HIV infection (29–32).

**INK128 Inhibits CCR5 Levels.** We next evaluated the effects of INK128 on activation of HIV in the chronically infected U1 cell line, which carries two copies of the HIV provirus per cell (33). Under basal conditions, U1 cells express low levels of HIV, but HIV expression is enhanced by stimulation with the phorbol ester PMA or by exogenous addition of Tat (34, 35). We cultured U1 cells in the presence of INK128 in the absence and presence of 10 nM PMA or 1 μg/mL Tat. INK128 was used at concentrations ≤10 nM because optimization experiments showed inhibition of cell proliferation at higher concentrations, consistent with the increased drug sensitivity of U937 cells (parent cell line of U1) to TOR-KIs compared with primary cells (36). As expected, unstimulated U1 cells produced low levels of HIV p24, but production was increased by addition of PMA or Tat. Importantly, INK128 inhibited p24 production in untreated cells as well and cells treated with PMA or Tat (Fig. 4). RT-PCR analyses showed that INK128 inhibits synthesis of full-length unspliced HIV mRNA (Fig. S2). Together, these data suggest that INK128 inhibits transcription, both basal and induced, of the HIV LTR.

**INK128 has Favorable Drug Interactions with Current Antiretroviral Classes.** Available ARTs target the HIV lifecycle steps of entry, reverse transcription, integration, and maturation. The observation that INK128 reduces CCR5 density and inhibits R5 HIV entry suggested that INK128 could enhance the antiviral activity of the CCR5 antagonist Maraviroc. In addition, by targeting virus transcription, INK128 could have favorable interactions with inhibitors of reverse transcription (RTIs), integration (IIs), and protease (PIs). We therefore evaluated the antiviral potency of each ART class in the presence and absence of INK128. We conducted these assays in activated PBLs infected with R5 HIV BaL and treated with various dilutions of Maraviroc (CCR5 antagonist), Efavirenz (RTI), Raltegravir (II), and Indinavir (PI). INK128 was used at low concentrations (≤EC50) to better detect changes in ART potency (Table S1). In experiments with two donors, INK128 enhanced the antiviral potency of Maraviroc by five- to sixfold, and had no negative effect on the potency of the other tested ARTs. Together, these data suggest that INK128 enhances the antiviral activity of Maraviroc, and it has favorable, nonantagonist drug interactions with the other existing ART classes.

**INK128 Inhibits HIV Replication in Humanized Mice.** We next evaluated the anti-HIV activity of INK128 in vivo, using NOD/SCID/IL2Rγnull (NSG) mice reconstituted with human PBLs and infected with HIV BaL. In pilot experiments, in which uninfected huPBL-NSG mice were treated with daily i.p. injections of INK128 at 0.5, 1, 3, 5, and 7 mg/kg for 2 wk, the 7 mg/kg dose was associated with wasting and death. Thus, we evaluated the antiviral activity of INK128 at 0 (PBS) (n = 6 mice), 1 mg/kg (n = 5), 3 mg/kg (n = 5), and 5 mg/kg (n = 5). Treatment was initiated immediately after virus injection and continued once daily for 14 d. Treatment had no adverse effects on the weight of the animals compared with controls (Fig. S3). Two mice, one in the control group and one in the 5 mg INK128/kg group, died in the course of the experiment. We could not determine the cause of death in the two animals, but incidental death, often the result of graft-versus-host disease from the transplanted human cells, is frequent in this animal model (37).

On day 7 after infection, control mice (n = 6) had mean plasma HIV RNA (copies per mL) of 3.3 × 10^6 (range, 2.1 × 10^6 to 5.2 × 10^6) (Fig. 5A). In INK128-treated mice, mean HIV RNA (copies/mL) were 1.2 × 10^6 (range, 1.7 × 10^6 to 4.5 × 10^6; n = 5; \( P = 0.3 \)), 8.5 × 10^5 (range, 3.5 × 10^5 to 1.7 × 10^6; n = 5; \( P = 0.008 \)) and 3.8 × 10^5 (range, 1 × 10^5 to 1 × 10^6; n = 4; \( P = 0.009 \)) at 1, 3, and 5 mg/kg/day doses, respectively. On day 14 after infection, mean plasma HIV RNA values were 1.2 × 10^6 (range, 2.4 × 10^5 to 2.4 × 10^6) in controls; and 1.1 × 10^6 (range, 5.2 × 10^5 to 2.1 × 10^6; \( P = 0.9 \)), 2.5 × 10^5 (range, 1.4 × 10^5 to 3.8 × 10^5; \( P = 0.03 \)) in INK128-treated groups. On day 28, mean plasma HIV RNA values were 1.2 × 10^6 (range, 2.4 × 10^6 to 2.4 × 10^6) in controls; and 1.1 × 10^6 (range, 5.2 × 10^5 to 2.1 × 10^6; \( P = 0.9 \)), 2.5 × 10^5 (range, 1.4 × 10^5 to 3.8 × 10^5; \( P = 0.03 \)) in INK128-treated groups. On day 28, mean plasma HIV RNA values were 1.2 × 10^6 (range, 2.4 × 10^5 to 2.4 × 10^6) in controls; and 1.1 × 10^6 (range, 5.2 × 10^5 to 2.1 × 10^6; \( P = 0.9 \)), 2.5 × 10^5 (range, 1.4 × 10^5 to 3.8 × 10^5; \( P = 0.03 \)) in INK128-treated groups.

![Fig. 3](https://www.pnas.org/ cgi/doi/10.1073/pnas.1511144112)

Heredia et al.
and $5 \times 10^3$ (range, $1.3 \times 10^3$ to $8 \times 10^3$; $P = 0.01$), at 1, 3, and 5 mg/kg/day doses, respectively. Consistent with reductions in viremia, infected mice treated with INK128 had higher CD4/CD8 ratios than did controls (Fig. 5B). Although CD4/CD8 ratios on day 7 were somewhat variable, day 14 ratios were significantly higher than controls. Day 14 mean CD4/CD8 cell ratios were 0.04 (range, 0.03–0.06) in control mice and 0.11 (range, 0.06–0.18; $P = 0.01$), 0.18 (range, 0.14–0.24; $P = 0.01$), and 0.76 (range, 0.5–1.14; $P = 0.01$), at 1, 3, and 5 mg/kg/day doses, respectively.

Together, these data demonstrate that INK128 suppresses viremia of the HIV reference strain BaL in a preclinical animal model. INK128 reduced plasma viremia by more than 2 log_{10} units, a decrease in viral load comparable to that achieved with EFdA, a potent NRTI in clinical trials, in a similar experimental setting (38).

**Discussion**

ART has transformed HIV infection into a chronic condition that requires life-long therapy (39). Patients on therapy can fail treatment, among other factors, because of the emergence of drug resistance. Most ART drugs target viral proteins, which can rapidly mutate and develop resistance under drug pressure. In contrast, targeting cellular proteins that HIV necessitates for successful completion of its lifecycle may slow emergence of resistance because cellular proteins have lower mutation rates than do viral proteins.

We have previously shown that targeting of cellular mTOR with rapamycin, which inhibits mTORC1 but not mTORC2, reduces CCR5 expression and R5 HIV entry in primary cells (11, 13, 15, 40). Roy et al. showed that rapamycin represses basal transcription of the HIV LTR without significantly affecting Tat-mediated transactivation (12). Consistent with these activities, rapamycin effectively inhibited the replication of R5, but not X4, HIV in primary PBLS (11, 13, 15). Nicoletti et al. have shown that rapamycin inhibits R5 HIV replication in a mouse model (41). In a recent human study, HIV-infected kidney transplant recipients treated with rapamycin had lower frequencies of lymphocytes containing HIV DNA than those treated with other immunosuppressive drugs, suggesting an anti-HIV effect of rapamycin (42). Together, these data validate rapamycin as an inhibitor of HIV, at least for R5 HIV strains.

Unlike rapamycin, the newly developed TOR-KIs inhibit both mTORC1 and mTORC2 (16–19). Using INK128 as a prototype TOR-KI (25–27), we demonstrate inhibition of CCR5 expression and inhibition of R5 HIV entry. INK128 potently inhibited basal transcription as well as transcription induced by PMA and by Tat. This broad antitranscriptional activity of INK128 is consistent with inhibition of mTORC2, whose activity is important for phosphorylation of PKC isoforms (including isoforms α and θ) (9, 20–22), and, in turn, induction of NF-κB (23, 24). By interfering with NF-κB induction, INK128 may prevent recruitment of the host transcription factor P-TEFb to the HIV LTR, thereby decreasing virus transcription (23, 43, 44). In infectivity assays using primary PBLS, INK128 inhibited both R5 and X4 HIV, laboratory-adapted and primary isolates, with EC_{50} values < 50 nM. Moreover, INK128 enhanced the antiviral potency of the CCR5 antagonist Maraviroc, probably by decreasing CCR5 levels, and had favorable antiviral interactions with inhibitors of reverse transcription, integration and protease. Thus, combinations

**Fig. 4.** INK128 inhibits HIV activation in chronically infected cells. Latently HIV infected U1 cells were cultured in the presence of various concentrations of INK128 for 1 h. Cultures were then untreated (A), or treated with HIV inducers PMA (B) and Tat protein (C). HIV production was measured by p24 ELISA in the culture supernatants on day 3. Data are means ± SD of three experiments.

**Fig. 5.** INK128 reduces plasma HIV RNA in humanized mice. Five- to seven-week-old NSG mice were intraperitoneally (i.p.) injected with PBLS (10^7 per mouse) from healthy donors. Three weeks later, successfully engrafted mice were i.p. injected with 15,000 TCID_{50} of HIV BaL. Immediately after virus challenge, i.p. treatment with INK128 or PBS was initiated and continued daily for 14 d. Plasma HIV RNA (copies per mL) was measured by quantitative RT PCR on days 7 and 14 (A). CD4/CD8 cell ratios on days 7 and 14 retroorbital blood samples were determined by Flow Cytometry Analysis (B). Short bars indicate geometric means. n.s. indicates non significant.
of ARTs and INK128 (or other TOR-KIs) might increase the antiviral potency of current ART regimens.

Oral administration of INK128 in mice has high absorption and bioavailability, with doses of 3 mg/kg giving a Cmax of 190 nM in plasma (25). These data suggest that anti-HIV drug levels can be achieved in vivo. Indeed, we show that INK128 reduces plasma viremia by more than 2 log10 units in humanized mice. This magnitude of virus suppression is similar to that achieved by EFdA, a potent NRTI in clinical development, in humanized mice (38). Thus, INK128, and perhaps other TOR-KIs, may have anti-HIV activity in vivo.

A counterintuitive, yet important, property of TOR-KIs is that their inhibition of both mTORC1 and mTORC2 is better tolerated by normal PBLs than targeting of mTORC1 alone with allosteric inhibitors (26, 45). It is possible that mTOR may have a noncatalytic scaffolding function that is suppressed by allosteric inhibition, but not with the catalytic inhibitor (45). It is also possible that catalytic inhibitors may have a more transient effect on blocking the kinase activity of mTOR, sufficient for anti-HIV activity but not for cellular toxicity. In agreement, INK128 did not decrease proliferation of primary PBLs at concentrations of up to 1 μM in our assays. Moreover, daily administration of INK128 inhibited HIV viremia in humanized mice without obvious toxicity, as determined by changes in body weight, over a 2-wk period.

Mechanistically, mTOR controls host protein synthesis mainly at the translation level (9). However, our data show that TOR-KI inhibition of HIV gene expression occurs at the transcriptional level, suggesting an indirect effect of the drug. It is possible that TOR-KIs interfere with translation of components in the protein machinery involved in HIV transcription, such as those in Tat protein "super-elongation complex" (46–48).

Our study has several limitations. One limitation is that antiviral studies in humanized mice were done with HIV BaL, which is a drug-sensitive, laboratory-adapted HIV strain. In future studies we plan to evaluate INK128 in mice infected with HIV primary isolates, particularly isolates from patients with resistance to ARTs. Another limitation is that we did not perform toxicity tests of INK128 in mice. We did not observe significant changes in weight in treated animals (Fig. S3), but in the absence of data on blood chemistry profiles or blood counts we cannot rule out potential toxicities. Moreover, we did not evaluate sufficient doses of INK128 to determine the therapeutic window, a critical parameter to determine its potential clinical utility of TOR-KIs against HIV. Future studies should determine the therapeutic window and dosing schedules for INK128 and additional TOR-KIs (18, 49–54) so as to identify the most effective and safest approach to inhibit HIV.

In addition to inhibiting the HIV lifecycle at transcription and at CCR5-mediated entry, TOR-KIs may help control HIV through immunomodulatory mechanisms. In particular, TOR-KIs may enhance both the quality and quantity of memory CD8 T cells (55, 56) and also T-cell responses promoted by myeloid dendritic cells (57).

TOR-KIs may help simplify treatment in the growing population of HIV patients with cancer (39, 58) by simultaneously targeting both conditions. In summary, targeting of cellular mTOR may help control HIV through CCR5-mediated entry, TOR-KIsmay help control HIV through positive selection using immunomagnetic beads following the manufacturer’s directions (Invitrogen). We analyzed cell fusion by measuring cytoplasmic dye exchange between fluorescent dye-labeled CD4 lymphocytes (targets) and transfected 293T cells expressing the R5 HIV Env or X4 HIV Env (effectors) using Flow Cytometry Analysis. This methodology is described in detail in SI Methods.

Quantification of CD4, CCR5, and CXCR4. Quantification of CCR5, CXCR4, and CD4 was done as described (59) using the following antibody clones: clone 45531 (CCR5), clone 12G5 (CXCR4), and clone RPA-T4 (CD4). For CCR5 and CXCR4, lymphocytes were first gated on CD3 (clone UCHT1) and CD4 (clone RPA-T4). For CD4, lymphocytes were gated using CD3 (clone UCHT1) in combination with CD8 (clone SK1). All antibodies were from BD Biosciences except for the CCR5 antibody, which was from R&D Systems. The methodology is fully described in SI Methods.

Real-Time PCR for Detection of Early Products of Reverse Transcription and of Integrated HIV DNA. Activated PBLs were infected with R5 and X4 HIV strains using a MOI of 0.01. Infected cells were cultured in the presence of IL-2 and different concentrations of INK128. Cell aliquots were collected at 16 and 72 h for analyses of early products of reverse transcription and of integration, respectively, by real time PCR. Detection of early products of reverse transcription was done with primers specific for the R-U5 region (60). For detection of integrated HIV DNA, we used a real-time nested Atu-HIV PCR assay previously described (61, 62), with the modifications described in SI Methods.

Semi-quantitative RT-PCR for Detection of Cellular HIV mRNA. Total cellular RNA was isolated using the Qiagen RNA Extraction Kit (Qiagen). RNA was then treated with DNase I, Amplification Grade (Invitrogen), and reverse transcribed with SuperScript III First-Strand Synthesis Supermix (Invitrogen) using hexamer primers. An aliquot of the cDNA was used as a template for PCR amplification of full length, unspliced HIV cDNA using primers US.1a and US.2a, and another aliquot for amplification with primer pairs specific for housekeeping [beta]-actin sequences (63).

Generation of Humanized Mice, Quantification of Plasma HIV RNA and Lymphocyte Subsets. Animal protocols were approved by the Institutional Animal Care and Use Committee, University of Maryland School of Medicine. NSG mice (5–7 wk) were infected intravenously (i.v.) with the laboratory-adapted HIV strain HIV BaL or HIV HXB2; primary isolates HIV 92BR020, 92UG031, 93BR029, and 93UG082; and multidrug resistant molecular clone NL4329129 (26). We obtained the NIH AIDS Repository. Primary isolate 2044 was from Paul Clapham (Windeyer Institute), and 1633 and 1638 from the Institute of Human Virology (University of Maryland School of Medicine). Maraviroc, efavirenz, raltegravir, and indinavir were from the NIH AIDS Repository. Primary isolate 1204 was purchased from ApexBio. Proliferation of PBLs was measured by the MTT kit (Roche), following the manufacturer’s directions. PBL infection assays were performed as described in SI Methods.

Cell Fusion Assay. CD4 cells were isolated from PBL cultures maintained for 7 d in the presence of different concentrations of INK128. Isolation of CD4 cells was done by positive selection using immunomagnetic beads following the manufacturer’s directions (Invitrogen). We analyzed cell fusion by measuring cytoplasmic dye exchange between fluorescent dye-labeled CD4 lymphocytes (targets) and transfected 293T cells expressing the R5 HIV Env or X4 HIV Env (effectors) using Flow Cytometry Analysis. This methodology is described in detail in SI Methods.

Supporting Information

Heredia et al. 10.1073/pnas.1511144112

SI Methods

MTT Assays. Cell proliferation was measured by a colorimetric MTT test (Roche). This test is based on the reduction of the yellow colored MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to blue formazan by mitochondrial dehydrogenases. The quantity of formazan produced (absorbance at 490 nm) is directly proportional to the number of living cells. Briefly, cell aliquots were seeded in 96-well plates (100 μL) and incubated with 10 μL of MTT solution for 4 h at 37 °C. A solubilization solution (50 μL) was added and plates incubated overnight at 37 °C. MTT conversion to formazan by mitochondrial dehydrogenase was assayed by optical density at 490 nm measured in an ELISA plate reader.

Infecitivity Assays. PBLs were separated from buffy coats by density centrifugation over Ficoll-Hypaque (Sigma), and activated by culture in the presence of 1 μg/mL anti-human CD3 antibody (clone x35, Fisher Scientific) and 2 μg/mL anti-human CD28 antibody (Clone CD28.6, eBioscience) for 3 d. Activated cells were infected by incubation with virus at a multiplicity of infection (MOI) of 0.001 for 2 h. Infected cells were washed three times with PBS and cultured in 5% CO2 at 37 °C, in RPMI/10% FBS supplemented with 50 units/mL IL-2 (Roche) and antiviral drugs, in 96-well flat-bottom plates at a density of 2 × 10^5 PBLs per 200 μL. Following 3 d of culture, the medium of half the plates was replaced with fresh medium containing IL-2 and antiviral drugs. On day 7, viral replication was measured by p24 ELISA (Coulter) in culture supernatants and cell viabilty was measured by MTT assays.

Cell Fusion Assay. CD4 cells were isolated from PBL cultures by density centrifugation over Ficoll-Hypaque (Sigma), and activated by culture in the presence of 1 μg/mL anti-human CD3 antibody (clone x35, Fisher Scientific) and 2 μg/mL anti-human CD28 antibody (Clone CD28.6, eBioscience) for 3 d. Activated cells were infected by incubation with virus at a multiplicity of infection (MOI) of 0.001 for 2 h. Infected cells were washed three times with PBS and cultured in 5% CO2 at 37 °C, in RPMI/10% FBS supplemented with 50 units/mL IL-2 (Roche) and antiviral drugs, in 96-well flat-bottom plates at a density of 2 × 10^5 PBLs per 200 μL. Following 3 d of culture, the medium of half the plates was replaced with fresh medium containing IL-2 and antiviral drugs. On day 7, viral replication was measured by p24 ELISA (Coulter) in culture supernatants and cell viabilty was measured by MTT assays.

Quantification of CD4, CCR5, and CXCR4. Before staining, PBLs were washed twice with PBS and incubated in blocking buffer (PBS containing 2% human serum, 5% horse serum, and 0.1% sodium azide) for 30 min at room temperature. Cells were then stained with the antibodies for 30 min at room temperature, washed twice with PBS, and acquired on a FACS Calibur (BD Biosciences) using Cellquest software (BD Biosciences). Immunofluorescence intensity was measured as an estimate of the average number of molecules on the cell surface. Fluorescence was measured using the Quantikine assay (BD Biosciences), which produces a regression line from a series of Quantibrite-phycocerythrin (PE) bead standards (BD Biosciences). The mean number of surface molecules for a cell labeled with a PE antibody was then determined from the FL-2 value of the cell using this linear regression and taking into account the PE/antibody ratio for each antibody (1:1 in our reagents).

Real-Time PCR for Detection of Early Products of Reverse Transcription and of Integrated HIV DNA. DNA was isolated using Mininiblood kit (Qiagen). PCR amplification was performed using Quantitect SYBR Green PCR Kit (Qiagen) in a LightCycler (Biorad). Detection of early products of reverse transcription was done in reactions containing 100 ng of DNA and the primer pair 5'-GCTCTCTGGCTAATGGAAC-3' and 5'-TGAC- TAAAAGGGTCTGAGGAT-3' (R/U5 region) (60). Samples were also amplified with primers for the housekeeping gene α-tubulin. Both sets of PCR reactions were done at an annealing temperature of 56 °C. Amplified products were analyzed by denaturation/renaturation to verify the specific Tm. The PCR cycle at which the signal entered the exponential range was used for quantification, and HIV copy numbers were corrected for those of α-tubulin. Standard curves for HIV and α-tubulin copy numbers were generated by analyzing serial dilutions of plasmds carrying the corresponding sequences. For detection of integrated HIV DNA, we used a real-time nested Alu-HIV PCR assay previously described (61, 62), with the following modifications. The first PCR used 100 ng of DNA template and primers Alu (5'-GCGTCCAAAGTGGCTATTACAG-3') and Gag (5'-GCTCTCGACCACATCTCTTCC-3') for 25 cycles. From this reaction, 1/20 of amplified product was used as template for the nested PCR with primers LTR-R (5'-GCTCTCAAAAGCTTTGCTGA-3') and LTR-U5 (5'-GACACTGACAAAAGGTCCTGA-3'), as described (61, 62) except for the annealing temperature, which was 61 °C in our reactions. As a standard curve for relative quantification of integrated DNA, the Alu-gag was first run using serial dilutions of DNA isolated from HIV infected PBLs (diluted in HIV-negative DNA).

Quantification of Plasma HIV RNA. For quantification of HIV RNA, viral RNA was extracted from 40 μL of plasma samples using Qiagen viral RNA Minikit (Qiagen). RNA was converted to cDNA using SuperScript III Supermix (Invitrogen). cDNA was amplified with HIV gag consensus primers (64), using Quantitect SYBR Green PCR kit (Qiagen) in a LightCycler (Biorad). Reactions were heated at 50 °C for 2 min, 95 °C for 15 s, followed by 35 amplification cycles (94 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s). A standard curve was prepared by serial dilutions of RNA extracted from plasma of an HIV patient with known HIV RNA copy number (HIV VQA RNA Quantification Standard; NIH AIDS Repository, catalog no. 3443). Peripheral blood CD4/CD8 ratios were determined by staining of whole blood with FITC-conjugated mouse anti-CD4 and APC-conjugated
mouse anti-CD8 monoclonal antibodies (BD Pharmingen), followed by Flow Cytometry Analysis.

**Statistics Analyses.** EC$_{50}$ values were determined by variable slope nonlinear regression analysis. Unpaired two-tailed $t$ tests were used to check for statistical significant differences between INK128 EC$_{50}$ values of R5 HIV versus X4 HIV in infectivity assays. Nonparametric Mann–Whitney tests were used to compare each treatment group and control group in animal studies. Statistical analyses were performed using GraphPad Prism (version 4.0). $P < 0.05$ was considered significant.

---

**Fig S1.** INK128 inhibits replication of multidrug resistant HIV molecular clone NL43$_{29129}$–2. Activated PBLs were infected for 2 h using a MOI of 0.001. Infected cells were cultured in IL-2 medium and the indicated concentrations of INK128. On day 7, virus production was measured by p24 ELISA in the culture supernatants (A), and cell viability was measured by MTT (B). Data are means ± SD of two independent experiments using two different donors. Data are normalized to values in cultures without INK128 (100%).

**Fig S2.** INK128 inhibits HIV transcription in U1 cells. U1 cells were cultured in the presence of 10 nM PMA and the indicated concentrations of INK128. After 2 d, cells were collected, mRNA isolated, quantified, reverse transcribed and amplified by real time PCR using unspliced HIV cDNA primer pair US.1a/US.2a and housekeeping (beta)-actin primers. For quantification, standard curves for unspliced HIV cDNA and [beta]-actin sequences were generated by performing 10-fold serial dilutions of mRNA isolated from PBLs acutely infected with HIV BaL. PCR amplification was performed using Quantitect SYBR Green PCR Kit in a LightCycler. Negative controls consisted of mixture reactions without the reverse transcription step. Data are means ± SD of two experiments, expressed as relative HIV mRNA expression compared with cultures containing no INK128 after normalization to [beta]-actin levels.
Fig S3. INK128 treatment does not result in weight loss in humanized mice. (A) Mice weight at the beginning of experiment (day 0) and after 14 d of daily treatment (i.p.) with INK128. (B) Comparison of changes in body weight (day 0 to day 14) between INK128-treated mice. Nonparametric statistical analyses of the data were performed using the Mann–Whitney u test (GraphPad Prism Software); \( P < 0.05 \) was considered significant. Short bars indicate geometric means. n.s. indicates non significant.

Table S1. Activity of INK128 against primary isolates of HIV-1 in PBMCs

<table>
<thead>
<tr>
<th>Primary Isolate</th>
<th>Coreceptor tropism</th>
<th>Geometric mean INK128 EC_{50}, nM (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 93BR029</td>
<td>R5</td>
<td>4.73 (2.78–8.05)</td>
</tr>
<tr>
<td>HIV-1 92UG031</td>
<td>R5</td>
<td>10.15 (3.73–27.60)</td>
</tr>
<tr>
<td>HIV-1 93UG082</td>
<td>R5</td>
<td>2.89 (0.82–10.13)</td>
</tr>
<tr>
<td>HIV-1 92BR020</td>
<td>R5</td>
<td>1.03 (0.48–2.19)</td>
</tr>
<tr>
<td>HIV-1 2044</td>
<td>X4</td>
<td>36.72 (13.38–100.8)</td>
</tr>
<tr>
<td>HIV-1 1633</td>
<td>X4</td>
<td>17.47 (6.89–44.24)</td>
</tr>
<tr>
<td>HIV-1 1638</td>
<td>X4</td>
<td>21.20 (3.25–138.4)</td>
</tr>
</tbody>
</table>

*EC_{50} values were determined by variable slope nonlinear regression analysis using GraphPad Prism software. Data are from three experiments, each with a different donor.
Table S2. Fifty percent effective concentrations (EC\textsubscript{50}) of Maraviroc (MVC), Efavirenz (EFV), Raltegravir (RAL), and Indinavir (IND) against HIV-1 BaL, in the absence and presence of INK128 in PBMCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MVC EC\textsubscript{50} (95% CI)</th>
<th>EFV EC\textsubscript{50} (95% CI)</th>
<th>RAL EC\textsubscript{50} (95% CI)</th>
<th>IND EC\textsubscript{50} (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No INK128</td>
<td>1.78 nM (0.67–4.76)</td>
<td>0.52 nM (0.31–0.87)</td>
<td>0.65 nM (0.34–1.13)</td>
<td>3.84 nM (3.21–4.57)</td>
</tr>
<tr>
<td>+3 nM INK128</td>
<td>0.54 nM (0.20–1.50)</td>
<td>0.51 nM (0.34–0.75)</td>
<td>0.81 nM (0.49–1.14)</td>
<td>3.14 nM (2.31–4.27)</td>
</tr>
<tr>
<td>+10 nM INK128</td>
<td>0.30 nM (0.20–0.45)</td>
<td>0.51 nM (0.33–0.78)</td>
<td>0.57 nM (0.24–1.34)</td>
<td>3 nM (1.10–8.18)</td>
</tr>
</tbody>
</table>

Prism software. Data are from two experiments, each with a different donor. *EC\textsubscript{50} values are geometric means, determined by variable slope nonlinear regression analysis using GraphPad.