Indirubin 3'-Monoxime, from a Chinese Traditional Herbal Formula, Suppresses Viremia in Humanized Mice Infected with Multidrug-Resistant HIV

Alonso Heredia, Senthilkumar Natesan, Nhut M. Le, Sandra Medina-Moreno, Juan C. Zapata, Marvin Reitz, Joseph Bryant, and Robert R. Redfield

EDITOR: Resistance of HIV to drugs compromises the success of antiretroviral therapy (ART), highlighting the need for drugs with novel targets and mechanisms of action. Most ART drugs target viral proteins. Targeting cellular, rather than viral, proteins may delay the emergence of HIV resistance as cellular proteins are less likely to mutate under drug pressure than are viral proteins. Cellular human positive transcription elongation factor (P-TEFb), composed of cyclin-dependent kinase 9 (CDK9) and cyclin T1, regulates RNA Polymerase II dependent transcription of cellular and integrated HIV genes.^{1–6} Consistent with these findings, the crystal structure of Tat bound to P-TEFb has revealed intimate contacts between Tat and the T loop of CDK9, as well as considerable interactions between Tat and cyclin T1.⁷ Targeting of P-TEFb with the CDK inhibitor flavopiridol inhibits HIV expression,⁸ but has unfavorable toxicities.^{9–11} Indirubin, another CDK inhibitor,¹² is the active ingredient of the traditional Chinese herbal formula Dan Gui Long Hui Wan, used to treat chronic myelogenous leukemia and psoriasis.¹³ We have previously demonstrated that indirubin-3'-monoxime (IM), a derivative of indirubin with improved solubility, is an inhibitor of CDK9 and P-TEFb function that blocks HIV gene expression in the absence of toxicity.¹⁴ IM inhibited both sensitive and drug-resistant strains of HIV. The anti-HIV activity of IM *in vitro* has also been confirmed by others.¹⁵

We now report on the *in vivo* anti-HIV activity of IM in a preclinical mouse model, NOD/SCID/IL-2R γ^{null} (NSG) mice engrafted with human peripheral blood lymphocytes (PBLs) and infected with HIV.¹⁶ Human PBL-engrafted mice were intraperitoneally (ip) injected with 20,000 units of 50% tissue culture infective dose (TCID₅₀) of HIV, followed by daily ip treatment with IM (10–40 mg/kg/day, dissolved in 10% Cremophor EL) or phosphate-buffered saline (PBS) (in 10% Cremophor EL solution) for 18 days. IM was purchased from Cayman Chemicals (Ann Arbor, MI) and Cremophor EL from Sigma (St. Louis, MO). IM doses were based on previous reports demonstrating lack of toxicity.^{17–19} Blood samples, drawn from the retroorbital vein on days 9 and 18 after infection, were analyzed for plasma HIV RNA copy number (quantitative RT-PCR) and human CD4/CD8 ratios

(flow cytometry analysis). Nonparametric statistical analyses of the data were performed using the Mann-Whitney *U* test (GraphPad Prism Software, La Jolla, CA); $p < 0.05$ was considered significant.

In a first experiment, we evaluated the *in vivo* activity of IM against multidrug-resistant molecular clone NL43₇₃₀₃₋₃, which carries the reverse transcriptase (RT) gene amplified from plasma of a patient with multidrug-resistant HIV.²⁰ This virus has mutations 41L, 67N, 210W, 215Y, 69D, 44D, and 118I, conferring resistance to thymidine analogs (AZT, d4T), guanosine analog (ABC), and adenosine analogs (ddI and TDF). On day 9, HIV RNA was undetectable in most samples (not shown). Day 18 data are shown in Fig. 1a. In mice given PBS ($n = 6$), mean plasma HIV RNA (copies/ml) was 7.6×10^6 (range, 2.5×10^6 to 7×10^7). In IM-treated mice, mean HIV RNA (copies/ml) was reduced to 1.7×10^6 (range, 3.4×10^5 to 1×10^7 ; $n = 5$; $p = 0.17$), 2.3×10^5 (range, 1.1×10^4 to 1×10^6 ; $n = 6$; $p = 0.002$), and 3.8×10^4 (range, 1×10^4 to 1.3×10^5 ; $n = 5$; $p = 0.004$) at 10, 20, and 40 mg/kg/day doses, respectively. The mean CD4/CD8 cell ratio in mice given PBS was 0.021 (range, 0.01 to 0.03). In contrast, the ratios were significantly higher in IM-treated mice. The mean CD4/CD8 ratios were 0.24 (range, 0.14 to 0.34; $p = 0.007$), 0.34 (range, 0.16 to 0.55; $p = 0.004$), and 0.19 (range, 0.07 to 0.32; $p = 0.007$) at the 10, 20, and 40 mg/kg doses, respectively. IM did not significantly impact body weights in treated animals ($p > 0.1$ in all three IM treatments compared to PBS treatment) or have any apparent toxicity. Together, these data demonstrate that IM reduces multidrug-resistant HIV NL43₇₃₀₃₋₃ replication *in vivo*.

We confirmed the *in vivo* activity of IM using another virus, multidrug-resistant molecular clone NL43₅₂₅₃₄₋₂ (Fig. 1b). NL43₅₂₅₃₄₋₂, also derived from a patient with multidrug resistance, carries mutations 41L, 210W, 215Y, 184V, 69 insertion, and 74V.²⁰ The presence of the 69 insertion and the 184V mutation, together with thymidine analog mutations, confers high-level resistance to all nucleoside reverse transcriptase inhibitors (NRTIs), including the cytosine analogs 3TC and FTC.²¹ IM was used at doses of 10 and 20 mg/kg/day. As with NL43₇₃₀₃₋₃, plasma NL43₅₂₅₃₄₋₂ RNA levels were undetectable on day 9 (not shown). On day 18, PBS-treated

FIG. 1. Indirubin-3'-monoxime (IM) reduces replication of multi-drug-resistant HIV in humanized mice. Six- to 8-week-old female mice were intraperitoneally (ip) injected with peripheral blood lymphocytes (PBLs) (10^7 per mouse) from healthy donors. Three weeks later, successfully engrafted mice were ip injected with 20,000 TCID₅₀s of HIV molecular clones NL4-3₇₃₀₃₋₃ (A) or NL4-3₅₂₅₃₄₋₂ (B). Immediately after virus challenge, ip treatment with IM or phosphate-buffered saline (PBS) was initiated and continued daily for 18 days. Upper panel shows plasma HIV RNA (copies/ml) measured by quantitative RT-PCR on day 18; samples giving undetectable results were assigned the lower detection limit (DL) of the assay (150 copies/ml). Middle panel depicts CD4/CD8 cell ratios on day 18 retroorbital blood samples, determined by flow cytometry analysis. Lower panel depicts body weights. Short bars indicate geometric (upper panel) and arithmetic (middle and lower panels) means. N.S., nonsignificant.

mice ($n=8$) had a mean HIV RNA (copies/ml) of 1.2×10^5 (range, ≤ 150 to 1.8×10^6). IM significantly suppressed HIV RNA to a mean of 2.7×10^3 (range, 150 to 8×10^4 ; $n=6$; $p=0.028$) at 10 mg/kg, and to a mean of 1×10^3 (range, 150 to 3.5×10^5 ; $n=7$; $p=0.023$) at 20 mg/kg. Mean CD4/CD8 ratios were 1.3 (range, 0.10 to 4.80) in PBS-treated mice, 1.01 (range, 0.40 to 2.1; $p=0.4$) at 10 mg IM/kg, and 2.51 (range, 0.65 to 4.68; $p=0.11$) at 20 mg IM/kg. Again, IM treatment did not impact body weight or have any identifiable adverse effects (not shown).

Together, the data demonstrate that IM suppresses viremia of two reference multidrug-resistant HIV molecular clones in a preclinical animal model. Although IM reduced plasma viremia by more than 2 log₁₀ units in both virus infections, HIV RNA levels were still detectable and relatively high in

some of the treated animals. This suggests that treatment with one drug alone may not be sufficient to completely inhibit plasma viremia in this animal model. In agreement, single treatment with EFdA (a potent NRTI in clinical development) decreased plasma viremia by ~ 2 log₁₀ units, yet to detectable levels, in a similar mouse model.²² Other limitations of the animal model used are the absence of multilineage hematopoiesis and of primary immune responses.²³ Thus, any potential contribution of IM to HIV treatment in patients should be viewed in the context of combined ART and of a whole human immune system.

Consistent with reductions in viremia and decreased CD4⁺ T cell depletion, NL43₇₃₀₃₋₃-infected mice treated with IM had significantly higher CD4/CD8 ratios than did PBS-treated controls. Although we did not measure CD4/CD8 ratios in

uninfected mice, ratios ranging between ~ 0.5 and 2 have been reported by others in uninfected NSG mice.^{16,22,24} In NL43₅₂₅₃₄₋₂-infected mice, IM caused a significant change in viremia but not in CD4/CD8 ratios. That the CD4/CD8 ratio did not change in infection with NL43₅₂₅₃₄₋₂ might be due to low virus replication levels (mean HIV RNA copies/ml of 1.2×10^5 in PBS-treated NL43₅₂₅₃₄₋₂ versus 7.6×10^7 in PBS-treated NL43₇₃₀₃₋₃ mice) and thereby reduced CD4⁺ T cell killing, consistent with the reduced viral fitness associated with the 184V mutation in NL43₅₂₅₃₄₋₂.²⁵

In clinical trials of indirubin (parent compound of IM) in China, daily oral doses of 150–450 mg were effective against chronic myelogenous leukemia.¹³ Treatment was well tolerated and the main side effect was gastrointestinal tract problems. However, indirubin pharmacokinetic data were not reported. Currently there are only limited animal data on the toxicity of IM,^{17–19} and no validated human or animal data on pharmacokinetics, making it difficult to predict what doses will inhibit HIV in patients. In our experiments, IM was administered via ip to reduce variability often observed with oral administration. Since oral indirubin is effective against leukemia and IM is more soluble than indirubin, we expect that oral administration of IM will be effective against HIV in patients in the absence of toxicity. In future studies we plan to evaluate the pharmacokinetics and pharmacodynamics of IM. We will also evaluate toxicity by examining blood chemistry profiles and blood counts as well as toxic lesions in different organs, both in IM treatment alone and in combination with ARTs.

In summary, these data demonstrate inhibition of multidrug-resistant HIV by targeting viral gene expression, a step of the viral cycle not targeted by current ARTs, with IM *in vivo*. Despite the limitations of the animal model,²³ IM, and perhaps flavopiridol analogs²⁶ and recently developed mimetic CDK9 inhibitors,²⁷ may provide effective approaches for controlling HIV, particularly drug-resistant strains, in patients.

Acknowledgments

This work was supported by research funds from the Institute of Human Virology.

Authors contributions: A.H., S.N., M.R., J.B., and R.R.R. designed the experiments; A.H., S.N., S.M., J.Z., and N.M.L. performed the experiments; A.H., M.R., J.B., and R.R.R. contributed to data analysis, discussion of results, and writing of the article. We thank Lanea George (Animal Facility, IHV) for outstanding technical assistance. We thank George L. Drusano (Institute of Therapeutic Innovation, College of Medicine, University of Florida) for critical reading of the article and input on future pharmacokinetic studies.

Animal protocols were approved by the Institutional Animal Care and Use Committee, University of Maryland School of Medicine.

HIV-1 molecular clones carrying RT sequences amplified from the plasma of patients with multidrug resistance were obtained from Dr. Robert Shafer (Stanford University School of Medicine, Stanford, CA) through the NIH AIDS Repository (Gaithersburg, MD).

Author Disclosure Statement

No competing financial interests exist.

References

1. Kao SY, Calman AF, Luciw PA, and Peterlin BM: Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* 1987;330(6147):489–493.
2. Dingwall C, Ernberg I, Gait MJ, *et al.*: HIV-1 tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure. *EMBO J* 1990;9(12):4145–4153.
3. Marciniak RA and Sharp PA: HIV-1 Tat protein promotes formation of more-processive elongation complexes. *EMBO J* 1991;10(13):4189–4196.
4. Yang X, Gold MO, Tang DN, *et al.*: TAK, an HIV Tat-associated kinase, is a member of the cyclin-dependent family of protein kinases and is induced by activation of peripheral blood lymphocytes and differentiation of promonocytic cell lines. *Proc Natl Acad Sci USA* 1997;94(23):12331–12336.
5. Zhu Y, Pe'ery T, Peng J, *et al.*: Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation *in vitro*. *Genes Dev* 1997;11(20):2622–2632.
6. Wei P, Garber ME, Fang SM, Fischer WH, and Jones KA: A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* 1998;92(4):451–462.
7. Tahirov TH, Babayeva ND, Varzavand K, Cooper JJ, Sedore SC, and Price DH: Crystal structure of HIV-1 Tat complexed with human P-TEFb. *Nature* 2010;465(7299):747–751.
8. Chao SH, Fujinaga K, Marion JE, *et al.*: Flavopiridol inhibits P-TEFb and blocks HIV-1 replication. *J Biol Chem* 2000;275(37):28345–28348.
9. Salerno D, Hasham MG, Marshall R, Garriga J, Tsygankov AY, and Grana X: Direct inhibition of CDK9 blocks HIV-1 replication without preventing T-cell activation in primary human peripheral blood lymphocytes. *Gene* 2007;405(1–2):65–78.
10. Biglione S, Byers SA, Price JP, *et al.*: Inhibition of HIV-1 replication by P-TEFb inhibitors DRB, seliciclib and flavopiridol correlates with release of free P-TEFb from the large, inactive form of the complex. *Retrovirology* 2007;4:47.
11. Liu X, Shi S, Lam F, Pepper C, Fischer PM, and Wang S: CDKI-71, a novel CDK9 inhibitor, is preferentially cytotoxic to cancer cells compared to flavopiridol. *Int J Cancer* 2012;130(5):1216–1226.
12. Hoessel R, Leclerc S, Endicott JA, *et al.*: Indirubin, the active constituent of a Chinese antileukemia medicine, inhibits cyclin-dependent kinases. *Nat Cell Biol* 1999;1(1):60–67.
13. Xiao Z, Hao Y, Liu B, and Qian L: Indirubin and meisoindigo in the treatment of chronic myelogenous leukemia in China. *Leuk Lymphoma* 2002;43(9):1763–1768.
14. Heredia A, Davis C, Bamba D, *et al.*: Indirubin-3'-monoxime, a derivative of a Chinese antileukemia medicine, inhibits P-TEFb function and HIV-1 replication. *AIDS* 2005;19(18):2087–2095.
15. Toossi Z, Wu M, Hirsch CS, *et al.*: Activation of P-TEFb at sites of dual HIV/TB infection, and inhibition of MTB-induced HIV transcriptional activation by the inhibitor of CDK9, Indirubin-3'-monoxime. *AIDS Res Hum Retroviruses* 2012;28(2):182–187.
16. Nakata H, Maeda K, Miyakawa T, *et al.*: Potent anti-R5 human immunodeficiency virus type 1 effects of a CCR5

- antagonist, AK602/ONO4128/GW873140, in a novel human peripheral blood mononuclear cell nonobese diabetic-SCID, interleukin-2 receptor gamma-chain-knocked-out AIDS mouse model. *J Virol* 2005;79(4):2087–2096.
17. Selenica ML, Jensen HS, Larsen AK, *et al.*: Efficacy of small-molecule glycogen synthase kinase-3 inhibitors in the postnatal rat model of tau hyperphosphorylation. *Br J Pharmacol* 2007;152(6):959–979.
 18. Sugihara K, Okayama T, Kitamura S, *et al.*: Comparative study of aryl hydrocarbon receptor ligand activities of six chemicals in vitro and in vivo. *Arch Toxicol* 2008;82(1):5–11.
 19. Ding Y, Qiao A, and Fan GH: Indirubin-3'-monoxime rescues spatial memory deficits and attenuates beta-amyloid-associated neuropathology in a mouse model of Alzheimer's disease. *Neurobiol Dis* 2010;39(2):156–168.
 20. Johnston E, Dupnik KM, Gonzales MJ, *et al.*: Panel of prototypical infectious molecular HIV-1 clones containing multiple nucleoside reverse transcriptase inhibitor resistance mutations. *AIDS* 2005;19(7):731–733.
 21. Larder BA, Bloor S, Kemp SD, *et al.*: A family of insertion mutations between codons 67 and 70 of human immunodeficiency virus type 1 reverse transcriptase confer multinucleoside analog resistance. *Antimicrob Agents Chemother* 1999;43(8):1961–1967.
 22. Hattori S, Ide K, Nakata H, *et al.*: Potent activity of a nucleoside reverse transcriptase inhibitor, 4'-ethynyl-2-fluoro-2'-deoxyadenosine, against human immunodeficiency virus type 1 infection in a model using human peripheral blood mononuclear cell-transplanted NOD/SCID Janus kinase 3 knockout mice. *Antimicrob Agents Chemother* 2009;53(9):3887–3893.
 23. Akkina R: New generation humanized mice for virus research: Comparative aspects and future prospects. *Virology* 2013;435(1):14–28.
 24. Roy U, McMillan J, Alnouti Y, *et al.*: Pharmacodynamic and antiretroviral activities of combination nanoformulated antiretrovirals in HIV-1-infected human peripheral blood lymphocyte-reconstituted mice. *J Infect Dis* 2012;206(10):1577–1588.
 25. Back NK, Nijhuis M, Keulen W, *et al.*: Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme. *EMBO J* 1996;15(15):4040–4049.
 26. Ali A, Ghosh A, Nathans RS, *et al.*: Identification of flavopiridol analogues that selectively inhibit positive transcription elongation factor (P-TEFb) and block HIV-1 replication. *ChemBioChem* 2009;10(12):2072–2080.
 27. Van Duyne R, Guendel I, Jaworski E, *et al.*: Effect of mimetic CDK9 inhibitors on HIV-1-activated transcription. *J Mol Biol* 2013;425(4):812–829.

Address correspondence to:
Alonso Heredia
Institute of Human Virology
725 West Lombard Street
Baltimore, Maryland 21201

E-mail: aheredia@ihv.umaryland.edu