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Authors

Kadiyala, Gayatri Nikhila
Telwatte, Sushama
Wedrychowski, Adam
et al.

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Differential susceptibility of cells infected with defective and intact HIV proviruses to killing by obatoclax and other small molecules

Gayatri Nikhila Kadiyala[a,b], Sushama Telwatte[a,b], Adam Wedrychowski[a,b], Julie Janssens[a,b], Sun Jin Kim[a,b], Peggy Kim[b], Steven Deeks[a], Joseph K. Wong[a,b] and Steven A. Yukl[a,b]

Objectives: Some drugs that augment cell-intrinsic defenses or modulate cell death/survival pathways have been reported to selectively kill cells infected with HIV or Simian Immunodeficiency Virus (SIV), but comparative studies are lacking. We hypothesized that these drugs may differ in their ability to kill cells infected with intact and defective proviruses.

Design: To investigate this hypothesis, drugs were tested *ex vivo* on peripheral blood mononuclear cells (PBMC) from nine antiretroviral therapy (ART)-suppressed individuals.

Methods: We tested drugs currently in clinical use or human trials, including auranofin (p53 modulator), interferon alpha2A, interferon gamma, acitretin (RIG-I inducer), GS-9620/vesatolimod (TLR7 agonist), nivolumab (PD-1 blocker), obatoclax (Bcl-2 inhibitor), birinapant [inhibitor of apoptosis proteins (IAP) inhibitor], bortezomib (proteasome inhibitor), and INK128/sapanisertib [mammalian target of rapamycin mTOR] [c]1/2 inhibitor). After 6 days of treatment, we measured cell counts/viabilities and quantified levels of total, intact, and defective HIV DNA by droplet digital PCR (Intact Proviral DNA Assay).

Results: Obatoclax reduced intact HIV DNA [median = 27–30% of dimethyl sulfoxide control (DMSO)] but not defective or total HIV DNA. Other drugs showed no statistically significant effects.

Conclusion: Obatoclax and other Bcl-2 inhibitors deserve further study in combination therapies aimed at reducing the intact HIV reservoir in order to achieve a functional cure and/or reduce HIV-associated immune activation.

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Keywords: apoptosis, Bcl-2, DNA, HIV, IAP, interferon, mTOR, PD-1, proteasome, RIG-I, TLR7

Introduction

Antiretroviral therapy (ART) can suppress HIV replication and reduce mortality and morbidity, but it does not cure HIV or fully restore health [1]. Persistent cellular reservoirs such as latently infected $CD4^+$ T cells [2–4] allow viral recrudescence after stopping ART. Many infected cells also continue to express viral products during ART [5], which likely contribute to the immune activation/inflammation, organ diseases, and reduced life expectancy that are observed despite years of suppressive ART [1]. Although the mechanisms of HIV persistence are not fully understood, latently HIV-infected cells appear to have multiple mechanisms that help evade killing and promote survival.

[a]Department of Medicine, University of California, San Francisco, and [b]Department of Medicine, San Francisco Veterans Affairs Medical Center, San Francisco, CA, USA.

Correspondence to Steven A. Yukl, 4150 Clement Street, 111W3, San Francisco, CA 94121, USA.

E-mail: Steven.Yukl@ucsf.edu

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HIV infection can trigger the death of both infected and uninfected cells through apoptosis, pyroptosis, and possibly necroptosis [6–9]. HIV RNA and/or DNA can be sensed by intracellular pattern recognition receptors (PRR) such as toll-like receptors (TLRs) [10,11] and RIG-I-like receptors (RLRs) [12], which can trigger antiviral defenses such as interferons (IFN) to induce cell death through apoptosis. Incomplete products of reverse transcription can also trigger pyroptosis through recognition by IFI16 [7], and HIV integration can induce death through activation of a DNA-dependent protein kinase, leading to phosphorylation of p53 [13,14]. In addition, HIV protease, Vpr, and Env can also stimulate apoptosis [15].

However, some HIV-infected cells may also escape death through various mechanisms. HIV preferentially infects memory $CD4^+$ T cells, which normally upregulate antiapoptotic proteins such as Bcl-2, inhibitor of apoptosis proteins (IAP), and cFLIP to prevent activation-induced cell death and promote long-term survival [16,17]. During early infection, some HIV proteins (such as Vpr, Nef, and Tat) can also exert antiapoptotic effects [18]. For example, Tat induces CFLAR (cFLIP) in primary T cells [19] and upregulates NF-κB-dependent apoptosis inhibitors, including Bcl-2, XIAP and cIAP-1 (BIRC2) [20]. Tat also binds p53 and prevents it from binding to PTEN, leading to downregulation of PTEN, activation of PI3K/Akt, and inhibition of apoptosis [21,22]. In Jurkat T cells, Vpr increases expression of Bcl-2 and decreases Bax expression [23]. HIV Nef can bind to and activate PI3K, which inhibits phosphorylation of Bad and blocks apoptosis [21,22]. Tat, Nef, and Env can also inhibit autophagy, which plays a role in intracellular defenses to pathogens and is essential for MHC loading [12].

Models of latent HIV infection have also demonstrated alterations in transcription of cellular genes regulating apoptosis. For example, ACH2 cells upregulate anti-apoptotic XIAP relative to CEM cells [24], and knockdown of pro-apoptotic factors (Bax, FADD) prolongs survival in HIV-infected Jurkat cells [25]. Productively and latently in vitro-infected primary $CD4^+$ T cells upregulate BIRC5 (survivin) relative to uninfected cells [26], whereas latently infected cells upregulate FAS, FASLG, BIRC2, and XIAP [27].

Although lower HIV expression in latently infected cells may allow them to escape killing, therapies that increase HIV expression ['latency reversing agents' (LRAs)] do not necessarily lead to cell death in primary cell models [28,29] or *in vivo*. For example, multiple clinical trials with LRAs have shown induction of cell or plasma HIV RNA but little or no cell killing as measured by HIV DNA or Quantitative Viral Outgrowth Assays [30–36]. It is unclear why HIV expression can lead to cytotoxicity in some circumstances (acute infection) but not others (reactivation from latency). It is possible that these LRAs do not effectively induce the levels and/or types of HIV RNA necessary for recognition by intracellular defenses or extracellular immune responses, which may be dysfunctional. However, cell death may also be impaired by antiapoptotic mechanisms that occur during the development of memory or latent infection. If so, other therapies may be required to counter these mechanisms.

Many different small molecules have been reported to induce selective killing of HIV-infected cells and/or to reduce some measure of HIV-infected or Simian Immunodeficiency Virus (SIV)-infected cells, such as proviral DNA or inducible reservoirs [9,17]. These molecules include p53 modulators [37], interferons [38–42], RIG-I inducers [43], TLR7 agonists [44,45], PD-1 blockers [46], Bcl-2 inhibitors [47–50], IAP/XIAP inhibitors [27,51–53], BIRC5 inhibitors [26], DDX3 inhibitors [54], PI3/Akt inhibitors [55,56], proteasome inhibitors [57], mammalian target of rapamycin (mTOR) inhibitors [58,59], TREM1 inhibitors [60,61], an inhibitor of HIV Rev and inflammation [14,62], and autosis-inducing peptides [63] (see Table, Supplemental Digital Content 1, http://links.lww.com/QAD/D183; please note that these drugs are not approved for use in reducing HIV reservoirs). If these agents prove well tolerated and effective, they could be used along with ART or other therapies in strategies aimed to reduce HIV-associated immune activation or cure HIV. A major benefit of these drugs is that their effect probably does not depend on latency reversal or functional extrinsic immune responses. However, these agents could also be used to sensitize or 'prime' latently infected cells for apoptosis prior to latency reactivation ('prime, shock, and kill' [47]) and/or immune-based therapies. Alternatively, they could be used to reduce the HIV reservoir prior to treatment with latency promoting agents ('kill, block, and lock') [12].

However, prior studies with these agents have used very different methods, and there have been no systematic studies to compare the degree to which these drugs selectively kill HIV-infected cells. The efficacy and selectivity for HIV-infected cells may depend on the degree to which HIV infection and/or latency leads to differential expression of specific cellular target genes, or if the killing is dependent on the expression of HIV RNA or pro-apoptotic HIV proteins. Therefore, we hypothesized that drugs acting through different mechanisms would have varying ability to kill HIV-infected cells, and that these effects may vary in cells infected with intact or defective [64,65] proviruses. To investigate these questions, we selected drugs with varying mechanisms (prioritizing those that have been used in humans) and compared their ability to selectively reduce total, intact, or defective HIV DNA after ex-vivo treatment of PBMC from ART-suppressed PWH. Although most drugs exhibited inconsistent effects, we found that the Bcl-2 inhibitor obatoclax reduced intact but not total or defective HIV DNA.

Table 1. Demographic and clinical characteristics of the study participants.

PID	$CD4^+$ nadir	Estimated years ART exposure	Estimated min. length of viral suppression (years)	CD4%	CD4	CD8%	$CD8^+$	VL	Comments (VL)	ART regimen
0156	328	19	14.8	38	965	51	1290	<40	Not detected	DTG, FTC/TAF
2046	10	29.6	22.8	24	335	34	476	<40	Detected	BIC/FTC/TAF
0176	662[a]	13.5	19.5[a]	30	830	42	1161	<40	Not detected	DTG, FTC/TAF
2027	172	30.8	22.6	12	471	73	2902	<30	Detected	DTG/3TC
2256	86	29.3	17.2	24	366	49	752	<30	Detected	RPV/TAF/FTC, DTG
3147	4	29.7	15.1	31	585	26	500	<30	Not detected	BIC/FTC/TAF
2747	357	15.5	5.1	37	562	29	447	<30	Not detected	DTG, FTC/TAF
2146	99	27.7	18.5	26	475	48	887	<30	Not detected	BIC/FTC/TAF
1713	50	12.2	11.8	33	708	50	1078	<30	Not detected	BIC/FTC/TAF

ART, antiretroviral therapy; VL, viral load.
[a]Elite controller and long-term nonprogressor.

Materials and methods

Study participants

Blood samples were obtained from nine ART-suppressed individuals (Table 1). Two of the nine participants were recruited from the San Francisco Veterans Affairs Medical Center (SFVAMC), whereas the remainder were recruited from the observational Study of the Consequences of the Protease Inhibitor Era (SCOPE/Options) cohort from the University of California, San Francisco (UCSF). Inclusion criteria included $CD4^+$ T-cell counts at least 300 cells/μl and viral load less than 50 copies/ml for at least 6 months. All participants provided written informed consent. The study was approved by the IRB of UCSF and the SFVAMC.

Peripheral blood mononuclear cell isolation and cell culture

Peripheral blood mononuclear cells (PBMC) were recovered by Ficoll density centrifugation from fresh venous blood. Blood (100–120 ml) was spun for 10 min without braking at 1000*g* and the plasma layer was removed. The remaining blood was resuspended in PBS, layered onto Ficoll, and spun without braking for 20 min at 160*g* and again for 10 min at 350*g*. The buffy coat of PBMC was resuspended and rested overnight in Roswell Park Memorial Institute medium (RPMI) with L-glutamine, penicillin, streptomycin, 15–20% fetal bovine serum, and the antiretrovirals nevirapine and indinavir. Aliquots of 6×10^6 PBMC (predicted to have ~600 proviruses [66]) were cultured in 6-well plates at 10^6 cells/ml and treated with individual drugs or negative control [e.g. dimethyl sulfoxide (DMSO)] for 6 days in order to allow time for death of infected cells and degradation of HIV DNA [43]. We chose PBMC instead of $CD4^+$ T cells because PBMC constitute a more representative and 'physiologic' mix of cells, which should account for effects of the drugs on other infected cell types or immune effectors. In addition, the higher numbers of PBMC allow testing of more drugs, which would otherwise not be feasible without leukapheresis. Cell counts and viabilities were measured by trypan blue staining at day 6. The following drugs (all of which are in clinical use or human trials) were tested: auranofin (redox and p53 modulator); interferon α2A; interferon γ; acitretin (RIG-I inducer); GS-9620/vesatolimod (TLR7 agonist); nivolumab (PD-1 blocker); obatoclax (Bcl-2 inhibitor); bortezomib (proteasome inhibitor); birinapant (IAP inhibitor); and INK128/sapanisertib (mTOR [c]1/2 inhibitor). Initial drug concentrations were chosen based on levels attainable in plasma (if known) or previously reported for use *in vitro*. For some drugs, doses were reduced over time (see Table, Supplemental Digital Content 2, http://links.lww.com/QAD/D184) if initial experiments suggested an effect on viability. For a few participants, there were not enough PBMC to test all drugs (see Supplemental Digital Content 2, http://links.lww.com/QAD/D184).

Nucleic acid extraction

For the first three participants, total cellular DNA and RNA were extracted from each well with Trireagent (Molecular Research Center, Cat# TRI 18) using the alternative protocol for DNA isolation with back extraction [5]. After the first three participants, the fume hood subsequently developed problems with low airflow, so nucleic acids were extracted from the remaining six participants using the Qiagen AllPrep DNA/RNA/miRNA Universal Kit. DNA was eluted in 60 μl EB buffer, and the concentration and quality were measured using the NanoDrop spectrophotometer. The DNA was then applied to a Qiashredder column and spun for 2 min at maximum speed to facilitate incorporation into droplets for Digital Droplet PCR (ddPCR) [67].

Quantification of HIV DNA and cell equivalents

The Intact Proviral DNA Assay [IPDA; duplex ddPCR for the HIV Packaging Signal (Psi) and Rev Response Element (RRE) regions] was performed as described previously [68,69]. DNA from each culture well was tested in 5–10 replicate ddPCR wells with a DNA input of 450–750 ng per well. HIV DNA copies were normalized to cell

numbers by ddPCR assay for copies of the human Telomerase Reverse Transcript gene (*TERT*; used for the first participant) [70] or a duplex ddPCR assay for two regions of the human *RPP30* gene [68] (used for the remaining participants when the primer/probe sequences became available). Both gene copy number assays had a DNA input of 2–7 ng per well. For additional rigor, HIV DNA levels were also normalized to cell numbers using the mass of DNA input per well (calculated from the DNA concentration and input volume) [71].

As the correction for cellular DNA 'shearing' (using the RPP30) may itself introduce bias or skew the raw data on intact HIV DNA levels, for additional rigor, we calculated levels of intact HIV DNA both without and with correction for shearing. In the eight individuals for whom we had RPP30 data, intact HIV DNA levels were corrected for DNA shearing using the following equation:

$$\textit{measured intact HIV DNA} = \textit{actual intact} - \textit{fraction sheared} \times \textit{actual intact}, \text{ or}$$

$$\textit{actual intact HIV DNA} = \textit{measured intact DNA} \times \frac{1}{1 - \textit{fraction sheared}}$$

$$\textit{corrected intact HIV DNA} = \textit{measured intact DNA} \times \frac{1}{1 - \left(\frac{Avg(Q1 + Q4)}{Q2 + Avg(Q1 + Q4)}\right)}$$

$$\textit{corrected intact HIV DNA} = \textit{measured intact DNA} \times \frac{Q2 + Avg(Q1 + Q4)}{Q2}$$

where Q2 is the number of 'unsheared' droplets in which both regions of RPP30 were detected (Fam+Vic+, quadrant 2) and average(Q1+Q4) is the average of the numbers of single positive 'sheared' RPP30 droplets in quadrant 1 (Fam+Vic-) and quadrant 4 (Fam-Vic+). Levels of total Psi (Psi+RRE− + Psi+RRE+), total RRE (Psi-RRE+ + Psi+RRE+), intact (Psi+RRE+), 3′-defective (Psi+RRE−), and 5′-defective (Psi-RRE+) HIV DNA copies per million cells were then expressed as percentages of the DMSO control.

Statistical analysis

Levels of total, intact, and defective HIV DNA were compared between each drug well and the DMSO control using the Wilcoxon signed rank test. All statistics were performed using GraphPad Prism (version 8). To account for testing multiple drugs, we calculated the Benjamini–Hochberg critical value (i/m^*Q, where i = rank of P value, m = total number of tests, and Q = chosen FDR of 0.05).

Results

Statistically significant reductions in viability were not detected, though there were trends

The median viability in the DMSO control was 88.6% (see Figure, Supplemental Digital Content 3, http://links.lww.com/QAD/D185). When expressed as a percentage of the DMSO control, the median viabilities for each drug (including data across all doses) were: auranofin = 91.1%; interferon α2A = 99.7%; interferon γ=102.2%; acitretin = 94.5%; GS-9620 = 99.2%; nivolumab = 99.6%; obatoclax = 96.4%, birinapant = 101.6%; bortezomib = 99.3%; and INK128 = 88.1% (see Figure, Supplemental Digital Content 4, http://links.lww.com/QAD/D186). Compared with DMSO, there was a nonsignificant trend towards lower viability with auranofin ($P = 0.078$), obatoclax ($P = 0.055$), and INK-128 ($P = 0.094$).

Reductions in total Psi+ or RRE+ HIV DNA were not detected

Though median levels of total HIV DNA were sometimes lower (P = NS) with some drugs than DMSO (particularly for total Psi+ HIV DNA), we did not detect any statistically significant differences between any drug and the DMSO control in levels of total Psi+ HIV DNA or total RRE+ HIV DNA, regardless of whether the HIV DNA was normalized to cell numbers using gene copy numbers (see Figure, Supplemental Digital Content 5, http://links.lww.com/QAD/D187, and Fig. 1) or DNA mass (see Figure, Supplemental Digital Content 6, http://links.lww.com/QAD/D188).

Obatoclax reduced levels of intact HIV DNA

Although median levels of intact HIV DNA were lower with auranofin, IFNα2A, acitretin, birinapant, bortezomib, and INK-128 than DMSO (P = NS), we observed a statistically significant reduction only with obatoclax (Fig. 2). Without correction for shearing, obatoclax significantly reduced intact HIV DNA regardless of whether it was normalized to cell numbers using gene copy numbers (median = 29.9%; $P = 0.0039$; Fig. 2a and Supplemental Digital Content 7, http://links.lww.com/QAD/D189) or DNA mass (median = 27.2% of DMSO; $P = 0.0039$; see Figure, Supplemental Digital Content 8, http://links.lww.com/QAD/D190), and even after correction for multiple comparisons (Benjamini–Hochberg critical value = 0.005 for both). For INK-128, there was a trend toward lower intact HIV DNA when normalized by gene copy numbers (median = 36.3%; $P = 0.094$) but not DNA mass (median = 35.6%, $P = 0.22$). The reduction in intact HIV DNA observed with obatoclax persisted when the intact HIV DNA was corrected for shearing (median = 41.3%; $P = 0.016$; Fig. 2b) or if we excluded data for participants in whom the cell viabilities for a given drug were less than 90% of the DMSO control (median = 46.7%; $P = 0.031$; see Figure, Supplemental Digital Content 9, http://links.lww.com/QAD/D191).

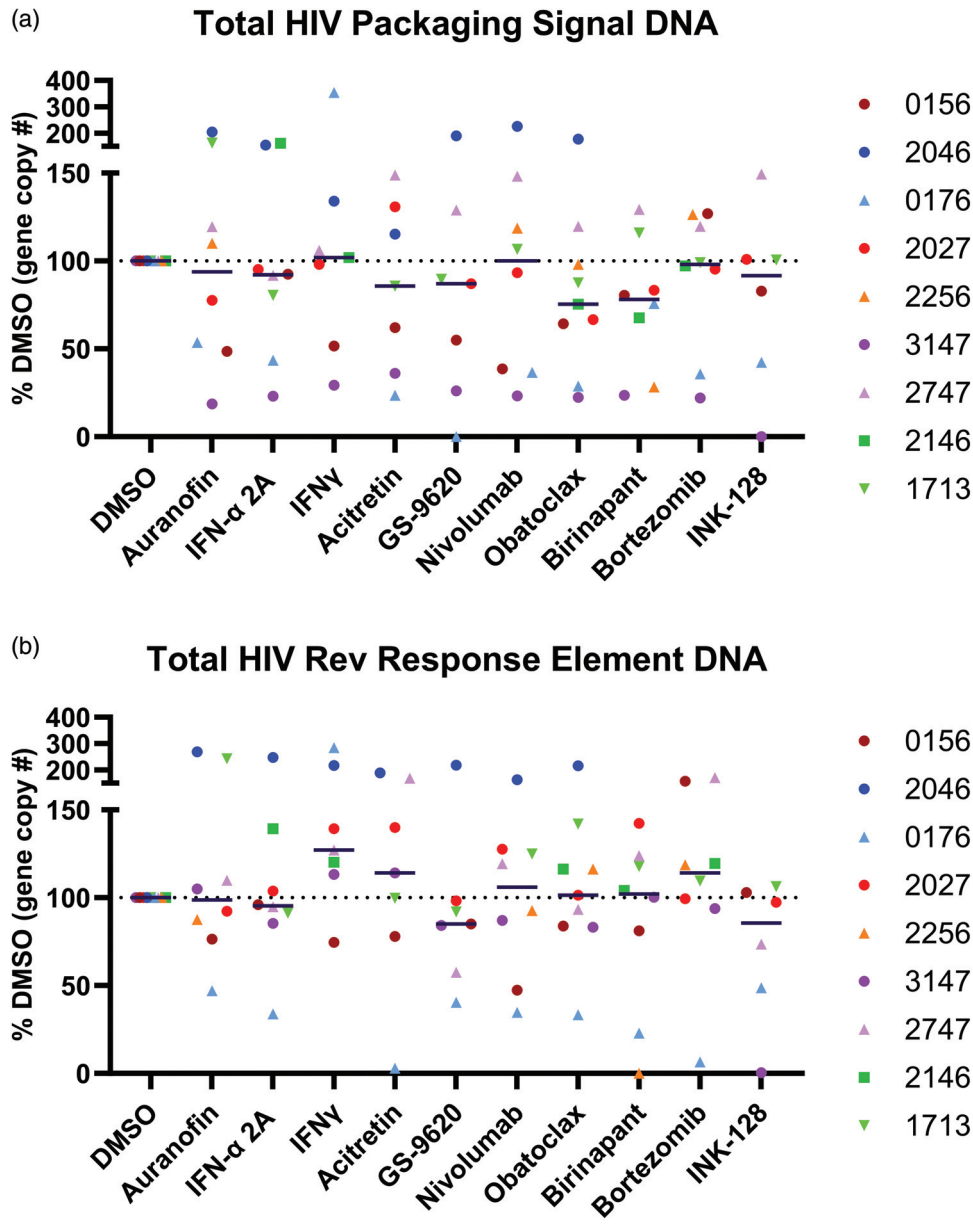

Fig. 1. Total HIV DNA levels, as normalized by human gene copy numbers. After 6 days of treatment with individual drugs (*x*-axis), cellular DNA was extracted from each well. HIV DNA was measured using the Intact Proviral DNA Assay, a duplex ddPCR assay for the HIV Packaging Signal (Psi) and Rev Response Element (RRE) regions. Total levels of (a) HIV Psi DNA (Psi+RRE− + Psi+RRE+) and (b) HIV RRE DNA (Psi-RRE+ + Psi+RRE+) were normalized to cell numbers by a separate ddPCR for copy numbers of the human *TERT* or *RPP30* genes. Results for each drug were further normalized to percentage of the DMSO control (*y*-axis) and compared with DMSO using the Wilcoxon signed rank test. Bars indicate medians.

Reductions in defective HIV DNA were not detected

We did not detect any statistically significant differences between any drug and the DMSO comparator in levels of either 3′ defective HIV DNA (obatoclax median = 83.4%; $P = 0.82$) or 5′ defective HIV DNA (obatoclax median = 94.8%; $P > 0.99$), regardless of whether the HIV was normalized to cell numbers using gene copy numbers (Fig. 3 and Supplemental Digital Content 10, http://links.lww.com/QAD/D192) or DNA mass (see Figure, Supplemental Digital Content 11, http://links.lww.com/QAD/D193).

Discussion

To our knowledge, this study is the first to compare the degree to which different small molecules acting through

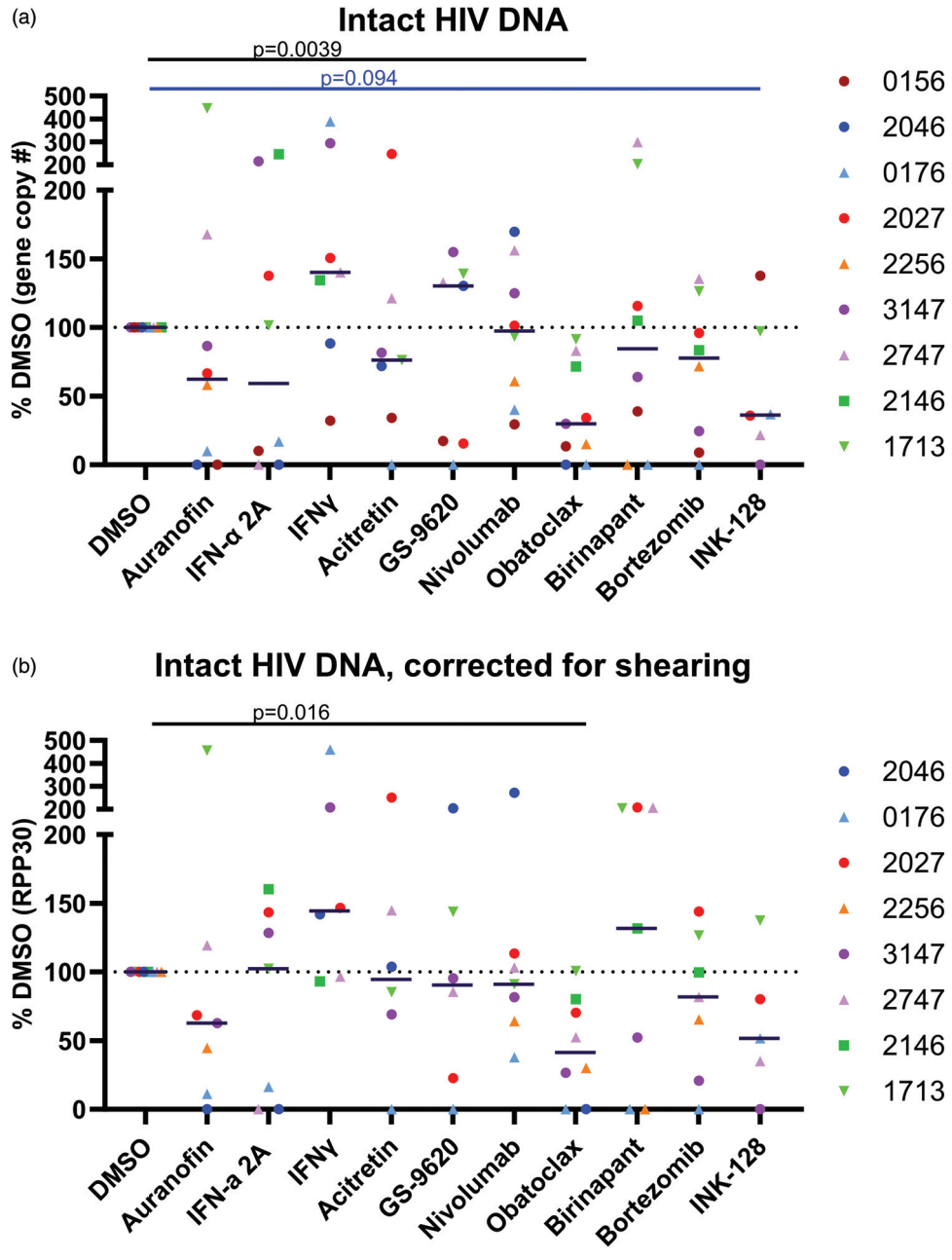

Fig. 2. Intact HIV DNA levels, without and with correction for DNA shearing. After 6 days of treatment with individual drugs (*x*-axis), levels of 'intact' HIV DNA (Psi+RRE+) in PBMC were measured by IPDA, normalized to cell numbers by ddPCR for human gene copy numbers (a), and further corrected for total DNA shearing (b) using the results of the duplex ddPCR for RPP30. Results for each drug were further normalized to percentage of the DMSO control (*y*-axis) and compared with DMSO using the Wilcoxon signed rank test (*P* values). Bars indicate medians. PBMC, peripheral blood mononuclear cells.

various mechanisms can induce selective ex-vivo killing of HIV-infected cells, as measured by reduction in the level of HIV DNA per million cells, and the first to compare how different drugs affect cells infected with intact and defective proviruses. Each of the drug classes we studied has at least one prior publication supporting the ability to induce selective killing of cells infected with HIV or SIV, or to reduce some measure of the number of HIV/SIV-infected cells. To our surprise, we did not find any reduction in total HIV DNA with any of the drugs studied, and only obatoclax caused a statistically significant reduction in intact HIV DNA. Discrepancies from prior studies are likely due to differences in the study participants or methods, including whether the drugs were tested *in vivo* or *ex vivo* or *in vitro*, as well as the target cell type, virus, drug, dose, treatment duration, and assay read-out.

Potential discrepancies could also reflect the limitations of our study, which are worth noting. First, we tested these drugs *ex vivo*, which may not correspond to what happens

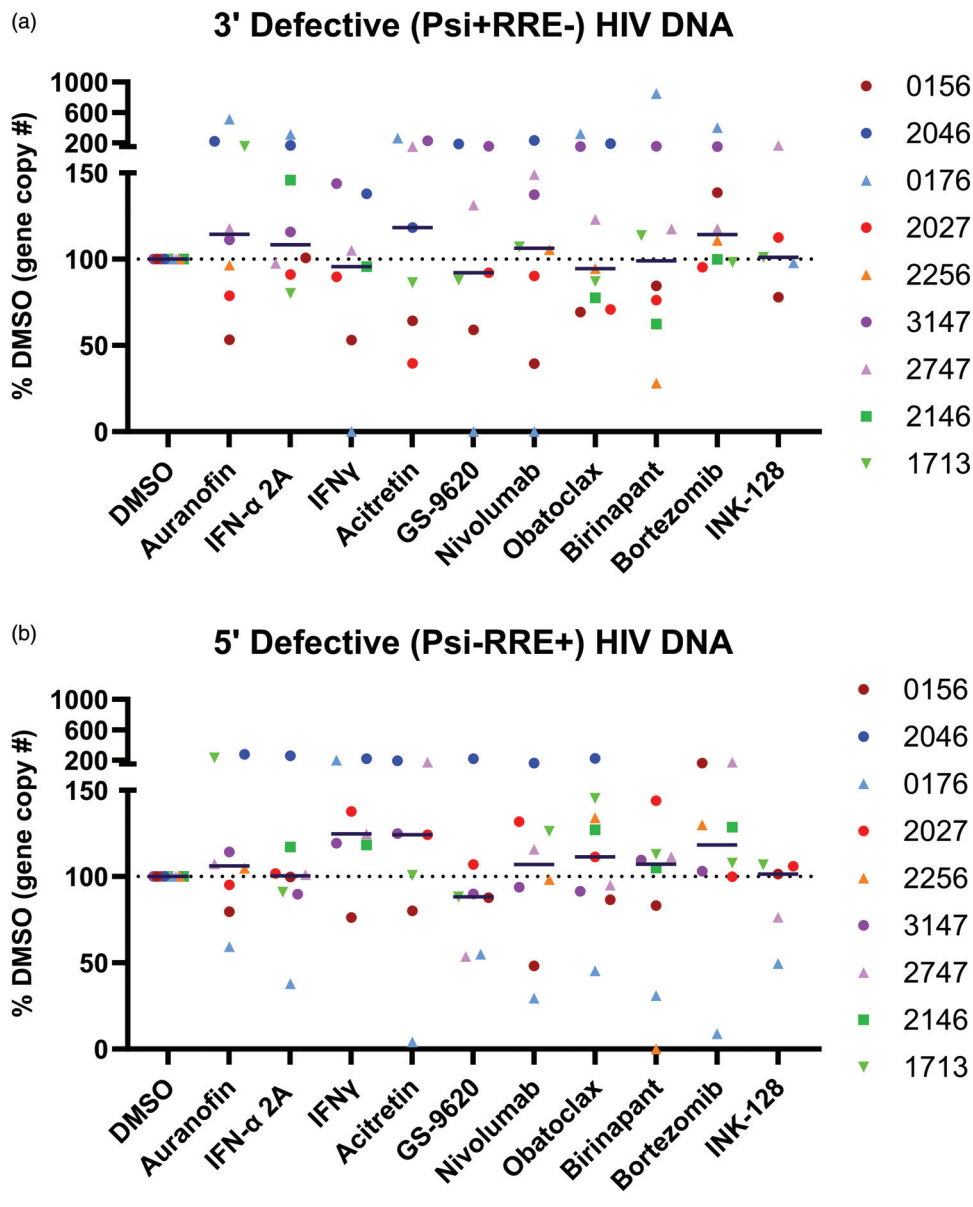

Fig. 3. Levels of defective HIV DNA, normalized by human gene copy numbers. After 6 days of treatment with individual drugs (*x*-axis), levels of (a) 3′ defective (Psi+RRE−); and (b) 5′ defective (Psi-RRE+) HIV DNA in PBMC were measured by IPDA and normalized to cell numbers by a separate ddPCR for human gene copy numbers. Results for each drug were further normalized to percentage of the DMSO control (*y*-axis) and compared with DMSO using the Wilcoxon signed rank test. Bars indicate medians. PBMC, peripheral blood mononuclear cells.

in vivo. Second, we tested PBMC from a relatively small number of study participants, which limits the power to detect small effects. Third, although we started with drug doses that can be achieved in the plasma or were used in prior studies, some drugs may have reduced viability in the initial experiments, requiring dose reductions in subsequent experiments. Fourth, we did not investigate treatment durations other than 6 days. Finally, we observed considerable dispersion in HIV DNA levels, with occasional samples showing HIV DNA levels greater than the DMSO control. These high values did not approach statistical significance and likely reflect the effects of chance, such as the starting concentrations of infected cells in the drug and DMSO wells or chance variations in measuring HIV levels (especially the low levels of intact HIV DNA) or cell equivalents. Of note, the error of measurement increases exponentially when calculating ratios of HIV DNA copies to cell numbers, with or without shearing correction, and then ratio to DMSO control. Alternatively, there could be biological differences between study participants or subsets of their reservoirs (for example, different clones). All these effects should make it more difficult to observe a consistent reduction in intact HIV DNA, as was observed for obatoclax.

Despite these limitations, we found that the Bcl-2 inhibitor obatoclax caused a statistically significant reduction in intact HIV DNA. This effect does not seem to be explained by a generalized induction of apoptosis in all cells, for the following reasons: obatoclax reduced intact DNA levels as a proportion of total cells, and relative to DMSO control; the reduction in intact HIV DNA was much greater than any reduction in viability; obatoclax had little or no effect on viability at lower doses; obatoclax reduced intact HIV DNA even after correction for total DNA shearing (an effect of cell death); and the effect persisted even when we limited the analysis to samples with high cell viability.

Our finding that obatoclax reduced intact HIV DNA has both similarities and differences from other published studies using Bcl-2 inhibitors. The Bcl-2-selective inhibitor venetoclax was shown to selectively promote apoptosis and death of HIV expressing J-Lat 10.6 cells and to reduce HIV DNA in primary $CD4^+$ T cells infected *in vitro* [72]. In another study, venetoclax (ABT-199) reduced total HIV DNA and IUPM in a primary cell model, but not in cells from ART-suppressed people (unless combined with reactivation and HIV-specific CTL) [48]. In two studies published during the last few months, venetoclax monotherapy decreased plasma viremia and intact HIV DNA in a humanized mouse model of acute HIV infection [49], reduced total and intact HIV DNA in $CD4^+$ T cells *ex vivo* [50], and delayed viral rebound in humanized mice [50]. Finally, obatoclax was recently shown to activate caspase 8 and preferentially induced apoptosis in latently infected cell lines [73].

We found that obatoclax reduced intact HIV DNA but not 5′ defective or 3′ defective HIV DNA. The levels of defective HIV DNA were much higher than intact HIV DNA, suggesting we had a greater power to detect any decrease in defective HIV DNA, which was not observed. Our findings suggest that cells infected with intact and defective proviruses may differ in the mechanisms that govern their persistence and survival. Defective proviruses may differ in the expression or function of viral genes, and infection with intact proviruses may have different effects on the expression of host cell genes. For example, cells infected with intact proviruses may have higher expression of Bcl-2 or may depend more on Bcl-2 for their survival. In one primary cell model of latency, Bcl-2 expression was found to be higher in cells that expressed Gag than those that did not [74]. Moreover, two different studies found that the 'translation competent reservoir' ($CD4^+$ T cells from ART-suppressed individuals that can be induced to express Gag protein after ex-vivo activation [48] or protein kinase C agonist treatment [75]) was enriched for cells that expressed more Bcl-2. High Bcl-2 expression may be selected by functional activity of the HIV protease, which has been implicated in the HIV cytopathic effect. HIV protease has been shown to cleave the host protein procaspase 8 to yield a novel protein, Casp8p41, which activates Bak to cause apoptosis [76,77]. Bcl-2 inhibits this process by binding to and inactivating Casp8p41, which may allow cells expressing high levels of Bcl-2 to escape killing and survive [78].

Cells infected with intact and defective proviruses may also differ in the extent of HIV transcription and the sequence of these transcripts, which could lead to differences in recognition by cellular PRRs and the subsequent mechanisms that trigger apoptosis. Moreover, obatoclax may itself induce HIV transcription. In a prior conference abstract, we reported that ex-vivo treatment with obatoclax seemed to increase various HIV transcripts after 24 h [79]. Although we did not observe a significant increase in HIV RNA after 6 days of obatoclax treatment in this study, it is possible that obatoclax caused an early increase in HIV transcription but that these cells were more likely to die before the 6-day time point.

The selective effect of obatoclax on cells with intact proviruses could also reflect a greater dependence of these cells on Bcl-2 to resist killing by extrinsic immune cells, such as the $CD8^+$ T cells and natural killer (NK) cells present in PBMC. Bcl-2 overexpressing cells are more resistant to killing by $CD8^+$ T cells *in vitro*, and the Bcl-2 inhibitor venetoclax has been shown to induce ex-vivo killing of HIV-infected cells (decrease HIV DNA and IUPM) when combined with HIV-specific $CD8^+$ T cells and either bryostatin or T-cell activation [48]. In another study, monotherapy with venetoclax enhanced killing of HIV-infected cells by autologous NK cells or $CD8^+$ T cells after in-vitro HIV infection [49].

The ability of Bcl-2 inhibitors such as obatoclax and venetoclax to promote selective killing of cells with intact proviruses may make them particularly useful in studies aimed to reduce the HIV reservoir in hopes of achieving a functional cure and/or reducing HIV-associated inflammation and immune activation. Future studies should examine the effect of various Bcl-2 inhibitors, alone and in combination with other drugs that induce selective killing of HIV-infected cells or modulate HIV expression (latency reversing agents or latency-promoting agents), during treatment ex-vivo and in animal models. As venetoclax is Food and Drug Administration (FDA)-approved for leukemia and lymphoma, future studies should investigate its effect on intact/defective HIV DNA and the latent reservoir using prospective samples from HIV+ patients who will be treated with venetoclax for these cancers. In addition, it will be exciting to see the results of an ongoing clinical trial of venetoclax to reduce the HIV reservoir, entitled 'Administration of Venetoclax to Promote Apoptosis of HIV-infected Cells and Reduce the Size of the HIV Reservoir Among People Living with HIV on ART' (ClinicalTrials.Gov # NCT05668026).

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Data availability: data are shown in graphical form in the Figures and Supplemental Digital Content. The tabulated datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

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Conflicts of interest

There are no conflicts of interest.

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