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Induction of autophagy by PI3K/MTOR and PI3K/MTOR/BRD4 inhibitors suppresses HIV-1 replication

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Running title: PI3K/MTOR inhibitors suppress HIV-1 replication

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Abstract

In this study, we investigated the effect of the dual phosphatidylinositol 3-kinase/mechanistic target of rapamycin (PI3K/MTOR) inhibitor dactolisib (NVP-BEZ235), the PI3K/MTOR/bromodomain-containing protein 4 (BRD4) inhibitor SF2523 and the BET inhibitor JQ1 on the productive infection of primary macrophages with human immunodeficiency type-1 (HIV). These inhibitors did not alter the initial susceptibility of macrophages to HIV infection. However, dactolisib, JQ1 and SF2523 all decreased HIV replication in macrophages in a dose dependent manner via degradation of intracellular HIV through autophagy. Macrophages treated with dactolisib, JQ1 or SF2523 displayed an increase in LC3B lipidation combined with SQSTM1 degradation without inducing increased cell death. LC3B-II levels were further increased in the presence of pepstatin A suggesting that these inhibitors induce autophagic flux. RNA interference for ATG5 and ATG7, and pharmacological inhibitors of autophagosome-lysosome fusion, and of lysosomal hydrolases, all blocked the inhibition of HIV. Thus, we demonstrate that the mechanism of PI3K/MTOR and PI3K/MTOR/BRD4 inhibitor suppression of HIV requires the formation of autophagosomes, as well as their subsequent maturation into autolysosomes. These data provide further evidence in support of a role for autophagy in the control of HIV infection and open new avenues for the use of this class of drugs in HIV therapy.

Although CD4+ T cells are the predominant human immunodeficiency virus type-1 (HIV††) reservoir, other cell types including macrophages and microglial cells also serve as occult sites of HIV persistence (1). These infected cells are broadly disseminated across numerous tissues, including sites that may be relatively inaccessible to host defenses or treatment strategies, such as the lymphatic tissue and the central nervous system, where concentrations of antiviral drugs are lower than in peripheral blood (2,3). HIV can spread through cell-to-cell transmission in the presence of antiretroviral therapy (ART) concentrations that effectively inhibit extra-cellular viral infection of susceptible cells (4). Moreover, as
infected cells can persist undetected by the immune system, and are unaffected by ART, lifelong treatment is required for continued viral suppression. Extensive efforts are underway to develop effective strategies for eliminating these reservoirs. One strategy, often called “shock and kill”, has emerged as a potential strategy to eradicate HIV but effective drugs are still lacking. Thus, there is an urgent need for the development of new therapeutic strategies.

HIV survival is dependent on its ability to exploit host cell machinery for replication and dissemination and to circumvent cellular processes that prevent its growth. One such mechanism of avoiding the innate immune system is the ability of HIV to inhibit or subvert macroautophagy (hereafter referred to as autophagy). Autophagy is a degradation pathway that occurs at basal levels in all cells whereby cytosolic double membrane-bound compartments termed autophagosomes engulf and sequester cytoplasmic constituents such as sub-cellular organelles and microbial pathogens. These autophagosomes then fuse with lysosomes forming autophagolysosomes (autolysosomes), resulting in the degradation of the engulfed components, generating nutrients and macromolecular precursors. Autophagy is upregulated in response to stress such as starvation, drug treatment and infection. Of the more than 35 human autophagy-associated genes currently known to be involved in autophagy, 10 are known to be essential for HIV replication (reviewed in 5). However, although HIV may require the early stages of autophagy, it must control the antiviral proteolytic and degradative late stages of autophagy to avoid its degradation.

In HIV-infected macrophages, Nef binds to and sequesters beclin 1 (BECN1) to the Golgi complex leading to the phosphorylation and cytosolic sequestration of transcription factor EB, culminating in the blocking of the degradative stages of autophagy and the accumulation of autophagic factors (6-8). Despite the down-modulation of autophagy by HIV, inducers of autophagy including 1α,25-dihydroxycholecalciferol (9,10), amino acid starvation (11), hydroxamate histone deacetylase inhibitors (12), sirolimus (8,9), toll-like receptor (TLR) 8 ligands (13), romidepsin (12), and a cell-permeable autophagy-inducing peptide termed Tat–beclin (derived from the region of BECN1 that interacts with HIV Nef and conjugated to the basic region of HIV Tat) (8), overcome the imposed phagosome maturation block leading to inhibition of HIV. Therefore, autophagy shows promise as a target for drug therapy.

The phosphatidylinositol 3-kinase (PI3K) pathway is a major focus for drug development due to its function as a key regulator of cell growth and survival, and cancer genetic studies suggest that the PI3K pathway is frequently altered in human tumors (14); PIK3CA (encoding phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) is the second most frequently mutated oncogene, and PTEN (phosphatase and tensin homolog) is one of the most frequently mutated tumor suppressor genes (15,16). Key targets of PI3K include the AKT serine/threonine kinase 1 (AKT1) and the mechanistic target of rapamycin (MTOR) pathway. Drugs targeting the PI3K/AKT1/MTOR axis include pan-PI3K inhibitors, isoform-selective PI3K inhibitors, sirolimus and analogues, active-site MTOR inhibitors, and dual-PI3K/MTOR and AKT1 inhibitors. Dactolisib (BEZ235, NVP-BEZ235) is an imidazooquinoline derivative that has dual ATP-competitive inhibition of PI3K and MTOR (17). By inhibiting MTOR through binding at the ATP-cleft, dactolisib prevents the phosphorylation of the ribosomal protein S6 kinase B2 (RPS6KB2) and other MTOR complex 1 (MTORC1) downstream effectors, leading to a release of autophagy inhibition by MTORC1. It is also effective in blocking AKT1 activation as it prevents the feedback activation of PI3K signaling normally observed with MTOR inhibitors, such as sirolimus (18). Dactolisib affects cell growth through PI3K/AKT1 and MTORC1 inhibition (19,20), and induces autophagy in tumor cells (21,22). Moreover, dactolisib also interferes with the growth of lymphocytic choriomeningitis virus through the inhibition of viral budding (23) and rescued mice from a lethal influenza infection (24).

The bromodomain (BRD) and extra terminal domain (BET) family of proteins (BRDT, BRD2, BRD3 and BRD4) are potential new candidates in the “shock and kill” strategy...
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for HIV eradication (25). BET BRD4 inhibitors act as acetylated lysine mimetics and thus disrupt the binding interaction of BET proteins with acetylated lysine residues on histones such as H3K27 (26). BET bromodomain inhibitors such as JQ1 (27) reactivate the transcription of HIV in latent T cell and monocyte infection models (28-30). Thus, design of PI3K/MTOR/BRD4 inhibitors where BRD4 inhibitors can induce activation of latent virus and PI3K/MTOR inhibitor can induce autophagy could be an effective strategy to eradicate HIV infected T cells or macrophages. SF2523 is a novel and only existing PI3K/MTOR/BRD4 inhibitor (31). SF2523 is i) a pan PI3K inhibitor at Kᵣ of 19 nM and 35 nM for PIK3CA and PIK3CD respectively; ii) inhibits BRD4 binding to MYCN promoter equivalent to JQ1 (1 µM) and blocks MYCN transcription; iii) pharmacodynamically inhibits AKT activation by blocking phosphorylation of AKT at Ser⁴⁷³ in macrophages; iv) blocks BRD4 binding to acetyl-lysine binding site on histone H3K27; v) blocks M1-M2 macrophage transition and augments immunity (32), and vi) has shown potent in vivo tumor activity against a large number of tumor types with no observable toxicity (33,34). SF2523 has been formulated for oral absorption, has excellent pharmacokinetic and absorption, distribution, metabolism, and excretion properties and is slated to enter clinical trials in the near future (CA192656). These characteristics in a single pill opened new opportunities to explore this drug for HIV therapy as it has a potential to induce both the “shock” and the “kill” arms of the “shock and kill” treatment strategy.

In this study we investigated the effect of dactolisib, JQ1 and SF2523 on the susceptibility of macrophages to HIV infection. We report that these inhibitors have no impact on the initial infection events, but through the induction of autophagy induce the degradation of intracellular viral particles that leads to a reduction in viral release. Moreover, our data demonstrate that the dactolisib-mediated autophagic degradation of HIV requires the nucleation and formation of autophagosomes and their subsequent maturation, which leads to a decrease in virion release.

**Results**

**Dactolisib induces autophagy in human primary macrophages**

Previous studies demonstrated that addition of dactolisib to continuous transformed cell lines or primary carcinomas leads to the induction of autophagy (18,19,22,35-39). However, because the effects of drugs on primary cells can be different than those observed in continuous cell lines, we investigated the ability of dactolisib to induce autophagy in primary human macrophages. During autophagy, cytosolic microtubule-associated protein 1 light chain 3 beta (MAP1LC3B or LC3B) I is converted to LC3B-II by an ubiquitin-like system that involves autophagy-related (ATG) 7, ATG3 and the ATG12–ATG5 complex. The ATG12–ATG5 complex ligates LC3B-II to the nascent autophagosome membrane through phosphatidylethanolamine with the LC3B-II associated with the inner membrane degraded after fusion of the autophagosome with lysosomes. Therefore, the conversion of LC3B-I to LC3B-II and its turnover is an indicator of autophagy induction and flux (40). Exposure of macrophages to dactolisib for 24 h led to a dose dependent increase in LC3B-II similar to that observed with sirolimus, an inducer of autophagy through inhibition of MTORC1 (Fig. 1A).

To verify that the increase in LC3 lipidation in dactolisib treated cells versus control cells represents increased autophagic flux rather than an accumulation of LC3B-II, the degradation of the polyubiquitin-binding protein sequestosome 1 (SQSTM1 or p62) was quantified. Inhibition of autophagy leads to an increase in SQSTM1 protein levels while autolysosomes degrade SQSTM1- and LC3-positive bodies during autophagic flux (41). In these experiments, dactolisib treatment led to a dose-dependent decrease in SQSTM1 protein levels corresponding to the induction of autophagic flux (Fig. 1A). Of interest, dactolisib treatment led to the dose-dependent increase in the expression of beclin 1 (BECN1) (Fig. 1A). Unlike other BH3-only proteins, the overexpression of BECN1 does not function as a pro-apoptotic molecule, but instead leads to
increased autophagy (42). As both a decrease in SQSTM1 and an increase in BECN1 levels correlate with enhanced autophagy, the observed decrease in the SQSTM1:BECN1 protein ratio in dactolisib treated cells suggests augmented autophagy induction (Fig. 1A) (40).

To further establish the induction of autophagic flux in dactolisib treated macrophages, and not simply the activation of LC3B transcription, additional experiments were conducted with macrophages in the presence of the established acid protease inhibitor, pepstatin A. Pepstatin A prevents the degradation of LC3B-II during autophagosome-lysosome fusion, resulting in an accumulation of LC3B-II over time as autophagy is initiated and sustained. Macrophages were treated with 10 µg/mL pepstatin A prior to dactolisib treatment, and lysed after subsequent 24 h dactolisib treatment. Blots of cell lysates confirmed autophagic flux, with significant LC3B-II accumulation relative to basal conditions in the vehicle control (Fig. 1B). These data indicate that in the presence of dactolisib treatment autophagy goes to completion.

Although we observed an increase in autophagic markers in the absence of visible pyknosis, karyorrhexis, or plasma membrane blebbing, it was important to confirm that the cells were not undergoing cell death at the concentrations being used, as the induction of excessive autophagy can cause cell death (43) and dactolisib exerts cytotoxic effects in cancer cells (18). Importantly, we did not observe significant dactolisib-mediated cell death as measured by lactate dehydrogenase (LDH) release at the concentrations used in this study (Fig. 1C). Moreover, based on the selective thermal denaturation of apoptotic ssDNA using low heat and formamide and subsequent detection using a monoclonal antibody, a more specific indicator of apoptosis than TUNEL (44), we also observed no significant difference in ssDNA accumulation using Welch's unequal variances t test (P > 0.05; Fig. 1D).

**Dactolisib decreases HIV release from human macrophages**

Our laboratory’s data and that of others indicate that autophagy is necessary for HIV to establish a productive infection (5). However, once a permissive infection is established HIV down-regulates autophagy to facilitate cell survival and to promote viral replication (6,7). Previous studies have shown that micromolar concentrations of LY 294002, which inhibits class I PI3K as well as MTORC1, casein kinase 2, and polo like kinase 1 (45), suppresses viral infection of macrophages post-viral entry and post-reverse transcription but prior to HIV gene expression (46). Conversely, wortmannin, an inhibitor that is used at nanomolar concentrations in cell culture to inhibit autophagy (40), and inhibits PI3K and PLK1, but not MTORC1 at these concentrations (45), fails to have any effect on HIV replication in macrophages (9). Therefore, as the pharmacological induction of autophagy is known to inhibit HIV (5,8-10,12,13,47), we next determined whether dactolisib-induced autophagy influenced HIV infection and replication in primary macrophages by comparing the extent to which dactolisib treatment influences HIV p24 antigen accumulation in the supernatants of productively infected macrophages. Dactolisib induced a dose-dependent decrease in HIV p24 antigen release into the culture supernatants that became significant by day 3 post-infection at all tested concentrations (P < 0.01; Fig. 2A). Interestingly, even the low dose of 25 nM dactolisib was sufficient to decrease extracellular HIV p24 antigen accumulation by 83% (P = 0.003). Moreover, the magnitude of the inhibition increased until cultures were discontinued on day 10 post-infection (Fig. 2A). Importantly, we observed that at 10 d post-HIV-infection dactolisib induced no significant cytotoxic effects at the concentrations used (Fig. 2B). Moreover, we also observed no significant difference in ssDNA accumulation at the same time point (Fig. 2C).

To understand how dactolisib affects HIV replication, we examined sequential steps of viral replication. Treatment of cells with dactolisib did not significantly affect binding of HIV to dactolisib-treated cells, as measured by ELISA of cell-associated p24 Gag protein (P > 0.3; Fig. 3A). Moreover, the quantity of trypsin-resistant intracellular p24 Gag in cells exposed to virus for 5 h in both untreated cells and dactolisib-treated cells were similar (P > 0.29;
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Fig. 3B). As controls, sirolimus and maraviroc were used. Sirolimus is known to down regulate C-C motif chemokine receptor 5 (CCR5) expression as well as to induce autophagy through the inhibition of MTOR (48) and maraviroc is a CCR5 antagonist (49); thus, they provide a control for CCR5 binding. Collectively, these results suggest that dactolisib has no effect on either HIV binding or entry.

We next measured viral infection using qPCR for the presence and quantity of strong-stop HIV DNA (with LTR R/U5 primers) 8 h post-infection. Macrophages were treated with dactolisib for 4 h prior to infection with HIV. Following 8 h of infection, dactolisib had no effect on HIV reverse transcription ($P > 0.1$; Fig. 3C). As Nef is expressed in abundance during the early phase of HIV infection (50), we also analyzed the translation of Nef by immunoblotting. We observed a slight, but non-significant increase in Nef production in dactolisib treated cells over vehicle controls ($P > 0.18$; Fig. 3D). We then assessed for productive HIV infection by assaying for Tat activity using TZM-bl cells. TZM-bl cells were pretreated with dactolisib for 4 h then exposed to HIV. At the concentrations tested, dactolisib had no significant inhibitory effect on the productive infection of TZM-bl cells with HIV ($P > 0.5$; Fig. 3E).

In our next series of experiments, we assessed whether the decrease in cell culture supernatant of HIV p24 antigen release was due to dactolisib inducing the production of replication-incompetent viral particles. 1 ng p24 antigen from cell-free supernatants derived from cells treated with dactolisib and infected for 10 d was used to infect fresh macrophages in the absence of dactolisib. We observed no difference in HIV-replicative fitness post-dactolisib treatment indicating that the virus being released is replication competent ($P > 0.19$; Fig. 3F). Next, we examined the effect of dactolisib on HIV replication at early time points post-infection by treating macrophage cultures exposed to HIV with dactolisib at 4 h pre-exposure, at the time of infection and at 3, 5, and 7 days post-infection. At every time point, each concentration of dactolisib induced a significant decrease in cell culture supernatant HIV p24 antigen accumulation (Fig. 3G) in the absence of toxic cellular effects.

**Dactolisib induces autophagy in HIV-infected macrophages by inhibiting MTOR and activating unc-51 like autophagy activating kinase 1**

To determine whether the dactolisib-mediated decrease of HIV p24 antigen release could be attributable to autophagy, we assessed the ability of dactolisib to induce autophagy in HIV-infected macrophages. 24 h exposure of HIV-infected macrophages to dactolisib led to a dose-dependent increase in LC3B-II and BECN1 and a dose-dependent decrease in SQSTM1 and the SQSTM1:BECN1 protein ratio suggesting that dactolisib overcomes the HIV-imposed autophagy inhibition and induces autophagy (Fig. 3H).

The (unc-51 like autophagy activating kinase 1 (ULK1) kinase complex functions at the initial stages of the canonical autophagy pathway and induces autophagy by phosphorylating beclin 1 and activating PIK3C3 (51-53). Under nutrient-rich conditions MTORC1, consisting of MTOR, the regulatory associated protein of MTOR complex 1 (RPTOR) and MTOR associated protein LST8 homolog (MLST8), phosphorylates ULK1 at Ser577 and binds it through RPTOR. Under conditions of stress such as nutrient deprivation or TLR signaling MTORC1 is inhibited leading to global dephosphorylation of ULK1, dissociation of ULK1 from MTORC1 and the induction of autophagosome formation. Therefore, we examined whether ULK1 is involved in dactolisib-mediated autophagy by determining whether dactolisib treatment causes the inhibition of MTOR and thereby the dephosphorylation and activation of ULK1. Dactolisib induced significant and dose-dependent dephosphorylation and activation of ULK1 as monitored by phospho-ULK1 (Ser577) specific antibodies (Fig. 3I). We next examined whether dactolisib causes global inhibition of MTORC1 by measuring the phosphorylation status of two well-known MTOR substrates, ribosomal protein S6 kinase, 70kDa, polypeptide 2 (RPS6KB2) and eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1). Dactolisib treatment induced the
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Dephosphorylation of RPS6KB2 and EIF4EBP1 in a similar manner to ULK1 (Fig. 3I) suggesting that dactolisib inhibits MTOR during HIV infection.

**Dactolisib-mediated autophagy inhibits HIV dissemination from human macrophages**

To determine whether dactolisib-induced autophagy contributes to the dactolisib-mediated decrease of HIV p24 antigen release, we assessed the effect of ATG5 and ATG7 silencing on HIV infection post-dactolisib treatment. Both ATG5 and ATG7 RNAi were effective in silencing their respective genes over the 10 d infection protocol (Fig. 4A) and are efficient at inhibiting LC3B lipidation and the degradation of SQSTM1, and thus autophagy (Fig. 4B)(12). ATG5 silencing reduced the inhibitory effect of 250 nM dactolisib from 94% at day 10 post-infection in the shNS cells (P < 0.001) to 32% in shATG5 cells, which was not significantly different from the vehicle treated shATG5 cells (p = 0.085; Fig. 4C). Similarly, ATG7 silencing reduced the inhibitory effect of 250 nM dactolisib to just 18% in shATG7 cells (Fig. 4C). Importantly, in the presence of shRNA-insensitive ATG5 or ATG7 ORF transcripts, the dactolisib mediated decrease in extracellular supernatant HIV p24 antigen accumulation was similar to the non-targeting negative control shRNA (Fig. 4C).

We next investigated whether autophagosome acidification, a late stage event during autophagy, is required for the dactolisib-mediated autophagic decrease in HIV release. During autophagy, lysosomes fuse with autophagosomes to form autolysosomes. Macrophages were treated with bafilomycin A1, an inhibitor of the vacular H⁺ ATPase and autophagosome-lysosome fusion, and subsequently infected with HIV. Bafilomycin A1 reversed the dactolisib-mediated decrease of extracellular HIV accumulation (Fig. 5) suggesting that the acidic pH of autolysosomes is required for the autophagy-mediated control of HIV.

After lysosomes fuse with autophagosomes to form autolysosomes, lysosomal hydrolases degrade the sequestered components, which are then released into the cytosol through the actions of lysosomal efflux permeases. We investigated whether lysosomal hydrolases are important for the dactolisib-mediated decrease in HIV release through autophagy using SID 26681509, a novel thiocarbazate specific inhibitor of the lysosome hydrolase cathepsin L. Importantly, in the absence of dactolisib, SID 26681509 induced no net inhibition of HIV (Fig. 5). Moreover, in the presence of dactolisib, SID 26681509 reversed the observed dactolisib-mediated decline in extracellular accumulation of HIV (Fig. 5).

We further examined the effect of dactolisib and the role of autophagy on intracellular HIV p24 antigen levels at a late time point post-infection by treating macrophage cultures exposed to HIV with dactolisib at 7 days post-infection in the presence or absence of bafilomycin A1. As expected, HIV p24 antigen levels were increased in the presence of bafilomycin A1 alone (Fig. 6). However, at the concentrations tested, dactolisib induced a significant decrease in intracellular HIV p24 antigen by day 10 post-infection (P < 0.05; Fig. 6). Importantly, in each case, bafilomycin A1 abrogated dactolisib-mediated degradation of HIV. Collectively, these data suggest that dactolisib induces the degradation of HIV through the induction of autophagy, which leads to a reduction in the number of released virions.

**PI3K/AKT1/MTOR/BRD4 inhibitor-mediated autophagy inhibits HIV dissemination from human macrophages**

To assess whether BRD4 inhibitors or PI3K/BRD4 inhibitors would also induce autophagy and inhibit HIV infection, we examined the effects of JQ1 and SF2523 on HIV infection. Macrophages were pretreated with pepstatin A and then treated with JQ1 or SF2523 for 24 h. Both drugs induced a dose-dependent significant accumulation of LC3B-II relative to basal conditions indicative of autophagic flux (Fig. 7A). Both JQ1 and SF2523 also induced the dose-dependent decrease in HIV p24 released into the culture supernatants that became significant by day 3 post-infection at all tested concentrations (P < 0.01; Fig. 7B) with the magnitude of the decrease increasing until cultures were discontinued at day 10 post-infection (Fig. 7B). Importantly, no significant
increase in LDH release was observed at 10 d post-HIV-infection (Fig. 7C).

To determine whether the JQ1 and SF2523-mediated decrease of HIV p24 antigen release could be attributable to autophagy, we assessed the ability of JQ1 and SF2523 to induce autophagy in HIV-infected macrophages. 24 h exposure of HIV-infected macrophages to JQ1 and SF2523 led to an increase in LC3B-II and BECN1 and a decrease in SQSTM1 and the SQSTM1:BECN1 protein ratio suggesting that both JQ1 and SF2523 can overcome the HIV-imposed autophagy inhibition and induce autophagy (Fig. 7D). We also examined whether ULK1 is involved in JQ1 and SF2523-mediated autophagy by determining whether JQ1 and SF2523 treatment causes the inhibition of MTORC1 and thereby the dephosphorylation and activation of ULK1, RPS6KB2, and EIF4EBP1. Both JQ1 and SF2523 induced significant dephosphorylation and activation of ULK1, RPS6KB2, and EIF4EBP1 (Fig. 7E) suggesting that JQ1 and SF2523 both inhibit MTOR during HIV infection.

We next examined the effect of SF2523, and JQ1 on intracellular HIV p24 antigen levels at a late time point post-infection by treating macrophage cultures exposed to HIV with SF2523, and JQ1 at 7 days post-infection in the presence or absence of bafilomycin A1. At the concentrations tested, both SF2523 and JQ1 induced a significant decrease in intracellular HIV p24 antigen by day 10 post-infection (P < 0.05; Fig. 8, A and B). As expected, bafilomycin A1 abrogated the PI3K inhibitor mediated degradation of HIV (Fig. 8, A and B). Collectively, these data suggest that dactolisib, along with SF2523 and JQ1, all induce the degradation of HIV through the induction of autophagy, which leads to a reduction in the number of released virions.

Discussion

HIV infection of macrophages and CD4+ T cells induces autophagy (7,54) and peripheral blood mononuclear cells (PBMC) from elite controllers contain significantly more autophagic vesicles and express more autophagic markers than normal progressors (55). PBMC from elite controllers are also more responsive to sirolimus, with treatment leading to an enhanced autophagic response and a greater reduction in virion production (55), illustrating the importance of autophagy in the cellular antiviral response. Therefore, modulation of autophagy processes is an important mechanism for controlling microbial pathogens (56,57), and the inhibition of HIV through pharmacologic induction of autophagy highlights this role (5,8,9,12,13). However, despite autophagy being an innate antiviral defense mechanism, viruses may also hijack autophagy for their efficient replication in a cell-type and virus specific manner. HIV utilizes autophagosomal membranes as a scaffold for Gag processing and the production of nascent virions (6), while controlling the antiviral proteolytic and degradative late stages of autophagy to avoid its own degradation (6,7,58,59). To our knowledge, these data are the first to describe the effect of a dual PI3K/MTOR inhibitor or PI3K/MTOR/BRD4 inhibitor on HIV infection of macrophages, an important site of HIV infection and persistence. Although dactolisib, JQ1 and SF2523 had no effect on viral binding, entry, reverse transcription, or protein synthesis events, they dose-dependently inhibited HIV through the degradative autophagy pathway that requires the formation of autophagosomes and their subsequent maturation into autolysosomes.

The PI3K/AKT1 pathway regulates a number of different cellular processes including cell proliferation, RNA processing, protein translation, autophagy and apoptosis. In order to replicate in host cells, many viruses evolved strategies to stimulate PI3K activity. In the case of HIV, productive infection of macrophages requires PI3K activity, which triggers key cellular events typically observed during cell survival and activation, namely PTEN reduction, and the membrane localization and increased activity of AKT1 (46,60). These cellular alterations, together with the previously reported reduction in tumor protein 53 (TP53) activity caused by its direct binding to intracellular HIV Tat (60), helps to mechanistically explain the extended survival phenotype of HIV-infected macrophages. Additionally, these cellular effects contribute to viral production and the establishment of macrophages as long-lived viral reservoirs, which are inaccessible to retroviral
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Although dactolisib interferes with T cell activation and function it does so at concentrations >100 nM (65), which is less than the dose required to induce autophagy and inhibit HIV. Additional studies are required to examine the effect of dactolisib on healthy human cells in the long-term, particularly important considering the lifelong dependence of HIV-positive persons on antiretroviral therapy.

Targeting host factors used by HIV is a highly attractive antiviral strategy. Unlike current antiviral treatments targeting viral proteins, such as reverse transcriptase, protease and integrase, this strategy can minimize viral escape. During infection, HIV Tat recruits the super elongation complex (SEC) to overcome promoter-proximal pausing of RNA polymerase II. BRD4 competitively blocks this Tat–SEC interaction on the HIV promoter. By dissociating BRD4 from the HIV promoter, BRD4 inhibitors allow Tat recruitment of SEC to stimulate HIV elongation in the absence of global T cell activation (28-30). As BRD4 is also a conserved negative regulator of autophagy, the BET inhibitor JQ1 induces autophagy through BRD4 inhibition (69). Hence blocking of BRD4 and the PI3K/MTOR pathway could reactivate HIV and induce autophagy in infected macrophages, providing an effective HIV therapy regimen.

Currently available antiretroviral therapy has greatly improved life expectancy and quality of life for those infected with HIV. However, multi-drug resistance continues to increase, emphasizing the importance of identifying novel strategies to improve HIV treatment. Since autophagy works at the host cellular level to improve intracellular killing of both replicating and non-replicating HIV the development of resistance is unlikely. The appeal of dactolisib as an anti-HIV therapeutic agent is considerable. Conventional cancer treatment utilizes chemotherapy at the maximally tolerated dose (MTD), or the highest dose shown to be both cytotoxic and tolerable for the patient. Clinical studies including trials of dactolisib alone in pancreatic neuroendocrine tumors (70) and bladder cancers (71) showed that dactolisib was poorly tolerated as the MTD was 300 mg in the phase I study (72). These
studies did demonstrate limited clinical efficacy with several patients with stable disease but given the poor tolerability, these trials did not advance. Dactolisib also has low oral bioavailability with highly variable systemic exposure due to poor aqueous solubility. In cancer therapy, this low oral bioavailability, combined with the reported toxicities, prevent dose escalation to where efficacy could potentially be achieved (73). Thus, to avoid these toxicities and achieve a dose likely to be impact the PI3K/MTOR pathway, a formulation that markedly enhances the oral bioavailability or delivery of the drug via a non-oral route could be considered. In the present study, we show that the concentrations required to inhibit HIV are far lower than those required to induce cell death of tumor cells. In total, the findings presented here suggest that drugs targeting the PI3K/AKT1/MTOR pathway be considered as a potential treatment approach for persons infected with HIV, and that dactolisib may be repurposed as an anti-HIV drug.

Experimental procedures

Ethics Statement – Venous blood was drawn from HIV seronegative subjects using a protocol that was reviewed and approved by the Human Research Protections Program of the University of California, San Diego (Project 09-0660) in accordance with the requirements of the Code of Federal Regulations on the Protection of Human Subjects (45 CFR 46 and 21 CFR 50 and 56) and was fully compliant with the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from subjects prior to their participation.

Cells and reagents – Monocyte derived macrophages were obtained from peripheral blood mononuclear cells as previously described (13). All experiments were performed in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS (Sigma), and 10 ng/mL colony stimulating factor 1 (Peprotech). TZM-bl cells were obtained through the AIDS Research and Reference Reagent Program, contributed by Drs John C. Kappes and Xiaoyun Wu and Tranzyme Inc. (74). Cell viability was estimated using the LDH Cytotoxicity Detection Kit PLUS and the single-stranded DNA (ssDNA) ELISA (Chemicon) as described previously (75).

Dactolisib (BEZ235, NVP-BEZ235) was purchased from Selleck Chemicals. Bafilomycin A1, SID 26681509 and sirolimus were purchased from Sigma. JQ1 was a gift from James Bradner, Dana-Farber Cancer Institute, Boston, MA. SF2523 was synthesized as described earlier (34). Bafilomycin A1 was used at 10 nM and SID 26681509 at 50 nM with pretreatment for 1 h before addition of dactolisib or sirolimus. Maraviroc was purchased from Toronto Research Chemicals and was used at 10 nM.

Virus – HIV Ba-L was obtained through the AIDS Research and Reference Reagent Program, contributed by Dr. Suzanne Gartner and Dr. Robert Gallo (76,77). Virus stocks and titers were prepared as previously described using the Alliance HIV p24 antigen ELISA (Perkin Elmer) (78). Macrophages were infected with $10^5$ TCID$_{50}$/mL HIV Ba-L per $5 \times 10^5$ cells as described previously (13). Viral binding and entry was assessed as described previously (9). Productive infection of TZM-bl cells 48 h post-HIV exposure was detected using the β-Gal Staining Set (Roche).

Immunoblotting – ATG5 (D5G3), ATG7 (D12B11), BECN1 (3738), EIF4EBP1 (53H11), phospho-EIF4EBP1 (Thr$^{37/46}$; 236B4), RPS6KB2 (49D7), phospho-RPS6KB2 (Thr$^{389}$; 9205), ULK1 (D9D7), and phospho-ULK1 (Ser$^{757}$; 6888) antibodies were obtained from Cell Signaling. SQSTM1 (ab56416), HIV p24 (39/5.4A), and HIV Nef (3D12) antibodies were obtained from Abcam. β-actin (ACTB; AC-74) and microtubule-associated protein 1 light chain 3 beta (LC3B; NB100-2220) antibodies were obtained from Sigma and Novus Biologicals respectively. Cell lysates were prepared using 20 mM HEPES, 150 mM NaCl, 1 mM EDTA supplemented with 1% (v/v) 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (all Sigma) and 1% (v/v) Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Cell lysates were resolved using 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol buffered 12% polyacrylamide gel (Novex) and transferred to 0.2 μm pore-size PVDF membranes (Thermo Scientific), followed by detection with alkaline phosphatase tagged secondary antibodies (Invitrogen) and 0.25 mM disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2′-(5-
chlorotricyclo[3.3.1.1^{3,7}]decan]-4-yl]-1-phenyl phosphate supplemented with 5% (v/v) Nitro-Block II (both Applied Biosystems). Relative densities of the target bands compared to the reference ACTB bands were calculated using ImageJ (NIH). Each data point was normalized to the vehicle then log₂ transformed.

**shRNA transduction** — Lentiviral transduction of macrophages with MISSION lentiviral particles containing shRNAs targeting **ATG5** (SHCLNV-NM_004849/TRCN0000150940), **ATG7** (SHCLNV-NM_006395/TRCN0000007584), or scrambled non-target negative control (SHC002V) was performed according to the manufacturer's protocol (Sigma). SHC002V was used as non-targeting negative control as it activates the RNA-induced silencing complex and the RNAi pathway, but does not target any human gene allowing the examination of the effects of shRNA transduction and RNAi activation on gene expression. Macrophages were transduced with non-specific scrambled shRNA (shNS) or target shRNA and selected using 3 mg L⁻¹ puromycin (Gibco). Five days later, cells were analyzed for target gene silencing and used in experiments. Validation was performed by phenotypic rescue using custom shRNA-insensitive open reading frame (ORF) cDNA lentiviral vectors (LentiORF; GeneCopoeia). For rescue experiments, macrophages were transduced with target shRNA with a neomycin resistance marker and LentiORF constructs. Selection was performed using 0.1 g L⁻¹ G418 (Gibco) combined with 3 mg L⁻¹ puromycin (for ATG7) or 0.2 g L⁻¹ hygromycin B (Gibco) (for ATG5) as selection agents (12).

**Quantitative real-time PCR (qPCR)** — Strong-stop HIV DNA quantification was measured using qPCR with the LightCycler 1.5 instrument and FastStart DNA Master SYBR Green I (both Roche Applied Science). Primers and run conditions were as previously described (78). Data were analyzed using the Pfaffl method (79). The ratio between HIV LTR DNA and polymerase (RNA) II (DNA directed) polypeptide A (POLR2A) was calculated and normalized so that HIV LTR DNA in untreated cells equals 1.00. Data was then log₂ transformed.

**Statistics** — Data were assessed for symmetry, or skewness, using Pearson’s skewness coefficient. Fold change data were log₂ transformed to convert the ratio to a difference that better approximates the normal distribution on a log scale. Comparisons between groups were performed using the paired, two-tailed, Student's *t* test. However, if the ratio of the standard deviations between two groups is greater than 2, then the Welch's unequal variances *t* test was used. Differences were considered to be statistically significant when *P* < 0.05 and is indicated on plots and charts with *. All data are presented as individual data points representing individual donors on scatter plots overlain with the mean ± 95 % confidence interval (CI), unless stated otherwise.
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Conflict of interest: DLD declares a financial conflict of interest with SignalRx Pharmaceuticals. The relationship between DLD and SignalRx has been reviewed by the conflict of interest office within UC San Diego. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions: Conceived and designed the experiments: GRC RSB SJ DLD SAS. Performed the experiments: GRC RSB SDH. Analyzed the data: GRC RSB SDH SAS. Contributed reagents/materials/analysis tools: GRC DLD SAS. Wrote the paper: GRC RSB SDH SJ DLD SAS.
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PI3K/MTOR inhibitors suppress HIV-1 replication

FOOTNOTES
††Abbreviations used are: AKT serine/threonine kinase 1, AKT1; antiretroviral therapy, ART; autophagy-related, ATG; beclin 1, BECN1; bromodomain, BRD; bromodomain and extra terminal domain, BET; β-galactosidase, β-gal; C-C motif chemokine receptor 5, CCR5; eukaryotic translation initiation factor 4E binding protein 1, EIF4EBP1; human immunodeficiency virus type-1, HIV; lactate dehydrogenase, LDH; maximally tolerated dose, MTD; mechanistic target of rapamycin, MTOR; mechanistic target of rapamycin complex 1, MTORC1; microtubule-associated protein 1 light chain 3 beta, MAP1LC3B; peripheral blood mononuclear cells, PBMC; phosphatidylinositol 3-kinase, PI3K; phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit, PIK3C; phosphotase and tensin homolog, PTEN; phosphatidylinositol 3-phosphate, PtdIns3P; polo like kinase 1, PLK1; polymerase (RNA) II (DNA directed) polypeptide A, POLR2A; ribosomal protein S6 kinase, 70kDa, polypeptide 2, RPS6KB2; regulatory associated protein of MTOR complex 1, RPTOR; sequestosome 1, SQSTM1; super elongation complex, SEC; 50% tissue culture infective dose, TCID50; toll-like receptor, TLR; unc-51 like autophagy activating kinase 1, ULK1.
FIGURES

FIGURE 1. Dactolisib induces autophagy in human macrophages.
(A) Macrophages were treated for 24 h with dactolisib. Left, representative immunoblots of LC3B isoforms, BECN1 and SQSTM1 using antibody to LC3B, BECN1, SQSTM1 and ACTB. Right, densitometric analysis of immunoblots. n = 4.
(B) Macrophages were pretreated with pepstatin A before incubation with dactolisib for 24 h. Left, representative immunoblots of LC3B isoforms using antibody to LC3B, and ACTB. Right, densitometric analysis of immunoblots. n = 4.
(C) Aliquots of supernatant from (A) were spectrophotometrically tested for LDH using the LDHPLUS assay. n = 4.
(D) Quantification of the number of cells with apoptotic ssDNA using the ssDNA ELISA 96 h after dactolisib treatment. n = 4.
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FIGURE 2. Dactolisib inhibits HIV replication.

(A) Macrophages were incubated with increasing concentrations of dactolisib before exposure to HIV. ELISA performed for supernatant HIV p24 antigen over 10 d. Data are reported as mean ± s.e.m., n = 4.

(B) After 10 d HIV infection, aliquots of supernatant from part (A) were spectrophotometrically tested for LDH using the LDH PLUS assay. n = 4.

(C) After 10 d HIV infection, cells from (A) were fixed and permeabilized and the percentage of cells with apoptotic ssDNA quantified by ELISA. n = 4.
FIGURE 3. Dactolisib does not affect HIV entry or release.

(A) Macrophages were treated with 250 nM dactolisib, 100 nM sirolimus, or 10 nM maraviroc for 4 h before exposure to HIV. Binding was measured at 3 h post exposure by washing cells extensively, then lysing and analyzing p24 by ELISA. n = 4.

(B) Macrophages treated with 250 nM dactolisib, 100 nM sirolimus, or 10 nM maraviroc for 4 h before exposure to HIV. Entry was measured at 5 h post exposure by washing cells extensively, followed by trypsinization, lysing and subsequent analysis of p24 by ELISA. n = 4.

(C) Macrophages were treated with 250 nM dactolisib for 4 h before exposure to HIV. DNA was extracted at 8 h post-infection for qPCR analysis of pre-integration strong-stop HIV DNA. POLR2A was amplified as a control. Results are expressed as the ratio between the HIV LTR DNA and POLR2A, normalized to the vehicle control then log2 transformed. n = 4.

(D) Macrophages were treated with 250 nM dactolisib for 4 h before exposure to HIV. Macrophages were lysed at 5 h post exposure and subjected to immunoblotting for both HIV Nef and ACTB. n = 4.

(E) Percentage of TZM-bl cells productively infected with HIV after 4 h treatment with 250 nM dactolisib, nM SF2523, nM JQ1, or 10 nM maraviroc for 4 h before exposure to HIV. n = 4.

(F) Macrophages were incubated with 1 ng p24 antigen from the 10 d aliquots of cell-free supernatants post-250 nM dactolisib treatment for four hours then cultured for 10 days with ELISA performed for HIV p24 antigen. Data are reported as mean ± s.e.m., n = 4.
Macrophages were incubated with 250 nM dactolisib at different time points with respect to infection with HIV. ELISA performed for HIV p24 antigen over 10 d. Data are reported as mean ± s.e.m., n = 4.

HIV-infected macrophages were treated for 24 h with dactolisib. Left, representative immunoblots of LC3B isoforms, BECN1 and SQSTM1 using antibody to LC3B, BECN1, SQSTM1 and ACTB. Right, densitometric analysis of immunoblots. n = 4.

HIV-infected macrophages were treated for 24 h with dactolisib. Left, representative immunoblots of phospho-ULK1 (Ser757), phospho-EIF4EBP1 (Thr37/46), phospho-RPS6KB2 (Thr389), total ULK1, total EIF4EBP1, total RPS6KB2 and ACTB. Right, densitometric analysis of immunoblots. n = 4.
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FIGURE 4. Dactolisib-mediated decrease of HIV p24 antigen release from macrophages requires ATG5 and ATG7.

(A) Macrophages were transduced with ATG5 shRNA (shATG5), ATG7 shRNA (shATG7), or scrambled shRNA (shNS). Samples were pooled and analyzed by western blotting. Left, representative western blots permed using antibodies raised to ATG5, ATG7, and ACTB. Right, densitometric analysis of western blots. n = 4.

(B) Macrophages were transduced as in part A with the corresponding rescue shRNA-insensitive open reading frame (ORF) lentiviral vectors, then were treated with 250 nM dactolisib for 24 h, harvested and analyzed for autophagy proteins. Representative western blots of LC3B isoforms, ATG5, ATG7, and SQSTM1 using antibodies raised to LC3B, SQSTM1, ATG7, and ACTB is presented. n = 4.

(C) Macrophages transduced as in part A and B were incubated with 250 nM dactolisib or 100 nM sirolimus for 4 h before HIV infection under continuous drug treatment for 10 d. ELISA performed for supernatant HIV p24 antigen content. Data are reported as mean ± s.e.m., n = 4.
FIGURE 5. Dactolisib-mediated decrease of HIV p24 antigen release from macrophages requires the completion of autophagic flux. Macrophages were incubated with bafilomycin A1, SID 26681509 or vehicle prior to treatment with 0-100 nM dactolisib for 4 h before HIV infection under continuous drug treatment for 10 d. ELISA performed for supernatant HIV p24 antigen content. Data are reported as mean ± s.e.m., $n = 4$. 
FIGURE 6. **Dactolisib induces the lysosomal degradation of intracellular HIV.**

Macrophages were infected with HIV for 7 d then treated with 250 nM dactolisib in the presence or absence of 10 nM bafilomycin A₁ for 3 d.

(A) ELISA was performed for both extracellular and intracellular HIV p24 antigen at 7 and 10 d post-infection. *n* = 4.

(B) Top, representative immunoblots of HIV p24 antigen using antibody to HIV p24 antigen and ACTB. Bottom, densitometric analysis of immunoblots. *n* = 4.
FIGURE 7. SF2523 and JQ1 induce autophagy and inhibit HIV replication.

(A) Macrophages were pretreated with pepstatin A before incubation with JQ1 and SF2523 for 24 h. *Left*, representative immunoblots of LC3B isoforms using antibody to LC3B, and ACTB. *Right*, densitometric analysis of immunoblots. *n* = 4.

(B) Macrophages were incubated with increasing concentrations of JQ1 and SF2523 before exposure to HIV. ELISA performed for supernatant HIV p24 antigen over 10 d. Data are reported as mean ± s.e.m., *n* = 4.

(C) After 10 d HIV infection, aliquots of supernatant from cells in part (B) were spectrophotometrically tested for LDH using the LDHplus assay. *n* = 4.

(D) Macrophages were infected with HIV for 7 d then treated with 250 nM JQ1 or 500 nM SF2523 for 24 h. *Left*, representative immunoblots of LC3B isoforms, BECN1 and SQSTM1 using antibody to LC3B, BECN1, SQSTM1 and ACTB. *Right*, densitometric analysis of immunoblots. *n* = 4.

(E) Macrophages were infected with HIV for 7 d then treated with 250 nM JQ1 or 500 nM SF2523 for 24 h. *Left*, representative immunoblots of phospho-ULK1 (Ser757), phospho-EIF4EBP1 (Thr37/46), phospho-RPS6KB2 (Thr389), total ULK1, total EIF4EBP1, total RPS6KB2 and ACTB. *Right*, densitometric analysis of immunoblots. *n* = 4.
FIGURE 8. **SF2523 and JQ1-mediated induction of autophagy inhibits HIV replication.**

(A) Macrophages were infected with HIV for 7 d then treated with 250 nM JQ1 or 500 nM SF2523 in the presence or absence of 10 nM bafilomycin A1 for 3 d. ELISA was performed for both extracellular and intracellular HIV p24 antigen at 7 and 10 d post-infection. \( n = 4 \).

(B) Macrophages were infected with HIV for 7 d then treated with 250 nM JQ1 or 500 nM SF2523 in the presence or absence of 10 nM bafilomycin A1 for 3 d. Left, representative immunoblots of HIV p24 antigen using antibody to HIV p24 antigen and ACTB. Right, densitometric analysis of immunoblots. \( n = 4 \).
Induction of autophagy by PI3K/MTOR and PI3K/MTOR/BRD4 inhibitors suppresses HIV-1 replication
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