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The MTORC1/PI3K Inhibitor NVP-BEZ235 Inhibits HIV-1 Infection in Primary Human Cells Through the Induction of Autophagy

A thesis submitted in partial satisfaction of the requirements for the degree
Master of Science

in

Biology

by

Rachel Sue Bruckman

Committee in charge:

Professor Stephen Spector, Chair
Professor Michael David, Co-Chair
Professor Stephen Hedrick

2014

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The thesis of Rachel Sue Bruckman is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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University of California, San Diego

2014

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ACKNOWLEDGEMENTS

I would like to take this opportunity to extend my utmost gratitude and respect to my advisor, Dr. Stephen Spector, as well as the many esteemed members of his laboratory with whom I had the privilege of sharing these past two years.

Dr. Spector has been an excellent advisor and support system during this project, welcoming me into his lab with open arms. His willingness to share knowledge from his rich background has made working in his lab both interesting and thought-provoking, while his humility and kindness has made it a joy.

I would also like to give special thanks to my mentor during this time, Dr. Grant Campbell, who not only patiently trained me in the lab skills required to attempt this project, but provided honest counsel throughout the duration of my time as a student. I am appreciative of his input and contributions to this project. His constructive criticism and guiding confidence have been invaluable, and will serve as a foundation for continued learning for years to come.

The additional members of the Spector lab have been nothing short of helpful and generous, for which I am appreciative and grateful. I have learned a great deal working alongside each one of them.

Next, I would like to thank the additional members of my committee, Dr. Michael David and Dr. Stephen Hedrick for their input and support during my defense.

And lastly, to my friends and family, whose continued patience and understanding throughout many missed dinners and busy weekends is unspeakably appreciated, and whose love and support means the world.

ABSTRACT OF THE THESIS

The MTORC1/PI3K Inhibitor NVP-BEZ235 Inhibits HIV-1 Infection in Primary Human Cells Through the Induction of Autophagy
by

Rachel Sue Bruckman

Master of Science in Biology

University of California, San Diego, 2014

Professor Stephen Spector, Chair
Professor Michael David, Co-Chair

Since the 1980s, and arguably earlier, human immunodeficiency virus type-1 (HIV) has wreaked havoc on the lives of millions and devastated entire communities as a result of HIV-induced Acquired Immunodeficiency Syndrome (AIDS). Despite the advent of highly active antiretroviral therapy (HAART), HIV infection remains prevalent worldwide and is particularly threatening in the face of emerging drug-resistant HIV strains, against which there is limited treatment. Autophagy is an essential and ubiquitous catabolic cellular pathway, by which complex proteins and organelles are digested and recycled in this concerted process of self-preservation. Until recently the role of autophagy in modulating the immune-response has been under appreciated, but

is now known to help fight against infectious diseases; increased autophagic activity of a cell results in increased pathogen recognition and subsequent inhibition. However, in the case of productive HIV infection, autophagy is down-regulated, thereby promoting viral replication and cell survival. NVP-BEZ235, a dual mammalian target of the rapamycin complex 1 (MTORC1)/phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) class I inhibitor, has been shown in cancer cell lines to induce autophagy and is examined here as a potential therapeutic agent against HIV infection. Using protein markers microtubule-associated protein 1 light chain 3 beta (LC3B) and sequestosome 1 (SQSTM1) to measure autophagic flux, I demonstrate that NVP-BEZ235 induces autophagy in primary human macrophages and $CD4^+$ T cells while inhibiting HIV infection in an autophagy-dependent manner. These findings suggest that NVP-BEZ235 may be a useful adjunct to current antiretroviral therapy, and has the potential to be used in an HIV cure strategy.

I. INTRODUCTION

Although unconnected at the time, Christian de Duve was awarded the Nobel Prize in Physiology (shared with George Palade and Albert Claude) for his characterization of the lysosome (gart et al., 1955) in 1974, around the same time that human immunodeficiency virus type-1 (HIV) was beginning to spread amongst the unsuspecting human population. Forty years later, de Duve expanded on his discovery to describe an additional intracellular recycling process which he termed “autophagy”, meaning “self-eating”. Autophagy is a ubiquitous catabolic cell survival mechanism distinct from programmed cell death, which sequesters proteins and retired organelles in a ubiquitin-like manner in order to recycle these cellular components for further use (Maiuri et al., 2007). Although autophagy converges with the lysosomal degradative pathway, it is a vital cellular homeostatic pathway distinct from that of the lysosome (Gordon et al., 1988), which has only recently begun to receive dedicated attention (Klionsky, 2007).

There are several types of autophagy: chaperone-mediated, microautophagy, and macroautophagy. This thesis limits itself to the examination of macroautophagy (hereafter referred to as autophagy), which is representative of typical autophagic processes. While the complete molecular picture of autophagic induction and control mechanisms remains unknown, numerous autophagy-related (ATG) genes have been characterized in yeast, and many have equivalents found in mammals (Yang and Klionsky, 2010, Wang et al., 2003, Mizushima et al., 2010). Upstream regulation of autophagy in humans is controlled by the unc-51 like autophagy activating kinase 1 (ULK1), which is regulated by autophagy-related 13 (ATG13), although the precise role of this complex in autophagy remains unclear (Yang and Klionsky, 2010). The highly

conserved mammalian target of rapamycin (MTOR) complex 1 (MTORC1) functions as a master regulator at this point in the pathway by phosphorylating ULK1 and ATG13, inhibiting autophagy during nutrient-rich conditions and in response to cytokine signaling. Conversely, the inhibition of MTORC1 allows autophagy to progress, particularly under conditions of stress (Ganley et al., 2009, Takeshige et al., 1992). During conditions of MTORC1 inhibition, the autophagic process begins *de novo* with the formation of the phagophore which is expanded by the ATG5–ATG12-ATG16L complex to form the autophagosome responsible for the sequestration of cytosolic contents. The autophagosome may then fuse with an endosome, forming an amphisome, or may then fuse with a lysosome for degradation, or both.

Although autophagy has been largely studied in the context of cell survival, its role in immunity has gained increased attention over the past decade (Mizushima et al., 2008, Schmid et al., 2007). In addition to its role in cell health and survival under stress (Yang et al., 2011, Ogata et al., 2006), autophagy has been implicated in a wide array of human diseases including cancer, neurodegenerative disorders, inflammatory bowel disease and infectious diseases (Levine and Kroemer, 2008, Rioux et al., 2007, Degenhardt et al., 2006). In the case of infectious diseases, autophagy has been identified as an important mechanism for pathogen detection and removal (Sanjuan et al., 2007, Levine and Kroemer, 2008, Yano et al., 2008, Choi et al., 2013). Recent studies have demonstrated that autophagy plays an important role against a wide variety of viral targets by responding to innate Toll-like receptor (TLR) signaling and contributing to the generation of an adaptive immune response through enhanced major histocompatibility complex (MHC) I and II antigen presentation (Richards et al., 2013, Ravidran et al., 2014). Until recently, the role of autophagy in the pathogenesis of HIV

infection was not appreciated but has since been shown to be down-regulated in the presence of HIV (Espert et al., 2009, Zhou and Spector, 2008, Spector and Zhou, 2008).

During different stages of infection, HIV modulates autophagy to promote viral replication while prolonging cell survival. During initial infection there is an induction of autophagy that promotes viral replication (Espert et al., 2009, Kyei et al., 2009). However, once a permissive infection is established, HIV inhibits autophagic flux to promote virus survival by preventing viral detection and degradation (Zhou and Spector, 2008, Espert et al., 2009, Kyei et al., 2009). Knowing this, it was hypothesized that by overcoming this down-regulation of autophagy with pharmacological inducers of autophagy, HIV may be detected and targeted for destruction. Our lab has shown in multiple studies that induction of autophagy is sufficient to significantly inhibit HIV *in vitro*, and additional studies have addressed the importance of autophagy in detecting and expelling other pathogenic infections (Campbell and Spector, 2011, Campbell and Spector, 2012, Shoji-Kawata et al., 2013, Campbell and Spector, 2013, Choi et al., 2013). Conversely, HIV induces autophagy in neighboring, uninfected CD4+ T cells, through HIV Env proteins which trigger cell death through an autophagy related mechanism (Espert et al., 2006).

For the reasons stated, autophagy is an attractive target for HIV drug therapy. NVP-BEZ235 is an imidazo[4,5-*c*]quinolin derivative that shows dual ATP-competitive inhibition of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and MTORC1, and has been shown to induce autophagy in studies using cancer cell lines (Maira et al., 2008). Mutations in the PI3K/AKT1/MTORC1 pathway are common in various human cancers (Liu et al., 2009), and NVP-BEZ235 has shown promise as a powerful agent against neoplastic tumors while exhibiting limited toxicity *in vivo* (Serra et al., 2008, Maira et al., 2008, Chiarini et al., 2010). By inhibiting MTORC1 through binding at the

ATP-cleft, NVP-BEZ235 prevents the phosphorylation of the 70kDa ribosomal protein S6 kinase polypeptide 2 (RPS6KB2) and other MTORC1 downstream effectors, leading to a release of autophagy inhibition by MTORC1 (Chiarini et al., 2010). While studies have shown NVP-BEZ235 affects cell growth through PI3K/AKT1 and MTORC1 inhibition (Fan et al., 2010, Serra et al., 2008), and the subsequent induction of autophagy in tumor cells (Chang et al., 2013, Liu TJ et al., 2009), only one study to our knowledge has examined the effects of NVP-BEZ235 on microbial pathogens. In that report, inhibition of PI3K/AKT1 through NVP-BEZ235 was shown to interfere with the growth of lymphocytic choriomeningitis (LCMV) through inhibition of viral budding (Urata et al., 2012), but how the drug acted was not examined. Additionally, the effect of NVP-BEZ235 on other viruses, specifically HIV, remains unknown. Furthermore, the effect of NVP-BEZ235 on autophagy has yet to be examined in human primary cells, which provide the most representative human *in vitro* model for HIV infection. This study, therefore, was designed to examine the effect of NVP-BEZ235 on autophagic processing in human primary cells and the therapeutic potential of NVP-BEZ235 against HIV. My findings show that NVP-BEZ235 effectively inhibits HIV infection through the induction of autophagy in both primary human macrophages and $CD4^{+}$ T cells.

II. MATERIALS AND METHODS

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 08-1613) 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). Written informed consent was obtained from all blood donors prior to their participation.

Cell Isolation and Culture

Human peripheral blood mononuclear cells (PBMCs) were obtained from HIV seronegative whole blood using Ficoll-Pacque PLUS (GE Healthcare) density gradient centrifugation. Macrophage cultures were obtained by incubating PBMCs for 24 h in RPMI 1640 (Gibco) supplemented with 2 mM Glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin (all Gibco), 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gemini Bioproducts), and 10 ng/mL macrophage colony stimulating factor (MCSF, Peprotech), after which non-adherent cells were removed and the adherent population further incubated for 10 d at 5% CO_2, 37°C in macrophage media. All macrophage cultures were maintained in this MCSF-supplemented media for the duration of the experiments.

Human $CD4^+$ T cells were differentiated by expansion in growth media: RPMI 1640 supplemented with 20% (v/v) FBS, 2 mM Glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 50 ng/mL interleukin-2 (IL2; Peprotech). $CD4^+$ T cells were activated using 50 μg/mL PHA-P (Sigma) for 48-72 h, washed in Dulbecco's phosphate

buffered saline (DPBS; Gibco), resuspended in growth media, and maintained at 5% CO_2, 37°C.

Reagents and cytotoxicity

NVP-BEZ235 (S1009; Selleck Chemicals) was prepared at 1 mg/mL in DMSO (Sigma), diluted to working concentrations in 100% ethanol (Gold Shield), and stored at -80°C until use. Pepstatin A and SID 26681509 (Sigma) were prepared in ethanol, used at 10 μg/mL and 50 nM final concentration. Bafilomycin A_1 (Sigma) was reconstituted in DMSO and used at 100 nM final concentration. Cells were pretreated with bafilomycin A_1 or SID 26681509 for 1 h before addition of NVP-BEZ235.

Cell death was estimated using the lactate dehydrogenase Cytotoxicity Detection Kit^{PLUS} and the Cell Proliferation Reagent WST-1 (both Roche) in tandem. Prior to administration of WST-1, culture supernatants were collected for use in the Cell Cytotoxicity Assay LDH (Roche). Cell viability and cytotoxicity was then measured spectrophotometrically according to the manufacturer's instructions.

HIV-1 infection and detection

HIV_{Ba-L} was obtained through the AIDS Research and Reference Reagent Program (Gartner et al., 1986, Popovic et al., 1988). HIV_{NL4-3} was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: p83-10 from Dr. Ronald Desrosiers (Gibbs et al., 1994). Virus stocks were prepared as previously described (Campbell et al., 2010).

Macrophages were pretreated with NVP-BEZ235 24 h before infection with HIV_{Ba-L} at a multiplicity of infection (MOI) of 0.004 for 24 h. Cells were then washed 3 × with DPBS and fresh media NVP-BEZ235 administered. Aliquots of cell supernatants

were collected 3, 5, 7, and 10 d post-infection, at which time media was replenished and drugs re-administered. Cell culture supernatants were screened for p24 antigen release as a measure of viral replication using the Alliance HIV p24 antigen ELISA (Perkin Elmer).

After 48-72 h treatment in PHA-supplemented media, $CD4^+$ T cells were washed 3 × with DPBS and resuspended in IL2-supplemented media at a concentration of 1 × 10^6 cells/mL. 1 h post-DPBS wash, cells were infected with HIV_{NL4-3} (MOI = 0.0004) and incubated for 96 h, after which cells were washed 3 × with DPBS and resuspended to 1 × 10^6 cells/mL (day 0). After an additional 24 h, PHA-P-activated and DPBS-washed PBMCs were added to HIV-infected cell cultures at a ratio of 2:1, and drug treatment added to total culture. Cell culture supernatants were collected at 4, 7, and 10 d cultures at which time media was replenished to maintain 1 × 10^6 cells/mL and drug treatments re-administered. Viral infection was measured by p24 antigen release into the supernatant and quantified using the Alliance HIV p24 antigen ELISA (Perkin Elmer).

Western and Immunoblotting

Cell lysates were prepared using a solution of 20 mM 4-(2-hydroxyethyl)-1-piperasineethanesulfonic acid, 1 mM ethylenediaminetetraacetic acid (both Gibco), 150 mM NaCl, 1% (v/v) 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (both Sigma) and 1% (v/v) Halt protease inhibitor cocktail (Thermo Scientific). Lysates were run on 2-[bis(2-hyxroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol buffered 12% polyacrylamide gels (Novex) and then transferred to 0.2 μm PVDF membranes (Thermo Scientific). Experiments were conducted in duplicate with three independent blood donors.

Proteins were detected using monoclonal antibodies: β-actin (ACTB; Sigma; AC-74), microtubule-associated protein 1 light chain 3 beta (LC3B; Novus Biologicals NB100-2220) and sequestosome 1 (SQSTM1; Abcam ab56416), and later detected using the WesternBreeze chemiluminescence kit (Novex). Densitometric analysis of resulting protein bands was performed using ImageJ software (NIH), normalizing relative band densities to corresponding ACTB bands.

Statistical analysis

Data are shown as mean values ± standard error of the mean (s.e.m). Comparisons between groups were performed using the Student's *t*-test, with differences considered to be statistically significant when $P < 0.05$.

III. RESULTS

NVP-BEZ235 induces autophagy in primary macrophages

NVP-BEZ235 has been shown to induce autophagy in a number of carcinoma cell lines (Serra et al., 2008, Maira et al., 2008, Chiarini et al., 2010); however, the impact of NVP-BEZ235 on autophagy in primary cells is unknown. Therefore, we first investigated the effect of NVP-BEZ235 in human primary macrophages. To study the effects of NVP-BEZ235 on primary immune cells, macrophages were cultured with 10-250 nM NVP-BEZ235 for 24 h, after which cells were washed, lysed, and proteins resolved using polyacrylamide gel electrophoresis and Western blotting. During the formation of the nascent autophagosome, cytosolic LC3B (LC3B-I) is conjugated to phosphatidylethanolamine by in an ubiquitin-like process involving ATG3, ATG7 and the ATG5–ATG12 complex resulting in autophagosome-associated LC3 (LC3B-II). When autophagosomes fuse with lysosomes, forming autolysosomes, the intraluminal LC3-II is degraded, and the cytoplasmic surface LC3-II is delipidated and recycled. Considering autophagy is a ubiquitious protein recycling process, the conversion of LC3B-I to LC3B-II and its turnover (as measured by the (LC3B-II:LC3B-I):ACTB protein ratio) demonstrates the progression of autophagy (Mizushima and Yoshimori, 2007). LC3B lipidation in response to NVP-BEZ235 treatment was increased at 10 nM, the lowest dose tested. Moreover, the effect on LC3B-II:LC3B-I ratio was dose-dependent and was maximal at 100 nM (6.8 fold increase; Figure 1A) with no additional lipidation observed at 250 nM.

To further establish the induction of autophagic flux in NVP-BEZ235 treated macrophages, and not simply the activation of LC3B transcription and/or the accumulation of autophagosomes, additional experiments were conducted with macrophages in the presence of the lysosomal protease inhibitor, pepstatin A. Pepstatin A prevents the degradation of LC3B-II during of 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 the addition of NVP-BEZ235 , and lysed after subsequent 24 h NVP-BEZ235 treatment. The accumulation of LC3B-II was increased in the presence of pepstatin A (Figure 2B), indicative of autophagic flux. An additional marker for autophagic flux is the degradation of the polyubiquitin-binding protein SQSTM1. SQSTM1 associates with the contents of the developing autophagosome only to be degraded following lysosome fusion; a decrease of SQSTM1, therefore, is indicative of a continuous progression of autophagy. Similar to LC3B lipidation, NVP-BEZ235 induced a decrease in SQSTM1 levels at 10 nM and was maximal at 100 nM (5.3 fold decrease; Figure 1C) with no further degradation observed at 250 nM. Interestingly, the extent of the induction of LC3B lipidation and SQSTM1 degradation was greater for NVP-BEZ235 than for the molar equivalent of the MTOR inhibitor rapamycin.

NVP-BEZ235 prevents and inhibits HIV infection in macrophages through autophagy

Our laboratory's findings and that of others indicate that autophagy plays an important role in establishing a productive infection with HIV (Brass et al., 2008, Campbell and Spector, 2011; 2013). However, once a permissive infection is established HIV down-regulates autophagy to facilitate cell survival and to promote viral replication (Kyei et al., 2009, Campbell and Spector, 2013). Previous studies have shown that by increasing autophagic flux using pharmacological inducers of autophagy, it is possible to overcome the HIV-induced inhibition of autophagy and inhibit HIV production from macrophages (Campbell and Spector, 2011, Campbell and Spector, 2012). We therefore evaluated if NVP-BEZ235 has HIV-inhibitory effects by comparing the extent to which NVP-BEZ235 treatment influenced p24 antigen release into the supernatants of

productively infected macrophages. NVP-BEZ235 induced a dose-dependent inhibition of HIV with 10 nM, the lowest dose tested, being sufficient to significantly inhibit HIV by day 3 post-infection; this inhibition continued until cultures were discontinued at day 10 post-infection. At 100 nM, NVP-BEZ235 almost completely inhibited HIV p24 release, exhibiting an over 90% decrease in HIV p24 by day 10 post-infection in a representative donor, compared to the untreated control (Figure 3). At doses of NVP-BEZ235 higher than 100 nM, further inhibition of HIV was not observed (data not shown).

To establish that the inhibition of HIV by NVP-BEZ235 was dependent on autophagic flux, we first inhibited autophagosome acidification using bafilomycin A_1, a late stage autophagy inhibitor that prevents autophagosome-lysosome fusion and subsequent maturation of the autolysosome. Pretreatment with bafilomycin A_1 reversed the NVP-BEZ235-mediated inhibition of HIV, which suggests that the autophagosome-lysosome fusion and subsequent maturation of the autolysosome is required for the autophagy-mediated control of HIV.

Once autolysosomes are formed, the sequestered components are degraded by lysosomal hydrolases, such as cathepsin L. We investigated whether these lysosomal hydrolases are important for NVP-BEZ235-mediated inhibition of HIV through autophagy using the thiocarbazate SID 26681509, a novel and specific inhibitor of the lysosome hydrolase cathepsin L. Importantly, unlike many other cathepsin and protease inhibitors, SID 26681509 does not inhibit the HIV protease. While macrophages capable of autophagic completion showed effective inhibition of HIV when treated with NVP-BEZ235, the HIV-inhibitory effects of NVP-BEZ235 were reversed when autophagy was blocked with SID 26681509. Thus, autophagy going to completion is required for the NVP-BEZ235-mediated inhibition of HIV (Figure 4).

NVP-BEZ235-induced inhibition of HIV in $CD4^+$ T cells

The hallmark of HIV is the infection and destruction of $CD4^+$ T cells. While macrophages play a significant role in establishing HIV infection and infection of the central nervous system, $CD4^+$ T cells have a critical role in HIV pathogenesis. $CD4^+$ T cell counts are commonly used clinically, a count of $<200/mm^3$ indicative of progression to AIDS. It was therefore of interest to determine if NVP-BEZ235 can also inhibit productive HIV infection of $CD4^+$ T cells.

Activated $CD4^+$ T cells were treated with NVP-BEZ235 for 24 h and assessed for LC3B lipidation and SQSTM1 degradation by immunoblotting. $CD4^+$ T cells showed both the degradation of SQSTM1 and increased conversion of LC3B-I to LC3B-II, confirming autophagic flux in these cells (Figure 5). Likewise, NVP-BEZ235 showed similar HIV-inhibitory effects in $CD4^+$ T cells in a dose-dependent manner (Figure 6).

NVP-BEZ235 shows no cytotoxic effects as determined by LDH release

NVP-BEZ235 is undergoing clinical trials as a therapeutic cancer-targeting drug with an acceptable toxicity profile, yet its cytotoxic effects in normal primary immune cells remain unknown. It was also unknown if NVP-BEZ235 could elicit harmful effects on cell stasis during HIV infection. Therefore, cell toxicity was measured at the end of HIV-infection of both macrophages and $CD4^+$ T cells.

Using lactase dehydrogenase (LDH) detection in culture supernatants as an indicator of cell death, aliquots of supernatants on day 10 of infection were collected and screened using the LDH^{PLUS} cytotoxicity assay. Spectrophotometric measurements showed little to no harmful effects on cell survival as a result of NVP-BEZ235, in either primary macrophages or $CD4^+$ T cells (Figure 7).

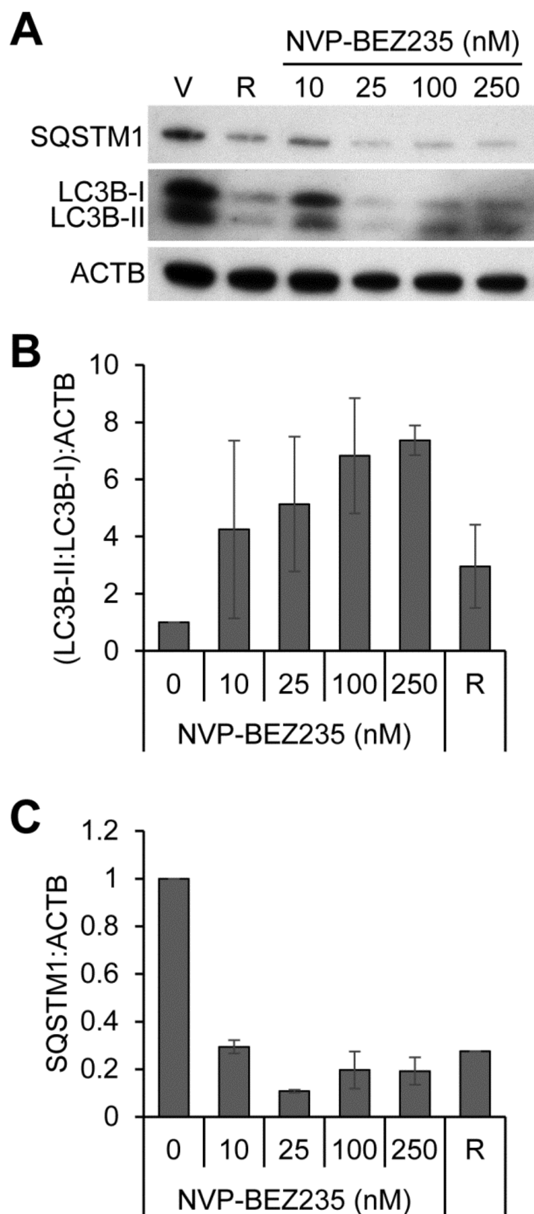

FIGURE 1. NVP-BEZ235 induces autophagy in human macrophages. Macrophages were treated with increasing concentrations of NVP-BEZ235, 100 nM rapamycin (*R*), or vehicle (*V*) for 24 h before being harvested and analyzed by Western blotting. (A) Immunoblot of LC3B isoforms and SQSTM1 using antibody to LC3B, SQSTM1 and ACTB. (B) Densitometric analysis of LC3B isoforms (LC3B-II:LC3B-I and normalized to ACTB). NVP-BEZ235 induces an increase in LC3B-II:LC3B-I indicative of increased autophagic flux. (C) Densitometric analysis of SQSTM1 (normalized to ACTB). NVP-BEZ235 induces a decrease in detectable SQSTM1 indicative of increased autophagosome turnover and autophagic flux. Data is shown as mean ± s.e.m. from a single donor performed in duplicate. Similar results were observed from four independent experiments.

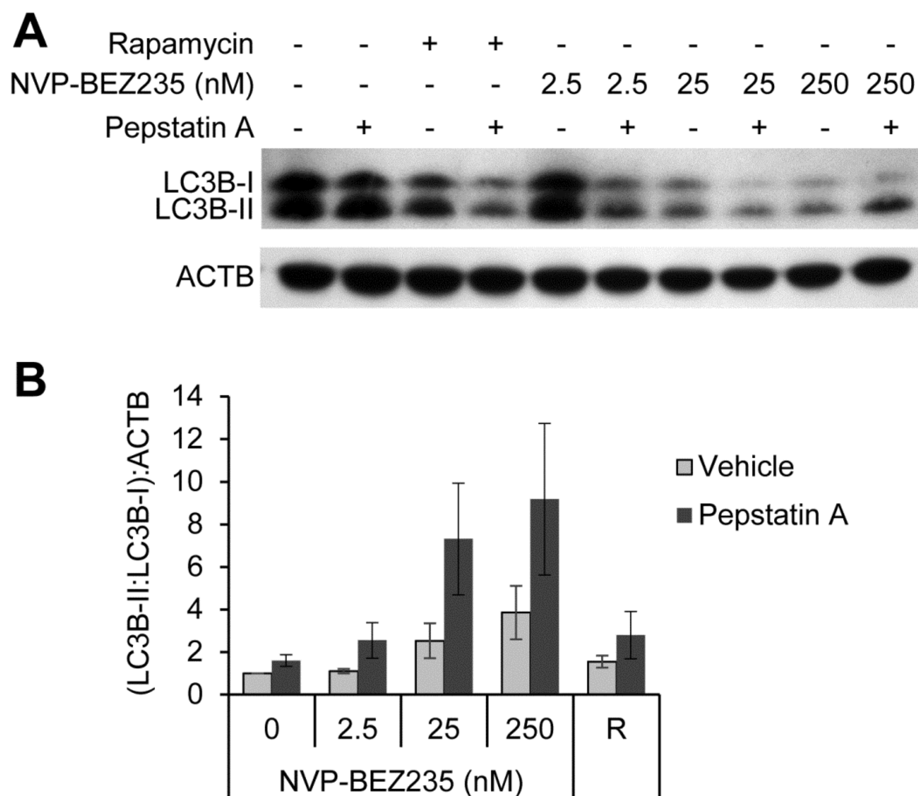

FIGURE 2. Confirmation of autophagic flux in macrophages using acid protease inhibitor, pepstatin A. Macrophages were treated with increasing concentrations of NVP-BEZ235, 100 nM rapamycin (*R*), in the presence or absence of pepstatin A for 24 h before being harvested and analyzed by Western blotting. (A) Immunoblot of LC3B isoforms using antibody to LC3B, and ACTB. (B) Densitometric analysis of LC3B isoforms (LC3B-II:LC3B-I and normalized to ACTB). Pepstatin A increases the NVP-BEZ235 induced LC3B-II:LC3B-I ratio indicative of decreased autophagosome turnover and autophagic flux. Data is shown as mean ± s.e.m. from a single donor performed in duplicate. Similar results were observed from two independent experiments.

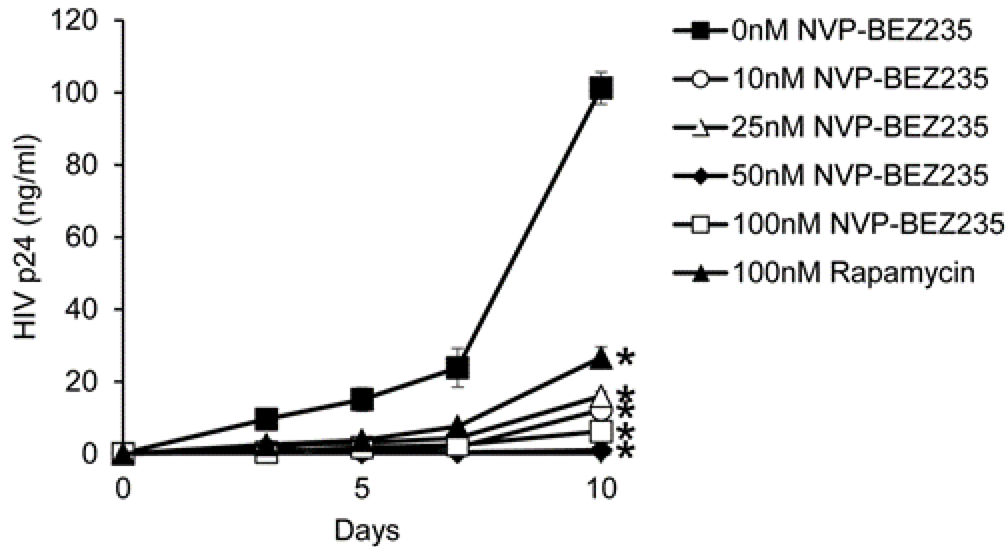

FIGURE 3. NVP-BEZ235 effectively prevents and inhibits HIV infection in macrophages. Macrophages were pretreated with NVP-BEZ235 24 h prior to infection with $HIV_{Ba\text{-}L}$. Release of HIV p24 antigen into the cell supernatant at days 0, 3, 5, 7, and 10 was detected by ELISA. NVP-BEZ235 inhibits HIV p24 release into the cell supernatant in a dose-dependent manner. Data is shown as mean ± s.e.m. from a single donor performed in triplicate. Similar results were observed from three independent experiments.* $P < 0.05$

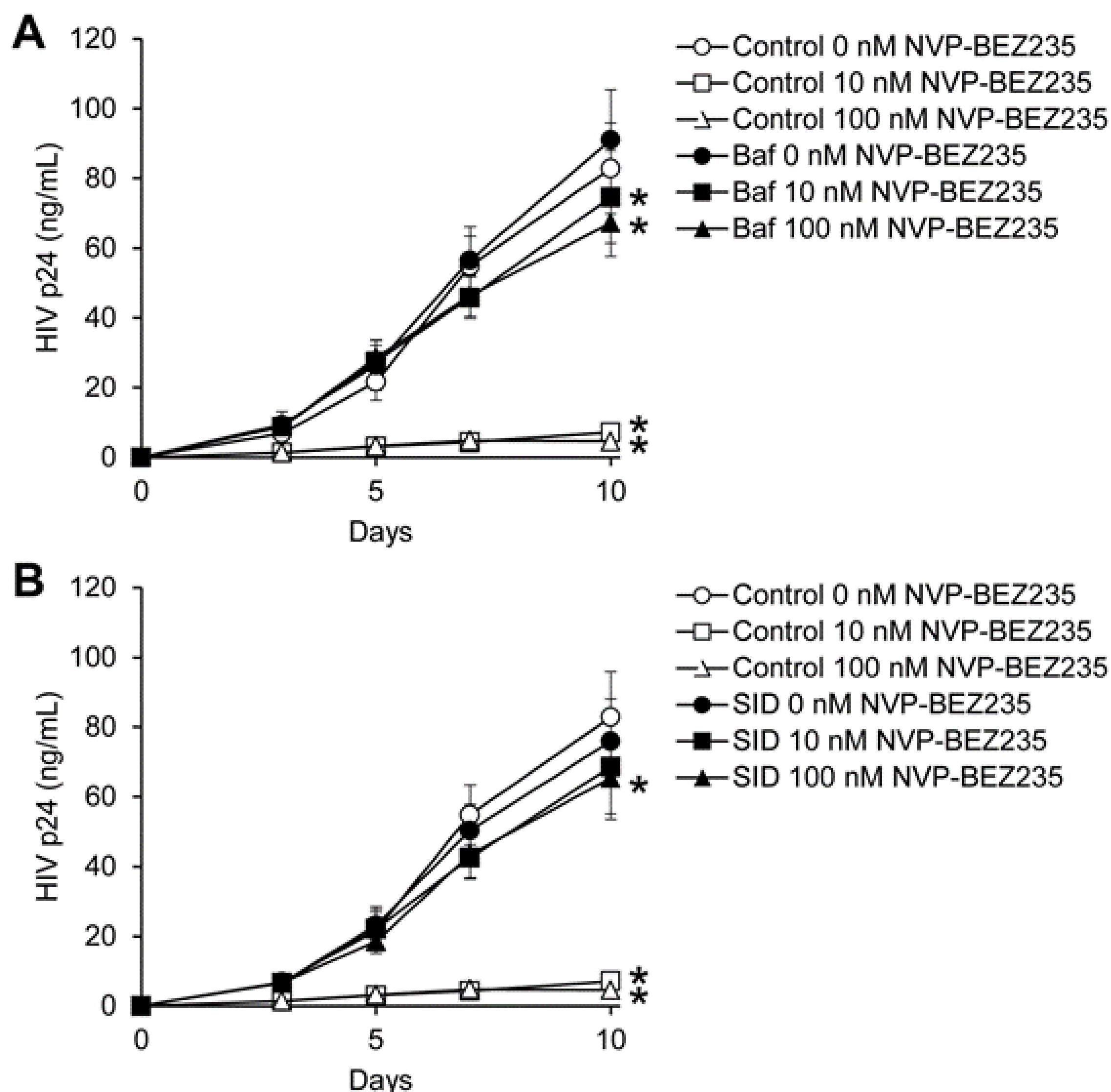

FIGURE 4. NVP-BEZ235 inhibits HIV infection in an autophagy-dependent manner. Macrophages pretreated with (A) bafilomycin A_1 (*Baf*), or (B) SID 26681509 (*SID*) or vehicle control (*Control*) prior to treatment with NVP-BEZ235 for 24 h before infection with HIV_{Ba-L}. Cells were then washed and incubated with NVP-BEZ235 in the continued presence of bafilomycin A_1 or SID 26681509 for 10 d. Viral replication was measured by HIV p24 release into the cell supernatant detected by ELISA. NVP-BEZ235-induced inhibition of HIV was reversed in the presence of both bafilomycin A_1 and SID 26681509. Results are reported as mean ± s.e.m., n = 5. * $P < 0.05$.

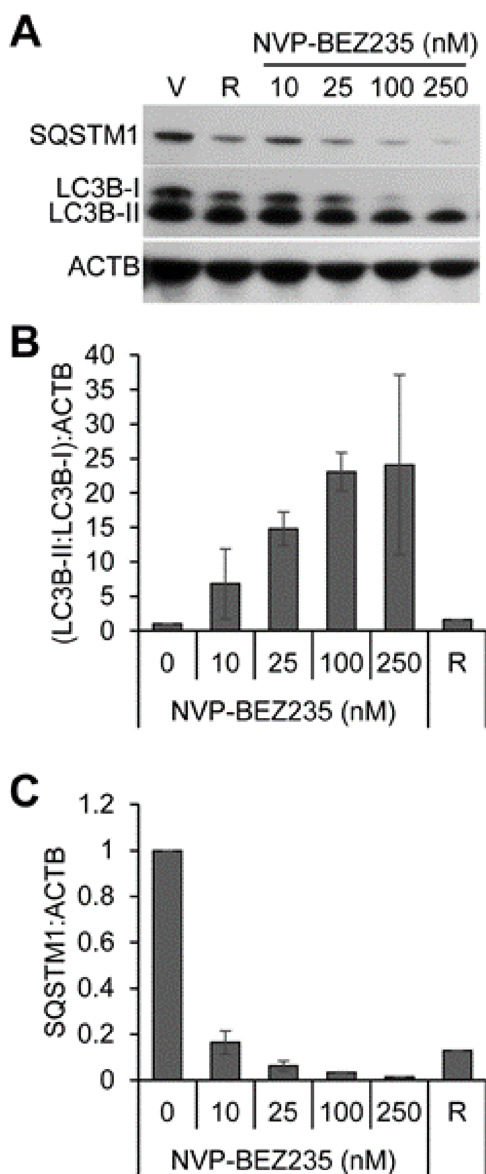

FIGURE 5. NVP-BEZ235 stimulates autophagy induction in CD4⁺ T cell population. PHA and IL2 treated CD4⁺ T cells were treated with increasing concentrations of NVP-BEZ235, 100 nM rapamycin (*R*), or vehicle (*V*) for 24 h before being harvested and analyzed by Western blotting. (A) Immunoblot of LC3B isoforms and SQSTM1 using antibody to LC3B, SQSTM1 and ACTB. (B) Densitometric analysis of LC3B isoforms (LC3B-II:LC3B-I ratio normalized to ACTB). NVP-BEZ235 induces an increase in LC3B-II:LC3B-I indicative of increased autophagosome number. (C) Densitometric analysis of SQSTM1 (normalized to ACTB). NVP-BEZ235 induces a decrease in detectable SQSTM1 indicative of increased autophagosome turnover and autophagic flux. Data are shown as mean ± s.e.m. from a single donor performed in duplicate. Similar results were observed from three independent experiments.

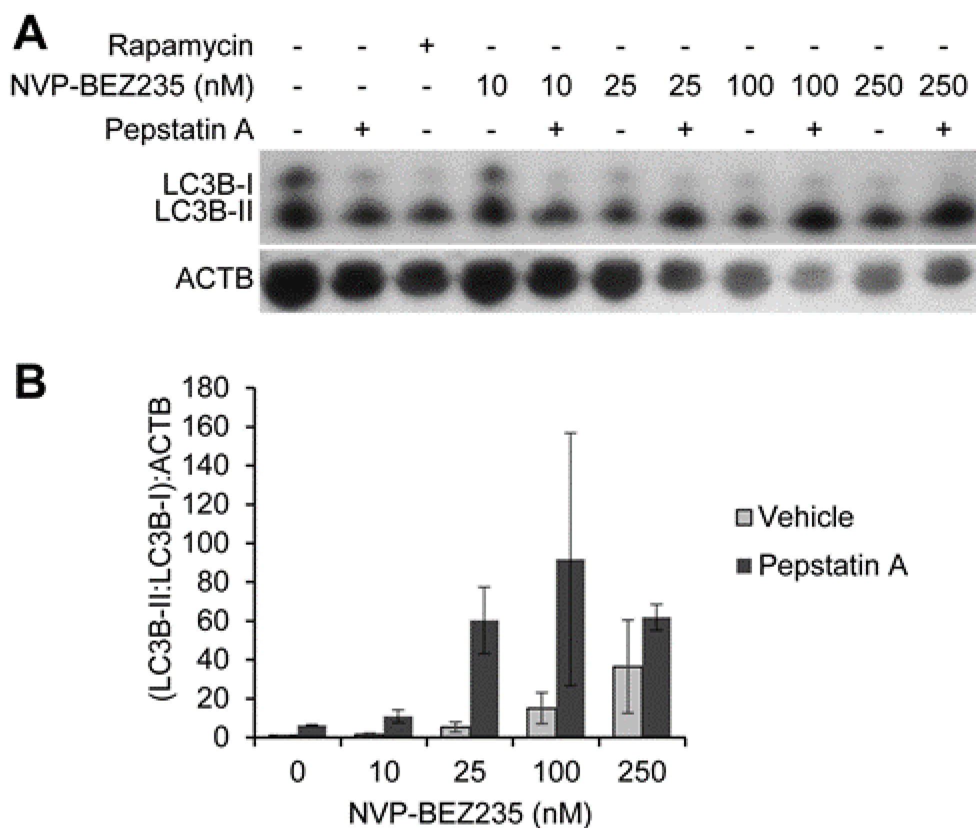

FIGURE 6. Confirmation of autophagic flux in CD4⁺ T cells using acid protease inhibitor, pepstatin A. PHA and IL2 treated CD4⁺ T cells were treated with increasing concentrations of NVP-BEZ235, 100 nM rapamycin (*R*), in the presence or absence of pepstatin A for 24 h before being harvested and analyzed by Western blotting. (A) Immunoblot of LC3B isoforms using antibody to LC3B, and ACTB. (B) Densitometric analysis of LC3B isoforms (LC3B-II:LC3B-I and normalized to ACTB). Pepstatin A increases the NVP-BEZ235 induced LC3B-II:LC3B-I ratio indicative of decreased autophagosome turnover and autophagic flux. Data is shown as mean ± s.e.m. from a single donor performed in duplicate.

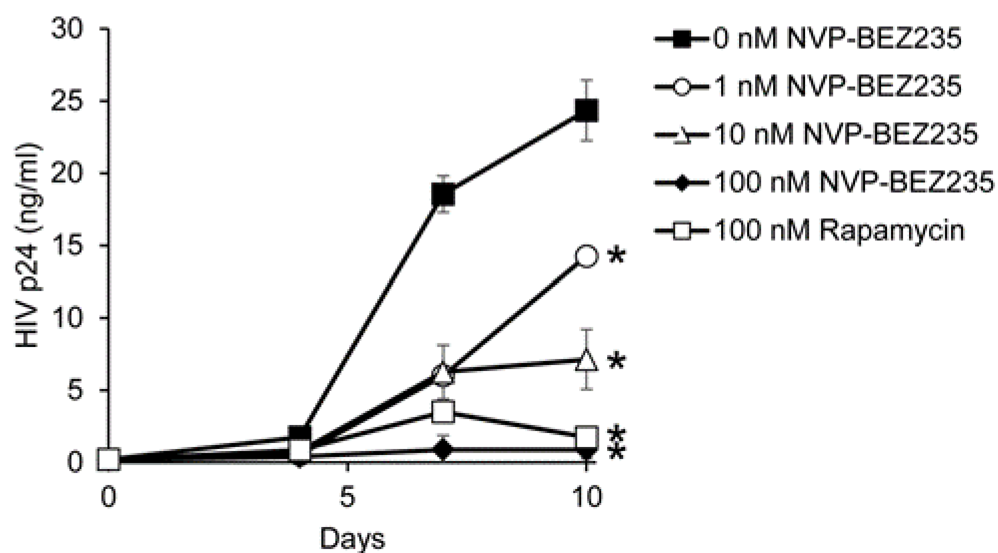

FIGURE 7. $CD4^+$ T cells treated with NVP-BEZ235 show significantly decreased HIV p24 release. PHA and IL2 stimulated $CD4^+$ T cells were infected with $HIV_{NL\text{-}43}$, washed and treated with NVP-BEZ235 (Day 0). Release of HIV p24 antigen into the cell supernatant at days 0, 4, and 10 was detected by ELISA. NVP-BEZ235 inhibits HIV p24 release into the cell supernatant in a dose-dependent manner. Results are reported as mean ± s.e.m. from a single donor performed in triplicate. * $P < 0.05$.

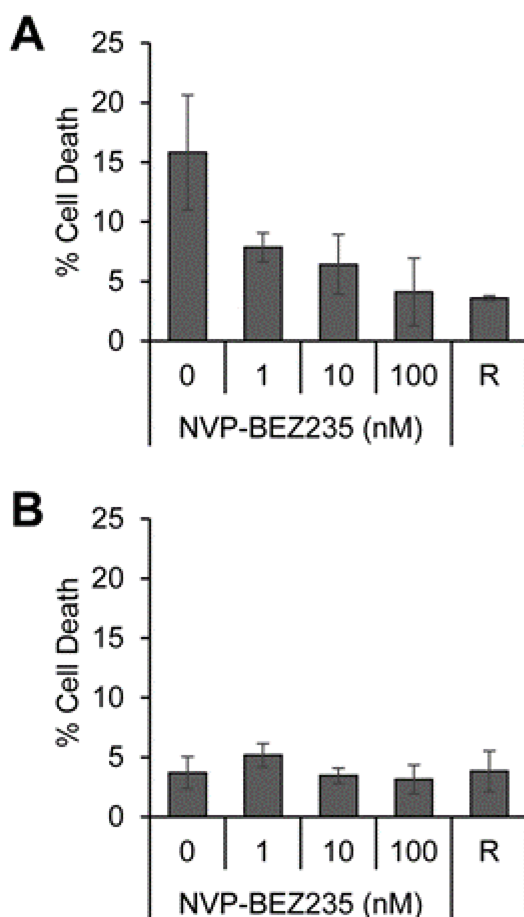

FIGURE 8. NVP-BEZ235 is not cytotoxic in HIV-infected cultures of $CD4^+$ cells and macrophages. Culture supernatants were collected 10 d post-HIV-infection of NVP-BEZ235 treated $CD4^+$ (A) T cell and (B) macrophage cultures, and were tested for lactate dehydrogenase (LDH) spectrophotometrically using the LDH^{PLUS} assay. NVP-BEZ235 treatment elicited no significant increase in cell death. Results are reported as mean ± s.e.m. from a single donor performed in triplicate. Similar results were observed from three independent experiments for the macrophage cultures.

IV. DISCUSSION

No virus has affected the human population in recent history as swiftly and dramatically as HIV. The World Health Organization estimates that as of 2012 HIV infection had caused the death of 1.6 million adults and children worldwide, with ~1.4 million people afflicted with HIV in the United States (WHO, 2012). With the advent of highly active antiretroviral therapy (HAART), life expectancy and quality of life for those infected with HIV have dramatically improved (Hammer et al., 1996, Autran et al., 1997). However, drug resistance continues to plague HIV-infected persons (Richman et al., 2004), making it advantageous to identify new strategies to improve HIV treatment.

Innate immunity, once believed to have a minor role in immune protection, has become increasingly recognized as the essential mediator of initial and rapid protection against invading microbial pathogens. Through the dissection of innate antiviral immune mechanisms, it becomes possible to understand how and to what effect viruses circumvent these processes. This understanding can then lead to the exploitation of these innate antiviral immunity mechanisms, which may clear the way for the development of new drugs targeting and enhancing these immunoprotective pathways. This approach is particularly beneficial as it will potentiate the host's own innate immunity to destroy the invading pathogen. One such strategy centers around the understanding and modulation of autophagy, which has gained recognition as an important cellular mechanism for controlling microbial pathogens (Mizushima et al., 2008, Levine and Kroemer, 2008). The induction and modulation of autophagy through pharmacological means to enhance HIV treatment is attractive and novel as autophagy works at the host cellular level to improve intracellular killing of both replicating and non-

replicating HIV within endosomes. A common target for autophagy modulation is MTORC1, which inhibits autophagosome formation through its phosphorylation of ULK1; therefore, by inhibiting MTORC1, autophagy is permitted to progress. The present data demonstrate that the dual PI3K/MTORC1 inhibitor NVP-BEZ235 does just that—inhibits MTORC1 and subsequently induces autophagy in human primary cells, as indicated by the accumulation of LC3B-II when protein degradation is inhibited. Moreover, these data indicate an ability to pharmacologically induce autophagy in order to increase degradation of intracellular viral components, or xenophagy. Autophagy, therefore, represents an opportunity to help cells help themselves. Additionally, viral resistance is unlikely to develop. Rather than targeting a disease agent exhibiting a highly mutated and diverse profile, as is the case in HIV, autophagy is a highly conserved cell survival process which can clear intracellular pathogens through non-specific sequestration (Meijer et al., 2007).

Exogenous treatment of NVP-BEZ235 supports this hypothesis, as it was shown to consistently inhibit HIV in an autophagy-dependent mechanism. The appeal of NVP-BEZ235 as an anti-HIV therapeutic agent is considerable: as a cancer drug, NVP-BEZ235 has been shown in early *in vivo* mouse trials to be well-tolerated (Maira et al., 2008). While NVP-BEZ235 has been shown to be cytostatic in the context of tumors, it does so with limited toxicity (Maira et al., 2008). In this research, NVP-BEZ235 was shown to have limited cytotoxicity in HIV-infected cells. This suggests NVP-BEZ235 inhibits HIV at some point in the viral replication cycle rather than as a consequence of autophagy-induced death (or autosis) of infected cells. Additionally, NVP-BEZ235 exhibited a similar cytotoxicity profile on uninfected cells (data not shown); however, additional studies are required to examine the effect of NVP-BEZ235 on healthy human cells long-term. This is particularly important considering the lifelong dependence of HIV-

positive persons on antiretroviral therapy. Furthermore, it remains uncertain at which stage(s) of viral replication HIV is inhibited, or if NVP-BEZ235 somehow affects HIV binding and cell entry. Additional experiments examining CCR5 expression, the presence of strong-stop HIV DNA, and transcription of viral *Nef* in NVP-BEZ235 are needed to fully understand the mechanism of NVP-BEZ235-induced HIV inhibition. However, the initial findings presented here show NVP-BEZ235 significantly inhibits HIV in human primary cells, and may be useful as adjunctive treatment for HIV infected persons.

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