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Dysregulation of ER stress and autophagic responses by the anti-retroviral drug Efavirenz

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Running Title: Dysregulation of ER stress and autophagy by Efavirenz

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ARVd: anti-retroviral drugs, BBB: blood brain barrier, cART: combination anti-retroviral therapy, ER: endoplasmic reticulum, IRE1 α: inositol requiring kinase 1 α, mTOR: molecular target of rapamycin, PREK: protein kinase-like ER kinase, PHBME: primary human brain microvessel endothelial cell, PI3P: phosphatidylinositol 3-phosphate, SEAP: secreted embryonic alkaline phosphatase, UPR: unfolded protein response.

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ABSTRACT

Increasing evidence demonstrates that the anti-retroviral drugs (ARVds) used for HIV treatment have toxic effects resulting in various cellular and tissue pathologies; however, their impact on the cells composing the blood-brain barrier (BBB) is poorly understood. The current study was focused on ARVds, used either in combination or alone, on the inductions of ER stress responses in human brain endothelial cells. Among studied drugs (Efavirenz, Tenofovir, Emtricitabine, Lamivudine, and Indinavir), only Efavirenz increased ER stress via upregulation and activation of PERK and IRE1α. At the same time, Efavirenz diminished autophagic activity, a surprising result as the induction of ER stress is typically linked to enhanced autophagy. These results were confirmed in microvessels of HIV transgenic mice chronically administered with Efavirenz. In a series of further experiments, we identified that Efavirenz dysregulated ER stress and autophagy by blocking the activity of the Beclin-1/Atg14/PI3KIII complex in regards to synthesis of phosphatidylinositol 3-phosphate (PI3P), a process which is linked to the formation of autophagosomes. Because autophagy is a protective mechanism involved in the removal of dysfunctional proteins and/or organelles, its inhibition can contribute to the toxicity of Efavirenz and/or the development of neurodegenerative disease in HIV patients treated with this drug.

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INTRODUCTION

The impact of a dysfunctional blood-brain barrier (BBB) on the development of neurological diseases has been highlighted in several studies (Grammas et al., 2011; Zlokovic, 2008). Alterations of BBB integrity have been implicated in multiple sclerosis (Larochelle et al., 2011), amyotrophic lateral sclerosis (Rodrigues et al., 2012), Parkinson's disease (Kortekaas et al., 2005), Alzheimer's disease (Erickson and Banks, 2013; Kelleher and Soiza, 2013), and brain infection by HIV (Mishra and Singh, 2014; Nakagawa et al., 2012). BBB alterations in HIV patients include alterations of tight junction protein expression and integrity (Boven et al., 2000; Dallasta et al., 1999; Ivey et al., 2009; Strazza et al., 2011), increased permeability (Boven et al., 2000), enhanced expression of extracellular matrix degrading enzymes such as matrix metalloproteinase-2 and -9 (Eugenin et al., 2006; Louboutin et al., 2010), and increased inflammatory cell migration (Hurwitz et al., 1994). In addition, dysfunctional BBB was proposed to participate in the accumulation of amyloid-beta in HIV-infected brains (Andras and Toborek, 2013).

The introduction of combined anti-retroviral therapy (cART) has changed the outcome and prognosis of HIV infection. What was once a fatal disease is now controlled, and the infected patients survive. With increased survival, it is estimated that patients over 50 years old represent approximately 50% of all HIV infected individuals in the USA (Vance, 2010). In these long-term survivors, the infection itself is controlled but several pathologies are observed, such as cardiovascular, lipid, metabolic, and neurological disorders (Clifford and Ances, 2013; Deeks et al., 2013; Galescu et al., 2013; Kebodeaux et al., 2013; Lake and Currier, 2013). Before the advent of cART, neurological disorders in HIV patients were often associated with severe

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cognitive dysfunction, such as HIV-associated-dementia. Currently, neurological disorders are rather associated with mild and slow progressive degeneration of cognitive and motor functions (Clifford and Ances, 2013); this susceptibility is correlated with age (Becker et al., 2004). While persistent (albeit at low rates) HIV replication in the brain may be responsible for neurocognitive alterations observed in infected individuals, the toxicity of anti-retroviral drugs (ARVds) is also likely to contribute to neurodegenerative disorders in HIV patients. Indeed, ARVds were described to disrupt the mechanisms of phagocytosis and production of amyloid-beta (Giunta et al., 2011), impact mitochondrial function and DNA replication (Apostolova et al., 2011; Blas-Garcia et al., 2010; Bollmann, 2013; Brinkman et al., 1999), induce oxidative stress (Manda et al., 2011), and stimulate cellular stress responses (Apostolova et al., 2013). Protease inhibitors used in HIV treatment have been associated with the development of dyslipidemia (Overton et al., 2012) and the inhibition of normal proteasome function (Piccinini et al., 2005). Several studies have linked the use of ARVds, and in particular Efavirenz, to hepatotoxicity via multiple mechanisms, including alterations of calcium homeostasis, mitochondrial damage, enhanced pro-inflammatory cytokine levels, and the interference with the cannabinoid receptor CB1 (Apostolova et al., 2013; Apostolova et al., 2011; Blas-Garcia et al., 2010; Gallego-Escuredo et al., 2010; Hecht et al., 2013). However, the toxicity and impact of those drugs has not been extensively studied in the context of the BBB.

The unfolded protein response (UPR)/endoplasmic reticulum (ER) stress and autophagy are the major pathways of cellular response to a variety of stressors. For example, induction of ER stress is an important mechanism to remove misfolded proteins, cope with calcium imbalance, and/or cope with alterations of redox potential and glucose deprivation. Autophagy is closely linked to ER stress and serves multiple purposes in the cell, including degradation of aggregated proteins,

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recycling of organelles, and/or destroying intracellular pathogens (Criollo et al., 2010; Nardacci et al., 2014; Qin et al., 2010). Dysregulation of these responses can have a drastic impact on cellular homeostasis and, due to their link to the apoptosis pathway, can result in cell death.

The goal of the present study was to identify the impact of ARVds, used in combination or alone, on induction of ER stress and autophagy in brain microvasculature. Our results demonstrate that Efavirenz alone, or in combination with other ARVds, induces ER stress via stimulation of inositol requiring kinase 1 α (IRE1α) and protein kinase-like ER kinase (PERK) signaling. Importantly, we also demonstrate that Efavirenz dysregulates autophagy by affecting the ability of the Beclin-1/Atg14/PI3KIII complex activity to synthesize phosphatidylinositol 3-phosphate (PI3P), a process which is linked to the formation of autophagosomes.

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MATERIAL AND METHODS

Cell culture and treatment with ARVds

Brain endothelial cells used in this study are of the hCMEC/D3 cell line, which represent well characterized BBB endothelial cells (Weksler et al., 2013). They were cultured in EBM-2 media (Lonza) supplemented with VEGF, IGF-1, EGF, basic FGF, hydrocortisone, ascorbate, gentamycin and 0.5 % FBS (Lonza) on cell culture dishes coated with rat-tail collagen I (BD Bioscience) in 5% CO_2 humid incubator at 37 ^{o}C. Cells were exposed to the following combinations of ARVds: cART1 (Efavirenz 10 μM, Emtricitabine 5 μM, and Tenofovir 1 μM) or cART2 (Atazanavir 1 μM, Ritonavir 60 nM, Emtricitabine 5 μM, and Tenofovir 1 μM) in serum-free and antibiotic-free EBM-2 basal media. These concentrations reflect physiological level of the drugs (Boffito et al., 2011; Droste et al., 2005; Marzolini et al., 2001; Stahle et al., 2004; Valade et al., 2014). For example, successful therapy was observed in patients with plasma concentrations of Efavirenz between 1000 and 4000 μg/L, which translates to 3.17 and 12.67 μM. Higher treatment failure was associated with plasma concentrations below this level and CNS toxicity was linked to levels above this range (Marzolini et al., 2001). ARVds were acquired from the NIH AIDS Reagent Program. Cultures were also exposed to Tunicamycin (0.1 μM, a positive control for induction of ER stress), Rapamycin (0.5 μM, a positive control for induction of ER stress) or vehicle (DMSO 0.1%). Primary human brain endothelial cells (Cell Systems) were cultured in the CSC complete media on dishes coated with CSC attachment factor (all from Cell Systems), followed by treatment with ARVds in serum-free CSC medium without antibiotics. SVG P12 cells were cultured in DMEM supplemented with 10% FBS (Gibco) and were exposed to ARVds in DMEM without serum and antibiotic.

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Secreted embryonic alkaline phosphatase (SEAP) assay

Cells were seeded at a density of 6 x 10^4 cells per well on a 96-well plate and incubated for 24 h. Plasmid pSELECT-Zeo-SEAP (Invivogen) encoding for SEAP was amplified in E. coli and purified using Midi Plasmid kit (Sigma Aldrich). Transfections were performed for 6 h using Lipofectamine 2000 (Invitrogen) in a ratio 3:1 with 0.1 μg of plasmid diluted in OPTI/MEM medium (Gibco) per each well. Then, cells were washed with EBM-2, incubated overnight in complete media, and exposed to ARVds in EBM-2 without serum and phenol red for 48 h. Supernatants were transferred to a 96-well plate (Thermo/Nunc) and assayed for SEAP activity using a SEAP reported assay kit (Invivogen) according to the manufacturer's instructions. Data were normalized by staining cells with DRAQ-5 (Abcam).

Autophagy activity assay

Cells were seeded at 6 x 10^4 cells/well on 96-well white plates (Thermo/Nunc) coated with collagen for 24 h, followed by treatment with ARVds for 48 h. Then, cells were washed twice with warmed PBS and incubated for 1 h with Cyto-ID detection dye and Hoechst 33342 (Enzo Life Sciences). Cells were subsequently washed twice in assay buffer and the absorbance was read on a Spectramax Gemini EM microplate reader (Molecular Devices) (Ex 480 nm; cut-off 495 nm; Em 510-565 nm). Cyto-ID signal was normalized to account for cell number using Hoechst 33342 signal (Ex 350 nm; cut-off 420 nm; Em 450-475 nm).

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Immunoblotting, immunoprecipitation, and immunostaining

Cells were exposed to ARVds in 100 or 150 mm dishes, washed, and lysed in RIPA buffer (Santa Cruz Biochemical) supplemented with protease/phosphatase inhibitors (Cell Signaling). Protein concentration was assessed using BCA protein assay kit (Pierce) and 30 μg proteins was loaded per each lane on precast TGX 4-20% gradient gels (Bio-Rad). Transfer was performed using Trans-blot Turbo Apparel with nitrocellulose transfer packs (Bio-Rad). Membranes were blocked by 4% BSA solution in TBS with 0.1% Tween-20 and then incubated with primary antibodies in 4% BSA blocking solution. The antibody dilutions were: anti-Atg2a (1:100); anti-CaMKKβ, anti-ATF6a, and anti-p-CaMKKβ (all 1:500); anti-p-PERK (1:600); anti-CHOP, anti-p-PKCɵ, and anti-ATF4 (all 1:750), anti-tubulin and anti-actin (Sigma) (both 1:10000); all remaining antibodies (1:1000). Signals were detected using Licor imaging system. For two-color imaging, membranes were incubated using anti-rabbit 800CW and anti-mouse 680LT antibodies (Licor) (1:30,000) washed with TTBS and imaged on an Odyssey CLx scanner (Licor). ECL detection was performed with anti-rabbit light chain HRP antibodies (1:10,000) (Jackson ImmunoResearch) and ECL reagent (GE Healthcare). Proteins G Magnetic Beads (Cell Signaling) were used for immunoprecipitation. Immunostaining was performed on cells grown on collagen-covered round coverslips (Fisher Scientific) or on isolated microvessels heat-fixed on slides. Samples were fixed using 4% paraformaldehyde (Santa Cruz), permeabilized using 0.1% triton X-100 solution, and blocked using NATS solution (20% FBS and 0.5% tween-20 in PBS). Samples were then exposed to primary antibodies at 37 ^{o}C in a humidified chamber and washed. Alexa-488 and -594 secondary antibodies (Invitrogen) and DRAQ-5 (Cell Signaling) were used to visualize the signals. Imaging was performed on an Olympus Fluoview 1200 microscope using a 60x oil immersion lens. Immunofluorescence and ECL quantification was

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performed using ImageJ software. Image Studio 4.0 (Licor) was used for Odyssey CLx acquisition quantification.

XBP-1 mRNA analysis

mRNA was harvested using RNA isolation kit (Qiagen) and reverse transcribed by a Reverse Transcriptase System (Promega) with random primers. Then, a XBP-1 fragment was amplified using primers 5'-TTACGAGAGAAAACTCATGGCC-3' and 5'-GGGTCCAAGTTGTCCAGAATGC-3'. The PCR product was resolved on a 6% acrylamide gel (19:1 ratio, BioRad) for the XBP-1 full length product (289 bp) and spliced product (263 bp).

Quantification of calcium and PI3P

Cells were seeded on a 96-well plate and exposed to ARVds for 48 h in serum-free media. Following 3 washes with HBSS buffer without calcium, cells were incubated with Fluo-4-AM (Invitrogen) at 10 μM for 40 min. Then, they were washed twice with HBSS buffer and fluorescence was read on a Spectramax Gemini EM microplate reader (Ex: 480 nm, cut-off: 495 nm, Em: 520 nm).

PI3P concentration was determined using a PI3P Assay Detection kit (Echelon). Cells were incubated with ARVds on 150 mm plates for 48 h and PI3P was isolated according to the supplied protocol. ELISA was then performed according to manufacturer's instruction and the signals were read on a Spectramax 190 microplate reader (Molecular Devices).

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Animal studies

All animal procedures have been carried out in accordance to NIH guidelines in the PHS animal welfare approved facilities. They were approved by the University of Miami Animal Care and use Committee. Tg26 HIV transgenic mice on a C57BL/6 background were acquired from Dr. Roy L. Sutliff (Emory University). Males, 10-12 week old, were administered with Efavirenz (10 mg/kg) or Etravirine (6.6 mg/kg) via gavage for 30 days. The dosing of both drugs is consistent with therapeutic dosing in humans (Bristol-Myers Squibb, 2015; Janssen Therapeutics, 2014). Etravirine is a new generation of non-nucleoside reverse transcriptase inhibitors (NNRTI), which has demonstrated far less toxicity in patients than Efavirenz (Nguyen et al., 2011). Control mice received vehicle. Drugs were dissolved in DMSO and mixed with saline at a final ratio of 25% DMSO and 75% saline. Following treatment period, mice were euthanized and perfused with saline. Brains were harvested and frozen in liquid nitrogen. Brain microvessels were isolated using a technique previously described by our laboratory (Park et al., 2013; Seelbach et al., 2010), lysed in RIPA buffer and homogenized using a hand held homogenizer (Kontes) or analyzed for expression of ER stress and autophagy markers by immunoblotting or spread on a slide and fixed for immunostaining.

Statistical analysis

The data were analyzed using GraphPad Prism software and experimental treatments were compared pairwise with control treatments using either one way or two way ANOVA, followed by Turkey's multiple comparisons test, Fisher LSD or student's t-test. Value of $p<0.05$ was considered significant.

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RESULTS

Induction of ER stress in cells exposed to ARVds

Toxicity of ARVds to human brain endothelial cells may result in stimulation of ER stress. Because induction of ER stress reduces the overall protein synthesis, this hypothesis was addressed by employing an assay based on expression of a secreted form of alkaline phosphatase (SEAP) (Kitamura and Hiramatsu, 2011). Cells were transfected with an expression plasmid for SEAP and incubated with a combination of ARVds, namely Efavirenz, Tenofovir, and Emtricitabine (abbreviated cART1 in this manuscript) and Ataznavir, Ritonavir, Emtricitabine, and Tenofovir (abbreviated cART2) in basal media. After 48 hours, supernatants were analyzed for SEAP by detecting alkaline phosphatase activity. As indicated in **Figure 1A**, exposure to cART1 and Tunicamycin (positive control) resulted in a significant reduction of SEAP activity as compared to vehicle or cART2. While cART2 also induced ER stress in brain endothelial cells, this effect was not as pronounced as in cART1-treated cells.

In the next series of experiments, we investigated the impact of cART1 on expression or activation of ER resident proteins (IRE1α, PERK, and ATF-6), which initiate the induction of ER stress. Exposure to cART1 for 48 h enhanced the expression of IRE1α and PERK phosphorylation as compared to cART2 or DMSO (**Figure 1B**). Tunicamycin produced similar responses; however, it also resulted in activation of ATF-6. These results were then confirmed by analysis of the expression of downstream mediators implicated in the communication between the ER and the nucleus. Indeed, increased phosphorylation of eIF2α, splicing of XBP-1 mRNA, and increased levels of ATF-4 were observed in cultures exposed to cART1 or Tunicamycin (**Figure 1C**). In addition, the expression of ER stress effectors CHOP and BiP was elevated in

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response to cART1, and highly enhanced in Tunicamycin-treated cultures (**Figure 1D**). Taken together, these results indicate that exposure of brain endothelial cells to cART1 induces ER stress, and implicates at least two pathways of ER stress activation, IRE1α and PERK.

Exposure to cART1 reduces autophagic activity

ER stress and autophagic pathways are linked by several signaling branches; therefore, induction of ER stress often leads to the activation of autophagy. Therefore, we evaluated autophagic activity in our experimental system. Cells were exposed to ARVds in basal media as in experiments presented in Figure 1, followed by determination of the activation of a widely-used autophagic marker, LC3b, which is processed from a 16 kDa (LC3bI) to a 14 kDa (LC3bII) migrating isoform as a result of autophagic activation (Karim et al., 2007; Zhou et al., 2011). Contrary to the expected results, a significant decrease in processing of LC3bI to LC3bII was observed in cells exposed to cART1 as compared to vehicle, indicating a decrease in autophagic activity by this ARVd combination (**Figure 2A**). We also detected a decrease in total LC3b (LC3bI plus LC3bII) level normalized to tubulin (**Figure 2B**) when compared to vehicle (DMSO)-treated controls. To further examine the autophagy response, we evaluated the impact of ARVds on the subcellular localization of LC3b and wipi-1, a protein that is recruited during autophagosome maturation (Proikas-Cezanne and Pfisterer, 2009). Cells were transfected with a plasmid encoding for a GFP-tagged wipi-1, recovered for 24 h, and exposed to ARVds in basal media for 48 h. While a drastic increase in the formation of wipi-1 foci was observed in Tunicamycin and Rapamycin treated cells (**Figure 2C**, arrows), such effects were not observed in cART1-exposed cells. In these cells, a weak and uniform wipi-1-positive immunostaining was detected. A concurrent increase in LC3b foci was also observed in cultures exposed to the

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positive controls (Rapamycin and Tunicamycin), but not in cells exposed to cART1 (**Figure 2C**, arrows). Taken together, these results demonstrate that cART1 leads to a decrease in autophagy in brain endothelial cells.

Efavirenz is the component of cART1 responsible for the dysregulation of ER stress and autophagy pathways

We next focused on identifying whether a single or multiple component of cART1 is/are responsible for the apparent dysregulation of ER stress and autophagy. Previous publications indicated that Efavirenz could induce ER stress and autophagy in other models (Apostolova et al., 2013; Apostolova et al., 2011; Purnell and Fox, 2014). However, our observations indicated a reduction in autophagy upon ARVd treatment.

Cells were exposed to the ARVds composing cART1 individually or in pairs and tested for the induction of ER stress by evaluation of IRE1α expression levels and for autophagy by determination of the LC3bII/tubulin ratio. Only the pairs of drugs containing Efavirenz (**Figures 3A** and **3B**) or Efavirenz alone (**Figures 3C** and **3D**) mimicked the effects of cART1 by inducing ER stress and, at the same time, reducing autophagy. Since these observations are contrary to other publications, we analyzed LC3b expression in response to different concentration of Efavirenz. We observed that with concentrations as low as 1 μM we still observed a reduction of LC3b expression and LC3bI/II conversion (**Figure 3E**).

To confirm these results, we next analyzed and quantified LC3b foci formation in cells exposed to Efavirenz (10 μM) or control treatments. The analyses were performed using foci picker 3D, a plugin for the ImageJ software. Using this method, we demonstrated a significant reduction in both the numbers of foci (**Figure 4A and B**) per cell and the percentage of cells with foci when

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exposed to Efavirenz as compared to vehicle (**Figures 4A and C**). In addition, co-exposure to Rapamycin did not affect the Efavirenz-induced stimulation of ER (measured by IRE1α immunoblotting) or reduction of autophagy (measured by LC3b foci or by immunoblotting) (**Figures 4A-4E).**

Efavirenz dysregulates ER stress activity and autophagy in other BBB cells and in brain microvessels of HIV transgenic mice

To further support the observation that treatment with Efavirenz mediated dysfunction of autophagic processes at the BBB level, studies were performed using two additional BBB cell types, namely primary human brain microvascular endothelial cells (PHBME) and an astrocytic cell line, SVG P12. Exposure to Efavirenz (10 μM) markedly elevated levels of ER resident protein IRE1α in both PHBME (**Figure 5A**) and SVG P12 cells (**Figure 5C**). In addition, a slight but significant reduction in autophagic activity was observed in both cell types as measured with Cyto-ID detection dye (**Figures 5B and D**). In PHBME cells, the expression of LC3bI and LC3BII was not altered (**Figure 5A**); however, a prominent decrease in the expression and conversion of LC3bI to LC3bII was observed in SVG P12 cells (**Figure 5C**). Efavirenz-induced induction of ER stress was also confirmed in brain microvessels of HIV transgenic mice exposed to this drug for 30 days. The treatment resulted in a significant upregulation of ER stress effector protein BiP, confirming the *in vitro* results. In contrast, similar exposure to Etravirine, a second generation non-nucleoside reverse transcriptase inhibitor, did not affect BiP expression levels (**Figure 6A**). Importantly, we also detected a significant reduction in LC3b-specific immunoreactivity and the number of foci in brain microvessels of mice treated with Efavirenz (**Figure 6B**), compared to vehicle or Etravirine.

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Efavirenz does not alter expression of proteins bridging ER stress and autophagy pathways

Several proteins and signaling pathways, which can bridge between ER stress and autophagy, were analyzed in order to identity a mechanism of Efavirenz-induced disconnection between ER stress and autophagy. First, we determined expression of the ASK1/TRAF2 complex, since this complex is activated by IRE1α and Efavirenz induced a significant increase in IRE1α expression. Surprisingly, treatment with Efavirenz did not alter expression of TRAF2 and slightly increased ASK1 expression as compared to vehicle (**Figure 7A**). Similarly, no change in JNK expression, a kinase responsible for the phosphorylation of Bcl-2, was observed (**Figure 7A**). A common trigger of ER stress and autophagy induction is the alteration of intracellular Ca^{++} levels. Consistent with observations by others (Apostolova et al., 2013), a significant increase in cytoplasmic calcium levels following treatment with Efavirenz was detected (**Figure 7B**). However, calcium-induced common mediators of autophagy, such as phosphorylation of PKCϴ or CaMKKβ, were not affected by Efavirenz exposure (**Figure 7C**).

We next evaluated expression and/or activation of several proteins implicated in the autophagy pathway. Mammalian target of Rapamycin (mTOR) is a central cell-growth regulator that integrates growth factor and nutrient signals. mTOR phosphorylation was reduced in cells exposed to Rapamycin, providing a stimulus for autophagy induction; however, no apparent changes in mTOR levels or its phosphorylation were observed in cells exposed to Efavirenz. In addition, treatment with Efavirenz did not affect expression levels of several downstream autophagy effector proteins, such as ULK1, or Atg5 and Atg12, which are implicated in the formation of the autophagic vesicle (**Figure 7D**). We also did not observe any changes in co-immunoprecipitation of Bcl-2 with Beclin-1, which could have an inhibitory effect on autophagy

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progression (**Figure 7E**). Overall, these results indicate that reduction of autophagic activity by Efavirenz is not due to decreased expression of proteins involved in the induction of autophagy or the signaling pathways which bridge autophagy with ER stress.

Efavirenz inhibits autophagy at the vesicle nucleation stage

The lack of changes in the expression of the mediators of autophagic activity suggested that Efavirenz-induced dysregulation of autophagy may be related to a blockage in the formation of autophagic vesicles. Therefore, we evaluated the formation of the complex composed of Beclin-1, Atg14 and PI3KIII, which is implicated in the membrane nucleation stage of autophagy. As indicated in **Figure 8A**, the complex was formed properly under all treatment conditions as PI3KIII and Atg14 co-immunoprecipitated with Beclin-1.

Because the Beclin-1/Atg14/PI3KIII complex controls synthesis of PI3P, which is implicated in the formation of the autophagosome, we next measured cellular levels of PI3P. PI3P levels were significantly reduced in cells treated with Efavirenz as compared to all other conditions (**Figure 8B**), indicating a blockage which may be responsible for decreased autophagy upon treatment with this drug. To confirm these findings, the expression levels and interaction of Atg9 and Atg2a was analyzed, as these proteins are recruited in response to the PI3P synthesis. In line with previous results, the expression of both proteins remained unchanged in Efavirenz-exposed cell; however, the association of Atg2a to Atg9 was reduced (**Figures 8C** and **8D**). We then extended these analyses to evaluate the levels of sequestosome 1 (SQSTM1)/p62, a protein that is consumed during autophagy. There was a significant accumulation of SQSTM1/p62 in brain endothelial cells treated with Efavirenz, indicating an inhibition in the autophagy pathway (**Figure 8E**). These results clearly indicate that exposure to Efavirenz blocks autophagy

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progression by inhibiting the Beclin-1/Atg14/PI3KIII complex activity to synthesize PI3P, preventing the recruitment of the downstream proteins and the nucleation of autophagic membranes.

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DISCUSSION

The benefits resulting from the usage of ARVds in the treatment of HIV are undeniable. They limit the progression of the disease and enable infected patients to survive while restricting the infection. However, with prolonged survival, these patients develop a variety of comorbidities, which are frequently linked to drug toxicity (Gakhar et al., 2013). Despite the control of the infection, the virus is never fully suppressed and circulating HIV proteins are present, albeit at low concentrations. This continuous exposure can lead to complications since some HIV proteins, such as Tat, can induce cellular stress, as well as inflammatory and oxidative responses (Toborek et al., 2003). Furthermore, the surviving patients are aging, making them more susceptible to ARVd toxicity and placing them at a higher risk of developing neurological problems. These facts highlight the importance of evaluating and preventing the toxicity of ARVds, especially because patients are likely to be on these medications for the rest of their lives.

In the present study, we focused on two combinations of ARVds: Efavirenz, Tenofovir, and Emtricitabine (referred in the manuscript as cART1) and Atazanavir, Ritonavir, Emtricitabine, and Tenofovir (referred as cART2). Both cART1 and cART2 are drug combinations that are recommended as the initial regimens for anti-retroviral-naïve patients (Department of Health and Human Services, 2014). In addition, cART1 components constitute the single tablet regimen (Atripla). The drugs evaluated in this study were from different categories of ARVds, namely, non-nucleoside reverse transcriptase inhibitors (NNRTI; Efavirenz and Etravirine), nucleoside reverse transcriptase inhibitors (NRTIs; Tenofovir and Emtricitabine), and protease inhibitors (PI, Atazanavir and Ritonavir).

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Potential side effects of ARVds include hyperbilirubinemia, lipodystrophy, diarrhea, hypersensitivity, lactic acidosis, peripheral neuropathy, neuropsychiatric disorders and pancreatitis (Blas-Garcia et al., 2011; Domingo et al., 2012; Gakhar et al., 2013; Gutierrez Mdel et al., 2011). Several reports also demonstrated hepatotoxicity and the blockage of proteasome activity by HIV protease inhibitors (Carr, 2003; Liang et al., 2001). In addition, secretion of pro-inflammatory cytokines (Diaz-Delfin et al., 2011) and neurotoxicity has been associated with the usage of NNRTI, namely Efavirenz and Nevirapine (Decloedt and Maartens, 2013). The neurotoxic impact of these drugs may be mediated, at least in part, by inhibition of creatine kinase and cytochrome C complex IV activity in the brain (Streck et al., 2011; Streck et al., 2008), mitochondria dysfunction (Purnell and Fox, 2014), and/or iNOS upregulation (Apostolova et al., 2014). Our studies focused on the toxicity of these drugs to brain endothelial cells as these cells are the main component of the BBB, and play a critical role in the protection of the brain against the development of neurodegenerative disorders. Mechanistically, we focused on the induction of ER stress and autophagic responses.

ER stress is involved in maintaining cellular homeostasis and resolving dysregulation in protein folding, calcium and redox imbalance, and glucose deprivation. Furthermore, given its role in monitoring protein folding, the process is often activated following viral infection. Proper indication of ER stress responses induces expression of protein chaperones and enhances the degradation of ER-associated proteins (Xu et al., 2005). On the other hand, aberrant ER stress may lead to cell death and apoptosis. ER stress is induced by at least three signaling pathways, initiated by PERK, IRE1α, and ATF-6 (Chambers and Marciniak, 2014). Therefore, these pathways were evaluated in our model systems exposed to different cART1 and cART2 drug combination. IRE1α and PERK, but not ATF-6, were induced by cART1, with the following

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studies indicating the role of Efavirenz in this process. Induction of ER stress was associated with increased expression of ER resident chaperones BiP and CHOP. Efavirenz-induced ER stress was initiated as early as 6 hours post exposure and lasted as long as 96 h (data not shown). In addition, it was reversible upon removing Efavirenz from the culture media (data not shown). Novel observations of the present study indicate that exposure to Efavirenz (alone or in combination; concentration range of 1-20 μM) results in induction of ER stress and reduced autophagic activity in BBB cells, namely in brain endothelial cells and astroglia. These findings were unexpected because induction of ER stress is usually associated with an increase in autophagic activity, as there are several mediators joining the two pathways. Therefore, it was surprising that treatment with Efavirenz resulted in a decrease in the autophagic activity of BBB cells as demonstrated by the reduced cleavage of LC3b, and a drastic reduction in the formation of LC3b foci**,** even with the addition of Rapamycin. Our results also contrast with literature reports in which Efavirenz was demonstrated to induce autophagy in hepatocytes, neurons and human umbilical vein endothelial cells (Apostolova et al., 2013; Apostolova et al., 2011; Purnell and Fox, 2014; Weiss et al., 2015). This discrepancy indicates a highly unique response of the BBB cells to this drug, and may indicate specific toxicity of Efavirenz towards the cerebrovascular system. Indeed, while some literature reports are based on treatment with Efavirenz of up to 50 μM (Apostolova et al., 2013; Apostolova et al., 2011), we observed significant toxicity of this drug at 20 μM and 100% cell death at 30 μM after a 24h exposure of primary human brain microvessels endothelial cells (data not shown). These individual responses and variable level of sensitivity to Efavirenz-induced toxicity may be related to the mitochondrial profile in different cell types, since Efavirenz affects mitochondrial functions. While our *in vitro* experiments were conducted in basal media, which may affect drug toxicity,

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Efavirenz-induced ER stress and reduced autophagy activity were also observed in brain microvessels of HIV transgenic mice chronically administered with a physiologically relevant dose of this drug, further demonstrating the relevance of our findings.

Autophagy is a protective mechanism of protein recycling and removal of defective proteins and organelles (Boya et al., 2005; Liang, 2010); therefore, a decrease in autophagy activity can potentiate Efavirenz toxicity. As the mechanism of dysregulation of ER stress and autophagy, we identified that the complex composed of Beclin-1/Atg14/PI3KIII is deficient in the synthesis of PI3P in cells exposed to Efavirenz. This deficiency results in a reduced efficiency in the nucleation step of the autophagy pathway, as demonstrated by the reduction in Atg9/Atg2a association and accumulation of protein SQSTM1/p62.

In line with several studies demonstrating that alterations of calcium metabolism can induce autophagy (Decuypere et al., 2013; Decuypere et al., 2011; Ghislat et al., 2012), we also considered this mechanism in the present study. Although an increase in cytoplasmic calcium levels was observed in brain endothelial cells exposed to Efavirenz, this increase did not result in stimulation of autophagy. This apparent disconnection is consistent with the fact that Efavirenz increases calcium levels by depletion of mitochondrial or ER calcium stores (Apostolova et al., 2013), which can inhibit autophagy (Engedal et al., 2013; Gordon et al., 1993). Indeed, several calcium-related mediators which may affect autophagy responses, such as CaMKKβ (Xi et al., 2013) or PKCϴ (Zhang et al., 2009), were not significantly affected by Efavirenz exposure.

In summary, our findings demonstrate that exposure to Efavirenz, but not to other common ARVds, induces ER stress responses but decreases autophagic activity in BBB cells and brain microvessels (**Figure 9**). This dysregulation of ER stress and autophagic pathways is associated with a reduced PI3P synthesis activity. The observed changes may contribute to the dysfunction

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of brain endothelial cells, contributing to cerebrovascular toxicity frequently observed in HIV-infected patients.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Bertrand and Toborek

Conducted experiments: Bertrand

Performed data analysis: Bertrand and Toborek

Wrote or contributed to the writing of the manuscript: Bertrand and Toborek

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FOOTNOTE

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FIGURE LEGENDS

Figure 1. cART1 induces ER stress. (A) Brain endothelial cells transfected with a plasmid coding for SEAP were incubated with either vehicle (DMSO, control), cART1, cART2, or Tunicamycin (positive control) for 48h in basal media. Alkaline phosphatase levels in supernatant were detected. (B) Expression and phosphorylation of ER stress inducers detected in cultures exposed to the same treatment as in (A). Cells were lysed and analyzed by immunoblotting for IRE1α, ATF-6, phosphorylated PERK (P-PERK), and total PERK. (C) Expression of ER stress-related signaling molecules eIF2α, phosphorylated eIF2α (P-eIF2α), and ATF-4 evaluated by immunoblotting. In addition, splicing of XBP-1 mRNA was analyzed using reverse transcriptase and PCR amplification. Cultures were treated as in (A). (D) Immunoblot for ER stress-related chaperones CHOP and BiP in cultures treated as in (A). The numbers above the images indicate densitometric measurements of specific band intensity vs. vehicle-treated cells. The values were normalized to housekeeping protein tubulin or to total (i.e., non-phosphorylated) expression of individual proteins. The data are mean ± SEM, n=3-16. ***p<0.001 vs. DMSO; ###p<0.001 vs cART1. Tuni: Tunicamycin; Rapa: Rapamycin.

Figure 2. cART1 reduces autophagy activity. Brain endothelial cells were treated as in Figure 1. (A) Immunoblotting detection of autophagy marker LC3b. Graph reflects the ratio of LC3bII to LC3bI for 4 independent assays. (B) LC3b total expression levels in the same cultures as in (A) normalized to tubulin expression. (C) Representative confocal images of immunostaining for wipi-1 and LC3b. Cells were transfected with an expression vector for a GFP tagged wipi-1, immunostained for LC3b, and nuclei were labelled with DRAQ-5. The data are mean ± SEM;

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n=4. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle; #p<0.05, ##p<0.01. Tuni: Tunicamycin; Rapa: Rapamycin.

Figure 3. Efavirenz is responsible for ER stress induction and autophagy reduction. Brain endothelial cells were exposed to either vehicle (DMSO), cART1, tandems of drugs composing cART1 (Efavirenz, Emtricitabine, and/or Tenofovir), Tunicamycin, or Rapamycin for 48 h. (A) Levels of IRE1α and (B) LC3b levels were analyzed by immunoblotting. (C) Immunoblotting for IRE1α in cells exposed to individual drugs composing cART1. Levels were normalized to Tubulin expression. (D) Quantification of LC3b to tubulin ratio under the same conditions as in (C). (E) Western blot for LC3b in endothelial cells treated with different concentrations of Efavirenz. The data are mean ± SEM, n=3. *p<0.05, **p<0.01, ***p<0.001 vs. Vehicle. Efa: Efavirenz; EM: Emtricitabine; Teno: Tenofovir; Tuni: Tunicamycin; Rapa: Rapamycin.

Figure 4. Quantification of Efavirenz-induced autophagy disruption. (A) Immunostaining for LC3b in brain endothelial cells exposed to the indicated drugs for 48h, lower images are enlarged fragments of the upper images indicated by the white squares. Arrows denote examples of LC3b immunoreactive foci. (B) Quantification of LC3b foci formation per cell and (C) percentage of cells with LC3b-positive foci. (D) Co-exposure to Rapamycin does not protect against Efavirenz-induced ER stress and (E) autophagic disruption. The data are mean ± SEM; blots, n=3-4; foci quantification, n = at least 200 cells/group. *p<0.05, **p<0.01, ***p<0.001 vs. DMSO; ##p<0.01 vs Efavirenz. Efa: Efavirenz; Rapa: Rapamycin; Tuni: Tunicamycin.

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Figure 5. Confirmation of Efavirenz-induced dysregulation of ER stress and autophagy responses in BBB cells. Primary human brain endothelial cells were exposed to Efavirenz, vehicle (DMSO), and Rapamycin (positive control), followed by immunoblotting for IRE1α levels and LC3b expression (A) and Cyto-ID detection for autophagosome formation (B). Similar analyses of IRE1α levels, LC3bI/LC3bII conversion and total expression (C) and Cyto-ID detection (D) in SVGP12 cells treated as in (A and B). The data are mean ± SEM; n=8. **p<0.01 vs. Vehicle. Efa: Efavirenz; Rapa: Rapamycin.

Figure 6. Efavirenz induces dysregulation of ER stress and autophagy responses in brain microvessels of HIV transgenic mice. Mice were administered with Efavirenz (Efa), Etravirine (Etra) or vehicle for 30 days as described in the Materials and Methods. (A) BiP was detected by immunoblotting (left panel) and quantified (right panel). (B) LC3b-positive immunostaining (left panel) was evaluated and quantified for foci formation. The data are mean ± SEM; 5 mice per group, 18-20 microvessels per mouse. **p<0.01, ***p<0.001 vs. Vehicle; ##p<0.01 vs Efavirenz.

Figure 7. Efavirenz does not affect expression of proteins of the autophagy pathway but increases intracellular calcium. Cells were exposed to Efavirenz, Tunicamycin, and Rapamycin. (A) Immunoblot for the expression levels of proteins associated with IRE1α-dependent induction of autophagy, ASK1, TRAF2, and JNK. (B) Detection of intracellular

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calcium level using Fluo-4-AM. (C) Immunoblot depicting the expression of calcium-dependent CaMKKβ and PKCɵ and their phosphorylated forms (p-CaMKKβ and p-PKCɵ) levels. (D) Expression of proteins implicated in the autophagic pathway, mTOR, phosphorylated mTOR (p-mTOR), ULK1, Atg5, and Atg12. Actin and Tubulin were detected as house-keeping proteins. (E) Immunoblot analysis of Bcl-2 expression in lysates and in Beclin-1 immunoprecipitated samples. The data are mean ± SEM, n=16. *p<0.05, ***p<0.001 vs. Vehicle.

Figure 8. Efavirenz inhibits autophagosome nucleation events and leads to the accumulation of SQSTM1/p62. Cells were exposed to the indicated drugs for 48h. (A) Analysis of the formation of the Beclin-1 complex implicated in the progression of autophagy. Top image represent input levels of Beclin-1 as determined by immunoblotting, while the bottom three images represent immunoprecipitation of Beclin-1 and co-immunoprecipitation of PI3 kinase class III and Atg14. (B) Cytosolic PI3P levels are decreased in Efavirenz-treated cultures. (C) Association of Atg2a with Atg9 is decreased by Efavirenz. Top images represent co-immunoprecipitation of Atg2a with Atg9. The lower images represent levels of Atg2a and Atg9 in cell lysates as determined by immunoblotting. (D) Quantification of Atg2a co-immunoprecipated with Atg9 shown in (C). (E) Accumulation of SQSTM1/p62 levels in Efavirenz-treated cultures as determined by immunoblotting. Graph represents quantification of SQSTM1/p62 normalized to tubulin levels. The data are mean ± SEM, n=3-5. *p<0.05, ***p<0.001 vs. Vehicle. BCLN1: Beclin-1; Efa: Efavirenz; Rapa: Rapamycin; Tuni: Tunicamycin.

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Figure 9. Summary of the input of Efavirenz on ER stress and autophagy signaling in brain endothelial cells. Green color depicts an increase in expression or activation. Red color represents inhibition of activity or processing.

Figure 1

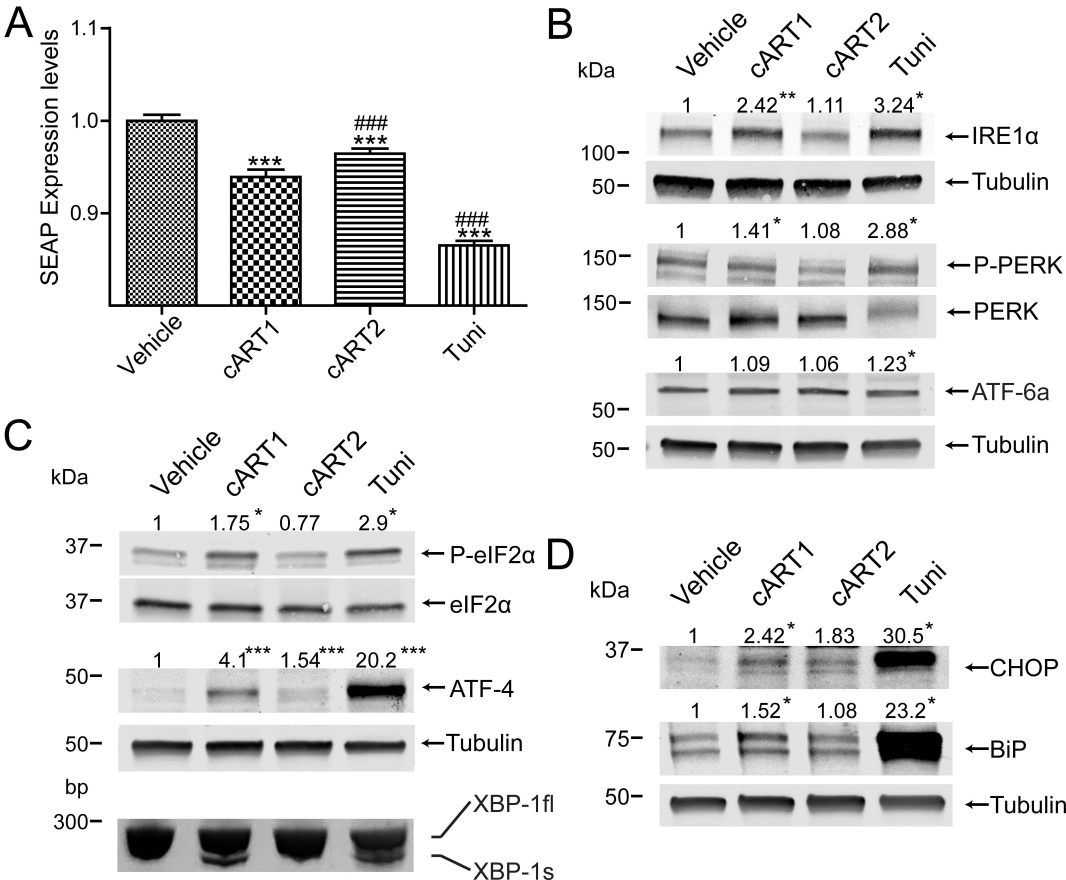

A
SEAP Expression levels
1.1
1.0
0.9
***
###
***
###
***
Vehicle
cART1
cART2
Tuni
B
kDa
Vehicle
cART1
cART2
Tuni
1
2.42**
1.11
3.24*
IRE1α
100
50
Tubulin
1
1.41*
1.08
2.88*
150
P-PERK
150
PERK
1
1.09
1.06
1.23*
ATF-6a
50
50
Tubulin
C
kDa
Vehicle
cART1
cART2
Tuni
1
1.75*
0.77
2.9*
37
P-eIF2α
37
eIF2α
1
4.1***
1.54***
20.2***
50
ATF-4
50
Tubulin
bp
300
XBP-1fl
XBP-1s
D
kDa
Vehicle
cART1
cART2
Tuni
1
2.42*
1.83
30.5*
37
CHOP
1
1.52*
1.08
23.2*
75
BiP
50
Tubulin

Figure 2

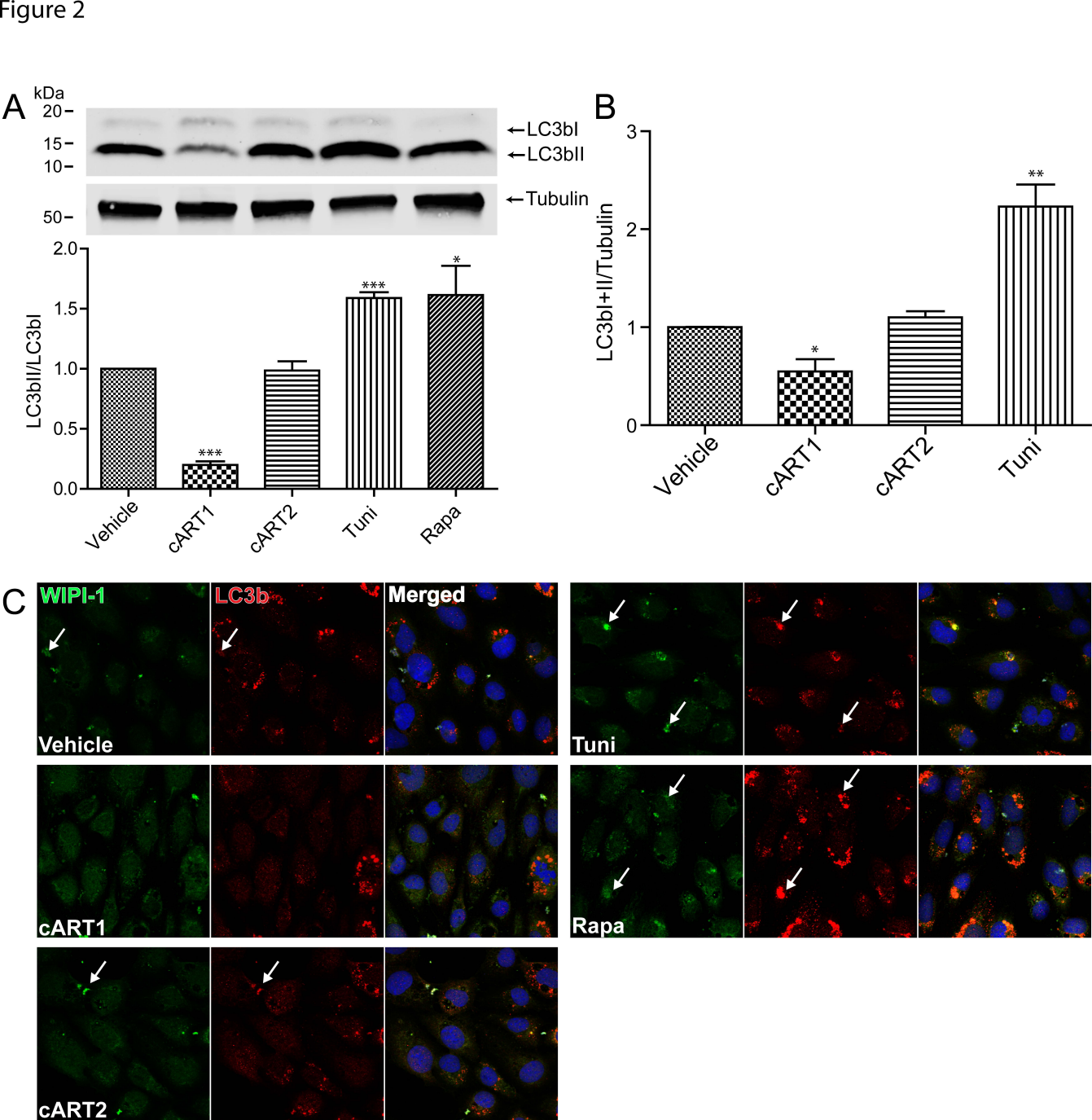

A
kDa
20
15
10
50
LC3bI
LC3bII
Tubulin
LC3bII/LC3bI
2.0
1.5
1.0
0.5
0.0
***
***
*
Vehicle
cART1
cART2
Tuni
Rapa
B
LC3bI+II/Tubulin
3
2
1
0
*
**
Vehicle
cART1
cART2
Tuni
C
WIPI-1
LC3b
Merged
Vehicle
Tuni
cART1
Rapa
cART2

Figure 3

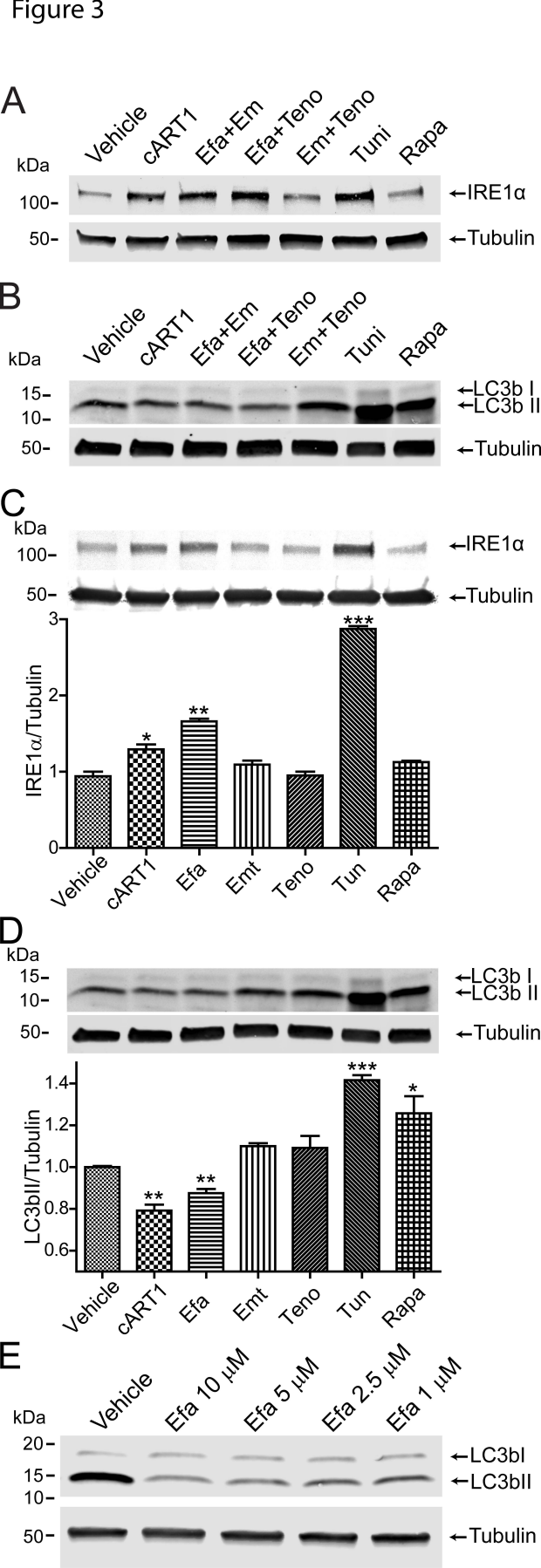

A
Vehicle
cART1
Efa+Em
Efa+Teno
Em+Teno
Tuni
Rapa
kDa
100-
50-
←IRE1α
←Tubulin
B
Vehicle
cART1
Efa+Em
Efa+Teno
Em+Teno
Tuni
Rapa
kDa
15-
10-
50-
←LC3b I
←LC3b II
←Tubulin
C
kDa
100-
50-
←IRE1α
←Tubulin
IRE1α/Tubulin
3
2
1
0
*
**
***
Vehicle
cART1
Efa
Emt
Teno
Tun
Rapa
D
kDa
15-
10-
50-
←LC3b I
←LC3b II
←Tubulin
LC3bII/Tubulin
1.4
1.2
1.0
0.8
0.6
**
**
***
*
Vehicle
cART1
Efa
Emt
Teno
Tun
Rapa
E
Vehicle
Efa 10 μM
Efa 5 μM
Efa 2.5 μM
Efa 1 μM
kDa
20-
15-
10-
50-
←LC3bI
←LC3bII
←Tubulin

Figure 4

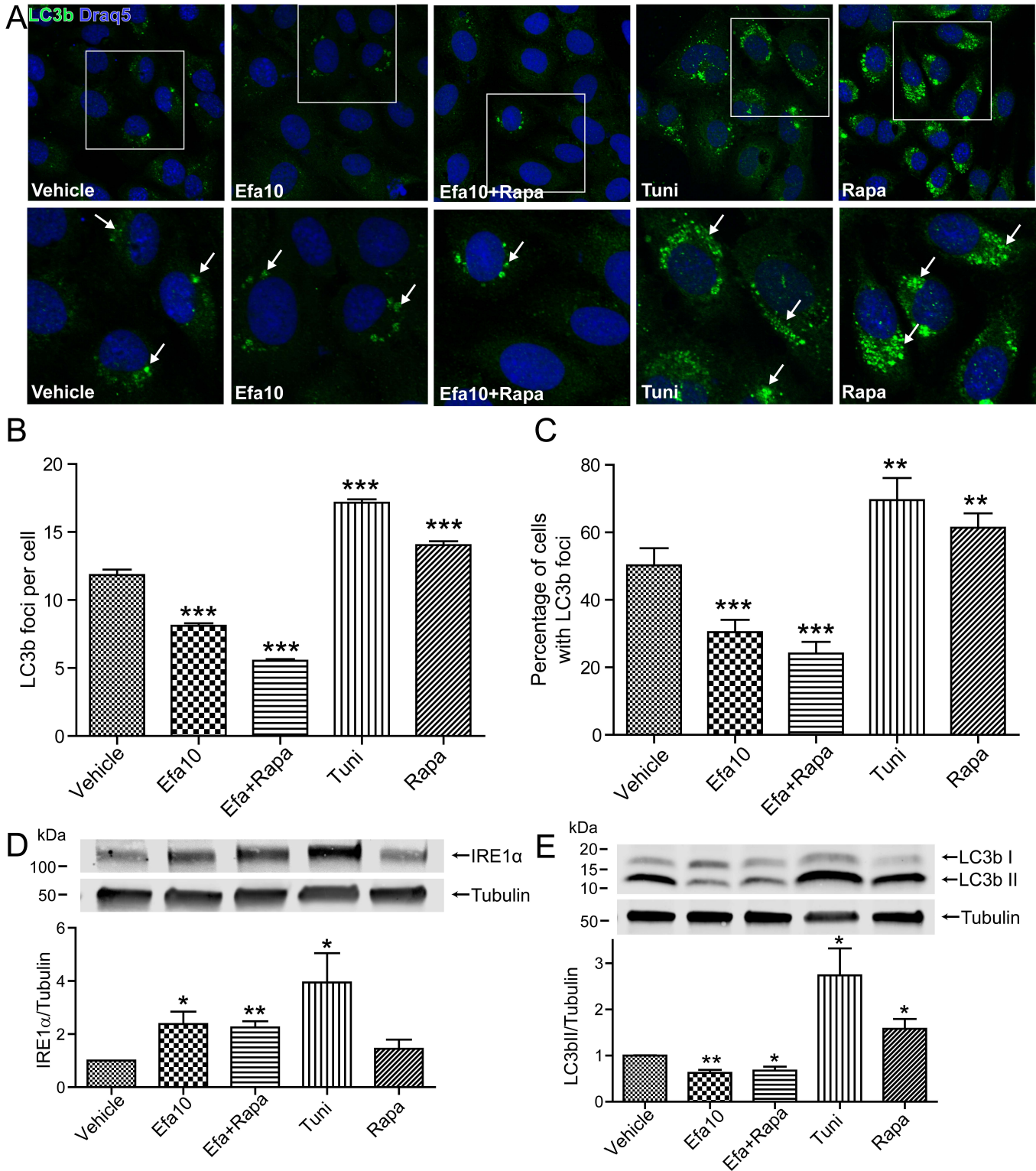
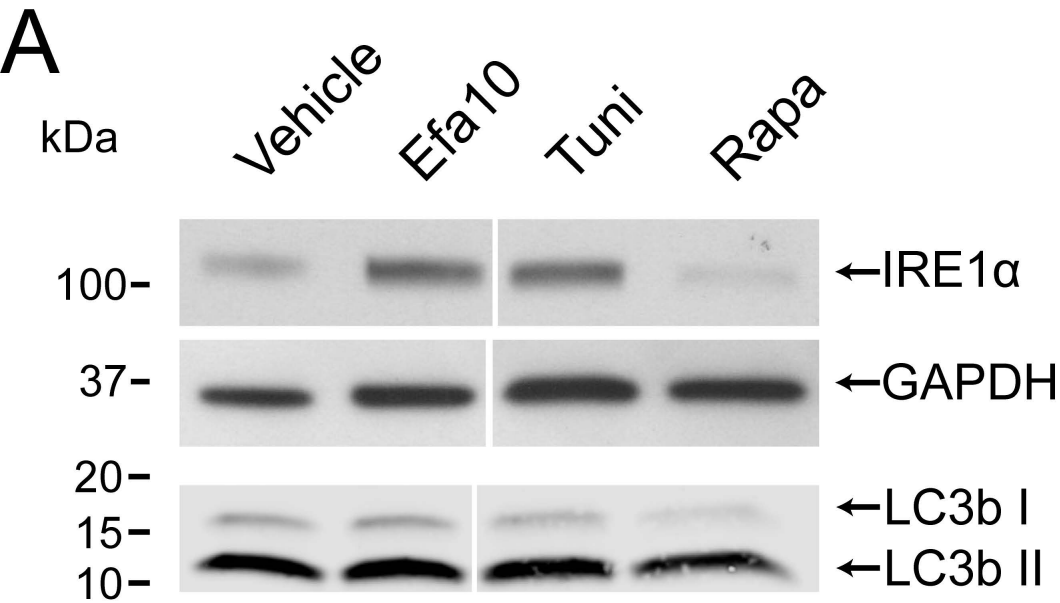
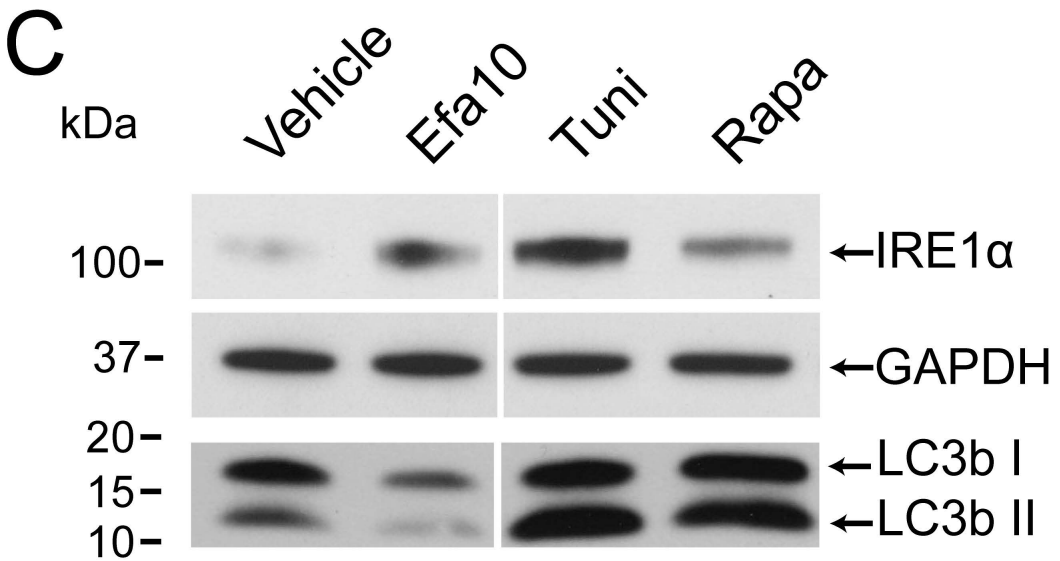
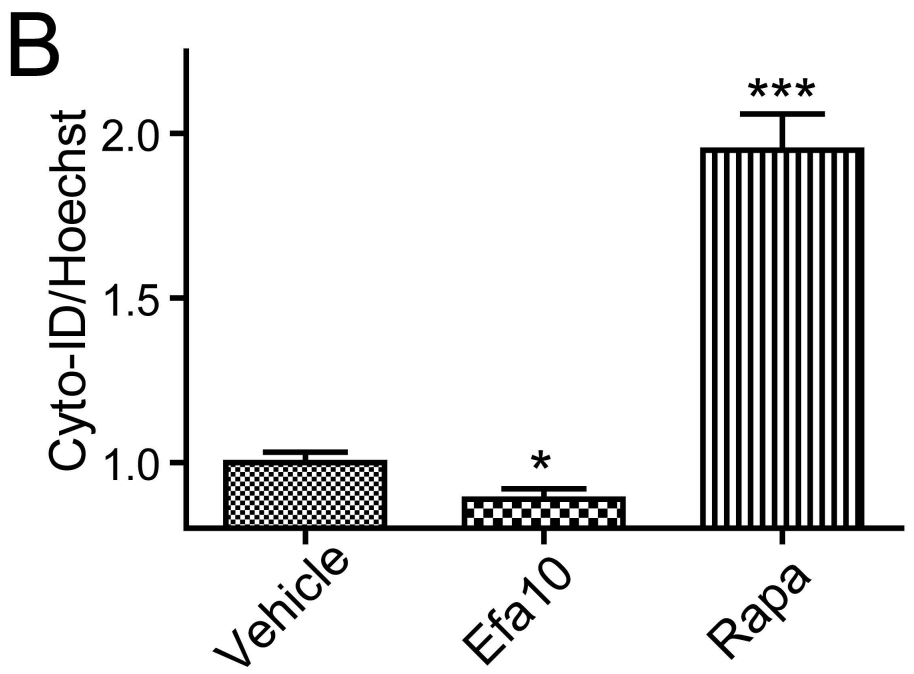

A
LC3b Draq5
Vehicle
Efa10
Efa10+Rapa
Tuni
Rapa
B
LC3b foci per cell
Vehicle
Efa10
Efa+Rapa
Tuni
Rapa
C
Percentage of cells with LC3b foci
D
kDa
IRE1α
Tubulin
IRE1α/Tubulin
E
LC3b I
LC3b II
Tubulin
LC3bII/Tubulin

Figure 5


A
kDa
Vehicle
Efa10
Tuni
Rapa
100-
37-
20-
15-
10-
←IRE1α
←GAPDH
←LC3b I
←LC3b II


C
kDa
Vehicle
Efa10
Tuni
Rapa
100-
37-
20-
15-
10-
←IRE1α
←GAPDH
←LC3b I
←LC3b II


B
Cyto-ID/Hoechst
2.0
1.5
1.0
***
*
Vehicle
Efa10
Rapa

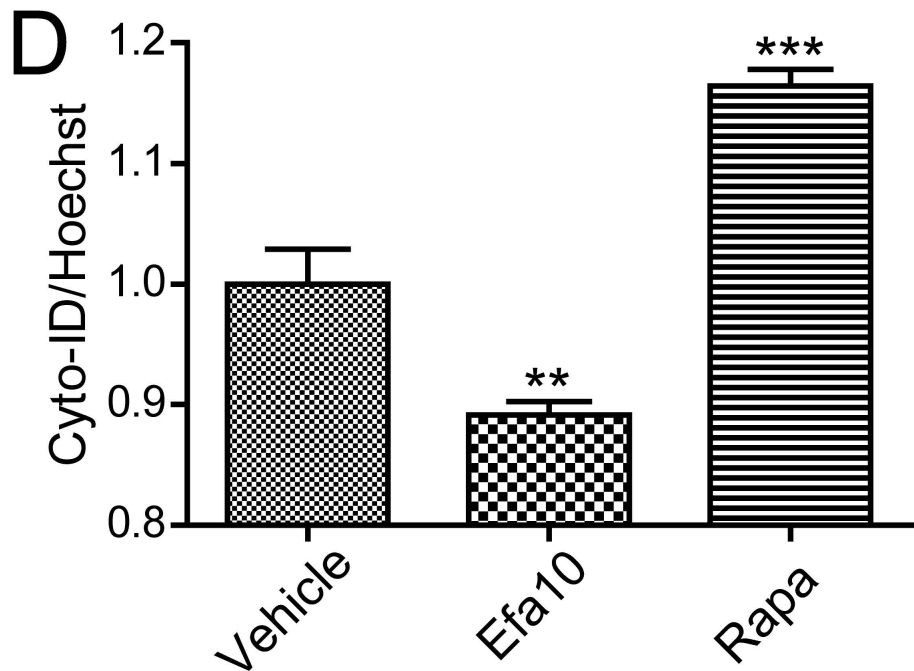

D
Cyto-ID/Hoechst
1.2
1.1
1.0
0.9
0.8
***
**
Vehicle
Efa10
Rapa

Figure 6

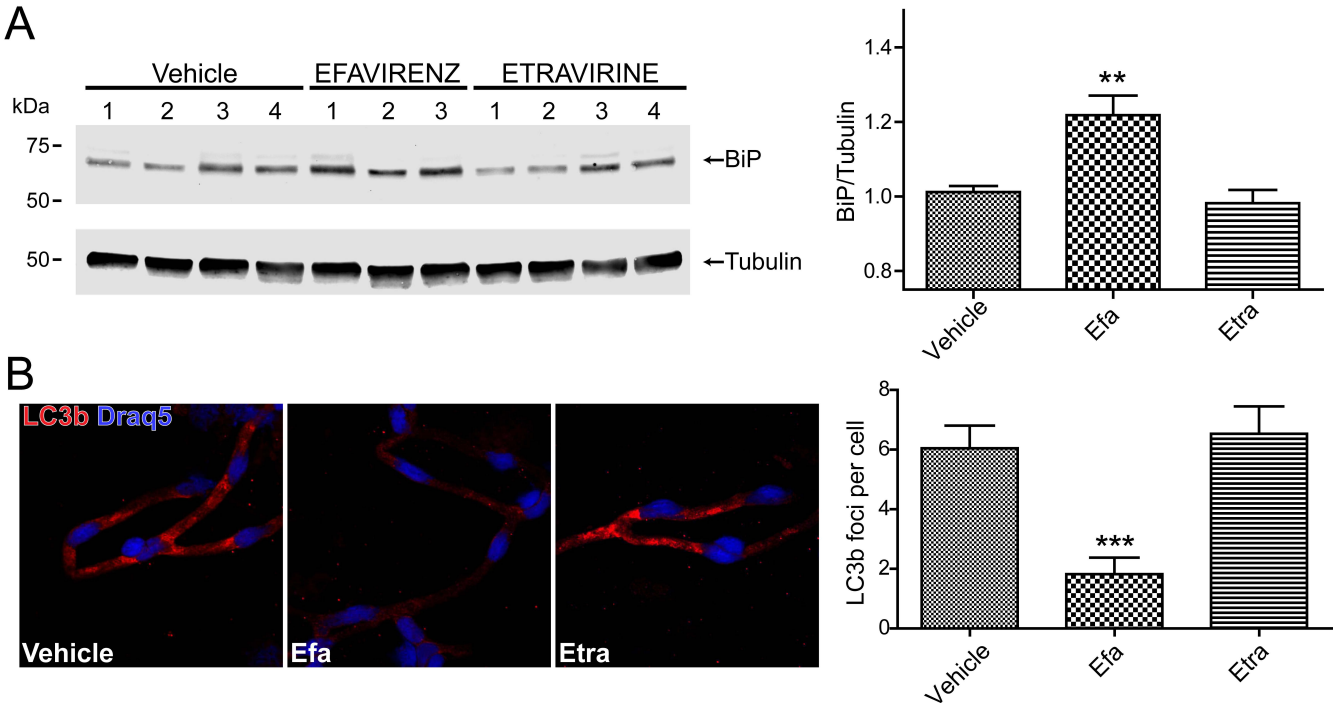

A
Vehicle
EFAVIRENZ
ETRAVIRINE
kDa
1 2 3 4 1 2 3 1 2 3 4
75
50
←BiP
50
←Tubulin
BiP/Tubulin
1.4
1.2
1.0
0.8
**
Vehicle
Efa
Etra
B
LC3b Draq5
Vehicle
Efa
Etra
LC3b foci per cell
8
6
4
2
0
***
Vehicle
Efa
Etra

Figure 7

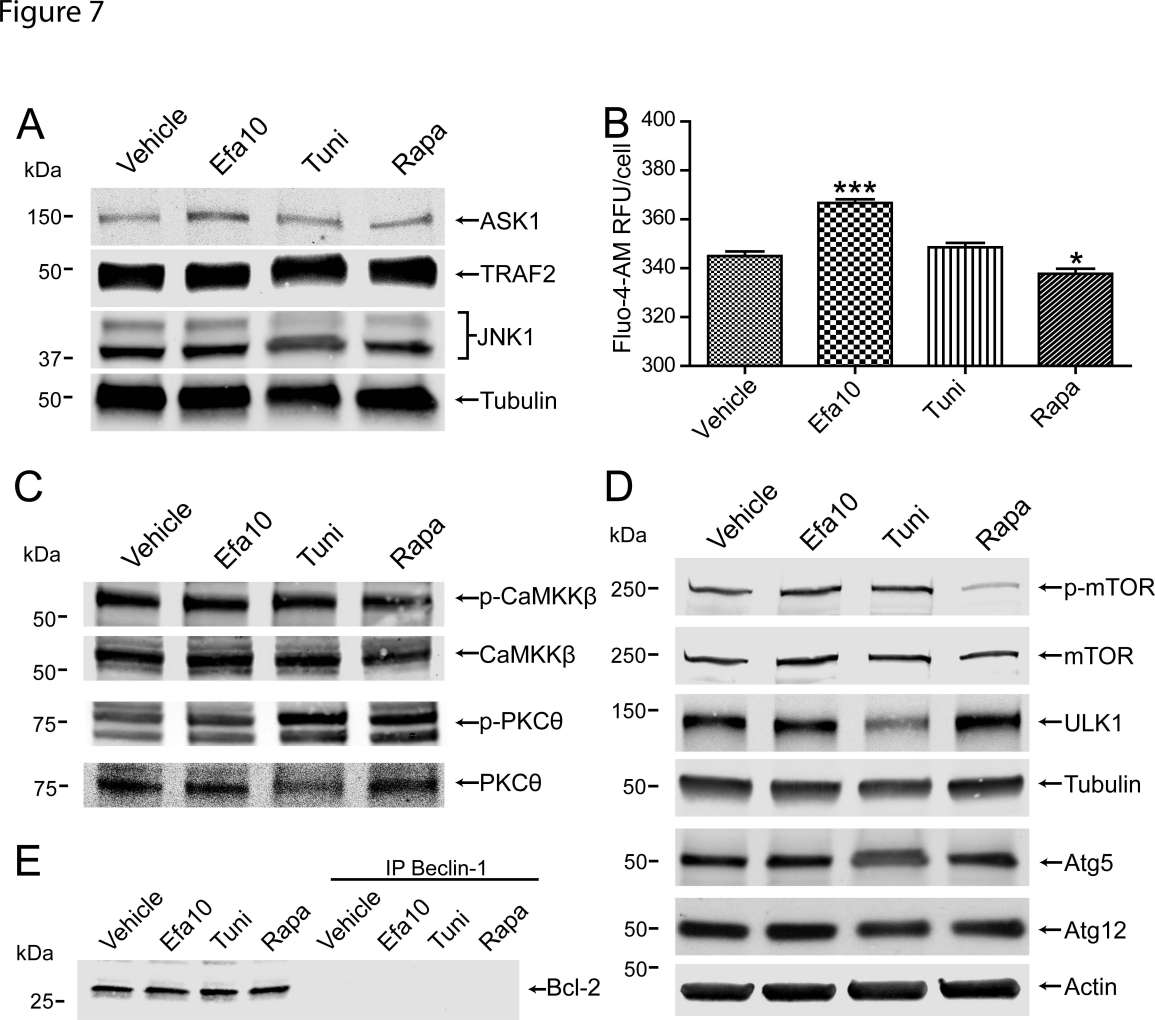

A
kDa
Vehicle
Efa10
Tuni
Rapa
150-
50-
37-
50-
←ASK1
←TRAF2
JNK1
←Tubulin
B
Fluo-4-AM RFU/cell
400
380
360
340
320
300
***
*
Vehicle
Efa10
Tuni
Rapa
C
kDa
Vehicle
Efa10
Tuni
Rapa
50-
50-
75-
75-
←p-CaMKKβ
←CaMKKβ
←p-PKCθ
←PKCθ
D
kDa
Vehicle
Efa10
Tuni
Rapa
250-
250-
150-
50-
50-
50-
←p-mTOR
←mTOR
←ULK1
←Tubulin
←Atg5
←Atg12
←Actin
E
IP Beclin-1
Vehicle
Efa10
Tuni
Rapa
Vehicle
Efa10
Tuni
Rapa
kDa
25-
←Bcl-2

Figure 8

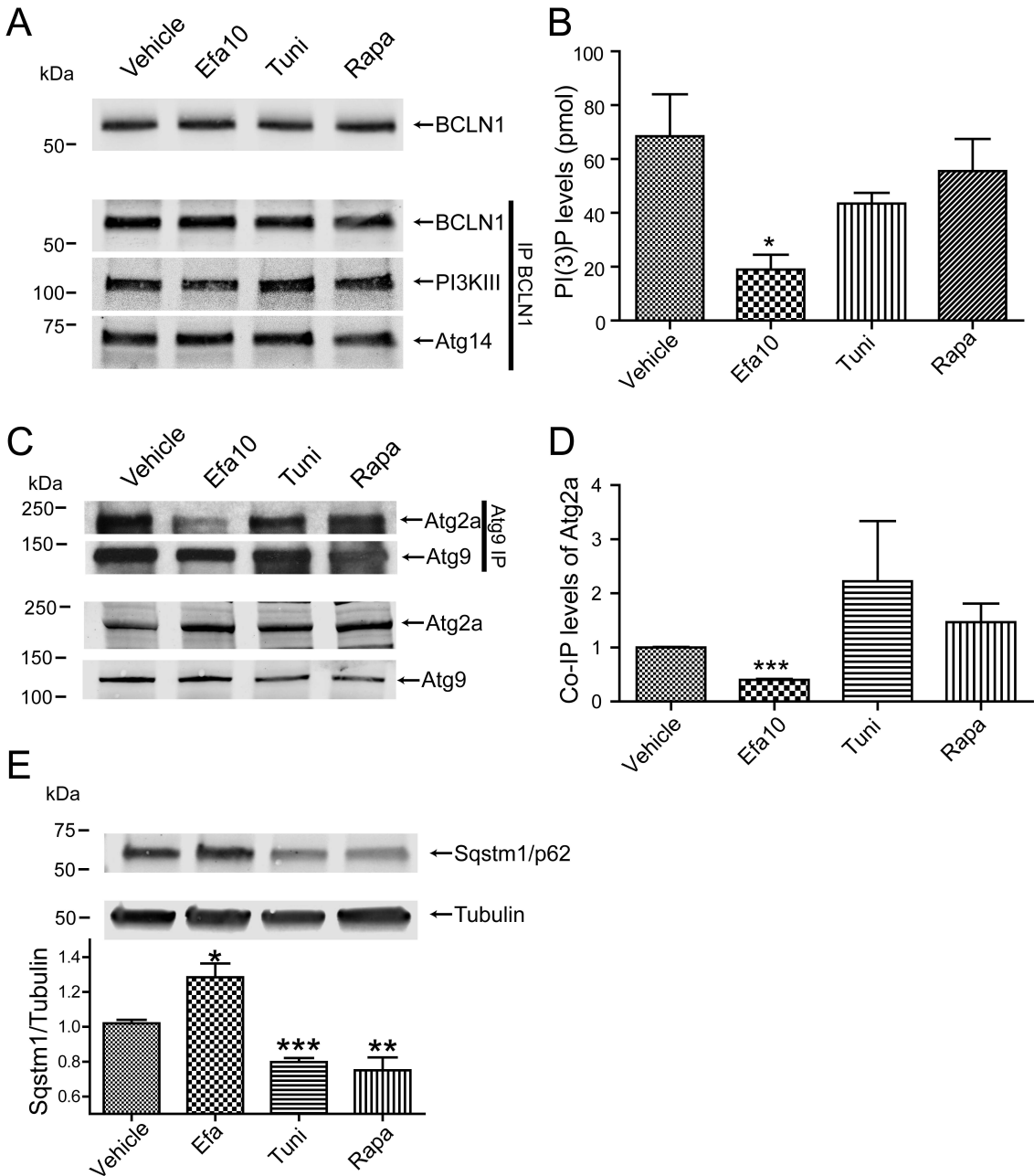
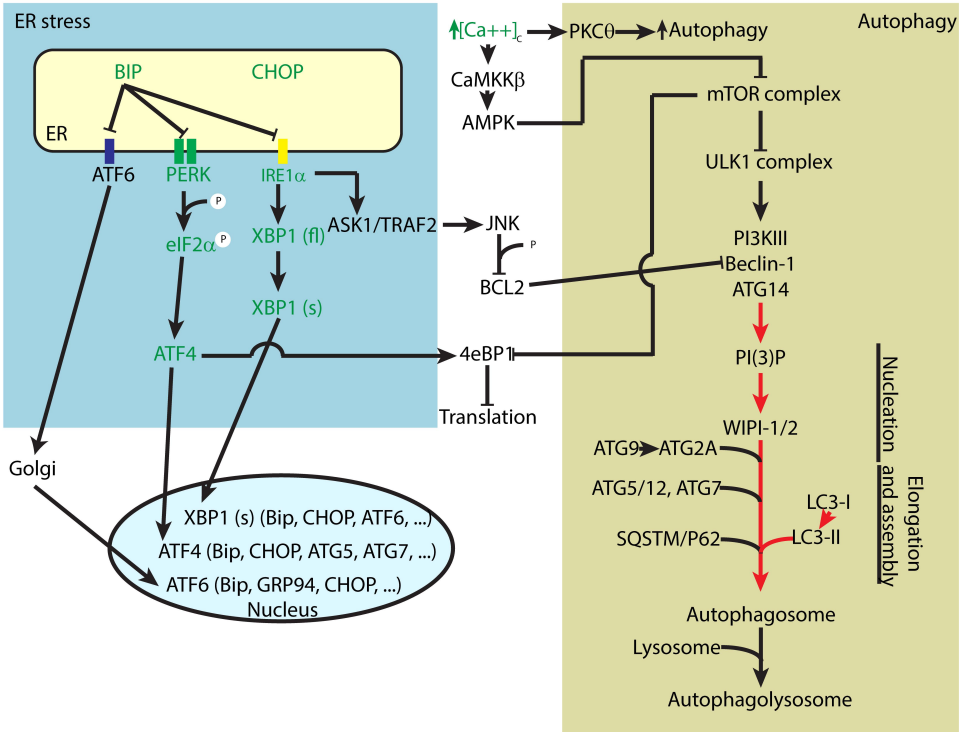

A
kDa
Vehicle
Efa10
Tuni
Rapa
50
←BCLN1
50
←BCLN1
100
←PI3KIII
75
←Atg14
IP BCLN1
B
PI(3)P levels (pmol)
100
80
60
40
20
0
*
Vehicle
Efa10
Tuni
Rapa
C
kDa
Vehicle
Efa10
Tuni
Rapa
250
150
←Atg2a
←Atg9
Atg9 IP
250
150
100
←Atg2a
←Atg9
D
Co-IP levels of Atg2a
4
3
2
1
0
***
Vehicle
Efa10
Tuni
Rapa
E
kDa
75
50
←Sqstm1/p62
50
←Tubulin
Sqstm1/Tubulin
1.4
1.2
1.0
0.8
0.6
*
***
**
Vehicle
Efa
Tuni
Rapa

Figure 9


ER stress
BIP
CHOP
ER
ATF6
PERK
IRE1α
eIF2α
P
XBP1 (fl)
XBP1 (s)
ASK1/TRAF2
JNK
P
BCL2
ATF4
4eBP1
Translation
Golgi
XBP1 (s) (Bip, CHOP, ATF6, ...)
ATF4 (Bip, CHOP, ATG5, ATG7, ...)
ATF6 (Bip, GRP94, CHOP, ...)
Nucleus
[Ca++]c
PKCθ
Autophagy
CaMKKβ
AMPK
Autophagy
mTOR complex
ULK1 complex
PI3KIII
Beclin-1
ATG14
PI(3)P
WIPI-1/2
ATG9
ATG2A
ATG5/12, ATG7
LC3-I
SQSTM/P62
LC3-II
Nucleation and assembly
Elongation
Autophagosome
Lysosome
Autophagolysosome