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Morphine counteracts the antiviral effect of antiretroviral drugs and causes upregulation of p62/SQSTM1 and histone modifying enzymes in HIV-infected astrocytes

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Abstract

Accelerated neurological disorders are increasingly prominent among the HIV-infected population and are likely driven by the toxicity from long-term use of antiretroviral drugs. We explored potential side-effects of antiretroviral drugs in HIV-infected primary human astrocytes and whether opioid co-exposure exacerbates the response. HIV-infected human astrocytes were exposed to the reverse transcriptase inhibitor, emtricitabine alone or in combination with two protease inhibitors ritonavir and atazanavir (ERA) with and without morphine co-exposure. The effect of the protease inhibitor, lopinavir alone or in combination with the protease inhibitor, abacavir and the integrase inhibitor, raltegravir (LAR) with and without morphine co-exposure was also explored. Exposure with emtricitabine alone or ERA in HIV-infected astrocytes caused a significant decrease in viral replication and attenuated HIV-induced inflammatory molecules, while co-exposure with morphine negated the inhibitory effects of ERA, leading to increased viral replication and inflammatory molecules. Exposure of FTC alone or in combination with morphine caused a significant disruption of mitochondrial membrane integrity. Genetic analysis revealed a significant increase in the expression of p62/SQSTM1 which correlated with an increase in the histone-modifying enzyme, ESCO2, after exposure with ERA alone or in combination with morphine. Furthermore, several histone-modifying enzymes such as CIITA, PRMT8 and HDAC10 were also increased with LAR exposure alone or in combination with morphine. Accumulation of p62/SQSTM1 is indicative of dysfunctional lysosomal fusion. Together with the loss of

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Author Contributions:

M.R. performed and analyzed the experiments shown in figures 1 through 5 and supplementary figures S1–S2 and wrote the manuscript. J.L. performed and analyzed the experiments shown in Figure 4A–B, 5A, and supplementary figure 2B, and assisted in the editing of the manuscript. C. R.O. provided assistance regarding the experiment shown in Figure 3 and assisted in writing the introduction and method sections. S.P. provided assistance regarding the experiment shown in Figure 4B. M.K. provided assistance regarding the experiment shown in supplementary figure 2B. F.K. provided technical expertise and assisted in the editing of the manuscript. N.E.-H. designed, conceived, analyzed and coordinated the study and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Conflict of Interest:

The authors declare that they have no conflict of interest.

mitochondrial integrity and epigenetic changes, these effects may lead to enhanced viral titer and inflammatory molecules contributing to the neuropathology associated with HIV.

Keywords

antiretroviral drugs; opioid; autophagy; scaffold protein; arginine methyl transferase

Introduction

The introduction of combined antiretroviral drugs (ARVs) more than 20 years ago has resulted in the immediate prevention of Human Immunodeficiency Virus (HIV) disease progression and has led to a significant increase in the life expectancy of infected people (Samji et al., 2013). Within the brain, residing glia (astrocytes and microglia) act mostly as a reservoir for HIV, maintaining a low level of HIV replication in the presence of antiretroviral drugs (Palmer et al., 2008). However, since antiretroviral drugs are not able to completely eradicate HIV from infected individuals, the increased lifespan has resulted in continuous brain exposure to virions and viral proteins, leading to a chronic state of inflammation and accumulation of neurological damage (Canizares et al., 2014; Rao et al., 2014). Although dispersion of many antiviral drugs into the central nervous system (CNS) is limited by poor penetration of the blood brain barrier (BBB) (Bertrand and Toborek, 2015; Decloedt et al., 2015; Ene et al., 2011), long-term antiretroviral-related toxicity in the CNS is considered another likely contributor to neurodegeneration and in accelerated aging associated with HIV (Akay et al., 2014; Kranick and Nath, 2012; Shah et al., 2016).

As patients are living longer, chronic pain has become a common manifestation affecting as much as 39 to 85% of people living with HIV/AIDS (Merlin et al., 2016). Co-morbidity with opiates are not only associated with recreational use but also with medicinal use related to opioid addiction and for the relief of pain in HIV-infected individuals (Al-Hasani and Bruchas, 2011). Several researchers have shown that opioids can have significant adverse interactive effects with many antiretroviral drugs that can contribute to nonadherence and poor clinical outcomes in this high-risk population (Iribarne et al., 1998; Kumar et al., 1996). Some studies have indicated that even low concentrations of ARVs that penetrate the BBB could have toxic effects (Robertson et al., 2012; Robertson et al., 2010) which can be further exacerbated by co-exposure to opioid drugs. Few studies have examined the adverse effects of opiates (morphine) on the use of ARVs in attenuating HIV replication and viral-induced inflammatory molecules in HIV-infected glial cells. Furthermore, limited information is available about the underlying mechanisms regulating drug-drug interactions between opioids and ARVs potentially leading to neuronal dysfunction (Hauser and Knapp, 2014; Maubert et al., 2015). In the current study, we explore the potential interactive effects of various antiretroviral drugs when combined with morphine. With changes in inflammatory molecule secretion and the influence of mitotoxicity, we propose a potential role of the adaptor protein p62/SQSTM1 and the autophagy pathway in mediating ARV neurodegeneration in the HIV population and drug abusing population.

Material and Methodology

HIV infection and treatments of human astrocytes

Primary human astrocytes (catalog #: 1800, ScienCell, Carlsbad, CA, USA) were grown to ~80% confluency and infected with HIV-1_{SF162} (p24 = 1 ng/mL; from Dr. Jay Levy through the NIH AIDS Research and Reference Reagent Program; Germantown, MD, USA) followed by exposure with 10 μ M each of emtricitabine, ritonavir, atazanavir (ERA) or lopinavir, abacavir, raltegravir (LAR) alone or in combination with 500 nM of morphine sulfate (Sigma-Aldrich, St. Louis, MO, USA) for 3, 5, and 7 days. HIV infection was quantified by p24 levels using a p24 antigen ELISA (ZeptoMetrix, Buffalo, NY, USA) and by RT-PCR using the long terminal repeat (LTR) primers (F-3): 5'-TTTGTTATATTTTGTGAGTTTGTAT-3' (nucleotide position: 200 to 224, 9285 to 9309), reverse primer (R-1), 5'-CAAAAAACTCCCCAACTCAAATCTA-3' (nucleotide position: 496 to 472, 9581 to 9557). Concentration of the antiretroviral drugs are based on previously published literature by others (Cohen et al., 2017; Ene et al., 2011; Kravcik et al., 1999; Nooka and Ghorpade, 2017) and cell viability assay. Morphine at a concentration of 500 nM has been previously shown to fully activate the μ -opioid receptor as well as to enhance HIV mediated neurotoxicity (El-Hage et al., 2005; Gurwell et al., 2001; Hauser et al., 1996; Stiene-Martin and Hauser, 1991).

ELISA

Levels of interleukin (IL)-6, monocyte chemotactic protein-1 (MCP-1), regulated upon activation normal T-cell expressed and secreted (RANTES), and tumor necrosis factor alpha (TNF- α) were measured by ELISA at 3, 5, and 7 days post-treatment (R&D Systems, Minneapolis, MN, USA). The O.D. was read at A450 on a Synergy HTX plate reader (BioTek, Winooski, VT, USA).

Real Time Polymerase chain reaction (PCR)

Relative abundance of mRNA was assessed by SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in 20 μ L real-time PCR reactions with gene specific primers using a Bio-Rad CFX96 real time system. All data were normalized to GAPDH and presented as 2^{-Ct} for fold-change values.

Cellular Membrane Integrity

Intracellular energy balance was evaluated using a mitochondrial ToxGlo assay (Promega, Madison, WI, USA). Briefly, a fluorogenic peptide substrate (bis-AAF-R110) was added to the cells and fluorescence was measured. Then, an ATP detection reagent was added to the cells and ATP levels were determined by luminescence. Fluorescence and luminescence were measured using a Synergy HTX plate reader (BioTek).

Reactive oxygen species (ROS)

ROS production were measured using the indicator 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2 DCFDA; Invitrogen, Carlsbad, CA, USA), which is de-acetylated to dichlorofluorescein (DCF). After treatments,

fluorescence was measured at $\lambda_{\text{ex}} = 485$ nm and $\lambda_{\text{em}} = 520$ nm using a Synergy HTX plate reader (BioTek).

Cell viability assay

Viability was assessed using a live/dead fluorescence assay (ScienCell) which yields two-color discrimination of the population of live cells (green) from the dead cells (red). Cells were imaged using an inverted fluorescence microscope (Zeiss, Germany), manually quantified and reported as percent of viability.

RNA Expression profiling

Human Cellular Stress Responses (catalog #: PAHS-019Z, Qiagen; Valencia, CA, USA) and Human Epigenetic Chromatin Modification Enzymes (catalog #: PAHS-085Z, Qiagen) RT² Profiler PCR Arrays were used for RNA profiling. Ct data were interpreted from the manufacturer's website (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). Relative expression was calculated using the $\Delta\Delta\text{CT}$ method with five housekeeping genes and compared with the expression in control cells.

Western blotting

Membranes were probed with primary antibodies against p62/SQSTM1, LC3B, Beclin1, and β -actin as internal control. Primary antibodies were followed by incubation with the respective secondary antibody conjugated to horseradish peroxidase. Immunoblots were exposed to SuperSignal West Femto Substrate (Thermo Scientific, Waltham, MA, USA) and visualized using a ChemiDoc imaging system (Bio-Rad). Protein expression was calculated using ImageJ software (National Institutes of Health (NIH), Bethesda, MD, USA).

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) techniques followed by Bonferroni's post hoc test for multiple comparisons (GraphPad Software, Inc., La Jolla, CA, USA). Alpha level of $p < 0.05$ was considered significant.

Results

Antiviral effects of emtricitabine (FTC) and lopinavir (LPV) in HIV-infected astrocytes co-exposed with morphine.

It is accepted that despite having poor penetration into the CNS and providing incomplete protection against HIV replication in brain reservoirs, combination antiretroviral therapies (cART) can improve cognition and reduce the prevalence of HIV-associated neurological complications. Despite those successes, long-term use of antivirals can have detrimental effects leading to enhanced neurotoxicity in HIV-infected individuals. We assessed the effectiveness of the reverse transcriptase inhibitor, emtricitabine (FTC; low CNS penetrance (Ene et al., 2011; Lahiri et al., 2016)) with and without morphine co-exposure (Fig. 1 A). Exposure to FTC significantly reduced HIV infection; however, co-exposure with morphine reverted this inhibition by three days post treatment. Similar effects were detected on days 5 and 7, albeit not significantly. Release of inflammatory molecules were detected by ELISA

and interestingly, although viral titer was attenuated, expression of MCP-1 was enhanced with FTC alone and with morphine by days 3, 5 and 7 of post-treatment (Fig. 1B). Separately, HIV-infected primary human astrocytes were exposed to the protease inhibitor, lopinavir (LPV; high CNS penetrance (Ene et al., 2011; Lahiri et al., 2016)) with and without morphine co-exposure. Exposure to LPV had no inhibitory effect on HIV titer (Fig. 1A) yet enhanced HIV-induced MCP-1 secretion only on day 3 (Fig. 1B). Co-exposure with morphine showed minimal interactive effect on viral titer (Fig. 1A) and showed minor, albeit significant, decrease in IL-6 secretion (Fig. 1B). Overall the data shows reduced efficacy in the antiviral response of FTC in HIV-infected astrocytes when co-exposed with morphine.

Enhanced mitochondrial damage and cytotoxicity with emtricitabine (FTC) and lopinavir (LPV) in HIV-infected astrocytes co-exposed with morphine.

Antiretroviral toxicity has been partly attributed to mitochondrial damage and oxidative stress molecule release (Chandra et al., 2009; Kline et al., 2009; Manda et al., 2011; Papparella et al., 2007). In addition, mitochondrial dysfunction has been confirmed as a great contributor to the occurrence of neurodegenerative diseases (Baloh, 2008; Choi et al., 2011; Dodson and Guo, 2007; Squitieri et al., 2006; Wang et al., 2008). To this end, we assessed effects of FTC and LPV on mitochondrial membrane integrity in HIV-infected astrocytes and determined whether co-exposure with morphine alters these responses. To assess cellular toxicity, we used the bis-AAF- R110 substrate which cannot cross intact membranes of live cells, giving reduced signal in viable cells. HIV infection exerted high levels of cytotoxicity compared to uninfected cells, whereas exposure with FTC alone or combined with morphine significantly enhanced cytotoxicity when compared to HIV-infected or uninfected cells, suggesting damage to the membrane (Fig. 2A). In contrast, significant toxicity with LPV alone or combined with morphine was undetectable. To discern whether the cytotoxicity observed was correlated with mitochondrial toxicity or primary necrosis, ATP levels were determined by measuring luminescence which was proportional to ATP. Levels of ATP remained unchanged, despite a significant increase of 35% in cytotoxicity suggesting that the increase may not necessarily be due to mitochondrial toxicity but rather to necrosis (Fig. 2A). This data correlates with published literature stating LPV toxicity has been linked more closely to the macrophage/microglia cell type (Chen et al., 2009; Lagathu et al., 2007; Zhang et al., 2014). Since mitochondria harbor the bulk of oxidative pathways (Andreyev et al., 2005; Murphy, 2009), we also assessed whether FTC and LPV cause changes to ROS in HIV-infected astrocytes and whether co-exposure with morphine alters the responses. HIV infection caused significant increases in ROS production when compared to uninfected cells, while exposure with FTC alone or with morphine further enhanced, albeit not significantly, the production of ROS in HIV-infected astrocytes (Fig. 2B). The overall data shows significant damage in membrane integrity with FTC and LPV alone or in combination with morphine. We also found that the damage to membrane integrity with FTC or LPV treatment did not correlate with viral titer levels (Fig. 1 A), suggesting that other factor(s) may be responsible for the damage.

Suppressive effect of cART was reverted by co-exposure with morphine in HIV-infected astrocytes.

Since ARVs are not prescribed as a monotherapy but rather in combination, we combined 3 different types of antiretrovirals in two sets. The first consisted of the reverse transcriptase inhibitor, emtricitabine, in combination with two protease inhibitors, ritonavir and atazanavir (ERA). The second set consisted of the protease inhibitor, lopinavir, in combination with the protease inhibitor, abacavir and the integrase inhibitor, raltegravir (LAR). HIV-infected astrocytes were exposed for 3, 5, and 7 days to the ERA or LAR, with or without morphine co-exposure. Exposure to ERA showed a ~2-fold attenuation in viral replication by LTR RT-PCR (Fig. 3 A), while co-exposure with morphine reverted the antiviral response and heightened viral replication by 3.5-fold when compared to ERA-treated cells. Likewise, exposure with LAR attenuated viral replication by 1.5-fold (Fig. 3 A); however, co-exposure with morphine did not alter the antiviral response of LAR. Assessment by p24 ELISA showed similar patterns (Fig. 3B). Next, the release of inflammatory molecules were detected by ELISA and showed a time-dependent increases in the release of the chemokines MCP-1, RANTES and the cytokines IL-6 and TNF- α by HIV-infected astrocytes (Fig. 3C). Although these secretions were attenuated with ERA, co-exposure with morphine negated the response leading to a significant increase of 3-fold and 3.6-fold in MCP-1, RANTES, respectively after 5 days post-treatment (Fig. 3C). HIV-induced secretion of IL-6 and TNF- α were not significantly attenuated with ERA, while co-exposure with morphine further enhanced cytokine release at 5 and 7 days post-treatment (Fig. 3C). Similar results were seen with LAR; however, co-exposure with morphine showed no further changes in inflammatory responses (Fig. 3C). Overall, the data shows a drug-drug interactive effect between morphine and cART in astrocytes leading to failure in the attenuation of viral replication and inflammatory molecule secretion by ERA but not LAR, potentially contributing to HIV-associated neuropathology.

Increased levels of the ubiquitin-binding scaffold protein, p62/SQSTM1 by exposure with ERA and morphine in HIV-infected astrocytes.

Cytokines can contribute to viral control by upregulating the antiviral immune response (Chevalier et al., 2011; Muller et al., 1994). However, chemokines such as RANTES and MCP-1 can increase the target cell pool for HIV by recruiting the primary target cells, CD4+ T cells and monocytes, respectively. Alternately, pro-inflammatory cytokines such as IL-6 and TNF- α produced during HIV infection can increase antiviral immunity but can also induce the transcription factor, nuclear factor kappa B (NF- κ B) which enhances HIV replication (Alcami et al., 1995; Osborn et al., 1989; Vallabhapurapu and Karin, 2009). Since sustained inflammatory molecule release and viral replication may induce stress and cytotoxicity, potentially contributing to HIV-associated neuropathology, RNA from HIV-infected astrocytes exposed for 3 and 7 days with ERA and LAR, with and without morphine co-exposure were used to explore cellular stress responses. We identified a significant increase in the mRNA levels of the ubiquitin-binding scaffold protein, p62/SQSTM1, in HIV-infected astrocytes exposed with ERA alone which was further upregulated in combination with morphine at 7 days post-treatment (Fig. 4A). The data was confirmed with RT-PCR using primers specific for p62/SQSTM1 and showed an

approximate 2-fold increase with ERA and a synergistic increase of about 8-fold when combined with morphine (Fig. 4B). Upon protein analysis, we detected a significant upregulation in the expression of p62/SQSTM1 in HIV-infected astrocytes exposed to ERA that was further increased with morphine (Fig. 4C, D). Interestingly, the upregulation in p62/SQSTM1 was not detected in HIV-infected astrocytes exposed to LAR alone or in combination with morphine (Fig. 4). Given p62/SQSTM1 is involved in autophagosome maturation and lysosomal clearance, we measured expression levels of two autophagy related proteins Beclin1 and LC3 (Fig. 4). Since p62/SQSTM1 is a multifunctional, stress-induced protein involved in the regulation of inflammatory responses and redox homeostasis, we believe that the high level of p62/SQSTM1 in HIV-infected astrocytes exposed to ERA and morphine could be the consequence of increased toxicity with ERA (Supplemental data 1). This data shows that co-exposure with ERA and morphine increases p62/SQSTM1 at RNA and protein levels which correlate with the increased release of inflammatory molecules and increased toxicity in HIV-infected astrocytes.

Increased levels of histone-modifying enzymes and G-protein coupled receptors by exposure of LAR and morphine in HIV-infected astrocytes.

Studies have shown a relationship between modulation of DNA methylation and the subsequent expression of the histone-modifying enzymes and antiretroviral therapy (Rosea et al., 2017; Trejbalova et al., 2016). RNA from HIV-infected astrocytes exposed for 3 and 7 days with ERA and LAR, with and without morphine co-exposure were used to explore epigenetic changes. The 84-gene array revealed a 3-fold increase in the mRNA expression of the histone-modifying enzyme ESC02 in HIV-infected astrocytes exposed with ERA alone and a 4-fold increase when combined with morphine at day 7. Separately, exposure with LAR alone caused about a 5-fold increase in the histone-modifying enzymes HDAC10 and KAT2A, about 8-fold increase in the histone-modifying enzymes AURKC and CIITA, and about a 12-fold increase the histone-modifying enzyme PRMT8 (Fig. 5A) at day 7. The expression of several of these genes including HDAC10, KAT2A, SETD6, and PRMT8 were further increased by co-exposure with morphine at day 7. The data was confirmed with RT-PCR using primers specific for ESC02, HDAC10, KAT2A, AURKC, CIITA, PRMT8 and SETD6, and showed a similar trend (Fig. 5B-H). While some of these enzymes and changes in their expression have been linked to HIV infectivity (Boulanger et al., 2005; Invernizzi et al., 2006; Kanazawa et al., 2000; Ran et al., 2017), a strong link has yet to be reported in conjunction with morphine. Of note, these epigenetics changes were also examined at day 3; however, differences in gene expression were minimal for both sets of ARV with or without morphine (Supplementary data 2).

Evidence has suggested a direct role of the μ -opioid receptor-1 (MOR-1) in HIV replication and pathogenesis (Dever et al., 2012) and given that astrocytes express opioid receptors as well as HIV co-receptors (Dever et al., 2012; El-Hage et al., 2005), we explore the possible interaction between cART and the G-protein coupled receptors utilized by both HIV and morphine. We detected significant increases in expression of the MOR-1 variants (MOR-1A, MOR-1K, and MOR-1X) after exposure with LAR alone that were further increased by co-exposure with morphine at day 7 but not detected at day 3 (Supplemental data 2). Gene expression changes of the MOR variants upon exposure to ERA alone or in combination

with morphine were insignificant. CCR5 expression, an HIV co-receptor, was increased with both sets of cART alone and in combination with morphine.

Overall, the data shows increased gene expression of several histone-modifying enzymes after exposure with LAR and morphine, which could be involved with LAR-induced suppression of HIV titer and attenuation in the release of inflammatory molecules in HIV-infected astrocytes. The changes in the expression of the opioid receptor variants and CCR5 may or may not be directly correlated with the changes in the epigenetic genes but still warrant further investigation.

Discussion and Conclusion

In this study we explored the effects of antiretroviral drugs and the possible interaction with the opiate morphine in HIV-infected human astrocytes. Despite the great success of antiretrovirals in increasing the lifespan of HIV-infected individuals, the life expectancy of treated patients remains 10–30 years shorter than the normal population (2008; Lohse et al., 2007). There have been numerous reports demonstrating that antiretroviral drugs, such as zidovudine, lamivudine, indinavir, and abacavir can induce oxidative stress, modulate the mechanisms of phagocytosis, and impact mitochondrial function and DNA replication (Apostolova et al., 2011; Bertrand and Toborek, 2015; Blas-Garcia et al., 2010; Brinkman et al., 1999; Giunta et al., 2011; Manda et al., 2011). In addition, therapeutic dosages of nelfinavir and saquinavir have been shown to cause an inhibition of normal proteasome function (Piccinini et al., 2005). Furthermore, antiretroviral-induced neurotoxicity was recently linked to the autophagy pathway as shown by the dysregulation of ER stress and the inhibition of autophagosome formation by blocking the activity of the Beclin-1/Atg14/PI3KIII complex in regard to synthesis of phosphatidylinositol 3-phosphate by the non-nucleoside reverse transcriptase inhibitor, efavirenz (Bertrand and Toborek, 2015). Although effective in reducing viral load and preventing severe forms of neurological disorders in HIV populations, milder forms of HAND remain prevalent regardless of treatment (Ellis et al., 2007; Harezlak et al., 2011). We observed that co-exposure to morphine reverted the reduction of viral titer by emtricitabine, ritonavir and atazanavir (ERA), which correlated with significantly increased production of RANTES, MCP-1, and TNF- α in HIV-infected astrocytes (Fig. 3). These observations agree with a previous study published by Vaidya et al., 2016. Accordingly, they used mathematical and experimental data from simian immunodeficiency virus (SIV)-infected macaques to calculate the susceptibility rate to SIV infection and reduced antiretroviral efficacy (with both early and late ART initiations) in cells co-exposed to morphine. They showed that with intermediate levels of drug efficacy, ART failed to control the viral load in morphine-dependent animals (animals injected intramuscularly with morphine for approximately 18 weeks) but not in morphine non-dependent animals (Vaidya et al., 2016). They suggested that the effect of morphine on HIV infectivity, despite the presence of ARV, may be due to an increase in HIV co-receptors CCR5 and CXCR4 in the periphery. As shown by others, heterologous cross desensitization between the MOR-1 and the CCR5 receptor may lead to downstream signaling interactions that could directly affect HIV infectivity and immune responses against HIV (Chen et al., 2004; Melik Parsadaniantz et al., 2015). In our studies, LAR exposure in combination with morphine increased the mRNA expression levels of different MOR variants and the CCR5

co-receptor (Supplementary data 2); however, these modulations did not significantly change HIV infectivity or secretion of inflammatory molecules (Fig. 3). These observations may be attributed to the potential involvement of modulations to viral latency by LAR at transcriptional levels. As shown previously, epigenetic variations of proviral and cellular DNA may be associated with the establishment of HIV latency (Kumar et al., 2015). In terms of epigenetic modulations, exposure to LAR and morphine caused a significant increase in several histone-modifying enzymes (Fig. 5). We speculate that these epigenetic changes potentially correlate with a decrease in viral titer and a decrease in the secretion of inflammatory responses in HIV-infected astrocytes. For example, the increased mRNA levels of methyltransferase SET Domain Containing 6 (SETD6) detected in Fig.5, was shown by others to repress the NF- κ B system (Chang et al., 2011; Levy et al., 2011), and to regulate both inflammatory gene expression and HIV replication (Fiume et al., 2012; Gangwani and Kumar, 2015; Pitha, 2011; Swingler et al., 1994). Another gene in the methyltransferase family, SET domain bifurcated 1 (SETDB1) was shown to methylate the HIV Tat protein and to recruit inhibitory proteins complex to the HIV-1 genome causing a repression of HIV-1 transcription (Van Duyne et al., 2008). The protein arginine methyltransferases (PRMT)-8, a plasma membrane-associated protein mainly expressed in brain tissues, was also increased with co-exposure of LAR and morphine (Fig. 5). Although PRMT8 has not been shown to be associated with HIV replication, the overexpression of PRMT6, another protein within the family, was linked with the reduction of Tat transactivation in HIV-1 production and viral replication (Xie et al., 2007). Low expression levels of PRMT6 in peripheral blood mononuclear cells derived from chronically infected and non-infected HIV individuals correlated with enhanced CD4⁺ T-cell exhaustion due to a permanent state of cell activation, even under antiretroviral drug treatments (Bogoi et al., 2018). Although HIV individuals receiving cART exhibited a slightly higher expression of PRMT6 compared with HIV patients not receiving cART, PRMT6 protein levels were lower than the expression observed in non-HIV infected individuals (Bogoi et al., 2018). Significant expression levels of the histone deacetylase 10 (HDAC10) was also detected with LAR alone and in combination with morphine (Fig. 5). HDAC10 has been reported to be downregulated by HIV-1 virus-associated envelope glycoprotein (vEnv) which lead to enhanced infectivity of progeny viruses (Ran et al., 2017). A study by Ran et al. confirmed the role of HDAC10 in HIV infectivity by generating HDAC10 knockdown in Jurkat T-cells followed by infection with HIV. Both viral integrated DNA level and the amount of produced p24 in the supernatant from the HDAC10 knockdown Jurkat T-cells were significantly higher than in control cells. This suggest that reduced HDAC10 expression level facilitated the early steps towards to the establishment of HIV infection. Despite numerous evidence by others showing epigenetic as a mechanism for regulating HIV, we still don't know how epigenetic changes modulate viral infection and inflammatory molecules in a morphine-induced system, and further studies to examine the possible association between LAR and morphine within an HIV latency model are necessary to confirm whether these results are due to epigenetic changes.

Since we did not observe a strong link between ERA exposure and epigenetic changes, and based on previous investigations (Lapierre et al., 2018; Rodriguez et al., 2017), we suspected that increases in HIV titer and inflammatory molecules by ERA in the presence of morphine

could be mediated through other mechanisms, such as the autophagy pathway. Autophagy is a dynamic process of degrading and clearing intracellular proteins and organelles (Moreau et al., 2010; Wang and Hill, 2015; Zhou and Spector, 2008). Although autophagy is considered a cytoprotective response to viral factors, it plays a complex role in HIV infection which can either induce or inhibit autophagy in permissive cells (Dever et al., 2015; El-Hage et al., 2015; Rodriguez et al., 2017; Wang et al., 2012; Zhou and Spector, 2008). Our group has reported that HIV infection in microglia triggers increased autophagosome formation but blocks fusion with the lysosome, thereby halting the autophagic process and leaving engulfed virions intact (El-Hage et al., 2015). HIV-induced neuronal damage and astrocytic toxicity have been shown to involve suppression of autophagy, supporting its role in HIV-induced neuropathology (Ellis et al., 2007). Evidence shows that exploitation of autophagy by viruses may be induced through a MOR stimulated signaling pathway (Zhao et al., 2010). Despite autophagy clearing intracellular pathogens including viruses, autophagosome formation, in many cases, promotes viral replication and assembly (Jackson, 2015) while also inhibiting the fusion of autophagosomes with lysosomes (El-Hage et al., 2015). p62/SQSTM1 can facilitate the clearance of non-ubiquitinated proteins and protein aggregates by autophagy. Additionally, either an accumulation or a reduction of p62/SQSTM1 can result in the impaired degradation of proteasome substrates (Seibenhener et al., 2004). We identified significant increases in mRNA and protein levels of p62/SQSTM1 in HIV-infected astrocytes exposed to ERA alone which increased upon co-exposure with morphine. High levels of p62/SQSTM1 are indicative of dysregulation in autophagic maturation/lysosomal fusion which could be related to the increased viral production and increased inflammatory molecule production by the host (Fig. 3). Altered function of p62/SQSTM1 may ultimately lead to neurodegenerative disorders associated with HIV (Du et al., 2009; Kuusisto et al., 2001, 2002). This supports the concept that induction of autophagy is the primary mechanism for increased viral production in cells co-treated with morphine and cART.

In conclusion, we report that the combination of the protease inhibitor, lopinavir with the protease inhibitor, abacavir and the integrase inhibitor, raltegravir (LAR) modulate several histone-modifying enzymes that have been associated with decreased HIV infection. Concurring with our observations that LAR decreases HIV titer and cytokine and chemokine release, even in the presence of morphine. On the other hand, the combination of the reverse transcriptase inhibitor, emtricitabine with the protease inhibitors ritonavir and atazanavir (ERA) in the presence of morphine reversed the reduction of HIV by the ARVs alone. These observations together with accumulation of p62/SQSTM1 and possible modulation of the autophagy pathway could be the triggering cause of enhanced neurotoxicity by these antiretroviral drugs in HIV-infected human astrocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

HIV replication and secretion of inflammatory molecules in HIV-infected astrocytes exposed to emtricitabine (FTC) and lopinavir (LPV) alone or in combination with morphine. HIV replication in human astrocytes was measured using HIV p24 Gag protein ELISA (a). Values were determined from standard curves and are presented as the mean $\pm$ the standard error of mean (S.E.M.) of three independent experiments. Corresponding cell culture supernatants were used to detect the levels of MCP-1, TNF- α , IL-6, and RANTES by ELISA (b). Values were determined from standard curves and are presented as the mean $\pm$ the S.E.M. of three independent experiments ($p < 0.05$ * vs. HIV, \$ vs. HIV+FTC, & vs. HIV+FTC+Mor, % vs. HIV+LPV)

Figure 2.

Mitochondrial damage and cytotoxicity measurements in HIV-infected astrocytes exposed to emtricitabine (FTC) and lopinavir (LPV) in viral-infected astrocytes co-exposed with morphine. Mitochondrial damage in human astrocytes was measured using a mitochondrial fluorescence and luminescence assay (a). Data are presented as percent of increased compared to untreated control cells. ROS production in human astrocytes was assessed by DCF fluorescence at the indicated time points after indicated treatments (b). Error bars show the S.E.M. of three independent experiments. Cellular viability was assessed by live/dead cell fluorescence assay. Data are presented as percent viability compared to untreated control cells (c). Error bars show the S.E.M. of three independent experiments ($p < 0.05$ * vs. HIV, \$ vs. HIV+FTC, & vs. HIV+FTC+Mor, % vs. HIV+LPV)

Figure 3.

HIV replication and secretion of inflammatory molecules in HIV-infected astrocytes exposed to ERA and LAR alone or in combination with morphine. HIV replication in human astrocytes was measured using qRT-PCR- and LTR-specific primers (a). Data are represented as relative fluorescence units (RFU); GAPDH was used as internal control. Error bars show S.E.M. for three independent experiments. HIV replication in human astrocytes was measured using HIV p24 Gag protein ELISA (b). Values were determined from standard curves and are presented as the mean $\pm$ the S.E.M. of three independent experiments. Corresponding cell culture supernatants used for p24 ELISA were used to detect the levels of MCP-1, TNF- α , IL-6, and RANTES by ELISA (c). Values were determined from standard curves and are presented as the mean $\pm$ the S.E.M. of three independent experiments ($p < 0.05$ * vs. HIV, § vs. HIV+ERA, & vs. HIV+ERA+Mor, % vs. HIV+LAR)

Figure 4.

Expression of human cellular stress genes and autophagy-related genes in HIV-infected astrocytes exposed with ERA and LAR alone or in combination with morphine. Relative mRNA expression of selected genes was measured using the human cellular stress responses RT2 profiler PCR array following the indicated treatments for 7 days (a). Data are presented as expression relative to untreated control cells. mRNA levels of p62/SQSTM1 were assessed by qRT-PCR following indicated treatments for 3 days and 7 days (b). Values were determined by the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH. Whole cell lysates from human astrocytes following 7 days of the indicated treatments were subjected to immunoblotting with antibodies against p62/SQSTM1, LC3, and Beclin1 protein (c, d). Densitometry was performed for quantification, and the ratio of each protein to β -actin are presented graphically. Error bars show the S.E.M. of three independent experiments ($p < 0.05$ * vs. HIV, \$ vs. HIV+ERA, & vs. HIV+ERA+Mor, % vs. HIV+LAR)

Figure 5.

Expression of histone-modifying enzymes and G-protein coupled receptors in HIV-infected astrocytes exposed with ERA and LAR alone or in combination with morphine. Relative mRNA expression of selected genes was measured using the human epigenetic chromatin modification enzymes array following the indicated treatments for 7 days (a). Data are presented as expression relative to untreated control cells. mRNA levels of the indicated genes were assessed by qRT-PCR following indicated treatments for 7 days (b–h). Values were determined by the $2^{-\Delta\text{CT}}$ method and normalized to GAPDH. Error bars show

S.E.M. for three independent experiments ($p < 0.05$ * vs. HIV, \$ vs. HIV+ERA, & vs. HIV+ERA+Mor, % vs. HIV+LAR)

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