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Protease inhibitors, saquinavir and darunavir, inhibit oligodendrocyte maturation: Implications for Lysosomal Stress

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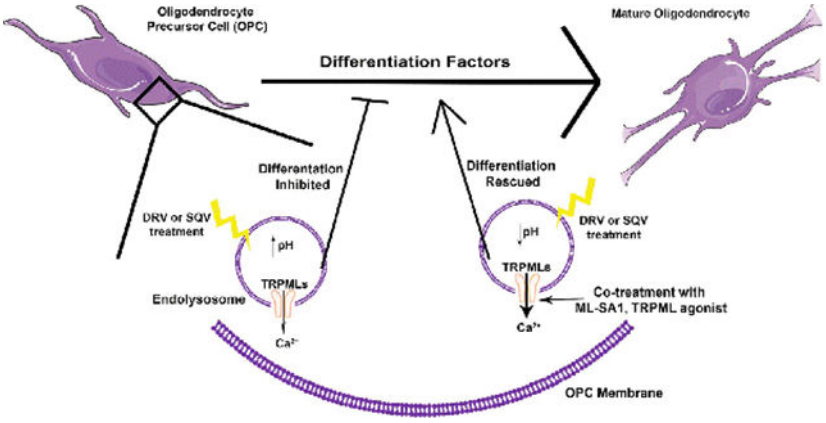
Graphical Abstract



Keywords

Antiretroviral therapy; oligodendrocyte; white matter; endolysosome

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Introduction

Human immunodeficiency virus-1 (HIV-1) continues to have a significant impact throughout the United States and globally. The introduction of antiretroviral (ARV) therapy (ART) has dramatically changed the course of HIV infection, shifting from a life-limiting illness characterized by fatal opportunistic infections and malignancies to a chronic disease associated with a near-normal life expectancy (FAUCI AND MARSTON 2015). In addition to its direct impact on the immune system, HIV affects the central nervous system (CNS) leading to a range of neurocognitive dysfunction collectively known as HIV-associated neurocognitive disorders (HAND) (SAYLOR *et al.* 2016). Cross-sectional studies report persistence of HAND in approximately 30–50% of ART-treated, HIV+ patients and while the prevalence of the most severe form, HIV-associated dementia (HAD), has diminished, milder forms of the disease account for the continued prevalence of HAND (HEATON *et al.* 2010).

In the post-ART era, the shifting clinical manifestations of HAND are accompanied by changes in pathologic observations which have transitioned from a prominent subcortical pathology characterized by neuronal loss, astrogliosis, and microgliosis to more subtle cortical and hippocampal alterations in synaptic number, functional connectivity, and neuroinflammation (SAYLOR *et al.* 2016). Despite the change in the neuropathology observed, white matter alterations and dysfunction continue to persist in HIV+ individuals taking ART (LANGFORD *et al.* 2003; EVERALL *et al.* 2009; MULLER-OEHRING *et al.* 2010; TATE *et al.* 2010). MRI and diffusion tensor imaging studies have demonstrated numerous alterations including thinning of the corpus callosum, reduction in blood flow to white matter, and loss of volume from the superior longitudinal fasciculus, superior corona radiata, and the internal capsule (HOARE *et al.* 2010; GONGVATANA *et al.* 2011; TATE *et al.* 2011; CORREA *et al.* 2015). Complementing these imaging findings, transcriptome analysis of frontal cortex from HAND patients who were virally suppressed through ART implicates persistent white matter changes despite viral control (BORJABAD *et al.* 2010). Transcripts critical for oligodendrocyte maturation, myelination, and maintenance, including myelin basic protein (MBP), myelin transcription factor 1, and myelin-associated oligodendrocyte basic protein (MOBP), were downregulated in patients on ART who were also diagnosed with HAND, despite reversal of proinflammatory gene signatures and restoration of synaptic gene signatures (BORJABAD *et al.* 2010). Thus, white matter pathologies continue to persist in the post-ART era; however, the mechanism underlying these observations is still unclear.

When adhered to, ART suppresses viral replication to undetectable levels in the periphery; however, low levels of inflammation and viral reservoirs are known to persist in the CNS. Furthermore, ARVs themselves may directly contribute to HAND pathogenesis, including the white matter abnormalities that are consistently observed.

Myelin is synthesized by mature oligodendrocytes which are generated from oligodendrocyte precursor cells (OPCs) in a well-characterized progression (MILLER 2002). OPCs, recognized by staining with the A2B5 antibody or expression of NG2 or PDGFRα, transition to immature oligodendrocytes expressing galactocerebroside (GalC)

and begin to extend actin filaments to elaborate processes (RAFF *et al.* 1978; RANSCHT *et al.* 1982; RAFF *et al.* 1983; BANSAL *et al.* 1989; HART *et al.* 1989; NISHIYAMA *et al.* 1996; BARACSKAY *et al.* 2007). Upon contact with neurons, these cells upregulate expression of myelin proteins including proteolipid protein (PLP) and myelin basic protein (MBP, and ultimately ensheath axons with the lipid- and protein-based myelin membrane. Our group has previously reported that the ARVs from the protease inhibitor (PI) class, ritonavir and lopinavir, inhibit oligodendrocyte maturation *in vitro* (e.g. reduction in GalC+ and MBP+ cells), and ritonavir reduced expression levels of myelin proteins (e.g. cyclic nucleoside phosphodiesterase (CNPase) and myelin oligodendrocyte glycoprotein (MOG)) *in vivo* (JENSEN *et al.* 2015). At present, the mechanisms mediating these effects are uncertain, though numerous candidates exist including the integrated stress response (ISR), oxidative stress, and organellar stress (AKAY *et al.* 2012; GANNON *et al.* 2017; STERN *et al.* 2018). In particular, lysosomal stress has been implicated in Tat- and gp120-mediated neuronal toxicity (CHEN *et al.* 2013; BAE *et al.* 2014; FIELDS *et al.* 2015) but the role that it may play in ARV-driven oligodendrocyte damage is currently unclear.

Here, we investigated the effects of two PIs, darunavir and saquinavir, on oligodendrocyte differentiation *in vitro*. We observed concentration-dependent inhibition of oligodendrocyte maturation and myelin protein production with both darunavir and saquinavir. Furthermore, our findings suggest that endolysosomal de-acidification mediates the ability of these ARVs to negatively regulate oligodendrocyte differentiation. Together, these data highlight the influence of proper endolysosome function during oligodendrocyte maturation and suggest that reducing lysosomal pH may provide a valid therapeutic option to prevent oligodendrocyte injury.

Materials and Methods

Chemicals and Reagents

The following antibodies used in this study were purchased from the indicated vendors: Sigma-Aldrich (St. Louis, MO): alpha-tubulin (α-tubulin; Cat# T5168 RRID: AB_477579) propidium iodide (Ca# P4170); BioLegend (San Diego, CA): myelin basic protein (MBP; Cat# 808401 RRID:AB_2564741); Invitrogen (Waltham, MA): Lysostracker Red DND-99. Additional antibodies were: galactocerebroside (GalC) mouse hybridoma supernatant (GalC H8H9) (Ranscht et al., 1982), A2B5 mouse hybridoma supernatant (BARACSKAY *et al.* 2007), myelin basic protein (MBP, rat hybridoma supernatant) and proteolipid protein (PLP, rat hybridoma supernatant) (kind gifts of Dr. Virginia Lee, University of Pennsylvania, Philadelphia, PA).

Preparation of primary rat oligodendrocyte precursor cell cultures

All experiments were performed in accordance with the guidelines set forth by The Children's Hospital of Philadelphia and The University of Pennsylvania Institutional Animal Care and Use Committees. Primary rat oligodendrocytes (OPCs) were isolated from brains of postnatal day 1 Sprague Dawley rats (Charles River Laboratories, Wilmington, MA RRID: RGD_737891) and plated on T75 flasks (JENSEN *et al.* 2015). We purified the OPCs using the "shake-off" method (MCCARTHY AND DE VELLIS 1980). Briefly, once OPCs

reached confluency on T75 flaks, they were rotated on an orbital shaker set to 250 rpm and incubated overnight at 37°C. The following day, cells were filtered using a 20μm nylon net (Merck Millipore, Darmstadt, Germany), followed by centrifugation at 1500 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 5 mL Neurobasal medium supplemented with B27 and incubated on a bacteriological petri dish for 15 mins at 37°C and 5% CO_2. The supernatant was collected and centrifuged at 1500 rpm for 5 mins at 4°C. The pellet was resuspended in Neurobasal medium supplemented with B27 and growth factors: PDGF (2ng/mL), NT3 (1ng/mL), and FGF (10ng/mL) and plated on 24-well plates with coverslips or 10 cm dishes.

Drug Treatments

Primary rat OPCs were grown in 24-well plates with coverslips or in 10 cm dishes, for immunocytochemistry or immunoblotting respectively, until they reached approximately 70% confluency. In order to differentiate OPCs into mature oligodendrocytes, growth medium was replaced with differentiation media consisting of 50% DMEM, 50% Ham's F12, Pen/Strep, 2 mM glutamine, 50 μg/mL transferrin, 5 μg/mL putrescine, 3 ng/mL progesterone, 2.6 ng/mL selenium, 12.5 μg/mL insulin, 0.5 μg/mL T4, 0.3% glucose, and 10 ng/mL biotin. At the time of differentiation, cells were treated with vehicle (DMSO), saquinavir (500 nM, 2.5 μM, or 5 μM), darunavir (450 nM, 4.5 μm, or 15μM), or ML-SA1 (20 μM) for 72 hours before staining or protein collection (FEIGENSON *et al.* 2009).

Immunofluorescence

Primary OPCs were prepared and stained as follows for GalC and MBP. Coverslips were removed from wells, rinsed with PBS, and incubated with primary antibody for immature oligodendrocyte marker, GalC (1:5 dilution in DMEM/F12) for 30 mins at room temperature. Coverslips were rinsed with PBS and incubated in Rhodamine-Red goat anti-mouse IgG3 secondary antibody (1:200 dilution in DMEM/F12; Jackson ImmunoResearch, West Grove, PA) for 30 mins at room temperature. Cells were fixed using 4% paraformaldehyde for 10 mins, followed by a blocking/permeabilization step consisting of 0.5% BSA and 0.1% Triton-X 100 in PBS for 30 mins. Cells were incubated in primary antibody for mature oligodendrocyte marker, MBP (1:1 dilution in PBS) for 30 mins at room temperature and then incubated in FITC-conjugated goat anti-rat secondary antibody (1:200 dilution in PBS; Jackson ImmunoResearch, West Grove, PA) for 30 mins at room temperature. Lastly, cells were incubated in DAPI (1:10,000 dilution in PBS; Thermo Fisher Scientific, Waltham, MA) for 5 mins and mounted on coverslips with Vectashield Antifade Mounting Medium (Vector Laboratories, Burlingame, CA). Primary OPCs were prepared and stained as follows for A2B5 and propidium iodide: coverslips were removed from wells, rinsed with PBS, and incubated with propidium iodide (5 μM in PBS). Coverslips were rinsed with PBS and incubated with primary antibody for OPCs, A2B5 (1:1 dilution in DMEM/F12) for 30 mins at room temperature. Coverslips were rinsed with PBS and incubated in FITC-goat anti-mouse IgM secondary antibody (1:200 dilution in DMEM/F12; Jackson ImmunoResearch, West Grove, PA) for 30 mins at room temperature. Coverslips were incubated with DAPI and mounted with the same method for GalC and MBP stained coverslips. Cells were imaged using a Keyence BZ-X-700 digital fluorescent microscope (Keyence Corporation, Itasca, IL) affixed with UV, FITC, Cy3, and Cy5 filters. Images were

captured at 40x magnification and were hand-counted to quantify the number of immature and mature oligodendrocytes. Specifically, averages were calculated for GalC and MBP across 20 fields/coverslip with 2–4 coverslips per treatment condition for each biological replicate.

Lysotracker Red analysis

The number of lysosomes was analyzed using 50 nM Lysotracker Red DND-99 (Invitrogen). Differentiated OLs were incubated with Lysotracker Red for 30 minutes at 37°C. Cells were then stained with GalC antibody and DAPI as mentioned above. Images were acquired on DMi8 Leica confocal microscope equipped with a 40x objective (Leica Microsystems, Wetzler, Germany) with an additional 2x electronic zoom. Imaging fields were chosen at random, 6 fields per coverslip and 2–3 coverslips per condition. Images were analyzed using Image-J particle analyzing software.

TUNEL Assay

To assess cells committed to apoptotic death, a TUNEL staining protocol (modified from (GAVRIELI *et al.* 1992) was utilized. Cells were fixed with ice cold methanol for 10 minutes, washed with PBS, and permeabilized with 0.1% Triton-X 100 and 0.5% BSA in PBS for 30 mins. Positive control coverslips were generated during this time by incubating in DN buffer (30 mM Trizma base pH 7.2, 140 mM sodium cacodylate, 4 mM magnesium chloride, and 0.1 mM dithiothreitol) for 2 mins, followed by DNase (1:200 in DN buffer) for 10 mins. All coverslips were washed with PBS and then placed in TDT buffer (30 mM Trizma base pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 2 mins. Cells were incubated for 1 hour at 37°C with TdT and biotin-UTP in TDT buffer (6 μL of each in 1 mL TDT buffer). After a subsequent PBS wash, cells were placed in TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 mins and then 2% BSA solution for 30 mins. Finally, cells were incubated with rhodamine-conjugated streptavidin for 20 mins, rinsed in PBS, incubated in DAPI for 5 mins, and then mounted on slides with Vectashield. Visualization and counting were performed as previously described (GAVRIELI *et al.* 1992).

Immunoblotting

Whole cell extracts of primary rat oligodendrocyte cultures were prepared with ice cold lysis buffer (25 mM Tris (pH 7.4), 10 mM EDTA, 10% SDS, 1% Triton-X 100, 150 mM NaCl containing protease and phosphatase inhibitor cocktails (PIs; Roche Diagnostics) followed by sonication and centrifugation at 10,000 rpm at 4°C for 30 mins. Protein concentrations were determined by spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA). Protein (7.5 μg) was loaded onto a 4–12% Bis-Tris gradient gels for separation. After separation, proteins were transferred to Immobilin-FL membranes and blocked in 5% milk for 30 mins at room temperature. Membranes were incubated overnight at 4°C with primary antibodies in TBS-T and 5% BSA. Primary antibodies to the following antigens were used: MBP (SMI-99, 1:1000 dilution), PLP (1:1000 dilution)and a-tubulin (1:10,000 dilution). Following three washes in TBS-T, membranes were incubated with corresponding antigen specific fluorescent probe-conjugated secondary antibodies (1:10,000 dilution) in TBS-T and 5% BSA. Membranes were visualized using an Odyssey Infrared Imaging System (LiCOR).). Densitometric analysis of band intensities were conducted using the Odyssey

Infrared Imaging System or by Fiji (NIH RRID: SCR_002285). All bands were normalized to the loading control as specified in each experiment.

Statistical Analysis

For all experiments, primary OPC cultures prepared from independent litters represent independent biological replicates, which were treated with vehicle or drug in the indicated combinations. An untreated condition (UT) was also included within each biological replicate and all results were normalized to this UT value to account for variability in the percentage of differentiation amongst biological replicates. Repeated-measures one-way analysis of variance (ANOVA) was used to analyze these data to account for inherent correlations present within a single biological replicate. Data were analyzed using GraphPad Prism statistical software (version 8.0; RRID: SCR_002798) and were graphically presented as fold change or percent total from UT ± SEM with the UT condition represented as a dotted line.

Results

The protease inhibitors, darunavir and saquinavir, inhibit oligodendrocyte maturation

Our group has previously demonstrated that ART compounds of the PI class, ritonavir and lopinavir, inhibit oligodendrocyte (OL) maturation *in vitro* (JENSEN *et al.* 2015). Based on these findings, we examined whether other PIs, specifically darunavir (DRV) and saquinavir (SQV), exert similar effects on OL differentiation. DRV is the only currently recommended frontline PI for adults in the United States, while SQV is part of the preferred second-line ART regimens for adults, adolescents, pregnant women, and children (WHO 2016). DRV and SQV concentrations were based on reported plasma and cerebrospinal fluid (CSF) levels in humans (DRV Cmax = 4.5μM, SQV Cmax = 2.5 μM), as measurements in human brain parenchyma have not been performed (VERMEIR *et al.* 2009).

Using our well-established cell culture model (SEE *et al.* 2004), OPCs were placed in differentiation media and treated with therapeutically relevant concentrations of DRV (450 nM, 4.5 μM, 15 μM) or SQV (500 nM, 2.5 μM, 5 μM). After 72 hours, cultures were stained for lineage specific proteins: A2B5, GalC and MBP for OPCs, immature and mature OLs, respectively (RAFF *et al.* 1978; RANSCHT *et al.* 1982; BANSAL *et al.* 1989; BARACSKAY *et al.* 2007). Representative images of treated OLs after 72 hours of differentiation demonstrated concentration-dependent decreases in numbers of immature and mature OLs in both DRV- and SQV-treated cultures compared with vehicle-treated controls (Fig. 1a). Specifically, the number of immature (GalC-positive; Fig. 1b) and mature (MBP-positive; Fig. 1c) OL cells were decreased in a concentration-dependent manner DRV- and SQV-treated cultures. We have represented this in figure 1 as a fold change normalized to the UT group and in the figure legend we record percent GalCpositive/DAPI-positive or MBP-positive/DAPI-positive. These percentages are typical of this well-established model (CHEW *et al.* 2005; FEIGENSON *et al.* 2009; FEIGENSON *et al.* 2011; DAI *et al.* 2014; BEYER *et al.* 2018) and the PI-treated groups have significantly fewer immature and mature cell counts than vehicle control groups. Additionally, we observed no differences in DAPI+

cell counts following DRV or SQV treatment compared with the DMSO-treated vehicle controls (Total Cell Number; Fig. 1d).

Neither saquinavir nor darunavir induce changes in OPC number or cell death

In order to determine whether the decreases in immature and mature OL numbers following SQV or DRV treatment were due to a reduction in OPC maturation or an increase in cell death, we examined terminal UTP Nick end labeling (TUNEL) positive cell counts to examine apoptotic cell death, and propidium iodide negative cell counts to examine overall cell viability. We also stained cells for OPC marker, A2B5 to examine changes in OPC number following DRV or SQV treatment. Representative images of treated OLs after 72 hours of differentiation demonstrated no change in numbers of OPCs and cell viability in both DRV- and SQV-treated cultures compared with vehicle-treated controls (Fig. 2a). Representative images of treated OLs 72 hours after differentiation displayed no increase in TUNEL+ cell counts (Fig. 2b). Specifically, the number of OPCs (A2B5-positive; Fig. 2c) remained unchanged in DRV or SQV-treated cultures compared with DMSO-treated vehicle controls and there was no significant decrease in cell viability (propidium iodide-negative; Fig 2d) in DRV or SQV-treated cultures compared with DMSO-treated vehicle controls. Finally, we observed no differences in the number of TUNEL+ cell counts even at the highest concentration of DRV or SQV tested when compared with DMSO-treated vehicle controls (Fig. 2d). In this assay, DNase was used as a positive control, resulting in a significant increase in TUNEL+ cells compared with the DMSO-treated vehicle control (Fig. 2d).

Saquinavir and darunavir inhibit myelin protein production

Since both SQV- and DRV-treated cultures contained fewer GalC+ and MBP+ cells, we next examined whether these drugs altered levels of the key myelin proteins, MBP and PLP. Cultures were treated at the time of differentiation, and protein was collected after 72 hours, and processed for immunoblot analysis. All three concentrations of DRV (Fig. 3a) and SQV (Fig. 3b) resulted in significant decreases in MBP and PLP protein levels compared with vehicle-treated controls. These data are consistent with our immunofluorescence observations in Figure 1, with fewer MBP+ cells and decreases in MBP and PLP protein abundance observed across all concentrations of SQV and DRV.

Darunavir and saquinavir decrease the number of acidic lysosomes and the TRPML1 agonist, ML-SA1, rescues darunavir and saquinavir-driven inhibition of oligodendrocyte maturation

We next aimed to determine whether either SQV- or DRV-mediated inhibition of OL differentiation is able to be rescued. While ART drugs are typically classified according to their mechanism of action of controlling HIV-1 viral levels, they can also be categorized as weak acids or bases. ART drugs that are considered weak bases, including DRV and SQV, can accumulate in endolysosomes and induce endolysosomal de-acidification (HUI *et al.* 2019). De-acidification of endolysosomes has been associated with neuronal dysfunction in several neurodegenerative conditions; however, how this could potentially impact OL maturation and function is unknown. Differentiating OLs that were exposed to either DRV or SQV and the number of acidic lysosomes were assessed via Lysotracker Red staining. We

observed a significant decrease in Lysotracker Red+ puncta at both the medium and highest concentrations for DRV and SQV in GalC+ and GalC− cells, demonstrating a reduction in the acidity of lysosomes following drug treatment (Fig. 4a–b). Since activation of TRPML1 channels is known to acidify the endolysosomes (BAE *et al.* 2014), we co-administered the TRPML1 agonist ML-SA1 (20μM) in the absence or presence of either DRV or SQV. We performed a dose-response curve of ML-SA1 testing a range of concentrations from 1 to 100 μM and observed no effect of ML-SA1 by itself on OL maturation or cell viability at these concentrations (data not shown). We observed a full recovery of OL differentiation for both DRV and SQV (Fig. 4c–e) at the lowest concentration of the ARVs, a partial rescue at the medium concentration for DRV-treated cultures, and no effect on the highest concentration for either ARV examined (Fig. 4c–e). Together, these data demonstrate that de-acidification of endolysosomes by DRV or SQV plays a role in their ability to hinder OL differentiation and maturation *in vitro*.

Discussion

The introduction of ART has changed the landscape of HIV/AIDS, as fewer patients progress to AIDS due to controlled viral suppression, thus expanding the lifespan of HIV-positive individuals. However, regardless of ART adherence HAND still persists; thus, a better understanding of the side effects of ART is necessary as patients remain on ART regimens for the entirety of their increased lifespans. In particular, white matter alterations as measured by diffusion tensor imaging and MRI remain important clinical determinants in HIV+ individuals on ART (LANGFORD *et al.* 2003; EVERALL *et al.* 2005; MULLER-OEHRING *et al.* 2010; TATE *et al.* 2010; JENSEN *et al.* 2019).

In the present study, we investigated the effects of ARV class PIs, DRV and SQV, on oligodendrocyte maturation. DRV and SQV inhibited oligodendrocyte differentiation concentration-dependently. Pharmacological activation of TRMPL1 on endolysosomes with ML-SA1 resulted in protection against DRV and SQV-induced oligodendrocyte maturation inhibition, *in vitro*, at low concentrations. The effect of SQV was more concentration-dependent than that of DRV. SQV at 450 nM induced a 50% decrease in GalC− and MBP-positive cells, respectively, which is similar to the effect of DRV at 500 nM. Both of the physiologically relevant concentrations for SQV (2.5 μM) and DRV (4.5 μM) had similar effects decreasing GalC− and MBP-positive cells, respectively by 75%. Interestingly, the highest concentration of DRV tested (15 μM) had similar effects on oligodendrocyte maturation as the physiologically relevant concentration; whereas, the highest concentration of SQV tested dramatically decreased GalC− and MBP- positive cells by 95%. Furthermore, the immunoblotting results corroborated the immunocytochemistry as key myelin proteins MBP and PLP expression werewere also decreased. Maturing OPCs treated with increasing concentrations of DRV or SQV showed no change in number of DAPI+ nuclei or A2B5+ cell counts. This is consistent with our well-established model as OPCs do not continue to proliferate in differentiation media (FEIGENSON *et al.* 2009; FEIGENSON *et al.* 2011). Furthermore, cultures treated with increasing concentrations of DRV or SQV displayed no increased TUNEL+ cells compared with vehicle-treated controls, indicating that OPCs do not have increased dsDNA breaks committing them to the apoptotic cell death cascade. To further rule out other cell death mechanisms, we stained cells with propidium iodide and

observed no increase in propidium iodide-positive+ cell counts compared with vehicle-treated controls. Thus, DRV or SQV-treated maturing OPCs are not dying in this *in vitro* model, and the lack of mature cells suggests that OPCs are inhibited from differentiating into mature OLs by these protease inhibitors.

These data provide novel, compelling evidence for DRV and SQV-induced oligodendrocyte maturation defects. Furthermore, these data add to previous numerous studies showing deleterious effects of PIs *in vitro,* peripherally and within the CNS (LAGATHU *et al.* 2007; ELLIS *et al.* 2008; CHANDRA *et al.* 2009; TOUZET AND PHILIPS 2010; MANDA *et al.* 2011; BRANDMANN *et al.* 2012; JENSEN *et al.* 2015; STERN *et al.* 2018). These studies could be helpful to clinicians when deciding which ARV drug to administer, especially in younger patients who are still developing myelin, and might be on ART for decades.

In addition to characterizing the effects of DRV and SQV on oligodendrocyte maturation, we identified a potential mechanism that mediates this effect. Both protease inhibitors significantly decreased the number of acidic lysosomes via lysotracker red staining, in both GalC+ and GalC− cells following the 72 hour differentiation period. Furthermore, we found that when co-treating maturing OPCs with DRV or SQV and the TRPML1 agonist ML-SA1, oligodendrocyte maturation was rescued fully at the 450 nM and 500 nM concentrations of DRV and SQV, respectively. When maturing OPCs were co-treated with the physiologically relevant concentrations of DRV and ML-SA1, oligodendrocyte maturation was partially rescued. These results suggest that these ARV agents de-acidify endolysosomes in maturing OPCs which in turn prevents OPC maturation into an OL and, to our knowledge, is the first study to demonstrate the critical role of proper endolysosomal pH in regulating OL differentiation. While it is possible that ML-SA1 could also be acting on the related ion channels, TRPML2 and TRPML3, RNA-Seq data from the CNS has shown that neither of these ion channels are highly expressed throughout the OL lineage (ZHANG *et al.* 2014). And we have not rule out the possibility that the release of ions such as Ca2+ from the endolysosomes due to TRPML1 activation has effects elsewhere in the cell that inhibit the damage caused by the PIs. These will be addressed in future studies.

While endolysosomes have long been recognized as degradative organelles, they are quickly emerging as important signaling hubs that integrate nutrient availability with specialized cellular functions (SETTEMBRE *et al.* 2013). Mutations in key lysosomal signaling molecules, RagA or Ragulator component Lamtor4, result in reduced myelination, which is rescued by inactivation of the transcription factor EB (TFEB), a master regulator of lysosomal biogenesis and autophagy (MEIRELES *et al.* 2018). Additionally, deficiencies in the mobilization of cholesterol from the endolysosome causes delayed myelination at early postnatal days or loss of myelin proteins in aged OLs (YU AND LIEBERMAN 2013). Thus, it seems likely that disruption to endolysosome function during differentiation would negatively impact OPC differentiation, though the downstream consequences of increased endolysosomal pH in OPCs are still unclear. Since OPC differentiation relies on the turnover and degradation of proteins which become obsolete at the mature OL stage, it is likely that the inability of the endolysosome to fuse with the autophagosome due to increased pH could interfere with macroautophagy, which is currently being investigated in our laboratory.

It is probable that other cellular mechanisms are also mediating effects of DRV and SQV, specifically the integrated stress response (ISR) and/or oxidative stress. Our group has shown evidence for ISR activation in neurons and astrocytes of HAND patients as well as in neurons in culture treated with different classes of ARV compounds (LINDL *et al.* 2007; AKAY *et al.* 2012; GANNON *et al.* 2017; STERN *et al.* 2018). Additionally, there is evidence for ISR activation in oligodendrocytes in other neuroinflammatory disease models such as multiple sclerosis (HUSSIEN *et al.* 2014; WAY AND POPKO 2016; ROMERO-RAMIREZ *et al.* 2017). Furthermore, we have previously shown that the PIs, ritonavir and saquinavir, and the NRTI, zidovudine (AZT), evoke the generation of reactive oxygen species (ROS) in primary rat cortical neurons leading to death (AKAY *et al.* 2014). This study reported that these ARV drugs induced the endogenous antioxidant response (EAR) and that pretreatment with monomethyl fumarate, an activator of the EAR pathway, partially blocked neuronal death (AKAY *et al.* 2014). We have also shown that ARV agents, ritonavir and zidovudine, induced ROS production in maturing OPCs, and that ROS are capable of halting oligodendrocyte maturation (FRENCH *et al.* 2009; REID *et al.* 2012; JENSEN *et al.* 2015). It is possible that these additional mechanisms are playing a role in DRV or SQV-driven inhibition of OL maturation concurrently or downstream of endolysosomal stress.

In summary, these data add to a growing body of evidence that suggest a role for ART-mediated persistence of HAND. Specifically, these data support a role for ART-mediated persistence of myelin abnormalities observed in HAND individuals. Furthermore, we implicate endolysosome de-acidification as a potential mediating mechanism in DRV or SQV-induced oligodendrocyte maturation defects. In addition to the development of new therapeutics with fewer harmful side effects, we must consider adjunctive therapies designed to alleviate neuronal dysfunction and preservation of myelin formation and maintenance.

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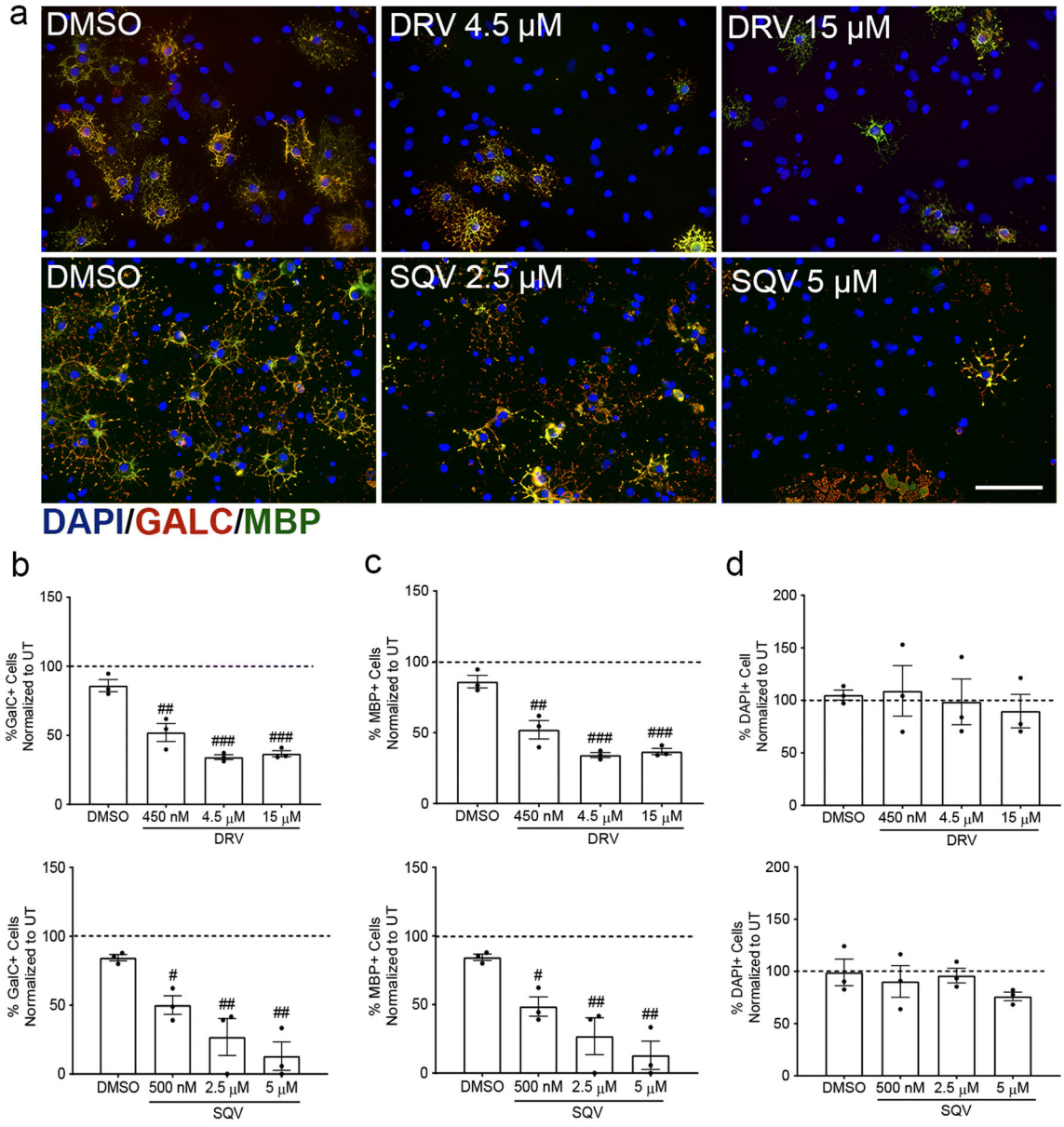

Fig. 1.

Saquinavir or darunavir treatment reduces oligodendrocyte maturation in a concentration-dependent manner. a) Representative photomicrographs of primary rat oligodendrocyte cultures co-treated with differentiation medium and either saquinavir or darunavir for 72 hour and stained for GalC, MBP and DAPI; scale bar = 50 μm. b) Quantification of immature oligodendrocytes (percent GalC-positive cells). c) Quantification of mature oligodendrocytes produced in this same paradigm (percent MBP-positive cells). d) Quantification of total cell number (DAPI-positive cells). The following average percent GalC+/DAPI+ or MBP+/DAPI+ for each group were typical for this well-established model: UT:19.97%; DMSO:17.73%; 450 nM DRV:10.64%; 4.5 μM; DRV:7.74%; 15 μM DRV:7.71%. UT:22.99%; DMSO:20.00%; 500 nM SQV:10.26%; 2.5 μM SQV:6.81%; 5 μM SQV:3.50%. Repeated Measures Analysis of Variance followed by Dunnett's *post hoc*. n =

3, independently prepared cultures. #p<0.05, ##p<0.01, ###p<0.001, compared with DMSO vehicle

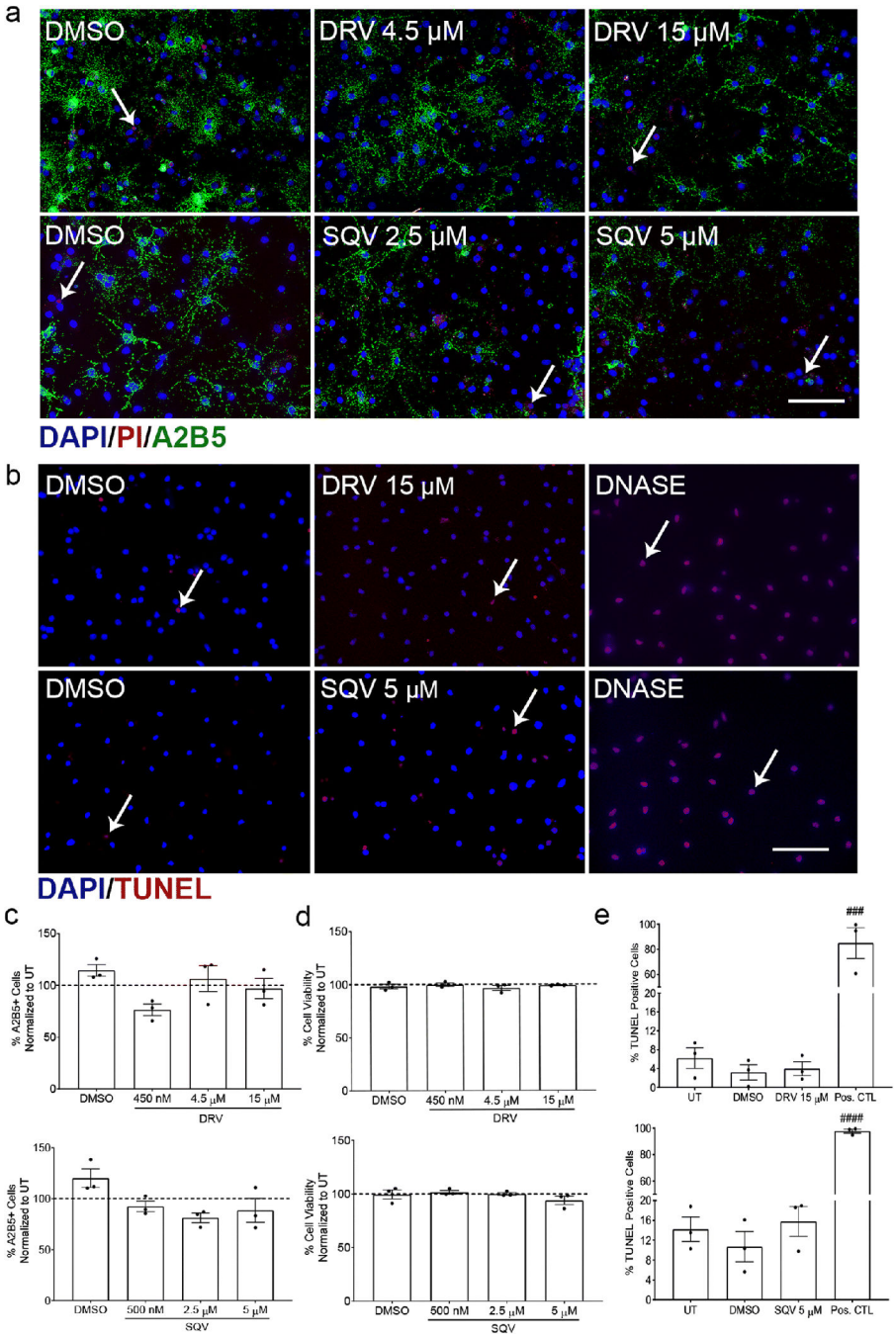

Fig. 2.

Neither saquinavir or darunavir alters OPC number or decreases viability. a) Representative photomicrographs of primary rat oligodendrocyte cultures co-treated with differentiation medium and either saquinavir or darunavir for 72 hour and stained for markers of OPCs (A2B5+), and viability (PI−); scale bar = 50 μm. b) Representative photomicrographs of primary rat oligodendrocyte cultures co-treated with differentiation medium and either saquinavir or darunavir for 72 hour and stained for dsDNA breaks, via the TUNEL assay, indicating apopotic cell death. c) Quantification of OPCs (percent A2B5-positive cells) d)

Quantification of cell viability (propidium iodide-negative cells). e) Quantification of TUNEL-positive cells. DNase was used as a positive control for the TUNEL assay. n = 3, independently prepared cultures. Repeated Measures Analysis of Variance followed by Dunnett's *post hoc* ###p<0.001, ####p<0.0001, compared with DMSO vehicle

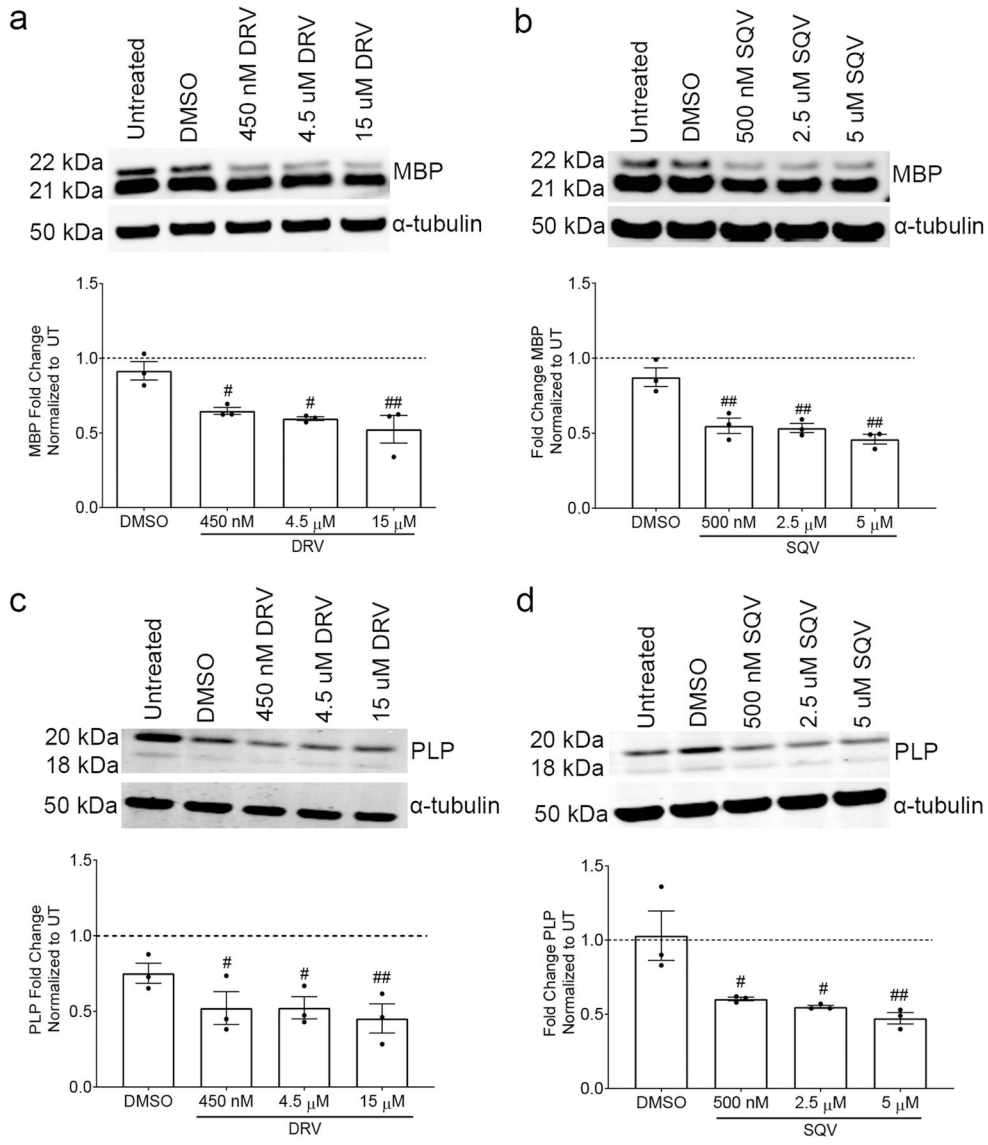

Fig. 3.

Saquinavir and darunavir reduce expression of myelin basic protein (MBP) and proteolipid protein (PLP). OPCs were treated with saquinavir or darunavir at the time of differentiation and harvested for protein after 72 hours. a) Representative western blot image for MBP and quantification of band intensities normalized to loading control, α-tubulin, and then normalized to untreated for oligodendrocyte cultures treated with darunavir. b) Representative western blot image for MBP and quantification of band intensities normalized to loading control, α-tubulin, and then normalized to untreated for cultures treated with saquinavir. c) Representative western blot image for PLP and quantification of band intensities normalized to loading control, α-tubulin, and then normalized to untreated for oligodendrocyte cultures treated with darunavir. d) Representative western blot image for PLP and quantification of band intensities normalized to loading control, α-tubulin, and then normalized to untreated for cultures treated with saquinavir. n = 3, independently prepared

cultures. Repeated Measures Analysis of Variance followed by Dunnett's *post hoc*. #p<0.05, ##p<0.01, compared with DMSO vehicle

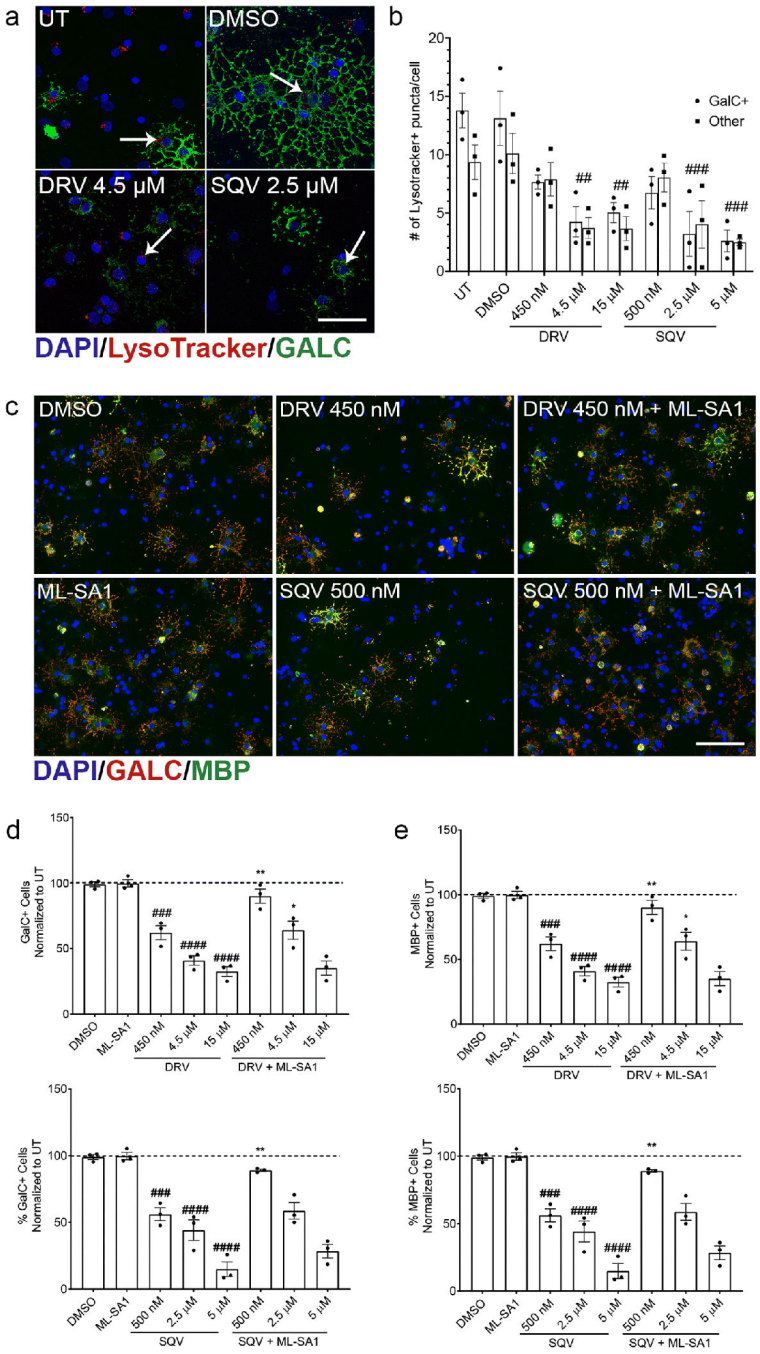

Fig. 4.

Darunavir and saquinavir significantly decrease the number of acidic lysosomes in maturing OPC cultures. TRMPL1 channel agonist, ML-SA1, partially rescues darunavir- and saquinavir-induced inhibition of oligodendrocyte differentiation.a) Representative photomicrographs of primary rat oligodendrocyte cultures treated with saquinavir or darunavir during an oligodendrocyte differentiation paradigm for 72 hours and labeled with lysotracker red and GalC; scale bar = 50 μm. b) Quantification of the number of acidic lysosomes in GalC-positive cells and non-Galc+ labeled cells. Two-way Repeated Measures

Analysis of Variance followed by Sidak's *post hoc*. n = 3, independently prepared cultures. ##p<0.01, ###p<0.001, compared with DMSO vehicle. c) Representative photomicrographs of primary rat oligodendrocyte cultures treated with saquinavir or darunavir during an oligodendrocyte differentiation paradigm and co-treated with ML-SA1 (20 μM) for 72 hours and labeled with GalC; scale bar = 50 μm. d) Quantification of immature oligodendrocytes (percent GalC-positive cells. e) Quantification of mature oligodendrocytes (percent MBP-positive cells). Repeated Measures Analysis of Variance followed by Sidak's *post hoc*. n = 3, independently prepared cultures. ###p<0.001, ####p<0.0001, compared with DMSO vehicle. *p<0.05, **p<0.01, compared with drug treatment