

NEUROTOXIC EFFECTS OF HIV-1 GP120 AND INTERACTION WITH
ALLOPREGNANOLONE

by

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ABSTRACT

KIMBERLY THORNTON: NEUROTOXIC EFFECTS OF HIV-1 GP120 AND INTERACTION WITH ALLOPREGNANOLONE

(Under the direction of Dr. Jason Paris)

With increasing incidence of HIV and its associated neurocognitive disorders, there has emerged a need for an antiviral with improved delivery to the CNS. In searching for drug candidates that meet these criteria, some have noted that certain steroid hormones like estrogen and progesterone that naturally accumulate in the CNS show neuroprotective effects in cells exposed to HIV proteins. This investigation focused on testing the potential protective effects of the progesterone metabolite, allopregnanolone (AlloP), against HIV protein gp120-mediated neurotoxicity. Experiments were conducted using SH-SY5Y neuroblastoma cells that were exposed to varying concentrations of AlloP (0-100 nM) in conjunction with one of three gp120 conditions (vehicle, X4 gp120 at 500pM, R5 gp120 at 500 pM). Once treated, plates were imaged at 24-hour intervals for 48 hours and assessed for proportion of cell death. Analysis of the results found that AlloP had no significant influence over cell death and that gp120 only induced a significant amount of cell death once deglycosylated. Further

investigation will be needed to assess the influence of AlloP and natural steroid products on the direct and indirect glial-mediated neurotoxic mechanisms of HIV-1 gp120.

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LIST OF ABBREVIATIONS

AlloP	Allopregnanolone
ANI	Asymptomatic Neurocognitive Impairment
ANOVA	Analysis of Variance
BDNF	Brain-Derived Neurotrophic Factor
cART	Combined Antiretroviral Therapy
CCR5	C-C Chemokine Receptor Type 5
CDC	Centers for Disease Control and Prevention
CD4	Cluster of Differentiation 4
CNS	Central Nervous System
CXCR4	C-X-C Chemokine Receptor Type 4
DMEM	Dulbecco's Modified Eagle Medium
gp120	Viral Envelope Glycoprotein 120
HAD	HIV-Associated Dementia
HAND	HIV-Associated Neurocognitive Disorders
HIV	Human Immunodeficiency Virus
MNI	Mild Neurocognitive Impairment

PNGase F	Peptide: N-glycosidase F
p53	Tumor Protein p53
RNA	Ribonucleic Acid
Tat	Trans-Activator of Transcription

1. INTRODUCTION

Despite recent advancements in care and treatment, Human Immunodeficiency Virus (HIV) continues to be a worldwide epidemic. Globally, 1.8 million new cases of HIV emerged in 2016, making the total number of recorded cases 36.7 million (CDC, 2018). Of these 36.7 million, 19.5 million were currently receiving medications to treat their HIV, varying cocktails of drugs known collectively as combined antiretroviral therapy (cART; CDC, 2018). These drugs work by inhibiting the replication of the virus to reduce the amount of HIV in the body (viral load) and, if effective, this “viral suppression” (defined as having less than 200 copies of HIV per mL of blood) should allow for continued immune system function and alleviation of symptoms (CDC, 2018). In spite of the widespread use of cART for the treatment of HIV, half of HIV positive individuals still experience HIV associated neurocognitive disorders (HAND; Saylor 2016).

HIV associated neurocognitive disorders (HAND) are the manifestation of HIV infection in the brain that generally result in cognitive, behavioral, motor, and autonomic disturbances (Eggers, et al., 2017). HAND is divided into the categories of asymptomatic neurocognitive impairment (ANI), mild neurocognitive impairment (MNI), and HIV-associated dementia (HAD). In its earlier stages, HAND can cause trouble with concentration, memory, and executive functions; as the disease progresses, however, HAND can begin to affect mood and motor functions (Eggers, et al, 2017). Before the advent of cART, it is estimated that 20-30% of HIV patients with uncontrolled HIV

developed HAND symptoms (Zayyad, et al., 2015). Despite the advancements in treatment that cART provides in the current era, because the prevalence of HAND increases over time following infection, it is currently estimated that 20-50% of HIV patients develop these disorders (Eggers, et al., 2017). While the chief impairments differ between the pre-cART (motor, cognitive, and verbal) and post-cART (memory and executive function) eras, high rates of mild impairment continue to persist through all stages of infection despite improved viral suppression due to cART (Heaton, et al., 2010). Though currently under investigation, it is suspected that these symptoms may be caused by the accumulation of potentially neurotoxic HIV proteins in the central nervous system (Zayyad, et al., 2015).

Among HIV proteins, gp120 is of core importance in the spread of the virus throughout the body. gp120 is a glycoprotein on the outer layer of the virus (viral envelope) that facilitates its entry into the host's immune cells (Yoon, et al., 2010). Equally as important in the virus's spread are two of the body's receptors that help facilitate viral entry: CXCR4 and CCR5. Though not the body's only co-receptors for HIV-1, CXCR4 and CCR5 appear to be the most predominant and have consequently been the most frequent targets of antiviral medications. Viral gp120 is typically dependent on only one of those receptors for entry, with CCR5-dependent strains of HIV-1 being responsible for most infections on the global scale and CXCR4-dependent strains being the dominant cause of Western infections (Vicenzi, et al., 2013). By binding to immune cell receptors CD4 and either CXCR4 or CCR5, gp120 facilitates virion fusion with the cell. Once infected, perivascular macrophages may cross the blood-brain barrier and shed their gp120 (which is non-covalently bound to gp41 on the outer viral

membrane; Hong, et al., 2015). This free gp120 can go on to activate an inflammatory response in microglia and astrocytes, alter mitochondrial dynamics in neurons, and cause direct toxic effects on astrocytes and neurons (Avdoshina, et al., 2016; Grovit-Ferbas, et al., 2010; Hong, et al., 2015; Podhaizer et al., 2012) As such, it is thought that gp120 may contribute to the development of HIV-associated cognitive dysfunction (Grovit-Ferbas, et al., 2010).

The problem of HIV-associated neurocognitive dysfunction is further exacerbated by the difficulty of efficiently delivering cART to the central nervous system (CNS). This occurs because many antiviral drugs used in cART are the substrates of brain-to-blood transporters like P-glycoprotein that pump substances out of the brain, blocking entry of most drugs (Hong, et al., 2015; McRae, 2016). HIV proteins like gp120, through their inflammation of neural tissue, increase the activity of these transporters, thus decreasing the ability of antiretroviral drugs to accumulate in the CNS (Hong, et al., 2015). The lack of therapeutics accumulating in this area leads to increased viral load and immune activation in the CNS despite suppression of blood plasma HIV RNA (Chan, et al., 2016; Hong, et al., 2015). In laboratory tests, patients with suppression of plasma HIV RNA still showed microglial activation, the extent of which was found to be associated with worse performance on cognitive functioning tests (Chan, et al., 2016). These facts point to the need for an adjunctive therapeutic that will accumulate in the CNS and can protect the brain.

Various steroid hormones have been assessed for their neuroprotective effects on gp120 toxicity in culture. In general, estrogens, progestins, and androgens all exert neuroprotective effects in the form of promoting anti-apoptotic factors, upregulating

antioxidant enzymes, and downregulating inflammatory factors (Diotel, et al., 2018). Specifically, testosterone (an androgen) and its related molecules have been shown have neuroprotective effects under low-energy and apoptotic conditions when present while their absence has been linked to oxidative damage under stress conditions (Zarate, et al., 2017). Estrogen, whose receptors are widely distributed in the brain, often acts as a regulatory molecule in various processes but can also shield neurons from damage caused by reactive oxygen species (Brooke, et al., 2002; Zarate, et al., 2017). Like estrogen, progesterone and its metabolites also serve as regulatory and proliferation-inducing molecules in the brain naturally but have been shown to exert beneficial effects in animal models of neurodegeneration and brain damage (Zarate, et al., 2017). Particularly, progesterone and its reduced metabolite, allopregnanolone, have shown promise in reducing inflammation, including that associated with HIV infection, in the nervous system (Zarate, et al., 2017).

Production of progesterone and its metabolites are thought to be part of the nervous system's natural response to damage; the metabolite allopregnanolone (AlloP) in particular has been widely recognized for its neuroprotective effects, which, unlike progesterone, also extends to reduction of excitotoxicity, another product of HIV infection in neurons that likely contributes to its neurodegenerative effects (Guennoun, et al., 2015; Paris, et al., 2016). While AlloP has already been found to reduce calcium influx-induced neuronal cell death associated the HIV protein Tat and both estrogen and progesterone have been found to have somewhat protective effects in response to both Tat and gp120, AlloP has yet to be tested for its efficacy as a protective agent against

gp120-induced cell death, also thought to be partially the result of calcium influx (Paris, et al., 2016).

In this investigation, we hypothesized that gp120 would be neurotoxic to SH-SY5Y cells that were differentiated to a dopaminergic-like phenotype and that allopregnanolone would attenuate gp120-mediated cell death.

2. MATERIALS AND METHODS

2.1 - Cell Culture

SH-SY5Y human neuroblastoma cells were obtained from ATCC (#CRL-2266, Manassas, VA) and cultured in 89.5% Ham's DMEM/F-12 medium (#11320-033, Thermo Fisher Scientific, Waltham, MA), 10% fetal bovine serum (#SH30071.03, Thermo Fisher Scientific), and 0.5% PSF (Penicillin, Streptomycin, Amphotericin B; #15240-062, Thermo Fisher Scientific). Cells were incubated (37°C, 5% CO₂) and media was replaced every 2-3 days until ready for differentiation at ~80% confluency.

2.2 - Differentiation

Six 24-well plates were seeded at 100,000 cells/well with DMEM/F-12 medium. After 24 hours, Differentiation Media #1, prepared from 50mG Retinoic Acid (#D2650-100ML, Sigma-Aldrich, Saint Louis, MO) and 33.3mL of 95% Ethanol (#793183, Sigma-Aldrich), was added to each well at a 1:500 ratio with growth media. After 5 days, Differentiation Media #2, prepared from 10µG of BDNF (#SRP3014-10UG, Sigma-Aldrich) and 1mL of Ham's DMEM/F12, was added to each well at a 1:200 working dilution in growth media supplemented with 0.5mL of PSF. 3-4 days later, the plates were tested/imaged. Throughout differentiation, media was exchanged every 48 hours.

2.3 – Chemicals

Allopregnanolone, obtained from Sigma-Aldrich (# P8887, St. Louis, MO), was dissolved in DMSO and diluted to concentration in media (final DMSO concentration, 1:10,000). X4_{III}B gp120 (#1041) and R5_{ADA} gp120 (#1081) were obtained from ImmunoDx (Woburn, MA) and diluted to concentration in ddH₂O.

2.4 – GP120 Deglycosylation

In trials with deglycosylated gp-120, PNGase F was used to remove N-glycans from gp120. The gp120 was incubated with PNGase F (600 U / 10 µg protein) in 1x glycobuffer at 37°C for 24 hours per manufacturer's protocol (New England Biolabs, Ipswich, MA).

2.5 - Treatment

For the nondeglycosylated experiments, each row of wells was treated with 5µL of either 0nM (DMSO vehicle), 1nM, 10nM or 100nM allopregnanolone. Every two columns were treated with 2.5µL of either distilled water, 500pM X4 gp120, or 500pM R5 gp120. In the trials with deglycosylated gp120, separate plates were used to test the X4 and R5 variants. Every pair of wells was treated with a different concentration of gp120 (vehicle, 0.1pM, 1pM, 10pM, 100pM, 1000pM) and one well in each pair was treated with either 10nM allopregnanolone or the vehicle (DMSO). Treatment chemicals were added immediately prior to first imaging.

2.6 - Imaging and Assay

A live/dead assay was conducted following treatment. Hoechst (blue) and propidium iodide (red) dyes were mixed with new growth media at a ratio of 1 drop per mL and added to the wells on the first day of imaging. Following initial treatment, plates were imaged every 24 hours over a span of 48 hours. The numbers of total cells and dead cells for each well at each time period were counted using the images produced, and percent cell death was calculated.

2.7 - Statistical Analysis

Separate two-way analyses of variance (ANOVA) were used to assess the effects of gp120 and allopregnanolone conditions on percent cell death and percent change at 24 hours. Fisher's Protected Least Significant Difference *post hoc* tests determined group differences following main effects. Analyses were considered significant when $p < 0.05$.

3. RESULTS

3.1 - Finding 1: Irrespective of GP120, Cell Death Increased Over 48 Hours

SH-SY5Y cells incubated with propidium iodide and Hoeschst demonstrated significant cell death over 48 hours, irrespective of gp120 exposure [$F(2,140) = 626.68$, $p < 0.05$] (Fig. 1). The proportion of dead cells at 24 and 48 hours significantly differed from every other time-point ($p < 0.0001$ vs. all time-points; Fig. 1).

3.2 – Finding 2: AlloP Did Not Interact with GP120 to Influence Cell Death

Given that incubation with live/dead dyes induced cell death irrespective of gp120 treatment, the 24-hour time-point was considered independently in order to assess the potentially-protective effects of AlloP. Neither gp120 (X4 or R5) nor AlloP (0-100 nM) significantly influenced the proportion of cell death (Fig. 2) or the percent change in death from control (Fig.3). Despite this, there was an apparent influence of AlloP (1 or 10 nM) to reduce the proportion of cell death in non-treated SH-SY5Y cells (Fig. 2A, 3A) and in those exposed to X4 gp120 (Fig. 2B, 3B); however, this did not reach statistical significance.

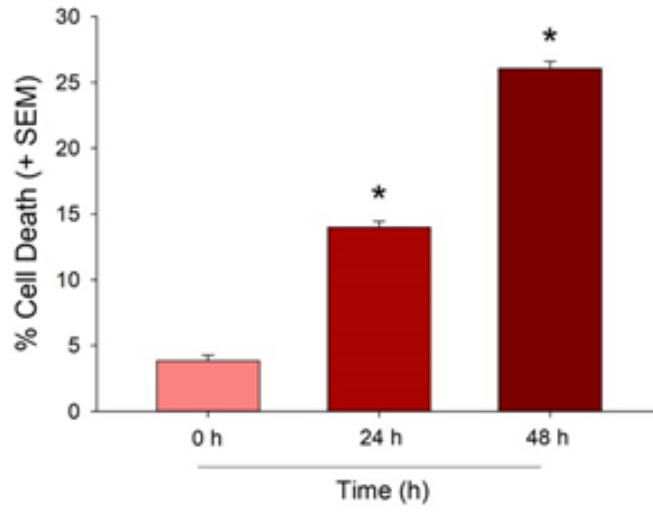


Figure 1: Total percent cell death over a time period of 48 hours. An asterisk (*) denotes statistical significance when compared to control.

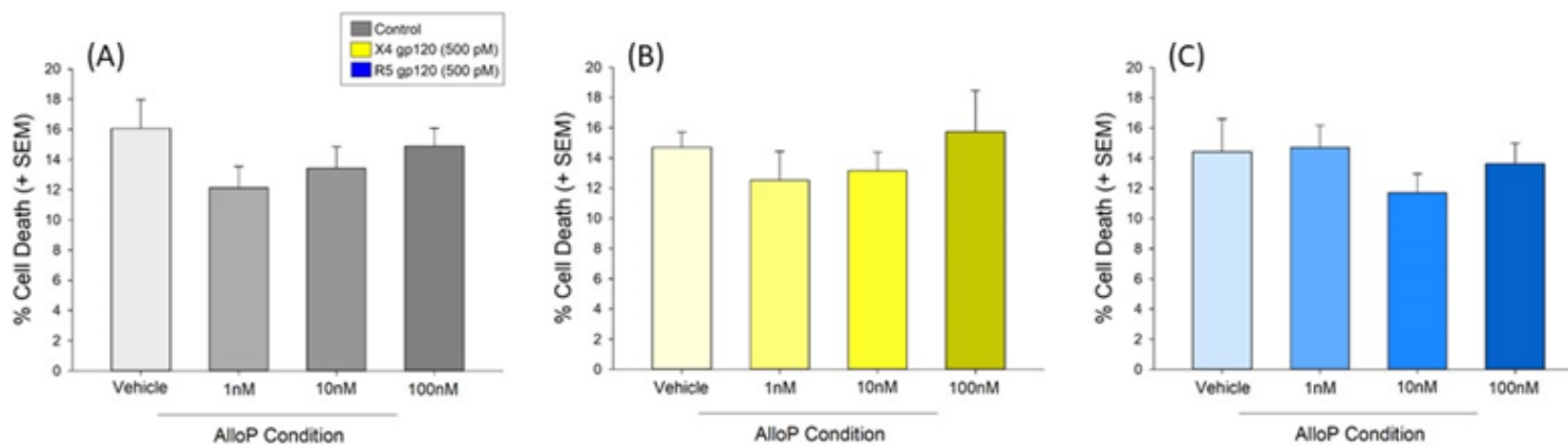


Figure 2: Percent cell death at differing concentrations of AlloP for cells exposed to gp120 control (2A), X4 gp120 (2B), R5 gp120 (2C).

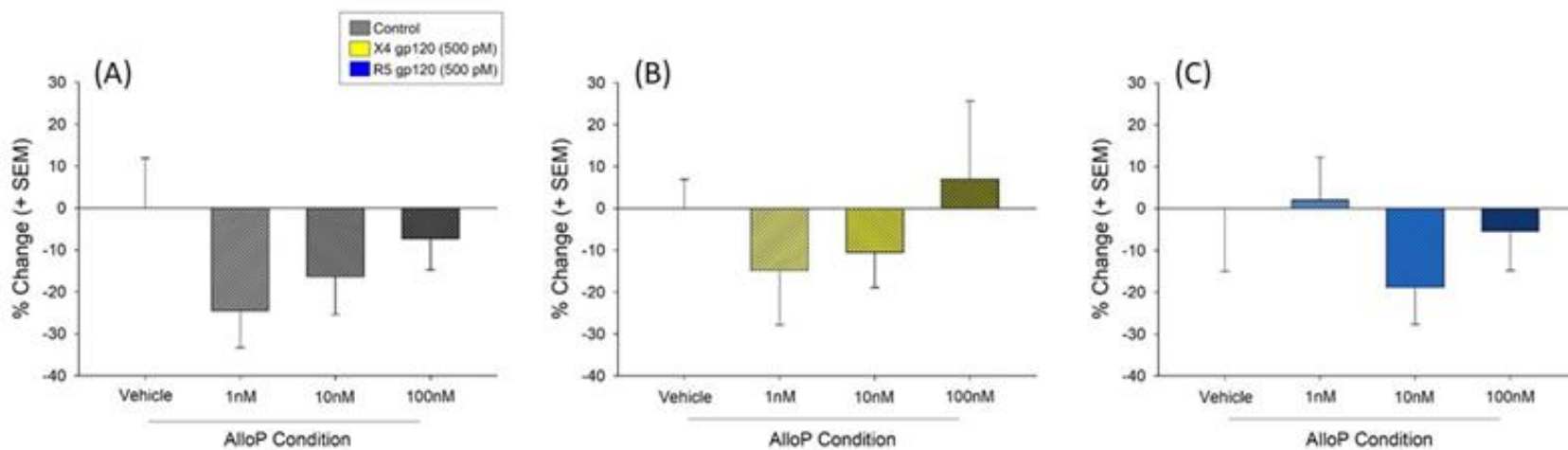


Figure 3: Percent change in cell death from vehicle at differing concentrations of AlloP for cells exposed to gp120 control (3A), X4 gp120 (3B), and R5 gp120 (3C).

3.3 – Finding 3: Deglycosylating GP120 Increased Neurotoxicity

The lack of a significant relationship between AlloP and reduction in cell death led to the consideration of gp120's glycosylation as potential confounding factor and the subsequent testing of deglycosylated gp120 with AlloP. Analysis of aggregate data from both X4 and R5 gp120 found that treatment of SH-SY5Y cells with gp120 led to statistically significant increases in neuronal cell death [$F(5,156) = 2.401, p < 0.05$], with wells containing 1pM and 10pM gp120 exhibiting significantly greater cell death than control ($p = 0.0041$ and $p = 0.0333$, respectively; Fig. 4). Inspection of X4 and R5 data separately hints that X4 gp120 (Fig. 4A) and R5 gp120 (Fig. 4B) may reach peak toxicity at differing concentrations of 1pM and 10pM, respectively, though this finding was not considered statistically significant.

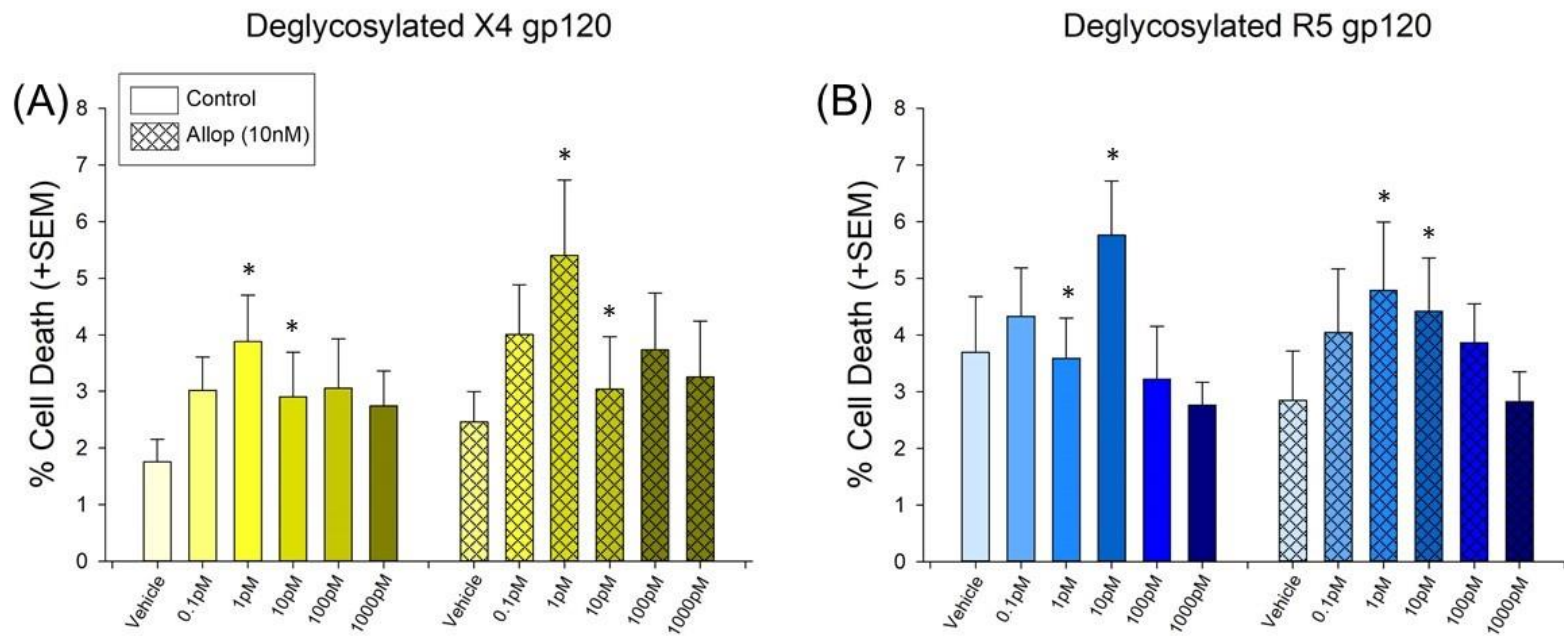


Figure 4: Percent cell death in cells exposed to varying concentrations of X4 gp120 (4A) and R5 gp120 (B) with or without exposure to 10nM AlloP. An Asterisk (*) signifies statistical significance from control.

4. DISCUSSION

The hypotheses that gp120 would be neurotoxic to dopaminergic SH-SY5Y cells and that allopregnanolone would attenuate gp120-mediated cell death were upheld and refuted, respectively. While plates exhibited significant cell death over 48 hours, neither gp120 (X4 or R5) nor AlloP influenced the proportion of cell death or percent change in death from control to a statistically significant degree. In the deglycosylation trials, however, gp120 (X4 and R5, 1 or 10pM) caused significant increases in neuronal cell death over control which was not influenced by AlloP.

The initial findings conflicted with a sizeable body of prior works stating that gp120 is toxic in neural cells. Previous findings indicate that the presence of gp120 leads to neuronal atrophy through proapoptotic pathways and processes commonly associated with mitochondrial impairment, particularly oxidative stress, calcium homeostasis disruption, and proapoptotic transcription factor p53 activation (Avdoshina, et al., 2016; Bardi, et al., 2006). Also notable is gp120's potential to exhibit neurotoxicity by binding tightly to neuronal-specific tubulin in the axon and interfering with the intracellular transport system (Avdoshina, et al., 2017). This is to say nothing of purported indirect neurotoxic effects of gp120, such as increased permeability of the blood-brain barrier and endothelial cells to toxins and upregulation of pro-inflammatory cytokines in glial cells, that could not be accounted for in this investigation because of the lack of glial and other tissues in the SH-SY5Y model (Chen, et al., 2016; Rozzi, et al., 2017). This surprise of

gp120's apparent lack of neurotoxicity in the face of numerous previous studies that had concluded to the contrary led us to investigate whether the glycosylation of gp120 had an effect on its ability to induce apoptosis. We hypothesized that perhaps by removing bulky sugar moieties attached to certain amino acids in the gp120 protein, shown to provide resistance to antibody-mediated neutralization and other immune mechanisms that would not be factors in the SH-SY5Y model, gp120 would show increased toxicity due to less steric hinderance in binding to cell receptors (Gram, et al., 2002; Koch, et al., 2003). Consistent with previous findings that certain deglycosylated mutants of gp120 displayed greater infectivity, we found that deglycosylated gp120 did result in an increase in cell death in a concentration-dependent manner (Huang, et al., 2011).

Though not considered significant in this study, steroid hormones such as estradiol, progesterone, and its metabolite AlloP have all exhibited neuroprotective qualities when exposed to various HIV-1 proteins in numerous prior investigations. While estrogens have been shown to attenuate gp120- and Tat-induced oxidative stress and progesterone has been shown to exert protective effects in response to both gp120 and Tat, AlloP has only been found to demonstrate neuroprotective effects against HIV-1 Tat in investigations conducted thus far (Paris, et al., 2014; Paris, et al., 2016; Wallace, et al., 2006). Because previous studies indicated that AlloP attenuated Tat-mediated calcium influx, one of the protein's means of inducing apoptosis, in primary mouse neurons, it was believed that AlloP could be effective against gp120 toxicity, also thought to be partially the result of calcium influx (Paris, et al., 2016).

Perhaps AlloP's failure to demonstrate significant neuroprotective effects against gp120 in this experiment should not be considered too much of a surprise. The study that

prompted further investigation of AlloP's potential protection against gp120 toxicity with its finding that AlloP reduces Tat-induced calcium influx only reported partially attenuated calcium influx following treatment with AlloP (Paris, et al., 2016). Additionally, the current study's choice of the SH-SY5Y model (human neuroblastoma cells) for investigation differed from that of the prior findings it was prompted by (mouse co-cultured neurons and mixed glia; Paris, et al., 2016). This choice of the SH-SY5Y model also resulted in the loss of any potential neuroprotective effects of AlloP conveyed by glial cells, which have been demonstrated to contribute significantly to the steroid-induced decrease of gp120 neurotoxicity, at least in studies with estrogen (Zemlyak, et al., 2005).

While the present work is of interest, it must be considered with some caveats, particularly in regards to model selection and assay methods and materials. Were this experiment to be repeated, we would recommend that the model neuron cultures include glial cells in order to examine the possible neuroprotective effects conveyed by glia. Additionally, the use of either different diagnostic criteria or different assay methods or materials would be recommended; the dyes used in this experiment, Hoechst and propidium iodide, have been noted in prior cases to possibly impact cell viability, with Hoechst possibly inhibiting topoisomerase I activity and high levels of iodide being linked to apoptosis in SH-SY5Y cells (Zhang, et al., 1999; Zhang, et al., 2017). Use of propidium iodide in apoptosis assays is also known to result in a significant number of false positives that could have prevented a truly accurate assessment of cell death (Rieger, et al. 2010).

In conclusion, exposure to deglycosylated X4/R5 gp120 resulted in a significant increase in the proportion of cell death in SH-SY5Y neuroblastoma cells and exogenous administration of AlloP did not attenuate these effects. Given that AlloP-mediated neuroprotection may involve glial-neuronal interactions, investigations using timelapse microscopy and primary models of neurons and mixed glia are required in order to further assess its potential protective effects over gp120-mediated neurotoxicity.

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