Using a simulated blood-brain barrier to investigate potential modulators of HIV-1-associated neuro-inflammatory processes in vitro

ABSTRACT:
Background
Neuroinflammation is a central feature of HIV-1 infection and HIV-1 Associated Neurocognitive Disorders. Standard anti-retroviral (ARV) and accompanying therapies are limited in their capacity to limit neuro-inflammation. Thus, it is of importance to expand the search for potential anti-inflammatory adjuvant therapies to include natural products. The use of complimentary/natural medicines within the HIV+ population is widespread, especially in developing countries. We have identified a new candidate complimentary medicine for use in this context. Grape seed-derived Proanthocyanidolic Oligomers (PCO) have significant anti-inflammatory action in the peripheral compartment in the context of e.g. skeletal muscle injury, but have not been investigated in the context of either neuro-inflammation or HIV/AIDS. Here its efficacy as an anti-inflammatory modality in this context was investigated in an in vitro co-culture model simulating the Blood Brain Barrier (BBB).

Methods
Single cultures of human astrocytes, HUVECs and primary human monocytes, as well as co-cultures (BBB), were stimulated with HIV-1 subtype B & C Tat protein and/or HL2/3 cell secretory proteins after pre-treatment with PCO extract. Effects of this pre-treatment on pro-inflammatory cytokine secretion and capacity for monocyte migration across the simulated BBB were assessed.

Results
In accordance with the existing literature, B Tat protein was more pro-inflammatory than C Tat protein, validating the accuracy of our model. PCO pre-treatment resulted in a significantly dampened IL-1β (P<0.0001) response to the stimulation with HIV-associated proteins, as well as a modulated monocyte chemo-attractant protein-1 (P<0.0001) response – which is a major role player in HIV-associated neuro-inflammation – and decreased capacity for CD14+ monocytes to migrate across the simulated BBB (P<0.0001). Additionally, PCO pre-treatment decreased both GFAP (P<0.001) and HSP-27 (P<0.001) expression in the astrocytes of the BBB. Conclusions
Novel data presented here illustrate that PCO is able to blunt the MCP-1 and IL-1β response to HIV-1 proteins in single cultures of human astrocytes and HUVECs, as well as in an in vitro simulation of the BBB. In addition, PCO was able to limit monocyte transmigration across the simulated BBB in response to HIV-1 proteins generated by HL2/3 cells. This suggests that grape seed-derived PCO should be considered as a complimentary anti-neuroinflammatory drug in the context of HIV/AIDS.

Keywords:
monocyte, migration, inflammation, proanthocyanidin, HL2/3, Blood-brain barrier, Grape seed extract, polyphenol, MCP-1
INTRODUCTION

Neuro-inflammation plays a key role in the neuropathogenesis associated with the Human Immunodeficiency Virus type 1 (HIV-1), the causative agent of Acquired Immune Deficiency Syndrome (AIDS). The virus establishes itself within the Central Nervous System (CNS) soon after an individual becomes infected (Yao et al., 2010), initiating an inflammatory cascade which eventually results in the development of a broad spectrum of neurological disease states collectively referred to as HIV-1 Associated Neurocognitive Disorders (HAND). Data presented in a recent multi-centre cohort study describe persistently high rates of neurocognitive impairment at all stages of HIV-1 infection irrespective of whether a patient is on modern anti-retroviral treatment and immune reconstitution regimes (Heaton et al., 2011). Thus it is evident that an early preventative approach to limiting the extent of neuroinflammation, is essential to positively influence the longer-term prognosis in terms of not only HAND, but also HIV-1 disease progression.

The utility of standard Anti Retro Viral (ARV) treatments in directly preventing HAND is limited. Apart from the fact that they were not intended or designed for this purpose, their relative ineffectiveness in this context is mainly due to two reasons: firstly, they are administered relatively late in the context of neuro-inflammatory changes associated with HIV-1 infection and secondly, different ARV drugs differ in their ability to cross the Blood Brain Barrier (BBB) and penetrate the CNS effectively (Strazielle and Ghersi-Egea 2005). While some ARV drugs are able to do so and reduce viral replication - reducing neuro-inflammation in an indirect manner - these may be associated with adverse side effects such as neurotoxicity (Cavalcante et al., 2010). Thus, although certain classes of ARV drugs are very effective in reducing viral load within the CNS, the consequent inflammation associated with CNS infection cannot be effectively treated with ARV drugs alone. Additionally, astrocytes, which are non-productively infected with the virus, still shed a number of neurotoxic viral proteins e.g. Tat, Rev, and Nef, without infectious virion production (Williams et al. 2009). ARV therapy does not target these accessory proteins, and therefore is not able to reduce their neurotoxic/neuroinflammatory effects. As a result, much attention has been given to the identification of possible adjuvant therapies that can either be administered concurrently with ARV drugs or given much earlier, before ARV roll-out. For example, chloroquine, simvastatin and minocycline has recently been shown to attenuate HIV-1 glycoprotein 120 (gp120) -mediated brain inflammation by modulating the interleukin-1β ( IL-1β) and inducible nitric oxide synthase (iNOS) responses in an in vivo rat model (Ashraf et al. 2014). However, more widespread use of some of these pharmaceutical preparations may result in drug resistance in the context of their original application – e.g. exacerbating drug-resistant malaria – so that not all of these are ideal solutions to the problem. It is therefore warranted that not only conventional Western medicine is considered to address the problem of HIV-associated neuroinflammation, but also traditional therapies. The use of complementary medicines especially within the HIV+ population is widespread (Mills et al., 2005; Morris 2001) especially in developing countries (Smith and van Vuuren 2014) although few have been investigated for efficacy of these therapies specifically in the context of neuro-inflammation, while others have been shown to be potentially detrimental in this context. We have identified a commercially available plant-derived product which may have the potential to alleviate both the chronic systemic inflammation and neuroinflammation associated with HIV infection. Grape seed-derived Proatho Cyanidolic Oligomers (PCO) comprised of catechin or epicatechin monomers, have been shown in a rat model of skeletal muscle injury, to improve antioxidant status and modulating inflammatory cytokine responses, in both tissue and circulatory compartments.
In the same model, PCO supplementation was shown to substantially reduce neutrophil infiltration into injured muscle and to facilitate an earlier switch in monocyte phenotype from a pro- to an anti-inflammatory phenotype, which was associated with faster resolution of inflammation and faster tissue regeneration (Kruger and Smith 2012; Kruger et al., 2014). Additional beneficial effects within the CNS that have been reported for PCO include protection against 12-O-Tetradecanoyl Phorbol-13-Acetate (TPA) induced lipid peroxidation and DNA fragmentation in the brain (Bagchi et al., 1998), reduction in brain oxidative stress in adult and middle-aged rats as well as inhibition of peritoneal macrophages (Devi et al., 2011) and protection of primary glial cells against nitrosative/oxidative stress (Roychowdhury et al., 2001). An additional benefit of the in vivo studies mentioned above, as well as a number of other in vivo studies and toxicity studies (Bagchi et al., 2000; Bagchi et al., 2002; Kim et al., 2006) is that they provide proof that PCO indeed crosses the blood-brain barrier – an important consideration in the research approach, since many anti-inflammatory/antioxidant compounds are limited in their therapeutic use for the very reason that they do not cross the BBB (Gilgun-Sherki et al., 2001).

Indeed, more evidence of beneficial effects of PCO on inflammatory processes can be found in mechanistic studies, albeit not in the context of HIV-neuroinflammation specifically. For example, PCO inhibited the Mitogen-Activated Protein Kinase (MAPK) pathway’s extracellular signal-related kinase 1/2 (ERK1/2), c-Jun N-terminal Kinases (JNK) and p38 kinases, which are involved in the generation of an inflammatory response (Kim et al., 2011). Also PCO was reported to inhibit the activation of caspase-1 (also known as interleukin-1 converting enzyme) (Zhang et al., 2013).

The above mentioned effects on signalling pathways and in vivo processes – although not assessed in the context of HIV-associated neuroinflammation - are all relevant to inflammation and thus HIV-associated neuroinflammation (Yao et al., 2010), (Bethel-Brown et al., 2011; Yadav et al., 2010; Walsh et al., 2014), suggesting potential benefits for PCO in this context. Furthermore, both in vivo toxicity studies (Bentivegna and Whitney 2002) and in vitro cytotoxicity studies (Bagchi et al., 2002) have proven PCO consumption to be safe, with no reports of adverse effects.

Therefore, the current study aimed to investigate the efficacy of PCO as an anti-neuroinflammatory therapy, utilizing individual human cell lines as well as an in vitro simulation of the BBB. In addition to investigating the effect of PCO pre-treatment on inflammatory cytokine responses to HIV-associated proteins, the ability of PCO to limit HIV-1-induced monocyte transmigration across the simulated BBB was assessed. To our knowledge we are the first to report significant anti-inflammatory effects of PCO in the context of HIV-associated neuroinflammation and elucidate potential mechanisms by which this is achieved.

MATERIALS AND METHODS

Materials

Full length synthetic HIV-1 Trans-activator of transcription (Tat) (derived from clinical isolates of HIV-1 subtype B [GenBank: M93258.1] and C [GenBank: FJ765005] proteins were synthesized and purified as previously described (Siddappa et al., 2006) and provided by Professor Ranga Udaykumar of the Jawaharlal Nehru Centre for Advanced Scientific Research (Bangalore, India). Legend MAX ELISA kits to detect monocyte chemoattractant protein-1 (MCP-1) in cell culture supernatants and FITC-conjugated anti-human CD14 were purchased from Biolegend (USA), and Alpha LISA kits to assess IL-1β secretion were purchased from Perkin Elmer (Waltham, MA, USA).
dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide for the MTT assay was purchased from Sigma-Aldrich (South Africa). Commercially available grape seed-derived PCO (OxiprovinTM) was obtained from Brenn-O-Kem Pty Ltd (Wolseley, South Africa). Grape seed-derived PCO aqueous solution was prepared by dissolving a commercially available PCO powder in warm distilled water (1 mg/ml) and then sterile filtered using a filter of pore size 0.22 µm.

Cell culture

Single cultures of primary human cerebrocortical astrocytes (Sciencell, USA) and Human Umbilical Vein Endothelial Cells (HUVECs) (Lonza, Germany) were maintained at 37°C in a humidified 5% CO₂ in high glucose Dulbecco’s modified eagle’s medium (DMEM) (Gibco, Life Technologies Corp., USA) supplemented with 10% Foetal Calf Serum (FCS) (Biochrom, Germany), with added 1% N2 Supplement (Gibco, Life Technologies Corp., USA) and complete Endothelial Growth Medium (EGM) (Lonza, Germany) for astrocytes and HUVECs respectively.

HL2/3 cells are HeLa derived cells producing high levels of Gag, Env, Tat, Rev and Nef proteins and were obtained through the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH: HL2/3 from Dr. Barbara K. Felber and Dr. George N. Pavlakis). HL2/3 cells were maintained in high glucose DMEM (Life Technologies Corp., USA) supplemented with only 10% FCS (Biochrom, Germany). Cells were routinely subcultured before reaching confluence and all experiments were conducted on low passage counts.

Cell numbers were determined using a haemocytometer following trypsinization and trypan blue staining. For all single culture experiments and MTT assays, all cell types were seeded in 6-well cell culture plates at a density of 500000 cells per well.

In order to simulate the blood-brain barrier, co-cultures of human astrocytes and HUVECs were established on opposite sides of fibronectin (BD Biosciences, USA) coated 3 µm pore size tissue culture inserts (BD Biosciences, USA), using the method described earlier (Eugenin and Berman 2003).

All cell culture experiments were done in triplicate and repeated a minimum of three times.

PCO Dose Response Cell Viability Assay

Considering that our specific PCO compound had not been tested on the cell types used in this study before, the optimal dose for in vitro use – defined as the highest dosage which does not result in a significant reduction in cell viability as assessed by % MTT reductive capacity – was determined. Briefly, HUVECs and primary human monocytes (selected from human PBMCs by adherence) were incubated with 50, 100 and 200µg/ml PCO extract for 1, 2, 3, 4 and 24 hours. At the end of the incubation period, the medium was removed from the 6 well plates and the cells were washed twice with PBS. Cell viability was assessed using a modified version of the MTT assay described by Gomez and colleagues (Gomez et al., 1997). MTT (0.01 g/ml) was dissolved in PBS, and 500 µl was added to each well dish. Cells were subsequently incubated for 1 h at 37°C in an atmosphere of 5% CO₂. After the incubation period, cells were washed twice with PBS, and 1 ml of HCl–isopropanol–Triton (1% HCl in isopropanol; 0.1% Triton X- 100; 50:1) added to each well and gently agitated for 5 min. This lysed the cell membranes and liberated the formazan pigment. The suspension was then centrifuged at 131 x g for 2 min. The optical density (OD) was determined spectrophotometrically at a wavelength of 540nm and the values expressed as percentages of control.

Full length HIV-1 subtype B & C Tat protein stimulation

Tat proteins were reconstituted in Tris-Cl buffer (20mM, pH8) and diluted supplemented with 1 mM DTT. Human astrocytes, HUVECs, primary human monocytes and co-cultures were pre-treated for 4h and 24h respectively prior to HIV-1 Tat protein stimulation.
After pre-treatment, all cells were stimulated with B Tat and C Tat (in separate wells) (10 ng/ml) for 2.5h and 24h, after which culture supernatants was collected and stored at -80°C for subsequent batch analyses. Appropriate controls were included for all conditions and treatments.

**HL2/3 cell stimulation**

Firstly, HL2/3 cell conditioned media was prepared: HL2/3 cells were seeded into six well plates at 200 000 cells per well and allowed to adhere to the culture surface. Once the HL2/3 cells had adhered, culture media was refreshed. HL2/3 conditioned media was collected at 2.5h and 24h from separate cultures. Secondly, the method described above for B and C Tat stimulation with and without PCO pre-treatment was then repeated, using HL2/3 cell conditioned media collected at 2.5 and 24 h as inflammatory stimulus instead of added B or C Tat.

This experiment was repeated in the co-culture system, but only with a final 4h incubation – the 24h incubation was omitted due to lowered HUVEC cell viability at this time point in the single culture experiments (refer to results). Briefly, HL2/3 cells were seeded into 24 well plates at 50 000 cells/well and allowed to adhere. Simultaneously, BBB cultures were treated with PCO for 4h prior to stimulation. Culture media was then refreshed, after which the BBB co-culture inserts were transferred to the wells containing the adherent HL2/3 cells. BBB co-cultures were exposed to the HL2/3 cells for a period of 2.5h, after which culture supernatants were collected and stored at -80°C until subsequent batch analyses.

**Pro-inflammatory cytokine & chemokine analysis**

MCP-1 concentration was measured in all supernatants by a conventional ELISA kit (Biolegend, USA), used according to the manufacturer’s instructions. IL-1β concentration was measured in all co-culture supernatants by AlphaLISA (PerkinElmer, Waltham, MA, USA) according to the manufacturer’s instructions.

**Assessment of in vitro monocyte migration capacity**

Monocyte transmigration was assessed in the BBB co-cultures by adding human PBMCs (3x10⁵ cells) to the top of the insert, allowing the cells to migrate in response to the various stimuli (i.e. HL2/3 cell products, of which some are well-known chemotactic agents (Albini et al., 1998) for 2.5h, after which the BBB inserts and cells in the bottom of the well were fixed in 4% paraformaldehyde and stained with a FITC-anti-human CD14 antibody, which is a monocyte specific marker. All CD14⁺ monocytes on top of the entire insert (unmigrated) and on the bottom of the culture well (migrated) were counted using a fluorescent microscope (Leica, Germany). The ability of PCO to modulate this response was investigated by again pre-treating BBB co-cultures for 4h prior to stimulation. Cells in suspension were not quantified, since we have previously shown that cell counts in these compartments are a constant for the model and independent of interventions/treatments (Kruger et al., 2014).

**Immunocytochemistry**

Additional membranes containing the BBB cells (i.e. treated the same as the migration assay inserts but not the same ones that were stained for CD14) were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Membranes were simultaneously incubated with an appropriate dilution (1:10000) of a chicken polyclonal antibody to Glial Fibrillary Acidic Protein (GFAP) (Abcam, UK) and a mouse monoclonal antibody to Heat Shock Protein 27 (HSP-27) (Abcam, UK) overnight at 4°C, followed by 5 washes with Phosphate Buffered Saline (PBS). Cells were then incubated simultaneously with anti-chicken FITC (Abcam, UK) and anti-mouse Pacific Blue (Molecular Probes, Life Technologies Corp., USA) secondary antibodies for 1 hour at room temperature, followed by 5 washes with PBS. Fluorescent images were captured at constant sensitivity setting to standardise fluorescent imaging between samples, using a Carl Zeiss Confocal Laser Scanner.
Scanning Microscope (LSM) 780 Elyra S1 with Super-Resolution (SR)-Structured Illumination Microscopy (SIM) super resolution platform. Results are expressed as mean fluorescent intensity.

Statistical analysis
All statistical analyses were performed using Graphpad Prism Version 5 software (Graphpad Software, La Jolla, CA, USA). Results are expressed as mean ± SD. One- or two-way analysis of variance (ANOVA) as relevant, followed by a Bonferroni post hoc test, was used to assess differences between time points and/or experimental groups. Differences were considered to be of statistical significance when \( P < 0.05 \).

RESULTS

PCO dose-dependent cell viability
For monocyte mono cultures, no difference in % MTT reductive capacity between control and treatment groups was evident (Figure 1c), suggesting that PCO had no effect on viability of this cell type. In astrocytes there was a moderate, yet statistically significant reduction in cell viability in the 200µg/ml treated group at the 24h time point only (Figure 1a). For HUVECs, a marked reduction in %MTT reductive capacity was observed across all treatment dosage groups at the 24h time point (Figure 1b). Although this may suggest increased sensitivity to PCO in this cell type specifically, further investigation indicated that the reduction in %MTT reductive capacity was probably not due to an increase in cell death, but rather due to a significant number of non-adherent living cells that were lost during the washing steps in the MTT assay protocol.

Anti-inflammatory effects of PCO elucidated in mono cultures
In the absence of HIV-associated proteins, control (unstimulated) astrocytes secreted low basal levels of MCP-1 at 6.5 hours, but no MCP-1 was detectable after 24 hours in culture (Figure 2a). The discrepancy between the 6.5 hr and 24 hr time points are not unexpected, given the relatively short half-life reported for MCP-1 \textit{in vivo} (10 minutes) (Smith \textit{et al.}, 2010). This result may thus indicate that the MCP-1 response in absence of HIV-associated proteins was only transient in nature. In contrast, after exposure to either B Tat or HL2/3 cell products, MCP-1 secretion increased significantly over time and was maintained up to the 24 hour point (all \( P < 0.0001 \) when compared to control). As expected, C Tat elicited no MCP-1 response. Pre-treatment with PCO in absence of HIV proteins reduced basal secretion of MCP-1. Additionally, PCO was able to blunt the response to B Tat and HL2/3 cell products in stimulated cells at both 6.5 and 24h (all \( P < 0.0001 \) when compared to control).

In HUVECs (Figure 2b), basal MCP-1 secretion followed a similar pattern to that seen in astrocytes at 6.5 hours, with the exception of C Tat, which also elicited a basal response in this cell type (\( P < 0.0001 \) when compared to control). In contrast to astrocytes, this basal secretion was maintained and even relatively enhanced at 24 hours. In the absence of HIV proteins, HUVECs responded similarly to astrocytes when PCO pre-treated. In the presence of all HIV protein stimuli employed, MCP-1 secretion increased continuously up to 24 hours. PCO was able to blunt the response in this cell type, reducing the MCP-1 response to non-detectable levels.

PCO modulates mediators of inflammation in a simulated BBB
When repeating the intervention protocols in a co-culture simulation of the BBB (consisting of astrocytes, HUVECs and monocytes) using the EGM media to ensure optimal survival of all cell types, the net effect of PCO that may be expected in an \textit{in vivo} situation becomes more evident (Figure 2c). In the absence of HIV-1 protein stimuli, PCO was able to reduce basal MCP-1 secretion by the BBB. Pre-treatment with PCO resulted in an attenuated MCP-1 response to HIV-1 B Tat (as anticipated C Tat did not elicit a response) (\( P < 0.05 \)) and significantly limited the HL2/3-induced response, so that...
MCP-1 levels did not differ from basal levels (P<0.0001). Monocyte cultures did not secrete detectable levels of MCP-1 under any of the experimental conditions, so most probably did not contribute significantly to this outcome.

Since treatment with HL2/3 conditioned media or co-culture with these cells (which contain Tat as well as other HIV proteins) resulted in the most pronounced inflammatory response in all experimental conditions up to this point, and since HL2/3 stimulation was deemed more disease relevant, HL2/3 cells were selected as only pro-inflammatory stimulus for all further experiments.

IL-1β levels were non-detectable in the single cultures of HL2/3 cells (data not shown), so that any IL-1β detected originated from the BBB. IL-1β secretion was evaluated in BBB cultures stimulated by co-culture with HL2/3 cells. Pre-treatment of the BBB cultures with PCO was able to effectively inhibit the IL-1β response following co-culture with HL2/3 cells, so that the response was similar to basal secretion levels (Figure 2d). TNF-α levels in co-culture supernatants were also assessed, however this cytokine did not prove to be a reliable marker of inflammation as all samples assayed came up negative (data not shown).

Monocyte migration across the BBB is effectively inhibited by PCO

Migration of primary human monocytes across the BBB was assessed, as well as the role of PCO as modulator of this process. The number of monocytes remaining on top of the trans-well filter insert (containing BBB) was named unmigrated cells, while those collecting in the bottom of the well are referred to as the migrated cells. Representative images of immunocytochemistry used to visualise monocytes for the purpose of quantification (Figure 3a) illustrate the marked differences in CD14+ monocyte counts between the experimental groups. Numerical data are presented in Figure 3b. As anticipated, HL2/3 stimulation resulted in a significant increase in monocyte migration across the in vitro BBB (ANOVA main effect P<0.0001). Pretreatment with PCO had no effect on migration in the absence of HIV proteins, but was able to effectively reduce the monocyte migration capacity in response to HL2/3 stimulation, maintaining it at basal levels.

Astrocyte intercellular signalling most affected by PCO

The expression of GFAP, an intermediate filament protein associated with the cytoskeletal protein network, as well as the small heat shock protein HSP-27, were measured by immunocytochemical staining of the astrocytes on the BBB membranes. PCO pre-treatment on its own resulted in a statistically significant increase in HSP-27(Figure 4). Expression of GFAP and HSP-27 was significantly increased post HL2/3 stimulation (P<0.001). PCO pre-treatment was associated with a reduction in GFAP expression to control levels and limited increases only in HSP-27 expression post HL2/3 stimulation (Figures 4 and 5).

Figure 1. Effects of the PCO extract used on cell viability in (a) astrocytes, (b) HUVECs and (c) monocytes.*** = P < 0.0001; ** = P < 0.001; * = P < 0.05
DISCUSSION

Neuroinflammatory processes are central to the aetiology, progression and prognosis of HAND, and considering the fact that standard ARV therapies are not able to effectively reduce neuroinflammation associated with HIV-1 infection, identification of alternative strategies have become a focus. In particular, natural products should be considered, given their widespread use. We are of the opinion that data generated in this study make a substantial contribution to this area.

Current data presents two major novel findings. Firstly, we illustrate that PCO largely inhibits monocyte infiltration across the BBB, by down-regulation of the MCP-1 and IL-1β responses. Secondly, an innovative feature of this study is the use of HL2/3 cells, in combination with a well-established in vitro model of the BBB, to simulate – in a more disease relevant manner than other non-infectious methods commonly employed – conditions at the neurovascular interface following HIV-1 subtype B infection. To our knowledge, we are the first to utilize this simulated BBB to assess (and successfully demonstrate) the efficacy of a complimentary medicine as an anti-inflammatory modality.

**HL2/3 cells mimic HIV-1 neuroinfection to produce a more HIV-relevant model**

Up to this point, HL2/3 cells have most commonly been used to investigate viral fusion dynamics in the context of HIV (Wexler-Cohen and Shai 2007). *In vitro* studies investigating neuroinflammation commonly use either infectious cultures or stimulation with single HIV-associated proteins or cells secreting single proteins. Here, we describe utilisation of the ability of HL2/3 cells for high-level production of a variety of HIV-associated proteins, including Gag, Env, Tat, Rev and Nef, to stimulate *non-infective* neuroinflammation *in vitro*. (We did not assess the exact concentration of HIV-associated proteins in the co-culture, since we could not find much literature reporting on physiological levels or *in vitro* levels of these proteins secreted; thus such a measurement would not assist in placing our results in context.) Our model is also more disease relevant than those using only single HIV-associated proteins, as evidenced by comparative data on responses to the Tat proteins we included in the first experiments of the current study. The simulated BBB co-culture, originally
used for testing chemokine and monocyte migration responses to HIV-1 (Eugenin and Berman 2003), was used here in a broader application to investigate efficacy of a natural extract as anti-inflammatory modality. Proof of the accuracy of the model to assess pro-inflammatory signalling is the fact that data generated from the HIV-1 subtype B and C Tat stimulation experiments – showing that subtype B is more inflammatory than subtype C – are congruent with previous reports in the literature (Mishra et al., 2007; Rao et al., 2008).

The combined use of the BBB model and HL2/3 cell stimulation – although technically complex – considerably improved the disease specific relevance and thus practical applicability of our results. For instance, using this model we can conclude that the effects of PCO reported here, are not limited to modulation of Tat-specific events only, but that it applies to inflammation associated with a much larger repertoire of HIV-1 proteins.

We would therefore like to recommend the use of this model for first-line in vitro evaluation of modulatory effects of newly discovered compounds and newly developed pharmaceuticals which may have the potential to modulate HIV-1 induced inflammatory processes within and surrounding the neurovasculature. **PCO modulates HIV-1 – associated neuroinflammation via the ERK1/2/JNK, p38 MAPK and NLRP3 pathways**

Turning our attention to the complementary medicine evaluated, to our knowledge our study is the first to provide scientific support for an anti-inflammatory effect of PCO in the context of HIV-associated neuroinflammation. Given the fact that the natural product currently endorsed by developing country governments for supplementation in people living with HIV, have been shown to be detrimental in this context (Fasinu et al., 2012; Müller et al., 2013) – leaving a gap in terms of adjuvant therapy for these patients – the current results have far reaching implications. Since many studies have been conducted on the effect of this particular PCO in the context of other diseases (Engelbrecht et al., 2007) and non-HIV models of inflammation (Myburgh et al., 2012; Kruger and Smith 2012; Kruger et al., 2014), showing only

![Figure 3. Representative images (a) and numerical data (b) illustrate the effect of PCO extract on migration capacity of CD14+ (FITC) primary human monocytes across a simulated BBB. Images were taken using a 40x objective. *** = P < 0.0001; ** = P < 0.001; * = P < 0.05](image-url)
positive effects and no undesired ones, our expansion of the knowledge highlights the need to focus resources on the continued development of this particular product.

In terms of potential mechanisms of action – which may be more widely applicable – available data and literature allows for discussion of potential mechanisms of PCO. Here we propose avenues by which PCO may modulate the pro-inflammatory signalling pathways in a simplified schematic presentation (Figure 6).

The β-chemokine MCP-1 is a central role player in HIV-associated neuro-inflammation. MCP-1 production in response to HIV-stimulation is facilitated via three routes, namely a) the ERK1/2/JNK pathway (Crystal et al., 2011), b) the p38 MAPK pathway (Fan et al., 2011) and c) caspase-1 activation via the NLRP3 inflammasome (Walsh et al., 2014). From the non-HIV literature, PCO is known to suppress the p38 MAPK pathway as well as the ERK1/2/JNK pathway (Kim et al., 2011), which is in agreement with our interpretation. However, the fact that all inflammatory responses to HL2/3 stimulation assessed in the current study seemed to be completely inhibited after PCO pre-treatment, suggests that it prevented HIV-associated activation of all three pathways. In particular the inhibition of the IL-1β response suggests that PCO also inhibit the NLRP3 inflammasome – caspase-1 pathway, which is the predominant pathway responsible for conversion of pro-IL-1β to IL-1β by caspase-1 (also called IL-1 converting enzyme) (Schroder et al., 2010). Inhibition of this pathway may also explain the anti-inflammatory function of PCO reported under non-HIV basal conditions, both here and by others (Kim et al., 2011; Sakurai et al., 2010; Terra et al., 2011).

Our data is in agreement with other studies elucidating potential mechanisms for PCO, but adds more mechanistic insight. For example, monocytes in circulation have been reported to switch to an anti-inflammatory phenotype which does not readily leave circulation to infiltrate tissue, after PCO treatment (Kruger et al., 2014) – our data shows that this phenomenon is also applicable to extravasation across

Figure 4. Representative images (a) and numerical data (b) illustrates the effect of PCO extract pre-treatment on HSP-27 expression (Pacific Blue) in primary human astrocytes in the simulated BBB. Magnification: 40x objective.

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05
the BBB. Others report down regulation of adhesion molecules (VCAM-1) by PCO (Sen and Bagchi 2001). Our observations of decreased attachment of specifically HUVECs after PCO pre-treatment, supports this notion. In addition to these two mechanisms described in the literature, our data propose a third target – prevention of the inflammatory cytokine signalling cascades related to MCP-1. Interestingly, our data is supported by relatively unexplained reports of decreases in pro-inflammatory cytokine levels in in vivo models after PCO treatment (Myburgh et al., 2012; Kruger and Smith 2012). Our data thus not only adds to the knowledge base related to HIV and neuroinflammation, but also contributes to interpretation of non-HIV results on inflammation and PCO.

**PCO modulates astrocyte inflammatory processes**

The intermediate filament protein GFAP is a widely recognised marker for astrocytes and its upregulation is used by many researchers to indicate reactive astrocytosis. In the context of HIV-1 Tat associated neurotoxicity, GFAP was identified as predictor of neurotoxicity. In the same study, downregulated expression of GFAP in astrocytes was indicative of modulation of the inflammatory processes in this cell type and thus increased neuronal cell survival (Zou et al., 2010). Our result of decreased GFAP expression after PCO pre-treatment, both in the presence and absence of HIV-1 associated proteins, is in agreement with this and further strengthens our data indicating an anti-inflammatory effect for PCO.

The small heat shock protein HSP-27 has been reported to increase as part of the cellular stress response and its overexpression is associated with activation of protective mechanisms during neuronal injury. For example in neuronal injury specifically, constitutive overexpression of HSP-27 has been linked to reduced apoptosis and caspase-3 induction (Vidyasagar et al., 2012). Thus, our observation that PCO pre-treatment, in

![Figure 5](image-url). Representative images (a) and numerical data (b) indicating the effect of PCO extract on GFAP expression (FITC) in primary human astrocytes of the simulated BBB. Magnification: 40x objective.

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05
the presence of HIV-1 associated proteins, is associated with a blunted HSP-27 response to HL2/3 stimulation may indicate that less cell damage was present, due to the anti-inflammatory effects already elucidated, so that a high degree of protection against e.g. apoptosis was not required. In addition, the PCO-induced increased HSP-27 expression in non-stimulated astrocytes suggests that PCO may confer resistance to apoptosis in these cells under basal conditions. Further investigation considering allostatic is required to confirm the longer term benefit of this effect in non-pathological states.

CONCLUSIONS

Current data illustrate that the combined use of HL2/3 cells and the simulated BBB presents an accurate, disease relevant in vitro model to study neuroinflammation in the context of HIV/AIDS.

CNS infiltration of both HIV-1 infected and uninfected monocytes is one of the main methods by which the virus enters and seeds the CNS as a viral reservoir to initiate neuroinflammatory processes. Thus, in order for any anti-inflammatory modality to be useful in this context, it would need to modulate this response, which PCO does very effectively by targeting multiple processes. Thus, extrapolating our data to a more clinical application, the overwhelming body of literature pointing towards several anti-inflammatory benefits of PCO, warrants further development of this extract as adjuvant therapy in the context of HIV-associated neuroinflammation in particular, but also as general anti-inflammatory supplement.

List of abbreviations

AIDS – acquired immunodeficiency syndrome
ARV – anti-retroviral
BBB – blood-brain barrier
CNS – central nervous system
CO₂ – carbon dioxide
DMEM – Dulbecco’s modified eagle’s medium
ERK - extracellular signal-related kinase
FITC - fluorescein isothiocyanate  
GFAP – glial fibrillary acidic protein  
gp120 - glycoprotein 120  
HAND – HIV-1-associated neurocognitive disorders  
HIV-1 – human immunodeficiency virus type-1  
HSP-27 – heat shock protein 27  
HUVECs – human umbilical vein endothelial cells  
JNK - c-Jun N-terminal kinase  
MAPK - mitogen-activated protein kinase  
MCP-1 – monocyte chemoattractant protein 1  
NLRP3 - – NOD-like receptor family, pyrine domain containing 3  
OD – optical density  
PBS – phosphate buffered saline  
PCO - proanthocyanidolic oligomers  
Tat – transactivator of transcription  
TPA - 12-O-tetradecanoylphorbol-13-acetate  
VCAM-1 – vascular cell adhesion molecule 1

**Competing interests**  
The authors declare no competing interests.

**Authors' contributions**  
CS and LDA jointly participated in study design. LDA carried out the experimental work under supervision of CS. CS and LDA both contributed to statistical analysis, data interpretation and manuscript preparation. Both authors read and approved the final manuscript.

**ACKNOWLEDGEMENTS**  
The authors gratefully acknowledge the South African Medical Research Council for financial support of the study. We are also grateful to the German Academic Exchange Service (DAAD), South African National Research Foundation and the Poliomyelitis Research Foundation for provision of student bursaries for LDA.

**REFERENCES**  


Cavalcante GI, Capistrano VL, Cavalcante FS, Vasconcelos SM, Macêdo DS, Sousa FC, Woods DJ and Fonteles MM. 2010. Implications of efavirenz for neuropsychiatry: a review. The International journal of
neuroscience. 120(12):739–745.


Submit your articles online at www.jresearchbiology.com

Advantages
- Easy online submission
- Complete Peer review
- Affordable Charges
- Quick processing
- Extensive indexing
- You retain your copyright

submit@jresearchbiology.com
www.jresearchbiology.com/Submit.php