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Inhibition of HIV-1 tat-induced transactivation and apoptosis by the divalent metal chelators, fusaric acid and picolinic acid—Implications for HIV-1 dementia

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ABSTRACT

The HIV-1 transactivator protein tat is pivotal to the pathogenesis of AIDS, exerting its effects on both viral and cellular gene expression. The basic structure of tat protein allows it to be secreted by HIV-1 infected cells and penetrate uninfected cells where it elicits its multifunctional biochemical effects. The main function of tat protein is viral transactivation which leads to the upregulation of transcription through complex interactions with RNA and host cell factors. Since HIV-1 has been widely implicated as a causative agent of HIV-1 dementia, the aim of our study was to investigate the ability of two novel metal chelators, fusaric acid (FA) and picolinic acid (PA) to firstly inhibit HIV-1 tat induced transcription and secondly, to minimize its cytotoxic effects as mediated via apoptosis. Biologically active tat protein is not freely available commercially. We therefore had to produce, isolate and purify our own protein. A cell culture system and flow cytometric techniques were used in our study. Exposure of CEM-GFP cells to exogenous recombinant tat protein induced transcription and apoptosis, and both processes were inhibited by FA and PA at concentrations that alone did not induce any cytotoxicity. Our data suggest that FA and PA may have therapeutic potential in the management of HIV-1 dementia.

1. Introduction

The progression of HIV-1 infection to AIDS is characterized by a steady decline in $CD4^+$ T cells that result in a suppressed immune system (Campbell et al., 2005). In addition, patients suffering from HIV infection and subsequently AIDS, often present with central nervous system abnormalities suggestive of neurotoxicity (Ferris et al., 2008; Woods et al., 2009). Interestingly HIV-1 does not infect neurons (Aksenov et al., 2009) and therefore its so-called neurotoxic effects have been postulated to be mediated indirectly. One of the possible candidates that have been identified to cause HIV-induced neurotoxicity is tat protein, the transactivator of the HIV-1 long terminal repeat (King et al., 2006).

HIV-1 enters the brain via the blood–brain barrier possibly through trans-endothelial migration (Buckner et al., 2006). Tat protein is produced in HIV-1 infected astrocytes, microglia and macrophages, and is subsequently secreted into the extracellular environment where it penetrates neighboring cells such as neurons. The mode of tat-induced neurotoxicity is thought to be via excitotoxic mechanisms that involve indirect and direct oxidative stress, increased intra-cellular calcium (Pocernich et al., 2005) and caspase-3 activation (Kruman et al., 1998). These mechanisms may lead to abnormal neuron structure and function in areas of the brain such as the hippocampus and cortex (Bonavia et al., 2001). These damaging effects may result in neuronal cell loss and eventual cognitive impairment as observed in HIV/AIDS patients (Pocernich et al., 2005).

The advent of HAART (highly active anti-retroviral therapy) has been successful in managing the impact of HIV on the immune system. However its efficacy to address HIV-associated brain disorders has been less impressive since a substantial percentage of HIV-1 infected individuals worldwide are still plagued by neurocognitive dysfunctions (Nath and Sacktor, 2006). It is in this regard that the present study focused on the ability of two transition metal ion chelating agents picolinic acid (PA) and fusaric acid (FA) to inhibit apoptosis and transactivation induced by HIV-1 tat protein. We used the CEM-GFP cell line that contains a GFP expression cassette under the control of subtype-B LTR. Using this cell line allowed us the advantage of investigating both apoptosis and transactivation simultaneously in the same cell line. The HIV-1 tat protein is a conserved zinc finger protein (Fernandez-Pol et al., 2001a)

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representing an indispensable regulatory component of the virus, thus making it a choice target for chelation therapy by PA and FA.

2. Materials and methods

2.1. Isolation and purification of tat protein

HIV-1 tat protein subtype C (101aa) was expressed in BL21 (DE3) *Escherichia coli* B cells encoding a T7 RNA polymerase gene under the control of a lac I promoter. We followed the isolation protocol described in Siddappa et al. (2006) with minor modifications. Briefly, tat protein was isolated and purified on a column (# 732-1010, BioRad, Hercules CA) used in tandem with ion exchange and affinity chromatography.

2.2. Cell culture

CEM-GFP (# 3655, NIH AIDS Research and Reference Reagent Program) cells were grown and maintained in RPMI 1640 (Lonza) medium containing 10% fetal calf serum (Biochrom) and 1% antibiotic solution of G418 (Invitrogen) 500 μg/ml. Viability of CEM-GFP cells was determined by the trypan blue exclusion test.

2.3. Transactivation assays for tat protein

CEM-GFP cells containing a GFP expression cassette under the control of subtype-B LTR were transfected with 10 μg/ml of tat protein formulated in Bioporter protein transfection reagent (Gene Therapy Systems, San Diego, USA) according to the manufacturer's instructions. Cells were treated with various concentrations of FA and PA 4 h after transfection. Twenty-four and 48 h later, cells were harvested by centrifugation and washed twice with phosphate buffered saline (pH 7.4). The presence of green fluorescence in the CEM-GFP cells was measured with a FacsCalibur flow cytometer (Becton Dickenson, San Jose, CA, USA). Cells were gated on the basis of forward versus side scatter. Green fluorescent protein positive cells were determined using a density plot of forward scatter versus FL-1. Cells with fluorescence intensity greater than 10^2 were considered to be GFP positive and gates were set accordingly to obtain percentages. A total of 10,000 events were scored per sample. The Cellquest Pro software was used for data analysis.

2.4. Apoptosis assay

Determination of levels of apoptosis induced by tat protein was conducted using the annexin V flous and caspase-3 kits (BD Pharmingen). Apoptosis induction by tat protein and treatments with FA and PA followed the same procedure as described for transactivation assays. Twenty-four hours after treatment, cells were harvested by centrifugation and washed twice with phosphate buffered saline. Cells were immediately stained with annexin V-FITC according to the manufacturer's protocol (BD Pharmingen). For caspase-3 determination, cells were washed twice with phosphate buffered saline and prepared according to the protocol provided by the manufacturers before being stained with the caspase-3 antibody. For both assays, cells were analyzed with a FacsCalibur flow cytometer (Becton Dickenson, San Jose, CA, USA). Instrument setup followed the manufacturer's recommendation and analysis of data was conducted using Cellquest Pro software.

2.5. Cell cycle analysis

Cells were harvested 24 h later by centrifugation and washed twice with phosphate buffered saline. Cells were fixed in 100% ethanol for 15 min followed by treatment with 0.1 mg/ml RNase A (Sigma) and propidium iodide (Sigma). Cells were analyzed with a FacsCalibur flow cytometer (Becton Dickenson, San Jose, CA, USA) and analysis of data was conducted using Cellquest Pro software. Cells were gated on the basis of forward versus side scatter and histograms were used to determine the percentage of cells in the respective phases of the cell cycle. A total of 10,000 events were scored per sample.

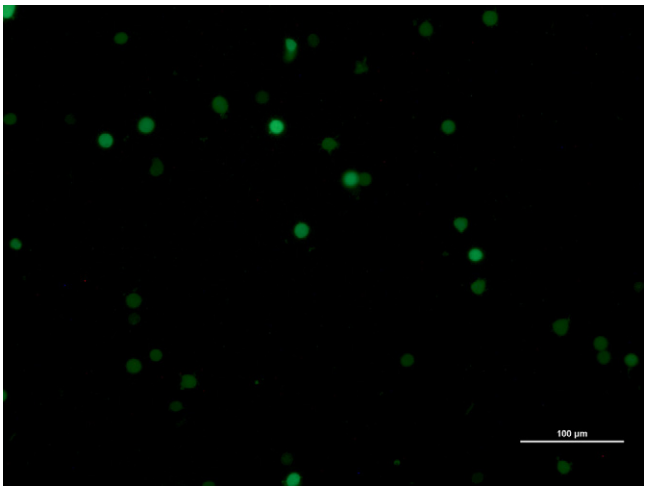

Fig. 1. CEM-GFP cells, containing a stably integrated GFP gene under the control of subtype-B LTR, were transfected with 10 μg/ml tat protein in 24-well plates. Cell cultures were grown for 24 h and then photographed using fluorescence microscopy.

3. Results

3.1. Fusaric acid and picolinic acid inhibit HIV-1 tat-induced transcription in CEM-GFP cells

We examined the effects of fusaric acid (FA) and picolinic acid (PA) on HIV-1 transcription in CEM-GFP cells. Exogenous tat protein (10 μg/ml) was used to induce transcription in CEM-GFP cells stably transfected with an integrated HIV-1 LTR-GFP. Tat-induced GFP expression was detected using fluorescence microscopy (Fig. 1) and quantified using flow cytometry. Exposure of CEM-GFP cells to tat protein for 24 h significantly stimulated transcription in these cells (Fig. 2A, $p < 0.001$). This stimulation was completely blocked by the addition of 0.1 and 0.3 μM FA, as well as 0.1 and 0.3 μM PA (Fig. 2A, $p < 0.001$). A similar result was obtained when CEM-GFP cells were exposed for 48 h (Fig. 2B).

3.2. Inhibition of transcription by fusaric acid and picolinic acid is not due to decreased cellular proliferation

Treatment of CEM-GFP cells with tat protein (10 μg/ml) alone or with tat protein and either FA or PA did not interfere with normal cell cycle kinetics as determined by flow cytometry. Examination of cell cycle data indicated no significant changes in the respective G0/1, S, G2/M phases (Table 1).

Table 1
Cell cycle analysis of CEM-GFP cells subjected to Tat protein for 24 h.

Treatment	G0/1-phase	S-phase	G2/M-phase
Control	61.47	24.54	13.38
Tat	60.84	27.21	11.82
0.1 μM fusaric acid	56.00	26.02	17.85
0.3 μM fusaric acid	65.58	20.43	12.59
0.1 μM picolinic acid	61.00	19.32	19.27
0.3 μM picolinic acid	59.76	18.18	20.71

Values indicate percentage of cells in different phases of the cell cycle.