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Ribotoxin restrictocin manifests anti-HIV-1 activity through its specific ribonuclease activity

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ABSTRACT

Restrictocin, a highly specific ribonuclease produced by *Aspergillus restrictus*, cleaves a single phosphodiester bond in a universally conserved stem and loop structure termed sarcin/ricin loop within the large ribosomal RNA of all organisms. In the current study, we demonstrate restrictocin to manifest anti-HIV-1 activity in two model cell systems. Using two mutants of restrictocin, we further show that the anti-HIV-1 activity of restrictocin is due to its specific ribonucleolytic activity. The study suggests that restrictocin is able to recognize region(s) within HIV-1 genome as its target. Restrictocin appears to have potential as a therapeutic antiviral agent against HIV-1.

1. Introduction

Ribotoxins are ribonucleolytic toxins produced by the fungus *Aspergillus* [1]. Restrictocin, α-sarcin and mitogillin are three well known members of the ribotoxin family [1–3]. The ribotoxins recognize and cleave a highly conserved stem and loop domain within the large ribosomal RNA of all organisms, termed as the sarcin/ricin domain [4]. The cleavage of 28S rRNA by ribotoxins causes release of an approximately 400 nucleotide-long fragment from the 3′ end, known as the α-fragment, which results in the impairment of EF-1 dependent binding of aminoacyl tRNA and GTP dependent binding of EF-2 to the ribosomal site, leading to a total collapse of protein synthesis machinery and eventually cell death [4,5]. Although, ribotoxins efficiently inhibit protein synthesis, they manifest poor cytotoxicity as they are not efficiently internalized. The IC_{50} of α-sarcin has been shown to be in the range of 0.4–10 μM for different cell lines [6]. However, ribotoxins potently inhibit protein synthesis in permeabilized cells, and therefore are much more toxic to them [7–9]. Restrictocin is a non-glycosylated, basic, single chain protein of 149 amino acids with a molecular weight of 16.8 kDa [2,10]. Ribotoxins, including restrictocin have remarkable structure and sequence specificity for their target. They only cleave a single phosphodiester bond in 28s rRNA sparing all other cellular RNAs. The specific target recognition ability of restrictocin has been shown to be through a histidine residue at position 49, substitution of which results in the loss of specificity though the RNase activity of the protein is maintained [8,9]. The catalytic apparatus of restrictocin is similar to that of pyrimidine specific RNases of the RNase A superfamily, and histidine at position 136 has been shown to be absolutely essential for the ribonucleolytic activity of restrictocin [9]. Restrictocin being a cytotoxic molecule, has been used successfully in the construction of active immunotoxins for site specific delivery to cancer cells [11,12].

Several ribonucleases belonging to ribonuclease A superfamily have also been shown to have anti-viral activity [13–19]. Bovine seminal ribonuclease (BS-RNase) was found to inhibit viral multiplication and syncytia formation in H9 virus infected cells without causing any effect on isolated HIV viral particles [16]. The amphibian RNase, onconase, has been reported to inhibit HIV-1 infection of H9 cells, and block viral replication in persistently HIV-1 infected cell lines [15,16]. Four recombinant RNases, namely eosinophil derived neurotoxin (EDN), an EDN variant with four amino acid extension at the N-terminus, -4EDN; RNase A; and angiogenin inhibited HIV-1 replication in PHA blasts [17]. Although EDN does not inhibit integration of HIV-1 into the host genome, it has been shown to inhibit ongoing viral replication in the cytosol as it degrades the newly produced viral RNA [15]. Recently, it was shown that soluble factors derived from T-cells, inhibiting X4 HIV-1 strain are the mixture of β-chemokines and two RNases, RNase 4 and

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angiogenin [18]. It, therefore, appears that RNases of diverse specificity and origin could be explored as potential therapeutics against HIV infection.

In the current study, we have investigated the activity of restrictocin against HIV-1. The study shows that restrictocin inhibits propagation of HIV-1 in host cells. Also, it is shown that the anti-HIV activity of restrictocin is due to its specific ribonucleolytic activity.

2. Materials and methods

2.1. Expression and purification of recombinant proteins

Restrictocin, its mutants, restrictocin-H49A and restrictocin-H136A, and human pancreatic ribonuclease (HPR) were expressed using appropriate plasmids in *Escherichia coli* strain BL21 (λDE3) [8,9,20]. All proteins were found to accumulate within the cytosol in the form of inclusion bodies, from where they were purified using the procedure described by Buchner et al. [21], with some modifications as described earlier [8,9,20]. The proteins were quantified by Bradford's method [22].

2.2. Anti-HIV activity assay using TZM-bl and CEM-GFP cells

HIV-1 NL4.3 was propagated in HEK 293T cells using pNL4.3 plasmid DNA, obtained from NIH AIDS and Reagent Programme, as described [23]. CEM-GFP, a $CD4^+$ T-lymphoblastoid reporter cell line expressing GFP under the control of HIV-1 LTR was also obtained from NIH AIDS and Reagent Programme and used as model system to assay the anti-viral activity of various proteins [24]. To study the anti-HIV activity of proteins, CEM-GFP cells were washed with RPMI-polybrene and 10% FCS, resuspended in RPMI containing polybrene and no serum and infected with HIV-1 at an MOI of 0.0125 for 4–5 h at 37 °C in a CO_2 incubator [25]. After the infection, cells were washed twice with incomplete RPMI medium, resuspended in complete medium and seeded at 2×10^5 cells/ml/well in 24 well plates. Different concentrations of proteins were added to different wells in triplicate and plates were incubated at 37 °C a CO_2 incubator. At the end of 7th day, cell suspensions were transferred from wells to tubes and centrifuged at $1000 \times g$ for 10 min. Supernatants were saved and used to determine p24 antigen levels, whereas the cell pellets were lysed by addition of passive lysis buffer and centrifuged at $15{,}300 \times g$ at 4 °C for 5 min. By determining the relative GFP fluorescence in supernatants of lysed cells, using a BMG FLUOstar Optima microplate reader anti-HIV activity of proteins was determined.

The anti-HIV activity of restrictocin was also assayed in HIV-1 infected TZM-bl cells which are modified HeLa cells that express CD4 and CCR5 rendering them susceptible to HIV infection. These cells also contain an integrated *E. coli lacZ* gene driven by the HIV-1 LTR. Upon infection with HIV-1, Tat is produced from the provirus which activates the *lacZ* reporter, resulting in the synthesis of β-galactosidase as well as luciferase in these cells. The HIV infection was quantified by measuring luciferase activity at 48 h post infection. To study the anti-HIV activity of proteins, TZM-bl cells, were seeded in 24 well plates and infected with HIV-1 at an MOI of 0.05. At the end of infection, cells were washed and different dilutions of various proteins were added to various wells in fresh medium. After 48 h, cells were washed and lysates were prepared using reporter lysis buffer (Promega, USA). Luciferase activity was assayed in the supernatants of the lysed cells. Relative luminescence was measured using a BMG FLUOstar Optima microplate reader and compared between control infected cells and treated infected cells to calculate percent inhibition of infection.

2.3. HIV-1 p24 antigen capture assay

HIV p24 levels were determined by sandwich ELISA using p24 antibody coated plates obtained from AIDS and Cancer Virus Program, NCI, Frederick, USA, following the manufacturer's instructions. HIV-1 inhibition was determined by calculating the relative p24 levels in infected samples treated with proteins compared with that in untreated infected controls.

2.4. Determination of HIV-1 integration by Alu-gag PCR

Integration of viral genome into host genome was determined by *Alu*-gag PCR which is a two step assay [26]. Total genomic DNA was isolated from HIV-1 infected cells, and used as the template in first round regular PCR with two primers that respectively anneal to *Alu* repeat element and *gag* gene. The sequences of two primers used in the first round PCR were, *Alu* Forward: 5′-GCCTCCCAAAGTGCTGGGATTACAG-3′ and *gag* Reverse: 5′-GTTCCTGCTATGTCACTTCC-3′. In the second round, real time PCR was done using the first round PCR product as the template with primers to the R and U5 regions within the HIV long terminal repeat, having the sequences, R forward: 5′-TTAAGCCTCAATAAAGCTTGCC-3′ and U5 reverse: 5′-GTTCGGGCGCCACTGCTAGA-3′. GAPDH was amplified as an internal control, using GAPDH forward: 5′-CAACAGCCTCAAGATCATCAG-3′ and GAPDH reverse: 5′-GAGTCCTTCCACGATACCAA-3′ primers. The integrated viral DNA in infected sample was quantified by comparing it with the uninfected sample. The relative integration of viral DNA in infected and protein treated samples as compared to infected control was calculated from Ct values of real time PCR as follows

$$\Delta \mathrm{Ct} = \mathrm{Ct}(\mathrm{target}) - \mathrm{Ct}(\mathrm{reference})$$

$$\text{Relative integration}\,(\Delta\Delta\mathrm{Ct}) = 2^{-(\Delta \mathrm{Ct\,sample} - \Delta \mathrm{Ct\,control})}$$

Infected untreated sample was taken as control and GAPDH sample as reference.

3. Results and discussion

3.1. Restrictocin inhibits replication of HIV-1 in host cells

The anti-HIV activity of restrictocin was investigated in HIV-1 infected T cell line CEM-GFP. Restrictocin showed a dose dependent inhibition of HIV-1 in CEM-GFP cells with an ID_{50} of 0.5 μM (Fig. 1A). The viability of CEM-GFP cells was not affected at the highest dose of restrictocin used (Fig. 1B). Further, the anti-HIV activity of restrictocin was assayed in another model cell line, TZM-bl. In HIV-1 infected TZM-bl cells also, restrictocin manifested a dose dependent inhibition of the virus with an ID_{50} of 2 μM (Fig. 2A). At the highest dose of restrictocin used, the viability of the HIV-1 infected TZM-bl cells was not significantly affected (Fig. 2B). Clearly, restrictocin manifests a dose-dependent inhibition of HIV replication in two model cell systems of HIV-1 infection, TZM-bl and CEM-GFP without causing significant toxicity.

3.2. Restrictocin treatment reduces p24 antigen levels in HIV-1 infected CEM-GFP cells

In the supernatant of untreated and restrictocin treated HIV-1 infected CEM-GFP cells, p24 levels were measured to further confirm the virus levels in these cells. The p24 levels were found to be reduced in a dose dependent manner in the supernatants of cells treated with restrictocin, corroborating the inhibition of virus replication observed in these cells (Fig. 3).