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Trichosanthin inhibits integration of human immunodeficiency virus type 1 through depurinating the long-terminal repeats

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Abstract Trichosanthin (TCS) is a type I ribosome-inactivating protein with potent inhibitory activity against human immunodeficiency virus type 1. However, the anti-viral mechanism remains elusive. By a well-accepted HIV-1 integration assay, we demonstrated that TCS prevents HIV-1 DNA integration in a dose dependent manner in cell culture. At the same condition, TCS fails to induce obvious cytotoxicity and is also unable to interference viral early events such as viral entry, uncoating or reverse transcription. The HIV-1 integrase can integrate HIV-1 long-terminal repeats into cellular chromosome. The interaction of TCS with these viral integration components was also examined, indicating that TCS does not interact with HIV-1 integrase by the GST-pull down assay, but binds to the long terminal repeats in a transient manner. We further revealed that TCS can efficiently depurinate HIV-1 long-terminal repeats, which may be responsible for the inhibitory activity on HIV-1 integration. In conclusion, we elucidated that TCS specifically inhibits HIV-1 integration by depurinating the long-terminal repeats.

Keywords Trichosanthin · Ribosome-inactivating protein · HIV-1 · Long-terminal repeat · Depurination · Anti-viral mechanism

Introduction

Trichosanthin (TCS) is a 27-kDa protein extracted from the root tuber of *Trichosanthes kirilowii* [1]. TCS is a type I ribosome-inactivating protein (RIP), which arrests protein synthesis via the *N*-glycosidase domain [2]. The protein has multiple documented functions, one of which is the anti-HIV effect [3]. TCS inhibited HIV replication in T lymphoblastoid cell line, and primary macrophages either in vitro or in vivo. Phase I and II clinical trials conducted in the US have found that TCS elicits a moderate increase in circulating CD4+ T cells and a significant decrease in p24 levels in AIDS patients failing treatment with antiretroviral agents such as zidovudine (AZT) [4–7]. In addition to TCS, many other RIPs, including Momordica anti-HIV protein (MAP30), pokeweed anti-viral protein (PAP), and Gelonium anti-HIV protein (GAP31), have been reported to inhibit HIV-1 replication in vitro [8].

The exact anti-HIV mechanism remains elusive. It was initially thought that TCS inhibits HIV by halting protein translation through RIP activity [9]. However, several mutants with low anti-HIV activity retained their ribosome-inactivating activity [10]. Furthermore, small amounts of TCS specifically inhibit HIV without obvious ribosome inhibiting effects, whereas large amounts induce apoptosis in HIV-1-infected cells [3, 11]. TCS also inhibits HIV-1 integrase in vitro [12], cleaves supercoiled DNA in vitro [13], is associated with chemokine receptors that are co-receptors for HIV-1 fusion, and stimulates the activation of chemokine receptors [14]. Until now, there is no evidence to precisely define the stage of infection arrested by TCS in cells. Here by quantitative real-time PCR method, we demonstrated that TCS specifically inhibits HIV-1 DNA integration in cell culture. Further studies revealed that TCS depurinates the long terminal repeats, which is the substrate of HIV-1 integrase and may be responsible for the virus-inhibitory activity.

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Materials and methods

Reagents, antibodies and plasmids

Rabbit anti-TCS polyclonal antibody was prepared as standard protocol. Anti-His antibody was from TianGen Biotechnology. AZT was from Sigma–Aldrich. The plasmids expressing His-IN (integrase) and GST-IN were kind gifts from Anna Cereseto (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy). GST-IN and His-IN were expressed in *E. Coli* and purified by GST•Bind Resin or His•Bind Resin according to the manufacturer's protocols (Novagen).

Protein purification

TCS was extracted from the root tubers of *T. kirilowii* as described [15]. The root tuber obtained from a local drugstore was homogenized with 50 mM Tris–HCl buffer at pH 6.8 (buffer A) using a high-speed blender. The homogenate was centrifuged to remove insoluble material. Then the protein fraction between 40 and 75% ammonium sulphate was collected, precipitated and dialyzed overnight. The resulting solution was applied to a CM-Sepharose C-50 column (Amersham Biosciences), washed with buffer A, and then eluted with buffer A containing 0.3 M NaCl. The elution peak was collected, and loaded on to the second column of Sephadex G-75 (Amersham Biosciences). TCS appeared in the second elution peak. Purity determination of TCS showed a single band of 27-kDa by SDS-PAGE.

HIV-1 integration assay

Env-pseudotyped HIV-1 was generated by co-transfection of 293T cells with env-deleted backbone HIV-1 plasmid pSG3.1Δenv and pSF162-env (Academy of Military Medical Sciences of China) to confirm single-round infection. The collected viral medium was mixed with different concentrations of TCS and then directly infected U937 cells (1×10^6 ml^{-1}). After 2 h, the cells were washed extensively by PBS and further incubated with fresh medium. The quantification of integrated HIV-1 provirus DNA in the cellular genome by real-time PCR was performed at 24 h-incubation and was followed as described [16]. During the first round PCR, integrated HIV-1 sequences were amplified with the HIV-1 LTR-specific primer (LTR-TAG-F, 5′-ATGCCACGTAAGCGAAACTC TGGCTAACTAGGGAACCCACTG-3′) and Alu-targeting primers (first-Alu-F, 5′-AGCCTCCCGAGTAGCTGGGA-3′ and first-Alu-R, 5′-TTACAGGCATGAGCCACCG-3′) that annealed to the conserved regions of Alu repeat element. Alu-LTR fragments were amplified from 1/10 of the total cell DNA in a 25-μl reaction mixture containing 1× PCR buffer, 3.5 mM $MgCl_2$, 200 μM dNTPs, 300 nM primers, and 0.025 units/μl of Taq polymerase. The first round PCR cycle conditions were as follows: a DNA denaturation and polymerase activation step of 10 min at 95°C and then 12 cycles of amplification (95°C for 15 s, 60°C for 30 s, 72°C for 5 min).

During the second round PCR, the first round PCR product was specifically amplified by using the tag-specific primer (tag-F, 5′-ATGCCACGTAAGCGAAACTC-3′) and the LTR primer (LTR-R, 5′-AGGCAAGCTTTATT-GAGGCTTAAG-3′). The second round PCR was performed on 1/25 of the first round PCR product in a mixture containing 300 nM of each primer, 12.5 μl of 2× SYBR Green master mixture (Tiangen Biotech) at a final volume of 25 μl, run on iQ5 Real-Time PCR Detection System (Bio-Rad). The second round PCR cycles began with DNA denaturation and a polymerase-activation step (95°C for 10 min), followed by 50 cycles of amplification (95°C for 15 s, 60°C for 60 s). For generating a linear curve the plasmid pSG3.1 containing the full-length HIV-1 DNA was used as a template. In the first round PCR the LTR-TAG-F and LTR-R primers were used and the second round PCR was preformed using the tag-F and LTR-R primers. The standard linear curve was in the range of 5 ng to 0.25 fg.

PCR analysis of early viral genes was performed at 6 h-incubation. The viral DNA segments from Gag or Nef were also amplified as described [16]. The viral Gag or Nef DNA sequences were amplified using specific primers: Gag specific primers, 5′-AGTGGGGGGACATCAAGCAGCCATG-3′ and 5′-TGCTATGTCAGTTCCCCTTGGTTCTC-3′; Nef specific primers, 5′-CCTGGCTAGAAGCACAAGAG-3′ and 5′-CTT GTAGCAAGCTCGATGTC-3′. The fragments were amplified from 10 ng of the total cell DNA in a 25-μl reaction mixture containing 1× PCR buffer, 3.5 mM $MgCl_2$, 200 μM dNTPs, 300 nM primers, and 0.025 units/μl of Taq polymerase. The PCR conditions were as follows: a DNA denaturation and polymerase activation step of 5 min at 95°C and then 10, 20 or 30 cycles of amplification (95°C for 45 s, 60°C for 30 s, and 72°C for 45 s).

Cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma–Aldrich) cell proliferation assay was used for cell viability measurement as standard protocol. The results were recorded by microtiter plate reader (Bio-Rad) at 570 nm with 650 nm as reference. Cell viability was calculated as OD570 of TCS-incubated group divided by OD570 of TCS-free group.

DNA binding assay

The sequences of 5′-biotin labeled oligonucleotides corresponding to HIV-1 U5 LTR were synthesized from Sangon Biotech: 5′-biotin-GTGTGGAAAATCTCTAGCAGT-3′ and 5′-ACTGCTAGAGATTTTCCACAC-3′. The oligonucleotides mixture diluted in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 50 mM NaCl were annealed at 95°C for 5 min and then they slowly cooled to the room temperature to generate 5′-biotin-LTR duplex. 1 nM 5′-biotin-LTR duplex was incubated with 10 nM TCS or 50 nM BSA in 10 mM Hepes-NaOH (pH 7.0), 2 mM $ZnCl_2$, 2% β-mercaptoethanol at 37°C for 30 min. The reaction mixture was then irradiated with a UV transilluminator (254-nm wavelength) from 3-cm above (2.4 MW/cm^2) at room temperature for 10 min [17]. The mixture was applied to gel mobility shift assay by LightShift Chemiluminescent EMSA Kit (Pierce).

Determination of adenine polynucleotide glycosylase activity

Adenine polynucleotide glycosylase activity was determined as previously described [18, 19]. The unlabeled HIV-1 LTR duplex was synthesized and annealed as above. Chromosomal DNA was purified from U937 cells by Chromosomal DNA Extraction kit (TianGen) and mechanically sheared. 10 μg LTR duplex or chromosomal DNA were incubated with 5 μg TCS or 5 μg BSA in 10 mM Hepes-NaOH (pH 7.0), 2 mM $ZnCl_2$, and 2% β-mercaptoethanol (final volume of 50 μl) at 37°C for 60 min. In one of the tubes, 20 μg of anti-TCS antibody was added. After incubation, 5 μl of 3 M sodium acetate (pH 5.2) and 2 vol of ethanol was added for 1 h at 4°C to precipitate the polynucleotides. The ethanol-soluble fractions were diluted with distilled water to 1 ml and then added with 0.4 ml of 0.14 M chloroacetaldehyde containing 0.1 M sodium acetate (pH 5.2). The samples were incubated at 85°C for 1 h. Adenine was converted to fluorescent 1, N^6-ethenoadenine. After cooling to room temperature, the fluorescence was measured by Hitachi F-4500 fluorescencemeter at an excitation wavelength of 310 nm and an emission wavelength of 410 nm. Adenine standard (Amresco) at the range from 1 pm to 10 nm was used for determination of the standard curve.

Results and discussion

TCS inhibits HIV-1 DNA integration in cell culture

To elucidate the anti-viral mechanism of TCS, we examined its effects on HIV-1 early events upon infection. The effect of TCS on HIV-1 integration was measured in cell culture. We used quantitative real-time PCR to quantify the provirus that integrated into cell chromosome as previous reports [16, 20, 21]. Only the integrated viral DNA was specifically amplified by two groups of primers: one is the primers for Alu repeats that dispersed in high frequency at cellular chromosome and the other is the primer for LTR region of HIV-1 DNA. As shown in Fig. 1a, TCS blocked HIV-1 integration in a dose-dependent manner. As TCS is a ribosome-inactivating protein, we further evaluated the possible toxicity. At the conditions of 2-h incubation, TCS was non-toxic as reflected by the MTT assay (Fig. 1b), indicating that the arrest of viral integration should not be due to the toxicity of TCS.

No inhibitory effect of TCS on total viral DNA

TCS significantly decreased the integrated HIV-1; however, the effect may also be due to blockage of upstream early events as viral entry, uncoating and reverse transcription. We then examined the effect of TCS on total viral DNA. Gag and Nef gene on HIV-1 DNA were

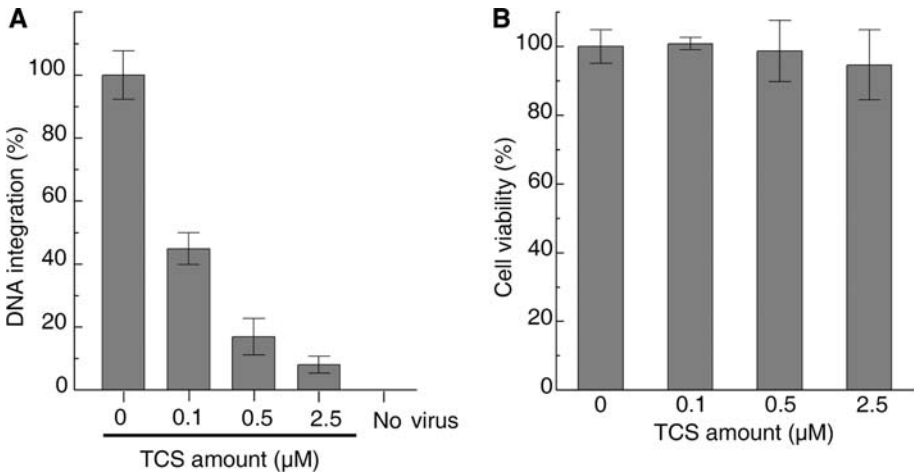

Fig. 1 TCS inhibits HIV-1 DNA integration without significant cytotoxicity. **a** U937 cells were incubated with HIV-1 that mixed with different amounts of TCS. The percentage of integrated viral DNA was assessed at 24 h post-infection. The data is shown as the mean ± SE (SEM; $n = 3$). **b** Effect of TCS on cell viability by MTT assay. The incubation condition of TCS with U937 cells was the same as above. The data is shown as the mean ± SE (SEM; $n = 5$)

selected for examination. As in Fig. 2, no inhibitory activity of TCS was observed on the production of total viral DNA. At the experimental conditions described the PCR was at the linear curve as is clearly indicated by results showing that the amount of the amplified DNA was increased with increasing PCR cycle numbers. Being a reverse transcriptase inhibitor, AZT blocked the viral DNA production as expected (Fig. 2). Thus, the results indicate that TCS selectively inhibits viral integration but not the upstream early events such as viral entry, uncoating or reverse transcription.

TCS transiently interacts with HIV-1 LTR

As TCS specifically inhibits viral integration, we then determined the interaction of TCS with HIV-1 integration components. First, the interaction of TCS with HIV-1 integrase was not detected by the GST pull-down assay (Fig. 3a). As an oligmer, His-integrase was pulled down by the beads-associated GST-integrase as expected [22]. Then we turned to study the interaction of TCS with HIV-1 LTR. LTR is the substrate of HIV-1 integrase. The binding of TCS to HIV-1 LTR was only detected under the treatment

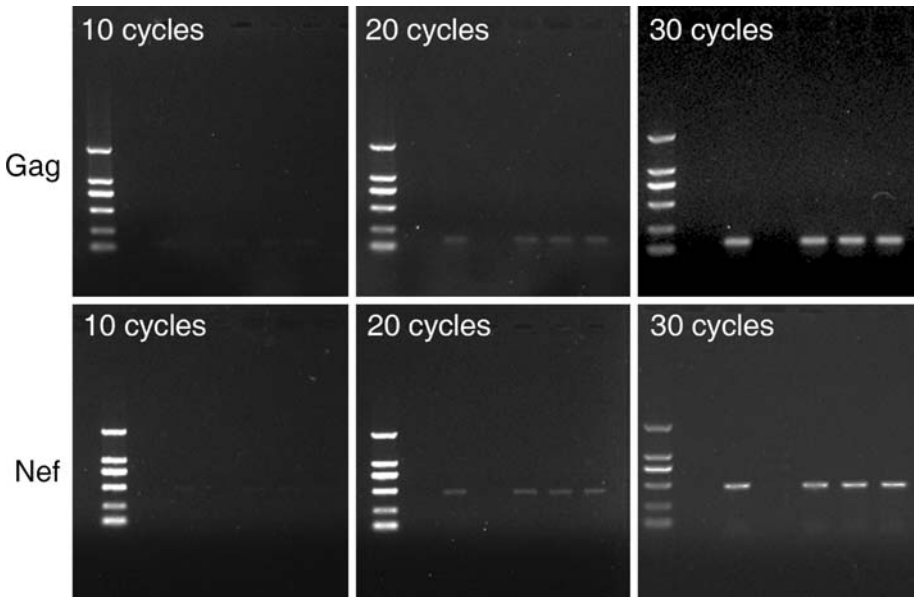

Fig. 2 Effect of TCS on total viral DNA in HIV-1 infected cells. U937 cells were infected with HIV-1 that mixed with different amounts of TCS or 2 μM AZT. A small segment from HIV-1 Gag or Nef was amplified with specific primers to indicate the total viral DNA at 6 h post-infection. The DNA markers indicate 2, 1, 750, 500, 250 and 100 bp from the top to the bottom. The *lanes* in each gel from left to right indicate DNA Marker, No virus, No TCS, 2 μM AZT, 0.1, 0.5 and 2.5 μM TCS, respectively

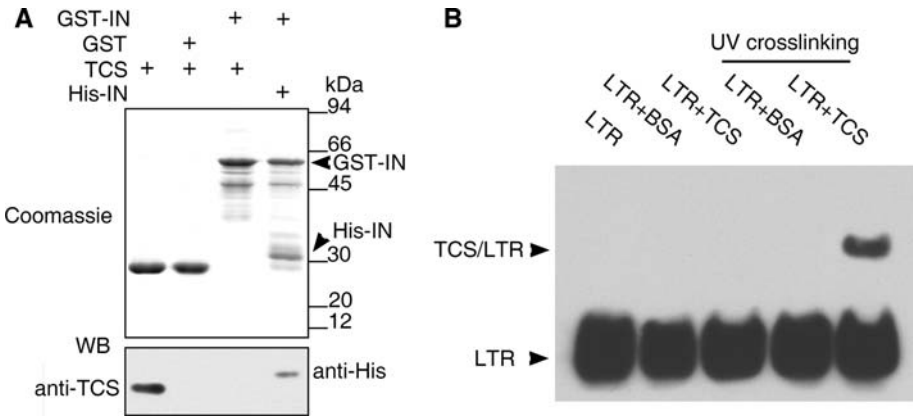

Fig. 3 TCS transiently interacts with HIV-1 LTR. **a** 10 μl Glutathione-sepharose beads with GST-IN were incubated with 5 μg TCS or 5 μg His-IN (positive control) overnight at 4°C. After extensive wash with PBS containing 5 mM DTT and 0.1% Nonidet P40, the bound proteins on beads were run in SDS-PAGE and further analyzed by Western blotting using anti-TCS antibody to detect the bound TCS or anti-His antibody to detect the bound His-IN. Glutathione-sepharose beads with GST were also used as a negative control. The migration positions of GST-IN and His-IN in SDS-PAGE are indicated. **b** Electrophoretic mobility shift analysis of TCS interaction with HIV-1 LTR duplex under the treatment of 254-nm UV-crosslinking. BSA was used as a negative control. The complex was analyzed by 6% PAGE gel and the biotin-labeled LTR duplex was detected by streptavidin–horseradish peroxidase conjugates. The migration positions of LTR and TCS/LTR complex are indicated

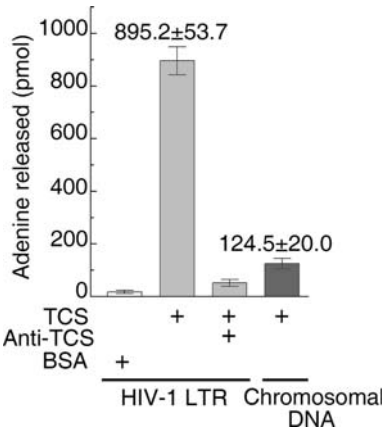

Fig. 4 Adenine DNA-Glycosylase activity of TCS on HIV-1 LTR. 10 μg HIV-1 LTR duplex or chromosomal DNA were incubated with 5 μg TCS at 37°C for 1 h. The released adenine was measured as described in experimental procedures. In one of the tubes, the activity of TCS was blocked by anti-TCS antibody. BSA was used as a negative control. The data is shown as the mean ± SE (SEM; $n = 3$)

of UV crosslinking [17], suggesting that TCS transiently interacts with LTR (Fig. 3b).

TCS depurinates HIV-1 LTR

Many RIP proteins were found widespread adenine DNA-glycosylase activity [19]. We thus performed a fluorimetric assay to measure the activity of TCS to depurinate the viral LTR [18, 19]. When compared with the chromosomal DNA, TCS was shown much higher adenine DNA-glycosylase activity on HIV-1 LTR (Fig. 4). The result suggests that TCS may act on HIV-1 LTR to interrupt viral integration.

TCS is an anti-viral peptide that was clinically applied in AIDS therapy [4–7]. We studied the anti-viral mechanism of TCS in cell culture, showing that TCS specifically inhibit HIV-1 DNA integration. It is the first to precisely define the stage of infection arrested by TCS in cells. The data is consistent with previous studies. TCS was previously reported to inhibit the activity of integrase in vitro [12]. Besides TCS, many anti-viral RIPs such as luffin, saporin, MAP30, and GAP31 were also reported potent inhibitory activity on HIV-1 integrase in vitro [12, 23]. MAP30 and GAP31 interfere both the 3′-end processing and strand-transfer reaction of integrase [23]. Thus, the cell culture data and previous in vitro data consistently support the notion that TCS inhibits viral infectivity through prevention HIV-1 integration.

Stirpe and co-workers reported that over 50 plant RIPs can remove adenine from various substrate, including DNA [19, 24]. These RIPs can be re-defined as polynucleotide: adenosine glycosidases. As a RIP, TCS was also reported polynucleotide: adenosine glycosidase activity on herring sperm DNA [19]. Here we further revealed that TCS efficiently depurinates HIV-1 LTR. The resulting abasic DNA is much less stable and more sensitive to strand cleavage than abasic RNA [25]. Also the abasic LTR is inappropriate for the activity of HIV-1 integrase [26]. Therefore, the action of TCS on viral LTR may be responsible for its inhibitory activity on integration.

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