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An inhibitor of c-Jun N-terminal kinases (CEP-11004) counteracts the anti-HIV-1 action of trichosanthin

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Abstract

Trichosanthin (TCS) is a type I ribosome-inactivating protein possessing multiple biological and pharmacological activities. One of its major actions is inhibition of human immunodeficiency virus (HIV) replication. The mechanism is still not clear. It is generally believed that this action is mediated via ribosome inactivation. Recently, we found that some TCS mutants with full ribosome inactivating activity were devoid of anti-HIV-1 effect. This suggested that there might be other mechanisms contributing to the anti-HIV-1 action. This study showed that a commonly used c-Jun N-terminal kinases inhibitor (CEP-11004) could counteract the antiviral action of TCS in C8166 cells. CEP-11004 alone had no effect on HIV-1 replication and TCS alone significantly inhibited this process. When CEP-11004 was used together with TCS, the antiviral action of TCS was much reduced. Two methods were used to assess viral replication. (1) By measuring the HIV-1 reverse transcriptase, TCS on the average reduced viral replication to $52 \pm 4\%$. With CEP-11004 pretreatment, TCS appeared to lose the HIV-1 inhibitory activity with viral replication stood at $101 \pm 7\%$. (2) By measuring HIV-1 p24, TCS reduced viral replication to $68 \pm 4\%$. With CEP-11004 pretreatment, TCS again seemed to lose its anti-HIV-1 activity with HIV-1 replication rose back to $101 \pm 4\%$. Both indexes indicated that CEP-11004 counteracted the antiviral action of TCS. Phosphorylation of JNK on the other hand was only slightly elevated by 1.5-fold by TCS and CEP-11004 inhibited this elevation. These results suggested that the anti-HIV-1 effect of TCS may be related to the MAPK signal process downstream from the point of CEP inhibition.
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Keywords: Trichosanthin; Ribosome inactivating proteins; HIV; c-Jun N-terminal kinases; MAPK; Anti-HIV activity; CEP-11004

Trichosanthin (TCS) is a single chain polypeptide classified as type 1 ribosome-inactivating protein (RIP). It is purified from the root tubers of *Trichosanthes kirilowii*. TCS is the first RIP found to inhibit human immunodeficiency virus (HIV) replication in vitro [1]. Later there were many other RIPs such MAP30, GAP31, DAP30, DAP32, TAP29, and trichobitacin reported to have similar action [2–5]. The exact mechanism is still not clear. It is generally believed that ribosome inactivation contributes to this action. However, not all RIPs have anti-viral activities. Two TCS mutants, TCS_{C19aa} and TCS_{KDEL}, retained full ribosome inactivation activity but lost most of the anti-HIV-1 activity [6].

It was reported that two RIPs, ricin A chain and α-sarcin, could activate the stress-activated protein kinase JNK1. These RIPs inflicted sequence-specific damage to the α-sarcin/ricin loop in the 28S rRNA [7]. Like ricin A chain and α-sarcin, TCS also has *N*-glycosidase activity that depurinates adenine 4324 of 28S rRNA [8]. This can damage eukaryotic ribosome and impair protein synthesis [9]. It appears that ribotoxic stress may play a role in the antiviral mechanism of TCS via the MAPK signal process. This study examined the effect of a commonly used JNK

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inhibitor (CEP-11004) on the anti-HIV-1 action of TCS. Viral replication was estimated by measuring the HIV-1 reverse transcriptase (RT) activity and HIV-1 p24 antigen. CEP-11004 is an Indolocarbazole analogue which is reported by Cephalon Inc. to inhibit MLK1-3 [10] and used as a JNK inhibitor [11–13].

Materials and methods

Chemicals and reagents. Natural TCS purified from the root tuber of *T. kirilowii* was obtained from Jinshan Pharmaceutical Limited Company (Shanghai, China). CEP-11004 was kindly donated by Cephalon Co. Phospho-plus SAPK/JNK (Thr183/Tyr185) antibody Kit (9250$^{\#}$) was bought from Cell Signalling Technology Inc. The colorimetric HIV-1 RT assay kit was purchased from Roche. Monoclonal anti-p24 antibody (P5F1) was developed by our laboratory. PVDF membrane (immobilon-P, 0.45 μM) was purchased from Millipore Corporation. Other reagents were mostly obtained from Sigma.

Virus and cell lines. C8166 and HIV-1_{IIIB} strain were kindly donated by Medical Research Council (MRC), AIDS Reagent Project, UK. C8166 cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco). HIV-1_{IIIB} was obtained from the culture supernatant of H9/HIV-1_{IIIB} cells. The 50% HIV-1 tissue culture infectious dose ($TCID_{50}$) in C8166 cells was calculated by the Reed and Muench method. Virus stocks were stored in small aliquots at −80 °C.

ELISA for HIV-1 p24 antigen. C8166 cells (4×10^5/ml) were pretreated with CEP-11004 (final concentration 0.4 μM) for 2 h before adding TCS and HIV-1. Cells (100 μl) were added in a 96-well plate containing 50 μl TCS solution followed by 50 μl HIV-1 solution (MOI = 0.05). The final concentrations of TCS will be 0.08, 0.4, and 2 μg/ml, respectively. Each experiment was done in triplicate. Six wells of cells treated with mock solution were used as positive control (HIV-1 infected) and negative control (without HIV-1), respectively. Plates were cultured at 37 °C with 5% CO_2 for 72 h. The level of HIV-1 p24 antigen in culture supernatant was determined by a sandwich ELISA as described previously [6].

HIV-1 RT assay. C8166 were pretreated with 0.4 μM CEP-11004 for 2 h and washed once with serum-free medium. Six hundred microliter of cells (3.0×10^5/ml) was added to a 24-well plate containing 600 μl TCS solution (or mock solution) followed by HIV-1 infection (MOI = 0.05). Each experiment was done in triplicate. Three wells of cells treated with mock solution were used as positive control (HIV-1 infected) and negative control (without HIV-1), respectively. Plates were cultured at 37 °C, 5% CO_2 for 72 h. HIV-1 particles in 800 μl of culture supernatant were precipitated by 30% PEG solution and then used for HIV-1 RT assay. HIV-1 RT was determined with a commercially available ELISA kit according to the instructions of the manufacturer [6].

c-Jun N-terminal kinase assay. Before protein extraction, C8166 cells were cultured in fresh complete medium for 24 h and then serum-starved for another 24 h. After serum-starvation, cells were re-suspended in serum-free medium at a density of 1×10^6/ml. Then TCS was added. After centrifugation, cells from each dish were lysed in 100 μl SDS sample buffer and heated for 5 min at 100 °C. The lysate was sonicated on ice. After centrifugation at 4 °C (12,000*g*), 5 μl of protein extract was left for protein concentration assay by the Bradford method. An equal amount of protein was separated by 10% SDS–PAGE gel electrophoresis and followed by Western blotting assay using a PhosphoPlus SAPK/JNK (Thr183/Tyr185) Kit according to the instruction of the manufacturer. JNK and p-JNK signal densitometry was performed with FluorChem 8000 (Alpha Innotech, San Leandro, CA) according to the manufacturer's instruction.

MTT assay for cell viability. Cytotoxicity was measured by MTT methods as described previously [6]. Briefly, 20 μl of MTT reagent was added to every well and the plate was incubated for 4 h at 37 °C and then incubated with 100 μl of 50% DMF–20% SDS overnight. After the formazum was dissolved completely, the plates were read on a Bio-Tek ELx 800 reader at 595 and 630 nm (A_{595}/A_{630}). The results were shown by absorbance values.

Results

CEP-110004 had no effect on HIV-1 replication

In the absence of TCS, HIV-1 replication is not affected by CEP-11004 irrespective of the method to determine viral replication (Fig. 1). HIV-1 replication is not significantly different from the control.

TCS inhibited HIV-1 replication but CEP-11004 counteracted this action

TCS significantly inhibited viral replication in a dose-dependent manner (Fig. 1). When the same experiment was performed in C8166 cells pretreated with CEP-11004, the

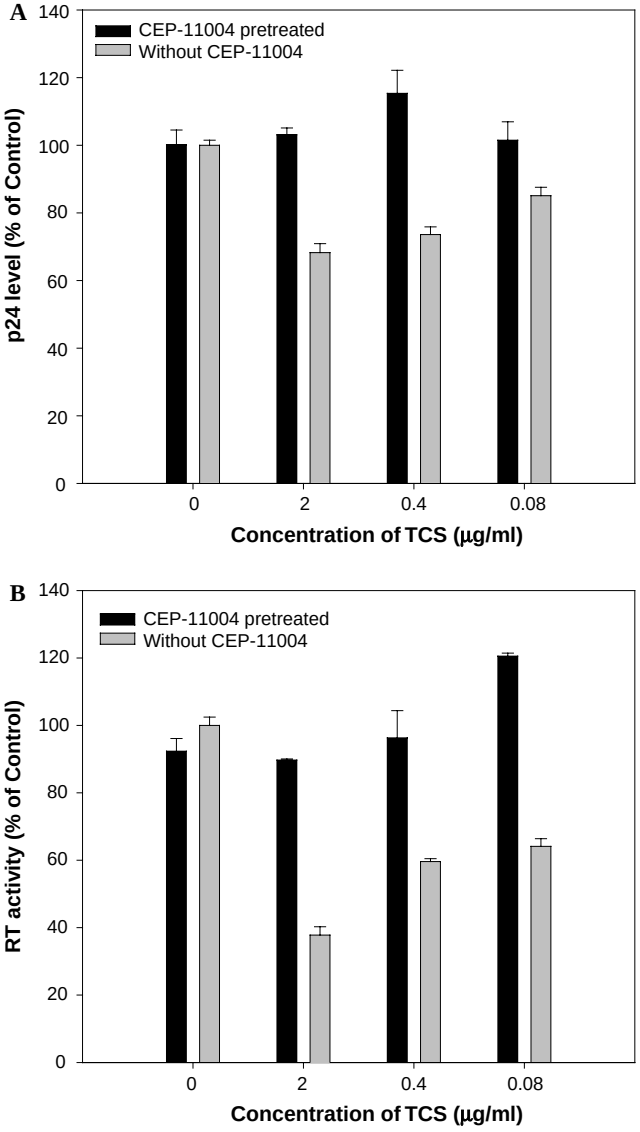

Fig. 1. A comparison of the anti-HIV-1 activity of TCS in the presence and absence of CEP-11004. Viral replication was assessed by p24 antigen (A) and RT activity (B). Values are expressed as means ± sem. There was significant difference between all CEP-11004 treated and non-treated group, $p < 0.05$.

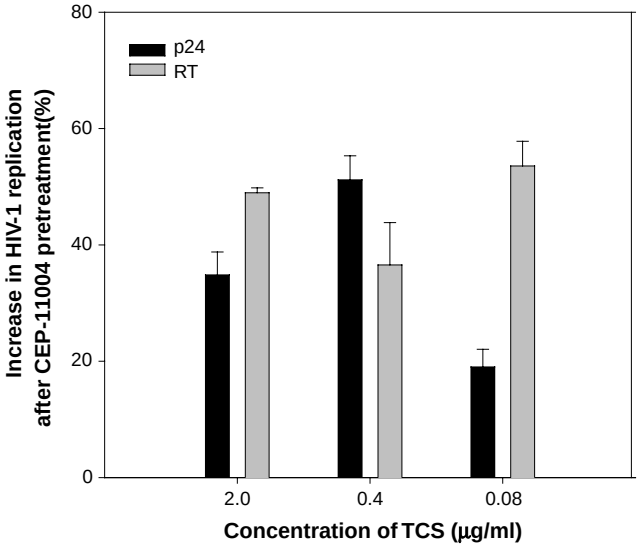

Fig. 2. Increase in HIV-1 replication after CEP-11004 pretreatment measured by HIV-1 p24 and RT. Values are calculated from the difference between the CEP-11004 treated and the non-treated group and are expressed as means ± sem.

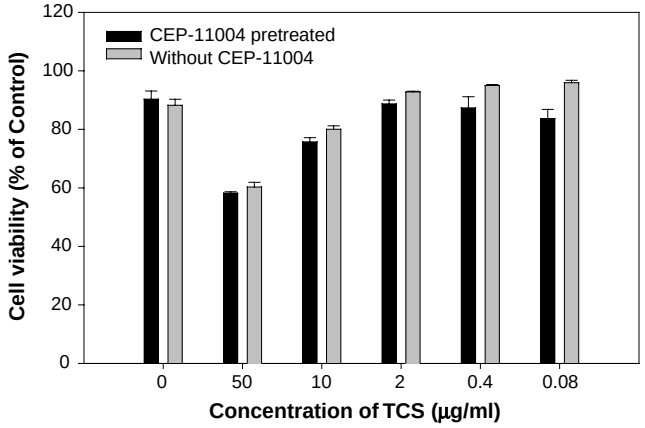

Fig. 3. Cell viability at different concentrations of TCS and in the presence and absence of CEP-11004. Values are expressed as means ± sem.

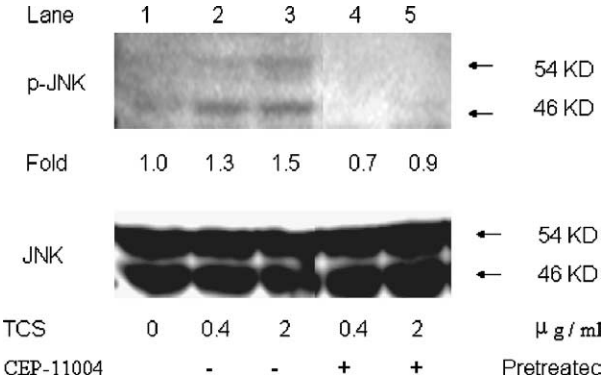

Fig. 4. Activation of JNK at different dosage of TCS. Lane 1, control; lane 2, 0.4 μg/ml TCS (without CEP-11004); lane 3, 2 μg/ml TCS (without CEP-11004); lane 4, 0.4 μg/ml TCS (CEP-11004 Pretreated); lane 5, 2 μg/ml TCS (CEP-11004 Pretreated).

inhibitory effect of TCS appeared to be much weakened. On the average (all dosage), TCS reduced viral replication to 68 ± 4% as measured by HIV-1 p24 and 52 ± 4% as determined by HIV-1 RT. On another group of cells pretreated with CEP-11004, TCS appeared to lose its anti-HIV-1 activity with HIV-1 replication rose back to 101 ± 4% as measured by HIV-1 p24 and 101 ± 7% as determined by HIV-1 RT. Significant increase in viral replication appeared in all TCS dosage group pretreated with CEP-11004 (Fig. 2). To avoid any molecular interaction between TCS and CEP-11004, cells assigned for HIV-1 RT assay were washed with serum-free medium after pretreatment with 0.4 μM CEP-11004. This was meant to remove excess CEP-11004 to avoid contact with TCS. The cells assigned for HIV-1 p24 assay did not receive the washing. The results from both groups did not differ too much, suggesting that intermolecular interaction may not be likely.

In these experiments, C8166 cell viability was stable for TCS dosage up to 2 μg/ml (Fig. 3). There was no significant effect of CEP-11004 on cell viability. On higher dosage above 10 μg/ml, cell viability began to fall.

JNK activation by TCS

Activation of JNKs was detected by Western blotting assay. TCS activated JNKs in a dose-dependent manner (Fig. 4). The activation can be blocked by CEP-11004 pretreatment.

Discussion

In one of our previous studies, it was demonstrated that ribosome inactivation may not be solely responsible for anti-HIV activity of TCS [6]. There is a need to look for alternative mechanism. The MAPK signal pathway can be a candidate. There were numerous studies showing activation of the MAPK pathways by RIP [7,14–17]. This study employed a commonly used JNK inhibitor CEP-11004 to test a hypothesis that TCS worked through the MAPK pathway. The anti-HIV-1 activity of TCS in the presence and absence of the inhibitor was examined. It was clearly shown that CEP-11004 counteracted the anti-HIV-1 action of TCS. An inhibitor of the MAPK pathway opposed the action of TCS suggesting that the action of TCS might involve the MAPK pathway. It can be speculated that components of the MAPK pathway downstream from the site of CEP-11004 inhibition can be candidates for TCS action. TCS activated one or more of these components leading to the anti-HIV-1 effects. Blocking the upstream of these components therefore abolished the anti-HIV-1 activity of TCS.

Another possibility to explain the present finding is that CEP-11004 interacts strongly with TCS, denaturing the protein or binds to its active site so as to neutralize the effectiveness of TCS. This is not very likely as

precautionary measures to prevent interaction were taken. After pretreatment with CEP-11004, cells were washed thoroughly before adding TCS. The results did not differ from those obtained from cells without washing. When high TCS dosage was used (50 μg/ml), cell viability decreased substantially (Fig. 3). The decrease was independent of CEP-11004. This showed that the action of CEP-11004 was selective. It counteracted the anti-HIV-1 but not the cytotoxic action of TCS. If indeed CEP-11004 bound to and/or denatured TCS, it would have abolished both the anti-HIV-1 and the cytotoxic activity. These results suggested that total inactivation of TCS by CEP-11004 through interaction was not likely. In addition there were no known studies reporting strong interaction between CEP-11004 and TCS.

In this study, activation of JNK was examined. TCS caused a small increase in phosphorylation of JNK which could be inhibited by CEP-11004. Whether this small rise can account for the TCS action is questionable.

There are evidences to suggest that MAPK may be a common denominator in TCS action and HIV-1 replication. Early studies showed that the MAPK signal pathway played an important role in HIV-1 replication. The pro-inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF-α) promote HIV-1 replication in U1 and peripheral blood monocytes. The cytokine and the stress response were mediated by P38 MAPK. In the presence of a specific p38 MAPK inhibitor (SB203580), HIV-1 replication induced by IL-1 and TNF-α was greatly suppressed [18]. It was also suggested that activation of ERK and JNK may contribute to the anti-HIV-1 activity of a plant protein jacalin [19].

Some RIPs like ricin A chain and α-sarcin were found to activate SAPK/JNK and its activator SEK1/JNKK/MKK4 probably through ribosome inactivation. This is the so-called ribotoxic stress response. This resulted in induction of the expression of immediate-early genes c-fos and c-jun [7]. c-Jun, a prominent member of the AP-1 transcription family, has been implicated in the regulation of a wide range of biological processes including development, differentiation, transformation, and apoptosis [20–23]. Other RIPs including onnamide A and theopederin B[14], Trichothecene mycotoxins [16], mistletoe lectin II [24], and shiga toxin [17] can also activate SAPK/JNK. In addition to RIP, antibiotic anisomycin, ultraviolet-B, and ultraviolet-C also activate JNK in a ribotoxic stress response manner [25,26].

There were numerous examples to show that RIP activated MAPK. HIV-1 replication was also shown to involve MAPK. The relationship between TCS MAPK and viral replication is so far lacking. This study showed support for MAPK as playing a role in the anti-HIV-1 mechanism of RIP.

In conclusion, the study showed that CEP-11004, a JNK inhibitor, counteracted the anti-HIV-1 action of TCS. This supports that the MAPK signal process may play a role in the anti-HIV-1 mechanism of TCS.

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