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Enhanced anti-tumor activity of trichosanthin after combination with a human-derived cell-penetrating peptide, and a possible mechanism of activity

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ABSTRACT

Trichosanthin (TCS), a type I ribosome-inactivating protein (RIP-I) and renowned Chinese traditional medicine, displays a broad spectrum of biological and pharmacological properties. Particularly, its anti-tumor activity has received a great deal of attention. However, the cellular mechanism for TCS uptake varies with different tumor cell lines, leading to discrepancies in its reported ability to penetrate cells. In this study, HBD, a human derived cell-penetrating peptide (CPP), was used to improve the delivery of TCS into several types of tumor cells, including HeLa cells. Recombinant TCS (rTCS) with or without the fused HBD peptide was expressed in *Escherichia coli* cells and successfully purified by Ni-NTA affinity chromatography. The cellular uptake efficiency of FITC-labelled-rTCS-HBD was observed in HeLa cells and compared with the uptake efficiency of non-HBD conjugated rTCS under the same conditions using laser confocal microscopy. Moreover, the IC_{50} value of rTCS-HBD in the tested tumor cells was much lower than that of rTCS, indicating that HBD could efficiently deliver the rTCS into tumor cells. When compared with rTCS, rTCS-HBD induced higher rates of apoptosis in HeLa cells as analyzed by flow cytometry. Furthermore, the apoptotic events observed in HeLa cells incubated with HBD-fused rTCS included activation of Caspase-9, decrease in the Bcl-2/Bax ratio, and cleavage of PARP. These results strongly suggest the participation of mitochondria in apoptosis. This report illustrates one possible method for achieving the efficient transport of TCS into cells using a CPP as a vector, and increases the likelihood that TCS can be used in the clinic.

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1. Introduction

Cancer is one of the deadliest diseases in humans, and its incidence and mortality rate continue to rise. As a result, it is critical that anti-tumor agents demonstrate greater efficacy in treating this disease. Trichosanthin (TCS) is a protein extracted from the root tubers of *T. kirilowii* Maxim, and a traditional Chinese medicine that has been used as an abortifacient for centuries in China [1,2]. TCS belongs to a family of structurally and functionally related toxin proteins, collectively referred to as ribosome-inactivating proteins (RIPs), which can be classified into three types [3,4]. TCS is a type I ribosome-inactivating protein (RIP-I) with a molecular weight of 27 kDa, and consists of a single chain containing 289 amino acids. Full-length TCS is processed to its mature form containing 247 amino acids, without putative secretory signal peptide and carboxyl-terminal sequence [5]. TCS inactivates ribosomes by removing a single adenine residue from position 4324 in the 28S rRNA subunit of eukaryotic ribosomes [6].

Numerous patients die from the effects chemotherapeutic drugs in addition to the cancer itself. This is mainly attributed to the high doses of drugs administered, and the fact that chemotherapeutic agents usually kill normal cells in addition to cancer cells, and suppress the immune system. A previous study showed that TCS is transported across the cell membrane by low density lipoprotein receptor-related protein 1 (LRP 1) [7], and this results in its differential permeability in different cell lines [7–13]. Cells with a high concentration of LRP1 on their outer membrane (e.g., trophoblasts and choriocarcinoma cells) are more sensitive to the effects of TCS [7]. However, cells lacking LRP-1 show poor internalization of TCS, which limits its potency. Therefore, efficient internalization of TCS into tumor cells is vital for achieving the desired therapeutic effect. Cell-penetrating peptides (CPPs) have been utilized for several years to carry macromolecules into cells. As recombinant

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RIPs and CPPs have been proven effective as transport agents [14,15], CPPs might be used to improve the transport and therapeutic efficacy of TCS.

The c-terminus of human extracellular superoxide dismutase (hC-SOD3, hereafter referred to as HBD), is a human-derived CPP with 27 amino acids (GPGLWERQAREHSERKKRRR ESECKAA), and was previously described by our group [16]. That study demonstrated that HBD was capable of transporting molecules such as apoptin into living cells both *in vitro* and *in vivo* without producing a toxic effect. Additionally, HBD has demonstrated its ability to enhance the anti-tumor effects of MAP30, another type I ribosome-inactivating protein [14]. Based on such findings, we predicted that integrating the c-terminus of TCS into HBD might facilitate the transport of TCS across cell membranes and enhance its toxicity to tumor cells.

In this study, we fused HBD with the C-terminus of recombinant TCS (rTCS) to improve the translocation efficiency of TCS. The recombinant fusion proteins with and without HBD were expressed and purified. The biological activities of both rTCS and HBD-conjugated rTCS were monitored using a *N*-glycosidase activity assay performed with a TNT® T7/SP6 Coupled Reticulocyte Lysate System. The cell penetration ability of HBD-conjugated rTCS was investigated in HeLa cells by confocal laser scanning microscopy, and its cytotoxicity was measured using the MTT assay. Apoptotic events in HeLa cells were detected by flow cytometry, and western blot studies were conducted to explore possible mechanisms for any improved anti-tumor activity.

2. Methods and materials

2.1. Materials

Plasmids pET-28a, pET-28a-HBD, pET-28b, and *Escherichia coli* strains DH5α and BL21 (DE3) were maintained in our laboratory. FITC was obtained from Sigma (St. Louis, MO, USA). RPMI 1640 growth medium and FBS were purchased from HyClone (Logan, UT, USA). BCA protein assay kits were supplied by Shanghai Sangon Biotech (Shanghai, China). HeLa, A549, 95D, and SMMC-7721 cells were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). RNasin® was from TAKARA, (Otsu, Shiga, Japan). Antibodies against caspase-8, caspase-9, PARP, and GAPDH were purchased from Proteintech (Wuhan, China). Primer molecules were synthesized by Sangon Biotech (Shanghai, China). Doubly distilled water purified with a Millipore purification system (Branstead, USA) to a specific resistance of >18 MΩ cm was used to prepare all solutions mentioned above.

2.2. Cell culture

Cells were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, streptomycin (100 U/mL), and penicillin (100 μg/mL); after which, they were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

2.3. Construction of expression vectors

Genomic DNA involving sequence encoding TCS was extracted from *T. kirilowii* Maxim by improved cetrimonium bromide (CTAB) methods [17]. The specific primer pairs used to generate the sequences for rTCS and rTCS-HBD from Genomic DNA are shown in Table 1, because the gene sequence encoding TCS does not contain any introns. The *rTCS* gene was inserted in the expression vector (pET-28a/b) between the *Nde* I and BamH I restriction sites. The *rTCS-HBD* gene was obtained by PCR, based on the known amino acid sequences of rTCS and HBD. The *rTCS-HBD* gene was then inserted in the expression vector (pET-28a/b) between *Nco* I and *Xho* I. The final plasmids (pET-28a-rTCS, pET-28a-rTCS-HBD, and pET-28b-rTCS-HBD) were checked for accuracy by DNA sequencing.

Table 1
Primers used to construct rTCS and rTCS-HBD proteins.

Name	Nucleotide sequence (5′ to 3′)	Orientation
rTCS	TTCATATGGATGTTAGCTTCCGTTTATCAGGTG	Upstream primer
	TTGGATCCTTATGCCATATTGTTTCTATTCAGCA GC	Downstream primer
rTCS-HBD	CCATGGGCGATGTTAGCTTCCGTTTATC	Upstream primer
	CTCGAGGGCGGCCTTGCACTC	Downstream primer

The underlined sequences in rTCS are *Nde* I and BamH I restriction sites, respectively. The underlined sequences in rTCS-HBD represent *Nco* I and *Xho* I restriction sites, respectively.

2.4. Expression and purification of rTCS and rTCS-HBD

The expression plasmids were transformed into *E. coli* BL21 (DE3) cells for production of recombinant proteins rTCS and rTCS-HBD. *E. coli* cells bearing the target gene were cultured for 8–12 h in 30 mL of Luria Broth (LB) culture medium containing kanamycin (50 μg/mL) at 37 °C. Next, the primary culture was inoculated into 200 mL of LB medium, and when the OD_{600} reading reached 0.8–1.0, IPTG was added at a final concentration of 1 mM. The *E. coli* were then cultured on a shaker for 16 h at 15 °C. Following culture, the bacteria were precipitated, resuspended in Tris-HCl buffer (20 mM Tris-HCl, 0.5 M NaCl, 5% glycerol, pH 8.5), and subjected to ultrasonication. The sonicated suspension was the centrifuged, and the cell lysate supernatant fraction was collected.

rTCS or rTCS-HBD contained in the supernatant fraction was purified using a Ni-NTA chromatography column. After washing the column with 20 mM imidazole, the desired protein was eluted with 200 mM imidazole. Imidazole was removed by dialysis against Tris-HCl buffer (20 mM Tris-HCl, 0.5 M NaCl, 5% glycerol, pH 8.5). 12.5% SDS-PAGE was performed to verify the purity of the isolated proteins.

2.5. Biological assays

The isolated proteins were assayed for their plasmid-cutting and *N*-glycosidase activities. For the plasmid-cutting activity assay, 2 μg of rTCS or rTCS-HBD was added to a 20 μL reaction system (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 100 mM NaCl, pH 8.0) with 1 μg of uncut plasmid DNA as the substrate, and incubated for 1 h at 37 °C. The banding pattern of the treated plasmid was analyzed by performing electrophoresis on a 1% agarose gel. The *N*-glycosidase activity of both proteins was measured using a cell-free translation system (Promega Corp; Fitchburg, WI, USA) containing rabbit reticulocytes and luciferase mRNA. Briefly, 2 μL of rTCS or rTCS-HBD of different concentrations (10^{-13} M or 10^{-9} M) was mixed with 12.5 μL of rabbit reticulocyte lysate, 0.5 μL of an amino acid mixture without methionine, 0.5 μL of an amino acid mixture without leucine, 1 μL of potassium chloride, 0.5 μL of luciferase control mRNA, 1 μL of RNasin® ribonuclease inhibitor, and 7 μL of ddH_2O (total volume: 25 μL). The system was then incubated in 30 °C water for 90 min. The amount of synthesized luciferase was detected using the luciferase assay system (Promega Corp).

2.6. FITC labelling of rTCS and rTCS-HBD

The prepared proteins were labelled with FITC using the following protocol: rTCS or rTCS-HBD was incubated with FITC (1 mg/mL, dissolved in DMSO) for 2 h at room temperature in the presence of 0.1 M sodium carbonate buffer (pH 9.0). The mixture was then dialyzed in PBS (pH 7.2) to remove any free FITC. The total protein/FITC ratio was determined by measuring light absorption at 280 nm and 488 nm, respectively, and the labelling ratio was calculated by the formula below. A BCA protein assay kit was used to determine concentrations of the labelled protein. When using the formula below, averages of 1.00 and 1.07 FITC molecules were bound to one molecule of rTCS and rTCS-HBD, respectively.

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