Antitumor effects of curcin from seeds of *Jatropha curcas*

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ABSTRACT

AIM: To study the antitumor effects of curcin from *Jatropha curcas*. **METHODS**: Antitumor activity of curcin was tested by MTT assay. The *N*-glycosidase activity of curcin was determined by characterization of R-fragment in gel. A cell-free system, rabbit reticulocyte lysate, was introduced to quantify the inhibitory activity of curcin on protein biosynthesis. **RESULTS**: The curcin had a powerful inhibitory action upon protein synthesis in reticulocyte lysate with an IC_{50} (95 % confidence limits) value of 0.19 (0.11-0.27) nmol/L. The IC_{50} (95 % confidence limits) of curcin on SGC-7901, Sp2/0, and human hepatoma was 0.23 (0.15-0.32) mg/L, 0.66 (0.35-0.97) mg/L, 3.16 (2.74-3.58) mg/L, respectively. Curcin was found to have no toxic to Hela cells and normal cells (MRC). After the rRNA of ribosome was treated with curcin and aniline at acidic condition, a cleaved R-fragment of approximately 450 nt appeared, but this fragment did not occur after treatment with curcin only. A comparison of the amino acid sequences of curcin, ricin A-chain and trichosanthin revealed that there were relatively high similarities among them. The percentages of homology between curcin and ricin A chain, between curcin and trichosanthin were found to be 54 % and 57 % respectively. Especially, the conserved residues forming the active sites of the A chain of ricin and trichosanthin occurred in curcin. **CONCLUSION**: Curcin has an obvious antitumor effect and its mechanisms are related to the *N*-glycosidase activity.

INTRODUCTION

Many plants contain proteins that are capable of inactivating ribosome and accordingly are called ribosome-inactivating protein (RIP). RIP are usually divided in 2 subgroups on the basis of their structure and functions: Type I RIP consisting of a single polypeptide chain with M_r 28 000-35 000 and alkaline isoelectric points (pI) of pH 8-10 with or without carbohydrates; type II RIP consisting of a catalytically active A chain linked to a cell-binding B chain. The A chain is the functional equivalent of a Type I RIP , and the B chain is a lectin[1]. For a long time the interest in RIP has been focused on developing antitumor drugs that selectively target to tumor cells[2]. Antitumor activity is related to *N*-glycosidase action, which cleaves the *N*-glycosidic bond of adenine A_{4234} of 28S rRNA. This makes ribosome unable to bind the elongation factors 1 or 2, consequently arresting protein synthesis[3,4]. In spite of having identical enzymatic activity, RIP has different effects on ribosome from different organisms. Interests in RIP, particularly in Type I RIP have been growing since they are used as components of 'immunotoxins', one type of hybrid molecules consisting of a toxic peptide chain linked to an antibody [5]. Immunotoxins will be promisingly used to eliminate such targets as harmful cell, neoplastic, immunocompetent and parasitic cells. However, there are some problems in the application of immounotoxins, such as

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poor stability, immunogenicity and promotion of vascular leak syndrome, which would raise serious questions on their application[6]. At present, it is useful to look for new RIP to identify those with the highest antitumor activity, to select the most suitable ones for humor therapy and to overcome the immune response that follows clinically oriented administration of RIP conjugates.

Distributed in many tropical and subtropical countries, *Jatropha curcas* L belongs to the family Euphorbiaceae. The toxicity of the whole seed from *Jatropha curcas* has been known for a long time. Its toxicity has been attributed to a protein component. A toxic protein was isolated from the seeds of *Jatropha curcas* by Felke (1914), and was designated as "curcin" by him. He proposed that the curcin was a kind of toxalbumin[7]. Barbieri (1993) reported the curcin was type I RIP, a single chain protein[1]. But it is indefinite that which curcin has the biological activities and enzymatic activities. The purpose of this study was to investigate the sensitivity of a variety of cultured cancer cells and normal cells to curcin, and to explore the application of curcin for the construction of immunotoxins.

MATERIALS AND METHODS

Materials The matured seeds of *Jatropha curcas* were harvested from Panzihua city, Sichuan Province, China in autumn. Curcin (M_r 28 200) was prepared from the seeds of *Jatropha curcas* as described[8,9] with slight modifications. Curcin was further purified using the Sephedex G-100 (Phamacia). Trichosanthin was purchased from Sigma Chemical Co, USA. Curcin and trichosanthin were analyzed by electrophoresis.

Cytotoxicity assays The cells used were SGC-7901 (gastric cancer cell line), Sp2/0 (mouse myeloma cell line), human hepatoma, Hela (carcinoma cell line) and MRC (human embryo lung diploid cell line) were provided by West China University of Medical Sciences. Cells were maintained as monolayer cultures in RPMI-1640 medium supplemented with 10 % fetal calf serum (Gibco BRL) and incubated at 37 °C in a humidified incubator at 5 % CO_2. Toxicity tests were described as before[10], five sorts of cells were seeded independent in a 96-well plate with the final volume 200 μL containing 1×10^4-1×10^5 cells per well. The plates were incubated at 37 °C for 48 h. Curcin in PBS was proportionally diluted with RPMI-1640, and 50 μL of each solution was added to triplicate wells (for negative reaction actidione was added to a final concentration of 0.05 mg/L, alternatively, for positive reaction RPMI-1640 was added). After 72 h, 0.1 mg (20 μL of 5 g/L) MTT was added to each well and incubated at 37 °C for 4 h. The medium was removed and 150 μL of Me_2SO was added into each well after the plate was shaken thoroughly for 1 min. The absorbances of the samples were measured at 570 nm with a spectrophotometer [Moded 3550 Microplate Reader (BIO-RAD)].

Assay for cell-free translation-inhibiting activity Protein synthesis was measured with a lysate of rabbit reticulocytes. Rabbit reticulocyte lysate was prepared as described by Sambrook[11] and protein synthesis was assayed as Merrick[12] and Sambrook[11]. The test sample (10 μL) was mixed with 10 μL of hot mixture [reaction mixture contained, total volume of 1000 μL, it include 250 μL phosphocreatine 100 mmol/L; 100 μL of amino acids (Q,W,D,N,A,G 2 mmol/L, other 1 mmol/L, minus Leucine); 20 μL of GTP 100 mmol/L; 50 μL of ATP 10 mmol/L; 200 μL of KCH_3COO 2 mol/L pH 7.5; 10 μL of $Mg(CH_3COO)_2$ 1 mol/L; 50 μL of creatine phosphate kinase 10 g/L, and 320 μL of H_2O], 25 μL of working rabbit reticulocyte lysate containing hemin 0.1 mmol/L, 2 μL of [^{3}H]leucine (74 kBq) and 3 μL of H_2O. The mixture was incubated at 37 °C for 30 min. Further incubation for 10 min allowed decolorization and tRNA digestion. One volume of the reaction mixture was then added to 40 % trichloroacetic acid with 2 % casein hydrolysate in a 96-well plate to precipitate radioactively labeled protein. The precipitate was collected on a glass fiber (Whatman GF/A filter), then washed and dried with absolute alcohol passing through a cell harvester attached to a vacuum pump. The filter was suspended in scintillant (4 % POP-0.04 % POPOP) and counted in an LS 6500 Beckman liquid scintillation counter.

Assay for *N*-glycosidase activity The assay was performed in accordance with the procedure of Endo *et al*[3] with some modifications. Rat liver ribosome was prepared as described by Speeding[13]. The 80 μL of reaction mixture, containing sample (curcin or trichosanthin) and buffer A (Tris-HCl 20 mmol/L pH 7.6, KCl 50 mmol/L, $MgCl_2$ 5 mmol/L, ribosome 1.5 $A_{260\ nm}$ value) was incubated at 37 °C for 10 min. The reaction was arrested by adding 150 μL of H_2O and appropriate volume of SDS to a final concentration of 0.5 %. One volume of phenol, equilibrated with Tris-HCl 0.1 mol/L pH 8, was added to extract RNA and the mixture was centrifuged at 13 000×*g* for 5 min. The supernatant was re-extracted with 1 volume of chloroform: isoamyl

alcohol (24:1, v:v). RNA in the supernatant was precipitated by adding 2.5 volumes of cold absolute ethanol and 0.1 volume of CH_3COONa 3 mol/L. Having been incubated at -20 °C for 30 min, RNA was pelleted by centrifugation and washed twice with cold 70 % ethanol, then dried by vacuum centrifugation. RNA was dissolved in 20 μL of DEPC-treated water. An aliquot of acidic aniline (aniline: acetic acid: DEPC-treated water, 1:1:3.5, v:v:v) was added and incubated at 60 °C for 10 min. RNA was recovered as described previously. Electrophoresis was carried out in 3.5 % urea-denaturing polyacrylamide gel at a constant voltage of 100 V. The RNA bands were visualized on a UV transilluminator and photographed with the BioImaging Systems (SYNGENE, a division of SYNOPTICS LTD).

RESULTS

Cell-free translation-inhibiting activity The purified curcin inhibited cell-free translation in the reticulocyte lysate system with an IC_{50} (95 % confidence limits) of 0.19 (0.11-0.27) nmol/L (Fig 1).

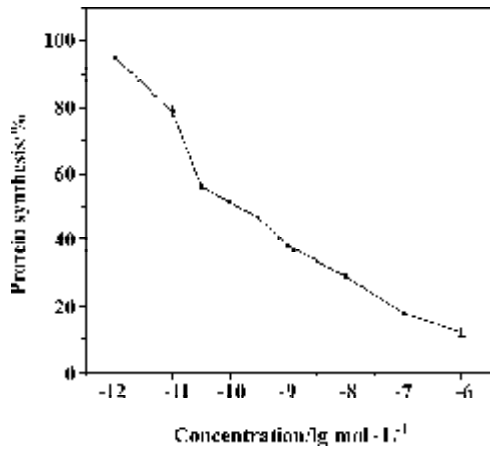

Fig 1. Inhibitory activity of curcin from *Jatropha curcas* in a cell-free protein synthetic system from rabbit reticulocyte lysate. *n*=3 wells. Mean±SD.

Cytotoxicity The curcin also inhibited protein synthesis of intact cells. The curcin inhibited protein synthesis of SGC-7901, Sp2/0 and human hepatoma at a low concentration; the IC_{50} (95 % confidence limits) of curcin on SGC-7901, Sp2/0, or human hepatoma were 0.23 (0.15-0.32) mg/L, 0.66 (0.35-0.97) mg/L, 3.16 (2.74-3.58) mg/L, respectively. The curcin also inhibited protein synthesis of Hela cells and normal cells (MRC), but only at concentrations thousand-fold higher than those in SGC-7901 (Fig 2).

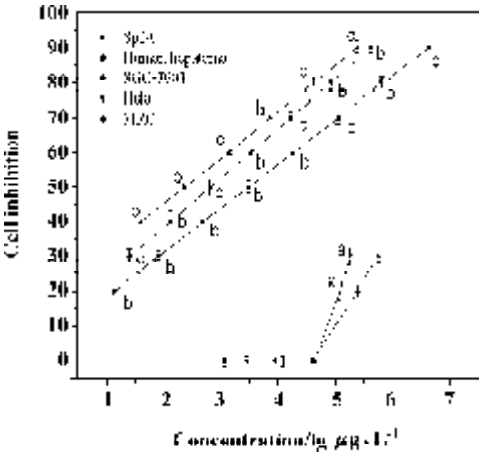

Fig 2. Determination of antitumor activity and cytotoxicity of curcin on SGC-7901, Sp2/0, human hepatoma, Hela cells, and normal cells (MRC). *n*=3 wells. Mean±SD. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* normal cell (MRC). Inhibition (%) by curcin=[1-($A_{570\,nm}$ value of curcin-treated culture/$A_{570\,nm}$ value of control culture)]×100 %. $A_{570\,nm}$ value of control was 1.17±0.05.

***N*-glycosidase activity** The *N*-glycosidase activity of curcin was examined by incubating ribosome with various amounts of curcin or trichosanthin, and the extracted rRNA was analyzed by gel electrophoresis. The characterizations of *N*-glycosidase activity are performed by exhibiting RNA fragment in gel. In Fig 3, lane 1 represents samples in the absence of toxin (control); lanes 2 and 3, samples treated with 5 ng curcin; lanes 4 and 5, samples treated with 10 ng trichosanthin. The arrow indicates the position of the RNA fragment after the ribosomal RNAs were treated by aniline. As shown in Fig 3, when the rRNA from curcin- treated ribosome was further treated by aniline at acidic pH, a cleaved fragment of approximately 450 nt appeared (lane 2), which is similar to that findings from trichosanthin/aniline-treated ribosome (lane 4). This fragment did not occur when ribosome was treated only with curcin or trichosanthin respectively. These results suggest that curcin has RNA *N*-glycosidase activity as trichosanthin does.

Activity domain of *N*-glycosidase in amino acid sequence of curcin A comparison of the amino acid sequences of curcin with other RIP, eg, ricin A-chain and trichosanthin, revealed that there existed relatively high similarity among them. The percentages of identity between curcin and ricin A-chain[14], and between curcin and trichosanthin[15] were found to be 54 % (156/287), 57 % (138/241) respectively (Fig 4).

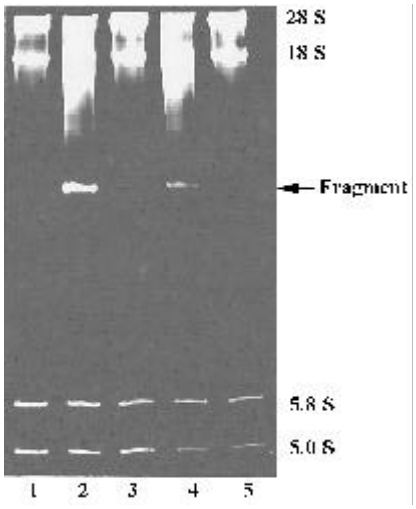

Fig 3. A 3.5 % urea PAGE analysis of rat liver ribosome RNA treated with purified curcin. Lane 1: control; Lane 2: treated with 5 ng curcin and aniline; Lane 3: treated with 5 ng curcin only; Lane 4: treated with 10 ng TCS and aniline; Lane 5: treated with 10 ng TCS only. The arrow indicates the R-fragment released as a consequence of ribosome-inactivating protein action after acid-aniline treatment.

It is interesting to note that the amino acids Asn-78, Tyr-80, Tyr-123, Arg-134, Gln-173, Glu-177, Arg-180, Glu-208, Asn-209 and Trp-211 formed jointly the activity site region of ricin A-chain, and the amino acids Tyr-14, Phe-17, Arg-22, Tyr-70, Tyr-111, Gly-128, Ala-148, Gln-156, Glu-160, Arg-163, Glu-189, Tro-192, and Leu-241 formed cooperatively the activity site region of trichosanthin (Fig 4). These amino acid residues are of importance to stabilize the interactions between the bases of RNA and RIP, which are involved in the N-glycosidase activity. Five amino acid residues, ie, Tyr-80, Tyr-123, Glu-177, Arg-180, and Trp-211, build up the active site of the A-chain of ricin based on the model of X-ray analysis[16]. In the model, Glu-177 and Arg-180 are particularly important. Glu-177 affects the speed of enzymatic reaction, and Arg-180 facilitated the breakage of *N*-glycosidic bond by donating proton to N_3 of adenine from substrate. Next to the active site of ricin A-chain, other five amino acid residues (Asn-78, Arg-134, Gln-173, Glu-208, and

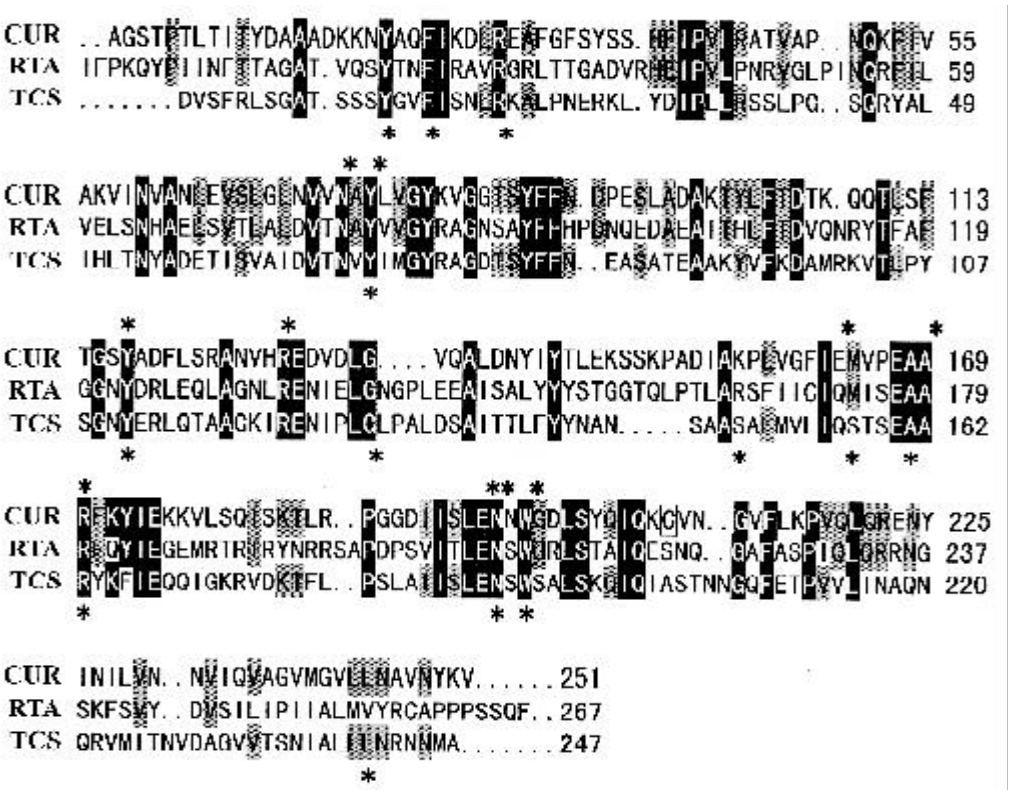

Fig 4. Comparison of amino acid sequence of curcin and other RIP. Sequence alignment was performed with the OMIGA program by using published sequence of RTA: ricin A-chain (*Ricinus communit*)[13], TCS: trichosanthin (*Trichosanthes kirilowii*)[14] and submitted to GenBank/EMBL/DDBJ sequence of CUR: curcin (*Jatropha curcas*) (the accession number AY069946). Identical amino acids of the three proteins are shown in black background, between two species are shown gray background. Activity sites in ricin A chain are shown with "*" in upside of sequence. Activity sites in trichosanthin are shown with "*" in underside. Cys-209 was shown in box and black.

Glu-209) are probably necessary to maintain its catalytic conformation[17,18]. The key active site residues of trichosanthin, including Gln-156, Glu-160, Arg-163, and Glu-189 are directly involved in catalysis, whereas Tyr-70 and Tyr-111 have a crucial role in binding the rRNA loop[19]. All amino acid residues forming the active sites of the A chain of ricin and trichosanthin were also found in curcin at corresponding positions (Tyr-20, Phe-23, Arg-28, Asn-74, Tyr-76, Tyr-117, Arg-128, Gly-134, Ala-155, Glu-167, Ala-169, Arg-170, Glu-196, Asn-197, Trp-199, and Leu-244), with the exception of one residue, Gln-173 in ricin and trichosanthin was replaced by Glu-163 in curcin. But Gln and Glu are similar in property except that Gln has an extra $-NH_3$ in side chain. These residues are consistent with the RIP activity, which might constitute the activity site region of curcin. It has been known that trichosanthin is homologous structurally to the ricin A chain, and it was found that the protein tertiary structure is even more conservative than the primary sequence. This similarity strengthens the notion that there could be a strong preservation of three-dimensional structure in these proteins with similar catalytic functions, especially with conserved critical amino acid residues in the region of the active site. Curcin possesses conserved residues of similar amino acids around the proposed active site of ricin A chain and trichosanthin. The results of those analyses indicate that curcin possesses an *N*-glycosidase activity, which is confirmed by the result of the activity assays.

DISCUSSION

Like other RIP, curcin possessed a cell-free translation inhibitory activating property. Its activity is higher than most RIPs, such as saporin (0.5 nmol/L), luffun A (1 nmol/L) and luffin B (4 nmol/L), trichosanthin (0.32 nmol/L)[1]. Moreover, it is clear that curcin has a higher RNA *N*-glycosidase activity than trichosanthin and its inhibition to protein synthesis might be more efficient than trichosanthin.

Previous work showed that curcin displayed very low toxicity to Ehrlich ascites cells[8]. In order to determine its potential application for the construction of immunotoxins for cancer therapy to a broad variety of cancer cells, we investigated the toxicity of curcin against the following cells: SGC-7901, Sp2/0, Human hepatoma, Hela cells and normal cells (MRC). Different effects of curcin on various cells examined were observed, with SGC-7901, Sp2/0 and human hepatoma being the most sensitive to curcin, and Hela cells being the most resistant to curcin. This implies that curcin is suitable for the preparation of immunotoxins. Many immunoconjugates of RIP and specific antibodies have been evaluated *in vitro* and *in vivo* as potential therapeutic agents for the treatment of cancer and autoimmune diseases. Immunotoxins have been applied to therapeutic uses and an important consideration for immunoconjugate assembly is the nature of the linkage between antibody and RIP. A disulphide linkage is usually thought to be essential for maximal cytotoxicity. Most type I RIP do not have any free cysteine residues, which necessitates for the modification of both antibody and RIP with chemical agents to produce the disulphide bond. Fortunately, curcin contains one cysteine residue and it may directly form a disulphide bond with an active antibody thiol group via adisulphide-exchange reaction. Therefore, curcin is a novel, cysteine-containing RIP, which might be ideal for the preparation of immunoconjugates with promising use as a chemotherapeutic agent for the treatment of various cancers.

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