Cytotoxic activity of ribonucleolytic toxin restrictocin-based chimeric toxins targeted to epidermal growth factor receptor

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Received 3 March 1997

Abstract Targeted toxins represent a new approach to specific cytocidal therapy. The ribonucleolytic protein toxin restrictocin is a potent protein synthesis inhibitor produced by the fungus *Aspergillus restrictus*. In the present study we have constructed two restrictocin based chimeric toxins where human transforming growth factor alpha (TGFα) has been used as a ligand. TGFα is a single chain polypeptide, which binds to epidermal growth factor receptor (EGFR) and causes proliferation in a large number of cancers. The ligand has been separately fused either at the amino terminus or carboxyl terminus of restrictocin, giving rise to TGFα-restrictocin and restrictocin-TGFα respectively. The fusion proteins were overexpressed in *Escherichia coli* and purified from inclusion bodies by a denaturation-renaturation protocol. Both the chimeric toxins actively inhibited eukaryotic protein synthesis in a cell free in vitro translation assay system. These chimeric toxins selectively killed human epidermal growth factor receptor positive target cells in culture. Among the two proteins, restrictocin-TGFα was more active than TGFα-restrictocin on all the cell lines studied.

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Key words: Delivery; Transforming growth factor α; Immunotoxin; Targeting

1. Introduction

One approach to cancer treatment that has received considerable attention in the past few years is to direct protein toxins to cancer cells [1,2]. Toxins have been genetically fused to antibodies and growth factors to generate recombinant immunotoxins or chimeric toxins that can selectively kill cells bearing specific receptors or antigens [1,2]. The potent inhibitory activity of bacterial toxins like *Pseudomonas* exotoxin (PE) and diphtheria toxin (DT) as well as the plant toxin ricin has been exploited for making chimeric toxins [1–4]. The choice of both the toxin and the target is extremely important in the construction of immunotoxins. An ideal target is either unique to the malignancy or is present in a large excess on the diseased cells to be eliminated [1]. Chimeric toxins have been made using a variety of ligands such as transforming growth factor alpha (TGFα), interleukin 2, interleukin 4, CD4, and single chain antibodies [1,2,4]. TGFα is a mitogenic polypeptide of 50 amino acids and has structure and biological properties similar to that of epidermal growth factor (EGF) [5]. Both TGFα and EGF bind to subdomain III of the extracellular portion of epidermal growth factor receptor (EGFR), causing receptor autophosphorylation and initiation of a cascade of biochemical reactions that ultimately leads to DNA synthesis and cell division [6,7]. Elevated expression of EGFR is a characteristic of several malignancies including those of breast, bladder, prostate, lung, cervical, thyroid, esophageal and ovarian origin [8]. The EGF receptor contains a specific internalization sequence which contributes to the rapid internalization of the ligand receptor complex. Overexpression on various cancers and rapid internalization of the EGFR makes this receptor a good target for the selective delivery of anticancer agents [8]. The factors that influence the potency of immunotoxins in cancer therapy are their specificity, cytotoxicity, tumor penetration, toxicity and immunogenicity [1,4]. Though highly potent and specific immunotoxins have been made, the toxicity and immunogenicity of these molecules has limited their application [2]. Studies are now focused on engineering the molecules further to resolve these issues. In addition, it would be helpful if new toxin molecules with low non-specific toxicity and immunogenicity are discovered, that can be used in the construction of chimeric toxins [1,2].

Ribotoxins are ribosome inactivating proteins produced by *Aspergillus*, that cleave a single phosphodiester bond on the 3′ side of G4325 in eukaryotic 28S rRNA [9]. The cleavage site is embedded in a purine rich single stranded segment of 14 nucleotides called the sarcin/ricin loop, which is one of the most strongly conserved regions of rRNA. Cleaved 28S rRNA is unable to participate in EF-1 dependent binding of aminoacyl tRNA and EF-2 catalyzed GTP hydrolysis and translocation during protein synthesis, leading to cell death [10]. Restrictocin, one of the members of the ribotoxin family, is a 149 amino acid single chain non-glycosylated polypeptide and a potent inhibitor of translation [11]. Restrictocin cannot enter into a cell on its own, and needs to be introduced inside the cell by artificial means to manifest its effect. The killing potency and absence of cell binding activity make restrictocin a suitable molecule to be used as a toxin moiety of an immunotoxin [12–14]. Previously we have expressed restrictocin in *E. coli* and shown that the recombinant toxin is as active as the native protein [15]. We have also shown that recombinant restrictocin has low toxicity, and is poorly immunogenic when tested in mice [14]. We have earlier made active chemical conjugates with recombinant restrictocin targeted at the human transferrin receptor [14]. Here we report on the construction and characterization of TGFα-based chimeric toxins of restrictocin targeted at the human epidermal growth factor receptor.

2. Materials and methods

2.1. Cell lines and cell culture

A431 (epidermoid carcinoma), A549 (lung carcinoma), K562 (erythroleukemia) (all human) and L929, a mouse fibroblast cell line, were obtained from ATCC. All the cell lines were maintained in exponential growth in RPMI 1640 medium supplemented with 2 mM

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PII S0014-5793(97)00333-5

glutamine and 10% fetal calf serum, at 37°C in a humidified atmosphere containing 5% CO_2.

2.2. Construction of plasmids

To construct pTGFα-restrictocin, plasmid pVC320, containing TGFα as a *Nde*I-*Nde*I insert, was digested with *Nde*I [16]. The purified insert was ligated into *Nde*I digested pRest, a T7 promoter based bacterial expression vector, that contains restrictocin as an *Nde*I-*Eco*RI fragment. The clone so generated contained DNA coding for TGFα at the 5′ end of restrictocin DNA. To construct pRestrictocin-TGFα, restrictocin DNA was amplified by PCR from the plasmid pRest to create *Nde*I sites at both the ends of the fragment [15]. PCR was used to create *Nde*I and *Eco*RI recognition sites respectively at the 5′ and 3′ ends of the TGFα fragment, using pVC320 [16] as template. Amplified fragment of restrictocin was digested with *Nde*I while TGFα fragment was digested with *Nde*I and *Eco*RI. Both the fragments were ligated in *E. coli* expression vector pVex11, digested with *Nde*I and *Eco*RI. This gave rise to pRestrictocin-TGFα, containing DNA coding for TGFα at the 3′ end of restrictocin. The correct clones were identified by restriction analysis and protein expression. *E. coli* strain DH5α was used for DNA manipulation.

2.3. Expression and purification of chimeric toxins

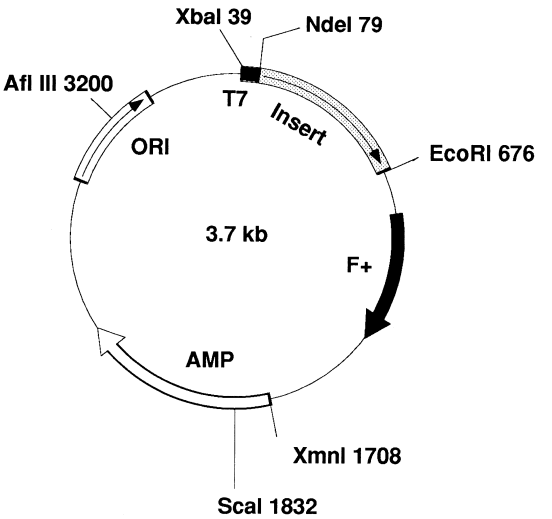
E. coli strain BL21 (λDE3) was used for protein expression. Cells were separately transformed with pTGFα-restrictocin or pRestrictocin-TGFα and grown in super broth containing 100 μg/ml of ampicillin at 37°C with shaking. At an OD_{600} of 2.0, the cells were induced with 1 mM IPTG, and 2 h later, they were harvested by centrifugation at 6000 rpm for 20 min. Inclusion bodies, isolated from the total cell pellet using the protocol described [17], were denatured in guanidine hydrochloride and reduced by dithioerythritol, followed by renaturation in refolding buffer containing arginine and oxidized glutathione. Renatured material was dialyzed against 50 mM phosphate buffer (PB), pH 6.5, containing 100 mM urea and purified on a S-Sepharose column using an FPLC system (Pharmacia). The fusion proteins were purified to homogeneity by gel filtration chromatography on a TSK 3000 column (LKB).

2.4. Ribonucleolytic activity of chimeric toxins

The ribonucleolytic activity of fusion proteins was assayed, in vitro, by measuring the inhibition of translation of endogenous globin mRNA of rabbit reticulocyte lysate in the presence of toxins. Rabbit reticulocyte lysate was prepared and the assay performed as described [18]. Incorporation of [^{3}H]leucine was measured as a function of toxin concentration.

2.5. Cytotoxicity and specificity of chimeric toxins

Activity of fusion proteins was evaluated on human carcinomas by assaying protein synthesis, in the absence and presence of various concentrations of toxins. After 48 h of incubation, with the toxin, adherent cells (A431, A549) were washed twice with leucine free DMEM and pulsed for 2 h with 0.25 μCi of [^{3}H]leucine. Suspension cells (K562) were directly labeled with 0.5 μCi of [^{3}H]leucine for 2 h. The cells were harvested on a glass fiber filter and counted using a LKB β-plate counter.



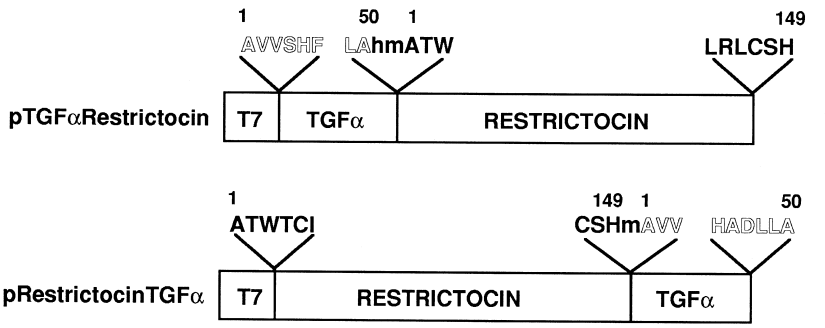


Fig. 1. Schematic representation of chimeric toxins. In pTGFα-restrictocin, DNA encoding TGFα has been fused at the 5′ end of restrictocin DNA, while in the case of pRestrictocin-TGFα, it has been fused at the 3′ end of restrictocin DNA. Amino acids, as single letter codes, shown in bold uppercase letters are of restrictocin, while those shown in outlined uppercase represent amino acids of TGFα. Extra amino acids introduced in the protein, because of the cloning strategy, are mentioned in lowercase letters. ORI: Bacterial origin of replication; F+: Bacteriophage origin of replication; AMP: ampicillin resistance gene; T7: T7 promoter.

2.6. Binding assay

The binding of fusion proteins to EGF receptor was evaluated by estimating their ability to displace iodinated epidermal growth factor bound to A431 cells. EGF was iodinated using iodogen method [19]. In a 24 well plate, 40 000 cells were plated per well and left overnight at 37°C to adhere. Cells were washed twice with binding buffer (RPMI 1640 containing 2 mM glutamine, 25 mM HEPES pH 7.2 and 0.1% BSA) and used for the assay.

3. Results

3.1. Construction and expression of chimeric toxins

Plasmids pTGFα-restrictocin and pRestrictocin-TGFα are T7 promoter based bacterial expression vectors, containing DNA encoding TGFα cloned respectively at the 5′ and 3′ end of restrictocin DNA. Fig. 1 shows the schematic representations of these constructs. The constructs were separately expressed in *E. coli* strain BL21 (λDE3), and in both cases, a protein of the expected molecular weight (22 kDa) was over-expressed as seen in the total cell pellet (Fig. 2, lane 1). Both the fusion proteins accumulated in spheroplast, in the form of insoluble inclusion bodies (Fig. 2, lane 2). The proteins were purified from the inclusion bodies by a three step purification protocol. In the first step, recombinant protein was isolated by denaturation of purified inclusion bodies and renatured in vitro. After renaturation, in the second step, chimeric toxins were purified on a cation exchange column (Fig. 2, lane 3). Proteins at this stage were almost 90% pure. In the last step, protein from the cation exchange column was further purified on a gel filtration column to separate aggregated multimers of the fusion proteins from the monomeric units (Fig. 2, lane 4). For all subsequent studies, monomeric protein obtained from the gel filtration column was used.

3.2. In vitro activity of chimeric toxins

Earlier we have shown that recombinant restrictocin potently inhibits protein synthesis in a rabbit reticulocyte based cell free translation assay system [15]. TGFα-containing chimeric toxins were also evaluated in vitro, for their translation inhibition activity. As shown in Fig. 3, both proteins effectively inhibited translation of endogenous globin mRNA in the assay. Inhibition was dose dependent and the amount of protein required to decrease protein synthesis to half (ID_{50}) was 0.15 and 0.40 nM for TGFα-restrictocin and restrictocin-TGFα respectively. TGFα-restrictocin and restrictocin-TGFα

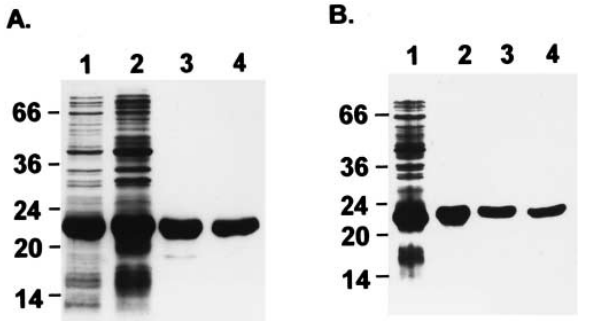


Fig. 2. Purification of chimeric toxins. A and B represent TGFα-restrictocin and restrictocin-TGFα respectively. A 12% reducing SDS-polyacrylamide gel was run and stained with Coomassie blue. Lane 1, total cell pellet; lane 2, inclusion bodies; lane 3, protein after S-Sepharose column; lane 4, protein after gel filtration. Molecular weight markers are shown $\times 10^{-3}$ Da.

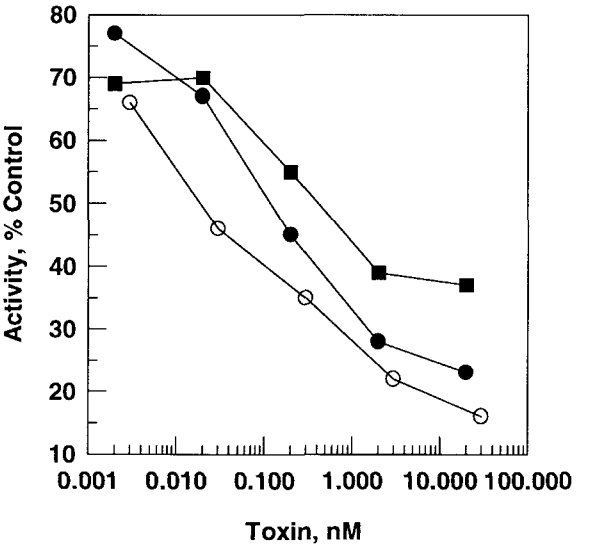


Fig. 3. Activity of chimeric toxins in cell free translation assay system. Rabbit reticulocyte lysate was incubated with different concentrations of TGFα-restrictocin (●), restrictocin-TGFα (■), and restrictocin (○), for 1 h at 30°C, and proteins were precipitated with 20% TCA. Precipitated material was collected on a glass fiber filter and counted on a beta counter for [^{3}H]leucine incorporation. The concentration of toxin required to inhibit protein synthesis by 50% (ID_{50}) was calculated by comparison with uninhibited protein samples.

were respectively six-fold and 16-fold less active than recombinant restrictocin, which had an ID_{50} of 0.025 nM in the same assay (Fig. 3).

3.3. Cytotoxicity and specificity of chimeric toxins

The cytotoxic potential of the two fusion proteins was evaluated on two target cell lines A431 and A549. The fusion proteins inhibited protein synthesis in target cells in a dose dependent manner (Fig. 4). Both restrictocin-TGFα and TGFα-restrictocin showed maximum activity on A431 cells, which express EGF receptor in excess, with ID_{50}s of 1.66 nM and 4.16 nM respectively. Restrictocin-TGFα was found to be 2.5-fold more active than TGFα-restrictocin (Table 1). On A549, a human lung carcinoma, restrictocin-TGFα showed activity similar to that on A431 but TGFα-restrictocin was found to have no cytotoxicity. On a EGFR negative cell line K562, both TGFα-restrictocin and restrictocin-TGFα were inactive. To check whether these chimeric toxins also recognize EGFR across the species, cytotoxic effect of these toxins was evaluated on a mouse fibroblast cell line L929. Both proteins had no cytotoxic effect on a murine cell line L929, as no inhibition of protein synthesis was observed even at a concentration of 200 nM, indicating the specific binding of the proteins to the human EGF receptor (Table 1). Restrictocin alone did not show any activity up to 115 nM (data not shown). Specificity of the chimeric toxins for the EGFR was checked by measuring their cytotoxic activity in the presence of free EGF. Excess of free EGF (1 μM) prevented the cytotoxic effect of fusion toxins (Fig. 4).

3.4. Binding of chimeric toxins

As TGFα-restrictocin and restrictocin-TGFα showed differential activity on target cell lines, their binding activities to EGF receptor were checked by measuring the ability of the

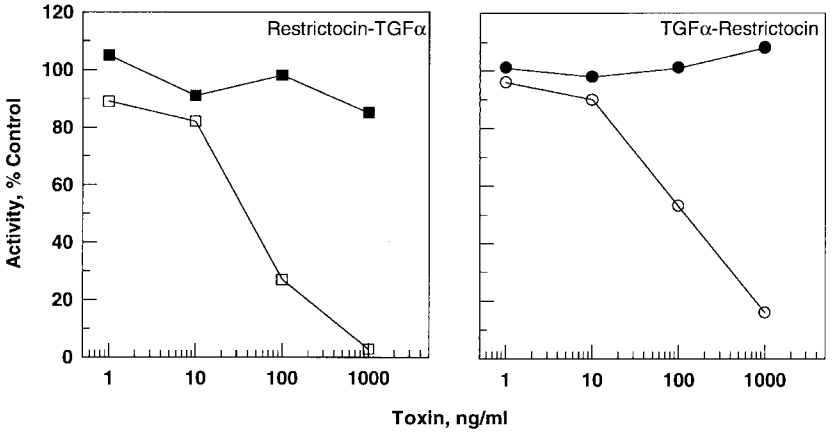


Fig. 4. Activity of chimeric toxins on A431 cell line. TGFα-restrictocin (circles) or restrictocin-TGFα (squares) was added to the cells in the absence (open symbols) or in the presence (closed symbols) of excess 1 μM EGF for 48 h at 37°C. [^{3}H]Leucine incorporation was measured as described. The results are calculated as percentage of total radioactivity incorporated in the cells incubated without chimeric toxin.

two fusion proteins to compete with [^{125}I]EGF for binding to the EGFR. As shown in Fig. 5, both proteins were found to be equally potent in their ability to bind to the EGF receptor. However, their binding activity was 50-fold lower than that of the EGF.

4. Discussion

EGFR has been used as a target in the past with PE and DT based chimeric toxins [8,16,20]. TGFα-PE40, a fusion protein in which TGFα is fused at the amino terminus of a truncated form of PE, has undergone clinical trials for the intravesical therapy of bladder cancer [21]. $DAB_{389}EGF$, which contains EGF fused at the carboxyl terminus of a truncated form of DT, has shown potent cytotoxic activity on various target cell lines [8]. These studies show that a cytotoxic agent which targets the EGF receptor can be highly selective and effective for the treatment of carcinomas, characterized by elevated EGF receptor expression. Previously we have demonstrated that recombinant restrictocin is poorly immunogenic and has low in vivo toxicity in mice [14]. It has also been used as a toxin component in the construction of immunotoxin [14]. In the present study we have explored the possibility of using restrictocin to construct chimeric toxin using TGFα as ligand. In the two fusion proteins made, the site of attachment of the ligand with respect to the toxin is different. TGFα-restrictocin and restrictocin-TGFα contain TGFα respectively at the amino and carboxyl ends of restrictocin. The constructs were expressed in *E. coli* and the proteins were purified to homogeneity. Although TGFα-restrictocin had 2-fold higher in vitro ribonucleolytic activity than restrictocin-TGFα, and they both bound to the EGFR with equal affinities, yet on target cells restrictocin-TGFα was found to be more toxic compared to TGFα-restrictocin. It clearly indicates that the preferred site of ligand attachment on restrictocin is at its carboxyl terminus. The site of attachment of the ligand in a chimeric toxin critically influences its cytotoxic potential. In DT based chimeric toxins, molecules where the ligand is attached at the carboxyl terminus are invariably more active than molecules where the ligand is present at the amino terminus [1]. In the case of PE, the ligand is attached at the amino terminus of the toxin, as the carboxyl terminus of the toxin contains an endoplasmic retention signal sequence and cannot be blocked [1].

Both chimeric toxins did not retain the full enzymatic activity of restrictocin or the binding activity of the ligand. This could be due either to misfolding of one protein with respect to the other or to a steric hindrance posed by the additional protein. However, despite the reduction in enzymatic and binding activities of its components, the fusion proteins manifested potent and specific cytotoxic activities on the target cells.

The cytotoxic activity of an immunotoxin or chimeric toxin involves its binding to the target, intracellular processing, and translocation to the intracellular target [1]. All these steps are critical. As both restrictocin based chimeric toxins, TGFα-restrictocin and restrictocin-TGFα bind EGFR with similar affinities, differences in their cytotoxic activities appear to be due to the differential intracellular processing and/or translocation. In this context, location of the binding ligand on

Table 1
Cytotoxic activity of TGFα-based chimeric toxins

Cell line	Origin	ID_{50} (nM)	
		TGFα-Restrictocin	Restrictocin-TGFα
A431	Epidermoid carcinoma	4.16	1.66
A549	Lung carcinoma	>40.00	1.87
K562	Erythroleukemia	>40.00	>40.00
L929	Mouse fibroblast	>200.00	>200.00

A431 or A549 cells were seeded at a density of 5×10^3 cells per well in 96 well plates, 16 h before the addition of toxin. K562 was seeded in 80% leucine free DMEM containing 18% RPMI 1640 and 2% serum, and used immediately. Toxin was diluted in Dulbecco's PBS containing 0.2% human serum albumin. ID_{50} is the amount of chimeric toxin required to decrease protein synthesis to 50% of control, where no toxin has been added.

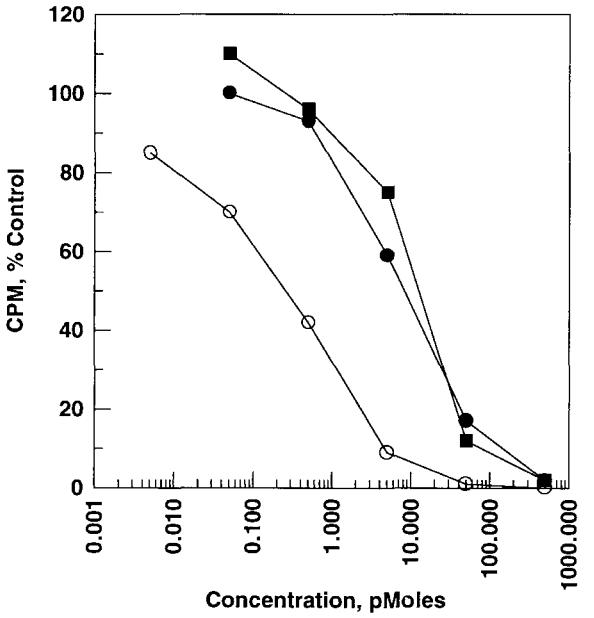


Fig. 5. Binding activity of chimeric toxins. ^{125}I-labeled EGF, at a concentration of 1.5 ng per well, was added with various concentrations of TGFα-restrictocin (●), restrictocin-TGFα (■) and EGF (○) on A431 cells, in 0.2 ml of binding buffer. Cells were incubated for 1 h at 4°C. At the end of incubation, cells were washed three times with binding buffer, and lysed with 10 mM Tris-HCl containing 0.5% SDS and 1 mM EDTA. The bound ligand was counted in a gamma counter (LKB).

restrictocin appears to be critical and a carboxyl terminal fusion appears to be tolerated better.

In conclusion, we have demonstrated that it is possible to make restrictocin-based chimeric toxins directed at the human EGF receptor. Chimeric toxins containing ligand at the carboxyl terminus of restrictocin had better cytotoxic activity. The molecules generated in the current study contain toxin and ligand fused in tandem without any linkers. Modification of these proteins by introducing a spacer between the two components may improve the folding of the molecule, thereby further improving their biological activities. As restrictocin has already been shown to have low in vivo toxicity and immunogenicity, it holds potential for being used as a toxin moiety in targeted therapy in the future.

Acknowledgements: The work was supported by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India. The technical assistance of Mr. Kevlanand is acknowledged. D.R. is a Senior Research Fellow of Council of Scientific and Industrial Research (CSIR).

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