*Annu. Rev. Pharmacol. Toxicol. 1992. 32:579–621*




# CYTOTOXIC CONJUGATES CONTAINING TRANSLATIONAL INHIBITORY PROTEINS

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KEY WORDS: targeting toxins, immunotoxins, chemotherapy, carrier molecules, growth factor conjugates

## INTRODUCTION

Cytotoxic drugs administered during conventional chemotherapy are taken up by sensitive nontarget organs besides the tumor cells and this often leads to unwanted side effects. These problems have initiated studies during the past decade to develop novel methods in which the therapeutic reagents could be directed to specific cell populations to widen the therapeutic window. The concept of targeting drugs was proposed nearly a hundred years ago by Paul Erlich, who envisioned the possibility of transporting toxic substances to tumor cells by carrier molecules (magic bullets). This novel approach became a reality with the advent of tumor selective monoclonal antibodies (Mabs) produced by somatic cell hybridization. Both cytotoxic drugs and toxin polypeptides have been chemically linked to Mabs. During the past decade various molecules have been produced and tested in a number of model systems (reviewed in 1–10). The range of targets includes (*a*) ex vivo treatment of bone marrow grafts to remove contaminating leukemic cells and alloreactive T cells, (*b*) administration of conjugates to inhibit tumor growth

in restricted anatomical spaces such as the peritoneum, and (*c*) systemic application of conjugates to inhibit diffused tumors and solid tumors. Success of the last two applications depends largely on the pharmacological and toxicological properties of the chimeric molecules. This article focuses on the most recent developments in targeting toxin polypeptides.

Immunotoxins are composed of a cytotoxic molecule linked to a carrier molecule by either a reducible or a nonreducible bond. The cytotoxic moiety is often a toxin polypeptide that catalytically inhibits a vital biosynthetic pathway. In general, the cytotoxic moiety used in targeting is a translational inhibitory protein but some investigators have also used enzymes such as phospholipases to destabilize the integrity of the tumor cell plasma membrane. Various carrier proteins have been investigated to deliver the toxin molecules, including tumor selective monoclonal antibodies, growth factors, and lymphokines.

## CHOICE OF TOXIN MOLECULES

Three types of toxin polypeptides are used in the preparation of immunotoxins (IT)[1]: (*a*) bacterial toxins (*b*) plant toxins and (*c*) fungal toxins. All three groups of molecules catalytically inhibit protein synthesis in eukaryotes but each at a distinct step in translation. Most of the work on the bacterial toxins was carried out on two highly toxic molecules, namely diphtheria toxin (DT) and pseudomonas exotoxin A (PE). Detailed studies on the structure/function of these two molecules are primarily responsible for the advances made in preparing highly effective chimeric molecules against tumor cells. Based on their biochemical characteristics, plant toxins can be grouped as Type I, single chain polypeptides that enzymatically inhibit translation, and Type II (e. g. ricin and abrin), which are heterodimers. The A chains of Type II toxins are the true toxic moieties, whereas the B chains contain binding sites for carbohydrates through which the A chain gains access to the interior of the cell. Fungal toxins such as alpha sarcin are also single chain proteins but are functionally different from Type I polypeptides. Alpha sarcin, for example, is a phosphodiesterase while the Type I toxins are N-glycosidases.

[1] Abbreviations used: CDR: complementarity-determining regions; DT: diphtheria toxin; EGF: epidermal growth factor; Fc: fragment crystallizable; GVHD: graft versus host disease; HAMA: human anti-murine antibody; IT: immunotoxins; KDEL: endoplasmic retention signal sequence; LDL: low density lipid; PAP: pokeweed antiviral protein; PE: pseudomonas exotoxin A; PEG: polyethyleneglycol; RIP: ribosomal inhibitory proteins; SCA: single chain antibodies; SPDP: N-Succinimidyl 3-(2- pyridyl)dithiopropionic acid; TCS: trichosanthin; TFR: transferrin

## RICIN

### *Structural Features*

Ricin is a toxic lectin isolated from castor beans. Its precursor is a single polypeptide composed of a signal peptide and two chains linked by a 12 amino acid joining peptide (J peptide). It is proteolytically cleaved to form the mature ricin of 62 kd containing an A chain (267 amino acids) and a B chain (262 amino acids) linked by a disulfide bond (11). The toxin is post-translationally glycosylated on both A and B chains with high mannose branched chains containing xylose and fucose. The A chain has two N-linked sugars at positions 10 and 236. The B chain has N-linked carbohydrates attached to residues 95 and 135. Heterogeneity in composition of sugars has been observed in all four sites (12). In the mature ricin molecule the A chain is linked to the B chain by a reducible bond between Cys 259 of the A chain and Cys 4 of the B chain. Ricin has been crystallized, and the three-dimensional structure has been elucidated by Robertus and colleagues (13, 14) at a resolution of 2.5 Å. Interaction between the subunits is mainly due to hydrophobic forces among various aromatic rings and aliphatic side chains.

The carboxy terminal of the B chain seems to be involved in association with the A chain. Three domains can be distinguished in the A chain by X-ray crystallography (14). Domain I consists of amino acids 1–117 dominated by a six-stranded B-sheet and two helices. Domain II encompasses residues 118–210 and is mainly made of alpha helices. The C-terminal end constitutes Domain III, and the striking feature of this part of the molecule is a stretch of hydrophobic residues located between positions 247 and 257. This region of the molecule is suggested to play a key role in the translocation of the toxic subunit into the cytoplasm.

The A chain of ricin is a specific N-glycosidase that catalytically hydrolyzes the glycosidic bond of adenosine residue 4324 in the 28S rRNA (15). Depurination results in irreversible inactivation of ribosomes and inhibition of protein synthesis. Ricin is such a potent toxin that a single A chain molecule can inactivate 1500 ribosomes per minute and once in the cytosol can kill the cell (16). The Km for reticulocyte ribosomes is about 0.1–0.2 $\mu$M and the Kcat about 1,500 $min^{-1}$. The depurination mechanism of ricin is predicted to be similar to that of adenosine monophosphate nucleosidase involving the formation of an oxycarbonium ion. Based on site-directed mutagenesis and X-ray diffraction studies, important residues of the A chain either in direct contact with the substrate or those involved in the actual catalysis have been identified. The catalytic site is present in a cleft; Tyr 80 and Tyr 123 are present at the top of the cleft. Neither is believed to be important for catalysis but both may contribute to the tight binding of the substrate. Substitution of

other residues present around the cleft such as Asn 209 and Trp 211 does not significantly change the enzymatic activity (17, 18).

Tight binding of the substrate (28S rRNA) is responsible for the partial breakage of the N-C bond (18). Two important changes involving either Glu 177 or Arg 180 have consistently been shown to dramatically decrease the biological activity of the A chain. The positive charge on Arg 180 is supposed to help in the partial breaking of the bond by ion pairing to the phosphate backbone. The destabilized N-C bond is then vulnerable to nucleophilic attack by a water molecule. Interestingly, intact ribosomes are better substrates than the synthetic oligomers homologous to the conserved region covering the depurination site. It is therefore likely that the ribosomal proteins may contribute to the proper presentation of the sensitive site in a manner facilitating catalysis or that some important regions of ricin A chain may interact with ribosomal proteins in stabilizing the enzyme-substrate complex. This issue is currently being investigated by genetic deletion analyses in many laboratories.

The B chain of ricin has two functions: (*a*) binding reversibly to galactose presented by cell surface glycopeptides and glycolipids, and (*b*) facilitating uptake of whole ricin by endocytosis and transport through the Golgi apparatus before translocation of the A chain into the cytosol (19). It is still contentious whether the two functions of the B chain are separable. Some studies indicate that toxin entry could be mediated by mechanisms independent of galactose recognition, but other evidence supports the galactose binding activity on the B chain as an important step in the entry to the cytosol (20–24).

## *Conjugates Prepared with Ricin and Ricin A Chain (RTA)*

Many immunotoxins using intact ricin and monoclonal antibodies or growth factors have been developed (25, 26). They are extremely cytotoxic to relevant target cells, but the specificities are suboptimal since B chains may bind to surfaces on nontarget cells. Another problem is the rapid clearance of ricin IT from the bloodstream (27). This is due at least partially to the clearance of native ricin by oligosaccharide-mediated uptake by the liver cells (28, 29). To circumvent this problem, immunotoxins have been made with chemically/enzymatically deglycosylated A chain (30, 31) or recombinant A chain. Chemical treatment destroys the mannose and other sugar residues on native A chain while recombinant A chain expressed in bacteria is free of B chain and completely lacks oligosaccharide side chains. These constructs have better pharmacological properties than conjugates made with native ricin A chain.

Several groups have obtained ricin A chain (rRTA) by genetic engineering

*Escherichia coli* at the level of 1.5 mg/liter of culture. O'Hare et al (33) cloned ricin A chain cDNA using vector pDs/3 and expressed the recombinant A chain with ten extra amino acids at the N-terminus in *E. coli* at the level of 2–3 mg/liter of culture. Piatak et al (34) obtained a correct 267 amino acid A chain from *E. coli* using several different host/vector systems at the level of 1–2% to 6–8% of total cell proteins. Frankel et al (35) used an expression system to produce a fusion protein containing ricin A chain linked to the enzyme $\beta$-galactosidase via a collagen fragment. Treatment of the fusion protein with collagenase released free ricin A chain that could be recovered from the enzyme by selectively absorbing the latter on an affinity column. In our laboratory, the coding region of the ricin A chain gene was cloned into the pET3b vector. The cloned fragment is flanked by the T7 promoter and terminator in this construction and expressed in *E. coli*, BL21(DE3). The production of purified A chain protein with 11 extra amino acids at the N-terminus was 80–90 mg/liter of culture or 24–26% of total cell proteins.

The specificities of the IT made from nRTA or rRTA are excellent due to the absence of nonspecific binding of B chain (27, 36–39). However, the cytotoxicities of these conjugates were relatively less (40) than intact ricin IT due to the loss of translocation functions asssociated with the B chain (40–43). Some of the immunoconjugates prepared with ricin A chain were not cytotoxic, whereas the same antibody linked to ricin was effective against target cells in the presence of lactose. Translocation signals associated with the B chain of ricin have been effectively used in potentiating ricin A chain conjugates. The B chain can be either delivered directly to cell bound A chain conjugates or indirectly by a second antibody homing onto the same cell. In both approaches however, lactose has to be included to prevent nonspecific toxicity. Alternatively, intact ricin could be modified (blocked ricin) by chemical modifications (41–43) that retain the translocation properties without the nonspecific toxicity mediated by the sugar binding sites. The cell binding, enzymatic specificity, and mechanism of cytosol entry between bacterial toxins and plant toxins are very different.

## SINGLE CHAIN RIBOSOMAL INHIBITORY PROTEINS (TYPE I RIP)

This class of proteins is used as an alternative in immunotoxin preparations and has many advantages in the treatment of several clinical diseases. Perhaps the best-studied single chain toxins are pokeweed antiviral proteins (PAP) isolated from the plant *Phytolacca americana,* saporin 6, isolated from the plant *Saponaria officinalis* and gelonin purified from *Gelonum multiflorum.* Similar proteins have been isolated from other plants (reviewed in 44) and are

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