Many diverse organisms like bacteria, plants and fungi, naturally produce toxic molecules, which play an important role in their survival by protecting them from predators and infecting agents. These naturally produced toxins are being investigated during the past two decades for several reasons. Firstly, the natural protein toxins produced by some of the bacteria are involved in their pathogenic mechanism(s) and are crucial in the severe diseases that they cause in man. This is seen in the case of diphtheria toxin (DT), *Pseudomonas* exotoxin (PE), tetanus toxin, botulinum neurotoxin and Shiga toxin (Montecucco and Scheavo, 1993; Sudhof *et al*, 1993). Shiga toxin is mainly produced by *Shigella dysenteriae*, which is the infective agent in dysentery, but Shiga-like toxins are also produced by enterohaemorrhagic strains of *E. coli*, giving rise to infections and diseases and causing serious health
problems (Olsnes et al, 1991). To manage, control, and even possibly eradicate such diseases, an understanding of these toxins and their action on cells is important. Secondly, naturally occurring protein toxins have an immense potential to be used as therapeutic molecules either independently or as components of targeted toxins consisting of a targeting moiety linked to the toxin. The recombinant single chain molecules thus produced are referred to as immunotoxins or chimeric toxins. For targeting the toxin to specific cells, antibody or antibody fragments, growth factors or peptide hormones have been used (Husain et al, 1997; Goyal et al, 2000; Bolognesi et al, 1996; Qi et al, 2003). Several naturally occurring protein toxins have been used to generate immunotoxins and chimeric toxins in attempts to find alternative ways to treat diseases like cancer (Olsnes et al, 1989). Thirdly, protein toxins are very useful tools in cell biology to study processes such as protein translocation across membranes, internalization by endocytosis, sorting along the endocytic pathway and exocytosis (Sandvig et al, 1991; Montecucco et al, 1993; Sudof et al, 1993).

There are two major classes of these natural toxins that covalently modify unique structural features of components essential for protein synthesis in eukaryotic cells, thereby arresting protein synthesis and causing ultimate cell death. For these toxins to exert their toxicity, a catalytically active fragment has to first enter the cell cytosol from where they can attack the relevant target site (Perentesis et al, 1992).

One of these classes is that of the bacterial ADP-ribosylating toxins, typified by diphtheria toxin (DT) and Pseudomonas exotoxin A (ETA), whose catalytic components cause ADP-ribosylation and subsequent inactivation of the essential elongation factor 2 of eukaryotic protein synthesis (Passador and Iglewski, 1994). The ribosylation occurs at a unique diphthamide, a post-tranlationally modified histidine
residue, which is present at the ribosome-binding site of elongation factor 2 (Iglewski and Kabat, 1975). The other class of protein-synthesis inhibiting protein toxins can be divided into two subclasses, each typified by a particular protein. One of these is α-sarcin, which belongs to a family of fungal ribotoxins, and the other is ricin, which is a member of a group of closely related plant proteins referred to as the ribosome-inactivating proteins (RIPs). Both members of this second class of protein-synthesis inhibiting protein toxins attack the ribosomal RNA (23-28S) of the major ribosomal subunit and cause covalent modifications in it by two catalytically different hydrolytic mechanisms. The fungal ribotoxins act as specific endonucleases while the ribosome-inactivating proteins act as specific RNA N-glycosidases, and both exert their action at a pair of adjacent nucleotides within a highly conserved sequence of a conserved loop near the 3′-end of 23-28S rRNA (Schindler and Davies, 1977; Chan et al, 1983; Fando et al, 1985). Such covalent modifications disrupt the integrity of the RNA that needs to be maintained within ribosomes to allow elongation factor binding and proper protein translation. Bacterial shiga toxin, produced by Shigella dysenteriae type I and the shiga-like-toxins (SLTs), produced by enterohaemorrhagic E. coli, that are functionally identical and very similar in sequence, were also later discovered to behave like plant RIPs (O’Brien et al, 1992). Thus, the bacterial ADP-ribosylating toxins covalently modify elongation factor 2 such that it cannot interact properly with the ribosomes, while the fungal ribotoxins and plant and bacterial RIPs covalently modify rRNA such that it disturbs elongation factor binding (Table 1).
### Table 1
PROTEIN TOXIN INHIBITORS OF PROTEIN SYNTHESIS

<table>
<thead>
<tr>
<th>Toxin family</th>
<th>Intracellular target</th>
<th>Reaction mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT and ETA</td>
<td>EF-2</td>
<td>ADP-ribosyltransferase</td>
</tr>
<tr>
<td>α-sarcin</td>
<td>23S-like ribosomal RNA</td>
<td>Endonuclease</td>
</tr>
<tr>
<td>RIP and SLT</td>
<td>26-28S ribosomal RNA</td>
<td>RNA N-glycosidase</td>
</tr>
</tbody>
</table>

Sites of action and reaction mechanisms of the major categories of protein-synthesis inhibiting protein toxins.

Perentesis *et al*, 1992
ADP-ribosylating toxins DT and ETA

The severity of infections caused by *Corynebacterium diphtheriae* and *Psuedomonas aeruginosa* is largely due to secretion of the potent bacterial exotoxins, DT and ETA, respectively. It was discovered as early as 1959 that the primary action of DT was to inhibit protein synthesis in susceptible cells (Strauss *et al*, 1959). Later, this was shown to be also true for ETA (Pavlovskis *et al*, 1972, Pavlovskis *et al*, 1974). The inhibition of protein synthesis by DT required NAD⁺ as a cofactor (Collier *et al*, 1964). The ADP-ribosyltransfer reaction catalyzed by DT and ETA occurs as follows (Honjo *et al*, 1969):

\[ \text{EF-2} + \text{NAD}^+ \rightarrow \text{ADP-ribosyl-EF-2} + \text{nicotinamide} + \text{H}^+ \]

The reaction is essentially irreversible under the pH and nicotinamide concentrations found *in vivo*. Subsequently, ETA was demonstrated to inactivate EF-2 by the same mechanism (Iglewski *et al*, 1975).

Both DT and ETA are released into the circulation of infected hosts as enzymatically inactive, single-chain proteins, after the removal of short, secretory leader peptides. DT is secreted as a single polypeptide of 535 amino acid residues (Greenfield *et al*, 1983). The toxin contains two intramolecular disulphide bonds and for activation it requires proteolytic cleavage at an arginine-rich site located in the first disulphide loop as well as reductive cleavage of the joining disulphide (Drazin *et al*, 1971). The resultant amino terminal 193 residue polypeptide, termed fragment A (Collier *et al*, 1969), is the ADP-ribosyltransferase and the 342 residue fragment B (Collier *et al*, 1971) mediates target cell surface binding and uptake (Collier *et al*, 1990).
The secreted form of ETA is a 613 residue protein with a molecular weight of 66,583 Da and four disulphide bonds (Middlebrook et al, 1984). This toxin also requires modification by proteolytic cleavage or a combination of denaturation or disulphide bond reduction for the expression of its enzymatic activity (Lory et al, 1980). In contrast to DT, the ADP-ribosyltransferase activity of ETA is found at the carboxyl terminus of the protein, while the amino terminal domain is responsible for cellular binding and uptake. Molecular and biological analyses of ETA have defined crucial domains of the toxin that are involved in cellular binding and transmembrane passage (Chaudhary et al, 1990). It has been shown that ETA is processed by a cellular protease to yield an N-terminal 28 kDa and a C-terminal 37 kDa fragment that are initially linked by a disulphide bond. Subsequent to cellular binding, the C-terminal fragment is translocated to the cytoplasm where it ADP-ribosylates EF-2 (Ogata et al, 1990).

**Fungal ribotoxins**

The search for new anticancer agents by scientists led to the isolation of a protein produced by the fungus *Aspergillus giganteus*, that inhibited the growth of several induced tumors (Olson et al, 1965). This protein was designated as α-sarcin for its anti-sarcoma activity. A similar protein, occurring as two closely-related isozymic forms designated restrictocin and mitogillin, was later isolated from the fungus *A. restrictus* (Goldin et al, 1966). α-Sarcin, restrictocin and mitogillin are small (molecular weight ~ 17,000 Da), basic, single subunit proteins that share a high degree of sequence homology (Sacco et al, 1983). Mitogillin and restrictocin differ from each other by only two residues and are more than 80% identical in sequence.
with α-sarcin (Fernandez et al, 1985). These endonucleases are single subunit proteins that lack a separate cell-binding domain.

The cytotoxic action of α-sarcin was found to be due to an irreversible inactivation of the ribosome (Schindler et al, 1977). The protein was effective against both prokaryotic (Schindler et al, 1977) as well as eukaryotic protein synthesis systems. Inactivation of ribosomes by this protein did not require a cofactor (Endo et al, 1982). It was demonstrated that α-sarcin causes cleavage of a single phosphodiester bond near the 3' terminus of the major rRNA of the large ribosomal subunit (Schindler et al, 1977). The cleavage releases a small RNA fragment, termed the α-fragment, from the end of the large RNA. This cleavage occurs in a highly conserved 13-residue purine-rich sequence that is present in the largest RNA of the larger ribosomal subunit; which is 23S RNA in bacteria (Hausner et al, 1987), 26S RNA in yeast (Miller et al, 1988) and 28S RNA in mammals (Endo et al, 1983). The proteins restrictocin and mitogillin were subsequently shown to possess an identical enzymatic activity (Fando et al, 1985).

α-Sarcin is a 150 amino acids long, highly basic protein, having 20 lysines, 4 arginines and 8 histidines. There are four cysteins linked by two disulphide bridges, between residues of positions 6 and 148, and 76 and 132 (Sacco et al, 1983). The amino acids His50, Glu96 and His137 have been shown to participate in catalysis (Lacadena et al, 1999). The fungal ribotoxin restrictocin, similar to α-sarcin, has two disulphide bonds between the cysteine residues 5-147 and 75-131. None of these four cysteins play a direct role in catalysis, and the presence of either one of these two disulphides is essential and sufficient to maintain the catalytically active configuration of this protein, although both disulphides are required for maintaining the stability
(Nayak et al, 1999). Among the putative active site residues of restrictocin, Tyr47 and His49 have been shown to be involved in target RNA recognition, Arg 120 may be anchoring the substrate in place in the active site, while His136 and Glu95 appear to be directly involved in the acid-base mechanism of RNA hydrolysis by this protein (Nayak et al, 2001).

**RNA N-glycosidases**

Although the toxicity of the seeds of the castor bean plant, *Ricinus communis*, was known since ancient times, it was only about a century ago that this toxic property was attributed to the protein ricin, the first RIP to be discovered. Soon after, abrin, another toxin produced by the unrelated plant *Abrus precatorius*, was discovered. Structurally and functionally similar toxins were later found from several other plants, and it was discovered that the toxicity of these proteins results from a protein synthesis inhibition (Lin et al, 1971). This entire group of plant proteins was designated as ribosome inactivating proteins (RIPs).

Experiments during the 1980s also revealed that a group of well-known bacterial toxins were also related to the plant RIPs; shiga toxin produced by *Shigella dysenteria* type I was the first of these bacterial toxins found to inactivate the large ribosomal subunit in a similar manner to that of plant RIPs. Shiga toxin, as well as the shiga-like-toxins (SLTs), a group of other functionally related bacterial toxins, including those produced by enterohaemorrhagic *E. coli*, are RIPs that are functionally identical and very similar in sequence (O’Brien et al, 1992). They consist of an A subunit of approximately 32 kDa in non-covalent association with a pentamer of B subunits of approximately 7.5 kDa each (Olsnes et al, 1981). The B subunits are
responsible for binding to eukaryotic cell surface glycolipid receptors (Lindberg et al, 1987). These toxins enter the cells by endocytosis and appear to be routed in a retrograde manner through the Golgi compartment and the endoplasmic reticulum before reaching the cytosol (Sandvig et al, 1992). The catalytically active A subunit of ST/SLTs has been shown to possess specific N-glycosidase activity that inactivates 28S rRNA by specific depurination, resulting in the inhibition of protein synthesis (Endo et al, 1988).

**Plant ribosome inactivating proteins**

The plant ribosome inactivating proteins can be broadly divided into two categories, the type 1 RIPs, which consist of a single polypeptide chain of about 30 kDa, and the type 2 RIPs, which consist of an enzymatically active A chain similar to type 1 RIPs, linked to a slightly larger B chain of about 35 kDa (Olsnes et al, 1973). The B chain has lectin-like properties with a specificity for sugars that have a galactose-like structure (Lord et al, 1994). Type 1 RIPs may or may not be glycosylated. The pI of all type 1 RIPs is invariably basic and usually greater than 9.5, while type 2 RIPs have a relatively neutral pI (Barbieri et al, 1993). Taking advantage of their high pI, type 1 RIPs are generally purified by cation-exchange chromatography on carboxymethyl or sulfopropyl-derivatized matrices (Stirpe et al, 1983), while type 2 RIPs are purified by affinity chromatography on Sepharose, acid-treated Sepharose or other galactose containing stationary phases followed by elution with galactose or lactose (Olsnes et al, 1982), exploiting the lectin properties of their B chains. The amino acid composition is known for a large number of RIPs (Falasca et al, 1982). Although most RIPs are glycoproteins, the carbohydrate moiety does not
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seem to play any major role in catalysis due to the following observations; (1) some RIPs such as saporin, tricosanthin and ricin A chain do not contain carbohydrates and yet their catalytic activity is not compromised, (2) the sugar composition of various RIPs varies in respect of quality and quantity, and (3) gelonin and ricin A chain could be partially deglycosylated without affecting their capability of inhibiting protein synthesis in a rabbit reticulocyte lysate (Stirpe et al, 1983).

Distribution of plant RIPs

RIPs are very widely distributed in the plant kingdom among the Angiospermae, both in monocots as well as dicots, and also in mushrooms (Lam et al, 2001) and an alga, Laminaria japonica (Liu et al, 2002). No RIP has been as yet identified from Gymnospermae. The level of RIPs expressed in plant tissues is also highly variable, and ranges from traces to hundreds of milligrams per 100 g plant tissue. In some plants, RIPs may be present in many or all the tissues examined, such as roots, leaves, stems, barks, seeds, flowers and fruits, while in others they may be confined to a single tissue. An interesting feature of some RIPs is the existence of different forms of that RIP in different plant parts, and sometimes in the same plant part; several isoforms of the RIP saporin were found in the seeds, leaves and roots of the Saponaria officinalis plant (Ferreras et al, 1993) with the highest protein synthesis inhibitory activity occurring in the mature seeds. The expression of at least some RIPs was observed to be enhanced in senescence and in conditions of stress (Stirpe et al, 1996). The two main RIPs from the leaves of the Phytolacca americana (pokeweed) plant were found to be synthesized with a seasonal pattern. Only small amounts of one of the isoforms, PAP (pokeweed antiviral protein) II were found in
pokeweed leaves in spring and its concentration was higher in summer. The levels of another isoform, PAP, however, appeared to be independent of the season, suggesting that synthesis of PAP occurs throughout the life span of the leaves, while the production of PAP II increases progressively with tissue ageing (Irvin et al, 1983). Distribution of the dianthin RIPs of the carnation plant was studied with the help of antibodies (Reisbig et al, 1983). The isoform dianthin 30 was found throughout the plant, while dianthin 32 was found only in leaves and growing shoots. Dianthins were much more concentrated in old leaves, where they represented 1-3% of the extractable protein, rather than in young leaves.

**Biosynthesis of RIPs**

The biosynthesis of RIPs have to be compatible with the enzymatic activity of these proteins as they are capable of damaging ribosomes. Three mechanisms were suggested to explain the "safe" synthesis of these proteins (Barbieri et al, 1993); one, that autologous ribosomes are relatively resistant to the action of RIPs; two, that the RIPs are synthesized as inactive proforms; and three, that RIPs are synthesized as inactive preproforms with N-terminal signal sequences that direct the nascent peptide chain into the lumen of the reticulum before it is finally folded in the active configuration. The biosynthesis of ricin has been studied in detail (Lord et al, 1985), and it was observed that during ricin biosynthesis in seeds, the N-terminal signal sequence of the prepro-ricin mediates that cotranslational translocation of the nascent precursor into the lumen of the endoplasmic reticulum. The signal sequence is cleaved during this step, following which pro-ricin is glycosylated and the disulphide bonds established. From the endoplasmic reticulum, the pro-ricin is transported via the Golgi
apparatus and the Golgi vescicles to the protein bodies, where pro-rinic is processed by an acid protease to its mature, active form.

**Mechanism of action of RIPs**

Endo and his coworkers discovered for the first time that the type I RIPs and the A chain of type II RIPs possess a unique RNA N-glycosidase activity and cleave the glycosidic bond of a highly conserved adenine$_{4324}$ of 28S eukaryotic mammalian rRNA (Endo et al, 1987). This base was also found to be just adjacent to the site of cleavage of rRNA by the group of fungal ribotoxins typified by $\alpha$-Sarcin (Endo et al, 1982), and restrictocin and mitogillin (Olson et al, 1965), that act as highly specific endonucleases and cleave the phosphodiester bond between G$_{4325}$ and A$_{4326}$ of rat liver RNA (Endo et al, 1982). When ribosomal RNA identity elements for ricin recognition and catalysis were studied, (Endo et al, 1991), it was observed that the target adenine residues were always located in a stem and loop sequence of rRNA having a GAGA sequence in the loop, suggesting that RIPs recognized this specific structure (Figure 1). However, the activity of ricin in depurinating purified 28S rRNA (Endo et al, 1988a) or a synthetic oligoribonucleotide fragment that mimics the target region of the 28S rRNA (Endo et al, 1988b), was much weaker as compared to its activity of depurinating intact ribosomes. This indicated that the RIP-ribosome interaction was much more complex than a simple recognition of primary RNA structure by the RIP. Ribosomes from fungi were found to be quite sensitive to RIPs. Yeast ribosomes were sensitive to both type I and II RIPs (Roberts et al, 1986), and ricin was shown to depurinate the major (26S) rRNA of yeast at the position corresponding to rat liver 28S A$_{4324}$ (Bradley et al, 1987).
Figure 1

TARGET SITE OF RIBOSOME INACTIVATING PROTEINS

A portion of rat 28S rRNA showing the target site of RIPS. RIPS cleave a specific N-glycosidic bond in the stem and loop structure in contrast to ribotoxins which cleave a specific phosphodiester bond.
Plant ribosomes displayed a variable sensitivity to RIPs from other plant species (Owens et al, 1973, Harley et al, 1982). Studies on the sensitivity of plant translation systems to autologous ribosomes initially indicated that RIPs did not affect ribosomes from their own plants (Reisberg et al, 1983) although later studies showed that some RIPs act on autologous ribosomes at concentrations much higher than those required to inhibit mammalian ribosomes (Harley et al, 1982, Taylor et al, 1990). All type I RIPs tested could inhibit protein synthesis by E. coli ribosomes (Habuka et al, 1990), although at a very much higher concentration than that needed to inhibit protein synthesis in rabbit reticulocyte lysate.

The plant RIP family contains only about nine invariant amino acid residues that come together in the active site. In ricin, these are residues Tyr80, Tyr123, Glu177, Arg180 and Trp211. Monzingo and Robertus, 1992, solved the X-ray crystal structure of ricin complexes with the adenosine analog formycin monophosphate (FMP) and the dinucleotide ApG. The substrate purine ring was found to be stacked between the rings of the two invariant tyrosine residues. The Arg180 bonded to N3 of the ring and Glu177 was found to lie near the ribose. Although Trp211 did not seem to make any specific contacts with the adenine, it was believed that this residue may be important for maintaining active site conformation or for interaction with larger polynucleotide substrates. A mechanism was proposed for RNA N-glycosidase activity of ricin (Monzingo et al, 1992, Ready et al, 1991) that incorporated both structural and kinetic analyses of ricin A chain (Figure 2). Tyr80 and Tyr123 lie above and below the adenine plane of the target adenine that gets stacked between them. Specific hydrogen binds are formed to the adenine ring as shown in Figure 2. Bond cleavage between adenine N9 and C1' of ribose is facilitated by protonation at
N3 by Arg180. Arg180, by transferring a proton to adenine, also attracts one proton from water and thereby activates the solvent as it develops a hydroxide character. The oxycarbonium ion that develops on the ribose is stabilized by ion pairing to Glu177. Activated water attacks the oxycarbonium, releasing the adenine-leaving group. The lower panel of Figure 2 shows an alternative of this mechanism, where hydrogen bonding serves as partial protonation of the leaving adenine, N9 helps to polarize the water and the product adenine is in the aromatic resonance form without any proton rearrangement.

Antiviral activity of plant RIPs

Beginning with the discovery of the type I RIP pokeweed antiviral protein as the principal antiviral component in crude extracts of pokeweed leaves that prevented the transmission of tobacco mosaic virus infection to plants (Duggar et al, 1925, Dallal et al, 1978), several RIPs from different sources were also shown to inhibit infection of test plants with diverse plant viruses, most of them being the type I RIPs. Although the actual underlying mechanism of this activity still remains to be elucidated (Peumans et al, 2001), three basic mechanisms have been proposed to explain this activity.

First, the RIPs may be acting directly on viral nucleic acids by means of their polynucleotide:adenosine glycosidase activity. Although the viral capsid may prevent a direct effect of RIPs on intact virus particles, as soon as viral particles are disassembled in the cell, their RNA or DNA may be attacked and deadenylated by the RIPs. It was also discovered that pokeweed antiviral protein could inhibit translation of capped viral RNAs without any ribosomal depurination (Hudak et al, 2000). The
Two variations of a plausible mechanism of action of RTA and the RIP family. The bond cleavage of stacked adenosine is facilitated by protonation by Arg180. Transition state oxycarbonium ion is stabilized by Glu177. Activated water attacks the oxycarbonium, releasing the adenine.
second hypothesis was that the RIPS may selectively gain entrance into virus-infected cells and destroy their protein synthetic machinery to prevent viral infection and spread to surrounding cells. Electron microscopic studies by Ready et al, 1986, revealed that pokeweed antiviral protein is synthesized in the cytoplasm of leaf cells and extruded across the cellular membrane into the cell wall matrix. The protein did not seem to be attached to the cell wall, but to be trapped between the cell wall matrix of the mesophyll cells. The hypothesis of a local cell suicide could therefore hold true only when the integrity of the cell plasmalemma is damaged. However, this model implies that viral infection is accompanied by severe cellular damage. Since protein synthesis will stop almost instantly when cells lose their structural integrity, depurination of ribosomes by RIPS could hardly have any direct effect (Peumans et al, 2001). Furthermore, it was observed that RIP-containing plants were not resistant against viruses. The third hypothesis proposed was that RIPS act in an indirect manner to activate the plant's defense system during viral infection. This seems to be the most probable mechanism for the antiviral activity of RIPS, even though evidence for such an antiviral activity of RIPS has only been obtained from transgenic tobacco plants expressing RIPS, and not from any plants that normally express RIPS (Lodge et al, 1993). Expression of PAP gave tobacco plants resistance against potato virus X and Y (Lodge et al, 1993) while expression of tricosanthin made transgenic tobacco resistant to turnip mosaic virus (Lam et al, 1996). However, all these RIPS also caused a very severe phenotype because of their toxicity towards these plants, and virus-resistant tobacco lines with a normal phenotype could only be obtained using PAPc, a PAP mutant that showed full antiviral activity but was not cytotoxic (Turner et al, 1997). Studies using active site mutants and deletion mutants of PAP led workers to
conclude that for antiviral activity, toxicity and depurination of ribosomes, an intact RNA glycosidase site is required, and an intact carboxyl terminus of PAP is needed for its toxicity and in vivo depurination of host ribosomes but not for its antiviral activity (Zoubenko et al, 1997, Smirnov et al, 1997, Zoubenko et al, 2000).

Much research has also been conducted to study the effects of RIPs on HIV, after it was discovered that PAP also inhibits HIV replication (Zarling et al, 1990). Rajamohan et al, 1999 demonstrated the anti-HIV-1 replication activity of some PAP isoforms, that also caused a depurination of HIV RNA. However, ricin A chain did not exert either activity. An inhibition of HIV-1 integrase enzyme activity was reported initially for the RIPs MAP30 and GAP31 (Lee-Huang et al, 1995) and later also for RIPs saporin and luffin (Au et al, 2000). The X-ray crystal structure of PAP isoform III, isolated from late summer leaves of the pokeweed plant, was solved recently at 1.6 Å resolution (Kurinov et al, 2003); molecular modeling studies of the interactions of PAP-III and PAP-I with a single-stranded RNA heptamer predicted a more potent anti-HIV activity for PAP-III due to its unique surface topology and more favorable charge distribution in its 20 Å-long RNA-binding active centre cleft, and PAP-III was indeed found to be more active than PAP-I in depurinating HIV-1 RNA (Kurinov et al, 2003). It was also recently reported that for tricosanthin, also a potent inhibitor of HIV replication, this antiviral activity could be counteracted by CEP-11004, a commonly used c-Jun N-terminal kinases inhibitor (Ouyang et al, 2006), suggesting that the anti-HIV-1 effects of TCS may be related to the MAPK signal process downstream from the point of inhibition caused by CEP. The involvement of this pathway was supported by the findings of Huang et al, 2006, who observed that Herpes simplex virus (HSV) infection caused an elevation of p38 MAPK and Bcl-2 in
Vero cells, while TCS could suppress this elevation while it reduced viral replication, implying that the MAPK family may play a role in the antiviral mechanism of TCS. The discovery of a novel interaction between PAP and eukaryotic translation initiation factors 4G and iso4G of wheat germ point to a potential indirect mechanism of this protein to access viral RNAs (Wang et al, 2006).

**RIPs and apoptosis**

The selective tumor-killing properties of RIPs as compared to normal cells have been known for quite some time. The potential that RIPs showed for cancer therapy as components of immunotoxins rekindled an interest in the study of these plant molecules. Many studies report that RIPs alone or immunotoxins made comprising the catalytic A chain of RIPs cause cell death by apoptosis (Kochi et al, 1993, Bolognesi et al, 1996, Brinkmann et al, 1997). The bacterial Shiga toxin, which is catalytically an RNA N-glycosidase like the plant RIPs, has also been shown to induce apoptosis (Kojio et al, 2000, Smith et al, 2003). A large variety of cells and cell lines could be affected by RIPs. For some of the RIPs, attempts have been made to delineate the apoptotic pathway and to propose a mechanism for cell death induction by these proteins. However, these pathways may not be mutually exclusive, and more investigation in this area is needed to determine the factor that triggers the RIP-induced signaling leading to cell death (Narayanan et al, 2005).

It was shown by Iordanov et al, 1997, that damage to 28S rRNA by the RIPs ricin and α-sarcin resulted in a novel pathway of kinase activation called ribotoxic stress response, resulting in the activation of SAPK/JNK and its activator, SEK1/MKK4. It was also shown that SAPK/JNK was not just due to protein synthesis
inhibition, but also due to signaling from the 28S rRNA damaged by RIPs. Further, not all protein synthesis inhibitors were able to elicit the ribotoxic stress response. It was thus proposed that this stress response was specific for those inhibitors that either induced damage to the α-sarcin/ricin loop of 28S rRNA or those that caused ADP-ribosylation of EF-2, thus arresting translation at the translocation step. Anisomycin, known to inhibit ribosomal peptidyltransferase action, also caused activation of these kinases, further confirming this hypothesis (Iordanov et al, 1997). A similar ribotoxic stress response was caused by bacterial Shiga toxin 1 resulting in the activation of p38 MAPK and JNK, ultimately resulting in the apoptosis of HCT cells. The catalytically inactive ST mutant E167D, lacking RNA N-glycosidase activity, was unable to induce this activation, indicating that the enzymatic activity of the toxin A chain was essential for apoptotic signaling (Smith et al, 2003). Hence, it appeared that at least one of the pathways induced by RIPs could be the ribotoxic stress response through their RNA N-glycosidase activity. However, the exact mechanism or the pathway by which the signals are transmitted from damaged 28S rRNA to the MAPK pathway needs to be investigated further (Narayanan et al, 2005).

In some studies of RIP-induced apoptosis induction, a mitochondrial pathway was implicated, by observations of a loss in mitochondrial membrane potential, rapid release of cytochrome c, caspase-9 activation, increased production of reactive oxygen species, and a rise in intracellular calcium levels (Narayanan et al, 2004, Wu et al, 2004, Zhang et al, 2001, Fujii et al, 2003). Shiga toxin-induced apoptosis could be inhibited by overexpression of Bcl-2, an anti-apoptotic protein acting at the level of the mitochondria (Jones et al, 2000). Ricin induced a rapid elevation of cellular calcium level in hepatoma cells (Hu et al, 2001). Tricosanthin induced high levels of
ROS production in human choriocarcinoma cells (Zhang et al., 2001); this event was shown to be dependent on extracellular as well as intracellular calcium levels and and ROS production paralleled calcium elevation, suggesting that ROS production might be a consequence of calcium signaling.

The fate of a cell to survive or die is regulated by a delicate balance between the levels of pro-and anti-apoptotic proteins within that cell (Lawen et al., 2003). Many pro-apoptotic factors are activated during both extrinsic as well as intrinsic apoptotic pathways. Apoptosis induction by RIP from *Agrostemma githago* was found to be associated with down-regulation of the anti-apoptotic Bcl-2 protein expression (Chiu et al., 2001). Both ST 1 and ST 2 caused a decrease in the cellular level of the Bcl-2 family anti-apoptotic protein, Mcl-1 (Erwert et al., 2003). Jones et al., 2000, demonstrated that treatment of epithelial cells with ST led to an increase in the levels of the pro-apoptotic protein, Bax. This role of RIPs in modifying the balance of pro­-and anti-apoptotic factors might be cell line dependent, as different cell lines express differing levels of these factors (Narayanan et al., 2005).

It was reported that treatment of U937 cells with ricin caused a marked decrease in intracellular levels of NAD$^+$ and ATP before inducing apoptosis (Komatsu et al., 2000). Some reports also suggest that a hyperactivation of poly-ADP-ribose-polymerase (PARP), a DNA repair enzyme, can cause a depletion in NAD$^+$ levels and so can induce mitochondrial damage and apoptosis (Yu et al., 2002, Chiarugi et al., 2002). It was shown that the RIPs saporin L2, gelonin and momordin have transforming activity which involves auto-ADP-ribosylation of PARP (Barbieri et al., 2003). It was also shown that these RIPs directly depurinated auto-modified PARP, releasing adenine from the ADP-ribosyl group. It was proposed that this depurination
could result in the inhibition of the DNA repair pathway as well as a lowering of PARP availability for further ADP-ribosylation, leading to a depletion of intracellular NAD$^+$ levels and an induction of apoptosis (Barbieri et al, 2003). However, Narayanan et al, 2004, demonstrated that PARP inhibitors could not rescue Jurkat cells from abrin-induced apoptosis, implying that this pathway may be relevant only in certain types of cell lines which are sensitive to alterations in cellular NAD$^+$ levels.

The non-specific nuclease activity of RIPs has also been suggested as a possible mode of their apoptosis induction (Narayanan et al, 2005), by causing DNA damage. There are several reports of the DNase-like activity of RIPs (Roncuzzi et al, 1996, Nicolas et al, 1997, 1997a, He et al, 2004). Nuclear localization studies of RIPs have yielded conflicting reports. Confocal microscopy studies using FITC-conjugated abrin revealed that there was no nuclear localization of this protein in SIHA cells after it entered the cytosol (Narayanan et al, 2005), although Bagga et al, 2003a, demonstrated that saporin-6 migrated to the nucleus of J774A.1 cells after initially staying in the cytosol. Cells treated with Shiga toxin also showed the toxin to be predominantly in the nuclear fraction (Sandvig et al, 1992, Suzuki et al, 2000).

The ability of RIPs to induce apoptotic rather than necrotic cell death is advantageous in their application in the construction of immunotoxins for targeted therapy in cancer, since necrosis causes a spillage of cellular content to the extracellular environment, which may result in harmful inflammatory responses. Apoptotic cells, on the other hand, are engulfed by macrophages (Bergamaschi et al, 1996).
Immunotoxins and RIPS

Immunotoxins are a group of artificially-made cytotoxic molecules generated for the purpose of targeting cancer cells. These molecules are composed of a targeting moiety, such as a ligand or an antibody, linked to a protein toxin moiety. Immunotoxins can be divided into two categories, the chemically conjugated immunotoxins and the chimeric toxins, the latter being constructed by the genetic fusion of ligand and toxin.

The rationale for using protein toxins to kill cancer cells is based on their extreme potency and ability to kill drug-resistant cells (Kreitman et al, 2003). These protein toxins may be bacterial or plant toxins. The bacterial Psuedomonas exotoxin and diphtheria toxin have both been extensively used for the construction of immunotoxins and chimeric toxins and have gone extensive clinical and preclinical trials (Kreitman et al, 1998). The major representative of the plant RIPS for immunotoxin and chimeric toxin construction so far has been the type II RIP ricin, although other RIPS such as saporin and gelonin have also been used to make active immunotoxins (Li et al, 2005).

A GnRH-PAP conjugate was shown to be cytotoxic to three different prostate cancer cell lines, supporting the feasibility of using such hormonotoxins as novel therapeutics for hormone-responsive cancers such as prostate cancer (Qi et al, 2003). When the binding and cytotoxicity of this conjugate was compared with that of a chimera of GnRH-PAP generated through recombinant DNA technology by Qi et al, 2004, it was observed that the chemical conjugate had much more potent binding and cytotoxicity towards GnRH-receptor bearing cells as compared to the chimeric toxin. The workers proposed that the generation of an active GnRH-PAP fusion protein may
not be possible as both ends of the GnRH molecule are needed for receptor binding while in the fusion protein only the N-terminal end was free for binding. That relative orientation of ligand and toxin in chimeric toxin constructs was important was also demonstrated by Rathore et al., 1997, who observed that putting the anti-TFR-scFv as targeting ligand at the C-terminus of fungal ribotoxin restrictocin resulted in chimeras that were much more potent cell-killers than those in which the ligand was placed at the N-terminus. However, the incorporation of a suitable proteolytically cleavable spacer between ligand and toxin led to the generation of highly potent restrictocin-based chimeras which showed similar cytotoxicity, irrespective of whether the targeting ligand was at the N- or C-terminus of the restrictocin molecule (Goyal et al., 2000).

A combination of chloroquine, an anti-malarial drug, and AZT, an anti-HIV drug, could enhance the cytotoxic and apoptotic effects of a saporin and human transferrin immunotoxin on K562 cells (Lizzi et al., 2005). From such findings, it was postulated that immunotoxins containing ricin-like toxins, that is, other RIPs, could be coupled with the use of common and cheap drugs for the treatment of cancer in HIV-infected patients. Recently, a novel fusion protein composed of part of the extracellular domain of the α-subunit of human muscle acetylcholine receptor and the plant ribosome inactivating protein gelonin was tested as a therapeutic agent for the treatment of Myasthenia gravis in an animal model (Hossan et al., 2006). No symptoms of M. gravis could be detected as determined one and seven weeks after the second application. It was proposed that this approach may be useful for the therapy of further autoimmune diseases by substituting other autoantigens for the AchR fragment in the fusion protein (Hossan et al., 2006).
The potential application of RIPs as the toxin component of chimeric toxins for targeted therapy of cancer makes it essential that systematic studies be carried out on various cancer cells and cell lines to determine the efficacy with which RIPs can kill these cells.

**Saporin**

Saporin represents a multigene family of RIPs isolated in the form of closely-related individually distinct isoforms from various parts of the *Saponaria officinalis* (soapwort) plant (Stirpe *et al.*, 1983). The first recombinant clone of saporin was obtained from the cDNA library from leaves of the soapwort plant (Benatti *et al.*, 1989). Saporin is a type I RIP, and is synthesized as a proform carrying a 24 amino acid long N-terminal signal sequence and a 22 amino acid long C-terminal extension (Benatti *et al.*, 1989). The mature protein is non-glycosylated, contains 253 amino acids, has a pI of 10.2 and runs as a 28-30 kDa band on an SDS-polyacrylamide gel. The mature protein also lacks cysteines and histidines.

Saporin is an N-glycosidase that depurinates 28S rRNA like other type I RIPs. Although Cavallaro *et al.* (1995) showed that saporin bound specifically to the α2-macroglobulin receptor (α-2 MR), also known as the low-density-lipoprotein-receptor-related protein (LRP), Bagga *et al.* (2003) have demonstrated that the sensitivities of both LRP-negative and LRP-positive cell lines were similar to saporin-6 toxicity, suggesting that the internalization of saporin may not be solely dependent on the expression of LRP on eukaryotic cells.

In recent years RIPs have also been tested for their activity on a wide variety of non-ribosomal substrates. Saporin L-1, present mainly in the leaves of *Saponaria*
officinalis, has been shown to depurinate various different types of RNA such as globin mRNA, E. coli RNA, TMV genomic RNA, yeast tRNA, bacteriophage MS2 RNA, DNA and also poly A (Barbieri et al, 1994). The pH, ionic and temperature conditions for these reactions were found to be different for different isoforms (Barbieri et al, 1996). The activity of other saporin isoforms was found to be variable on different substrates. It was discovered that other RIPs too could act on non-ribosomal substrates and it has thus been suggested that RIPs could also be classified as polynucleotide:adenine-glycosidases (PNAG) (Barbieri et al, 1997).

Saporin has also been shown to inhibit HIV-1 integrase activity (Au et al, 2000), along with other RIPs such as luffin, MAP30 and GAP31 (Au et al, 2000, Lee Huang et al, 1995), although the mechanism is still not known.

The crystal structure of saporin-6, purified from the seeds of Saponaria officinalis, has been solved (Savino et al, 2000). The protein is composed of two domains; the N-terminal domain is predominantly β-stranded and the C-terminal is largely α-helical. A ribbon diagram of the secondary structural elements of saporin-6 is depicted in Figure 3. A structure superimposition of saporin-6 with other RIPs such as momordin, pokeweed antiviral protein, and ricin A chain (Figure 4) shows that insertions and deletions in saporin-6 compared to these other proteins lie mainly in the random coil regions. Most of the secondary structural elements are comparable although the termini of these elements may be altered and deviations are mainly seen in some loop regions. The C-terminal region of saporin-6 has a two-stranded antiparallel β-sheet element, the two strands of which are connected by a short loop whose length is variable among RIPs, and is particularly shorter in saporin-6. Though the active site of saporin-6 is conserved and almost perfectly superimposable to that
Figure 3

STRUCTURE OF SAPORIN-6

A diagram of folding of saporin-6 (Savino et al, 2000). A-H indicate the eight α-helices.
Figure 4

STRUCTURE SUPERIMPOSITION OF VARIOUS RIPs

Stereo view of structures of saporin-6 (red), momordin (cyan), pokeweed antiviral protein (blue) and ricin A chain (green) superimposed. The loop between strands β7 and β8 is outlined in the box.

Savino et al., 2000
of other RIPs, the shorter loop present in the C-terminal region, covering the active site crevice, may be responsible for increased accessibility to substrates, thereby explaining the wide substrate variability of the protein (Savino et al, 2000). From chemical modification studies of surface lysine residues, a region near the C-terminal end was identified as a putative site for initial ribosome recognition, and three lysine residues, all close to each other in this region, and either conserved in other RIPs or replaced in them by positively charged residues, was predicted to map some of the contact area between saporin and the ribosome (Savino et al, 2000).

A study of the structural properties of saporin in the presence of denaturing agents and/or proteolytic enzymes has shown that saporin is extremely resistant to denaturation by high concentrations, upto 4M, of urea or guanidine, even at relatively high temperatures, upto 55°C. The guanidine-treated saporin is also not attacked by proteolytic enzymes (Santanche et al, 1997). Saporin, as well as other RIPs have been shown to induce cell death via apoptosis in various different cellular models (Bergamaschi et al, 1996, Komatsu et al, 1998, Zhang et al, 2001, Narayanan et al, 2004). Pathways and factors involved have been studied for some RIPs, though the apoptotic pathway caused by saporin is still unexplored. The extreme stability of saporin to denaturation and proteolysis and its apoptosis-inducing ability makes it an ideal candidate as the toxin component in immunotoxins and chimeric toxins.

An investigation of the role of the putative active site residues of saporin-6 Tyr^{72}, Tyr^{120}, Glu^{176}, Arg^{179}, and Trp^{208} and two invariant residues Tyr^{16} and Arg^{24} by alanine scanning mutagenesis revealed that for the RNA N-glycosidase activity residues Tyr^{16}, Tyr^{72} and Arg^{179} are absolutely critical and Tyr^{120} and Glu^{176} can be partially dispensed with, while residues Trp^{208} and Arg^{24} did not appear to be involved
in this activity (Bagga et al, 2003a). The residues Tyr$^{72}$, Tyr$^{120}$, Glu$^{176}$, Arg$^{179}$, and Trp$^{208}$ were found to be essential for genomic DNA fragmentation activity, while residues Tyr$^{16}$ and Arg$^{24}$ did not appear to be required for this activity. The key residues that were involved in catalytic RNA N-glycosidase activity of saporin-6 appeared to be functionally similar to homologous residues in ricin and other RIPs, although DNA fragmentation was found to be not entirely dependent on RNA N-glycosidase activity. The study indicated that saporin-6 possesses two distinct catalytic activities, that is, RNA N-glycosidase activity and genomic DNA fragmentation activity, and for its complete cytotoxicity, both activities were required (Bagga et al, 2003a). Internalization studies on saporin-6 indicated that initially the protein remained in the cytosol, but later migrated to the nucleus, although the protein does not contain any apparent nuclear localization signal. From these studies it could be concluded that the primary intracellular target of saporin-6 was rRNA, and the activity on genomic DNA was a later event in the cytotoxicity of the protein (Bagga et al, 2003a).

More than nine isoforms of saporin have been identified from various parts of the Saponaria officinalis plant, and they differ from each other in both physico-chemical as well as biological properties. Among these, saporin-6 constitutes the major protein of the preparation from seeds, accounting for approximately 0.4% of the whole seed weight or 7% of the total seed protein (Stirpe et al, 1983), and hence is usually called saporin, by analogy to other RIPs such as gelonin and dianthin.

A comparison of the amino acid sequences of all the saporin isoforms showed that they were highly homologous, with differences being observed only at thirteen positions. Some of these amino acid changes among isoforms are conserved changes
but at seven positions there is a change in the polarity of the amino acid residue among isoforms.

A comparative study conducted between recombinant saporin seed isoforms 5 and 6 produced in *E. coli*, showed saporin-6 to be approximately 10-fold more active in its cytotoxic activity on J774A.1 cells, as well as having higher N-glycosidic and genomic DNA fragmentation activity as compared to saporin-5 (Bagga *et al.*, 2003). The difference in the activity of these two isoforms could be attributed to amino acid differences that lie outside the proposed active site of the protein. The biological role of RIPs such as saporin in plants that express them are not known definitely, and understanding the differential levels of expression, distribution and differential catalytic activity of RIP isoforms may throw some light on this area.

The present study was conducted to understand the basis of differential catalytic activity of saporin isoforms 5 and 6, to understand the apoptotic, and integrase-inhibitory activities of this molecule, and to explore the role of its C-terminal residues in catalysis. An attempt was also made to construct first generation saporin-based chimeric toxins using IL13 as the targeting ligand. The study comprised the following objectives:

1. Investigation of the basis of differential catalytic activity in saporin isoforms.
2. Study of the mechanism of apoptosis induction by saporin.
4. Study of the role of C-terminus of saporin-6 in its catalytic activity.