Chapter 9
Ribosome Inactivating Proteins from Plants: Biological Properties and their Use in Experimental Therapy

Lucía Citores, Rosario Iglesias and José Miguel Ferreras

Abstract Ribosome-inactivating proteins (RIPs) are 28 S rRNA N-glycosidases isolated mainly from plants that irreversibly inactivate ribosomes, thereby impairing protein synthesis. In recent years, polynucleotide:adenosine N-glycosidase activity and induction of apoptosis have been reported and may have a particular significance. There are two classes of RIPs: type 1 RIPs, consisting of single-chain proteins, and type 2 RIPs, consisting of an A chain with RIP properties covalently linked to a B chain with lectin properties. Type 2 RIPs may be very toxic or non toxic, whereas type 1 RIPs are always non-toxic. Due to the diverse activities of RIPs, research has been conducted to investigate their use as antiviral and antitumor agents or as the toxic part of conjugates. Conjugates consist of a targeting portion such as an antibody, a lectin or a growth factor linked to a toxic portion. RIPs have been used as the toxic portion in conjugates that have been tested in several experimental therapies against various malignancies. Although some important disadvantages still need to be improved, recent clinical trials encourage the use of these conjugates as efficacious agents in the treatment of cancer and other diseases.

9.1 Introduction

Ribosome-inactivating proteins (RIPs) have been initially studied as proteins widely distributed in plants that inhibit protein synthesis in mammalian cell-free systems [1, 2]. However, work done in recent years revealed that RIPs can also be

L. Citores · R. Iglesias · J. M. Ferreras (✉)
Department of Biochemistry and Molecular Biology and Physiology, Faculty of Sciences, University of Valladolid, E-47005 Valladolid, Spain
e-mail: rosario@bio.uva.es

L. Citores
e-mail: luciac@bio.uva.es

R. Iglesias
e-mail: riglesia@bio.uva.es

E. F. Fang and T. B. Ng (eds.), *Antitumor Potential and Other Emerging Medicinal Properties of Natural Compounds*, DOI: 10.1007/978-94-007-6214-5_9,

found in algae, fungi and bacteria [3, 4]. It has been reported that RIPs can also inhibit protein synthesis in other animal and fungi cell-free systems, and some of them can inhibit protein synthesis in plants and bacteria [3]. They also display other important biological activities. Among them, the polynucleotide:adenosine glycosidase activity [5] and the ability to induce apoptosis in several types of animal cells [6] could play a significant role, perhaps more important than that of protein synthesis inhibition. Despite all the work done in the field of RIPs, evidence is not yet available for a convincing role played by RIPs in plants. They seem to play different roles in different species, so antiviral, antifungal, storage, programmed senescence, antifeedant, stress protection and development regulation roles have been proposed for these proteins [3]. Stirpe and Barbieri [1] have proposed to classify these proteins into two types: type 1 RIPs, consisting of single-chain proteins, and type 2 RIPs, consisting of an A (active) chain with RIP properties covalently linked to a B (binding) chain with lectin properties. The latter RIPs can enter cells more easily because the B chain allows the binding to sugar-containing cell surface receptors, and for this reason they could be potent toxins. However as discussed later, recently a surprising variety of RIP and related lectin structures have been found. For example, the type 2 RIPs from *Sambucus* are less toxic to animals than the type 1 RIPs from *Saponaria* [7]. Alternatively, four-chain type 2 RIPs [7, 8] and type 1 RIPs with an inner removable peptide [9] also have been reported.

Because of their diverse activities, RIPs either alone or as part of conjugates are good candidates for developing selective antiviral and anticancer agents. Conjugates consist of a targeting portion such as an antibody, a lectin or a growth factor linked to a toxic portion. RIPs have been used as the toxic portion in several conjugates that have been tested in experimental therapies against various malignancies. In agriculture, RIPs have been shown to increase resistance against virus and other parasites in transgenic plants [3].

9.2 Distribution

RIPs have been found mainly in flowering plants (angiosperms) and, to a lesser extent, in fungi, algae and bacteria (reviewed in [3]). To date, type 1 RIPs have been isolated from at least 70 species of plants of both monocotyledons and dicotyledons. They are distributed among all flowering plant families without a pattern permitting their location in a particular taxon. However, the highest number of RIPs is present in a small number of families, namely Caryophyllaceae, Cucurbitaceae, Euphorbiaceae, Phytolacaceae and Poaceae. RIPs have been found in all types of plant tissues. Some species contain type 1 RIPs in only one tissue of the plant and others contain RIPs in several parts of the plant. However, the fact that no RIPs have been found in a particular species, family or tissue does not mean that they do not contain them since the amount of RIP could be too low to be isolated or even detected. The detection method usually consists in assaying the inhibition of protein synthesis in a cell-free system such as a rabbit reticulocytes lysate. The presence

of inhibitors has been detected in greater than 70 plants but it has not been possible to determine if the protein synthesis inhibition is due to a RIP or to another inhibitor such as an RNase or a protease. Finally, the presence of RIPs depends on different factors like senescence, virus infection, development and stress [3]. Type 2 RIPs are less widespread but they have been isolated from at least 20 species of plants belonging to 10 different families. At least five species (*Sambucus ebulus*, *Sambucus nigra*, *Cinnamomum camphora*, *Momordica charantia* and *Iris hollandica*) contain both type 1 and type 2 RIPs.

RIPs are also present in bacteria [4], fungi [10] and algae [11] but to date the presence of these proteins seems to be limited to a few species. All these findings favour the hypothesis that RIPs are broadly distributed and therefore could play an important biological role in the RIP-producer organism.

9.3 Enzymatic and Biological Activities

9.3.1 rRNA N-glycosidase

Initially RIPs were studied as the most potent inhibitors of protein synthesis in mammalian cell-free systems [1, 2]. They have been shown to inactivate the 60S subunit of mammalian ribosomes in a catalytic and irreversible manner. The inactivated ribosomes are not able to carry out the elongation cycle in protein synthesis. Later it was also reported that RIPs inactivate ribosomes from other animal species and that some of them could also inactivate ribosomes from plants and bacteria [3]. The mechanism of protein synthesis inhibition was elucidated by Endo and Tsurugi [12]. They found that RIPs are 28S rRNA N-glycosidases (EC 3.2.2.22) that cleave the N-glycosidic bond between the adenine No. 4324 from the 28S rRNA and its ribose in the 60S subunit of rat ribosomes (or the equivalent adenine in sensitive ribosomes from other organisms) [3]. This adenine is located in the α-sarcin–ricin loop (SRL) that is crucial for anchoring the elongation factor G or the elongation factor 2 on the ribosome during mRNA–tRNA translocation in prokaryotes and eukaryotes respectively [13].

Most type 1 and type 2 RIPs depurinate ribosomes at one site (the adenine 4324), whereas other RIPs such as saporins, PAP-R and trichokirin depurinate the ribosomal RNA at multiple sites [14]. However, the depurinating activity seems to be greater on the adenine 4324 than at other sites [14].

9.3.2 Polynucleotide: Adenosine Glycosidase

Recently other substrates for RIPs have been reported [5]. Some RIPs release adenines from viral genomic RNAs of MS 2, TMV and AMCV [5]. Many RIPs can also depurinate polyadenylic acid. Most RIPs release adenines from rRNA and all

of them extensively depurinate herring sperm DNA [5]. Thus, Barbieri has proposed the name of polynucleotide: adenosine glycosidases for these proteins [5].

Some RIPs remove adenine from the poly(ADP-ribosyl)ated poly(ADP-ribose) polymerase (PARP), which is involved in DNA repair and apoptosis [15]. This damage to activated PARP may have a role in the inhibition of DNA repair by RIPs, which seems to be independent of the inhibition of protein synthesis [16].

9.3.3 Apoptosis

In addition to their N-glycosidase activity on nucleic acids, both type 1 and type 2 RIPs are also capable of inducing cell death by apoptosis [17]. RIP-treated cells exhibit the morphological and biochemical events associated with apoptosis. Emerging evidence suggests that induction of apoptosis does not directly correlate with the protein synthesis inhibition [18]. RIPs trigger apoptosis in different cell types via different mechanisms. Despite a large number of studies on RIP-induced apoptosis, the exact mechanism by which these toxins induce apoptosis is not very clear. Generally, the apoptosis induced by RIPs involves the caspase dependent mitochondrial pathway leading to loss of mitochondrial membrane potential, rapid release of cytochrome c, activation of caspase-9 and an increase in the production of reactive oxygen species (ROS) in cells [17]. However, the death receptor-mediated apoptosis pathway also seems to be involved in the killing of cells by some RIPs [6, 17].

9.3.4 Other Activities

Other activities associated with some RIPs are chitinase activity, topological activity on DNA, HIV integrase inhibitory activity, superoxide dismutase activity, DNase activity, and lipase activity (reviewed in [3]). Some of them may have a significant role as has been proposed for the lipase activity in ricin toxicity or the HIV integrase inhibitory activity in the antiviral properties of some type 1 RIPs.

9.4 Structure

Figure 9.1 summarizes the different structures of RIPs and related lectins. Type 2 RIPs may be dimeric (such as ricin) or tetrameric. In the latter case the two A-B dimers may be linked by their A chains (*Ricinus communis* or *Viscum album* agglutinins) or by their B-chains (*Sambucus* tetrameric type 2 RIPs). In this case the B-chain 1 alpha site does not bind sugar-containing compounds [8]. Type 2 RIP related lectins are proteins which do not show enzymic activity and show only

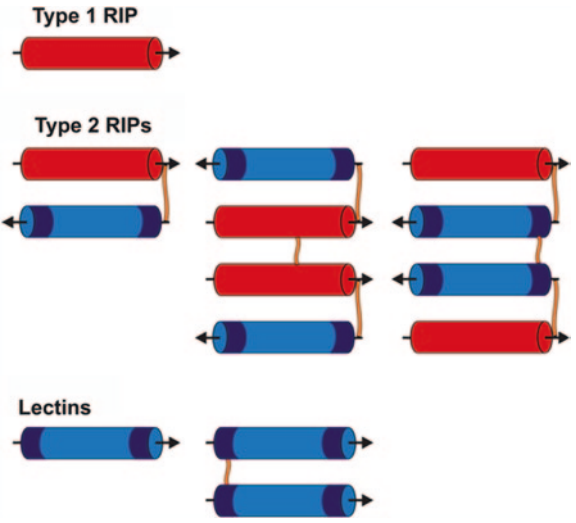

Fig. 9.1 Graphical representation of RIPs and related lectins. Type 1 RIPs consist of single-chain catalytic proteins whereas type 2 RIPs consist of catalytic(s) or A chain(s) (coloured in *red*) linked to binding or B chain(s) (coloured in *cyan*) by disulphide bond(s) (coloured in *orange*). The sugar binding subdomains 1 alpha and 2 gamma are coloured in blue. Pure lectins may have one or two B chains linked by a disulphide bond. The *arrow* indicates the C-terminal end

lectin activity. The structure of these lectins has a striking homology with that of the B chain of type 2 RIPs. In fact they are encoded by a truncated type 2 RIP gene which has lost almost all the sequence coding for the A chain. They can be monomeric (one single B-chain) or dimeric (two B-chains held together by a disulphide bridge). In this case a new cysteine appeared which is responsible for the dimerization of the two polypeptide chains through a disulphide bond. The different B chains vary with respect to sugar specificity. Type 2 RIPs from *Ricinus*, *Abrus* or *Adenia* are specific for galactose containing sugars and are very toxic. Type 2 RIPs and related lectins from *Sambucus* are much less toxic and may be specific for galactose, sialic acid or unable to bind sugars [8].

The structure of ebulin l (a heterodimeric type 2 RIP presents in *S. ebulus* leaves) is shown in Fig. 9.2. In the A chain, ebulin l has roughly the same positioning of key active site residues as type 1 RIPs and other type 2 RIPs such as ricin, abrin or volkensin [19]. Tyr 77 and 116, Arg 127, Glu 163, Arg 166 and Trp 197 are highly conserved in both type 1 and type 2 RIPs. Additionally all type 2 RIPs conserve the Cys 249 of ebulin l A chain that links it to the B chain by a disulphide bond. This Cys is absent in type 1 RIPs such as saporin or PAP. Some tetrameric type 2 RIPs (i.e., *R. communis* agglutinin) present a seven amino acid loop with an additional Cys (located in a position corresponding to Gly148 and 149 in ebulin l) that allows the formation of a disulphide bridge with another A chain [8]. The overall fold of the ebulin B chain is also very similar to that of type 2 RIP B-chains and lectins and is composed of two beta trefoil domains (I and II) with sugar-binding ability [19]. The domain I presents the 1 alpha sugar binding site (Trp 39, Asp 24, Gln 37, Asn 46 and Gln 47) and the domain II the 2 gamma sugar binding site (Asp 235, Phe 249, Asn 256 and Gln 257). These aminoacids are also well conserved in the B chain of other type 2 RIPs and lectins but the sugar-binding ability of the different B chains varies considerably. Tyr29 and

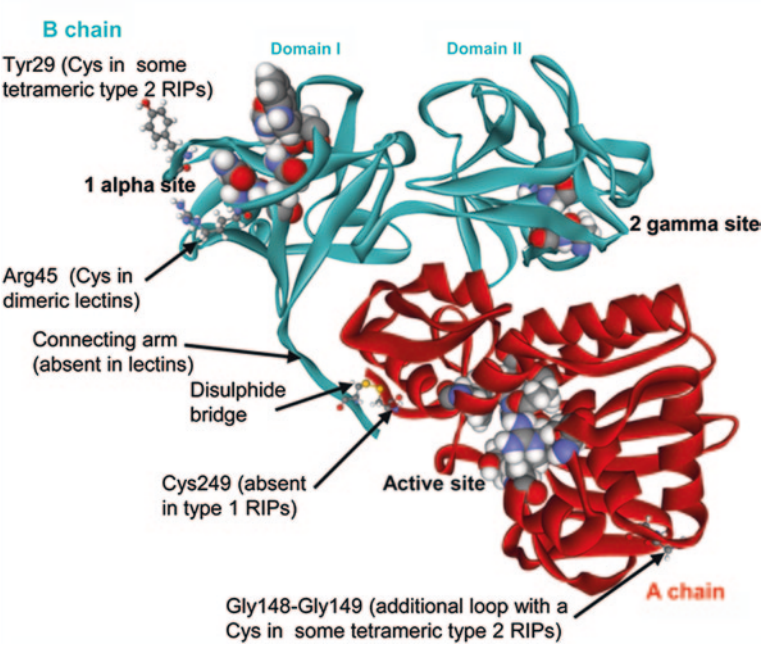

Fig. 9.2 Three-dimensional model of the type 2 RIP ebulin l (PDB ID: 1HWP). The A (*red*) and B (*cyan*) chains and the disulphide bridge linking both chains are indicated. The active site and sugar-binding sites are represented in CPK (*balls*). The residues in ebulin l that change in type 1 RIPs, tetrameric RIPs and dimeric lectins are represented in balls and sticks

Arg 45 are substituted by Cys in some tetrameric type 2 RIPs and dimeric lectins, respectively, thus allowing the formation of an additional disulphide bridge between B chains [8].

9.5 Toxicity and Intracellular Pathway of Type 1 and 2 RIPs

As previously indicated, type 1 RIPs are single-chain toxins (A-chain) with N-glycosidase activity while type 2 RIPs consist of an A-chain with N-glycosidase activity and a galactoside binding lectin (B chain) linked together by a disulphide bond. Type 1 RIPs have a high cell-free translation inhibitory potency but a low cytotoxicity (Table 9.1). Because of the absence of a B chain, type 1 RIPs, such as saporin, poorly enter into cells, consequently causing low toxicity to cells and animals. However, the presence of the B chain is not sufficient to confer a high level of cytotoxicity on all type 2 RIPs. Based on their toxicity to mammals, type 2 RIPs has been divided into two groups: the toxic and nontoxic type 2 RIPs. The former group include ricin, abrin, viscumin and

Table 9.1 Toxicity of type 1 and type 2 RIPs

	Rabbit lysate IC_{50} (nM)[a]	HeLa cells IC_{50} (nM)	Mouse LD_{50} (mg/kg)
Type 1 RIPs			
Saporin	0.037	2,300	4
PAP	0.037	33,000	2.6
Gelonin	0.4	34,000	40
Type 2 RIPs			
Ebulin l	0.15	64.3	2
Nigrin b	0.03	27.6	12
Ricinus communis agglutinin	0.046	n.d	>0.033
Sieboldin b	0.015	11.8	>1.6
Abrin	0.5	0.0037	0.00056
Ricin	0.1	0.00067	0.003
Viscumin	3.5	0.008	0.0024
Volkensin	0.37	0.0003	0.00138

The table shows the effects of type 1 and type 2 (either toxic or non-toxic) RIPs on protein synthesis by a cell-free system derived from rabbit reticulocytes lysates and their toxicity to intact HeLa cells and mice. The data were obtained from Refs. [1, 2, 8]

[a]Reduced toxin (only in type 2 RIPs): the toxin was pre-incubated with a reducing agent (such as 2-mercaptoethanol or dithiothreitol) before testing activity

volkesin, which are among the most potent plant toxins. In contrast, nigrin b, ebulin l, sieboldin b and *R. communis* agglutinin belonging to the latter group show little or no toxicity in intact cells and higher animals (Table 9.1). Their lack of toxicity has been attributed in part to a defective B chain with reduced affinity for the galactosides present at the surface of plasma membrane proteins and to a different intracellular routing and processing of the toxin [8].

The cell surface receptors differ from one RIP to another. However they share the need to enter the cytosol to exert their toxicity. The molecular mechanisms involved in intracellular traffic of RIPs have been studied extensively for toxic type 2 RIPs, especially for ricin, which has been taken as a model of highly toxic RIPs. Ricin enters target mammalian cells by receptor-mediated endocytosis and is transported to endosomes. From here part is recycled back to the cell surface, another part is transported to the lysosomes and degraded and only a small fraction is eventually translocated to the cytosol (Fig. 9.3). Only 5 % of the endocytosed toxin is transported to the trans-Golgi network [20] and then backward through the Golgi apparatus to the endoplasmic reticulum (ER). In ER the catalytic A chain is reductively separated from the cell-binding B chain. The A chain is then retrotranslocated to the cytosol by utilizing the ER-associated degradation pathway (ERAD) usually followed by misfolded proteins, which in the cytosol are polyubiquitinated and degraded by the proteasome. Once in the cytosol ricin A-chain escapes, in part, proteasomal degradation probably due to its low content in lysine residues. Once in the cytosol the enzymatic A chain inactivates the ribosomes [21].

Ricin toxicity is sensitive to brefeldin A and to low temperature. In contrast to ricin, the non toxic type 2 RIPs nigrin b and ebulin l follow a pathway that is

insensitive to brefeldin A and to temperatures below 37 °C indicating that transport from endosomes to the Golgi complex is not required for nigrin b and ebulin l A-chain translocation [8]. In fact, nigrin b was found to enter cells like ricin, but was more rapidly and extensively degraded, and when excreted by HeLa cells the nigrin b-derived material was completely inactive [22]. Thus, cell protein synthesis inhibition by nigrin b seems to be a consequence of the spontaneous translocation of nigrin b from the endosome when the extracellular concentration of RIP is high (Fig. 9.3).

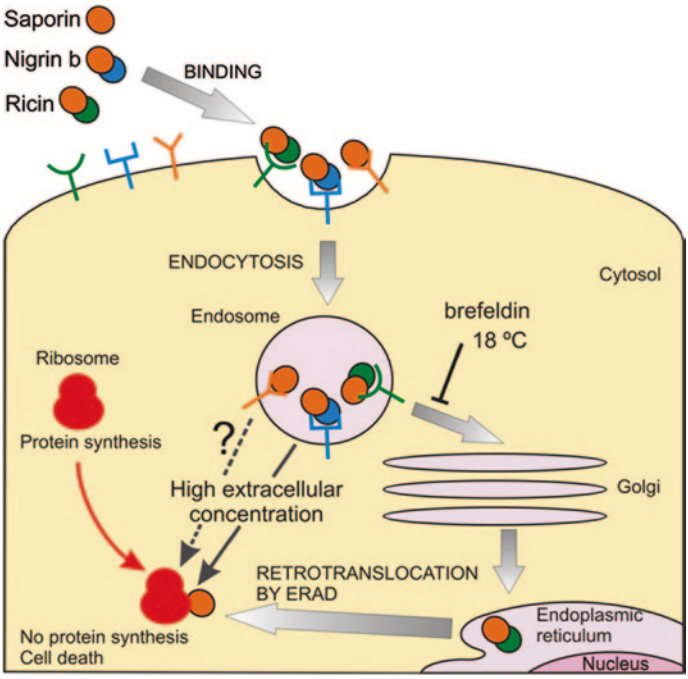

Fig. 9.3 Intracellular trafficking of ricin, nigrin b and saporin. Ricin binds to glycoproteins of the plasma membrane and internalizes into the cell. Only a small number of protein molecules are transported first to the Golgi network and then to the endoplasmic reticulum (ER). In the endoplasmic reticulum, the disulphide bridge is reduced and the A chain translocates to the cytosol by the endoplasmic reticulum-associated degradation (ERAD) pathway. In the cytosol, the A chain inactivates the ribosomes, inhibiting protein synthesis and causing cell death. This pathway is sensitive to low temperature and brefeldin A. Nigrin b can bind to different glycoproteins on the plasma membrane and internalizes into the cell. From endosomes, protein molecules are mostly transported to lysosomes for degradation. However, at much higher extracellular concentration, the saturation of the endosome with nigrin b can lead to a spontaneous release of nigrin b into the cytosol, causing ribosome inactivation. This pathway is not sensitive to low temperature and brefeldin A. Saporin binds in part to low-density lipoprotein receptor-related proteins and is internalized into the cell. Saporin cytotoxicity cannot be blocked by brefeldin A, indicating that the protein reaches the cytosol following a yet unknown intracellular route that does not involve transport to the Golgi. The orange circles represent the A chain of the three RIPs. Ricin B-chain is represented by green circles and nigrin b B-chain by blue circles

Less is known about the pathway followed by type 1 RIPs, which could vary, because of their different interaction with cells due to the lack of the lectin chain that facilitates binding to intact cells. However, studies done with saporin have shown a clear role for cell surface receptors belonging to the low density lipoprotein related receptor family in mediating saporin internalization in different cell lines [23]. Also, saporin cytotoxicity cannot be blocked by brefeldin A indicating that the protein reaches the cytosol following an intracellular route that does not include transport to the Golgi [23] (Fig. 9.3).

9.6 Use of RIPs in Experimental Therapy

Due to the diverse activities of RIPs, extensive research has been conducted to investigate their use as antiviral and antitumor agents. The most promising applications of RIPs in experimental medicine, especially in anticancer therapy, are related to their use as components of immunotoxins, conjugates or recombinant chimeras in which the enzymatic RIPs are linked to tumor targeting ligands or antibodies that mediate their binding and internalization by malignant cells.

On the down side, because of their cytotoxic nature, type 2 RIPs present a significant public health concern due to their potential use as bioterrorism agents. Ricin, for example, is a potent cytotoxin, easy to purify in large amounts, easy to handle and stable. For this reason extensive efforts have been made to develop a safe antidote or vaccine against this toxin [24].

9.6.1 Use of Unconjugated RIPs in Experimental Therapy

Interest in type 2 RIPs as anticancer agents began as early as 1970, when it was shown that ricin and abrin were more toxic to tumor cells than to normal cells [25]. Mistletoe extracts, based on their presumed immunostimulatory and antineoplastic effects, have been used in the complementary treatment of cancer patients, for more than 80 years. The active compound of these extracts is the type 2 RIP viscumin (MLs) which possess apoptosis-inducing effects on many types of cancers [26, 27]. Recently a recombinantly engineered rViscumin has been developed for application in cancer treatment and is being tested in phase I/II clinical studies [28]. Another type 2 RIP from *Ximenia americana*, termed riproximin, which is the active component of the plant material used in African traditional medicine to treat some forms of cancer, has shown potent antitumor activity in a rat metastasis model [29]. Therefore, it seems that type 2 RIPs such as viscumin, abrin or ricin bear apoptosis-inducing activities toward cancer cells by targeting different stages of apoptotic pathways [26].

Plants from Cucurbitaceae family, mainly belonging to the genus *Trichosanthes* and *Momordica*, have a long history of being used in old traditional Chinese

medicine. Trichosanthin is a type 1 RIP purified from *T. kirilowii* that has been known for around 30 years. Trichosanthin is known to possess a broad spectrum of biological and pharmacological activities, including abortifacient which terminates early pregnancy, anti-tumor, immunomodulatory, nuclease, and anti-human immunodeficiency virus activities. Recently, trichosanthin has been found to induce apoptosis, enhance the action of chemokines and inhibit HIV-1 integrase [30]. Also of interest as a medicinal candidate, alpha-momorcharin, a type 1 RIP, purified from *M. charantia* has demonstrated strong anti-tumor growth activity and anti-HIV function besides abortifacient activity [31] (see Chap. 2 in this book). In addition, recombinant luffin, a type 1 RIP from *Luffa cylindrica*, displays in vitro cytotoxicity against various tumor cell lines [32].

9.6.2 Use of RIPs for the Construction of Immunotoxins

Another application of RIPs is the linking of them to monoclonal antibodies or their fragments to obtain immunotoxins that are specifically toxic to target cells [33–36]. The antibody (or antibody fragment) [37] binds specifically to an antigen on the surface of the cell to be killed and the immunotoxin internalizes through endocytosis following the antigen intracellular pathway. Finally the toxin translocates to the cytosol and kills the cell by inhibiting protein synthesis or inducing apoptosis.

These kinds of therapeutic agents were first postulated by the German immunologist Paul Ehrlich in 1897 who envisaged them as “magic bullets” [38]. However, they did not become a reality until the development of the monoclonal antibody, protein purification and protein cross-linking technologies. In fact the first immunotoxin containing a RIP was reported by Youle and Neville in 1980 [39]. It consisted of a monoclonal antibody directed against the murine T cells Thy 1.2 antigen, covalently linked to ricin using the crosslinking agent m-maleimidobenzoyl-N-hydroxysuecinimide ester (MBS). The immunotoxin showed cell type specificity when the unwanted binding via the B chain was blocked with lactose. Unfortunately, the necessary use of lactose limited its use to ex vivo therapy [40].

Immunotoxins have been constructed with selected potent microbial and plant proteins. Among microbial proteins, *Pseudomonas* exotoxin or diphtheria toxin have been used, and among plant proteins, both type 1 and type 2 RIPs have been used.

Since the construction of the first immunotoxin, which was prepared by chemically linking the whole molecule of an antibody to the toxin, several kinds of these chimeras have been designed in order to improve several important aspects such as specificity, efficacy, in vivo stability, immunogenicity and unwanted side effects. From a strategic point of view, several types of immunotoxins have been designed [1]: direct immunotoxins, indirect immunotoxins (directed to a primary antibody) and bispecific antibodies (directed to the toxin and the target simultaneously). In order to prevent unwanted side effects immunotoxins have been

constructed with antibody fragments such as Fab' (obtained by pepsin digestion and mild reduction), Fab (obtained by papain digestion), Fv (containing only the two antibody variable domains, VH and VL, linked by a disulphide bridge) and scFv (single-chain variable fragment, containing the two antibody variable domains connected with a short linker peptide).

The choice of the crosslinker agent is also very important. Efforts have been made in order to optimize the efficient release of the toxic moiety when the target is reached. This question has been excellently reviewed recently by Dosio et al. [36]. The most used RIP for the construction of immunotoxins is ricin, but as discussed, it is extremely toxic. For this reason attempts have been made to reduce the non-specificity caused by the B-chain. Approaches are based on the blockage of the B-chain lectin binding ability [36] or the use of the deglycosylated ricin A-chain (the glycosylated ricin A-chain is toxic to macrophages and Kupffer cells) [36]. Another approach is to use type 1 RIPs instead of ricin. The most commonly used are saporin, PAP and gelonin [34]. Recombinant immunotoxins have also been designed consisting of single-chain variable fragments (scFv) genetically fused to a RIP such as gelonin [41].

Immunotoxins cause several undesirable side effects and toxicities. Some of these adverse effects are mild or moderate like fever, nausea, vomiting, diarrhoea, myalgia, edema and hypoalbuminemia. Other effects are severe and could pose a limitation to the therapeutic use of protein immunotoxins. An increased toxicity may result from immunogenicity as a result of the formation of human antimouse antibodies (HAMA) or anti-toxin-antibodies (HATA). These antibodies may prevent repeated cycles of therapy. The development of immunotoxins containing humanized antibodies or choosing smaller antibody formats containing only the variable domains may in part resolve these problems. Many efforts are being made also to decrease immunogenicity of the toxin moiety.

The most common toxicity in patients treated with immunotoxins, in particular ricin A chain-based ones, is vascular leak syndrome (VLS) characterized by an increase in vascular permeability caused by the weak binding of the immunotoxin to normal endothelial cells. Another typical toxicity related to immunotoxin administration is hepatotoxicity that is attributed to the binding of basic residues on the targeting Fv to negatively charged hepatic cells. Renal toxicity is also observed following treatment with immunotoxins. Moreover, successful treatment of solid tumors faces several obstacles including poor penetration into tumor masses and the immune response to the conjugate. Tumor progression is characterized by the formation of a neovasculature, which supplies tumor cells with oxygen and nutrients. The formation of new blood vessels (angiogenesis) is necessary for the growth and metastatic spread of solid tumor. Immunoconjugates containing RIPs targeting the tumor neovasculature have been designed and some of them have considerable potential for use in cancer therapy [7].

Immunotoxins have been included in experimental therapies against various malignancies, often achieving promising results but also often causing severe side effects in patients. Among RIPs, ricin, saporin and gelonin have been widely used

to construct anti-cancer immunotoxins. The subject has been dealt with in a number of recent reviews [33–37] and for this reason only some selected examples are shown in Table 9.2.

Briefly, clinical trials with RIP-containing immunotoxins have been conducted against hematologic malignancies and solid tumors. Clinical studies have been carried out using both blocked ricin and its A-chain (glycosylated or deglycosylated) linked to several monoclonal antibodies for the treatment of acute lymphoblastic leukemia, B cell non-Hodgkin's lymphomas, Hodgkin's disease, cutaneous T cell lymphomas, graft-versus-host disease, haploidentical stem cell transplantation and T cell non-Hodgkin's lymphoma. Regarding solid tumors clinical applications of ricin-based immunotoxins include melanoma, colorectal cancer, small cell lung cancer, leptomeningeal neoplasia and breast cancer. Additionally, type 1 RIPs such as PAP, saporin or gelonin have been used in immunotoxins tested in clinical trials in patients with acute lymphoblastic leukaemia, Hodgkin's disease and myeloid malignancies.

9.6.3 Use of Other RIP Conjugates

Targeting can also be performed using tumor-specific ligands. Cancer cells overexpress cell-surface receptors including growth factor, transferrin and interleukin receptors. Several conjugates consisting of a tumor-specific ligand (cytokine, growth factor, transferrin or peptide hormone) coupled to a toxin by genetic fusion or by chemical ligation have been produced. The targeting protein or ligand binds to cell

Table 9.2 Selected examples of clinically evaluated/under evaluation immunotoxins

Immunotoxin	Target	RIP	Target diseases	Phases
Combotox	CD19/CD22	dgA	NHL, ALL	I
IMTOX-25	CD25	dgA	HD, CTCL, GVHD, HSCT, melanoma	I,II
Anti-B4-bR	CD19	Blocked ricin	NHL	II,III
$F(ab')_2$BsAb	CD22	Saporin	NHL	I
BER-H_2-Saporin	CD30	Saporin	HD	I/II
Hum-195/rGel	CD33	Gelonin	AML, CML	I
B43-PAP	CD19	PAP	ALL	I
XomaZyme-Mel	Melanoma	Ricin A-chain	Melanoma	I,II,I/II
N901-bR	CD56	Blocked ricin	SCLC	I,II
Anti-CEA-bR	CEA	Blocked ricin	Colorectal cancer	I/II

The data were obtained from the references cited in the text
Abbreviations ALL (acute lymphoblastic leukemia); *AML* (acute myelogenous leukemia); *CEA* (carcinoembryonic antigen); *CML* (chronic myelogenous leucemia); *CTCL* (cutaneous T cell lymphoma); *dgA* (deglycosylated ricin A-chain); *GVHD* (graft-versus-host disease); *HD* (Hodgkin's disease); *NHL* (non-Hodgkin's lymphoma); *HSCT* (haploidentical stem cell transplantation); *SCLC* (small cell lung cancer)

surface receptors and are subsequently internalized by endocytosis, resulting in cell death. The toxin moiety requires internalization and translocation to the cytosol to achieve the cytotoxic effect by inactivating cytosolic protein synthesis and inducing apoptosis [35, 42].

The transferrin receptor, a cell membrane-associated glycoprotein involved in iron homeostasis and cell growth, has been explored as a target to deliver therapeutics into cancer cells due to its increased expression on malignant cells. Conjugates have been made using the plasma protein transferrin as a carrier fused to several RIPs such as ricin A-chain [43], saporin [43], nigrin b [7] and ebulin l [7]. These conjugates have demonstrated anti-tumor activity in several in vitro and in vivo models [43].

Epidermal growth factor receptor (EGFR) is overexpressed in many different types of solid tumors and is associated with metastasis and poor prognosis. Several anti-cancer conjugates targeting the EFGR have been developed. Recombinant saporin has been linked to EGF both directly and via an adapter. Both conjugates were very effective in inhibiting an oral squamous cell carcinoma cell line. More importantly, the authors observe a significant reduction in the number of colonies formed [44].

Fibroblast growth factor receptors (FGFRs) are a family of at least 12 different proteins. Many solid tumors express receptors binding FGF2. FGF2-saporin was the first recombinant fusion chimera based on saporin expressed in *E. coli* shown to be highly selective and cytotoxic towards FGF2 receptor-expressing cells [45]. The conjugate also showed significant anti-proliferation activity when tested in animal models of human ovarian teratocarcinoma or melanomas in a combination therapy [46].

Another antitumor therapy approach involves the vascular endothelial growth factor (VEGF). VEGF-gelonin fusions contain a highly selective carrier, targeting tumor endothelial cells. This fusion protein is able to inhibit tumor growth and metastasis dissemination [47].

The therapeutic efficacy of natural ligand-toxin conjugates can be limited by the intracellular trafficking pathway followed by endogenous ligands. To improve the therapeutic efficacy of ligand-drug conjugates, research groups are developing new approaches for engineering the ligands to be more effective drug carriers [42].

Finally, the use of pure lectins for targeting has also been proposed [7]. Conjugates containing nigrin b as the toxic portion and the mucin-binding lectins SELld or SELfd as the targeting molecule, proved to be effective in killing COLO 320 and HeLa cells. In contrast, the free lectins had a very small or no effect on cell viability. SELld and SELfd are type 2 RIPs-related dimeric lectins isolated from the leaves and fruits of *Sambucus ebulus* respectively. These results open the possibility of using pure lectins as targeting molecules for cancer therapy.

9.7 Conclusions and Perspectives

Since their discovery, RIPs have been the subject of extensive investigation due to their strong enzymatic activity and toxicity. These enzymes share common properties such as conserved active site residues and reaction mechanisms. RIPs are

N-glycosidases capable of inhibiting protein synthesis by depurinating rRNA. However, the diversity among RIPs and their activities toward different targets make it difficult to extrapolate results in attempting to characterize the biological role of RIPs in plants. Discovery of multiple enzymatic activities in some RIPs, which can depurinate not only ribosomal substrates but may also damage DNA or RNA of pathogens and host cells, makes the picture more complicated. RIPs are broadly distributed in plants; however there are no systematic screening studies to generalize their occurrence. Results from such studies might provide new information about the phylogenetic distribution, structure and functions of RIPs. Also solving the three-dimensional structure of more RIPs will facilitate the elucidation of RIP structure–function relationships and will add valuable information regarding the structural homology of the RIPs. Plants produce RIPs that are able to kill mammalian cells once delivered to the cytosol. In mammalian cells, both type 1 and type 2 RIPs have been related to apoptosis. The mechanisms by which apoptosis is activated by a particular RIP may differ and may be independent of protein synthesis inhibition. Therefore, the process of RIP uptake and transport in cells needs more investigation in order to understand the mechanism by which RIPs lead to cell death. Knowing how they target cells will make it possible to use them to our advantage in medicine. RIPs have a potential as therapeutic agents if the toxicity can be specifically directed. RIP-containing conjugates have been used in many experimental strategies against cancer cells, often showing excellent clinical activity, for example immunotoxins targeting hematological malignancies. A major limitation of immunotoxins is the development of neutralizing antibodies to both the toxic and the carrier portions of the conjugate together with the development of VLS and hepatotoxicity. However, there have been significant advancements in the design of new immunotoxins that reduce side effects on normal cells. From the progress over the past twenty years it is apparent that immunotoxins are having an increasing impact in experimental therapy.

Acknowledgments This work was supported by grants FISPI04/1279 to J.M.F. and BIO39/VA42/10 to L.C. We thank Judy Callaghan (Monash University, Melbourne, Australia) for correcting the manuscript.

References

1. Barbieri L, Battelli MG, Stirpe F (1993) Ribosome-inactivating proteins from plants. Biochim Biophys Acta 1154:237–282
2. Stirpe F, Barbieri L (1986) Ribosome-inactivating proteins up to date. FEBS Lett 195:1–8
3. Girbes T, Ferreras JM, Arias FJ, Stirpe F (2004) Description, distribution, activity and phylogenetic relationship of ribosome-inactivating proteins in plants, fungi and bacteria. Mini Rev Med Chem 4:461–476
4. Obrig TG (1997) Shiga toxin mode of action in E. coli O157:H7 disease. Front Biosci 2:d635–d642
5. Barbieri L, Valbonesi P, Bonora E, Gorini P, Bolognesi A, Stirpe F (1997) Polynucleotide: adenosine glycosidase activity of ribosome-inactivating proteins: effect on DNA, RNA and poly(A). Nucleic Acids Res 25:518–522

6. Polito L, Bortolotti M, Farini V, Battelli MG, Barbieri L, Bolognesi A (2009) Saporin induces multiple death pathways in lymphoma cells with different intensity and timing as compared to ricin. Int J Biochem Cell Biol 41:1055–1061
7. Ferreras JM, Citores L, Iglesias R, Jimenez P, Girbes T (2011) Use of ribosome-inactivating proteins from *Sambucus* for the construction of immunotoxins and conjugates for cancer therapy. Toxins (Basel) 3:420–441
8. Ferreras JM, Citores L, Iglesias R, Jiménez P, Girbés T (2010) *Sambucus* ribosome-inactivating proteins and lectins. In: Lord JM, Hartley MR (eds) Toxic plant proteins-series plant cell monographs, vol 18. Springer, Berlin, pp 107–131
9. Mak AN, Wong YT, An YJ, Cha SS, Sze KH, Au SW, Wong KB, Shaw PC (2007) Structure-function study of maize ribosome-inactivating protein: implications for the internal inactivation region and the sole glutamate in the active site. Nucleic Acids Res 35:6259–6267
10. Wang HX, Ng TB (2001) Isolation of pleuturegin, a novel ribosome-inactivating protein from fresh sclerotia of the edible mushroom *Pleurotus tuber-regium*. Biochem Biophys Res Commun 288:718–721
11. Liu RS, Yang JH, Liu WY (2002) Isolation and enzymatic characterization of lamjapin, the first ribosome-inactivating protein from cryptogamic algal plant (*Laminaria japonica* A). Eur J Biochem 269:4746–4752
12. Endo Y, Tsurugi K (1988) The RNA N-glycosidase activity of ricin A-chain. The characteristics of the enzymatic activity of ricin A-chain with ribosomes and with rRNA. J Biol Chem 263:8735–8739
13. Shi X, Khade PK, Sanbonmatsu KY, Joseph S (2012) Functional role of the sarcin-ricin loop of the 23S rRNA in the elongation cycle of protein synthesis. J Mol Biol 419:125–138
14. Barbieri L, Ferreras JM, Barraco A, Ricci P, Stirpe F (1992) Some ribosome-inactivating proteins depurinate ribosomal RNA at multiple sites. Biochem J 286(Pt 1):1–4
15. Barbieri L, Brigotti M, Perocco P, Carnicelli D, Ciani M, Mercatali L, Stirpe F (2003) Ribosome-inactivating proteins depurinate poly(ADP-ribosyl)ated poly(ADP-ribose) polymerase and have transforming activity for 3T3 fibroblasts. FEBS Lett 538:178–182
16. Sestili P, Alfieri R, Carnicelli D, Martinelli C, Barbieri L, Stirpe F, Bonelli M, Petronini PG, Brigotti M (2005) Shiga toxin 1 and ricin inhibit the repair of H_2O_2-induced DNA single strand breaks in cultured mammalian cells. DNA Repair (Amst) 4:271–277
17. Sikriwal D, Batra JK (2010) Ribosome inactivating proteins and apoptosis. In: Lord JM, Hartley MR (eds) Toxic plant proteins-series plant cell monographs, vol 18. Springer, Berlin pp 167–189
18. Das MK, Sharma RS, Mishra V (2012) Induction of apoptosis by ribosome inactivating proteins: importance of N-glycosidase activity. Appl Biochem Biotechnol 166:1552–1561
19. Pascal JM, Day PJ, Monzingo AF, Ernst SR, Robertus JD, Iglesias R, Perez Y, Ferreras JM, Citores L, Girbes T (2001) 2.8-A crystal structure of a nontoxic type-II ribosome-inactivating protein, ebulin l. Proteins 43:319–326
20. van Deurs B, Sandvig K, Petersen OW, Olsnes S, Simons K, Griffiths G (1988) Estimation of the amount of internalized ricin that reaches the trans-Golgi network. J Cell Biol 106:253–267
21. Lord JM, Spooner RA (2011) Ricin trafficking in plant and mammalian cells. Toxins (Basel) 3:787–801
22. Battelli MG, Musiani S, Buonamici L, Santi S, Riccio M, Maraldi NM, Girbes T, Stirpe F (2004) Interaction of volkensin with HeLa cells: binding, uptake, intracellular localization, degradation and exocytosis. Cell Mol Life Sci 61:1975–1984
23. Vago R, Marsden CJ, Lord JM, Ippoliti R, Flavell DJ, Flavell SU, Ceriotti A, Fabbrini MS (2005) Saporin and ricin A chain follow different intracellular routes to enter the cytosol of intoxicated cells. FEBS J 272:4983–4995
24. Reisler RB, Smith LA (2012) The need for continued development of ricin countermeasures. Adv Prev Med 2012:149737
25. Lin JY, Tserng KY, Chen CC, Lin LT, Tung TC (1970) Abrin and ricin: new anti-tumour substances. Nature 227:292–293
26. Fu LL, Zhou CC, Yao S, Yu JY, Liu B, Bao JK (2011) Plant lectins: targeting programmed cell death pathways as antitumor agents. Int J Biochem Cell Biol 43:1442–1449

27. Pryme IF, Bardocz S, Pusztai A, Ewen SW (2006) Suppression of growth of tumour cell lines in vitro and tumours in vivo by mistletoe lectins. Histol Histopathol 21:285–299
28. Zwierzina H, Bergmann L, Fiebig H, Aamdal S, Schoffski P, Witthohn K, Lentzen H (2011) The preclinical and clinical activity of aviscumine: a potential anticancer drug. Eur J Cancer 47:1450–1457
29. Voss C, Eyol E, Frank M, von der Lieth CW, Berger MR (2006) Identification and characterization of riproximin, a new type II ribosome-inactivating protein with antineoplastic activity from *Ximenia americana*. FASEB J 20:1194–1196
30. Shaw PC, Lee KM, Wong KB (2005) Recent advances in trichosanthin, a ribosome-inactivating protein with multiple pharmacological properties. Toxicon 45:683–689
31. Fang EF, Ng TB (2011) Bitter gourd (*Momordica charantia*) is a cornucopia of health: a review of its credited antidiabetic, anti-HIV, and antitumor properties. Curr Mol Med 11:417–436
32. Liu L, Wang R, He W, He F, Huang G (2010) Cloning and soluble expression of mature alpha-luffin from *Luffa cylindrica* and its antitumor activities in vitro. Acta Biochim Biophys Sin (Shanghai) 42:585–592
33. Becker N, Benhar I (2012) Antibody-based immunotoxins for the treatment of cancer. Antibodies 1:39–69
34. Polito L, Bortolotti M, Pedrazzi M, Bolognesi A (2011) Immunotoxins and other conjugates containing saporin-s6 for cancer therapy. Toxins (Basel) 3:697–720
35. de Virgilio M, Lombardi A, Caliandro R, Fabbrini MS (2010) Ribosome-inactivating proteins: from plant defense to tumor attack. Toxins (Basel) 2:2699–2737
36. Dosio F, Brusa P, Cattel L (2011) Immunotoxins and anticancer drug conjugate assemblies: the role of the linkage between components. Toxins (Basel) 3:848–883
37. Madhumathi J, Verma RS (2012) Therapeutic targets and recent advances in protein immunotoxins. Curr Opin Microbiol 15:300–309
38. Gensini GF, Conti AA, Lippi D (2007) The contributions of Paul Ehrlich to infectious disease. J Infect 54:221–224
39. Youle RJ, Neville DM Jr (1980) Anti-Thy 1.2 monoclonal antibody linked to ricin is a potent cell-type-specific toxin. Proc Natl Acad Sci U S A 77:5483–5486
40. Filipovich AH, Vallera DA, Youle RJ, Quinones RR, Neville DM Jr, Kersey JH (1984) *Ex-vivo* treatment of donor bone marrow with anti-T-cell immunotoxins for prevention of graft-versus-host disease. Lancet 1:469–472
41. Cao Y, Marks JD, Huang Q, Rudnick SI, Xiong C, Hittelman WN, Wen X, Marks JW, Cheung LH, Boland K, Li C, Adams GP, Rosenblum MG (2012) Single-chain antibody-based immunotoxins targeting Her2/neu: design optimization and impact of affinity on antitumor efficacy and off-target toxicity. Mol Cancer Ther 11:143–153
42. Yoon DJ, Liu CT, Quinlan DS, Nafisi PM, Kamei DT (2011) Intracellular trafficking considerations in the development of natural ligand-drug molecular conjugates for cancer. Ann Biomed Eng 39:1235–1251
43. Daniels TR, Delgado T, Helguera G, Penichet ML (2006) The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells. Clin Immunol 121:159–176
44. Heisler I, Sutherland M, Bachran C, Hebestreit P, Schnitger A, Melzig MF, Fuchs H (2005) Combined application of saponin and chimeric toxins drastically enhances the targeted cytotoxicity on tumor cells. J Control Release 106:123–137
45. Lappi DA, Ying W, Barthelemy I, Martineau D, Prieto I, Benatti L, Soria M, Baird A (1994) Expression and activities of a recombinant basic fibroblast growth factor-saporin fusion protein. J Biol Chem 269:12552–12558
46. Davol P, Beitz JG, Mohler M, Ying W, Cook J, Lappi DA, Frackelton AR Jr (1995) Saporin toxins directed to basic fibroblast growth factor receptors effectively target human ovarian teratocarcinoma in an animal model. Cancer 76:79–85
47. Smagur A, Boyko MM, Biront NV, Cichon T, Szala S (2009) Chimeric protein ABRaA-VEGF121 is cytotoxic towards VEGFR-2-expressing PAE cells and inhibits B16–F10 melanoma growth. Acta Biochim Pol 56:115–124

Lucía Citores studied Chemistry at the University of Valladolid, Spain, and completed her PhD at the same University in 1996. From 1997 to 2004 she worked at the Institute for Cancer Research in Oslo, Norway, as a postdoctoral researcher. During this time she became interested in the endocytosis and intracellular transport of fibroblast growth factors and their receptors. She returned to Spain in 2004, and for five years she stayed at the University of Valladolid as a postdoctoral researcher. In 2009, she was appointed as Non-civil servant tenured Lecturer in Biochemistry and Molecular Biology at the University of Valladolid and in 2011 she was appointed as Associated Professor in the same area. Her research interests have focused on distribution, activity and structure of ribosome-inactivating proteins and how they enter and intoxicate mammalian cells.

Maria Rosario Iglesias studied Biology at the University of León, Spain, and completed her PhD at the University of Valladolid, Spain, in 1989. From 1991 to 1992 she worked at the University of Windsor, Canada, as a postdoctoral researcher in several aspects of genetic alterations of plasma lipoproteins, focused on the study of the transciptional regulation of Apo B gen in HepG2 cells. She returned to Valladolid in 1992 and she was appointed as Associated Professor in Biochemistry and Molecular Biology there in 1998. Her research interests have focused on the activity, molecular cloning and expression of ribosome-inactivating proteins, as well as the role played by RIPs in plant defense response to biotic and environmental stress.

José Miguel Ferreras studied Biology at the University of León, Spain, and completed his PhD at the University of Valladolid, Spain, in 1989. From 1990 to 1991 he worked at the University of Bologna, Italy, as a postdoctoral researcher. He returned to Valladolid in 1991 and he was appointed as Associated Professor in Biochemistry and Molecular Biology. His research interests have focused on the distribution, activity and structure of ribosome-inactivating proteins and their use for the construction of immunotoxins and other conjugates for cancer therapy.