

Characterization of highly toxic type 2 ribosome-inactivating proteins from *Adenia lanceolata* and *Adenia stenodactyla* (Passifloraceae)

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Abstract

From the caudices of the Passifloraceae *Adenia lanceolata* and *A. stenodactyla*, two lectins called lanceolin and stenodactylin, respectively, were purified by affinity chromatography on CL Sepharose 6B. The lectins are glycoproteins with M_r 61,243 (lanceolin) and 63,131 (stenodactylin), consisting of an enzymatic A chain linked to a larger B chain with lectin properties, with N-terminal amino acid sequences similar to that of volkensin, the toxic lectin from *A. volkensii*. The lectins agglutinate red blood cells, inhibit protein synthesis both by a cell-free system and by whole cells, and deplete ribosomes and DNA, but not tRNA or poly(A). They are highly toxic to cells, in which they induce apoptosis, and to mice, with LD₅₀s 8.16 µg/kg (lanceolin) and 2.76 µg/kg (stenodactylin) at 48 h. Thus, lanceolin and stenodactylin have all the properties of the toxic type 2 ribosome-inactivating proteins and are amongst the most potent toxins of plant origin.

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1. Introduction

Ribosome-inactivating proteins (RIPs) (reviews in Peumans et al., 2001; Van Damme et al., 2001; Girbés et al., 2004; Hartley and Lord, 2004; Stirpe, 2004; Stirpe and Battelli, 2006) on the basis of their

structure are divided into type 1, consisting of a single chain with enzymatic activity, type 2, consisting of an enzymatic A chain linked to a B chain, which is a lectin specific for galactose, *N*-acetyl galactosamine or *N*-acetyl neuraminic acid (Van Damme et al., 1998) and a type 3, including proteins that are activated after proteolytic removal of a peptide segment with unknown function (review in Peumans et al., 2001).

RIPs are officially known as rRNA *N*-glycosidases (EC 3.2.2.22), because it was found that both type 1 RIPs and the A chains of type 2 RIPs

Abbreviations: RIPs, ribosome-inactivating proteins; hsDNA, herring sperm DNA

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inactivate ribosomes by removing a single adenine from rRNA (Endo and Tsurugi, 1987). However, subsequently it was found that RIPs remove adenine from various polynucleotides other than rRNA (Barbieri et al., 1997), and consequently the denomination of adenine polynucleotide glycosylase was suggested as a more appropriate one (Barbieri et al., 2001).

Type 1 RIPs do not bind to cells and do not enter easily into the cytoplasm, and consequently have a relatively low toxicity to cells and animals. The lectinic B chains of type 2 RIPs bind to galactosyl-terminated glycoproteins on the cell surface, allowing and facilitating the entry into cells of the A chains, which then exert their enzymatic activity on ribosomes and possibly other cellular structures. Thus, some type 2 RIPs are potent toxins, the best known being ricin, whilst others (list in Stirpe, 2004) have a much lower toxicity, comparable to that of type 1 RIPs. These differences may be due at least in part to different binding, entry into cells, intracellular routing, degradation and exocytosis of toxic and non-toxic lectins (Battelli et al., 1997, 2004).

RIPs are widely distributed in the plant kingdom (reviews in Van Damme et al., 2001; Gírbés et al., 2004), and are present also in bacteria (Shiga and Shiga-like toxins, Endo et al., 1988). In plants, type 1 RIPs are very frequent, and it is known that they are expressed at higher levels in plants belonging to some families (Caryophyllaceae, Cucurbitaceae, Euphorbiaceae). Fewer type 2 RIPs are known, and the number of the toxic ones is restricted to 10 (Stirpe and Battelli, 2006), ricin and abrin, known since Paul Ehrlich's time, and others found in the last 30 years. These toxins, or part of them, are used for the preparation of conjugates with antibodies ("immunotoxins") or other carriers, useful to eliminate in a selective manner cells target of the carrier used (Fracasso et al., 2004; Bolognesi and Polito, 2004). Unfortunately, toxins have been used also for homicidal purposes (e.g. Knight, 1979) and there are fears that could become weapons for warfare or bioterroristic attacks (Greenfield et al., 2002).

Among the toxic type 2 RIPs, there are two very potent toxins, modeccin and volkensin, found, respectively, in the roots of the Passifloraceae *Adenia (Modecca) digitata* (Refsnes et al., 1977; Stirpe et al., 1978) and *A. volkensii* (Barbieri et al., 1984; Stirpe et al., 1985). These two toxins, like other RIPs, are useful models to study the entry into cells, intracellular routing and processing of pro-

teins, and are especially interesting because they are retrogradely transported not only along peripheral nerves, like ricin and abrin (review in Wiley and Lappi, 1994), but also within the central nervous system (Wiley and Stirpe, 1988; Cevolani et al., 1995). This prompted us to investigate whether other plants belonging to the genus *Adenia* produce toxic type 2 RIPs similar to modeccin and volkensin. In a previous study, 10 plants of the genus *Adenia* were examined, and it was observed that in all of them there are lectins, six of which had the characteristics of type 2 toxic RIPs, those from three plants, *A. goetzii*, *A. lanceolata* and *A. stenodactyla* appearing particularly potent (Pelosi et al., 2005). We describe now the main properties of the toxic lectins from *A. lanceolata* and *A. stenodactyla*, hereupon referred to as lanceolin and stenodactylin, respectively.

2. Materials and methods

2.1. Safety precautions

The plant tissues used contained a high amount of very potent toxins, and hence stringent safety measures were required during handling. The plants should be handled carefully, avoiding direct or indirect contacts with skin or eyes, and tissues must be homogenized in closed equipment under a ventilated fume hood, to prevent inhaling aerosols that may be formed. The use of glass containers and of syringe needles should be avoided as far as possible.

2.2. Materials and animals

Plants were purchased from Exotica Botanical Rarities, Erkelenz-Golkrath, Germany, and were kept in the greenhouse of the Botanical Garden of the University of Bologna until use. Adenine, poly(A), tRNA and molecular mass markers were from Sigma Aldrich (St. Louis, MO, USA). Volkensin was prepared as described by Barbieri et al. (1984). Ricin was prepared as described by Nicolson et al. (1974) and other RIPs as described by Barbieri et al. (1987). Rabbit sera against ricin and type 1 RIPs were obtained as described (Strocchi et al., 1992). Reagents for electrophoresis and L-[4,5-³H]leucine were from GE Healthcare (Milan, Italy).

Mice and rabbits were purchased from Charles River Laboratories Italia s.r.l. (Milano, Italy).

2.3. Cell cultures

The cell lines used, namely murine 3T3 (fibroblasts), human NB100 (neuroblastoma) and BeWo (chorioncarcinoma) were maintained as monolayer cultures in RPMI 1640 medium supplemented with antibiotics and 10% calf serum (hereupon referred to as complete medium), in a humidified atmosphere containing 5% CO₂, at 37 °C. Subcultures were obtained by trypsin treatment of confluent cultures. The human JM cell line (monocyte-derived) was grown in suspension and treated with phorbol myristate to induce adhesion as described by Bolognesi et al. (1995).

2.4. Lectin purification

The procedure followed in previous work (Pelosi et al., 2005) was scaled up to process larger amounts of material. Briefly, parts of caudices (120–130 g) were homogenized in a blender with 5 ml/g of phosphate-buffered saline (PBS, 0.14 M NaCl containing 5 mM sodium phosphate buffer, pH 7.5). After overnight stirring at 4 °C, the homogenates were centrifuged at 2000g for 30 min at 4 °C, the supernatant was adjusted to 100% saturation with solid ammonium sulfate and centrifuged again. The precipitate was dissolved in, and dialyzed against PBS and loaded on an acid-treated Sepharose CL-6B column (29 × 5 cm). The retained protein was eluted as a single peak with 0.2 M galactose, dialysed against water and freeze-dried.

Stenodactylin was reduced in 50 mM Tris/HCl, pH 8.5 containing 2% 2-mercaptoethanol (v/v) for 2 h at room temperature, and its constituent chains were separated by chromatography on an acid-treated Sepharose CL-6B column (14 × 1 cm), equilibrated in PBS and eluted with 0.2 M galactose in PBS at 0.5 ml/min as described in Barbieri et al. (1980).

Separation of the various forms of lanceolin was attempted by affinity chromatography on acid-treated Sepharose CL-6B with elution by a galactose gradient. The chromatography was run in PBS at 4 °C.

2.5. Analytical procedures

Lectins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were incubated in sample buffer (40 mM Tris/HCl pH 6.8, 2% SDS, 0.005% bromophenol

blue) containing 5% (v/v) 2-mercaptoethanol (reducing conditions), or 1 mg/ml iodoacetamide (non-reducing conditions) for 20 min at 37 °C, and were analyzed on a PhastGel Homogeneous 20, together with molecular mass standards in a Phast System. The gel was stained with Coomassie Brilliant Blue, following the protocol recommended by the manufacturer (GE Healthcare). Proteins were also stained with the Glycoprotein Detection Kit for glycoproteins (Sigma). Molecular mass was also determined by HPLC/MS ESI onto a Waters (Waters, Millford, MA, USA) Alliance/zq 4000 apparatus. The proteins (0.5 mg/ml in water) were applied to a VydacTM 5 µm 214TP52 column (250 × 2.1 mm).

Isoelectric point was determined with a Phast System (GE Healthcare), according to the manufacturer's instructions.

2.6. Amino acid sequencing by Edman degradation

Dimeric lanceolin and the separated A and B chains of stenodactylin were reduced and *S*-pyridylethylated with 4-vinylpyridine as previously reported (Scudiero et al., 1995). The modified lanceolin and stenodactylin subunits, after desalification by ethanol precipitation, were run on SDS-PAGE and transferred to a poly(vinylidene difluoride) membrane (Applied Biosystems Foster City, CA, USA). The resulting protein bands were then carefully cut and directly subjected to Edman degradation on a Procise Model 491 sequencer (Applied Biosystems) for N-terminal sequencing (Scudiero et al., 1995). Amino acids were aligned with the program Clustal W (Thompson et al., 1994).

2.7. Preparation of antisera and ELISA assay

Antisera against lanceolin and stenodactylin were prepared essentially as described previously (Strocchi et al., 1992). Briefly, male New Zealand rabbits weighing 2–3 kg received 100 ng of each toxin, injected subcutaneously in 1 ml of incomplete Freund's adjuvant. Animals were boosted with 200 ng, 300 ng and 2 µg of toxin in PBS, each dose given 1 month after the previous injection. Blood was collected from an ear incision from day 7 to day 28 after the second and third injection and was allowed to clot for 1 h at room temperature in siliconized Vacutainer tubes. After 12–18 h at 4 °C, clot-free serum was pooled, centrifuged at 1500g for 20 min at 4 °C, divided into aliquots and stored at

–80 °C. Antisera against volkensin and ricin were prepared in a similar way. Rabbit antisera against type 1 RIPs were obtained as described (Strocchi et al., 1992). Experiments were performed with the authorization of the Ministero della Salute according to the Italian Accreditation of Laboratory Animal Care.

Cross-reactivity was determined using a modification of the enzyme-linked immunosorbent assay (ELISA) as described previously (Parente et al., 1993), except that wells were coated with 2 µg of antigen in 100 µl of 50 mM carbonate buffer, pH 9.0. In the case of type 2 RIPs, all additions after the antigen were in PBS containing 50 mM lactose, 50 mM mannose and 0.05% (w/v) Tween 20. The secondary anti-rabbit antibody was conjugated to alkaline phosphatase (Sigma). Absorbance at 405 nm was measured by a microtiter plate reader Multiskan EX (ThermoLabSystems, Basingstoke, UK).

2.8. Hemagglutinating activity

Hemagglutinating activity was determined in microtiter plates. Each well contained, in a final volume of 100 µl, 25 µl of a 4% suspension of human erythrocytes (0, Rh +), serial dilutions of the lectins and, when appropriate, the sugars tested for inhibitory activity.

2.9. Protein synthesis and enzymatic activity

The effect of the lectins on protein synthesis by a cell-free system (a rabbit reticulocyte lysate) and by cells was determined as described previously (Bolognesi et al., 2000, 2002), with the details given in the legend to the appropriate table or figure.

Adenine polynucleotide glycosylase activity was determined essentially as described by Barbieri et al. (2003) by measuring the adenine released from rat liver ribosomes, tRNA and poly(A).

2.10. Cell viability assay

The effect of RIPs on the viability of NB100 cells was evaluated *in vitro* by a colorimetric cell cytotoxicity assay (Promega CellTiter 96 Aqueous One Solution Cell Proliferation Assay), based on the cellular conversion of a tetrazolium salt into a colored formazan product that can be detected using an ELISA plate reader. Cells were checked for

viability and adjusted to 3×10^4 /ml in complete RPMI 1640 medium, and then 100 µl were distributed into triplicate wells of a 96-well microtiter plate. After 24 h, 100 µl of lanceolin or stenodactylin solutions were added to final concentrations ranging from 10^{-15} to 10^{-10} M. After 72 h of incubation at 37 °C in a humidified 5% CO₂ atmosphere, 20 µl of CellTiter 96 Aqueous One Solution Reagent was added. The absorbance at 490 nm was recorded after a further incubation of 1 h.

2.11. Toxicity experiments

The toxicity of the purified lectins was evaluated as described by Barbieri et al. (1984) using male Swiss mice weighing 20–22 g, receiving food and water *ad libitum*. Groups of five mice for each dose were injected i.p. with scalar doses of the toxins (3.9–11.18 µg/kg for lanceolin, 0.48–2.87 µg/kg for stenodactylin), dissolved in PBS. Total number of mice was 100. LD₅₀ values at 48 h and 7 days were calculated by the method of Spearman Kaerber as described by Finney (1964). The experiments were performed with the authorization of the Ministero della Salute according to the Italian Accreditation of Laboratory Animal Care, with an experimental protocol approved by the Ethical Committee of the University of Bologna.

3. Results

3.1. Purification of the lectins

By affinity chromatography on Sepharose CL-6B used in previous work, adapted to larger quantities of starting material, a peak of protein material was eluted with 0.2 M galactose from both *A. lanceolata* and *A. stenodactyla* caudices extracts. The yield was 100 mg, approximately, per 100 g of tissue. Proteins from both plants inhibited protein synthesis and agglutinated red blood cells (see below), thus confirming that they are toxic lectins, and are referred to as lanceolin, from *A. lanceolata*, and stenodactylin, from *A. stenodactyla*.

On SDS-PAGE lanceolin under non-reducing conditions showed two bands, a main band with mobility corresponding to M_r 63,000 and a faint, barely visible band corresponding to M_r 32,500, approximately (Fig. 1, lane 1). After reduction, two doublets (A1, A2 and B1, B2, for A and B chains, respectively) with M_r ranging from 27,000 to 33,400 (lane 2) were produced.

Lanceolin was then subjected to a further affinity chromatography on an acid-treated Sepharose CL-6B column eluted with a galactose gradient, with the aim to separate the two larger bands seen on gel electrophoresis. Only a partial separation into unequal peaks was obtained (Fig. 2; Fig. 1, lanes 6 and 7). It was ascertained that the most abundant form (lanceolin 1) was formed by the slowest migrating A chain (A1) and the fastest migrating B chain (B2), whereas a second form (lanceolin 2) was formed by the other two bands. Lanceolin 1 showed the highest affinity for the acid-treated

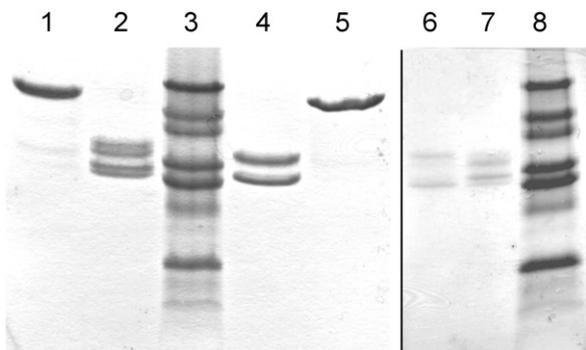


Fig. 1. Page electrophoresis of the toxins. Unreduced (lane 1) and reduced (lane 2) lanceolin, reduced (lane 4) and unreduced (lane 5) stenodactylin. Molecular mass markers (lanes 3 and 8) were, from top to bottom: bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa) and α -lactalbumin (14 kDa). Lanceolin after further affinity chromatography on acid-treated Sepharose CL-6B: reduced lanceolin 2 (lane 6) and lanceolin 1 (lane 7).

Sepharose and a slightly lower molecular weight. Due to a very low yield of perfectly separated forms, all further experiments, unless otherwise stated, were carried out using the unresolved mixture of lanceolins, as it is commonly practiced with other type 2 RIPs extracted from plant tissues, and only the biological properties of the separated A and B chains of stenodactylin were further analyzed.

Stenodactylin under non-reducing conditions gave a single band (Fig. 1, lane 5) with mobility corresponding to M_r 53,800, while after reduction it was resolved into two bands corresponding to M_r 24,800 and 30,000, hereupon referred to as the A and B chain, respectively (lane 4).

Molecular masses were also determined by HPLC/MS: 61,243 Da for lanceolin 1 (most abundant peak, see above) and 63,131 Da for stenodactylin.

On isoelectric focusing, the unresolved mixture of lanceolins showed three bands at pH 5.7, 5.5 and 5.4, and stenodactylin two bands at pH 5.0 and 4.8 (results not shown).

Both lectins appeared to be glycoproteins (results not shown).

3.2. Amino acid sequence analysis

The N-terminal amino acid analysis on lanceolin and stenodactylin subunits was performed by direct Edman degradation and the N-terminal amino acid sequences are reported in Fig. 3. The results confirm that both A and B chains of lanceolin are heterogeneous due to the presence of amino acid

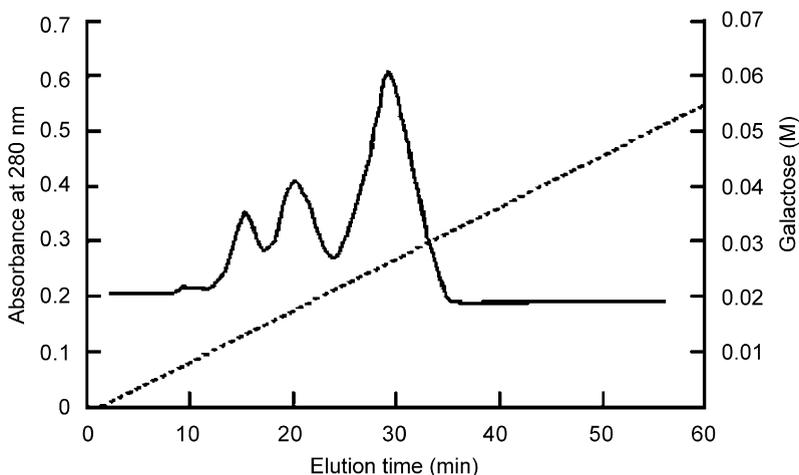


Fig. 2. Sepharose CL-6B chromatography with gradient elution of *A. lanceolata* isolectins. The column (4 × 1 cm) was eluted with a 0–0.15 M galactose gradient (total volume 250 ml).

substitutions. In fact, the first residue Val, found in A2, is missing in band A1. Furthermore, Phe 7 in band A2 is substituted with Leu 6 in band A1. In the case of B chains, Phe or Ser are present at positions 4 of band B1 and B2, respectively. Residues 16–17 were not assigned during the Edman degradation (Fig. 3).

In the case of stenodactylin, the N-terminal analysis allowed us to obtain the sequence of the first 22 and 21 amino acid residues for A and B chains, respectively (Fig. 3). Residues 18–20 of the B chain were not assigned. The sequence of stenodactylin B chain is identical to the sequence of band B2 of lanceolin, unless the insertion of Pro 2 and Val 3 (Fig. 3).

3.3. Immunological properties

The possibility was considered that the lectins could be similar to modeccin and volkensis, two immunologically similar toxic lectins previously purified from other *Adenia* species (Stirpe et al., 1978, 1985). Lanceolin and stenodactylin were tested with antisera against each other and with the serum against volkensis, the toxin from *A. volkensis*, and vice versa. Indeed, both lectins cross-reacted with the respective antisera and with the serum against volkensis, while volkensis gave a strong reaction with the antisera against both lanceolin and stenodactylin (Fig. 4). There was no cross-reaction with the serum against ricin, another type 2 RIP.

The lectins were also tested with antisera against some type 1 RIPs. Lanceolin and, to a lesser extent, stenodactylin gave some cross-reactivity with sera against momordin I and pokeweed antiviral protein (PAP-S), but did not react at all with the serum against saporin S6 (Fig. 4).

3.4. Hemagglutinating activity

Both lectins agglutinated human erythrocytes, the minimum agglutinating concentration being 3.77×10^{-6} and 7.9×10^{-7} M for lanceolin and stenodactylin, respectively. Agglutination was inhibited by 0.25 M galactose, methyl-galactose, melibiose and 0.125 M lactose, whereas 0.25 M arabinose, fructose, glucose, lyxose, maltose, mannose, ribose, sucrose and xylose had no effect on agglutination. Neither the separated A chain nor B chain of stenodactylin agglutinated erythrocytes.

3.5. Glycosylase activity

The lectins depurinated rat liver ribosomes, with an activity comparable to that of volkensis (Table 1). Stenodactylin was the most active, consistently with the results obtained with hsDNA as substrate (Pelosi et al., 2005). Since RIPs also deadenylate other polynucleotides, their activity was assayed on tRNA and on poly(A), on which both lectins were inactive

Alignment:

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Lanceolin A1      -FPKVILDCTRATVERYTQFI  20
Lanceolin A2      VFPKVIFDCTRATVERYTQFI  21
Stenodactylin A chain VFPKVIFDCTRATVERYTQFIM 22
Modeccin A chain  -FPKVTLDDTRATVESYTT
Volkensin A chain VFPKVPFDVPKATVESYTRFIR 22
                ****  :  .:****  **:**

Lanceolin B1      D--CPFGETTAYIVGRDXXCV  19
Lanceolin B2      D--CPSGETTAYIVGRDXXCV  19
Stenodactylin B chain DPVCPSGETTAYIVGRDXXXV  21
Modeccin B chain  EMICPSGETTAYIVGRGXGXV
Volkensin B chain DPVCPSGETTAFIVGRDGRCV  21
                **  *****:****

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Fig. 3. N-terminal amino acid sequences (A) and amino acid alignment (B) of the N-termini of S-modified lanceolin, stenodactylin, modeccin and volkensis subunits. The single letter code has been used for amino acids. Identical residues (*), conserved substitutions (:) and semi-conserved (.) substitutions are reported. X, unassigned amino acid positions. The N-terminal sequence of modeccin was from Montecucchi et al. (1989) and that from volkensis from Chambery et al. (2004).

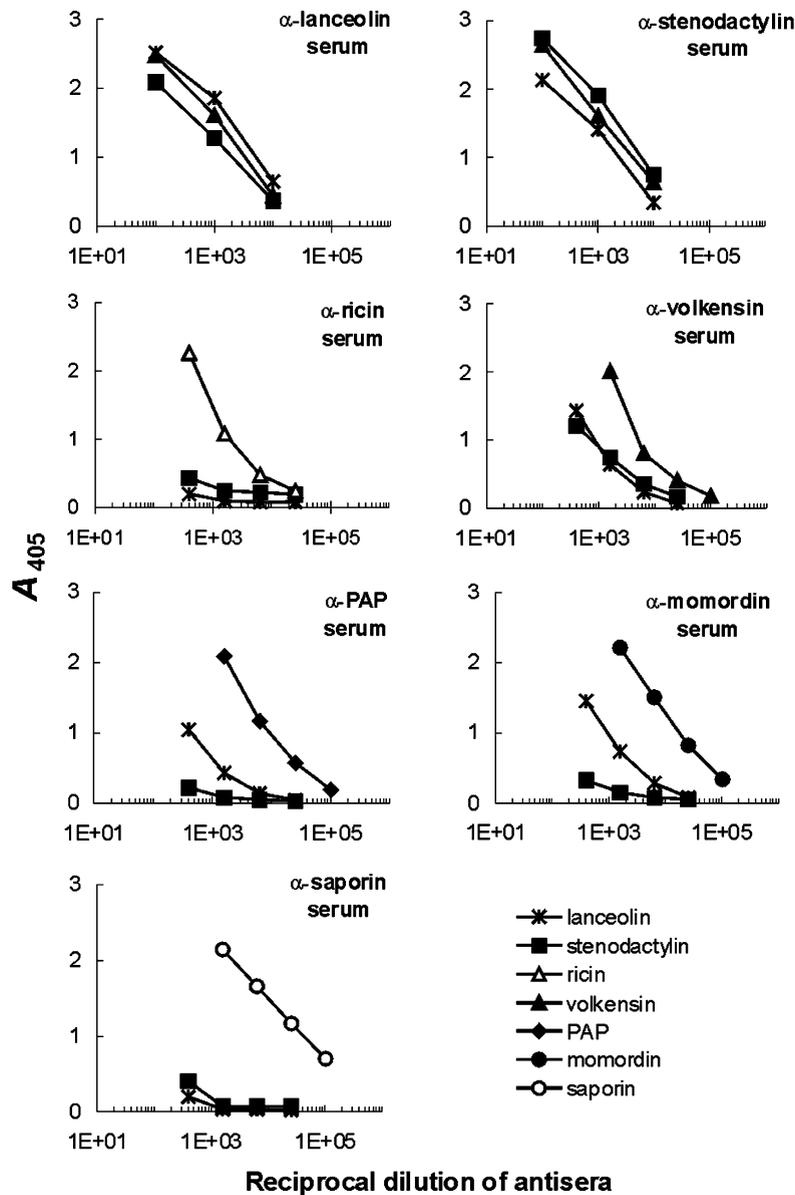


Fig. 4. Reactivity of lanceolin, stenodactylin and volkensin with the respective and other antisera. ELISA was performed as described in the text.

at the concentrations used for assays on ribosomes (results not shown).

3.6. Effect on protein synthesis and cytotoxicity

Both lectins inhibited protein synthesis by a rabbit reticulocyte lysate (Pelosi et al., 2005). The separated A chain of stenodactylin inhibited protein synthesis by a rabbit reticulocyte lysate, with an IC_{50} 4.4×10^{-8} M. The separated B chain did not

affect protein synthesis at concentrations up to 3×10^{-5} M.

The effect of the lectins on protein synthesis was tested on four cell lines in comparison with volkensin (Table 2), and it appeared that the inhibitory activity of stenodactylin was very close to that of volkensin, whereas the activity of lanceolin was somewhat lower.

In NB100 cells exposed to various concentrations of either lanceolin or stenodactylin, the loss of cell

viability was parallel to the inhibition of protein synthesis (Fig. 5). In a time-course experiment with stenodactylin, the inhibition of protein synthesis preceded the loss of cell viability, which was still more than 50% at 6 h after protein synthesis was completely inhibited (Fig. 6).

NB100 cells exposed to lanceolin and stenodactylin showed morphologic aspects of cell damage and cell death compatible with apoptotic and post-apoptotic lesions (Fig. 7).

3.7. Toxicity to mice

Mice receiving the lectins by i.p. injection died not before 24 h and never after 7 days. An acute and a delayed LD₅₀ was calculated at 48 h and 7 days, respectively, and were 8.16 µg/kg (95% confidence limits 7.11–9.37) and 6.78 µg/kg (4.91–9.36) for lanceolin. The acute LD₅₀ for stenodactylin was 2.76 µg/kg (2.12–3.58), whereas all mice receiving 1.21 µg/kg died within 7 days and a delayed LD₅₀

was not calculated for this toxin. Apoptotic and necrotic lesions in the liver and kidneys were observed in the dead animals and will be described in detail elsewhere.

4. Discussion

Our results demonstrate that *A. lanceolata* and *A. stenodactyla* contain a high amount of proteins that have the characteristics of galactose-specific lectins, in that they bind to Sepharose columns, from which are eluted by galactose, and agglutinate erythrocytes, the agglutination being inhibited by galactose or galactose-containing sugars. The lectins depurinate ribosomes and hsDNA, inhibit protein synthesis both in cell-free systems and in cells, and are highly toxic to mice, especially the stenodactylin, thus having all the characteristics of type 2 toxic RIPs.

The lectins are glycoproteins and, like other type 2 RIPs, consist of a smaller A chain and a larger B chain, which presumably are linked by a disulfide bond, since they can be separated by reduction. The A chain of stenodactylin inhibited protein synthesis, as expected, whereas the B chain did not agglutinate erythrocytes, a surprising result since the B chain of modeccin and volkensin had hemagglutinating properties (Barbieri et al., 1980; Stirpe et al., 1985). The lack of agglutinating capacity can be due either to conformational changes or to aggregation of the B chain after separation from the A chain. Alternatively, it is possible that the B chain is monovalent, and that the whole toxin can agglutinate cells only after formation of aggregates, as other type 2 RIPs do (e.g. viscumin, Olsnes et al., 1982).

Table 1
Adenine glycosylase activity on rat liver ribosomes

Enzymatic activity (pmol adenine released/10 pmol lectin/40 min)		
Lanceolin	Stenodactylin	Volkensin
0.78 (2)	4.55 (2)	2.01 (4)

Reaction mixtures contained, in a final volume of 200 µl: 0.05 M sucrose, 0.04 M KCl, 0.135 M NH₄Cl, 0.0025 M Mg acetate, 0.02 M ammonium acetate, 0.005 M Tris/HCl buffer, pH 7.8, 10 pmol lectin and 10 pmol ribosomes. Final pH was 7.0. Incubation was at 30 °C for 40 min. Values are mean results of the number of experiments given in parentheses.

Table 2
Effect of toxins on protein synthesis by cells

Toxin	Cells			
	BeWo	JM	NB100	3T3
	Inhibition of protein synthesis IC ₅₀ (M) ^a			
Lanceolin	4.4 × 10 ⁻¹³	1.0 × 10 ⁻¹³	6.9 × 10 ⁻¹³	1.9 × 10 ⁻¹³
Stenodactylin	1.3 × 10 ⁻¹³	2.8 × 10 ⁻¹⁴	3.3 × 10 ⁻¹³	2.0 × 10 ⁻¹⁴
Volkensin	1.7 × 10 ⁻¹³	2.6 × 10 ⁻¹⁴	4.0 × 10 ⁻¹³	9.7 × 10 ⁻¹⁴

Cells (2 × 10⁴/well) were seeded in 24-well microtitre plates in complete medium. After 24 h the medium was replaced with fresh complete medium containing lectins ranging from 10⁻¹⁵ to 10⁻¹¹ M. Control cells were incubated with complete medium alone. After 72 h of incubation and 4-h pulse with L-[4,5-³H]leucine (125 nCi/0.25 ml) the radioactivity incorporated was determined.

Protein synthesis by control cells was (d.p.m./well): BeWo 181,188 ± 25,838, JM 24,509 ± 3580, NB100 152,353 ± 23,060 and 3T3 41,006 ± 6369.

^aConcentration causing 50% inhibition.

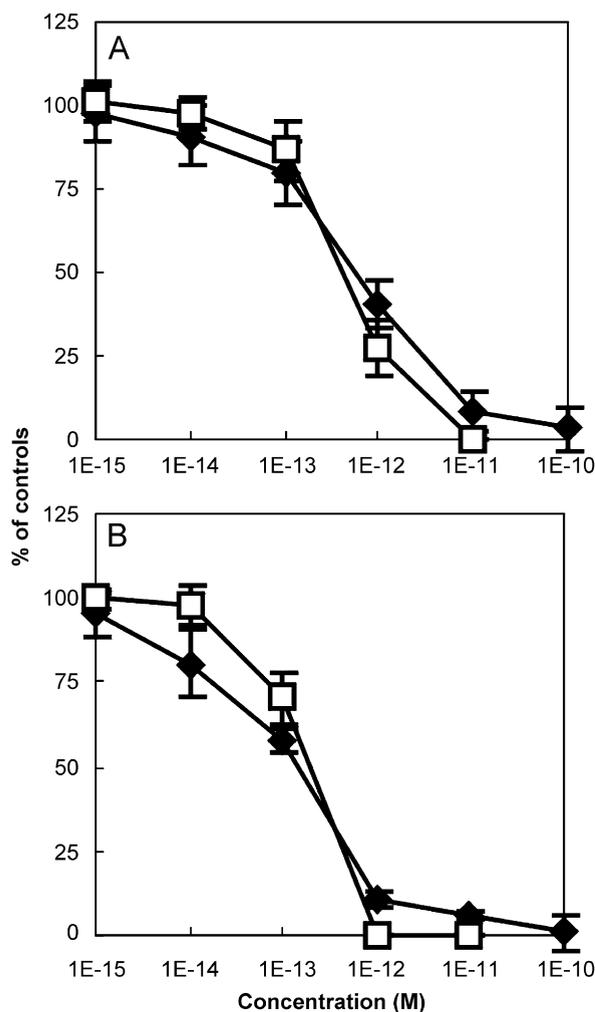


Fig. 5. Effect of lanceolin and stenodactylin on protein synthesis and viability of NB100 cells. A dose-response study. Experimental conditions are described in the text with lanceolin (A) and stenodactylin (B). (◆) Protein synthesis; (□) viability. Protein synthesis by control cells was (d.p.m./well): 142,429 ± 6423.

Lanceolin is present in two forms, like modeccin (Barbieri et al., 1980). By a homology search analysis of the N-terminal sequences, the doublets B1-B2 and A1-A2 of lanceolin shared a high percent identity with volkensin B chain and A chain, respectively (Fig. 3). In a first group, lanceolin A1 chain shares a 15/18 identity with modeccin A chain (Montecucchi et al., 1989) and both have a Phe as N-terminal amino acid. In a second group, lanceolin A2 shares 21/21 identity with stenodactylin A chain and both share 15/21 identity with volkensin A chain: these three sequences present an extra Val at the N-terminus. Similarities between these two groups are still significant with a somewhat

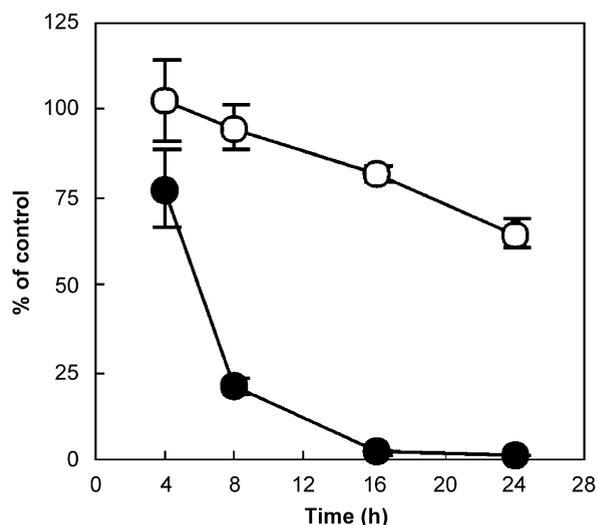


Fig. 6. Effect of stenodactylin on protein synthesis and viability of NB100 cells. A time-course study. Experiments were performed as in Fig. 5, with 10^{-11} M stenodactylin. (●) Protein synthesis; (○) viability. Protein synthesis by control cells was (d.p.m./well): 117,338 ± 7034.

overall identity of 12/20. Identities among known B chain sequences from Passifloraceae are strikingly stringent except for the first three N-terminal residues: stenodactylin and volkensin B chains present a sequence DPV, modeccin EMI and lanceolin only a D.

Interestingly, in the N-terminal amino acid sequences of both lanceolin (A1 and A2 bands) and stenodactylin A chains is present a cysteinyl residue at position 9 (position 8 for A1). It is known that in other type 2 RIPs, such as ricin and volkensin, a cysteinyl residue at the C-terminal end of the A chain forms an interchain disulfide bond with a cysteinyl residue at the N-terminal end of the B chain. It could be of interest to investigate the role of the additional Cys residue of lanceolin and stenodactylin A chains, since the reduction of the disulfide bridge inside the cell is an essential requirement for the toxicity of type 2 RIPs.

The two toxins are immunologically similar to each other and to volkensin as well, which is not surprising, in that they all came from plants belonging to the same Passifloraceae family and seem to have many similarities in their amino acid sequences. Lanceolin somewhat cross-reacts also with sera against type 1 RIPs momordin I and PAP-R, with which stenodactylin gives a very faint or no reaction. Both lectins do not react with sera against ricin and saporin S6.

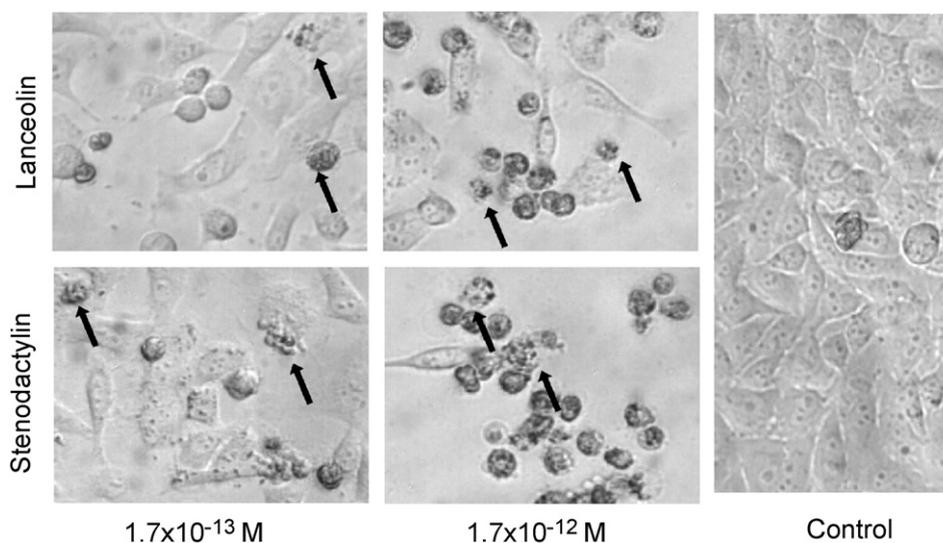


Fig. 7. Effect of toxins on NB100 cells morphology assessed by phase contrast microscopy. Cells (50,000/well) were seeded in 24-well plate and after adhesion were treated for 24 h with RIPs from *A. stenodactyla* and *A. lanceolata* at the indicated concentrations. Original magnification $\times 200$. Some apoptotic cells are indicated by arrows.

Stenodactylin has a high enzymatic activity with both ribosomes (present results) and hsDNA (Pelosi et al., 2005) as substrates and, consistently, is highly toxic to all cell lines tested, reaching levels never seen before with toxins of this type. It is noteworthy that the cell lines examined have a different sensitivity to the lectins, which varies from one cell line to another. The loss of viability of NB100 cells exposed to the toxins at 96 h parallels the inhibition of protein synthesis as commonly found with other type 2 RIPs (e.g. Wang et al., 2006).

Both toxins cause apoptotic lesions as early as after 24 h. All tested toxic type 2 RIPs, their isolated A chains and type 1 RIPs cause apoptosis, for which several mechanisms were proposed (reviews in Battelli, 2004; Narayanan et al., 2005).

When the time-course of the effects of stenodactylin on protein synthesis and viability was examined, it was seen that the inhibition of protein synthesis is a precocious event preceding the loss of cell viability, which is still 70% of normal 8 h after protein synthesis is completely inhibited.

The two lectins are highly toxic to mice, stenodactylin being more potent than, and thus different from, lanceolin, consistently with the effect on cells. Under this respect, it should be noticed that the dose of stenodactylin causing 100% death is very low, and comparable to that of volkensin (Stirpe et al., 1985), which makes these two lectins the most potent toxins of plant origin known so far.

This suggests that this high toxicity may be due to the killing of relatively few cells that are essential for life.

From previous and present results, it appears that several, and probably more, species of *Adenia* plants may contain a high level of potent toxins. These highly poisonous plants are freely sold, and could be a hazard to people handling them, especially children. Worst than that, parts of the plants and/or their toxins, either native or recombinant, could be used for criminal and even warfare or bioterrorism purposes, as it is feared for ricin (Bigalke and Rummel, 2005). Although these plants are not available in large amounts as castor bean seeds, from which ricin is extracted, the toxins could be cloned, expressed in bacteria and produced in quantities. The fact that both toxins do not cross-react with an anti-ricin serum indicates that the available anti-ricin sera or antibodies (Dertzbaugh et al., 2005) and presumably vaccines (Hassani et al., 2004) would not be useful for the detection of these toxins for forensic or other purposes, or for the prophylaxis and therapy of their toxic effects.

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References

- Barbieri, L., Zamboni, M., Montanaro, L., Sperti, S., Stirpe, F., 1980. Purification and properties of different forms of modeccin, the toxin of *Adenia digitata*. Separation of subunits with inhibitory and lectin activity. *Biochem. J.* 185, 203–210.
- Barbieri, L., Falasca, A.I., Stirpe, F., 1984. Volkensin, the toxin of *Adenia volkensii* (kilyambiti plant). *FEBS Lett.* 171, 277–279.
- Barbieri, L., Stoppa, C., Bolognesi, A., 1987. Large scale chromatographic purification of ribosome-inactivating proteins. *J. Chromatogr.* 408, 235–243.
- 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.
- Barbieri, L., Valbonesi, P., Bondioli, M., Lugo Alvarez, M., Dal Monte, P., Landini, M.P., Stirpe, F., 2001. Adenine glycosylase activity in mammalian tissues: an equivalent of ribosome-inactivating proteins. *FEBS Lett.* 505, 196–197.
- 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.
- Battelli, M.G., 2004. Cytotoxicity and toxicity to animals and humans of ribosome-inactivating proteins. *Mini Rev. Med. Chem.* 4, 521–523.
- Battelli, M.G., Citores, L., Buonamici, L., Ferreras, J.M., de Benito, F.M., Stirpe, F., Girbés, T., 1997. Toxicity and cytotoxicity of nigrin b, a two-chain ribosome-inactivating protein from *Sambucus nigra*: a comparison with ricin. *Arch. Toxicol.* 71, 360–364.
- Battelli, M.G., Musiani, S., Buonamici, L., Santi, S., Riccio, M., Maraldi, N.M., Girbés, T., Stirpe, F., 2004. Interaction of volkensin with HeLa cells: binding, uptake, intracellular localization, degradation and exocytosis. *Cell. Mol. Life Sci.* 61, 1975–1984.
- Bigalke, H., Rummel, A., 2005. Medical aspects of toxin weapons. *Toxicology* 214, 210–220.
- Bolognesi, A., Polito, L., 2004. Immunotoxins and other conjugates: pre-clinical studies. *Mini Rev. Med. Chem.* 4, 563–583.
- Bolognesi, A., Olivieri, F., Battelli, M.G., Barbieri, L., Falasca, A.I., Parente, A., Del Vecchio Blanco, F., Stirpe, F., 1995. Ribosome-inactivating proteins (RNA *N*-glycosidases) from the seeds of *Saponaria ocymoides* and *Vaccaria pyramidata*. *Eur. J. Biochem.* 228, 935–940.
- Bolognesi, A., Polito, L., Tazzari, P.L., Lemoli, R.M., Lubelli, C., Fogli, M., Boon, L., De Boer, M., Stirpe, F., 2000. In vitro anti-tumour activity of anti-CD80 and anti-CD86 immunotoxins containing type I ribosome-inactivating proteins. *Br. J. Haematol.* 110, 351–361.
- Bolognesi, A., Polito, L., Lubelli, C., Barbieri, L.A., Parente, A., Stirpe, F., 2002. Ribosome-inactivating and adenine polynucleotide glycosylase activities in *Mirabilis jalapa* L. tissues. *J. Biol. Chem.* 277, 13709–13716.
- Cevolani, D., Strocchi, P., Bentivoglio, M., Stirpe, F., 1995. Suicide retrograde transport of volkensin in cerebellar afferents: direct evidence, neuronal lesions and comparison with ricin. *Brain Res.* 689, 163–171.
- Chambery, A., Di Maro, A., Monti, M.M., Stirpe, F., Parente, A., 2004. Volkensin from *Adenia volkensii* Harms (kilyambiti plant), a type 2 ribosome-inactivating protein: gene cloning, expression and characterization of its A-chain. *Eur. J. Biochem.* 271, 108–117.
- Dertzbaugh, M.T., Rossi, C.A., Paddle, B.M., Hale, M., Poretzki, M., Alderton, M.R., 2005. Monoclonal antibodies to ricin: in vitro inhibition of toxicity and utility as diagnostic reagents. *Hybridoma* 24, 236–243.
- Endo, Y., Tsurugi, K., 1987. RNA *N*-glycosidase activity of ricin A-chain. Mechanism of action of the toxic lectin ricin on eukaryotic ribosomes. *J. Biol. Chem.* 262, 8128–8130.
- Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T., Igarashi, K., 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* 0157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA *N*-glycosidase activity of the toxins. *Eur. J. Biochem.* 171, 45–50.
- Finney, D.J., 1964. *Statistical Methods in Biological Assay*. Griffin, London, pp. 524–530.
- Fracasso, G., Bellisola, G., Castelletti, D., Tridente, G., Colombatti, M., 2004. Immunotoxins and other conjugates: preparation and general characteristics. *Mini Rev. Med. Chem.* 4, 545–561.
- Girbés, T., Ferreras, J.M., Arias, F.J., 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.
- Greenfield, R.A., Brown, B.R., Hutchins, J.B., Iandolo, J.J., Jackson, R., Slater, L.N., Bronze, M.S., 2002. Microbiological, biological, and chemical weapons of warfare and terrorism. *Am. J. Med. Sci.* 323, 326–340.
- Hartley, M.R., Lord, J.M., 2004. Cytotoxic ribosome-inactivating lectins from plants. *Biochim. Biophys. Acta* 1701, 1–14.
- Hassani, M., Patel, M.C., Pirofski, L.A., 2004. Vaccines for the prevention of diseases caused by potential bioweapons. *Clin. Immunol.* 111, 1–15.
- Knight, B., 1979. Ricin—a potent homicidal poison. *Br. Med. J.* 1, 350–351.
- Montecucchi, P.C., Lazzarini, A.M., Barbieri, L., Stirpe, F., Soria, M., Lappi, D., 1989. N-terminal sequence of some ribosome-inactivating proteins. *Int. J. Protein Peptide Res.* 33, 263–267.
- Narayanan, S., Surendranath, K., Bora, N., Surolia, A., Karande, A.A., 2005. Ribosome inactivating proteins and apoptosis. *FEBS Lett.* 579, 1324–1331.
- Nicolson, G.L., Blaustein, J., Etzler, M.E., 1974. Characterization of two plant lectins from *Ricinus communis* and their quantitative interaction with a murine lymphoma. *Biochemistry* 13, 196–204.
- Olsnes, S., Stirpe, F., Sandvig, K., Pihl, A., 1982. Isolation and characterization of viscumin, a toxic lectin from *Viscum album* L. (mistletoe). *J. Biol. Chem.* 257, 13263–13270.
- Parente, A., de Luca, P., Bolognesi, A., Barbieri, L., Battelli, M.G., Abbondanza, A., Sande, M.J.W., Siniscalco Gigliano, G., Tazzari, P.L., Stirpe, F., 1993. Purification and partial characterization of single-chain ribosome-inactivating proteins from the seeds of *Phytolacca dioica* L. *Biochim. Biophys. Acta* 1216, 43–49.

- Pelosi, E., Lubelli, C., Polito, L., Barbieri, B., Bolognesi, A., Stirpe, F., 2005. Ribosome-inactivating proteins and other lectins from *Adenia* (Passifloraceae). *Toxicon* 46, 658–663.
- Peumans, W.J., Hao, Q., Van Damme, E.J.M., 2001. Ribosome-inactivating proteins from plants: more than RNA *N*-glycosidases? *FASEB J.* 15, 1493–1506.
- Refsnes, K., Haylett, T., Sandvig, K., Olsnes, S., 1977. Modeccin—a plant toxin inhibiting protein synthesis. *Biochem. Biophys. Res. Commun.* 79, 1176–1183.
- Scudiero, R., Capasso, C., Del Vecchio Blanco, F., Savino, G., Capasso, A., Parente, A., Parisi, E., 1995. Isolation and primary structure determination of a metallothionein from *Paracentrotus lividus* (*Echinodermata*, *Echinoidea*). *Comp. Biochem. Physiol.* 11B, 329–336.
- Stirpe, F., 2004. Ribosome-inactivating proteins. *Toxicon* 44, 371–383.
- Stirpe, F., Battelli, M.G., 2006. Ribosome-inactivating proteins: progresses and problems. *Cell. Mol. Life Sci.* 63, 1850–1866.
- Stirpe, F., Gasperi-Campani, A., Barbieri, L., Lorenzoni, E., Montanaro, L., Sperti, S., Bonetti, E., 1978. Inhibition of protein synthesis by modeccin, the toxin of *Modecca digitata*. *FEBS Lett.* 85, 65–67.
- Stirpe, F., Barbieri, L., Abbondanza, A., Falasca, A.I., Brown, A.N.F., Sandvig, K., Olsnes, S., Pihl, A., 1985. Properties of volkensin, a toxic lectin from *Adenia volkensii*. *J. Biol. Chem.* 260, 14589–14595.
- Strocchi, P., Barbieri, L., Stirpe, F., 1992. Immunological properties of ribosome-inactivating proteins and of a saporin-IgG conjugate. *J. Immunol. Methods* 155, 57–63.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Van Damme, E.J.M., Peumans, W.J., Barre, A., Rougé, P., 1998. Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. *Crit. Rev. Plant Sci.* 17, 575–692.
- Van Damme, E.J.M., Hao, Q., Barre, A., Vandebussche, F., Desmyter, S., Rougé, P., Peumans, W.J., 2001. Ribosome-inactivating proteins: a family of plant proteins that do more than inactivate ribosomes. *Crit. Rev. Plant Sci.* 20, 395–465.
- Wang, B.-Z., Zou, W.-G., Liu, W.-Y., Liu, X.-Y., 2006. The lower cytotoxicity of cinamomin (a type II RIP) is due to its B-chain. *Arch. Biochem. Biophys.* 451, 91–96.
- Wiley, R.G., Lappi, D.A., 1994. *Suicide Transport and Immunolesioning*. R.G. Landes Company, Austin.
- Wiley, R.G., Stirpe, F., 1988. Modeccin and volkensin but not abrin are effective suicide transport agents in rat CNS. *Brain Res.* 438, 145–154.