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Cytotoxicity of ribosome-inactivating protein saporin is not mediated through α_2-macroglobulin receptor

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Abstract Saporin is a single chain ribosome-inactivating protein produced by the plant *Saponaria officinalis*. Several isoforms of saporin have been isolated from various parts of the plant. In the present study recombinant saporin isoforms 5 and 6 were produced in *Escherichia coli*. Saporin-6 was found to be more active than saporin-5 in its N-glycosidase, cytotoxic, and genomic DNA fragmentation activities. Earlier, saporin has been shown to bind low-density lipoprotein receptor-related protein (LRP), however, in this study the sensitivities of LRP-negative and LRP-positive cell lines were found to be similar towards saporin-6 toxicity suggesting the internalization of saporin not to be solely dependent on the expression of LRP on eukaryotic cells.

Key words: Ribosome-inactivating protein; Toxin; Ribosome; Immunotoxin; Ribotoxin

1. Introduction

Ribosome-inactivating proteins (RIPs) from plants are toxic translation inhibitors that inactivate ribosomes by catalyzing the hydrolysis of a specific N-glycosidic bond of large rRNA [1]. RIPs have been classified into two types; type I RIPs, e.g. saporin, trichosanthin and pokeweed antiviral protein (PAP), are single chain proteins and do not have a second cell binding domain unlike their type II counterparts that include ricin and abrin. Because of the absence of a binding domain, the single chain type I RIPs lack the non-specific cytotoxicity shown by the type II RIPs and are therefore toxins of choice for the construction of immunotoxins. The site of action of RIPs in 28S rRNA is located in a highly conserved, purine-rich stem and loop structure termed as α-sarcin/ricin loop. The target adenine residue, A4324 of rat 28S rRNA, comprises a part of tetranucleotide '$GA_{4324}GA$' in the loop [2]. Most of the single chain RIPs also remove an equivalent adenine residue, A2660, from 23S rRNA of *Escherichia coli* ribosomes [3]. The catalytic depurination disrupts the binding of elongation factors to the ribosomes, thus arresting the protein synthesis at the translocation step [4].

Saporin, isolated from the plant *Saponaria officinalis*, is one of the most active single chain RIPs. Saporin belongs to a multigene family that encodes its several isoforms, which differ in their physico-chemical as well as biological properties [5]. More than nine isoforms of saporin have been isolated from various parts of *S. officinalis* plant. Isoforms of saporin are designated by source tissue and the peak number in which they were obtained in the ion-exchange chromatography of the crude extract of the tissue [5,6]. Saporin-6 constitutes the major peak (peak 6) of preparation from seeds, accounting for approximately 0.4% of the whole seed weight or 7% of the total seed protein [6]. This preparation, however, contained a mixture of at least four forms of saporin-6 showing heterogeneity at positions 48 and 91 [7,8]. At position 48 either Asp or Glu, and at position 91 either Arg or Lys were found [7,8]. The different saporin isoforms have been shown to have immunological cross-reactivity [9]. Genomic clones of several saporin seed isoforms obtained by polymerase chain reaction (PCR) amplification of *S. officinalis* genomic DNA have been designated as genomic clone-1 to 5 [10]. The derivation of amino acid sequences of these clones revealed that genomic clone-1, 4 and 5 referred to saporin isoforms 1, 4 and 5 respectively, whereas genomic clone-2 and 3 referred to two of the four forms of saporin-6 having Asp48 and Lys91, and Glu48 and Lys91 respectively [10]. All these isoforms show a very high sequence similarity, and among the isoforms differences were observed only at 13 positions out of which seven positions show change in polarity or charge of amino acid. Among various isoforms only saporin-1 and saporin-3 from roots have been found to be glycosylated [5]. Saporin extracted from the seeds of *S. officinalis* has been shown to bind to α_2-macroglobulin receptor, also called as low-density lipoprotein receptor-related protein (LRP) [11]. LRP is expressed in many tissues and cell types, particularly in fibroblasts, monocytes and hepatocytes, and is responsible for the uptake and clearance of macromolecular complexes between proteinases and α_2-macroglobulin [11]. We have shown recently that the cytotoxic activity of saporin-6 results due to the combined manifestation of its N-glycosidase and internucleosomal DNA fragmentation activities [12].

In the present study, we have functionally compared two isoforms of saporin namely, saporin-5 and 6. The catalytic and cytotoxic activities of saporin-6 were found to be significantly higher than of saporin-5. However, the cytotoxicity of saporin was not found to be dependent on the expression of LRP. Also, the difference in the cytotoxic activities of the two isoforms was found to be solely due to the difference in their catalytic activities.

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2. Materials and methods

CHO-K1 and CHO-13-5-1 cell lines were a generous gift from Dr. David J. FitzGerald, N.I.H., USA.

2.1. Cloning, expression, purification and structural characterization of saporin isoforms

Genomic DNA, extracted from the seeds of *S. officinalis* was used as template to amplify DNA coding for the two saporin isoforms by PCR. The published sequence of saporin gene was used to design PCR primers [13]. The amplified DNAs were cloned into the expression vector pVex11, containing a T7 promoter, multiple cloning site and a T7 transcription terminator.

Saporin isoforms were expressed in BL21 (λDE3) strain of *E. coli*. Bacterial cells were transformed with the desired construct and grown at 37°C, in super broth containing 100 μg/ml ampicillin. At an OD_{600} of 2.0, the cells were induced with 1 mM isopropyl thiogalactose (IPTG), and harvested 2 h later. The recombinant proteins were purified from the inclusion bodies following the method of Buchner et al. [14] as described earlier [12]. Proteins were isolated from the inclusion bodies by denaturation, and after renaturation they were purified by successive chromatography on S-Sepharose and TSK3000 gel filtration columns [12].

The secondary structure of saporin isoforms was analyzed using circular dichroism (CD) as described earlier [12].

2.2. Assay for specific N-glycosidase activity

Rabbit reticulocyte lysate was taken as the source of ribosomes and treated with different concentrations of proteins at 30°C for 30 min [12,15]. The reaction was stopped with sodium dodecyl sulfate (SDS) and total RNA was isolated using Trizol reagent as per manufacturer's instructions. The RNA pellet was dissolved in water, and half of the RNA sample was treated with aniline-acetate. The aniline-treated and untreated samples were electrophoresed on 2% agarose gel and the RNA was visualized by ethidium bromide staining [12].

2.3. Assay for in vitro protein synthesis inhibition

The inhibitory activity of saporin isoforms towards in vitro protein synthesis was assayed as described [12,16]. Several dilutions of the toxin were incubated with rabbit reticulocyte lysate at 30°C for 60 min; the proteins were precipitated with 15% trichloroacetic acid and harvested on glass fiber filters. The dried filters were counted using a liquid scintillation counter. Activity was expressed as percentage of control where no toxin was added.

2.4. Assay for genomic DNA fragmentation

U937 cells were used to evaluate the genomic DNA fragmentation ability of saporin isoforms as described earlier [12]. DNA was isolated from saporin-treated and control cells, run in a 1.5% agarose gel and visualized by staining with ethidium bromide.

2.5. Cytotoxic activity of saporin isoforms

The cytotoxic activity of saporin isoforms was assayed on a variety of cancer cell lines. Adherent cells were plated at a density of 5×10^3 cells/well in a 96-well plate in 0.2 ml of RPMI/DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal calf serum (FCS) for 16 h. The medium was replaced with 0.2 ml leucine-free medium containing 2% FCS for evaluating the cytotoxicity. The suspension cells were seeded at 10^4 cells/well in 0.2 ml of leucine-free medium containing 2% FCS and used immediately. The cells were incubated with various concentrations of toxins, diluted in 0.2% human serum albumin (HSA) in Dulbecco's phosphate-buffered saline for 34 h followed by labeling with 0.75 μCi [^{3}H]leucine per well for 2 h. The cells after freezing and thawing were harvested on filtermats using a 96-well plate automated harvester and the filters were counted using an LKB β-plate counter. Activity was plotted as percentage of control where no toxin was added to the cells.

Cytotoxicity of saporin isoforms was assayed on HeLa cells infected with adenovirus as described by Fernandez-Puentes and Carrasco [17]. HeLa cells, 2×10^4 cells/well, were grown in a 96-well culture plate for 12 h at 37°C. The medium was replaced with 0.2 ml leucine-free RPMI containing 1% FCS. Adenovirus, propagated and isolated from HEK 293 cells, and different dilutions of saporin isoforms, in 0.2% HSA, were added to the cells and incubated for 5 h. The cells were labeled with 0.1 μCi [^{3}H]leucine per well for 2 h at 37°C, harvested onto filtermats and counted as described above.

3. Results and discussion

In the present study we have investigated the mechanism of saporin cytotoxicity using recombinant saporin-5 and saporin-6 isoforms of *S. officinalis*. Saporin-6 refers to the form having Asp at position 48 and Lys at position 91, thus representing genomic clone-2 of Barthelemy et al. [10]. The sequence of saporin-5, which corresponds to genomic clone-5 of saporin described by Barthelemy et al. [10], is identical to the deduced amino acid sequence of saporin leaf cDNA except that the latter has a Ser at position 99 whereas a Leu is present at this position in saporin-5.

3.1. Expression, purification and structural characterization

Both saporin-5 and saporin-6 proteins were overexpressed in *E. coli* BL21 (λDE3) cells and purified from the inclusion bodies. The typical yield of purified saporin-5 was in the range of 18–20 mg/l culture whereas that of saporin-6 was 3–5 mg/l

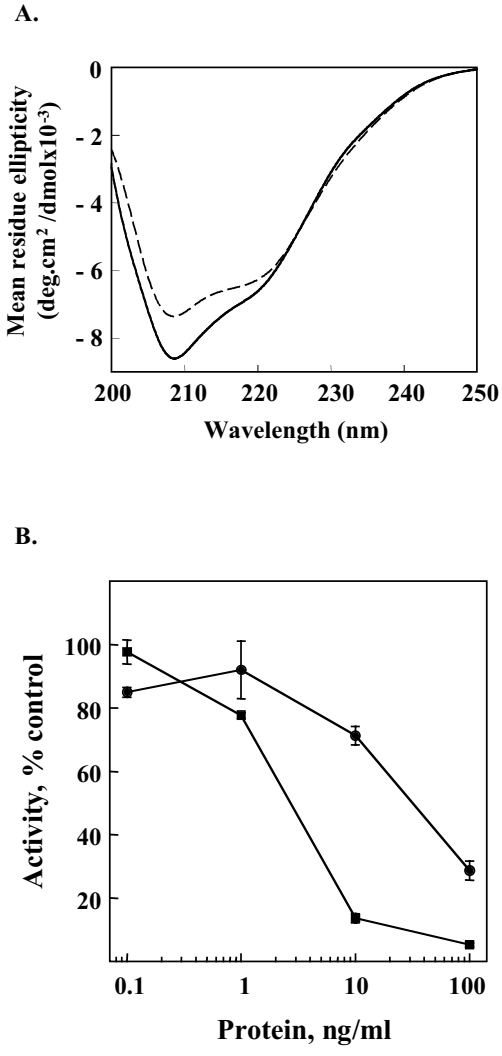

Fig. 1. Structural and catalytic properties of saporin isoforms. A: Far UV CD spectra. The spectra were acquired at a scan speed of 50 nm/min with a sensitivity of 5 mdeg and a response time of 1 s. Saporin-5 (dashed line) and saporin-6 (solid line). B: In vitro protein synthesis inhibitory activity. Rabbit reticulocyte lysate was treated with various concentrations of saporin isoforms, and incorporation of [^{3}H]leucine was measured into the newly synthesized proteins. Saporin-5 (●) and saporin-6 (■).

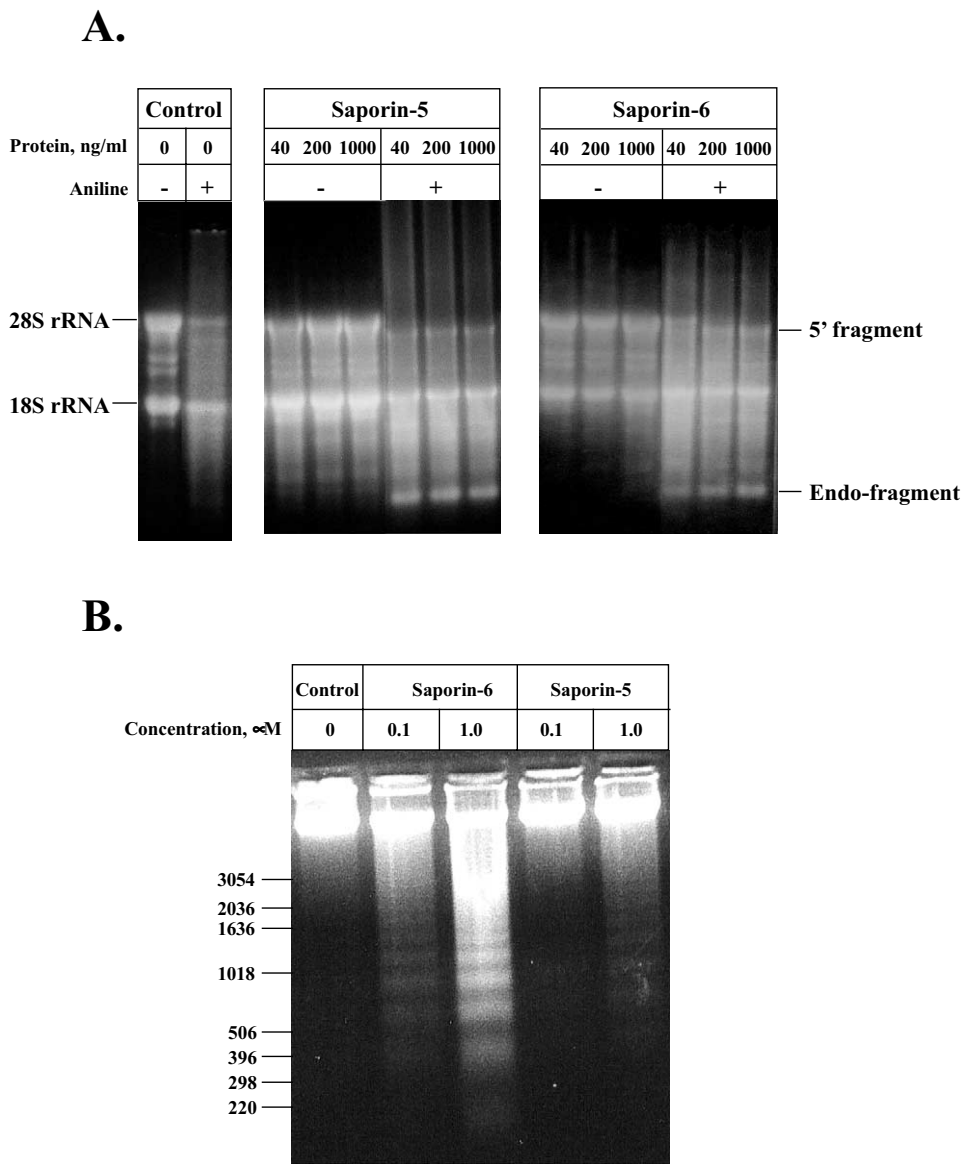

Fig. 2. Enzymatic activity of saporin isoforms. A: N-glycosidase activity. Rabbit reticulocyte lysate was treated with indicated concentrations of saporin isoforms. Total RNA was extracted, and aniline-treated and untreated RNA was resolved on a 2% agarose gel. B: Genomic DNA fragmentation activity. U937 cells were incubated with indicated concentrations of saporin isoforms for 48 h. Genomic DNA was isolated, run on a 1.5% agarose gel and visualized by ethidium bromide staining. Molecular weights in bp are indicated on the left.

culture. The purified saporin-6 was used to raise polyclonal antibodies in rabbits. These antibodies reacted equally well with saporin-5 and saporin-6 indicating similar antigenic determinants on both the isoforms (data not shown).

The secondary structure of saporin isoforms was evaluated by CD spectral analysis. Saporin-5 and saporin-6 showed similar CD spectra, characteristic of a compactly folded α+β structure, in the far ultraviolet (UV) range 200–250 nm (Fig. 1A). Compared to saporin-6, saporin-5 had a relatively higher β-sheet and a reduced α-helical content (Fig. 1A). These spec-

Table 1
Cytotoxicity of saporin isoforms on LRP-positive and LRP-negative cells

Protein	ID_{50} (μg/ml)			
	LRP-positive		LRP-negative	
	J774A.1	U937	HUT102	HeLa
Saporin-5	0.30 ± 0.009	55.0 ± 5.03	3.4 ± 0.35	> 80
Saporin-6	0.03 ± 0.005 (10)	6.5 ± 0.69 (8)	0.7 ± 0.04 (5)	15 ± 1.73 (> 5)

ID_{50} refers to the concentration of toxin required to inhibit cellular protein synthesis by 50%. All assays were carried out at least three times, and results are expressed as means ± S.E.M. The numbers in parentheses refer to fold activity of saporin-6 compared to saporin-5 in the same cell line.

Table 2
Cytotoxicity of saporin isoforms on permeabilized HeLa cells

Protein	ID_{50} (μg/ml)	
	+Adenovirus	−Adenovirus
Saporin-5	20.0 ± 2.33	> 80
Saporin-6	2.5 ± 0.18	> 80

ID_{50} refers to the concentration of toxin required to inhibit cellular protein synthesis by 50%. All assays were carried out at least three times, and results are expressed as means ± S.E.M.

tra are similar to that observed for dianthin-30 and ricin A-chain suggesting a similar structure for these functionally related proteins [18].

3.2. Protein synthesis inhibitory activity

The protein synthesis inhibitory activity of saporin isoforms was tested in an in vitro translation assay using rabbit reticulocyte lysate. The lysate permits the translation of globin mRNA when suitable energy source and amino acids are supplied to optimize the reaction. The lysate was treated with different concentrations of saporin-5 or saporin-6, and the inhibition of protein synthesis was measured as decrease in the incorporation of [^{3}H]leucine in nascent polypeptides synthesized. Both the isoforms caused a dose-dependent inhibition of protein synthesis (Fig. 1B). However, saporin-6 was found to be about 10-fold more active than saporin-5 in inhibiting in vitro protein synthesis (Fig. 1B). The respective ID_{50} values of saporin-6 and saporin-5 were 2.6 ± 0.11 and 30 ± 1.15 ng/ml. This agrees with the earlier observation suggesting that leaf isoforms have one order of magnitude lower inhibitory activity in rabbit reticulocyte lysate than the seed isoforms [5].

3.3. Specific N-glycosidase activity

RIPs cleave the N-glycosidic bond at A4324 of 28S rRNA when intact rat ribosomes are the substrate. This depurination makes the site susceptible to amine-dependent cleavage leading to the release of a 390 bases long 3′ rRNA fragment called as Endo-fragment [19]. To evaluate the specific RNA N-glycosidase activity of saporin isoforms, rabbit reticulocyte lysate was taken as the source of ribosomes. The total RNA was isolated from the toxin-treated lysate, treated with aniline and analyzed on agarose gels. Both the isoforms specifically released the 390 bases long Endo-fragment on aniline treatment at a concentration as low as 40 ng/ml (Fig. 2A). However, at lower concentration with saporin-5 the digestion of 28S rRNA was incomplete indicating saporin-5 to have a lower activity compared to saporin-6 in this semi-quantitative assay also (Fig. 2A).

3.4. Genomic DNA fragmentation

Saporin has been shown to induce cell death via apoptosis [20]. Internucleosomal degradation of DNA, reflected as DNA laddering, is a characteristic biochemical feature of apoptosis [12,20,21]. In this study the effect of saporin isoforms was compared on genomic DNA of U937 cells. U937 cells were treated with various concentrations of two saporin isoforms and after 48 h genomic DNA was isolated from the treated and untreated cells. As shown in Fig. 2B, DNA from cells treated with 0.1 μM saporin-6 was found to be fragmented, which intensified further at 1 μM concentration. Saporin-5 appeared to be less active in causing genomic DNA fragmentation and a much reduced fragmentation was observed with 1 μM protein (Fig. 2B).

3.5. Cytotoxic activity

The cytotoxic activity of saporin isoforms was studied on both LRP-positive and LRP-negative cell lines. The cell lines J774A.1 (mouse monocyte macrophage) and U937 (human histiocyte lymphoma) express LRP, whereas HUT102 (human cutaneous T-cell lymphoma) and HeLa (human epitheloid carcinoma) cells lack LRP [11]. The cells were treated with various concentrations of both saporin isoforms, and decrease in the incorporation of [^{3}H]leucine in toxin-treated cells as compared to the control was taken as the measure of cytotoxicity. Both the isoforms showed a dose-dependent toxicity on all the cell lines studied, however, the toxicity varied with the cell line (Table 1). Among the cell lines tested, J774A.1 was found to be the most sensitive cell line. The activity of saporin-6 was found to be 10-fold higher than of saporin-5 on J774A.1 (Table 1). Saporin-6 was found to be more active than saporin-5 on all other cell lines also; the difference of activity between the two isoforms varied between 5- and 20-fold depending on the cell line tested (Table 1). In this study the LRP-negative cell lines used were found to have sensitivities similar to LRP-positive cell lines towards saporin toxicity (Table 1).

To further investigate the involvement of LRP in the cytotoxic activity of saporin, the toxicity of saporin-6 was assayed on LRP-positive Chinese hamster ovary cell line CHO-K1, and its mutant CHO-13-5-1, which has been shown to have no detectable LRP mRNA or protein [22]. Both CHO-K1 and CHO-13-5-1 cells were found to have similar sensitivities towards saporin-6 toxicity. The respective ID_{50} values of saporin-6 on CHO-K1 and CHO-13-5-1 cells were 2.2 ± 0.15 and 3 ± 0.20 μg/ml. It has been shown that functional LRP is required to mediate *Pseudomonas* exotoxin (PE) entry and delivery to the cell interior [22,23]. The mutant CHO-13-5-1 cells showed > 100-fold resistance to PE compared to CHO-K1 cells (data not shown).

Saporin isolated from the seeds has been shown to bind to LRP [11], however, in the present study the sensitivities of LRP-positive and LRP-negative cells were found to be similar. Studies done on interactions of LRP and saporin have shown that anti-LRP antibodies and lipoprotein lipase, a known ligand of LRP, inhibit the cytotoxicity of saporin to U937 cells [11]. Also, studies with a conjugate of saporin and urokinase plasminogen activator (uPA) suggested the internalization of the conjugate to be mediated by saporin and LRP interactions [24,25]. Although the above observations suggest LRP as one of the receptors through which saporin enters, the lack of comparative studies with LRP-negative cell lines does not rule out other possible mechanisms of entry. It is widely accepted that type I RIPs and A-chain of type II RIPs can also enter the cell cytosol through passive mechanisms such as fluid-phase pinocytosis or non-specific cell engulfment [26,27]. Recent studies have shown that trichosanthin binds to LRP as well as another member of LDL receptor family, megalin [28], suggesting that RIPs can enter the cells through various receptors of the same family. Therefore, one of the reasons of similar cytotoxic activities of saporin isoforms in LRP-positive and LRP-negative cell lines could be the entry of toxin into LRP-negative cells through receptors or mechanisms oth-

er than LRP-mediated endocytosis. Similar sensitivities of CHO-K1 cells and its mutant CHO-13-5-1 cells, which lack LRP, to saporin-6 strongly suggest that binding and internalization of saporin is not mediated through LRP.

To find out if the difference in activities of saporin isoforms on various cell lines is due to a difference in their internalization efficiencies, the cytotoxicity of isoforms was tested on HeLa cells infected with adenovirus. The adenoviral infection makes the cells permeable and will eliminate any differences that might exist in the efficiency of saporin isoforms to enter the cells. The cells were treated with various concentrations of saporin-5 or saporin-6 in the presence or absence of adenovirus and decrease in the incorporation of [^{3}H]leucine, in toxin-treated cells, was taken as the measure of cytotoxicity. Both saporin-5 and saporin-6 showed a dose-dependent toxicity to the infected cells (data not shown). As shown in Table 2 saporin-6 was eight-fold more active than saporin-5 in adenovirus infected cells, whereas in uninfected cells ID_{50} could not be achieved up to 80 μg/ml for both the isoforms, indicating that the difference in the cytotoxicity is a reflection of the difference in the catalytic activity of the two isoforms (Table 2).

In conclusion, saporin-6 has been shown to have higher N-glycosidase and DNA fragmentation activities than saporin-5 that reflect in a similar difference in their cytotoxic activities. The difference in the activity of the two isoforms could be attributed to amino acid differences, which lie outside the proposed active site of the toxin. Of the 12 differences between saporin-5 and saporin-6, substitutions at positions 134(Q/K), 147(S/L), 149(S/F), 162(D/N), 188(I/T) and 196(N/D) result in change in polarity or charge of amino acid residue. Based on a putative model of saporin, Fabbrini et al. [24] had postulated the substitution of Lys134 of seed type saporin with Gln in leaf cDNA isoform to be responsible for difference in their activity. This residue was predicted to be located at a conserved surface loop of RNA binding domain. Crystal structure of saporin-6 showed that Lys134 is present in a hydrogen-bonded turn and Asp196 is present in a loop. Substitution of charged amino acids Lys134 or Asp196 in saporin-6 with uncharged amino acids Gln or Asn respectively in saporin-5 may result in a change in local structure as well as charge, which can affect the toxin interactions and activity on the ribosomes. The two isoforms do not appear to differ in their cell binding and internalization activities, and the binding of saporin to various cell lines does not appear to be only through LRP.

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