CHAPTER III

INTERACTION OF SRL WITH NORMAL AND LEUKEMIC CELL LINES AND ITS PHYSIOLOGICAL RESPONSE

"Discovery consists of seeing what everybody has seen thinking what nobody has thought."

Albert Szent...
3.1. INTRODUCTION

Importance of cell surface glycoconjugates came to the forefront of biochemical research after the realization that these molecules play crucial role in a variety of physiological functions such as fertilization, embryonic development, cell differentiation, cell adhesion/migration and apoptosis [Varki et al., 1999]. Glycosylation changes on the cell surface are often the hallmark of transformation of cells to malignant state. In other words cells express glycans with varied structures at different levels [Couldrey and Green, 2000; Yarema and Bertozzi, 2001; Dube and Bertozzi, 2005]. Malignant transformation is often associated with incomplete or neosynthesis of carbohydrate chains and are being referred to as tumor antigens due to abnormal expression of genes encoding glycosyl transferases [Dube and Bertozzi, 2005; Fuster and Esko, 2005; Wang, 2005]. Such alterations could have critical impact on variety of cellular interactions, which determine the metastatic behavior of the tumor cells [Varki A, 1999; Hakomori and Handa, 2002; Dube and Bertozzi, 2005]. In the past two decades a vast wealth of information has been accumulated that implicate the altered glycosylation during malignancy [Gorelik et al., 2001].

Characterization of altered cell surface glycosylation is a difficult task and requires precise knowledge of the cell surface glycan repertoire and also the analysis is complicated because of the considerable structural micro heterogeneity often seen in naturally occurring glycans [Varki A, 1993].
Lectins with their unique ability to recognize specific carbohydrate moieties have been widely used as molecular tools in lieu of monoclonal antibodies for the characterization of cell surface glycoconjugates [Heinrich et al., 2005; Amborsi et al., 2005]. Lectins have become popular tools because of the ease to purify some of these in substantial quantities and that they can easily be tagged to probing molecules such as fluorescent dyes, biotin etc to give stable derivatives, their interaction with cell surface ligands can be examined with greater specificity and sensitivity. It is possible to calculate the relative cell surface density of lectin-binding sites from these measurements when the volume of each cell is simultaneously determined in the flow cytometer by electrical sizing. The binding of lectins to cells of different size can be compared thereby in a standardized way [Siegert et al., 1984]. Considering the structural differences in the cell surface glycans of different cell types, lectins are also used in cell biology for identifying subpopulations [McCoy JP, 1986].

Agaricus bisporus lectin (ABL), phytohemagglutinin (PHA), concanavalin A (ConA), and wheat-germ agglutinin (WGA) were the earliest examples of lectins, successfully used for examining the surface carbohydrate topography of a variety of normal and neoplastic mammalian cells [Present CA and Kornfeld S, 1972; Chilson et al., 1984; Leca et al., 1986]. To date, several lectin receptors were isolated and characterized from normal and cancer cells using lectins with different sugar specificity.
Recently, WGA receptor of 141 kDa glycoprotein from human neutrophils [Solorzano et al., 2007] and Amaranthus leucocarpus lectin (ALL) receptor from human T lymphocytes [Porras et al., 2005] have been characterized. Also, receptors for Amaranthus leucocarpus have been isolated and characterized from murine naïve thymocytes and murine peritoneal macrophages, these studies have demonstrated the expression of these receptors only on resting cells but not on activated cells of murine peritoneal macrophages [Gorocica P, 1998: Porras et al., 2000]. Like wise, the activation of T-lymphocytes has been associated with changes in carbohydrates structures [Conzelmann et al., 1984; Lefrancois et al., 1985] apart from different receptors. For example, simple O-glycan [Neu5Acα2-3Galβ1-3 (Neu5Acα2-6) GalNAcα] is the principal oligosaccharide on CD43 of resting lymphocytes [Piller et al., 1988, 1991] whereas, differential expression of complex O-glycan [Neu5Acα2-3Galβ1-3(Neu5Acα2-3Galβ1-4GlcNacβ1-6) GalNAc] occurs on CD43 of activated lymphocytes. Interestingly, same O-glycan is also expressed on resting lymphocytes of patients suffering from Wiskott-Aldrich syndrome (WAS) [Piller et al., 1991]. In addition to detection and characterization of different glycan/glycoproteins from normal and cancer cell surfaces, there are numerous reports on the physiological effects of lectins on cancer cells [Minko, 2003; Gabor et al., 1998, 2002, 2004; Wirth et al., 1998, 2002; Kiss et al., 1997; Valentiner et al., 2002, 2003; Bangchonglikitbul, 2002; Lorea
et al., 1997; Lityńska et al., 2001; Schwarz et al., 1999] and even some of the studies report use of lectins in supplemental cancer therapy in humans [Fritz et al., 2004; Thies et al., 2005].

Previous chapter on glycan array analysis to establish the carbohydrate binding property of Sclerotium rolfsii lectin, interestingly revealed that the lectin binds vividly to a classic tetrasaccharide, [Neu5Aca2-3Galβ1-3 (Neu5Aca2-6) GalNAcα]. This glycan was referred to as MN antigen [Springer and Desai, 1982] and is part of CD43 which is expressed only on resting lymphocytes, whereas SRL exhibit negligible affinity to a related glycan, [Neu5Aca2-3Galβ1-3(Neu5Aa2-3Galβ1-4GlcNacβ1-6) GalNAc], which is also part of CD43 but is expressed on activated lymphocytes. This important sugar binding property of SRL can be expected to have differential binding towards resting and activated PBMCs. Considering these findings along with its ability to recognize cancer associated TF antigen and the variant forms, it was exciting to examine the interaction of S. rolfsii lectin with normal resting, activated PBMCs and leukemic cells.

Present chapter describes the exploitation of exquisite carbohydrate binding property of SRL in its interaction with human normal resting, activated and leukemic cells, and also the detection and characterization of the receptors through which it interacts and exerts various physiological effects.
3.2. MATERIALS AND METHODS

3.2.1. Materials

Bovine serum albumin (BSA), N-hydroxysuccinimido biotin, fluorescein isothiocyanate (FITC), mucin (porcine stomach, type III), fetuin, N-acetylgalactosamine, N-acetylgalactosamine, fetal calf serum (FCS), PHA-L, histopaque1077, trypan blue, RPMI (Roswell Park Memorial Institute) 1640 medium, DMSO, ponceu S, protease inhibitor complex (Cat # P-8340) and 4', 6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Chemical Co., St. Louis, USA. Streptavidin, 3-3′diaminobenzidine (DAB) chromogen/H₂O₂ substrate in buffered solution (pH 7.5) and standard protein marker (PMW-M) were purchased from Bangalore-Genei, India. Acrylamide and N, N-methylene diacrylamide were from Koch-Light Laboratories, England. N, N, N, N′-tetramethylene diamine and ammonium per sulphate were obtained from BDH Chemicals Ltd., Poole, England. Paraformaldehyde was purchased from ICN Biomedicals, Inc., Aurora, Ohio. Tritiated thymidine was procured from BRIT (Board of Radiation and Isotope Technology), India. The tissues culture flasks and 96 well plates were from NUNC (Denmark).

Glycosidic enzymes viz., O-glycosidase (Endo-α-N-acetylgalactosaminidase) from *Streptococcus pneumoniae*, recombinant protein expressed in *E. coli* (EC# 3.2.1.97; CAS# 9032-92-2), N-glycosidase (Peptide N-Glycosidase F; PNGase F) from *Elizabethkingia*
meningosepticum (EC# 3.5.1.52; CAS# 83534-39-8), neuraminidase type V from Clostridium perfringens (EC# 3.2.1.18; CAS# 9001-67-6) and α-1, 2 fucosidase (EC# 3.2.1.63; CAS# 9037-65-4) were purchased from Sigma Chemical Co., St. Louis, USA. Fluorescein isothiocyanate labeled and biotin labeled Amaranthus caudatus lectins (ACA) were purchased from EY laboratories Inc., San Mateo, CA, USA.

3.2.2. Purification of SRL

Sclerotium rolfsii lectin (SRL) was purified from the sclerotial bodies of the fungus according to the method of Swamy et al., [2001].

3.2.3. FITC labelling of lectin

FITC conjugated SRL used for binding studies and for fluorescent microscopy was prepared according to the procedure described by Goldman [1981]. Briefly, SRL (10 mg/ml) was incubated with FITC at the concentration of 25 μg/mg of protein in carbonate buffer (pH 9.5) with gentle stirring for 12 h, at 4°C. Unbound excess FITC was removed by extensive dialysis against TBS (pH 7.2) and stored at 4°C till further use. Biotinylated SRL was prepared by conjugating with N-hydroxysuccinimido biotin as described by Duk et al., [1994].

3.2.4. Preparation of periodate-BSA (p-BSA)

Periodate-BSA required for blocking non-specific lectin binding sites was prepared according to the method of Glass et al., [1981]. BSA
(fraction V) in 0.1 M sodium acetate buffer, pH 4.5 (4 g/100 ml) was treated with 10 mM periodic acid for 6 h at room temperature. Then excess of periodate was eliminated by adding glycerol to a final concentration of 10 mM and later the solution was dialyzed extensively against 10 mM PBS and subsequently against distilled water and freeze-dried.

3.2.5. Cell lines and Cell culture

The human T leukemia cell lines Molt-4 and Jurkat cells were obtained from American Type Culture Collection (ATCC) USA and cultured in RPMI 1640, supplemented with heat inactivated 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1 M pyruvate in a humidified atmosphere with 5% CO2 at 37°C. For all the experiments, cells of passage numbers 3 to 15 were used. The cell lines were revived and grown for three passages before performing any experiment. Cell number was assessed by trypan blue dye exclusion technique using a haemocytometer.

3.2.6. Isolation and activation of PBMCs

Normal human venous blood was drawn in sterile syringe and the PBMCs were separated by density gradient separation using ficoll hypaque solution. Heparinized blood (diluted with saline 2:1) was overlaid on ficoll hypaque solution in a ratio of 3:1 and was centrifuged at 2,000 rpm, for 30 min at room temperature, to separate three distinct layers. PBMCs form an opaque middle layer whereas RBCs settle down at the bottom. The middle layer was carefully removed and washed with RPMI 1640 medium twice.
The supernatant was discarded and the cells were resuspended in complete medium and $1 \times 10^6$ cells/500μl/well was seeded in 24 well plate. The cells were stimulated with phytohemagglutinin (PHA-L) at 5 μg/ml for 72 h at 37°C in an atmosphere of 5% CO₂.

### 3.2.7. Binding studies of SRL to cells by flow cytometry

Quantitative assessment of surface binding of SRL to different cells was studied by flow cytometry using FITC-SRL in comparison with *A. caudatus* agglutinin (ACA). Cells suspensions were washed thrice with cold phosphate buffered saline (PBS, pH 7.4) and adjusted to $1 \times 10^6$ cells/tube. Non-specific lectin binding sites were blocked by incubating the cells suspension with 3% p-BSA in PBS (200 μl/1x10⁶ cells) on ice for 1 h and subsequently washed once with cold PBS. After blocking, FITC-conjugated lectins (SRL and ACA) were added to get a final concentration of 2.5 μg/ml and incubated on ice for 1 h in dark. Cells were washed twice with cold PBS and fixed with freshly prepared 2% paraformaldehyde for 5 min at 4°C. Finally, cell pellet was thoroughly washed and resuspended in 500 μl of cold PBS. Flow cytometric analyses was performed using FACS Vantage (Becton Dickson San Jose, CA, USA) equipped with 488 nm argon laser and analyzed with Cell Quest-pro software by selective gating based on the parameters of forward and side scatter for determining percent positivity and mean fluorescence intensity (MFI). The unstained cells processed similarly was used as negative control and the positivity was set at 1%. Data was
acquired for 10,000 events and presented as a frequency histogram (X-axis; fluorescent intensity and Y-axis; number of events or cells). Three independent experiments were carried out for each of the lectin.

In order to confirm the carbohydrate mediated binding of lectin, FITC-SRL (2.5 µg/ml) was pre-incubated with mucin and fetuin (final concentration 12.5 µg in 200 µl), for 1 h at room temperature and was then added to the cell preparations and analyzed by flow cytometry.

3.2.8. Fluorescence microscopy

Localization of lectin binding sites on Molt-4, Jurkat, resting and activated PBMCs was determined by fluorescence microscopy. Cells were blocked with 1% p-BSA for 1 h at 4°C and stained by incubating with FITC-conjugated SRL (2.5 µg/ml in PBS) for 1 h at 4°C in dark. Cells were fixed with 2% freshly prepared paraformaldehyde for 10 min on ice followed by staining with DAPI for 10 min at 4°C. Extensive washing was done after each step using ice cold PBS. Cells were suspended in mounting medium and mounted on slides for visualization by fluorescent microscope. (Zeiss LSM 510, Jena, Germany) and images were captured at 40x magnification.

3.2.9. Isolation of membrane proteins

Membrane proteins from normal resting/activated PBMCs and leukemic cell lines (Molt-4 and Jurkat) were isolated by Mem-PER system kit method developed by Pierce (Cat # 89826) which is a non mechanical
alternate method to traditional membrane protein isolation techniques. The Mem-PER system consists of three reagents. *Reagent A* is a cell lysis buffer, *Reagent B*, detergent dilution buffer and *Reagent C*, membrane solubilization buffer. To isolate membrane proteins, cells (5 x 10⁶) were harvested by centrifugation at 2,000 rpm for 2-3 min at room temp, washed with PBS and pelleted. Each of the cells pellet was lysed at room temperature, by adding 150 µl of reagent A and 5 µl of 1x protease inhibitor complex and incubated for 10 min with intermittent vortexing. Membrane proteins were later solubilized on ice with reagent C diluted 2:1 with reagent B (total 450 µl) for 30 mins by vortexing at every 5 minutes. The solubilized protein mixture was centrifuged (9,000 rpm; 3 min; 4°C) to remove cellular debris. The clarified supernatant was incubated at 37°C for 10-20 min in a water bath and subjected to centrifugation (10,000 rpm; 2 min; room temperature) to separate distinct hydrophilic and hydrophobic protein fractions (Care must be taken to avoid use of incubator/oven to maintain temp). Phase partitioning resulted in two distinct layers, the hydrophilic proteins forming a layer at the top and the hydrophobic membrane proteins at the bottom, which were carefully separated and stored in separate micro-centrifuge tubes at 4°C till further use.

### 3.2.10. Concentration of membrane proteins by organic solvent precipitation

Since protein concentration in hydrophobic fraction was very less, organic solvents were used to precipitate and concentrate membrane proteins from the detergent phase prior to SDS-PAGE using
chloroform/methanol as described by Wessel and Flugge [1984]. Briefly, 400 µl of methanol was added to 100 µl sample, vortexed, centrifuged (9,000 rpm; 30 sec; room temp) and 200 µl of chloroform was added. After extensive mixing on a vortex mixer, samples were centrifuged (9,000 rpm; 30 sec; room temp). For phase separation, 300 µl of water was added to the samples, vortexed vigorously and centrifuged at 9,000 rpm for 1 min at room temperature. The upper aqueous phase was removed carefully without disturbing the protein at the interphase. Proteins were precipitated by adding 300 µl of methanol to the organic phase and the precipitated proteins were spinned down (9,000 rpm; 2 min; room temperature). Supernatant was discarded and the protein pellet was dried under a stream of air. The pellet was dissolved in 50 µl of 25 mM Tris-HCl buffer (pH 7.2) centrifuged and supernatant was used for protein estimation.

3.2.11. Protein estimation

The Folin and Wu based detergent compatible protein assay reagent kit (Bio-Rad Dc Cat # 500-0114) was used to quantify extracted membrane proteins.

3.2.12. Electrophoresis and western blotting

Membrane protein isolates of each cell types were prepared for electrophoresis by mixing with equal volumes of 2x sample buffer and briefly boiling in a water bath. Protein samples (40 µg) were subjected to SDS-PAGE on 10% polyacrylamide slab gels as described by Laemmli.
[1970] under reducing conditions for 1 h using 120 V constant voltages on a Hoefer Mighty small-II (USA) electrophoresis unit. Resolved proteins were transferred on to nitrocellulose membrane (Millipore Corporation) for 3 h at 75 mA, as described by Towbin et al., [1979] using semi-dry transfer cell in transfer buffer (25 mM Tris-HCl, 192 mM glycine made up in 20% v/v aqueous methanol). Efficiency of protein transfer was confirmed by staining the blots with ponceau S stain (0.1% ponceau in 5% acetic acid) and later destained by distilled water. Protein mixture (Bangalore Genei) consisting of lysozyme (14.3kDa), soyabean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), BSA (66 kDa) and phosphorylase b (97.4 kDa) were used as standard molecular weight markers for calibration.

3.2.13. Identification of SRL receptors

The glycoproteins on the nitrocellulose membrane were identified using biotinylated lectin after reaction with streptavidin-HRP [Rohringer and Holden, 1985]. The protein bands were visualized by diaminobenzidine (DAB) staining method [Hsu and Soban, 1982].

Prior to lectin probing, nonspecific binding sites were blocked by incubating with 3% p-BSA (In TBS) for overnight at 4°C. Blots were washed thrice with tris buffered saline containing 0.1% tween-20 (TBST) and incubated with biotinylated SRL (20 μg/ml in TBST) for 1 h at room temperature on a rocking platform. Blots were washed thrice with TBST to
remove unbound lectin and incubated with streptavidin-HRP (1:1000 in TBST) for 1 h at room temperature. After thorough washing with TBST, blots were allowed to react for 30 to 60 sec with DAB solution supplemented with H₂O₂. Finally, the blots were washed thrice with TBST and distilled water to arrest the reaction.

3.2.14. Characterization of lectin receptors on blots using glycosidases

Characterization of the SRL binding glycan moieties of the resolved membrane glycoproteins immobilized on nitro cellulose membrane was carried out using different glycosidases. Membrane protein blots were subjected to treatment with a specific glycosidase or a sequential cleavage using different glycosidases before probing with lectin.

3.2.14.1. O-glycosidase treatment

O-glycosidase treatment of membranes was performed with Endo-α-N-acetylgalactosaminidase according to the instructions of manufactures. Since chloride is reported to inhibit the enzyme, the p-BSA blocked blots were thoroughly washed with the reaction buffer (50 mM sodium phosphate buffer pH 5.0) and glycoproteins were digested with O-glycosidase (10 mU/ml) prepared in reaction buffer containing 5 μl/ml of 1x protease inhibitor complex at 37°C for 3 h. After digestion, membranes were thoroughly washed (5 times) with TBST (residual of phosphate gives more background with DAB).
3.2.14.2. N-glycosidase treatment

N-glycosidase treatment of membranes was carried out with Peptide N-glycosidase F (PNGase F) according to the method described by Li et al., [1993] with minor modification. After blocking and thorough washing with TBST, blots were incubated with PNGase F (6.16 U/ml) prepared in Tris-HCl buffer (200 mM, pH 7.2) containing 20% Triton X-100 (15% v/v in water) for 48 h at 37°C on rocking platform. Failure to add Triton X-100 will result in 3-fold reduction of enzyme activity.

3.2.14.3. Neuraminidase and Fucosidase treatment

Terminal sialic acid and fucose on glycans were cleaved by neuraminidase and fucosidase respectively. Neuraminidase was prepared in 25 mM Tris-HCl pH 7.2 and fucosidase was prepared in 20 mM Tris-HCl pH 7.5 containing 25 mM NaCl/Nitrocellulose blots containing membrane proteins were incubated either with neuraminidase (20 mU/ml) or fucosidase (4 mU/ml) at 37°C for 12 h.

After various glycosidase treatments, blots were thoroughly washed with TBST (5 times) and processed for lectin probing and the bands were visualized as described earlier.

3.2.15. Effect of SRL on human normal resting PBMCs

Proliferative/antiproliferative activity of SRL on human peripheral blood mononuclear cells (PBMCs) was determined by tritiated thymidine
incorporation assay, using freshly isolated human PBMCs as described by Wang et al., [1992] with some modifications. PBMCs were isolated from the freshly collected blood samples of healthy donors and the cells were maintained in RPMI 1640 complete medium as described previously. The cells were diluted with RPMI medium containing 10% FCS and then seeded (1x10^5 cells/50 μl/well) in 96-well microplates (NUNC Denmark). Various concentrations (10, 5, 2.5, 1.25 and 0.625 μg/ml) of SRL in RPMI 1640 medium was added to each well in a final volume of 100 μl and incubated at 37°C in a humidified, 5% CO₂ atmosphere for 72 h. Eighteen hours prior to the total incubation period, 1 μCi of tritiated thymidine was added to each well in a final volume of 10 μl and the incubation was continued for another 18 h. Finally, cells were harvested onto a glass filter paper (Amersham Biosciences) using cell harvester (NUNC, Denmark), washed thoroughly with distilled water and dried in micro-oven. Filter discs were transferred to scintillation vials (Tarsons, India) containing 600 μl of toluene-based scintillation cocktail. Finally the incorporated tritiated thymidine was measured by β-scintillation counter (Packard Bioscience Ltd.) as counts per minute (CPM).

In order to confirm the lectin-receptor mediated proliferative activity, assays were performed using SRL preincubated with mucin. SRL (10 μg/ml) was preincubated with mucin (12.5 μg in 100 μl) for 1 h at 37°C and used for cell proliferation assay. Appropriate controls containing cells without
lectin and cells with mucin alone were included. Results are representative of three independent experiments done in triplicate.

3.2.16. Effect of SRL on Molt-4 leukemic cell line

Proliferative/antiproliferative effect of SRL on Molt-4 leukemic cell line was determined by tritiated thymidine incorporation assay as described previously. Proliferative/antiproliferative effect of SRL on Molt-4 leukemic cell line was determined by tritiated thymidine incorporation assay as described previously. Cells were seeded (1x10^5 / well) in a 96-well tissue culture plate and allowed to acclimatize for 3h before lectin treatment Serial concentrations of SRL from 6.25 μg/ml to 100 μg/ml in 50 μl of RPMI medium was added to each well and incubated for 72 h. All other steps of assay remained same as described earlier. For lectin blocking assay, SRL (100 μg/ml) was preincubated with 12.5 μg of mucin in a total volume of 100 μl for 1 h at 37°C and used for assay.

3.2.17. Statistical analysis

Statistical analysis of the data was done using SigmaPlot 8.0 software to calculate the mean and standard deviation (SD). The P values were calculated using student's t-test or single factor ANOVA and considered significant when P < 0.05.
3.3. RESULTS

To understand the role of SRL in cellular response towards normal and leukemic cells, it is necessary to identify its glycoconjugate receptors and to characterize them. As an initial step in this approach, a flow cytometric and fluorescence microscopic techniques have been employed in order to know the quantitative and qualitative cell surface binding of SRL. Subsequently blotting techniques were used to identify the cell surface receptors of SRL and glycosidic cleavage experiments for characterizing carbohydrate nature of the compliment receptors. Finally, SRL mediated cellular response on both normal and leukemic lymphocytes (Molt-4) was carried out with a novel thymidine incorporation assay and demonstrated that the response is mediated by lectin-carbohydrate receptor interaction.

3.3.1. Flow cytometric analysis of SRL with human normal resting, activated and leukemic cells

Surface binding of SRL to human normal, activated and leukemic cells (Molt-4 and Jurkat) were analyzed using FITC-SRL by flow cytometry and the binding patterns were compared with *Amaranthus caudatus* lectin (ACA), which broadly exhibit similar sugar specificity [Rinderle et al.,
All the studies were carried out with paraformaldehyde fixed cells in order to have stable conditions during measurements and to exclude the possibility of endocytosis. Histograms of flow cytometric analysis of FITC-SRL and FITC-ACA interaction with resting/activated PBMCs, Molt-4 and Jurkat cells in comparison with respective unstained cells are presented in figure 1. It is evident from the data that more than 90% of gated cells were positive for SRL in all the cell types tested, whereas ACA did not show significant binding to any of these cells. It is also evident from the histograms that complete shift of the peak was observed for SRL at 2.5 μg/ml with all the 4 cell types indicating highest binding. However, ACA show least or negligible shift, demonstrating no binding at the same concentration. These results of lectin binding to different cell types summarized in table 1 and results of inhibitory effect of mucin on binding of SRL are given in figure 2. Preincubation of SRL (2.5 μg/ml) with mucin (12.5 μg) completely abolishes the binding of SRL. However, fetuin at similar concentration showed less inhibitory effect on SRL and the results are summarized in table 2. These results demonstrate that the interaction of SRL with these cells is mediated by cell surface carbohydrates apart from demonstrating the inhibitory efficacy of mucin and fetuin.
Figure 1. Interaction of SRL with human normal resting/activated PBMCs and two leukemic cell lines (Molt-4 and Jurkat) in comparison with ACA. All the cell types were stained with FITC conjugated SRL and ACA at a concentration of 2.5 µg/ml. The cells were prepared and stained as described under materials and methods. Unstained cells (UNS) were used as negative control. As depicted by histograms, SRL shows maximum binding with all the cell types, whereas ACA showed negligible binding.
Table 1. Flow cytometric analysis of SRL in comparison with ACA

<table>
<thead>
<tr>
<th>Cells</th>
<th>Untreated</th>
<th>SRL</th>
<th>ACA</th>
<th>Untreated</th>
<th>SRL</th>
<th>ACA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal PBMCs</td>
<td>1.50</td>
<td>100</td>
<td>7.46</td>
<td>13.78</td>
<td>558.38</td>
<td>15.80</td>
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<tr>
<td>Activated PBMCs</td>
<td>1.04</td>
<td>99.89</td>
<td>9.43</td>
<td>9.53</td>
<td>243.33</td>
<td>12.18</td>
</tr>
<tr>
<td>Molt-4</td>
<td>0.96</td>
<td>97.36</td>
<td>5.34</td>
<td>8.52</td>
<td>394.14</td>
<td>10.47</td>
</tr>
<tr>
<td>Jurkat</td>
<td>1.06</td>
<td>97.96</td>
<td>1.44</td>
<td>33.63</td>
<td>298.49</td>
<td>29.08</td>
</tr>
</tbody>
</table>

Table 2. Inhibitory effects of Mucin and Fetuin on cell surface binding of SRL as determined by flow cytometry

<table>
<thead>
<tr>
<th>Cells</th>
<th>SRL</th>
<th>SRL + Mucin</th>
<th>SRL + Fetuin</th>
<th>SRL</th>
<th>SRL + Mucin</th>
<th>SRL + Fetuin</th>
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</thead>
<tbody>
<tr>
<td>Normal PBMCs</td>
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<td>394.14</td>
<td>10.37</td>
<td>10.62</td>
</tr>
<tr>
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<td>0.74</td>
<td>92.15</td>
<td>298.49</td>
<td>35.09</td>
<td>44.15</td>
</tr>
</tbody>
</table>
Cell surface binding studies by flow cytometry revealed that both activated and leukemic cells showed reduced mean fluorescence intensity (MFI) as compared to normal resting lymphocytes (Table 1). This finding suggests that the number of surface ligands for SRL decreases or modified upon activation and transformation as seen in leukemic cell lines. Abolished or diminished binding of SRL after treatment with mucin or fetuin as compared to monosaccharides which failed to exert inhibitory effect even at 200 mM concentration strengthen our earlier findings of complex sugar specificity of the lectin.

3.3.2. Localization of lectin binding sites by fluorescence microscopy

Interaction of SRL with different cell types was also examined by fluorescence microscopy and the binding of free FITC-SRL and its mucin complex on different cells are presented in figure 3. These findings demonstrate uniform intense binding of the SRL on the cell surfaces and reduced binding in SRL-sugar conjugate which was also depicted in flow cytometry studies.
Figure 2. Interaction of SRL (---), SRL preincubated with mucin (---) and fetuin (---) with normal resting PBMCs (A), PHA-activated PBMCs (B), Molt-4 (C) and Jurkat (D) cells. Cells were either incubated with FITC-SRL (2.5 µg/ml), or SRL+mucin (12.5 µg) complex and SRL+fetuin (12.5 µg/ml) complex on ice for 1 h. Cells were then washed and fixed with paraformaldehyde before analysis by flow cytometry. The histogram overlay plots compared with untreated cells (----) showing complete inhibition of SRL binding to all the cells by mucin and lesser extent by fetuin.
Figure 3. Localization of lectin binding sites on human normal resting/activated PBMCs and leukemic cell lines (Molt-4 and Jurkat). Paraformaldehyde fixed cells after incubation with FITC-SRL, were observed under fluorescent microscope and photographed at 40 x magnification. DAPI was used for nuclear staining and mucin treated FITC-SRL was used for inhibition studies. For all the cells, intense labeling was seen with SRL while no binding was observed with mucin treated SRL.
3.3.3. Identification of SRL receptors by lectin blotting

Membrane proteins extracted from different cell types, were resolved by SDS-PAGE and further transferred on to nitrocellulose solid support. Resolved proteins were identified by ponceau staining to ascertain complete transfer of the proteins. After destaining, the protein bands were allowed to interact with biotinylated SRL and the receptors were visualized by streptavidin-HRP/DAB reaction. Profiles of SRL receptors from different cell types detected by biotinylated SRL were presented in figure 4 and the bands were compared with ponceau stained bands. As revealed from these blots not all, but only few protein bands selectively interacted from each of the cell types. SRL-receptors found on normal lymphocytes (figure 4, lane 1) are of high molecular weight range (Mr >80 kDa), where as none of these bands are seen in activated (lane 2) and leukemic Molt-4 and Jurkat cells (lane 3 & 4 respectively). At least 12 different bands (Mr 14-85 kDa) are common for activated (lane 2) and leukemic cell lines, Molt-4 (lane 3) with Mr of 85, 82, 73, 66, 62, 58, 54, 50, 40, 29, 27 and 14.5 kDa. Where as all of these bands, except bands corresponding to Mr 85 and 82 kDa are absent in Jurkat cells (lane 4) indicating its difference with Molt-4 cells. These leukemic cells also differ from the activated lymphocytes by having two additional bands corresponding to Mr 29 and 27 kDa (lane 2, 3 & 4). Further a band with Mr 73 kDa seen with Molt-4 and Jurkat cells occur as faint band in activated lymphocytes. These results clearly demonstrate the differential expression of SRL specific glycoproteins on normal resting, activated and leukemic cell lines.
3.3.4. Profiling and characterization of SRL receptors by enzymatic digestion

Membrane proteins isolated from human normal resting/activated and leukemic cell lines were resolved by SDS-PAGE and transferred on to nitrocellulose membrane. These proteins after glycosidic cleavage by either specific individual glycosidase or in combination were allowed to interact with biotinylated SRL and the profiles of receptors were compared with the enzyme untreated blot. Membrane proteins of different cells were subjected
to following glycosidic cleavages and the profiles of the SRL-receptors were characterized in terms of the specificities of glycosidases.

1) O-glycosidase cleavage (Figure 5B)

2) Neuraminidase cleavage followed by O-glycosidase (Figure 5C)

3) Fucosidase cleavage followed by O-glycosidase (Figure 5D)

4) N-glycosidase cleavage (Figure 5E)

O-glycosidase used in the present study is a highly specific enzyme, which hydrolyzes the N-acetylgalactosamine glycosidic linkage, liberating the core disaccharide Galβ1-3GalNAc from the serine or threonine residue present in glycoproteins or glycopeptides. But it does not cleave substituted Galβ1-3GalNAc like sialylated, fucosylated and GlcNAc substituted glycans. All the SRL reactive bands of normal lymphocytes observed in control blot (figure 5A; lane 1) lost their ability to interact with SRL after treatment with O-glycosidase (figure 5B; lane 1), indicating the presence of unsubstituted Galβ1-3GalNAc. On the other hand, no alterations in SRL binding pattern (figure 5B) were observed for the membrane proteins from activated (lane 2) and transformed lymphocytes, Molt-4 (lane 3) and Jurkat cells (lane 4) specifically in the region of Mr 27-85 kDa (27, 29, 40, 50, 54, 58, 62, 66, 73, 82 and 85 kDa). These results suggested that the SRL reactive membrane proteins of activated and leukemic lymphocytes carry substituted Galβ1-3GalNAc moieties in contrast to unsubstituted form on normal lymphocytes.
Figure 5. Characterization of SRL receptors isolated from human normal lymphocytes (1), PHA-activated (2), Molt-4 (3) and Jurkat (4) after treatment with different glycosidases. Membrane proteins (40 μg) were resolved by SDS-PAGE and blotted on to nitrocellulose membrane and specifically cleaved by glycosidase(s) before the interaction with biotinylated lectin and visualized with streptavidin-HRP/DAB reaction.

A) Control, without any enzyme treatment
B) After treatment with O-glycosidase
C) After treatment with neuraminidase followed by O-glycosidase
D) After treatment with fucosidase followed by O-glycosidase
E) After treatment with N-glycosidase
Treatment with neuraminidase followed by \( O \)-glycosidase (figure 5C), lead to failure of several membrane proteins (Mr 82, 85 and >85 kDa including very high Mr protein indicated with *) from normal lymphocytes to interact with SRL. Also bands corresponding to Mr 40, 82 and 85 kDa from activated lymphocytes were not reactive. In addition, several bands of Molt-4 and Jurkat were weakly reacted with SRL signifying some differences between activated and transformed lymphocytes. These results revealed that the disaccharide occurring on these receptors is masked by terminal sialic acid. Treatment with fucosidase followed by \( O \)-glycosidase allowed some of the bands (Mr 50, 54, 62 and 73 kDa) from normal lymphocytes (figure 5D; lane 1) to react with SRL indicating the fucosylated \( O \)-glycan, whereas the same glycans are defucosylated in activated and transformed lymphocytes because these bands were reactive without fucosidase treatment.

Aspartagine linked glycans of the membrane proteins were cleaved using \( N \)-glycosidase (PNGase F) and the profile of SRL interacting proteins after cleavage is presented in figure 5E. As seen from the blot, several bands of activated, Molt-4 and Jurkat cells (figure 5E; lane 2, 3 & 4) which were seen after other enzymatic treatments remained common indicating that all of these bands were recognized by SRL through its exclusive ability to recognize \( O \)-glycans. However, considering the sharpness of bands, it appears that the \( O \)-glycans which were probably buried and not accessible
for O-glycosidase action are exposed after N-glycosidase treatment. But with respect to bands appeared in lane 1 corresponding to normal lymphocytes is intriguing as the bands appear with these cells after N-glycosidase treatment (figure 5E; lane 1) are also seen after fucosidase and O-glycosidase treatment (figure 5D; lane 1). These findings revealed the differential expression of substituted forms of TF glycans on different glycoproteins of malignant cells, which are specifically recognized by SRL.

3.3.5. Mitogenic activity of SRL on human normal PBMCs

The lectin-mediated stimulation of thymidine incorporation which reflects the amount of DNA synthesis in the proliferating cells was used to determine the mitogenic activity of SRL. PBMCs isolated from the blood samples of healthy donors by ficoll-hypaque density gradient were used for the mitogenic assay. The stimulatory effect of SRL on the PBMCs at various concentrations of the lectin is presented in figure 6. An increase in thymidine incorporation by more than 2.8 fold (P<0.05) was found at 10 μg/ml concentration of SRL. The mitogenic activity of SRL is dose dependent and showed 1.13±0.10, 1.42±0.34, 2.06±0.46, 2.24±0.46 and 2.85±0.54 (in all the cases P<0.05) fold increase at 0.625, 1.25, 2.5, 5 and 10 μg/ml of the lectin concentration respectively. However, mitogenic activity remained constant beyond these concentrations even up to 100 μg/ml (data not shown) and it reaches plateau. On the other hand, PHA a known mitogen used as positive control, showed very high (>100 fold)
Figure 6. Mitogenic activity of SRL on human PBMCs. Cultured cells (1x10^6) were incubated with different concentrations of SRL (0.312-10 μg/ml) for 72 h. Mitogenic activity was expressed in terms of fold increase in proliferation determined from the thymidine incorporation measured as counts per minute (CPM) in lectin treated cells in comparison with unstimulated cells (CT). Parallely in a set, SRL treated with mucin (S+M) and only mucin without the lectin (M) were assayed. The data presented as mean ± SD of three independent experiments carried out in triplicates. * Indicates significant difference from control (CT) at P<0.05 and # indicates significant difference from SRL-mucin complex at P<0.05.
thymidine incorporation at 2.5 µg/ml concentration (data not shown). The mitogenic activity was specifically inhibited by pre incubation of SRL (10 µg/ml) with mucin at a concentration of 12.5 µg, which displayed 98% inhibition of mitogenic activity (figure 6). The inhibitory effect of mucin is also dose dependent and showed 60 and 80% inhibition at 3.125 and 6.25 µg respectively, which in turn demonstrated the carbohydrate mediated mitogenic activity of SRL.

3.3.6. Antiproliferative effect of SRL on Molt-4 leukemic cell lines

The effect of SRL on the growth of Molt-4 cells was determined by tritiated thymidine incorporation assay and the results are presented in figure 7. Percent proliferation of Molt-4 cells at different concentrations of SRL and mucin complexed with SRL were calculated by considering the untreated cells as 100%. Cells were treated with SRL concentrations ranging from 100 to 6.25 µg/ml at concentrations beyond 12.5 µg/ml SRL exhibited significant antiproliferative activity towards Molt-4 cells. As seen from the figure 7, SRL induces 68±0.69%, 59±0.91% and 58±2.23% (in all the cases \( P<0.05 \)) of inhibition on growth of Molt-4 cells at 100, 50 and 25 µg/ml concentration respectively. Further, mucin effectively blocked the inhibitory effect of SRL on the proliferation of Molt-4 cells. Mucin alone did not have any influence on the growth of Molt-4 cells, which indeed confirms the observed growth inhibitory effect of SRL. Different concentrations (0.625 - 25 µg) of mucin were checked on the growth inhibitory effect of SRL and an optimal 12.5 µg was found to be effective in blocking the activity. Cells
treated with SRL at 6.25 and 12.5 μg/ml concentrations exhibited a marginal increase in proliferation. SRL conjugated to mucin exhibited higher proliferative activity as compared to unconjugated SRL. These results demonstrated the potential antiproliferative activity of SRL at concentrations >12.5 μg/ml on Molt-4 cells which is mediated by specific surface carbohydrate ligands.

**Figure 7. Antiproliferative activity of SRL on Molt-4 leukemic cell line.** Cells (1x10⁴) were incubated with increasing concentrations (6.25 to 100 μg/ml) of SRL. Parallely in another set, cells were incubated with same concentrations of SRL but complexed with mucin. After 72 h, proliferation of the cells was determined by thymidine incorporation assay and the percent cell proliferation for each concentration of SRL and SRL-mucin complex was calculated by considering untreated cells as 100%. The data were presented as mean ± SD of three independent experiments carried out in triplicates. * Indicates significant difference from control (CT) at P<0.05 and # indicate significant differences from the respective controls at P<0.05 (ANOVA; n=9).
4. DISCUSSION

Carbohydrate binding properties of *S. rolfsii* lectin as revealed by hapten inhibition and enzyme linked lectinosorbent assay (ELLA) indicated its preferential affinity towards the O-linked glycoconjugates, specifically the disaccharide Galβ1-3GalNAc-O-ser/thr. Apart from these studies, glycan array analysis results provided valuable information on its extended carbohydrate binding property to recognize several substituted forms of the disaccharide Galβ1-3GalNAc-O-ser/thr is an important glycoconjugate of animal origin and is well characterized as an oncofetal antigen commonly referred as Thomsen Friedenreich antigen (TF) expressed on cells in a variety of cancers [Yu, 2007]. Also earlier studies in this laboratory have established that SRL interacts with a putative endogenous receptor, a glycosyl ceramide (glycosyl inositol phosphoryl ceramide; GIPC) in response to stress allowing the fungus to form sclerotial bodies [Swamy *et al.*, 2004]. These studies have demonstrated that SRL plays a key role in the development of the fungus. Considering its sugar binding property and its involvement in cellular development, it was interesting to investigate its interaction and physiological significance on different human cancer cells.

In this study, we investigated the interaction of SRL with normal and activated PBMCs and with two leukemic cell lines viz., Molt-4 and Jurkat by flow cytometry and florescence microscopy. After confirming the cell surface binding to these cells, lectin interacting membrane receptors were
identified and characterized with respect to carbohydrate epitopes using different glycosidases. Finally the effect of SRL on the growth of these cells was demonstrated by tritiated thymidine incorporation assay.

We have demonstrated the binding of SRL to human normal and activated PBMCs apart from its binding to leukemic cells, Molt-4 and Jurkat by flow cytometry, where in it shows equally well binding to all the four cell types which indicate all the four express SRL binding receptors. However, considering the differences in mean fluorescent intensity values (MFI) for each of the cell type, which varied remarkably suggested that normal lymphocytes express highest binding sites compared to activated and leukemic cells (Molt-4 and Jurkat). Flow cytometry results were further strengthened by florescence microscopic observations where uniform and intense FITC-SRL label was found on most of the surface for normal (resting) and Molt-4 cells; where as for activated lymphocytes and Jurkat cells partial but intense surface binding was observed. Surface binding of the lectin is mediated by lectin-carbohydrate interaction as demonstrated by the abolition of binding by mucin.

Interestingly, *Amaranthus caudatus* lectin (ACA) which has close similarities with SRL in sugar binding properties as compared to other TF recognizing lectins viz., PNA, ABL, Jacalin etc., showed remarkable deviations from SRL in binding to normal and leukemic cells. Both SRL and ACA have affinity towards α anomer of TF antigen, recognizing mucin core
2 preferentially over core 1 structure. However, ACA differ from SRL by recognizing monosaccharides, galactose and N-acetylgalactosamine [Rinderle et al., 1989]. With these subtle differences in carbohydrate recognizing properties with SRL, ACA showed minimal binding to any of the cell types in spite of its TF antigen binding property. From the observed differential binding of SRL and ACA, it may be speculated that these cells expresses variant forms of TF antigen in addition to TF antigen itself. These findings supported the earlier observation that the activated and transformed lymphocytes express different sets of unique glycoproteins or glycans as compared to resting lymphocytes [Nicolson, 1976; Speckart SF, 1978]. Lectin blot assays using biotinylated SRL for determining the expression of cell surface glycoproteins of human normal resting/activated lymphocytes and leukemic cells have demonstrated impressive differences. SRL binding receptors of high molecular weight proteins (Mr >85 kDa) were uniquely found on normal resting lymphocytes, which were totally missing from activated as well as leukemic cells suggesting glycan moieties of these glycoproteins undergo changes upon activation or transformation, probably trimmed or not completely synthesized, leading to modifications which are not recognized by SRL. The majority of SRL-receptors are common on activated lymphocytes, Molt-4 and Jurkat cells, except two protein bands (M, 82 & 85 kDa) which are absent in Jurkat cells. Another interesting observation is that a band with M, 97 kDa is uniquely observed in activated lymphocytes which could be a key surface protein marker of greater
significance for differentiating these cell types. Characterization of the glycan moieties of the SRL-interacting receptors from different cells using different glycosidases provided substantial information about the glycan nature. However, it was possible to describe the nature of glycoconjugates expressed on different cells on the basis of glycan array analysis of SRL. In normal lymphocytes, all the SRL receptor bands uniquely observed have lost the ability to interact with lectin after O-glycosidase treatment. Considering the specificity of the enzyme which essentially cleaves unsubstituted Galβ1-3GalNAc-O-ser/thr, these receptors essentially have unsubstituted TF antigen. Although TF antigen is a well documented pancarcinoma marker and is known to be over expressed on cells during various cancers, there are no reports on its occurrence on normal lymphocytes. Earliest report on the expression of this antigen in cryptic form on normal lymphocytes was by Wolf et al., [1989]. It was shown that the human peripheral blood lymphocytes reacted with PNA and anti TF monoclonal antibodies only after neuraminidase treatment. However, there are also reports available on expression of TF antigen in several normal tissues [Philipsen et al., 1991; Therkildsen et al., 1994; Carneiro et al., 1994], but it is not very clear whether the TF is present inherently on normal cells to smaller extent and is simply over expressed during transformation. Fucosidase cleavage followed by O-glycosidase impressively demonstrated the occurrence of several cryptic TF antigen masked by fucose and neuraminic acid in normal lymphocytes. Also the
same set of SRL interacting bands were seen in activated and leukemic cells, suggesting that defucosylation of the glycotopes occurs upon activation and transformation.

Another interesting observation is that a band corresponding to Mr 58 kDa in Molt-4 cells is intensely stained after fucosidase cleavage followed by O-glycosidase, which is not seen with other cells. It appears that this particular SRL receptor contains significant levels of fucosylated and sialylated TF disaccharide.

Upon N-glycanase treatment most of the bands observed for all the cells are common with those seen upon fucosidase and O-glycanase treatment, except a major band of Mr 29 kDa substantiating the specificity of SRL, which is known to bind only O-glycans but not N-glycans. In addition, all the bands appeared as sharp bands compared to other blots. Probable explanation for this is the O-glycans are not completely accessible for SRL binding, as the N-glycans could pose steric hindrance due to their large size.

Several monoclonal antibodies including mAb RS1-114 [Stein et al., 1989], mAb AH9-16 [Itzkowitz et al., 1989], mAB A78-G/A7 [Cao et al., 2000; Baldus et al., 2000, 2001], mAB (DAKO) and lectins such as PNA [Cooper and Reuter, 1983; Orntoft et al., 1985], Jacalin [Sumar N, 1990], ACA [Boland, 1991; Sata et al., 1992] and ABL [Yu, 1999] have been widely employed for detection and localization of TF antigen on variety of cancers. Due to microheterogeneity, several variant forms of TF antigens are
expressed in cancer cells [Dube and Bertozzi, 2005], which cannot be effectively detected by these molecules. Thus, SRL with its novel sugar binding properties provide critical insight into the molecular aspects of cancer especially as a glycan reading tool for monitoring aberrant glycosylation. These studies apart from demonstrating the nature of glycosylation changes on lymphocytes during mitogenic activation and transformation also indicate the potential use of SRL for exploring the cell surfaces.

The results of SRL receptor binding studies and the effect of SRL on the growth of cells clearly establish that SRL interacts with functionally associated membrane-glycoproteins of human leukemic cell lines leading to the inhibition of proliferation and an opposite effect in normal cells. This finding is in line with previous reports on fungal lectins from *Pleurotus ostreatus* [Wang et al., 2000a], *Tricholoma mongolicum* [Wang et al., 1995], *Agaricus bisporus* [Yu et al., 1993], *Grifola fondosa* [Kawagishi et al., 1990], other mushroom lectins [Parslew et al., 1999; Battery et al., 2002; Mahajan et al., 2002; Kent et al., 2003; Ngai and Ng, 2004] and also animal and plant lectins [Wang et al., 2000b] inhibiting in microgram concentrations, where as *Arisaema jacquemontii* lectin, inhibits the growth of various cancer cell lines at a concentration of 100 mg/ml [Kaur et al., 2006]. In addition to antiproliferative effect on human Molt-4 cell lines, SRL also exerts proliferative activity towards normal human PBMCs. There are several reports on mitogenic lectins from various sources [Miller, 1983;
To the best of our knowledge, this is the first example of fungal lectin exhibiting proliferative effect on normal and parallelly antiproliferative effect on leukemic lymphocytes. However, there are reports on lectins, which exhibit mitogenicity towards human/animal lymphocytes and antiproliferative activity towards other cancer cell lines [Wang et al., 2003; Singh et al., 2004; Ngai and Ng, 2004; Bains et al., 2005 a b] but not to leukemic lymphocytes. Thus, the property of antiproliferative activity of SRL which is similar to ABL [Yu, 1993] probably attributable to its amino acid sequence similarities [Demetres et al., 2007]. However, higher potency and mitogenic property may be related to the fact that it recognizes variant forms of TF disaccharide. The observed opposite effects of SRL on PBMCs and leukemic cells were not due to number of receptors, since flow cytometric analysis revealed that SRL strongly binds to normal lymphocytes followed by activated lymphocytes and leukemic cell lines, in contrast it detected maximum number of glycoproteins (receptors) from leukemic cells in blotting experiments, suggesting no obvious correlation between these two parameters (number of receptors and cellular effects). The ability of SRL to induce a weak (at 25 μg/ml) rather than strong antiproliferative response might be due to either recognition of TF antigen or its variant forms, which resulted in a higher number of available membrane receptors including specific receptor responsible for antiproliferative activity. Such hypothesis could be further supported by the fact that, these variant forms of TF antigen containing glycoproteins are abundantly expressed on cancer cells [Dube and Bertozzi,
Similarly, it is not known whether a different population of normal lymphocytes present in the experimental populations were stimulated by the SRL or will it recognize entirely different receptors which will eventually lead to weak mitogenic stimulations or it is a consequence of the lectin-induced proinflammatory cytokines. Though the initial step in mitogenic stimulation is binding of the lectin to the cell surface carbohydrate moieties, this is not sufficient, since certain lectins are non-mitogenic, even though they bind well to untreated or sialidase treated human lymphocytes [Ho et al., 2004].

Our present study demonstrated the potential inhibitory activity of SRL at microgram concentration on the in vitro proliferation of Molt-4 and warrant for an investigation on its potential use in cancer therapy. In addition, considering the immunomodulatory and sugar binding properties of SRL it may prove to be useful tool in cancer research, immunomodulation, glycobiology and as a chemotaxonomic marker for comparative evolutionary studies of fungal lectins.
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