Summary and scope of the present work

Lectins are carbohydrate binding proteins of non-immune origin that agglutinate cells and have at least one non-catalytic domain able to recognize and bind reversibly to specific mono and oligosaccharides. Two lectins from plant pathogenic fungi *Sclerotium rolfsii* (SRL) and *Rhizoctonia bataticola* (RBL) have been purified to homogeneity and have been extensively studied in our laboratory. The detailed glycan array analysis of lectins carried out at the consortium for functional glycomics, USA, has revealed their unique glycan binding specificity directed towards cancer associated glycans. SRL has exquisite specificity towards O-glycans and it recognizes Thomsen-Friedenreich antigen (TF) and its derivatives, which are known to be expressed in more than 90% of human malignancies. Hence the interaction of SRL is studied with human colon and breast cancer cells in comparison with normal mammary epithelial cells.

RBL, a potent mitogenic and cytotoxic lectin has shown unique specificity towards complex N-glycans and tandem repeats of sialyl Lewis antigens. These high mannose N-glycans are known to be part of CA-125, a well known ovarian cancer marker. Hence interaction of RBL with human ovarian cancer PA-1 cells was studied which revealed that, RBL induces strong cytotoxic effect on PA-1 cells by induction of apoptosis through the activation of intrinsic pathway.

Changes in the N-linked glycosylation on the cell surface are correlated with drug resistance and considering the unique sugar specificity of
RBL towards these cancer specific N-glycans, that potentially underlie anticancer drug resistance prompted us to study its interaction with drug resistant ovarian cancer OVICAR-3 cells. As RBL showed potent cytotoxicity towards ovarian cancer cells in vitro, it was necessary to check whether it exerts similar anti-tumour effect in vivo. Hence the in vivo studies in mice model were carried out to further exploit its clinical potential in combating ovarian cancer.

Considering the need, to search for a novel biomarker and therapeutic agent and to exploit the TF antigen specificity of SRL, the effect of Sclerotium rolfsii lectin (SRL) on the proliferation of human colon cancer HT-29 and DLD-1 cells was investigated. As SRL recognizes cancer specific carbohydrate antigen (TF and its derivatives), which are known to be over-expressed in cancerous especially in metastatic tissues, binding studies of biotin labelled SRL was performed by histochemistry which showed strong binding of SRL to cancerous and metastatic tissues and no or very weak binding to the normal tissues of human colon. Binding of FITC-SRL in presence or absence of TF expressing glycoprotein’s by flow cytometry suggested that, a total of 90% cells were positive for SRL when compared to the unstained cells. The presence of TF expressing glycoconjugates resulted in significant decrease in the binding of lectin to these cells and aBSM Bovine asialo mucin (aBSM) is found to be most effective inhibitor. Surface binding was also visualized by confocal microscopy after staining with FITC-
SRL. Uniform and intense fluorescence on the cell surface suggested the high expression of SRL recognizing receptors on the cell surface.

SRL inhibits the growth of human colon cancer cells in vitro. The presence of SRL in the culture of human colon cancer HT-29 and DLD-1 cells resulted in dose- and time-dependent inhibition of the cell growth. At 10 μg/mL SRL caused significant inhibition of HT-29 and DLD-1 cell growth. The growth inhibitory effects of SRL in HT-29 cells were blocked by the presence of TF-expressing, aBSM and to a lesser extent by bovine submaxillary mucin (BSM), suggesting that the growth inhibitory effect of SRL is mediated by its carbohydrate binding sites. SRL-mediated growth inhibition is a direct consequence of its cell surface binding and is sufficient to trigger the growth inhibitory effect as revealed by similar growth inhibitory effect of Sepharose-conjugated SRL on HT-29 cells as that of native SRL.

SRL induced apoptosis in HT-29 cells was demonstrated by the flow cytometry, caspase Glo-3/7 assay and western blot analysis. HT-29 cells treated with SRL showed a significant increase of the Annexin-V positive cells at 24 and 48h indicating the induction of early apoptosis. The dot plot analysis revealed that 56% cells were positive for early/late apoptosis after 48h treatment. Assessment of cellular caspase-3 activity in response to SRL treatment revealed that both HT29 and DLD-1 cells showed increase in the expression of the active caspase-3. Further an increase in caspase-3/7 activity by 2.79-fold was found in HT29 cells after treatment with SRL for 48 h when
compared with the untreated cells. The presence of inhibitors to caspase-8, caspase-9 or pan-caspase could all largely prevent SRL-induced caspase 3/7 activation, indicating the possible involvements of both the extrinsic and intrinsic apoptosis signaling pathways. The presence of pan-caspase inhibitor largely diminished the growth inhibitory effect of SRL on HT29 cells, indicating that induction of cell apoptosis is directly linked with SRL-mediated cell growth inhibition. The presence of SRL was found to cause a time-dependent increase of active caspase-8 and -9 and time-dependent decrease of their inactive forms and PARP cleavage. SRL also showed to induce a time-dependent increase of the expressions of FasL, FADD, and truncation of BID as evidenced by western blotting. These results suggest that, the mechanism of SRL induced apoptosis in HT-29 cells is mediated involving both extrinsic and intrinsic apoptotic pathway.

*In vivo* studies of SRL were performed by injection of SRL intra-tumorally in NOD-SCID mice bearing HT-29 xenografts, on every alternate day that resulted in total regression of the tumors. SRL caused significant reduction of the tumor size when compared with the control groups.. Toxicity studies of SRL at different concentrations revealed that, SRL showed no toxicity to the animals and is tolerable at lower doses, the concentration at which it showed effective anti tumor activity. However, at a higher dose (1000µg/ml) but it is toxic and non tolerable as revealed by *in vivo* studies

There is a need for the identification of novel agents that are relatively safe but can suppress growth of human breast cancer by targeting the cancer
associated antigens. Interaction of SRL was studied with human breast cancer cells in comparison with normal mammary epithelial cells. SRL revealed differential sensitivity towards human normal (HMECs and MCF-10A) and malignant (MCF-7 and ZR-75) breast epithelial cells. The histochemical studies of SRL showed strong binding to cancerous and metastatic human breast tissues and relatively weak binding to the normal tissues. This further supports the binding specificity of SRL to the TF-related glycans which are known to be over-expressed in cancerous and in particular metastatic tissues. The cell surface binding of SRL was evaluated by staining cells with FITC labeled SRL and analyzed by flow cytometry. Nearly all (99%) MCF-7 cells showed strong and uniform binding by SRL when compared to the unstained cells. The surface binding of SRL was blocked by pre-incubation of the lectin with TF-expressing glycoproteins. Interestingly, almost all HMECs cells also showed positive binding by SRL but with lower intensity. Surface binding to MCF-7 cells was also visualized by confocal microscopy after staining cells with FITC-SRL. Uniform and intense fluorescence on the cell surface suggested the high expression of SRL recognizing receptors on the cell surface.

SRL showed dose and time dependent inhibition of proliferation of breast cancer MCF-7 and ZR-75 cells. At 20 and 40μg/ml SRL caused significant growth inhibition in MCF-7 cells after 48h and 72h. The growth inhibitory effect of SRL in MCF-7 and ZR75 cells was blocked by the
presence of TF-expressing aBSM (100µg/ml), suggesting that the growth inhibitory effect of SRL is mediated by its carbohydrate binding sites.

In contrast to breast cancer cells, SRL showed a weak inhibition of normal breast epithelial MCF-10A and HMEC cell proliferation. The comparative cell proliferation studies with normal and cancer cells indicate that, SRL strongly inhibit proliferation of breast cancer cells and has relatively marginal effect on normal epithelial cells. It is interesting that Sepharose-conjugated SRL showed similar growth inhibitory effect on MCF-7 cells as that of native SRL indicating that SRL-mediated growth inhibition is a direct consequence of its cell surface binding that is sufficient to trigger the growth inhibitory effect.

SRL induced apoptosis in MCF-7 cells was assessed and demonstrated by Flow cytometry, confocal microscopy, Caspase Glo 3/7 assay and western blotting. SRL treated MCF-7 cells showed an increase of hypodiploid (sub G1) and G0/G1 population and decrease in the S and G2/M phase cell population after 24h. MCF-7 cells treated with SRL showed a gradual increase in early apoptotic cell population (Annexin-V positive and PI negative) by 21-fold, as compared to untreated cells suggesting that, SRL induces cell cycle arrest and apoptosis.

Assessment of cellular caspase-3/7 activity in response to SRL treatment revealed an increase in caspase-3/7 activity by 2.02 fold after 48h treatment when compared with the untreated cells. The presence of inhibitors
to each of caspase-8, caspase-9 or pan-caspase could all largely prevent SRL induced caspases-3/7 activation, indicating the possible involvements of both the extrinsic and intrinsic apoptosis signalling pathways. The presence of pan-caspase inhibitor largely diminished the growth inhibitory effect of SRL on MCF-7 cells, indicating that induction of cell apoptosis is directly linked with SRL-mediated cell growth inhibition. The confocal images of mitochondria in MCF-7 cells stained with mitochondrial probe, (MitoTracker Red CMXRos, showed time dependent decrease in fluorescent intensity representing mitochondrial membrane potential (ΔΨm) collapse suggesting that SRL causes depolarization and loss of mitochondrial membrane potential. SRL was found to cause a time-dependent increase of active caspase-8 and -9 and time-dependent decrease of their inactive forms and PARP cleavage. SRL also showed to induce a time-dependent increase in the expressions of FasL, FADD, and truncation of BID as evidenced by western blotting. These results suggest that, SRL induced apoptosis in MCF-7 cells is mediated through both extrinsic and intrinsic apoptotic pathways.

The effect of RBL induced cytotoxicity is studied using drug resistant OVCAR-3 cells, which shows that, RBL induces high toxicity in a dose and time dependent manner. The IC₅₀ value for RBL is 10µg/ml. The cytotoxic effect executed by RBL was effectively blocked by competing glycoconjugate such as asialofetuin. These findings demonstrated that the cytotoxic effect of RBL on human ovarian cancer cell line OVCAR-3 is
mediated by its carbohydrate binding site. RBL is known to induce cell death in human ovarian cancer PA-1 cells, hence the growth inhibitory effect of RBL was analysed by in vivo studies using human ovarian cancer cells PA-1 xenografts in NOD-SCID mice. Significant reduction of the tumor size was observed in RBL treated group when compared to control group. Reduction in the average tumor volume and weight was observed in RBL treated mice group. Analysis of different parameters like toxicity, total blood count and serum enzyme levels of the mice showed no considerable difference in RBL treated and untreated mice. The in vivo studies support the possible therapeutic application of RBL for ovarian cancer.

Specificity of SRL towards cancer associated carbohydrate antigen and its strong growth inhibitory effect on cancer cells by inducing programmed cell death or apoptosis and causing minimal damage to normal cells have provided further evidence and encourage to pursue pre clinical trials to explore its possible potential in cancer research and therapeutics. In a similar way the study also confirms potential of RBL in ovarian cancer therapeutics.