4. INTRODUCTION

The epithelial ovarian cancer constitutes 80-90 % of ovarian malignancies amongst the other tissue origins [Riman et al. 1998]. Ovarian cancer is one of the leading causes of cancer death among the gynecologic malignancies worldwide, as its incidence is increasing every year [Ferlay et al. 2010]. The reason for this is that most of the ovarian cancers are diagnosed at advanced stage as the disease is asymptotic at early stages and at the time of diagnosis most of the patients have a wide spread disease [Van Dalen A et al. 2000]. Although there are many chemotherapeutic drugs which have shown high response rate after surgery, patients have poor survivalnce (25 %), which further decrease to 5 % when patients are detected with stage III and IV [Gupta and Lis 2009]. Majority of patients die from the disease; when relapse occurs and patients develop clinical drug resistance, which is eventually fatal despite further treatment [Kaye SB, 1995].

Important drugs used in ovarian cancer therapy include the taxoids as well as anthracyclines and there are reports on resistance developed for these drugs [Kaye SB, 1993]. The resistance mechanism includes so-called Multidrug resistance which involves increased expression of P-glycoprotein (permeability glycoprotein, abbreviated as P-gp or Pgp) also known as multidrug resistance protein 1 (MDR1) or ATP-binding cassette subfamily B member 1 (ABCB1) or cluster of differentiation 243 (CD243) or ATP-dependent efflux pump is a 170 kDa glycoprotein that in humans is encoded by the ABCB1 gene. P-gp is a well-characterized ABC-
transporter (which transports a wide variety of substrates across extra and intracellular membranes) of the MDR/TAP subfamily. Thus increased expression of this protein would result in a lowered net accumulation of the drugs involved. The fundamental mechanisms by which this resistance develops could be described by pharmacological or cellular factors. Pharmacological factors include: decreased drug accumulation, enhanced drug inactivation and increased rates of repair of drug-DNA damage. Cellular factors contribute for developing resistance involving multiple mechanisms and one such example is DNA damage induced by a range of cytotoxic agents in ovarian cancer cells, the key to sensitivity/resistance may be the ability of these cells to engage the process of apoptosis. Several genes are involved in control of apoptosis; which include the mutations in tumour suppressor, p53 "the guardian of the genome" is also known as cellular tumor antigen p53 or phosphoprotein p53 or tumor suppressor p53 that in humans is encoded by the TP53 gene. The p53 protein is crucial in multicellular organisms, where it regulates the cell cycle and, thus, functions as a tumor suppressor, preventing cancers, which has been linked to cisplatin resistance, as well as for carboplatin. It is also demonstrated that there is direct association between cisplatin resistance and microsatellite instability (indicative of defective mismatch repair) [Anthoney A, 1995].

Changes in the glycosylation on the cell surface are correlated with drug resistance such as elevated expression or activity of ATP-binding cassette (ABC) transporters such as multidrug resistant protein 1 (MDR1/ABCB1) and breast cancer resistance protein BCRP/ABCG2. These
transporters are membrane glycoproteins containing N-glycans which get altered during cancer. Such glycosylation changes in N-linked glycans are also known to be involved in important events like transformation, invasion and metastasis [Rudd et al. 2001; Brooks, 2008]. The increased branching in the N-glycans is one of the common glycosylation changes associated with cancer, mainly the increase in β-1-6 branching results in the expression of tri- and tetra-antennary oligosaccharides with terminal sialylation [Dennis 1992; Dennis et al. 1987]. Expression of sialyl Lewisα, sialyl Lewisβ, and their isomers on the N- and O-linked oligosaccharides are also observed in various human malignancies that are involved in cancer progression. Expression of these altered glycans in breast, bladder, lung, ovary and colon cancer is correlated with poor prognosis and these changes are associated with advanced forms of malignancies [Gorelik et al. 2001; Powlesland et al. 2009; Nakamori et al. 1993; Davidson et al. 2000].

The detailed studies have demonstrated the involvement of these altered glycans in many pathophysiological conditions and hence they can be targeted by developing the pharmaceutical agents for the improved treatment of many diseases including cancer. Discovering new cancer specific glycans/alterations and understanding them in detail for their use in cancer therapy is now becoming one of the important aspects of cancer glycobiology. Molecules such as antibodies or lectins which can decipher the information encoded in these glycans are useful tools in cancer research.
Lectins from plants and mushrooms, which have specific affinity towards cancer associated antigens and with antitumor activity are finding application in cancer research and some of them are under clinical studies [Liu et al. 2010; Fu et al. 2011; Khan and Khan 2011; Singh et al. 2010].

Isolation of a mitogenic and immune stimulatory lectin from a phytopathogenic fungus *Rizhoctonia bataticola* (RBL) has been reported recently from our laboratory. RBL has complex sugar specificity and Glycan array analysis has revealed the exquisite specificity towards N-glycans, primarily recognizing high mannose, tri- and tetra- antennary complex N-glycans. Further RBL also recognizes tandem repeats of sialyl Lewis antigen. Since human ovarian cancer cells are known to express altered complex N-linked glycans, the interaction of RBL was studied with human ovarian cancer PA-1 cells. The results show that, RBL has a cytotoxic effect on PA-1 cells which can be effectively blocked by competing glycoproteins [Nagre et al. 2010; Pujari et al 2010]. Recently the detailed signaling mechanism involved in RBL-induced cell death of PA-1 cells *in vitro* has been elucidated and showed that the cytotoxic effect of RBL is due to the induction of apoptosis by the activation of intrinsic pathway [Eligar et al, 2012].

In the present study we report the effect of RBL on the proliferation of other human ovarian cancer OVCAR-3 cells, which are resistant to drugs like
cisplatin and adriamycin. The antitumor effect of RBL was studied in vivo by using NOD-SCID mice bearing PA-1 xenografts.

4.1 Materials

Bovine serum albumin (BSA), Asialofetuin, 2-mercaptoethanol, trypan blue, sodium azide, bromophenol blue, CM-cellulose, Acrylamide, bis-acrylamide, Tris, sodium dodecyl sulphate (SDS), and glycine were obtained from Sigma chemicals Co., St. Louis, USA. Sepharose-4B was from Pharmacia and Tissue culture grade plastic-ware were procured from BD Biosciences (USA). All other chemical used were of analytical grade.

4.2 Methods

4.2.1 Purification of RBL

RBL was purified from the fungal mycelia as described by Nagre et al; 2010. The following steps were used to purify RBL to homogeneity.

a. Extraction

*R. bataticola* cultures were grown in Byrde’s liquid synthetic media [Byrde et al. 1956] for 11 days, the mycelia mat was harvested, washed with distilled water and freeze-dried and powdered in a glass mortar. Mycelial powder (10 g) was suspended in extraction buffer (50 mM sodium acetate buffer, pH 4.3) and sonicated briefly at 4°C. The sample was kept for extraction at 4°C for overnight on a magnetic stirrer. The extract was
centrifuged (9,500×g, 30 min, at 4 °C), and supernatant was filtered using membrane filter (0.45 μm) and used as crude extract.

b. Ion-exchange chromatography on CM cellulose

The clear filtrate was passed through a CM-cellulose column (20×1.5 cm) equilibrated with extraction buffer and unbound fraction were washed using same buffer. Adsorbed proteins were eluted using 50 mM sodium acetate buffer pH 4.3 containing 500 mM NaCl. The fractions with highest OD (at 280 nm) and hemagglutinating activity were pooled and kept for dialysis against PBS.

c. Affinity chromatography on asialofetuin-Sepharose 4B

The lectin was further purified by affinity chromatography on an asialofetuin-Sepharose 4B column (10×1.3 cm) at 4°C. The lectin was applied to the affinity column equilibrated with PBS, the column was washed with PBS, and the affinity bound lectin was eluted using glycine-HCl buffer (100 mM, pH 2.0) containing 500 mM NaCl. Fractions containing lectin activity were pooled, dialyzed against PBS and stored at −20 °C for further studies.

4.2.2 Cell culture

Human ovarian cancer cell line OVCAR-3 was procured from ACTREC Advanced Center for Treatment Research and Education in Cancer and maintained in DMEM (Gibco) supplemented with 10% heat inactivated
Fetal calf serum (FCS), 1mM glutamine, 1 mM sodium pyruvate, 100mg/ml streptomycin and 100 units/ml penicillin at 37°C in 5% CO₂ and 95% humidified air.

4.2.3 Growth inhibitory studies

RBL induced cytotoxic effect was monitored by Sulforhodamine B (SRB) assay. OVCAR-3 cells were seeded in a 96 well plate at 5x10⁴ cells/ml and grown for 24h before the lectin treatment to study the dose and time dependent effect. Cells were treated with RBL at different concentrations (10 to 80µg/ml) in MEM (Minimum Essential media) with 0.5 % FCS for 24 and 48 h in a humidified atmosphere (37 °C, 5 % CO₂). After each time point 50µl of 50% TCA cold solution (4°C) was added to each well containing 200µl of medium containing cells so that a final concentration of 10% TCA is reached in each well. The 96-well plate was placed for 1h at 4°C to allow cell fixation. 70µl of 0.4% SRB (w/v) solution in 1% acetic acid was added to each well and left for 30min at room temperature. Plate was washed the with 1% acetic acid five times in order to remove unbound SRB. To solubilise bound SRB, 10 mM Trizma base( 200µl) was added and plates were placed on a plate shaker for at least 10min. Absorbance was read with a microplate reader at 492nm, subtracting the background measurement at 620 nm. The percent viable cell number was calculated with respect to controls considered 100 % viable.
4.2.4 Assessment of RBL toxicity

Since RBL has antiproliferative effect in vitro it was essential to know whether it is toxic, if used as a drug. Hence the effect of RBL was tested on clinically relevant parameters of toxicity, such as body weight, peripheral blood cell count and biochemical parameters in sera were analysed. Eighteen NOD-SCID mice were divided into three groups with six animals in each group. Groups I and II were injected intraperitoneally with a different concentration of RBL (25 and 50 μg/mouse), respectively and group III with PBS (control) on every alternate day for 9 days. The body weights of mice were measured on every alternate day. On the 11th day, blood was collected and different parameters of toxicity including total blood cell count, hemoglobin content, serum liver enzymes (aspartate transaminase and alanine transaminase) were measured.

4.2.5 Assessment of the effect of RBL on ovarian tumor growth in NOD-SCID mice

In-bred NOD-SCID mice were procured from Advanced Centre for Treatment, Research and Education in Cancer, India, and maintained on synthetic diet and used for the toxicity and anticancer studies with clearance from the ethical committee (IRB approval No.11/2009). PA-1 cells were suspended at $1 \times 10^7$ cells/ml in DMEM media and cell suspension (100 μl) was injected subcutaneously into two mice. After 4 weeks, the tumors from these donor mice were excised, chopped into 2–3 mm fragments and a single piece of tumor was transplanted subcutaneously into each of 18 NOD-SCID
mice. First, tumors were allowed to grow 5–6 mm, and then the mice were divided randomly into three groups with six animals in each group. Animals in groups I and II were injected with RBL (25 or 50 μg/mouse) on every alternate days for 9 days (a total of five injections). The animals in group III were injected with PBS [25 mM Phosphate-buffered saline, pH 7.5] and used as negative control. On the 14th day following the first dose, all the animals in groups I, II and III were photographed and sacrificed to measure the tumor weight and size.

4.2.6 Statistical analysis

Each experiment was performed at least three times, each time in triplicate. Results were analyzed by one-way ANOVA followed by ‘Newman-Keuls’ test for multiple comparisons using ‘StatsDirect’ software and data considered significant when $P < 0.05$.

4.3 RESULTS

4.3.1 Effect of RBL on the proliferation of human ovarian cancer cells

RBL induced antiproliferative effect upon binding in a dose and time dependent manner. The presence of RBL at different concentrations in the culture of human ovarian cancer cells resulted in decrease in the cell viability as time increases. Dose response profile showed 59.3 ±1.9 % (n = 3) and 72.4 ±10.7 % (n = 3) inhibition of OVICAR-3 cells at 10 and 20 μg/ml respectively after 24h, whereas 79.8±12.19% (n=3) and 82.8 ± 4% (n=3) inhibition of OVCAR-3 cells at 40 and 80 μg/ml respectively after 24h (Fig. 1). Time kinetics of RBL induced cytotoxic effect was analyzed for two different time intervals. RBL showed a time dependent decrease in the cell
viability with maximum effect at 48h. At 48h almost all cells were dead at 10 and 20µg/ml. This preliminary data suggest that RBL exhibits strong cytotoxic effect towards OVCAR-3 cells in a time and dose dependent manner which needs to be further analysed for the detailed mechanism for the RBL induced cell death.

Carbohydrate binding site mediated growth inhibition was confirmed by using glycoprotein such as asialo fetuin. RBL (10 µg/ml) was pre incubated with the asialo fetuin 100 µg/ml, before treating with the OVCAR-3 cells. Pre incubation of RBL with asialo fetuin resulted in significant inhibition of its cytotoxic effect induced by RBL and the cell viability increased to 55.4± 2.5% when compared to RBL treated cells, suggesting that the cytotoxic effect of RBL is mediated by its carbohydrate binding sites.

**Fig. 1: RBL causes dose-dependent cell death in ovarian cancer OVCAR-3 cells.** The cells were incubated without or with different concentrations of RBL for 24h. RBL-mediated cell growth inhibition is prevented by the presence of asialo fetuin. OVICAR-3 cells were incubated without or with10 µg/ml RBL in the presence of 100 µg/ml of asialo fetuin (AF) for 24h. The cell viability was assessed by Sulphorodamine B method. Data represent Mean±SD of triplicate determinations from three different assessments.
4.3.2 RBL suppresses growth of PA-1 tumor xenografts in mice.

Since RBL showed promising cytotoxic effect on ovarian cancer cells in *in vitro* studies, it was necessary to explore its clinical potential and hence pre-clinical studies were carried out in mice models using PA-1 xenografts. Tumor models were produced by injecting PA-1 cells in NOD-SCID mice and xenografts produced were treated with different concentrations of RBL for defined periods.

Intra-peritoneal injection of RBL in mice bearing PA-1 xenografts at 50μg/mouse (group II) on every alternate day resulted in significant reduction in tumor size (Fig. 2). A reduction in average tumor volume and weight was observed in RBL-treated mice (50 μg/mice) after 9 days (Fig. B). Tumor weight and volume were reduced to 0.100 ± 0.012 g (P=0.029) and 193.5 ± 52 mm³ (P=0.0036) in RBL-treated mice in Group-II in contrast to control mice that had a tumor weight and volume of 0.319 ± 0.043 g and 618.0 ± 190 mm³ respectively. RBL-treated mice in Group-I (25 μg/mouse) also showed a partial reduction in tumor weight and volume (Figure 5C and D) however the effect is significant when compared with the control groups after 14 days following first dose (Fig.11.B), suggesting RBL at afore mentioned concentrations has potential antitumor activity.
Fig. 2: RBL inhibits tumor growth in vivo. (A) NOD-SCID mice with PA-1 tumors were injected intraperitoneally with RBL (25, 50 μg/mice) or control PBS on alternate days for 9 days. Representative images of the animals after 14 days are shown. Tumors were excised (B), and tumor weight and volumes were compared between the groups (C and D).

4.3.3 RBL toxicity in mice

To assess whether RBL is toxic at afore mentioned concentrations at which it induced anti tumor activity, it was tested for its toxicity. The groups of mice were injected intra-peritoneally with increasing concentration of RBL
and different clinical parameters were assessed. Toxicity studies of RBL at concentrations of 25 and 50 μg/animal showed no difference in body weights, total blood cell counts and serum biochemical parameters when compared with group III (control). Analysis of toxicity parameters in these mice showed no considerable difference in RBL-treated and untreated groups. The results suggest that RBL showed no sign of toxicity to the animals at above mentioned concentration and hence is safe for its clinical use. The details of physical and biochemical parameters analyzed are represented in table.1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RBL 25μg/ml</th>
<th>RBL 50μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial body weight (g)</strong></td>
<td>23.46</td>
<td>23.26</td>
<td>23.36</td>
</tr>
<tr>
<td><strong>Final body weight (g)</strong></td>
<td>23.76</td>
<td>23.66</td>
<td>23.35</td>
</tr>
<tr>
<td><strong>Mortality</strong></td>
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<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>RBC (×10^6 /ccm)</strong></td>
<td>6.77</td>
<td>6.99</td>
<td>6.81</td>
</tr>
<tr>
<td><strong>WBC (×10^3 /ccm)</strong></td>
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<td>2.71</td>
<td>2.86</td>
</tr>
<tr>
<td><strong>Platelet count Lakhs/ccm</strong></td>
<td>7.12</td>
<td>8.04</td>
<td>7.41</td>
</tr>
<tr>
<td><strong>Hemoglobin(g/dl)</strong></td>
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<td>9.3</td>
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<tr>
<td><strong>AST(IU/L)</strong></td>
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<td>60.16</td>
<td>65.5</td>
</tr>
<tr>
<td><strong>ALT (IU/L)</strong></td>
<td>20.16</td>
<td>18</td>
<td>23</td>
</tr>
</tbody>
</table>

**Table 1: RBL toxicity in mice.** NOD-SCID mice were injected intraperitoneally with RBL (25, and 50 μg/mouse) and TBS on alternate days for 9 days containing 6 animals in each treatment. The Average body weight, total blood cell count, and biochemical parameters in serum were determined on 14th day and represented.
4.4 DISCUSSION

The present chapter reports the cytotoxic effect of an N-glycan specific lectin from *Rizhoctonia bataticola* on human ovarian cancer OVCAR-3 cells and its PA-1 tumor suppressing effect *in vivo*.

Alterations in N-glycosylation during transformation have been well studied in epithelial cancers that are associated with carcinogenesis, invasion and metastasis [Varki 1999; Dwek 1996]. Cell surface expression of branched N-glycans and high mannose tri- and tetra-antennary bisecting N-glycans on glycoproteins are observed in many cancers including ovarian cancer [Dennis et al. 1987; Machado et al. 2011; Abbott et al. 2008]. Some of these glycoproteins are considered as cancer markers. Tumor markers of ovarian cancer have been studied in detail, and CA-125 is one of the best studied ovarian tumor marker, which is known to express complex N-glycans that are recognized by RBL. However the relationship between chemoresistance and expression of N-glycans has been investigated in very few studies. Cisplatin-resistant head and neck cancer cells showed reduced β1,6-N-acetylglucosamine branches on N-linked glycans expressed on α5β1 integrin on cancer cells compared to the parent line [Nakahara S, 2003]. Lectins have been used to detect the alterations in N-glycan however; little information is available on the precise structural alteration of N-glycans in drug-resistant cells.

The unique sugar specificity of RBL towards these cancer specific N-glycans, that are correlated with anticancer drug resistance prompted us to
study its interaction with drug resistant ovarian cancer OVCAR-3 cells. The cytotoxic effect of RBL on human ovarian cancer PA-1 cells has been reported earlier [Nagre et al. 2010]. In the present study effect of RBL induced cytotoxicity is studied using a different ovarian cancer cell line that is OVCAR-3 that is resistant to anticancer drugs, Cisplatin and Adriamycin. The study shows that, RBL induces high toxicity in a dose and time dependent manner with an IC$_{50}$ value of 10 $\mu$g/ml. The cytotoxic effect of RBL was effectively blocked by competing glycoconjugate such as asialofetuin, supporting that effect is mediated by its carbohydrate binding site. The cytotoxic effect executed by RBL is of great clinical significance in the light of growing interest in fungal lectins during last decade due to their immunomodulatory, antiproliferative, antitumor and hypertensive activities. Further studies are essential in order to understand the detailed signaling mechanism of RBL mediated cell death for exploring its application as a possible drug against ovarian cancer which is drug resistant.

Lectins from higher fungi have been shown to possess antiproliferative activity in different cancer cells in vitro and in vivo [Khan and Khan 2011]. Lectins have been widely used as candidate antitumor drugs in human cancers in vitro; more importantly, some of them have been further explored and applied into the pre-clinical and clinical therapies in fighting against human cancers (Gupta et al., 2010; Thies et al., 2005). Lectins from Ricin-B family and proteins with legume lectin domains have been well-known to induce programmed death in cancer cells, referring to apoptosis and autophagic cell
death, *in vivo*. Unlike other reported fungal lectins, RBL is potent because of its ability to induce apoptosis at low concentrations and at early time points by activating the intrinsic apoptotic pathway in PA 1 cells [Eligar et al, 2012]. RBL is known to induce cell death *in vitro* in human ovarian cancer PA-1 cells, hence *in vivo* effect of RBL was analysed 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.

To date we have reported examples of lectins with therapeutic potential., Ricin-B family, especially ML-I and proteins with legume lectin domains such as ConA and *Phaseolus vulgaris* lectin (PHA) are also shown induce apoptotic cell death in various types of cancers; more interestingly, besides apoptosis-inducing effect, ConA also induces autophagic cell death in hepatocarcinoma [De Mejía and Prisecaru (2005), Lei and Chang (2007); Hoessli and Ahmad (2008), Liu et al. (2010)]. Many lectins have been gradually developing into potential antitumor drugs via targeting PCD pathways for cancer therapeutics (Fu. et al,2011).
In conclusion, RBL induces potent toxicity in OVICAR-3 ovarian cancer cells \textit{in vitro}. The sensitivity of these drug resistant cells towards RBL need to be further investigated in detail that would result in second-line therapy for recurrent carcinoma of the ovary. The \textit{in vivo} studies in NOD-SCID mice bearing PA-1tumor xenografts further support the potential of RBL for its clinical implications.