Chapter 1

Cytotoxicity and antiproliferation study of complexes \textit{in vitro} and \textit{in vivo}

Review of literature:

Cytotoxicity refers to the ability of chemicals or any agent (immune cells) to destroy certain living cells. Cells may respond in different ways when exposed to cytotoxic agents, it depends on types of cell, agents and different circumstances like medium, temperature, time, etc. (Bracci et al. 2014). When cells are exposed to cytotoxic agents, they may undergo either necrosis, a type of traumatic cell death in which they rapidly lose their membrane integrity and die or they can activate a genetically controlled program cell death, termed as apoptosis. Apoptosis is a type of safe and careful programmed cell death in which different biochemical changes occurs including membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies and finally engulfed by neighbouring cells or macrophages (Anilkumar et al. 1992). Cancer is one of the most diagnosed disease occurs due to disturbed balance between cell division and cell death, where too little apoptosis occurs, resulting abnormal cells that may not die. The mechanism of apoptosis is complex and involves many pathways and defects can occur at any steps of these pathways. Apoptosis plays an important role in the treatment of cancer as it is a popular target of many treatment strategies (Gerl and Vaux 2005). Several
literatures suggest that targeting apoptosis in cancer is feasible. Therefore, there is a need of cytotoxic agents that favour apoptotic cell death as there are many ways to evaluate cytotoxicity and most involve method to assess cell death is cell membrane integrity. Membrane integrity can be evaluated by using vital dyes such as trypan blue or propidium iodide (Cummings et al. 2012). Antiproliferative activity of a chemicals is refers to prevention or retardation in cell growth and division. Measurement of antiproliferative activity against malignant cells is also a convenient method for optimizing the activity of a particular agent mostly in case of anticancer drugs. The rate of cell proliferation and viability levels are excellent marker of cell health. Proliferation and viability analysis are crucial for cell growth and differentiation studies and are generally coupled with metabolic activities of the cells (Butler et al. 2014).

Presently, in cancer chemotherapy, cisplatin and related inorganic drugs are being frequently used to treat many cancer types (Reedijk 1996; Wong and Giandomenico 1999) Although several tribulations are associated with their use like high toxicity leading to side effects and limitations in dose/cycle that can be administrated (Chu 1994). Improved knowledge in platinum drugs design and administration procedures have some way to reduce the toxicity of these complexes. On the other hand, non-platinum metal based complexes have also been investigated for their possible use as anticancer drugs. In this context, Ruthenium (Ru) complexes have attracted much attention because of their unique properties like biocompatibility, stability and many more relevant characteristics. Ru is one of the most promising metals of transition
metal series and its complexes are emerging as unique anticancer drugs in preclinical developments and two complexes (NAMI-A and KP1019) are currently in clinical trials (Clarke et al. 1999; Sava et al. 1999). As compared to platinum drugs, ruthenium complexes exert lower general toxicity which has been attributed to the ability of ruthenium compounds to specifically act on cancer cells (Sava et al. 1989). Metals have been used as a medicine in several diseases and have an esteemed place in medicinal chemistry. In case of cancer a number of transition metal complexes have been screened for antitumor activity by established procedures (Guo and Sadler 1999). Transition metals represent the d block element which includes groups 3 - 12 on the periodic table. A transition metal is one which forms one or more ions which have incompletely filled d orbital. This property of transition metals resulted in the foundation of coordination complexes. Coordination compounds or metal complexes were consisting of a central metal atom which bonded with organic or inorganic moiety or anions surrounding it. In 1969 major breakthrough in this field was occurred when the anti-tumor activity of an inorganic complex cisplatin was discovered. Cisplatin has developed into one of the most frequently used and most effective cytostatic drug for treatment of solid carcinomas and some other cancers (van Rijt et al. 2009). Selective toxicity against cancer cells is a most important determinant and essential parameter for anticancer drugs. Several anticancer drugs are currently available for clinical therapy, but very few of them are effective against some types of cancer. Huge advancement in cell culture techniques resulted in establishment of various cancer cell lines and currently different cancer cell lines of
human as well as murine ones for the detection of cytotoxicity and antiproliferative property of a particular drug. Recently in 1992, colorimetric MTT assay in 96-well culture plates were developed, which adopted in the screening system in the NCI, based on a new idea i.e. disease-oriented screening (DOS) using about 60 human cancer cell lines (Tashiro 1992). This chapter includes short term cytotoxicity of newly synthesized transition metal-arene complexes against Dalton’s Lymphoma (DL) cells and further selected complexes were screened on different types of human cancer cell lines as well as normal cell lines and antiproliferation activity were further analyzed on most affected cell line. Further the data were validated on In vivo tumor model (DL bearing mice) system by measuring different parameters.

There are several assays involves colorimetric, fluorescence, or luminescence detection by measuring redox potential of metabolically active cells or by measuring ATP content. Measuring cytotoxicity and antiproliferative activity of agents/drugs is very crucial process in therapeutic anticancer drug development and accurately measure cytotoxicity can be a very valuable tool in identifying compounds that might create certain health benefits and ensure the safety margin in humans (Ferrante et al. 1999). Additionally, understanding the mechanisms involved in cytotoxicity can likewise give researchers a more in depth knowledge on the biological processes governing cell growth, proliferation and death. Cytotoxicity can be measured in a different ways, in which most cytotoxicity assays work on the basis of principle that live cells possess intact cell membrane and ability to exclude certain dyes like trypan blue, eosin, or propidium, whereas dead cells do not exclude due to
compromised cell membrane. It is very easy and short time process to calculate number of lives (with clear cytoplasm) or dead cells (with stained cytoplasm) in cell suspension (Strober 2015).

**Materials and Methods:**

**Materials:**

RPMI-1640, MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] and Ficoll Histopaque (HiSepTM LSM1077) were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai, India. Dulbecco Modified Eagle Medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin, L-glutamine, Trypsin-EDTA, tissue culture flask (surface area 25 cm², canted neck, vented cap) and 96 well plates (flat bottom) were procured from CELL clone™ Genetix Biotech Asia Pvt. Ltd. Cisplatin injection purchased from Cipla with given name cisplatin injection BP Cytoplatin-50 Aqueous, each 50 ml vial contains: cisplatin IP 50mg, sodium chloride IP 0.9% w/v and water. Trypan Blue dye for microscopy was purchased from LOBA CHEMIE PVT. LTD. Mumbai, India and the remaining chemicals were purchased from local firms and were of highest purity grade.

**Preparation of stock solutions of Transition metal-arene complexes:**

Stock solution (40 µg/µL concentration) of all twenty (20) Ru, Rh and Ir arene complexes were initially dissolved in DMSO and further dilutions were made in complete DMEM, in which the concentration of DMSO did not exceeded more than 0.1% v/v.
Cell lines and their maintenance:

A murine lymphoma cells namely Dalton’s Lymphoma cells designated as “DL” cells were used for short term (3h) toxicity and 24h cytotoxicity and antiproliferation activity test of complexes. Dalton’s Lymphoma (DL) cells are a transplantable murine T cell lymphoma that spontaneously originates in thymus of DBA strain (H-2d) of mice and can be maintained by serial transplantation in intraperitoneal cavity of one mouse to other mouse and it mimics some lymphoma and leukemia of human origin (Goldie and Felix 1951; Kumar and Singh 1996). Dalton’s Lymphoma (DL) cells were aspirated from intraperitoneal cavity of DL bearing mouse.

A-549 (Human Lung adenocarcinoma), MDA-MB-231 (Human Breast adenocarcinoma), HeLa (Human Cervical cancer), C6 (Rat Glioma) cancer cell lines and HaCaT (Human immortal keratinocyte), NIH-3T3 (mouse embryonic fibroblast) normal cell lines was obtained from National Centre for Cell Science (NCCS), Pune, India. These cell lines were grown in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% FBS, along with 100 unit/mL penicillin, 100 μg/mL streptomycin solutions. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were harvested when they reached 80-90% confluence and plated for subsequent experiments.

Maintenance of laboratory Animals:

Balb/c mice were maintained and inbred under standard laboratory conditions as per the guidelines of the Institutional Animal Ethical Committee (IAEC), Banaras Hindu University; at 25°C with 12:12 hours light/dark cycle and mice were fed standard
rodent chow with drinking water ad libitum. 12 to 14 week old healthy Balb/c male mice of 25-30 gram body weight (B. W.) were used for the experiments.

1. Short term toxicity against Dalton’s Lymphoma (DL) cells in vitro:

Methods:

These days numerous methods are available for the detection of cell death in which visual assessment by light microscopy provides a fast and economical means to detect cell death in a generalized fashion. This can be done on living samples (e.g. monitoring cell cultured conditions) or fixed or histological sections. Trypan blue dye exclusion method (Strober 2015) is most classical and frequent method for assessment of cell death and was adapted here to assess short term toxicity of 20 novel (twenty in number) Ruthenium (Ru), Rhodium (Rh) and Iridium (Ir) complexes against DL cells.

Principle:

Trypan blue dye exclusion method is usually used to determine the number of viable/dead cells present in a cell suspension. Trypan blue is diazo (-N=N-) dye and a vital stain used to selectively stain dead cells or tissue into blue colour. Live cells possess intact cell membrane and having very selective permeability for compounds. Intact cell membrane of live cells do not allow stain to inter inside the cells and shown with a clear cytoplasm under microscope while nonviable or dead cells are permeable and stained in blue colour due to compromised cell membrane and shown
as a distinctive blue colour under a microscope. This method is also described as dye exclusion method due to exclusion of live cells from staining.

**Reagents:**

1. **Preparation of 10X Phosphate Buffered Saline (PBS):**
   
   2 g KCl, 2.4 g KH$_2$PO$_4$, 80 g NaCl and 11.45 g Na$_2$HPO$_4$ were dissolved in approximately 800 mL of double dH$_2$O Adjust pH at 7.4 with 1 M NaOH or 1 M HCl and then make up to 1000 mL, autoclaved and stored at 4°C.

2. **Preparation of 0.4% trypan blue solution in PBS:**
   
   Trypan Blue dye (2 g) was initially dissolved in 400 mL 1X PBS and makeup to 500 mL after then filters it with a whatman filter paper and stored at 4°C.

**Trypan blue Protocol:**

This protocol describes Trypan Blue staining procedures which were used to discriminate between viable and dead cells on the basis of membrane integrity.

1. Cells were aspirated from intraperitoneal cavity of DL bearing mouse.

2. Cells were suspended and washed twice with PBS (Phosphate buffer saline).

3. $1 \times 10^6$ viable cells were treated and incubated with increasing concentration of the complexes at 37 °C for 3 h duration, separately.

4. Cells were then washed with PBS and mixed with 0.4% trypan blue dye in equal ratios and left for 5 minutes at 37°C.

5. After a brief incubation at 37 °C the live and dead cells were scored with the help of a neubers cell counter chamber/hemocytometer.
Live and dead cells were counted separately under inverted light microscope in a hemocytometer. The results are expressed as a percentage of dead cells and percent (%) of dead cells was calculated with following formula:

\[
\text{Percent (\%)} \text{ dead cells} = \left(\frac{\text{Number of blue cells}}{\text{Number of total cells}} \times 100\right)
\]

2. Cytotoxicity and antiproliferation activity of complexes against cancer cell lines and normal cell lines:

Method:
Cytotoxicity and proliferation analysis of cells were done with Tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT) colorimetric method which was described by Mosmann 1983.

Principle:
This assay involves the ability of viable (metabolically active) cells to convert a soluble tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), into an insoluble formazan crystal (Slater et al. 1963).

![Figure 1.1: Chemical structure of yellow soluble MTT and its conversion into insoluble formazan crystal product in living cells.](image)
MTT assay protocol:

The cytotoxicity and antiproliferative activity of two selected complexes were checked on five (5) different cancer cell lines [Dalton’s Lymphoma (DL), Human Cervical cancer (HeLa), Human Breast cancer (MDA-MB-231), Human lung cancer (A549), Rat glioma (C6)] and three (3) different normal cells [Human keratinocyte (HaCaT), Mouse embryonic fibroblast (3T3)] and Human peripheral blood mononuclear cells (PBMCs)].

1. Cells were trypsinised, counted and $1 \times 10^4$ cells seeded in 96-well culture plates and 3 to 4 replicates were used for control as well as treated groups.

2. After 24 h, culture medium was replaced by fresh medium containing various concentrations of complexes and incubated for 24 h in a 5% CO$_2$ humidified atmosphere.

3. After drug exposure, medium was removed and 100 µL medium and 10 µL of 5 mg/mL MTT in phosphate buffered saline (PBS, pH 7.4) was added to each well for an additional 2 h.

4. Medium and MTT were removed and 100 L of DMSO was added to dissolve the MTT formazan crystals.

5. The absorbance of samples was measured at 570 nm with ELISA plate reader.

The percent of the cell viability was calculated using the equation:

$$\text{Percent (\%) cell viability} = \left( \frac{\text{Mean O.D. of treated cells}}{\text{Mean O.D. of control cells}} \right) \times 100$$
Isolation of human peripheral blood mononuclear cells (PBMCs) and cytotoxicity test:

In addition, the cytotoxicity of both complexes was also checked against human PBMCs for 24h incubation period and IC$_{50}$ values of both complexes were evaluated.

Method:

1. 5 mL of heparinised peripheral blood sample from healthy male donors was collected freshly through venipuncture.

2. To this equal volume of DMEM was added in a sterile centrifuge tube.

3. Same proportion of blood was layered on the top of Ficoll Histopaque in 15 mL tube and centrifuged at 2000 rpm for 30 min.

4. After centrifugation, the whitish buffy coat (between plasma and Histopaque) was carefully aspirated with sterile syringe.

5. Isolated PBMCs were washed twice with DMEM, $2 \times 10^4$ cells were seeded and treated with both complexes at different concentrations for 24h incubation period.

6. After incubation, the medium was removed and the cells were washed with PBS and incubated again with 100 $\mu$L of medium containing 10 $\mu$L of MTT solution (5mg/mL) for 3h at 37°C.

7. The insoluble formazan crystals formed were then dissolved with 100 $\mu$L of Dimethyl sulfoxide (DMSO) and absorbances of the samples on plates were measured in microplate reader at 570nm.
8. The cytotoxicity of the complexes on PBMCs was calculated with respect to control using the same formula mentioned above and IC$_{50}$ (inhibitory concentration) values calculated and represented as mean ± SE for triplicates.

3. Analysis of antitumor property of D3 and D13 complexes in vivo:

Methods:

DL transplantation and treatment plan:

Under aseptic conditions, 1×10$^6$ viable Dalton’s Lymphoma (DL) cells in 1 mL of PBS were inoculated / transplanted intraperitoneally in mice to establish the ascites tumor model and day of DL transplantation count as day zero (0). Development of DL intraperitoneally was confirmed by abnormal swelling and increased body weight, which were clearly noticeable after day 10 of transplantation and generally DL bearing mice survived for only 18 ± 2 days. The success rate of DL development is 100%. (Goldie and Felix 1951). Working solutions (15 μg/μL) from stock of both Ruthenium-Arene complexes were freshly prepared in PBS and after diluting with PBS, desired concentrations were directly injected intraperitoneally in mice. Standard anticancer drug cisplatin were used as positive control. Initially, maximum tolerable dose (MTD) for positive control (cisplatin) and both complexes were determined and drugs were administered intraperitoneally (i.p., standard route for this model) at its MTD and lower dose.

DL transplanted mice were randomly divided into three groups (n = 6) as follows: Control group, D3 treated group and D13 treated group. Control group was further
divided into two subgroups; PBS control (as negative control) and cisplatin (5 mg/kg B.W) treated (as positive control) group. Likewise, D3 and D13 groups were divided into two subgroups; 5 mg/kg B.W and 10 mg/kg B.W. according to two different doses. Cisplatin, D3 and D13 were administered in respective groups at indicated doses on 6th, 12th and 18th day of DL transplantation and negative control group was given the same volume of vehicle.

**Antitumor test:**

The antitumor activity of both complexes (D3 and D13) were carried out by comparative study of survival days in DL bearing mice between treated and control groups with the help of Kaplan–Meier survival curve and also by computing Treated/Control (T/C) value in accordance with the U.S. National Cancer Institute (NCI) standard protocols for primary screening. Briefly, for the survival analysis, results are expressed as percent of mean survival time of treated animals (T) over mean survival time of the control (C) group (Ahluwalia et al. 1984). The ratio of T/C value represents as a percentage and if a compound has a T/C percentage ≥120%, termed as active complex (Boyd 1996; Andreani et al. 2008).

**T/C value was calculated according to the formula:**

\[
T/C \% = \left[ \frac{\text{Median survival days of treated (T) group}}{\text{Median survival days of control (C) group}} \right] \times 100
\]

According to NCI criteria, T/C % exceeding 120 and increased life span ≥20% indicate that the drug has significant antitumor activity. The antitumor efficacies of
both complexes were also compared with cisplatin, standard chemotherapeutic drugs as positive control.

**Measurement of body weight and belly size:**

Increments in body weight and belly size were consistently measured at day 0, 3, 6, 9, 12, 15 and 18 because in case of ascites tumor model, increasing body weight represent tumor mass and increment in belly size is equivalent to tumor volume growth. Belly size was measured with vernier calipers. The belly size (tumor volume) was calculated from two dimensional measurements (Yoshikawa et al. 1995) and formula:

\[
\text{Tumor volume (Cm}^3\) = \frac{a \times b^2}{2}
\]

Where \(a\) and \(b\) are the tumor length and width (mm) respectively.
Results:

1. Short term toxicity:

Short term cytotoxicity test were performed to screen out the potentiality of 20 complexes, trypan blue exclusion assay was performed against DL cells and the ensuing data are depicted in Figure 1.2. In general short term toxicities of complexes were increased as concentration increases and approximately 6-8% of dead cells were found in control, whereas frequency of dead cells increased in treated cells. The short term cytotoxicity of 12 complexes of series 1 was not much satisfactory and most probably at higher concentrations (>50 µg/mL), these compounds may exert much toxicity to the cells. Whereas series 2 and 3 complexes were exerted higher cytotoxicity as compare to complexes of series 1 as well as control cells. Although many of them exert significant cytotoxicity but two complexes designated as D3 and D13 appeared to be more potent than others in all series. Most satisfactory outcome was observed in D3 and D13 treated DL cells and this indeed indicates the effectiveness or supremacy of both Ru complexes over other complexes in 3 h short term toxicity test. On the basis of their cytotoxic potentiality, two complexes D3 and D13 were selected for further testing in a panel of cancer cells.
Figure 1.2: Percentage (%) of dead cells in response to different concentrations of 12 complexes of series 1 [A] and 8 complexes of series 2 & 3 [B] after 3 hrs. of incubation period (n=3). The results are expressed as the mean ± S.E. and compared with the control. Data were analyzed by One-way ANOVA followed by Tukey post hoc test. *p ˂ 0.05 was considered as statistically significant.

2. Cytotoxicity and Antiproliferation activity against different types of Human cancer cell lines and Normal cells:

The cytotoxicity and antiproliferation activity of selected two complexes D3 and D13 has been studied by MTT colorimetric assay after 24 h of incubation period, across the panel of five different cancer cell lines and three normal cells. To monitor
the action of the cytotoxic drugs, all cancer cell lines as well as normal cells were exposed with various concentrations of complexes for 24 h and IC₅₀ values were calculated. Initially the complexes were tested on two different types of murine cancer cells namely DL and C6 cells. The results indicate that both complexes showed significant cytotoxicity in these two cancer cell lines. The data were also compared with cisplatin which is widely used in cancer chemotherapy and both complexes showed higher cytotoxicity on C6 cells as compared to cisplatin. Further, the cytotoxic efficacy of D3 and D13 were assessed against different types of human cancer cell lines, breast (MDA-MB-231), lung (A-549) as well as cervical cancer (HeLa) cell lines. Both complexes exert significant cytotoxicity on breast and lung cancer cell lines and IC₅₀ value is nearly comparable to cisplatin whereas highest cytotoxicity was observed in case of HeLa cells. Among cell lines tested, D3 and D13 exhibited significance potential against HeLa cells and having lowest half maximal inhibitory concentration (IC₅₀) value 6.06 ± 0.26 and 5.76 ± 0.26 µg/mL respectively. However cytotoxicity against other cell lines was lower than HeLa cells and having IC₅₀ values 12.43 ± 0.54 and 8.74 ± 0.04 for DL, 6.85 ± 0.05 and 5.9 ± 0.09 for C6, 11.21 ± 1.13 and 10.8 ± 0.26 for A-549, 11.92 ± 0.31 and 13.53 ± 0.57 µg/mL for MDA-MB-231 of D3 and D13 complexes respectively.

Since complexes exerted very potent cytotoxicity against different types of cancer cell lines preferably at HeLa, provoked to see the effect of complexes on normal cells because most of the chemotherapeutic drugs exert high toxicity on normal cells along with cancer cells. Therefore D3 and D13 were tested against three normal cells
(human immortal keratinocyte designated as HaCaT and mouse embryonic fibroblast NIH-3T3 and normal human PBMCs) and found to be less cytotoxic relatively (to cancer cells). For both complexes IC<sub>50</sub> value on normal cell lines were >15 µg/mL and on PBMCs IC<sub>50</sub> value was 26.91 ± 1.21 for D3 and 23.98 ± 2.40 g/mL for D13 as depicted in table 1.1. Furthermore, cytotoxicity of cisplatin was also evaluated against five (5) cancer cell lines and one normal (HaCaT) cell line. The cytotoxicity of cisplatin was found to be very high in most of the cancer cell line which were used in these experiments but it also showed significant toxicity against normal cell line HaCaT. These results suggest that D3 and D13 possess great selectivity between cancer and normal cells and displays application potential in cancer chemoprevention and chemotherapy.

[A] Daltons Lymphoma (DL) cells

[B] Rat glioma (C6) cell line
Human breast cancer (MDA-MB-231) cell line

Human lung cancer (A-549) cell line

Human cervical cancer (HeLa) cell line

Figure 1.3: Cytotoxicity of D3 and D13 complexes against five (5) different types of cancer cell lines [A] DL [B] C6 [C] MDA-MB-231 [D] A-549 and [E] HeLa after 24 h of exposure (n = 3). The results are expressed as the mean ± S.E. and compared with control. Data were analyzed by One-way ANOVA followed by Tukey post hoc test. *p  0.05 was considered as statistically significant.
Figure 1.4: Cytotoxicity of D3 and D13 complexes against (A) HaCaT cell line (B) NIH-3T3 cell line and (C) Human PBMCs after 24 h of exposure (n = 3). The results are expressed as the mean ± S.E. and compared with the control. Data were analyzed by One-way ANOVA followed by Tukey post hoc test. *p  < 0.05 was considered as statistically significant.
<table>
<thead>
<tr>
<th>Complexes</th>
<th>Incubation period - 24 h</th>
<th>IC_{50} value (in μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DL</td>
<td>C6</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>3.94 ± 0.15</td>
<td>10.12 ± 0.31</td>
</tr>
<tr>
<td>Comp.1</td>
<td>11.95 ± 0.52</td>
<td>6.73 ± 0.15</td>
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<tr>
<td>Comp.2</td>
<td>10.73 ± 0.58</td>
<td>5.81 ± 0.19</td>
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</table>

ND: Not Determined

**Table 1.1:** IC_{50} ± S.E. values of cisplatin, D3 and D13 complexes after 24 h exposure across the panel of five (5) cancer cell lines and three (3) normal cells (n = 3).

**Comparative analysis of antiproliferation activity at different time periods of D3 and D13 on HeLa cells:**

In light of the cytotoxicity findings described above, the study was further extended on the antiproliferation efficacy of compounds D3 and D13 against HeLa cells for 24 h, 48 h and 72 h time periods and evaluated their IC_{50} values respective to their exposure time periods. Antiproliferative efficacy of complexes were found to be increased as exposure time increases and has decreased value of inhibitory concentration as exposure time increases (comparative graph and IC_{50} values are depicted in figure 1.5 and table 1.2 respectively). Together, these data suggest that D3 and D13 induced cytotoxicity as well as inhibit cell proliferation in HeLa cells.
3. Antitumor property of D3 and D13 complexes in vivo:

3.1. Enhancement of survival of DL bearing Mice:

*In vivo* study refers to real and natural physiological conditions, which can confirm the actual potency of complexes to act as anticancer drugs. Therefore, to evaluate the *in vivo* antitumor aspect of complexes, both complexes were tested on animal tumor model (Dalton’s Lymphoma bearing mice). Cisplatin, D3 and D13 complexes were administered intraperitoneally in tumor bearing mice. Results showed a significant inhibition of tumor growth compared to the control group. More importantly, median lifespan/survival days of tumor bearing mice treated with complexes were
significantly higher as compared to that of untreated tumor bearing mice suggesting that the treatment affected viability of cancerous cells, but have no effect/least effect on normal cells. Enhancement in the longevity of D3 and D13 treated groups as compared to the negative control and survival data were analyzed by Kaplan–Meier survival curve using log-rank statistics suggested a significant dose-dependent increase in survival by approximately 1.8 fold and 2.4 fold with dose of 5 and 10 mg/kg BW of D3 complexes, whereas D13 at both doses (5 mg/kg and 10 mg/kg BW) increased 1.8 fold and 2.5 fold longevity respectively (fig. 6). Furthermore, if compared with positive control (cisplatin treated tumor bearing mice) both complexes at 10 mg/kg BW increased approximately 1.4 fold of survivability. Consistent with these results, administration of drugs at same dose to normal mice did not cause any major side effects as observed and mice were survived normally. Thus, D3 and D13 could be used as a potential cancer therapeutic agent.

**Figure 1.6:** Kaplan–Meier survival curve for survival of Dalton’s lymphoma (DL) bearing mice as control and DL bearing treated mice with D3 and D13 at two different (5 and 10 mg/kg BW) doses. Graph was plotted and analyzed by Prism 6
software with log-rank analysis to examine the level of significance and P < 0.001 was obtained in between control and treated groups.

3.2. Evaluation of Treated/Control (T/C) values of complexes:

Maximum tolerable dose (MTD) of Cisplatin, D3 and D13 were analyzed and it was 5 mg/kg, 10 mg/kg and 10 mg/kg BW respectively. MTD data indicate that both Ru complexes were less toxic and more tolerable than Cisplatin drug. Antitumor activity of both complexes was assessed in accordance to National Cancer Institute (U. S.) standard protocol for in vivo primary screening. The Treated/ Control (T/C) values are given in figure 1.7 and Table 1.3. Data indicate the very high antitumor activity of both Ru complexes on DL bearing mice. At higher dose of D3 and D13, T/C values were 237% and 247% respectively when treated on the 6th, 12th and 18th day after DL transplantation. However, the T/C value of cisplatin was equivalent and nearly comparable to lower doses of both complexes.

<table>
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<tr>
<th>Experimental Conditions</th>
<th>No. of animals</th>
<th>Treatment days</th>
<th>Median survival time (days)</th>
<th>Range survival time</th>
<th>T/C %</th>
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<tr>
<td>Control -ve (PBS)</td>
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<tr>
<td>+ve (cisplatin)</td>
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<td>30-36</td>
<td>179</td>
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<td>32-37</td>
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<td>6th, 12th, 18th</td>
<td>47</td>
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**Table 1.3:** Median survival days and T/C value of Cisplatin, D3 and D13 towards Dalton’s Lymphoma (DL) bearing mice.
3.3. Regression in body weight and belly size growth pattern:

In ascites tumor model system, increment in tumor mass and tumor volume can be detected by body weight and belly size growth pattern respectively. Both D3 and D13 demonstrated tumor growth inhibition in respect to both body weight and belly size (Figure 1.8A and 1.8B). Increment in body weight of control, cisplatin (5 mg/kg BW), D3 (10 mg/kg BW) and D13 (10 mg/kg BW) treated groups were approximately 21.3g, 16.8g, 13.1g and 13.5g respectively. Whereas increment in belly size of control, cisplatin (5 mg/kg BW), D3 (10 mg/kg BW) and D13 (10 mg/kg BW) treated groups were approximately 15.7 cm$^3$, 11.8 cm$^3$, 9.4 cm$^3$ and 9 cm$^3$ respectively. Maximum inhibition in body weight and belly size growth rate was observed in higher dose of D3 and D13 treated groups. Clearly, D3 and D13 exhibited potential therapeutic efficiency in tumor bearing mice at the dose of 10 mg/kg. These results indicate that both complexes offer advantages of decreased side
effects and improved drug tolerance. It may suggest that the D3 and D13 are promising platform for safe and efficient cancer chemotherapy.

![Graph A](image1)

**Figure 1.8:** Effect of complexes on body weight (A) and belly size (B) growth pattern of DL bearing mice. Body weight of Dalton’s lymphoma (DL) bearing mice and treated mice from Day 0 to 18 days after DL transplantation. Values are expressed as mean ± SEM obtained from 3 different sets of experiments. *Denotes significant differences at the level of P < 0.05 between the control and treated groups.
Discussion:

After the discovery of anticancer activity of cisplatin, transition metal complexes received much attention and engrossed the researchers for the quest of alternative drugs. Cisplatin and its derivatives are well known anticancer drugs, which are still being used in more than 50% of the treatment regimes for cancer patients (Gasser et al. 2011; Dasari and Tchounwou 2014). In spite of their amazing anticancer activity, these platinum complexes having different drawbacks and limitations like severe systemic toxicity and drug resistance. It is generally accepted that the severe systemic toxicity is the consequences of the fact that the ultimate target of these complexes is DNA which omnipresent in all cells (Oberoi et al. 2013; Pizarro and Sadler 2009). Changes in chemical structure by using non-platinum metals can improved its tumor specificity and thereby reduce undesired side effects. With this idea, rigorous researches are enduring and it has been reported in many literatures that organometallic complexes like Ru, Rh and Ir exhibited cytotoxicity and also have impact on retardation of cancer cell growth (Gras et al. 2010). The cytotoxicity and growth inhibition of cancer cells are not the sufficient parameters to investigate an anticancer drug, it is also necessary to search specificity of a complex on particular type of cancer cells along with cytotoxicity and antiproliferative activity. Furthermore, cytotoxicity test of complex on normal cells will take one step ahead to precisely select right complex for particular type of cancer with general lower toxicity on body’s normal cells.
Effective complexes were selected through short term toxicity and results indicate that the ruthenium-arene complexes of series 2 and 3 show greatest cytotoxicity among all complexes were employed in short term toxicity test against DL cells. Further, in long term cytotoxicity and antiproliferation activity investigation, both Ru-arene complexes exhibit uppermost and preferential cytotoxicity against HeLa cells with least IC$_{50}$ value among all five cancer cell lines use in this study. More importantly, excellent results were obtained in case of cytotoxicity on normal cells in which both Ru-arene complexes did not show that much extent of cytotoxicity and showed 2-3 fold selectivity in killing cancer cells (HeLa) over normal cells. Although, the reason is not very clear at this point about differential cytotoxicities of Ru-arene complexes among cancer cells, it might be linked with numerous factors such as cellular uptake efficiencies and preferential interaction with DNA/RNA and protein targets which may depends on types of cancer cells. However the less cytotoxicity in normal cells surely depend on either one or more characteristic features of cancer cells like higher expression of some receptors and fast proliferative or higher metabolic properties like redox status and others. It was well established by extensive studies that cisplatin binds with DNA formed DNA-complex adduct (Sherman et al. 1985; Reedijk 1987). Cisplatin binds with N-7 position of the DNA base guanine which is a crucial first step for the cross linking by formation of a 1,2-intrastrand cross-link of adjacent guanine bases and leads to bending at 45° and unwinding of DNA. These distortions in the structure of DNA are ultimately anticipated to be responsible for the anticancer activity (Kratz et al. 1998).
The basic concept seems to hold that binding with N-7 position of Guanine is important for chemotherapy (Brookes 1990). Since the Ru-arene complexes were synthesized with fundamental concept of platinum complexes with huge modification in ligands as well as in structure therefore it is expected that their mode of action would also be different from cisplatin group of complexes. In anticancer Ru-arene complexes, arene ligands represent a relatively new group of ruthenium compounds with a mechanism of action different from NAMI-A which are currently on clinical trials (Morris et al. 2001; Aird et al. 2002; Alessio et al. 2004; Novakova et al. 2005). These types of complexes are stable in aqueous medium and do not hydrolyze, but the chloride ligand is readily lost and the complex is transformed into more reactive complex in water. It has also been shown that in cell-free media Ru-arene complexes preferentially binds to guanine residues in natural double-helical DNA (Wang et al. 2013). In addition to DNA binding activity, the ligands of these complexes involved to coordinate with guanine N-7 and non-covalent, hydrophobic interactions between the arene ligand and DNA, which may include arene intercalation and minor groove binding. Some Ru-arene complexes with single hydrocarbon ring (e.g. p-cymene and benzene) cannot interact with double-helical DNA by intercalation but more efficiently they can distort the conformation dsDNA distinctly by thermal destabilization (Chen et al. 2002; Novakova et al. 2003). Interestingly, there were other differences also seen in the repair mechanism of Cisplatin-DNA adduct and Ru-arene-DNA adduct. Ru-arene-DNA-adduct were removed by the different mechanism other than nucleotide excision repair which
were believed to be main process for repairing the cisplatin-DNA adduct (Kartalou and Essigmann 2001). Thus, these Ru-arene complexes may affect the cancer cells differently as a consequence it was proficient for selectivity and antitumor efficacy towards HeLa cells and exert negligible or least cytotoxicity on normal cells. Further, toxicity of cisplatin was also evaluated on different types of above cancer cell lines and on normal cell line (HaCaT). The comparable efficiencies and selectivity of Ru-arene complexes (D3 and D13) with cisplatin indicate that selected Ru-arene complexes are potential novel anti-tumor agents with marked degree of selectivity towards HeLa, cervical cancer cells. Further, it was shown by antiproliferative test at three different time periods (24, 48 and 72 h), both Ru-Arene complexes also exhibited antiproliferation activity against HeLa cells and with increasing incubation periods the antiproliferation efficacy of complexes were improved and comparatively less concentration of complexes were required for growth inhibition. The antiproliferation activity of Ru-arene complexes can be explained and correlated with the inactivation of topoisomerase II activity (topoisomerase II poisoning). Because many DNA binding anticancer drugs that target Topoisomerase II enzyme which is a major nuclear enzyme that participates in making of replication fork and also maintains the topology of DNA. In some literatures it was shown that some Ru complexes effectively block the activity of topoisomerase II by forming a ternary cleavage complex of DNA, drug and topoisomerase II (Vashisht Gopal and Kondapi 2001; Vashisht Gopal et al. 2002). Although antiproliferation activities of both Ru-arene complexes do not give direct
evidence as a cause for this kind of activity (topoisomerase II antagonism) but it may suggest that topoisomerase II antagonism like activity may partly account for the anticancer activity of this class of ruthenium complexes.

For the development of new therapeutics in nonclinical research, it is necessary to investigate complexes from *in vitro* to *in vivo* models and *in vivo* studies reflects more clear views the real potential of any drug. Among the transition metal compounds, ruthenium appears to be more potential candidate with different chemistry from cisplatin and its analogs. Hypoxic environment of many tumors may be one of the basics causes of cancer cell selectivity or preferences by reducing the Ru III to Ru II complexes species, which binds rapidly (McKeage et al. 1991). Ru complexes particularly from series 2 and 3 showed more potent activity than others in *in vitro* study. In *in vivo* experiment, both complexes were tested for antitumor action on DL bearing mice and also compared with cisplatin treated groups in different parameters like survival days, T/C value, body weight and belly size growth pattern. Before antitumor study, the major differences were observed in maximum tolerable dose (MTD) which indicates that as compared to cisplatin drug, both Ru-arene complexes are more tolerable or less toxic in *in vivo* condition. In the present studies, the effect both Ruthenium-arene complexes were found to be dose dependent manner and enhanced more than two fold in the life span of tumor bearing mice and also reduced body weight which represents tumor weight and belly size i.e. equivalent to tumor volume. Lower dose of both Ru-arene complexes demonstrate equivalent efficacy to cisplatin in life span increment and regression in tumor
growth. Similar *in vivo* study has been performed with ruthenium-DMSO complexes in which they reported that complexes increased longevity of tumor bearing mice and have also been effective against several murine models including a cisplatin resistant P-388 leukemia (Keppler et al. 1990). In another literature, effect of RAPTA-C was studied in EAC cells isolated from peritoneal cavity of tumor bearing mice and reported that RAPTA-C induced apoptosis in dose dependent manner (Chatterjee et al. 2008). The antitumor action of ruthenium complexes might be the consequences of reasonably well penetration within the cancer cells, binding efficiently to DNA and with proteins also (Frasca et al. 1996). It is also reported that some of them like Ru III modified its structural conformation after binding with DNA (Novakova et al. 1995). However dose dependent reductions in tumor growth were also reported by a Ru (II)-CNEB complexes by apoptotic induction through declination in lactate dehydrogenase and mitochondrial dysfunction without affecting the normal cells (Koiri et al. 2009).

Present study exhibited cytotoxic and antiproliferative activity of Ru-arene complexes against different types of cancer cells, preferably towards HeLa (Human cervical cancer) cells without affecting normal cells at particular concentration comparatively to cancer cells and antitumor efficacy by enhancing life span along with suppressing tumor growth of DL bearing mice. The observations that Ru-arene complexes possess anticancer activity *in vitro* and *in vivo* have significant implications for the future perspective in cancer management and drug development.