CHAPTER 6

IN-VITRO CELL LINE AND BIOCOMPATIBILITY STUDIES
Chapter 6: In-vitro cell line and biocompatibility studies

6. Introduction

In-vitro cell line studies overcome the drawbacks involved with animal experimentation and ease out the investigation in cancer arena. They are one of the superior models for examination of mechanisms involved in evolution and progression of cancer (Louzada et al, 2012). In-vitro cell line studies should enable researcher in understanding various cellular pathways, role of genetic and epigenetic factors, apoptosis and development of cancer and examination of cancer therapeutics (Kao et al, 2009). Results obtained from cell line studies can been replicated and applied to humans and such investigations have received consent from many biomedical and pharmaceutical organizations.

6.1. HepG2 liver cancer cell line as an in-vitro model

This cell line is popularly known as ‘hepatocellular carcinoma’ cell line and was discovered by Barbara Knowles and her colleagues in 1979 (Aden et al, 1979). HepG2 cell lines have been used in several areas of research like studies related to development, hepatotoxicity testing, hepatic oncogenesis and liver metabolism. HepG2 are epithelial cells which grow as monolayers and in small clusters, presenting a chromosome value of 55. This cell line has been obtained from liver tissue with distinguishable hepatocellular carcinoma (derived from a fifteen year old male subject). HepG2 secretes various components like albumin, fibrinogen, transferrin and does not lead to tumor generation in nude/SCID mice (http://hepg2.com). Many other cell lines and their variants have been stated in the literature which have their origin from human HCC like Hep3B, Huh7, PLC/PRF/5 etc. However, these cell lines have been mainly employed for study of Hepatitis virus C infections (von Hahn et al, 2006; Fukuhara et al, 2011). HepG2 cell line has been principally utilized for oncological studies of various synthetic compounds and drugs.

The cytotoxic activity of Cisplatin per se, has been investigated in HepG2 cell line in numerous studies. In a particular study, the combined effect of VX680 and Cisplatin in inhibiting the HepG2 cell line growth was observed by MTT assay and the changes in apoptosis and protein levels were observed by flow cytometry and western blotting respectively. It was observed that the compound under investigation acted synergistically with Cisplatin with dose and time dependent cytotoxic effect in the cytotoxicity assay with increased apoptosis and p53 protein expression as compared to drug alone (Yao et al, 2014).
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The *in-vitro* cytotoxicity of Cisplatin and various polymeric, lipid based formulations has been studied against number of cell lines (Imai et al, 1997; Truter et al, 2001). In some studies, it has been reported that the IC$_{50}$ of Cisplatin solution is higher than the formulations of Cisplatin, while in some cases it is lower (Burger et al, 2002).

In another study, the cytotoxicity of Gold nanoparticle-L aspartate structure loaded with Cisplatin was studied in HepG2 cell line (Tomuleasa et al, 2012). An increased susceptibility of HepG2 cells was demonstrated with nanoparticle formulations as compared to the free drug, probably due to the increased cellular uptake of nanocarriers on basis of their small size, shape and surface chemistry, leading to enhanced tumor accumulation and thus greater cytotoxicity.

The *in-vitro* cytotoxic activity of Cisplatin and its polymer or lipid based formulations has been demonstrated against number of murine tumor cell lines (Schechter et al, 1995). But there are no studies available in the literature on evaluation of the anticancer efficacy of albumin polymeric nanoparticles of Cisplatin. In the present work, efficacy of developed nanoparticulate formulations of Cisplatin was evaluated against human liver cancer cell line HepG2 using number of *in-vitro* assays in comparison to the pure drug.

### 6.2. Experimental

#### 6.2.1. *In-vitro* cytotoxicity testing of bovine serum albumin Cisplatin nanoparticles by Sulforhodamine B (SRB) assay

SRB assay was performed on optimized batch BSCSP1, Cisplatin and blank BSCSP1 formulation in different concentrations ranging from $10^{-4}$ to $10^{-7}$ M. Results for this assay are depicted in Table 6.2, Figs. 6.1 and Fig. 6.2.

#### 6.2.1.a. Principle of Sulforhodamine B assay

Sulforhodamine B (SRB) assay is one of the widely utilized techniques for estimation of *in-vitro* cytotoxicity of drugs. SRB is known to complex with cellular protein constituents, which have been prefixed with trichloroacetic acid (TCA) to the culture plates. SRB is a bright pink coloured dye, showing stiochiometric binding with the cancer cells, thus amount of bound dye is proportional to the cell concentration in this assay. As such SRB is unable to differentiate between live and dead cells after the assay. Despite this, the assay retains its property to detect...
the cytotoxicity effects related to drugs. Other advantages of this assay include high sensitivity, endpoint stability, suitability for large scale processing, simplicity and ability of performance in 96 well culture plates (Vichai and Kirtikara, 2006).

6.2.1.b. Procedure for estimation of cytotoxicity by Sulforhodamine B assay

- HepG2 cell line was grown in RPMI-1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine.
- Cells were inoculated into 96 well microtiter plates in 90 μL at plate densities depending on the doubling time of individual cell lines and the microtiter plates were incubated at 37°C, 5% CO₂, 95% air and 100% relative humidity for 24 hrs, prior to addition of experimental drugs.
- After 24 h, one plate of each cell line was fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition (Tz).
- Test formulations viz. Cisplatin, CDDP BSA nanoparticles and blank BSA nanoparticles were solubilized in purified water stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate was thawed to room temperature.
- Serial dilutions of the concentrate were made to provide a total of four drug concentrations ranging from 10⁻⁴ to 10⁻⁷ M plus control. Aliquots of 10 μl of these different drug dilutions were added to the appropriate micro-titer wells already containing 90 μl of medium, resulting in the required final drug concentrations.
- After addition of formulations, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 μl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4°C.
- Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent Growth was the ratio of average absorbance of the test well to the average absorbance of the control wells * 100.
- Using the six absorbance measurements [Time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels 10⁻⁴ to 10⁻⁷ M (Ti)], the percentage growth inhibition was calculated at each of the drug concentration levels. Percentage growth inhibition was calculated as: [(Ti-Tz)/(C-Tz)] x 100 for concentrations for which Ti ≥ Tz (Ti-Tz)
positive or zero and \[(Ti-Tz)/Tz\] x 100 for concentrations for which Ti < Tz \[(Ti-Tz)\] negative.

- The dose response parameters were calculated for each test article. Growth inhibition of 50% (GI50) was calculated from \[(Ti-Tz)/(C-Tz)\] x 100 = 50, which was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was calculated from Ti = Tz.

- The value of LC50 (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment was calculated from \[(Ti-Tz)/Tz\] x 100 = -50.

- The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an Elisa plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

- For the purpose of qualitative analysis, pictographic images of all the tested formulations BSCSP1, Cisplatin, Blank BSA formulation and plain HepG2 cell line were taken by a camera at the end of the assay and were compared with each other to determine the cytotoxicity exhibited by each tested formulation.

6.2.2. Determination of in-vitro cytotoxicity of CDDP Gelatin nanoparticles by SRB assay

The cytotoxicity of the optimized Cisplatin gelatin formulation GNCSP1, Cisplatin and Blank gelatin nanoparticles was tested using the same protocol as mentioned for BSA formulations. Results for this study are presented in Table 6.3 and Fig. 6.3.

6.2.3. Selection of nanoformulations for detailed in-vitro studies

The optimized human serum albumin drug loaded nanoformulations FDH8, FDL4 and HSTCSP1 (plain, ligand attached and TG linked respectively) and blank formulations of the same along with the Cisplatin were selected for detailed in-vitro studies on HepG2 cell line since, these were found to be superior over developed bovine serum albumin and gelatin
formulations in terms of their high negative surface charge, greater entrapment efficiency, better sustained drug release properties and low antigenicity.

*In-vitro* cytotoxicity assay and qualitative cell uptake by confocal microscopy was also performed simultaneously on B16F10 murine melanoma cell line (negative control) for comparative purpose with HepG2 cell line.

### 6.2.3.a. Procurement and maintainance of HepG2 and B16F10 cell lines

HepG2 and B16F10 cell lines were obtained from National center for cell sciences, Pune, India. The above cell lines were maintained in 10% DMEM (Cell Clone, Genetix Biotech Asia Pvt Ltd, India) with Fetal Bovine serum (GIBCO BRL, MD, USA), Penicillin (100 U/ml) and Streptomycin (100 g/ml). Cells were grown in humidified condition of 5% CO₂ at a temperature of 37°C. From the point of view of *in-vitro* cell line studies, the human serum albumin formulations were coded as mentioned in Table 6.1.

#### Table 6.1: Coding of nanocarrier formulations for *in-vitro* studies

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Name of the Formulation</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pure Cisplatin</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Plain Cisplatin HSA nanoformulation</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>Blank HSA nanoformulation</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>Galactosamine linked Cisplatin HSA nanoformulation</td>
<td>D</td>
</tr>
<tr>
<td>5</td>
<td>Blank Galactosamine linked HSA nanoformulation</td>
<td>E</td>
</tr>
<tr>
<td>6</td>
<td>TG linked Cisplatin HSA nanoformulation</td>
<td>F</td>
</tr>
<tr>
<td>7</td>
<td>Blank TG linked HSA nanoformulation</td>
<td>G</td>
</tr>
</tbody>
</table>

### 6.2.3.b. Determination of cell viability by Methyl-tetrazolium (MTT) assay

#### 6.2.3.b.1. Principle of MTT assay

MTT assay is a simple, non-radioactive colorimetric assay to measure cell cytotoxicity, cell proliferation or cell viability. MTT, a yellow coloured water soluble salt is converted by active, live cells into water insoluble dark blue formazan product (*Gupta and Gupta, 2004*). Since the conversion takes place only in viable cells, amount of formazan and consequently the colour produced is proportional to concentration of live cells present in culture plates after treatment.
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with cytotoxic compounds. From the results of absorbance, the IC$_{50}$ value is calculated i.e. the concentration of test compound at which a 50% reduction in optical density is observed as compared to control.

6.2.3.b.2. Preparation of HepG2 and B16F10 cells for MTT assay

The above cells with 90% confluency were washed with phosphate buffer saline to remove the maintaining medium DMEM. The monolayer of cell lines was washed with phosphate buffer saline till the washings were free from DMEM. The above monolayer was successfully removed using repeated washings with trypsin and pooled in an eppendorf tube. The single cell suspension without any mechanical lumps was obtained by mechanical disaggregation. The viability of the cells was determined by tryptan blue exclusion test. Cells with more than 95% viability were counted with a hemocytometer. The cells were diluted with DMEM medium supplemented with 10% fetal bovine serum (FBS) for seeding in 96 well culture plates.

6.2.3.b.3. Procedure for MTT assay

B16F10 and HepG2 cells were seeded in 96 well plates at a concentration of 4x10$^3$ cells/100 $\mu$l/well and incubated for 24 hours. These cells were then treated with pristine drug Cisplatin, Blank nanoparticles and Cisplatin polymeric nanoparticles for 24, 48, 72 and 96 hours at concentrations ranging from 0.1 to 100 $\mu$g/ml for determination of IC$_{50}$. Post treatment, the cells were washed with phosphate buffer saline and MTT (Sigma Aldrich, USA) was added in the plates at a concentration of 5 mg/ml. The formed formazan crystals were solubilized in DMSO and optical density was measured using ELISA microplate reader (Molecular devices, Spectra Max 190) at 540 nm after background correction at 690 nm. Results for this study are depicted in Table 6.4, Figs. 6.4, 6.5 and 6.6.

6.2.3.c. Colony formation assay for developed nanoformulations

6.2.3.c.1. Principle of colony formation assay

Colony formation assay was performed based on the fact that a single cancerous cell is able to form colonies consisting of atleast 50 units. Hence, this assay essentially tests the "unlimited multiplication" property of cancer cells (Franken et al, 2006). It is a qualitative technique to determine the cell death or reduction in cell number post treatment with cytotoxic compounds.
Hence, this method can be applied in determination of efficacy of tested antineoplastic compounds.

6.2.3.c.2. Procedure for colony formation assay

For colony formation assay, 35 mm petri plates were seeded with 600 cells/plate and allowed to proliferate for 48 hours. Later, these cells were treated for 24 hours with the sub-toxic concentrations of pure drug, blank and drug loaded formulations. Complete DMEM medium was added after PBS washings of the treated cells. Petri plates were incubated for approximately 3-4 days, cells were fixed using methanol and stained with 0.2% crystal violet solution for visual observation of colonies. Colonies with 50 or more number of cells were counted and percent inhibition potential for the colonies by the developed formulations were calculated (Results given in Figs. 6.7 and Fig. 6.8).

6.2.3.d. Wound scratch assay

6.2.3.d.1. Principle of wound scratch assay

Wounds in the tissues or cells endure a complex series of events to repair the damaged area. These events include increase in vascularization by angiogenic factors, extracellular matrix deposition and enhanced cellular proliferation. Hence, these assays can be employed on both qualitative and quantitative levels to estimate cellular migration and proliferation rates of cancer cells (Liang et al, 2007). The major steps involved here are creation of a "wound" or "scratch" in the monolayer of cancerous cells and measuring the "wound coverage" or the complete closure of the wound after a specified time period.

6.2.3.d.2. Procedure for wound scratch assay

B16F10 cells were seeded at a concentration of $4 \times 10^4$ cells/ml in 35 mm petri plates and allowed to grow upto 60-70% confluency. Sub-toxic concentrations of above mentioned formulations were added in the plates and incubated for 24 hours. Later, wounds were created in the above plates using a microtip. The cells were washed with PBS for 2-3 times to remove the peeled cells from the surface and initial wound width was measured using an ocular grid. After incubation period of 24 hours, the cells were fixed with methanol, observed for migration and the images were taken using an inverted microscope (Axiovision, Zeiss). Twenty five measurements were
recorded for each formulation and percent migration for each of the formulation was calculated considering migration in untreated control as 100%.

6.2.3.e. Determination of cellular morphology by Leighton tube assay

6.2.3.e.1. Principle of Leighton tube assay

Leighton tube study is carried in cell line studies to assess the extent of cellular damage such as destruction of the cell wall and release of cellular content upon treatment with anticancer agents/formulations. The changes in morphology of cells are detected qualitatively in this assay.

6.2.3.e.2. Procedure for estimation of cellular morphology by Leighton tube assay

B16F10 melanoma and HepG2 cells were grown in 35 mm petri plates on cover slips in DMEM medium. Sub-toxic concentrations of pure drug, blank albumin nanocarriers and drug loaded formulations were added in the above plates and incubated for 48 hours. Later, these cells were fixed with 70% ethanol and stained with hematoxylin and eosin. The cover slips were dipped lightly in xylene to remove excess stain and fixed on glass slides using DPX mounting medium. The changes in morphology of the cells post treatment were observed under the inverted light microscope. Results for this study are presented in Fig. 6.9.

6.2.3.f. Cell cycle analysis by flow cytometry

6.2.3.f.1. Principle of flow cytometric analysis

Cancer cell growth is known to consist of successive phases and these cells are characterized by certain processes during the cell division, DNA synthesis and replication. DNA selective dyes, upon staining with test compounds emit a particular fluorescence which is proportional to DNA content in each phase of the cells (Jayat and Ratinaud, 1993).

6.2.3.f.2. Procedure for cell cycle analysis by FACS

For cell cycle analysis, sub-toxic amounts of pure Cisplatin and developed nanoformulations were added to 35 mm plates containing sub-confluent B16F10 cells after 48 hours incubation period. These cells were collected, cleaned 2-3 times with PBS and fixed with chilled 70% ethanol. The obtained cell pellet was subjected to treatment with RNase (MBI Fermentas, USA) and stained with propidium iodide. Cell cycle analysis was performed on Becton-Dickinson
FACS scan and data was analyzed using MODFIT software (Results depicted in Fig. 6.10 and Fig. 6.11).

6.2.3.g. Determination of qualitative cellular uptake by confocal microscopy

6.2.3.g.1. Principle of confocal microscopy

Confocal microscopic studies are performed to determine the internalization of nanoparticles in the cancerous cells. These studies are of significance due to the fact that this property of cell uptake is associated with the therapeutic activity of nanoparticles i.e. more the uptake; greater is the ability to cause cancer cell death. This study generally employs fluorescent dyes for qualitative estimation of cell uptake of nanocarriers (Panyam et al, 2003).

6.2.3.g.2. Procedure for cellular uptake of nanoparticles by confocal microscopy

For this particular study, nanoparticles were loaded with FITC dye in the concentration of 0.2 mg/ml. B16F10 cells were allowed to reach 60% confluency and were treated with the plain dye and dye loaded albumin nanoparticles respectively for 2 hours. These cells were fixed with 1% paraformaldehyde subsequent to PBS washings. Further, the cells were treated with DAPI and washed thrice with PBS. The coverslips containing B16F10 cells were observed under microscope after treatment with 2.5% DABCO and were sealed. Acquisition was performed using confocal microscope at 63X. (LSM 510, Zeiss). Data was analyzed using LSM image browser software. Results for this study are illustrated in Fig. 6.12.

6.2.3.h. Determination of cellular uptake of nanoparticles by flow cytometry

Nanoparticle uptake is an important parameter in drug delivery to tumor cells in order to evaluate the amount of active in the intracellular compartment and its consequent activity. Nanoparticle uptake is generally determined by flow cytometric analysis of the fluorescently labelled nanoparticle formulations.

6.2.3.h.1. Procedure for quantitative cellular uptake by flow cytometry

Quantitative assessment was performed for uptake of nanoparticles loaded with coumarin-6 dye (0.6 mg/ml). B16F10 cells were grown in 35 mm plates till they attained 60-70% confluency. Confluent cells were treated with plain dye and dye loaded nanocarriers for specific time limit of 15 and 30 minutes. After harvesting, these cells were fixed using 1% PFA solution. Cells were
suspended in PBS solution subsequent to washings with the same solvent. Analysis was performed on FACS Calibur using CellQuest software. Results for this study are presented in Fig. 6.13.

6.2.3.i. Statistical analysis

All the measurements were performed in triplicate. Statistical analysis was performed using the software Graphpad Prism 5. Results were expressed as mean ± S.D. One-way ANOVA with Tukey’s multiple comparison test was used to determine the significant difference between the treatment groups and untreated control (UC).

6.2.4. In-vitro biocompatibility studies on the developed formulations by hemolytic assay

Hemolysis is a condition that can cause hemolytic anemia, renal failure or jaundice and leads to life threatening situations. It is a simple, cost effective methodology to estimate the effects of various particulate materials on biological membrane. The action of nanoparticles on biological membranes is important as nanomaterials form a part of medicinal devices and drug delivery systems for therapeutic and diagnostic purposes. This assay has been used for determination of hemolytic potential of various particles like silica, asbestos and more recently nanoparticles (Harrington et al, 1971; Wang et al, 2010).

6.2.4.a. Principle of hemolytic assay

In hemolytic assay, nanoparticles are incubated with blood; hemoglobin is released by damaged cells during the assay and converted to red-colored cyanmethemoglobin by use of specific reagents. The nanoparticles and undamaged RBCs are then removed by centrifugation, and the amount of cyanmethemoglobin in the supernatant is measured by a spectrophotometric method. This measured absorbance is compared to a standard curve to determine the concentration of hemoglobin in the supernatant. This hemoglobin concentration is then compared to that in the supernatant of a blood sample treated with a negative control to obtain the percentage of nanoparticle-induced hemolysis.

Ideally, the polymeric formulations intended for intravenous administration should exhibit minimum or no hemolysis since the formulation is indicated for therapeutic purpose. Hence, the aim of this study was to evaluate the hemolytic potential of developed albumin nanoparticles in
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comparison to pure drug. Similarly, a marketed injection of Cisplatin (Cytoplatin, Cipla Pvt Ltd, India) was also investigated for comparison with the prepared nanocarriers.

6.2.4.b. Procedure for hemolytic assay

Hemolytic assay on the developed formulations was performed as per a previously reported method with slight modifications (Tafaghodi et al, 2006). Briefly, 5 ml of human blood was collected in EDTA tube and centrifuged at 3000 rpm for 15 minutes to get a pellet of red cells. Supernatant was removed and the packed red cells were washed thrice with normal saline solution. Saline solution was added to the cells to form 50% hematocrit. Normal saline and Triton X-100 were used as controls for 0% and 100% haemolysis respectively. Synthesized nanoparticle formulations at various concentrations (0.5 mg/ml, 1mg/ml and 1.2 mg/ml) were incubated with the erythrocyte suspension at 37 °C for 60 minutes on a water bath. The reaction was stopped by addition of 25% (50 μl) Glutaraldehyde solution. The resultant dispersions were centrifuged at 3000 rpm for 15 minutes to remove the debris and final volume was made up. The supernatant was carefully separated and absorbance (A) of the supernatant was recorded at 540 nm by UV-spectrophotometric analysis (UV- JASCO Spectrophotometer) (Fig. 6.14). The percent haemolysis was calculated as

\[
\% \text{ Hemolysis} = \frac{\text{Absorbance of sample} - \text{Absorbance of control}}{\text{Absorbance of Triton X-100} - \text{Absorbance of control}}
\]

6.3. Results and Discussion

Surfactant free, biodegradable human serum albumin nanoparticles were developed in the present work using desolvation technique. Nanoparticles per se are synthesized with an aim to reduce the toxic effects of anticancer agents with enhanced cellular uptake and action. Hence, the prepared nanoformulations were deemed to be tested in human hepatocellular carcinoma cell lines to estimate their in-vitro effects in comparison to the pristine drug.

6.3.1. In-vitro cytotoxicity of bovine albumin nanoparticles by SRB assay

The optimized nanoparticulate formulation, drug and blank nanocarriers were subjected to cytotoxicity studies on HepG2 cells and the cell viability was tested after 48 hours as shown in Figs. 6.1, 6.2 and Table 6.2. The GI_{50} value of plain Cisplatin was found to be 3.6 μM. Blank bovine albumin nanoparticles exhibited a GI_{50} of more than 100 μM, indicating absence of
cytotoxicity of the placebo nanoformulations. The cytotoxicity of the developed formulations on HepG2 cells was found to be 49.5 μM, which was quite higher than that of pure drug. This was attributed to the sustained release properties of the nanoparticles, due to which they were not able to attain the cytotoxicity equivalent to that of Cisplatin in 48 hours.

The pictographic representation of cytotoxic assay is shown in Fig. 6.2. Fig. 6.2.a shows the image of plain HepG2 cancer cell line containing irregular shaped, clustered cells. It can be seen from the Fig. 6.2.b that Cisplatin exhibited cytotoxicity on the HepG2 cancer cell line as is evident from reduction in cell number, disruption in the cancer cell structure and their shrinkage. Cisplatin BSA nanoparticles (Fig. 6.2.d) also exhibited cytotoxicity on the cell lines but the effect was less as compared to the pure drug. This could be attributed to slow release of the Cisplatin from developed nanoparticles. Hence, the developed formulation was considered to be less cytotoxic as compared to the pure drug Cisplatin. Blank nanoparticles did not exhibit any cytotoxicity as seen from Fig. 6.2.c. This indicated that the drug loaded in the developed formulation was available for action against HepG2 cells but released in a slow manner to exhibit cell death.

Table 6.2: *In-vitro* cytotoxicity analysis of the developed BSA formulations

<table>
<thead>
<tr>
<th>Test Formulations</th>
<th>molar drug concentrations calculated from the graph</th>
<th>LC50</th>
<th>TGI</th>
<th>GI50</th>
</tr>
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<tr>
<td>Cisplatin</td>
<td>&gt;100</td>
<td>66.8</td>
<td>&gt;100</td>
<td>3.6</td>
</tr>
<tr>
<td>Blank BSA nanoparticles</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>CDDP BSA nanoparticles</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>49.5</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6.1: *In-vitro* cytotoxicity of BSA nanocarriers by SRB assay
6.3.2. *In-vitro* cytotoxicity of CDDP Gelatin nanoparticles by SRB assay

It can be seen from the Table 6.3 that Cisplatin showed cytotoxicity on the liver cancer cell line causing disruption in the cancer cell structure and their shrinkage. Also, Cisplatin gelatin nanoparticles exhibited cytotoxicity on the HepG2 cells, but the cytotoxicity was less as compared to pure drug. This could be attributed to slow release of the Cisplatin from developed gelatin nanoparticles. Blank nanoparticles do not exhibit any cytotoxicity as seen from Fig. 6.3. This suggested that the developed formulations exhibited sufficient cytotoxicity for treatment in liver cancer with concomitantly reduced toxicity of the nanoparticles and the probability of reduced formulation dosing frequency.
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Table 6.3: Cytotoxicity analysis of the developed Gelatin nanoformulations

<table>
<thead>
<tr>
<th>Test Formulations</th>
<th>μMolar drug concentrations calculated from the graph</th>
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<tr>
<td></td>
<td>LC₅₀</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Blank Gelatin nanoparticles</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CDDP Gelatin nanoparticles</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Fig. 6.3: SRB assay for gelatin nanoparticles

6.3.3. In-vitro cytotoxicity studies using MTT assay

Human serum albumin nanoparticles were taken further for detailed in-vitro analysis to determine their efficacy on human hepatocellular cell line with respect to Cisplatin. These studies comprised of MTT assay, Clonogenic assay, Wound scratch assay, Leighton tube assay, Cell cycle analysis, Confocal microscopy and Flow cytometry studies. Additionally, MTT assay and cellular uptake studies were also performed on B16F10 murine melanoma cell line to determine the selectivity of formulations for liver cancer.

6.3.3.a. MTT assay on the developed HSA nanoparticles of Cisplatin

Cisplatin loaded albumin nanoparticles were subjected to MTT assay to estimate the extent of cytotoxicity of the plain drug as well as drug loaded nanoparticles by correlating the values with
IC\textsubscript{50} obtained from the experiment. The IC\textsubscript{50} values for both HepG2 and B16F10 cell lines are as given in Table 6.4, Figs. 6.4 and 6.5. It was observed that Cisplatin was able to hamper the cell growth in a time and concentration dependent manner wherein almost 100% of the cells were killed after 96 hours. However, it can be seen from the table that the studied drug loaded albumin nanoparticles exhibited time and concentration dependent cytotoxicity on the both the cell lines under study in a controlled manner, which was slower as compared to pristine drug. This phenomenon was correlated to the dissolution profile of the formulations. Drug was found to release from the formulations in a sustained manner, with only a small proportion of drug being released at each time point. Hence, even after 96 hours, the IC\textsubscript{50} for pure drug and drug loaded nanoparticles did not match. The data warrants avoidance of repeated administration of Cisplatin and this would be of clinical significance because the developed formulations would prevent repeated administration of the active moiety and result in increased patient compliance.

Interestingly, it was seen that the 24, 48, 72 and 96 hour IC\textsubscript{50} values for all the tested drug loaded nanoparticles were significantly less for the hepatocellular carcinoma cell line as compared to B16F10 cell line (p<0.05). This could be substantial in proving the specificity of albumin nanoparticles towards hepatocellular carcinoma, rather than melanoma, due to which the cytotoxic action of the formulations was more on HepG2 cell line as compared to the murine melanoma cell line.

Formulations C, E and G were also tested in this assay to determine the cytotoxic potential of the placebo nanoparticles per se. It was observed that the IC\textsubscript{50} values for blank nanoparticles were not derivable, suggesting the absence of any cytotoxic potential of these formulations. This depicted the safety of polymers and other excipients used for fabricating the nanoparticles.

In case of studies on HepG2 cell line, it was observed that Cisplatin exhibited IC\textsubscript{50} values of 2, 0.4, 0.2 and 0.1 after MTT assay at 24, 48, 72 and 96 hours respectively. The low IC\textsubscript{50} values were associated with the high cytotoxicity and potency of this drug. Plain Cisplatin albumin nanoparticles exhibited higher IC\textsubscript{50} values of 10, 0.9, 0.8 and 0.7 μg/ml as compared to the pure drug for same time intervals, which was associated to sustained drug diffusion from the nanocarriers. The IC\textsubscript{50} values of ligand attached nanoparticles were compared with those of plain nanoparticles to determine the increase in selectivity to hepatocellular carcinoma due to active targeting (Fig. 6.6). It was seen that the 24 hours IC\textsubscript{50} for galactosamine anchored formulation
was higher as compared to the passively delivered albumin nanoparticles. However, beyond 24 hours, there was a decrease in the IC\textsubscript{50} values to 0.7, 0.2 and 0.1 μg/ml at 48, 72 and 96 hours respectively (p<0.05). This suggested that with passage of time, the formulation was more cytotoxic to the hepatoma cells, probably due to the enhanced entry in the cells due to faster internalization in the asialoglycoprotein receptors and enhanced activity on the liver cancer cells. This could prove the advantages offered by actively targeted polymeric formulations in treatment of cancerous cells. TG loaded formulations showed sufficient cytotoxicity on liver cancer cells where the IC\textsubscript{50} values were found to be 27.5, 12.5, 0.55 and 0.1 μg/ml at 24, 48, 72 and 96 hours respectively.

Based on the observations from MTT assay, the subsequent studies were performed with two inhibitory concentrations viz. IC\textsubscript{50} and IC\textsubscript{20} values for the developed albumin formulations. The IC\textsubscript{20} values were extrapolated from the graphs of % viability verses concentration for all the formulations under test.

Table 6.4: IC\textsubscript{50} values of developed albumin nanocarriers on HepG2 and B16F10 cell lines

<table>
<thead>
<tr>
<th>Formulation Codes</th>
<th>HepG2 cell line</th>
<th>B16F10 cell line</th>
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<tr>
<td></td>
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<td>48 hrs</td>
</tr>
<tr>
<td>A</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
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</tr>
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<td>27.5</td>
<td>12.5</td>
</tr>
<tr>
<td>G</td>
<td>ND</td>
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</table>

ND: Not determined  * Units for IC\textsubscript{50} values are in μg/ml
Chapter 6: In-vitro cell line and biocompatibility studies

(a) 24 hours MTT assay

(b) 48 hours MTT assay

(c) 72 hours MTT assay
Fig. 6.4: MTT assay on B16F10 cell lines for pure drug, blank and drug loaded formulations (a) 24 hrs (b) 48 hrs (c) 72 hrs (d) 96 hrs
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Fig. 6.5: MTT assay on HepG2 cell lines for pure drug, blank and drug loaded formulations (a) 24 hrs (b) 48 hrs (c) 72 hrs (d) 96 hrs

Fig. 6.6: Comparative evaluation of passively targeted verses ligand targeted albumin nanoparticles by MTT assay

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6.3.3.b. Colony formation assay on developed nanoparticles

Cancerous cells are prone to formation of colonies from a single cell due to their inherent property of uncontrolled division and proliferation. In the present work, clonogenic assay was performed at IC\textsubscript{20} and IC\textsubscript{50} concentrations of the formulations coded A to G.

A concentration dependent decrease in the formation of colonies was exhibited for both i.e. Cisplatin and Cisplatin loaded nanoparticles. Interestingly, the numbers of colonies were found to be less than 50 in number for both the above groups. Hence, the nanoparticle formulation was comparable in terms of efficacy to Cisplatin in inhibiting the cancer cell colony formation. Cancer cells show a self sufficiency towards growth signals. These results indicated that the cells lose their ability to replicate in the presence of Cisplatin and developed Cisplatin albumin nanoformulations. Cisplatin prevented the anchorage independent growth of HepG2 cells which was evident by the dose dependent decrease in the number of colonies in drug and formulation groups. CDDP acts on HepG2 cells by the growth arrest due to inhibition of p53 activation (Kai et al, 2013). The numbers of colonies formed in the blank formulation group were similar to the untreated control, due to absence of antineoplastic moiety and inherent cytotoxicity. The results for clonogenic assay are given in Figs. 6.7 and 6.8. Additionally, it was observed that the results for clonogenic assay were parallel for all the formulations viz. plain nanoparticles, TG linked and ligand attached nanoparticles. Hence, the nanoparticles were almost comparable to plain drug Cisplatin in hampering the clonogenic potential of HepG2 cells.

![Pictographic representation of clonogenic assay](image-url)
6.3.3.c. Wound scratch assay in HepG2 cells

Cellular migration is an important phenomenon in cancer progression since the metastasis of tumor occurs in this manner. In the present study, wound healing assay was performed at IC$_{20}$ and IC$_{50}$ concentrations to examine the potential of Cisplatin and the developed nanocarriers to inhibit the above process. However, satisfactory assay results could not be obtained due to the growth of HepG2 cells in form of clumps rather than monolayers, which was a peculiar property of HepG2 cells. Monolayer cell formation with discrete cells is essential to assess the migration of cells otherwise it leads to erroneous results.

8.3.3.d. Determination of cellular morphology by Leighton tube assay

Leighton tube assay was carried out for formulations A to G to assess the potential of nanosystems to cause changes in the cellular morphology of HepG2 cells compared to the pure drug. From Fig. 6.9, it can be seen that both the drug and drug loaded nanoformulations caused changes in cell structure, swelling of cells, spindle formation and consequent cell damage with equivalent efficacy. At lower concentrations of the drug and drug loaded formulations, shrinking of cells was seen with reduced number and at higher concentrations rounding of cells was observed. Additionally there was a decrease in the population of HepG2 cells in the treatment
groups compared to untreated control. However, blank nanoparticles acted parallel to the control group and did not depict any morphological changes.
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Fig. 6.9: Changes in the HepG2 cellular morphology by treatment groups
(a) Untreated control (b) A1 IC20 (c) A2 IC50 (d) B1 IC20 (e) B2 IC50 (f) C1 IC20 (g) C2 IC50
(h) D1 IC20 (i) D2 IC50 (j) E1 IC20 (k) E2 IC50 (l) F1 IC20 (m) F2 IC50
(n) G1 IC20 (o) G2 IC50

Pictographically, it can be observed that plain albumin nanoparticles and ligand attached nanoparticles were more effective in killing the cancer cells than TG linked nanocarriers, which was well in correlation with the results of the MTT assay. Also, since this assay was performed for 48 hours as per the standard protocol, it was possible to observe the morphological changes upto this point only.

6.3.3.e. Cell cycle analysis using flow cytometry

Cisplatin per se is known to induce apoptosis in cells by activation of Fas ligand on the tumor cell surface, by induction of caspase-3 or by destabilization of mitochondria caused due to reactive oxygen species generation (Alas et al, 2002). There is evidence that Cisplatin and
Cisplatin based therapeutics can interfere in the cell cycle progression of cancer cells. This drug is known to induce cell death either by inducing cytostatic effect or by delaying the progression of cells in cell cycle. Alkylating agents like Cisplatin are also known to cause programmed cell death by inhibiting the cells in G2 phase of cell cycle (Cepeda et al, 2007).

These results were reinstated by the present study wherein dose dependent increase in Cisplatin induced G2/M phase arrest was seen in HepG2 cells. In case of pure drug, with increase in dose, the number of cells in S phase were found to reduce with more number of cells in G0-G1 and G2 phase. Similar G1 cell arrest has also been observed with Cisplatin in p53 defective HeLa cells (Koprinarova et al, 2010).

However, in case of nanoparticle treated groups, the percentage of cells were more in all the phases of cell cycle, with more amount of accumulation in S phase, with increasing concentration of the formulations. The developed nanoparticles also exhibited dose dependent increase in the G2/M phase leading to cell death. The percentage of cells in the G2/M phase for the pure drug and nanoparticles were found to be significantly higher than the control group, indicating greater G2/M phase arrest. Also, the percentage of cells in G2/M phase were found to be less for nanoparticles than the pure drug group probably because of sustained release of drug from nanoparticles, leading to slower rate of action. Additionally, the concentration of developed nanoparticles was seen to increase in the S phase in nanoparticle formulation IC50 groups, suggesting their ability to hinder the production of DNA in S phase of cell cycle in addition to the cell division process. Hence, the developed nanoparticles were found to be superior to pure drug by acting on different phases of cell cycle. Results for cell cycle analysis are presented in Figs. 6.10 and 6.11.
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Fig. 6.10: Cell cycle analysis by Flow cytometry (a) Untreated control (b) A1 IC_{20} (c) A2 IC_{50} (d) B1 IC_{20} (e) B2 IC_{50} (f) D1 IC_{20} (g) D2 IC_{50} (h) F1 IC_{20} (i) F2 IC_{50}
6.3.3.f. Quantitative cellular uptake by confocal microscopy

In this particular study, the nucleus of the cells was stained using DAPI (blue coloured dye staining the nucleus) and FITC (green coloured dye just surrounding the cells) in overlay images. FITC alone is not able to transverse the cells due to its hydrophilic nature (Fig. 6.12). However, when the nanoparticles were loaded with this particular dye, fluorescence was observed in the B16F10 and HepG2 cells indicating that nanoparticles were able to enhance the penetration of FITC in cancer cells. These results indicate that nanoparticles are able to modulate the properties of antineoplastic drugs and enhance their uptake in cancer cells.

Additionally, the uptake of plain albumin nanoparticles (Formulation B) in HepG2 cells was more than B16F10 cells indicating the probable specificity of the formulations for hepatocellular carcinoma and not any other cancer. Similarly, the uptake of ligand attached nanoparticles (Formulation D) was more in HepG2 cells as compared to plain albumin nanoparticles. Hence, the uptake of nanocarrier formulations in HepG2 cancer cell lines was in the following order

\[
\text{B16F10 melanoma} \quad \text{<} \quad \text{HepG2 cells} \\
\text{Plain albumin nanoparticles} \quad \text{<} \quad \text{Ligand attached nanocarriers}
\]
**Fig. 6.12:** Qualitative cellular uptake of nanoparticles by confocal microscopy

(a) B16F10 cells (b) HepG2 cells
The cellular uptake of TG linked formulations was similar to that of plain albumin formulations, probably due to the similarity in the preparation of formulation and absence of targeting moiety in the formulations.

Hence, the results for this particular study exemplified better cellular uptake of nanoparticulate formulations as compared to pure drug due to their small size and surface properties. It also emphasized on the advantage of utilizing a targeting ligand for enhanced cancer cell activity and uptake in cells. The specificity of developed formulations for HepG2 cell line in comparison to B16F10 cell line was also illustrated by qualitative cell uptake study, which was also supported by the results from MTT cytotoxicity assay.

6.3.3.g. Qualitative cellular nanoparticles uptake by flow cytometry studies

Flow cytometric analysis was performed to evaluate the uptake of developed nanoparticles in HepG2 cells (Fig. 6.13). This study was performed only for plain albumin nanoparticles and their actively targeted counterpart.

Mean fluorescence intensity (MFI) of the plain albumin nanoparticles at a concentration of 2.5 μg/ml for 15 minutes was found to be 28.67 units, which was enhanced to 47.89 units after 30 minutes. Similarly, the MFI was 53.79 units and 91.26 units for similar time points at a concentration of 5μg/ml. Hence, both the albumin nanoformulations were uptaken in the hepatoma cells in a time and concentration dependent manner.

Parallel results were also observed for the ligand attached albumin nanoparticles where the MFI was found to increase with time and concentration of the formulations. However, it was seen that the ligand attached formulations showed better uptake potential as compared to plain nanoparticles. The obtained results were well in correlation with the earlier findings using confocal microscopy.

Hence, the developed albumin nanoformulations were readily taken up by the hepatoma cells and would prove effective for the therapy of hepatocellular carcinoma.
### Key
- HepG2 cells only
- Plain Nanoparticles 2.5 μg/15 min
- Plain Nanoparticles 2.5 μg/30 min
- Plain Nanoparticles 5 μg/15 min
- Plain Nanoparticles 5 μg/30 min
- Ligand Nanoparticles 2.5 μg/15 min
- Ligand Nanoparticles 2.5 μg/30 min
- Ligand Nanoparticles 5 μg/15 min
- Ligand Nanoparticles 5 μg/30 min

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**Fig. 6.13: Quantitative cellular uptake by flow cytometry**

### 6.3.4. *In-vitro* hemolytic assay for the developed nanoparticles

The results for hemolysis assay are given in Fig. 6.14. It was observed that at the highest concentration (1mg/ml) studied, the average % hemolysis values for TG enzyme, albumin and ligand galactosamine were 2.46, 2.66 and 2% respectively, indicating their safety for intravenous administration. The % hemolysis results for the marketed injection and pure solution were found to be 6.99 and 6.25% respectively indicating the hemolytic potential of these formulations was probably due to free drug present in the preparation. Nanoparticles contain the drug entrapped in polymer matrix and hence are expected to exhibit less hemolysis than the marketed preparations.
due to slower release of the drug from polymer matrix. It was seen that the plain albumin nanoparticles, ligand attached nanoparticles and TG albumin nanoparticles at the highest dose exhibited hemolysis values of 4.75 %, 4.28 and 5.19% respectively, which were less than those obtained for marketed preparation, although not statistically significant. Hence, the developed nanoparticles were found to be safe for intravenous administration due to low hemolysis values.

Fig. 6.14: *In-vitro* hemolytic assay for the developed nanoparticle formulations

6.4. Conclusion

Cisplatin is known to exhibit short term action on hepatoma due to its short half life and side effects. However, when this drug is encapsulated in albumin nanoparticles, the side effects of the drug are expected to reduce with better penetration into the hepatocellular carcinoma with enhanced cell uptake. This hypothesis was proved by various *in-vitro* studies like MTT assay, colony formation assay, morphology studies, cell cycle analysis and cellular uptake studies for the developed albumin formulations.

Results from *in-vitro* studies confirmed the availability of drug from the nanoformulations with time dependent cytotoxic action on hepatoma. The sustained release of Cisplatin for 96 hours from albumin nanocarriers lead to this particular phenomenon, causing prolonged cytotoxic action on HepG2 and B16F10 cells. As seen from the results of *in-vitro* studies, developed nanoparticles of albumin were found to be selectively cytotoxic to HepG2 cells.
Ligand targeted formulation was found to be superior to plain nanoparticles in terms of its cytotoxic potential and uptake in hepatoma cells, thus confirming the advantages offered by active targeting of drug moieties. In the cell uptake studies, most of the fluorescence was observed in the nucleus for this formulation, thus pointing the ability of ligand to cause transit of the nanoparticle formulations at cellular level. Overall the in-vitro efficacy exhibited by the Cisplatin loaded nanoparticles was in the following order

**Ligand attached nanoparticles > Plain nanoparticles > TG linked nanoparticles**

The above results suggest the ability of nanoparticles to cause a reduction in the dose of antineoplastic agents, with enhanced cellular activity and targeting potential. Additionally, developed nanocarriers were found to be biocompatible and safer than the pristine drug due to their low hemolytic potential, indicating suitability of developed polymeric nanoparticles for intravenous administration.