9.IN VIVO STUDIES

9.1 INTRODUCTION

Preclinical study models on particular animal are a game changer in study of tumour biology and response to our drug or formulation. Animal models are enlightened the target specificity and biodistribution of the drug molecule (1). In the verge of understanding and curing a diverse types of cancer, Animal modeling breakthroughs in the field of genetic engineering and offers tremendous strength to potentiate the informative role of in-vivo studies.

Most investigation of nanoparticulate systems has relied on parental route of administration to achieve targeted delivery to the lung, however the direct administration of LPHNPs into the airways has the advantage of circumventing systemic dilution and removal by other tissues and organs (2). In order to compare preparations on an equivalent basis during animal studies, it was necessary to ensure that in each case 100 percent of the total dose was delivered directly to the lung (3). The lung tissue very well takes HNCs, after Intratracheal administration so giving a bolus dose of the preparations at the bifurcation of the trachea will meet this requirement.

9.2 MATERIALS AND METHODS

All experiments and protocol described in the present study were approved by the Institutional Animal Ethical Committee (IAEC) of Maliba Pharmacy College, UkaTarsadia University, Bardoli wide protocol MPC/IAEC/02/2019 and with permission from committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

9.2.1 Intratracheal Instillation

The Intratracheal instillation of Cisplatin HNCs and cisplatin marketed formulations were carried out by well adapted method (4). Albino rats were selected for study because of the ease in their availability, handling and sampling. The study was carried out as accordance with the guidelines for the care and use of laboratory animals as adopted and promulgated by the animal ethics Committee. Animals will be anesthetized using urethane IP (1.2 mg/kg). Anaesthetized animals will be placed in

supine position on a 45 slanted support and a small middle incision will be made over trachea. The Trachea was exposed by blunt dissection of the sternohyosdeus muscle. A small hole will be made in the trachea between the fifth and the sixth tracheal rings using a 20-gauge needle. After suturing, Neosporine antibiotic powder is applied on surgical area to prevent microbial infection. A short (10 to 15 cm) length of PE50 tubing will be inserted into the hole and advanced to the bifurcation of the trachea. Drug formulation will be slowly instilled over a 1 min period using a 1 ml syringe attached to the PE50 tubing. Following instillation tubing will be withdrawn and a small drop of cyanoacrylate adhesive will be placed over the hole to seal the opening. The skin will be clothed with 3-0 Dexon sutures.

Animal will be removed from anesthesia and allowed to recover under a heating lamp. The rats were given amoxicillin (40 mg/kg) to prevent any infection and tramdolol to prevent postoperative pain every 4-6 hrs for 2 days. After recovery, animals will be housed in individual plastic cages with the access to food and water for the remainder of the study.

9.2.2 Broncho alveolar lavage (BAL) and Lung homogenate (LH)

Broncho alveolar lavage (BAL) was performed on anaesthetized and recannulated (as necessary) animals with 12ml PBS, pre-warmed to 37°C. For performing the lavage the Hamilton syringe connected to the PE50 tubing was replaced with a 3-way stopcock attached with two 20ml syringes. The tubing was reinserted through the cannula and advanced till the tracheal bifurcation. Fluid (PBS) was slowly injected into the lung via one syringe and then BAL withdrawn by gentle aspiration via the other (5). This BAL yielded between 7 to 11 ml liquid, which was centrifuged at 4.38 x 103 x g for 5 min. the supernatant was mixed with 10% Triton - x-100 in a ratio of 9:1 respectively to dissolve the HNCs (6) if required with the aid of gentle warming. It was then extracted and assayed by HPLC method for Cisplatin (Chapter 3).The lungs and the portions of tracheal below the instillation site were excised and homogenized (LH) in 10ml PBS containing 1% Triton-X-100 and the diffused drug was analyzed.

The drug concentration in the lung is the drug estimated in Lung homogenate (LH) and BAL fluid and pulmonary pharmacokinetic parameters were calculated on the basis of following definitions:

- **Tmax:** The time point at which maximum drug concentration is attained in lung homogenate (i.e. the time interval of Cmax).
- **AUC_{total}**: The area under the curve of drug concentration in lung vs. time, over the period of study (12hrs).
- **$t_{1/2}$** : Pulmonary half-life of drug is calculated by:

  1. Calculating the sum of the values of drug concentration in BAL and LH at individual sampling points.

  2. Regressing the calculated sum over the entire duration of study.

  3. Deriving the time point at which the sum of drug level is 50%compared to instilled quantity (i.e. deriving the median of the regression line).

9.2.3 L/B ratio, LDH and ALP estimation

Lung tissue was harvested as per the standard procedure for the organ removal with the exception that perfusion to complete exsanguination utilized only 3 ml of sterile, normal saline (7). Right lung was sharply separated from the bronchus, heart, thymic tissue, trachea, surgical clip if present, and any other adherent clot or fibrinous exudate. Isolated right lung tissue was weighed on a calibrated scale and theratio of lung weight to body weight (lung weight/body weight) was calculated

From the stored BAL fluid the enzymatic activities of lactate dehydrogenase (LDH) and Alkaline phosphatise (ALP) is estimated using commercial available kits from Abcam, Mumbai. Enzyme level in the BAL fluid is reported as fold increase compared to saline treated groups. Weights of lungs were reported as g/100 g body weight.

9.2.4 Histopathological examination of lung

Once BAL fluid was recovered lungs were inflated with the use of 2 ml phosphate buffer saline. Tissues were excised and it is fixed with 10 % formalin solution then embedded in paraffin. Various sections were cut using apparatus called microtome from MICROM. Sections are mounted on slides and then it is stained with haematoxylin and eosin. The sections were observed in inverted phase contrast microscope and it is processed by NIS software.

9.2.5 Acute Toxicity Study

In vivo acute toxicity studies on animals are an essential part of drug development process. Such acute toxicity studies are carried out for various objectives i.e.

- ✓ To determine the Median Lethal Dose (LD50) after a single dose administered through one or more routes, one of which is the intended route of administration in humans.
- ✓ To determine Maximum Tolerated Dose (MTD) and No Observable Effect Level (NOEL).
- ✓ To identify potential target organs for toxicity, determine reversibility of toxicity, and identify parameters for clinical monitoring.
- ✓ To help select doses for repeated-dose toxicity tests.

A number of methods are available to have an insight about the acute toxicity of any chemical or drug product. These include classical Litchfield and Wilcoxon method (Dosing of animals of both sex with increasing amounts of chemical and plotting dose-response curve to determine LD_{50}/MTD). This type of study has a disadvantage that it uses a large number of animals. So two methods are available now as alternatives which reduces the use of animals i.e. Fixed Dose Procedure (FDP) and Up-Down Procedure (UDP). Both methods produce data consistent with classical LD_{50} methods.

Among these methods Up-Down procedure requires the least number of animals (6-10) of single sex and provides results in terms of LD_{50} along with data for the hazard classification system, unlike FDP that does not estimate results in terms of LD_{50} value. Instead FDP gives better evaluation of the maximum tolerated dose of drug/drug product.

MTD of a drug can be defined as the highest dose of a drug or treatment that does not cause unacceptable side effects. The maximum tolerated dose is determined in clinical trials by testing increasing doses on different groups of people until the highest dose with acceptable side effects is found. Toxicity parameters to be considered include,

1. Mortality
2. Clinical pathology
3. Gross necropsy
4. Weight change
5. Signs of toxicity – convulsions, rashes, akinesia, licking, tremors

Drug doses at or below this level should not induce

- ✓ Overt toxicity, for example appreciable death of cells or organ dysfunction,
- ✓ Toxic manifestations that are predicted materially to reduce the life span of the animals except as the result of neoplastic development or
- ✓ 10% or greater retardation of body weight gain as compared with control animals.

In some studies, toxicity that could interfere with a carcinogenic effect is specifically excluded from consideration. For determination of MTD of siRNA loaded HNCs, fixed dose procedure of OECD Organization for Economic Cooperation and Development was used. Typical protocol includes administration of a drug/drug product in escalating doses through intravenous route and observing animals for any signs of toxicity.

9.2.6 Haemolytic study

9.2.6.1 Haemolytic study by UV method

Fresh blood from rats was collected in a heparin-coated tube. Erythrocytes were isolated from the heparinized human blood by centrifugation (2500 rpm for 15 min). The supernatant was discarded and the settled erythrocytes were re-suspended in phosphate buffer saline (pH 7.4). The mixture was centrifuged again, and precipitates (red cells) were rinsed again and again until the upper fraction of the liquid became transparent. The purified erythrocytes were then re-suspended in normal saline to obtain 2% (v/v) of the RBC suspension. Thereafter, 1.8 ml of the erythrocyte suspension was incubated with 0.2 ml of tested samples at 37°C for 30 min in an incubator shaker and then centrifuged at 2500 rpm for 5 min. Control samples of 0% lysis (in saline) and 100% lysis (in SLS containing double distilled water) were employed in all experiments. The percent hemolysis was measured by UV–Vis spectroscopic analysis of the supernatant at the wavelength of 545 nm. The mean value of three measurements using different samples was recorded. The percent hemolysis was calculated using equation below

$$\% \text{ Hemolysis} = \frac{(ABS - ABS_0)}{(ABS_{100} - ABS_0)} \text{ X } 100$$

9.2.6.2 Haemolytic study by Optical method

Fresh blood from rats was collected in a heparin-coated tube. Erythrocytes were isolated from the heparinized human blood by centrifugation (2500 rpm for 15 min). The supernatant was discarded and the settled erythrocytes were re-suspended in phosphate buffer saline (pH 7.4). The mixture was centrifuged again, and precipitates (red cells) were rinsed again and again until the upper fraction of the liquid became transparent. The purified erythrocytes were then re-suspended in normal saline to obtain 2% (v/v) of the RBC suspension. Then it is subjected to optical microscope and Shape of RBC is observed.

9.3 RESULTS AND DISCUSSION

9.3.1 Concentration of drug in LH and BAL

The in vivo evaluation was carried out by the estimation of the concentration of drug in BAL and LH after the administration of rehydrated HNCs formulations and of plain drug. The dose of 750 μg was intra-tracheally instilled. Amount of drug present in the LH was considered as the drug absorbed and available for the pharmacological response and the amount of drug present in the BAL was considered as drug not absorbed into the lung tissue but still retained in the bronchial spaces (in HNCs encapsulated form). Later represents a reservoir of drug that eventually would be absorbed by the lung tissue.

Table 9-1 Concentration of drug in Lung homogenate and BAL after intratracheal instillation

	Marketed formulation			**HNCs (R4)**		
Time (hrs)	**BAL (ng/ml)**	**Lung homogenate (ng/ml)**	**Total Concentration of drug in lungs (ng/ml)**	**BAL (ng/ml)**	**Lung homogenate (ng/ml)**	**Total Concentration of drug in lungs (ng/ml)**
2	237.12 ±2.79	415±4.89	652±8.94	655± 11.78	291±8.48	946±2.79
4	101 ±1.68	211±3.74	312±11.58	547± 9.28	423±14.83	1061±7.42
8	ND	97	97±5.83	361± 17.49	469±11.69	784±8.28
10	ND	ND	-	223± 16.94	551±15.93	774±17.38
12	ND	ND	-	112±	237±6.93	349±13.26

ND-Not detected & (Values are reported as Mean±SD)

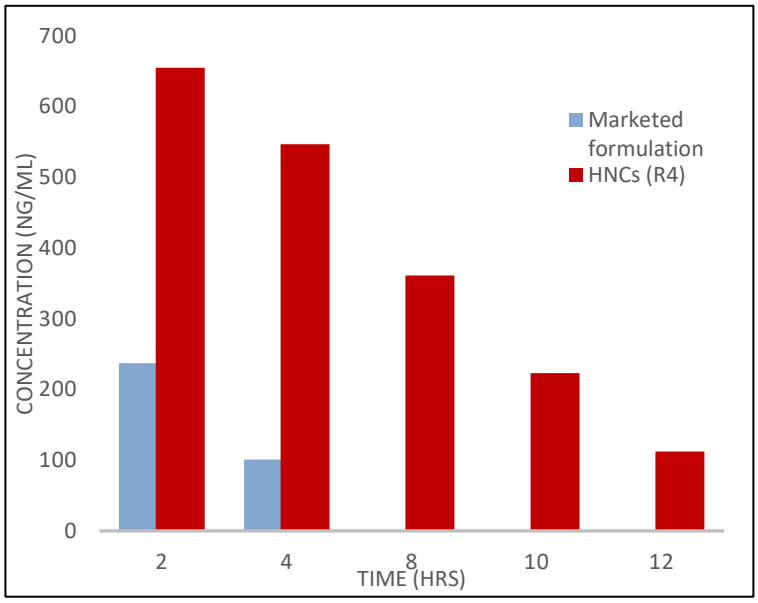


Figure9-1 Cisplatin concentration in Broncho alveolar lavage (BAL)

at different time interval

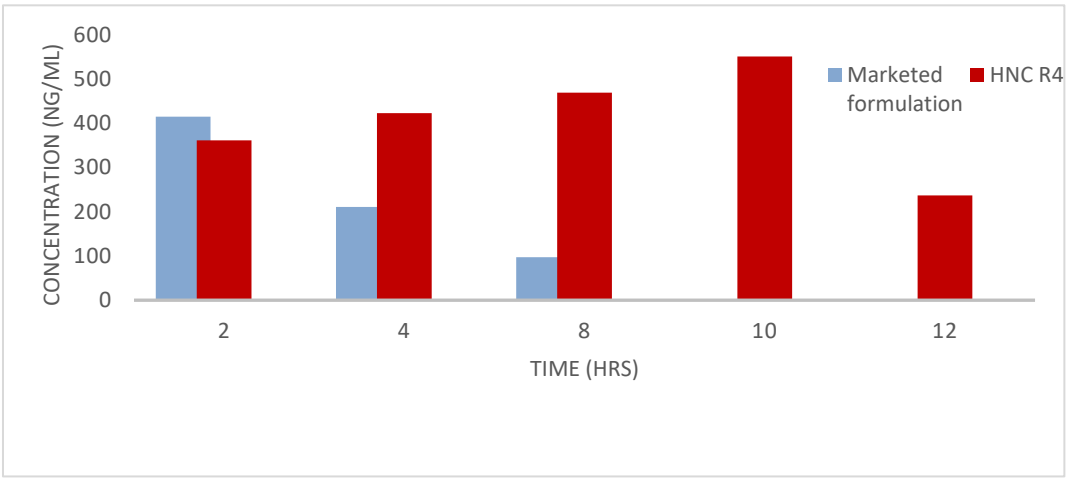


Figure9-2 Cisplatin concentration in Lung homogenate at different time interval

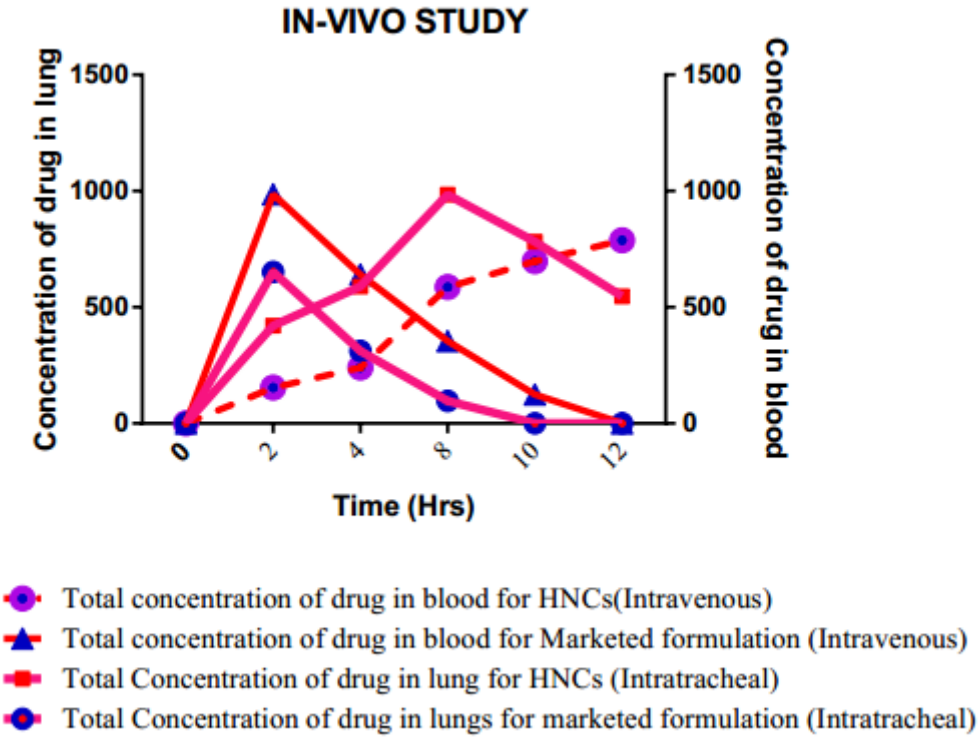


Figure 9-3Total concentration of drug in blood and lung (ng/ml) with different formulations at different time interval

Table 9-2Pulmonary and IV Pharmacokinetic Parameters

Formulation	AUCtotal (ng.h/ml)	Cmax (ng/ml)	Tmax (h)	$t_{1/2}$ (h)	MRT (h)
Marketed formulation (intra tracheal)	4619±121.79	652±5.69	2±0.05	8.06±0.1	4.40±0.16
HNCs R4 (intra tracheal)	11314±95.38	1061±9.27	12±0.1	2.20±0.16	11.41±0.07
Marketed formulation (intravenous)	11257±78.47	1220±14.83	2±0.2	1.11±0.08	3.45±0.17
HNCs R4 (intravenous)	8924±58.28	988±27.94	8±0.28	2.20±0.1	6.54±0.13

The pulmonary pharmacokinetic parameters were calculated and are represented in Table 9.2.A maximum t½ value of 8.06 hours was observed with Cisplatin HNCs compared to 2.20 hours with marketed formulation. Eventually, there was an increase in AUC for HNCs compared with the AUC of Marketed formulation. HNCs showed 2.44 times higher AUC values than marketed formulation. The Tmax values for HNCs was 4hours compared to marketed formulation was 2 hours, thereby confirming the maintenance of effective drug concentration with HNCs in lung tissue for prolonged period compared to marketed formulation

9.3.2 L/B ratio, LDH and ALP estimation

The weight of the lungs was normalized to 100 g of body weight for the purpose of comparison. For the saline treated animals L/B ratio were obtained 0.41 while the L/B ratio for the Lipopolysaccharides were noted as highest due to accumulation of extracellular fluids in mucosa of respiratory cells which shows oedema. L/B ratio is lowest for the HNCs R4 formulation which shows highest activity of formulation aginst NSCLC. As expected Activities of LDH and ALP is highest in Lipopolysaccharides model and developed Cisplatin caprylate shows similar activity when compared to saline control. Graph of the activities and L/B ratio is shown in figure 9.4. In figure 9.4,Y1 axis shows fold increase in activity of LDH and ALP where Y2 shows ratio of gm wt. of lung/100 gm of body weight.

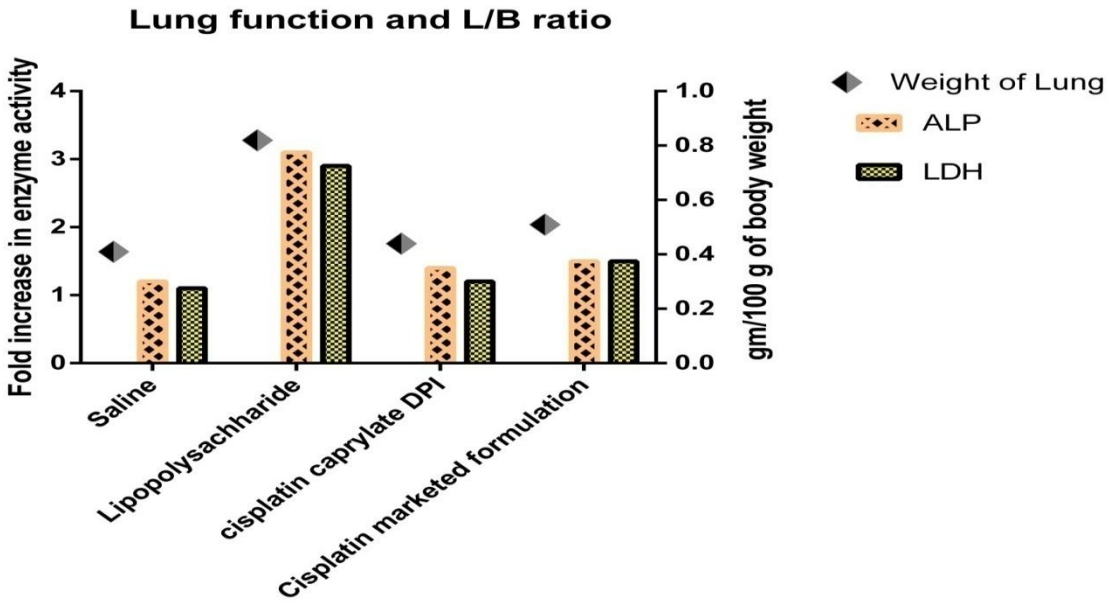
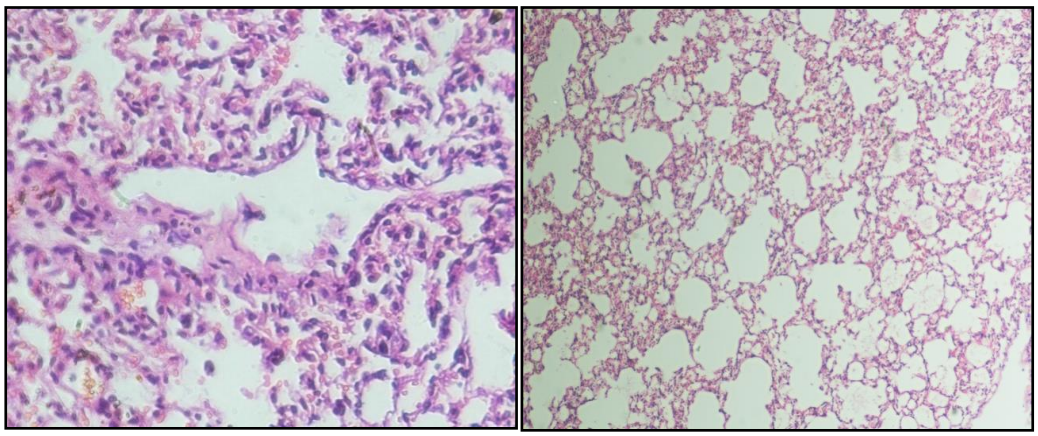


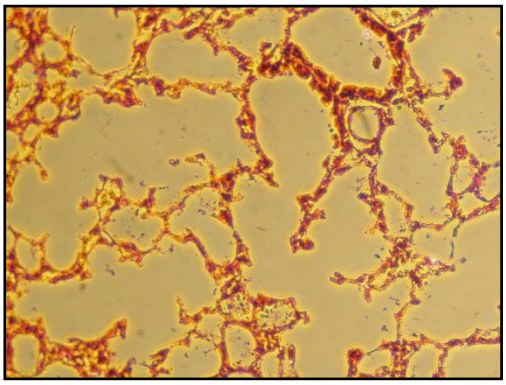
Figure 9-4enzyme activity and L/B ratio

9.3.3 Histopathological images

Histopathological studies were performed to determine the toxicity of Cisplatin caprylate DPI to the local lung tissues. Lipopolysaccharides shows high level of inflammation which is clarified by infiltrated leukocyte migration in bronchial epithelial tissues and degeneration of epithelial tissues caused by with necrosis and exudation. On other hand saline control and formulation treated samples doesn't shows any inflammatory infiltration. Images of histogram are shown in Figure 9.5.



Saline control HNCs P2



LPS positive control

Figure 9-5 histopathological images

9.3.4 MTD Estimation

Single animals were dosed in sequence usually at 48 hr interval. The first animal was dosed at a level selected from the sighting study. A period of at least 24 hr was allowed between the dosing of each animal. All animals were observed for at least 14 days for any signs of toxicity. If the animal survived, the second animal received a higher dose. If the first animal died or appeared moribund (Moribund status: being in a state of dying or inability to survive, even if treated), the second animal was administered a lower dose. Animals were euthanized by intraperitoneal injection of pentobarbital (50 mg/ml) after study or if moribund status (inability to ambulate, inflammation, anorexia, dehydration, or more than 20% weight loss) was observed. The weight of each animal was recorded immediately before intravenous injection, 1 day after injection, and at the end of study.

Table 9-3MTD Study: Dosing Protocol

Sr.No.	Group No.	Formulation	Dose
1	1	Normal saline	-
2	2	Cisplatin marketed solution	5 mg/kg of cisplatin
3	3A,3B,3C	Cisplatin caprylate HNCs	0.75 mg/kg of siRNA+5 mg/kg of cisplatin
4	4A,4B,4C	Cisplatin caprylate DPI (HNC R4)	0.75 mg/kg of siRNA+5 mg/kg of cisplatin

Table 9-4Results of MTD study

Sr.No	Group No	Weight (g) (Mean±SEM)			Observation	
		Initial	After 1 day	After 14 days	Toxicological Signs/symptoms	Mortality
1	1	30.1±0.13	29.5±0.15	28.4±0.14	None	None
2	2	29.2±0.12	30.1±0.25	29.4±0.16	None	None
3	3A	29.5±0.21	28.5±0.14	28.4±0.25	None	None
4	3B	32.4±0.24	30.4±0.19	29.1±0.18	None	None
5	3C	31.5±0.31	31.1±0.18	31.5±0.31	None	None
6	4A	29.7±0.25	28.9±0.34	29.5±0.19	None	None
7	4B	30.4±0.31	29.4±0.28	30.4±0.27	None	None
8	4C	31.8±0.22	30.4±0.23	31.2±0.26	None	None

All groups showed no sign of toxicity after administration of test substance. In all groups MTD values were considered as greater than maximum administered dose i.e. >0.75 mg/kg of siRNA for both; HNCs R4 and HNCs R3. For Cisplatin MTD was greater than administered dose of 5mg/kg.

9.3.5 Haemolytic study

Haemolysis was measured in using above mentioned method. From the results one can see that haemolytic properties of optimized formulation are below marketed Cisplatin injection. Comparative graphs of the haemolytic study are shown in figure. Even optical method confirms that the shape and concentration of the RBCs in the HNC R4 and marketed formulation are almost same.

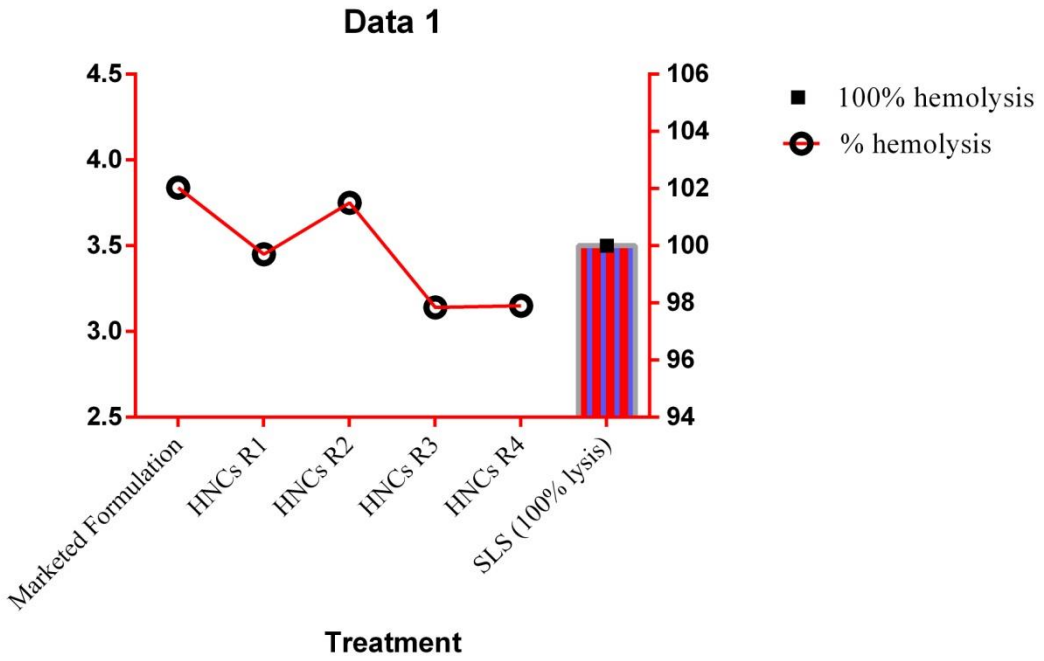


Figure 9-6Haemolysis by UV spectroscopy

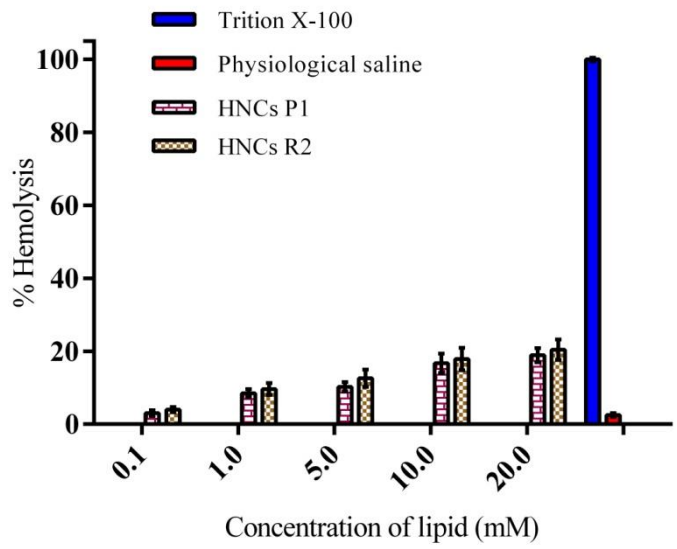
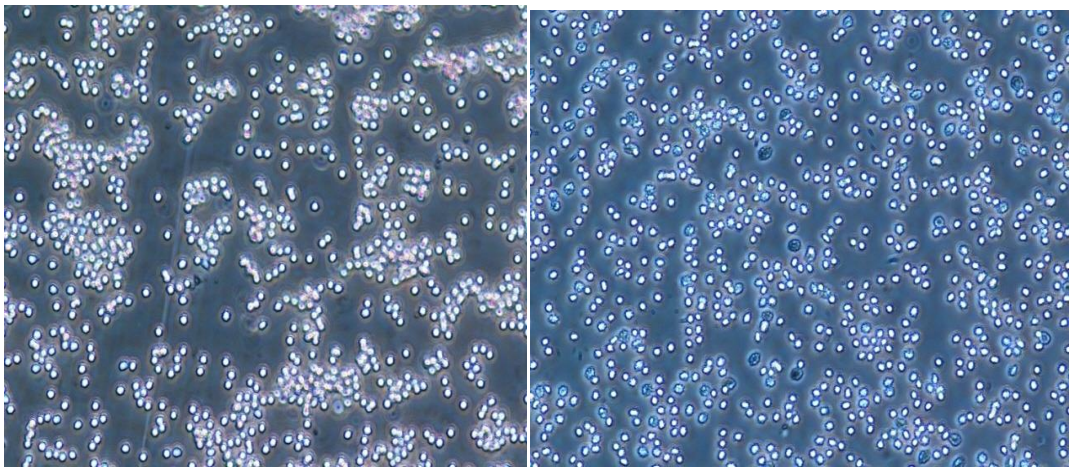
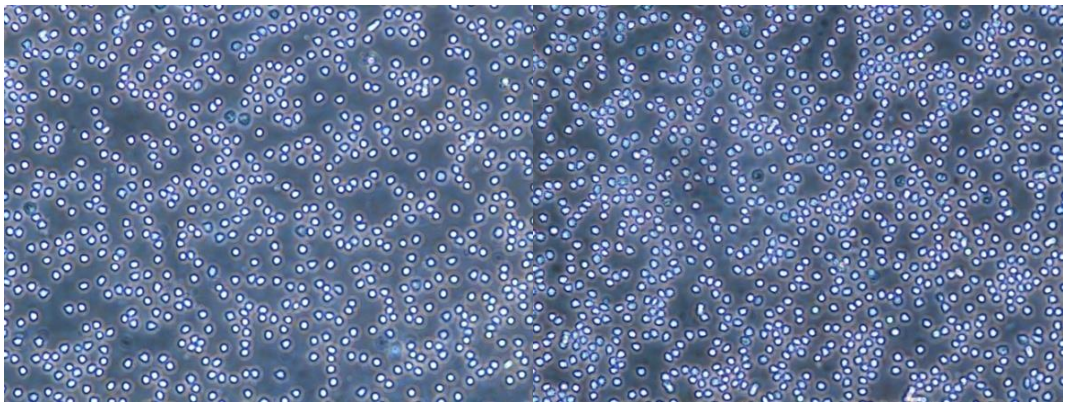


Figure9-7 Hemolytic aggregation study



Triton x100 Physiological saline



HNCs P1 HNCs R2

Figure9-8Haemolysis by Optical method

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