CHAPTER 5: IN VIVO TOXICITY STUDY

Contents

List of Table

5.1. 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.

1. To determine the Median Lethal Dose (LD_{50}) after a single dose administered through one or more routes, one of which is the intended route of administration in humans.
2. To determine Maximum Tolerated Dose (MTD) and No Observable Effect Level (NOEL).
3. To identify potential target organs for toxicity, determine reversibility of toxicity, and identify parameters for clinical monitoring.
4. 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) [1] and Up-Down Procedure (UDP) [2]. Both methods produce data consistent with classical LD_{50} methods [3, 4]. 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 [5]. 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 [6]

- 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 gemcitabine loaded liposomes, fixed dose procedure of OECDOrganization 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.

5.2. Description of the Methods

All experiments and protocol described in the present study were approved by the Institutional Animal Ethical Committee (IAEC) of Pharmacy Department, The M. S. University of Baroda 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.

5.2.1. Selection of Animals Species

Female Swiss Albino mice were used for the study as females are generally slightly more sensitive to such studies [4]. Healthy young adult animals (with 8-12 weeks age) which were nulliparous and non-pregnant were used for study.

5.2.2. Housing and Feeding Conditions

The temperature in the animal room was 20-25°C. Artificial lighting with the sequence of 12 hr light and 12 hr dark was kept in animal housing. The animals were housed individually. For feeding, conventional rodent laboratory diets was used with an unlimited supply of drinking water.

5.2.3. Preparation of Animals

The animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to dosing for acclimatization to the laboratory conditions.

5.2.4. Preparation of Doses

Test substances (Gemcitabine loaded Liposomes) were administered in a constant dose volume of 20 mL/kg by varying the concentration of the dosing preparation. (The dosing volume was chosen such that the volume did not exceed 2 mL/100g bodyweight). All doses were prepared prior to administration. Above certain dose, only liposomal carrier was tested to ascertain the safety profile of developed liposomal carrier systems.

Gemcitabine loaded lyophilized liposomal formulation was reconstituted with sufficient quantities of normal saline to produce gemcitabine concentrations desired for administration. All the test substances were sterilized by filtering through 0.2 µ membrane filter prior to administration.

5.2.5. Procedures

5.2.5.1. Administration of Doses

The test substances were administered via tail vein of animals using sterile single use disposable polystyrene syringes.

5.2.5.2. Sighting Study

The purpose of the sighting study was to allow selection of the appropriate starting dose for the main study. The test substance was administered to single animals in a sequential manner starting from DOSE first to DOSE last. The sighting study was completed when a decision on the starting dose for the main study was made (or if a death is seen at the lowest fixed dose).

The starting dose for the sighting study was selected from the fixed dose levels as described in Table 6.1. Starting dose selection was obtained from the available literature showing toxicological data for specific chemicals.

5.2.5.3. MTD Determination

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.

5.2.5.4. Numbers of Animals and Dose Levels

1. The action to be taken following testing at the starting dose level is indicated based on the observations. One of three actions will be required; either stop testing and assign the appropriate hazard classification class, test at a higher fixed dose or test at a lower fixed dose. However, to protect animals, a dose level that caused death in the sighting study was not revisited in the main study.

2. A total of five animals of female sex were used for each dose level investigated. The five animals were made up of one animal from the sighting study dosed at the selected dose level together with an additional four animals.

3. The time interval between dosing at each level was determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose was delayed until there was confidence of survival of the previously dosed animals. A period of 3 or 4 days between dosing at each dose level is recommended, if needed, to allow for the observation of delayed toxicity. The time interval may be adjusted as appropriate, e.g., in case of inconclusive response.

5.2.5.5. Observations

Animals were observed individually after dosing at least once during the first 30 min, periodically during the first 24 hr, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they needed to be removed from the study and humanely killed for animal welfare reasons or were found dead.

Observations included were changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern. Attention was directed to observations of tremors, convulsions,

salivation, diarrhea, lethargy, sleep and coma. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress were humanely killed.

Loss of weight, if more than 20% of initial, or death of animal was considered a positive response at short term outcome (during first 24 hr). For long term outcome death was used as a termination point to stop the test. The duration of observation was determined by the toxic reactions, time of onset and length of recovery period. The times at which signs of toxicity appear and disappear were considered important, especially if there was a tendency for toxic signs to be delayed [7]. All observations were systematically recorded, with individual records being maintained for each animal.

5.3. Results and Discussion

Liposomal formulations, RGD-grafted liposomes was administered intravenously to the female Swiss Albino mice with and without gemcitabine loading as given below (**Table 6.1**) during sighting study in single mice. Mice showed symptoms of toxicity at 125 mg/kg of Gemcitabine and hence 100 mg/kg was selected as MTD. At the dose 100 mg/kg of gemcitabine, lipid concentration was found to be about 1500 mg/kg forgrafted liposomes. Hence, to determine the starting dose for liposomal carrier only, 1500 mg/kg (RGD-Graftedliposomes) of lipids were used.

TABLE 5 1. Sighting Study: Dosing protocol

Sr.No.	Formulation	Dose (mg/kg)			
1.	RGD-Grafted liposomes	50 mg/kg of gemcitabine	75 mg/kg of gemcitabine	100 mg/kg of gemcitabine	125 mg/kg of gemcitabine -
2.	RGD-Grafted liposomes (Placebo)	500 mg /kg of Lipid	1000 mg /kg of Lipid	1500 mg/kg of Lipid	3000 mg/kg of Lipids
3.	Normal Saline	-	-	-	-

Except 125 mg/kg of Gemcitabine, all animals were found healthy and no sign of any toxicity was appeared. Results for sighting studies are summarized in **Table 6.2**.

TABLE 5 2 Results of Sighting Study

Formulation	Animal No.	Dose	Observation	
			Toxicological Signs/symptoms*	Mortality
RGD-Grafted liposomes	1.	50 mg/kg of gemcitabine	None	None
	2.	75 mg/kg of gemcitabine	None	None
	3.	100 mg/kg of gemcitabine	None	None
	4.	125 mg/kg of gemcitabine	Toxicity	None
RGD-Grafted liposomes(Placebo)	1.	500 mg /kg of Lipid	None	None
	2.	1000 mg /kg of Lipid	None	None
	3.	1500 mg/kg of Lipid	None	None
	4.	3000 mg/kg of Lipids	None	None

**Observations included were changes in skin and fur, eyes and mucous membranes, respiratory distress, symptoms related to autonomic and central nervous systems including tremors, convulsions etc., lethargy, and coma.*

After performing the sighting study maximum dose in each group was selected as a starting dose and main test was performed using the dosing protocol shown in **Table 6.3**. Based on sighting study, 100 mg/kg was selected as maximum dose for MTD study. Dosing sequences were limited to 3000 mg/kg for RGD-Grafted liposomes (Placebo). At these much higher doses, amount of gemcitabine loading would be much higher than that required for therapeutic efficacy and hence sufficient to prove safety profile for the developed gemcitabine delivery lipid carriers.

TABLE 5 3 MTD Study: Dosing Protocol

Sr.No.	Group No.	Formulation	Dose		
1.	1	Normal Saline	-		
2.	2	RGD-Grafted liposomes	100 mg/kg of gemcitabine		
3.	3A,3B,3C	RGD-Grafted liposomes(Placebo)	1500 mg/kg of lipids (3A)	2000 mg/kg of lipids (3B)	3000 mg/kg of lipids (3C)

Results for the MTD study is summarized in **Table 6.4**. MTD study was performed in group of five mice, where one mouse was collected from sighting study. 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. >100 mg/kg of gemcitabine for RGD-Grafted liposomes>3000 Mg/kg of total lipids for RGD-Grafted liposomes (placebo).

TABLE 5 4 Results for 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	29.7±0.38	30.3±0.17	None	None
2.	2	29.7±0.24	30.1±0.43	30.2±0.38	None	None
3.	3A	30.4±0.37	30.7±0.35	30.7±0.14	None	None
4.	3B	29.5±0.19	29.0±0.27	30.1±0.26	None	None
5.	3C	30.2±0.16	29.6±0.30	30.4±0.41	None	None

All excipientsused in the formulations are approved for intravenous route according to USFDA and were used at a concentration below IIG limit.Conclusively, the developed liposomal carrier is safe for in-vivo application.

From the results of the study, it can be concluded that optimized gemcitabine liposomal formulations were non-toxic at therapeutic concentrations.

5.4. References

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