Chapter 4

Oral acute toxicity study of a selected chaperone in rats

4 Introduction

Glucocerebrosidase (GCase), a GBA1 gene-encoded lysosomal enzyme, is a risk factor form Parkinson's disease (PD) (Schapira and neuroscience 2015). Disease-modifying treatment strategy for PD by stabilization of GCase enzyme by chaperones is of particular interest (Silveira, MacKinley et al. 2019). These are identified as potential therapeutic agents for disease-modifying therapy of PD due to their GCase stabilizing activity. Unfortunately, AMB is the only GCase chaperone that is entered into the clinical trial as a disease-modifying agent for PD. Despite the notion that GCase may have a role in PD etiology, there are presently no registered medications for the treatment of neuroprotection or neurorestoration in persons with GCase deficiency (Zeuner, Schäffer et al. 2019). In the previous study, the novel GCase chaperone [GC466, N-[(3-bromo-4-methoxyphenyl) methyl] cycloheptanaminehydrobromide] was identified. It binds firmly to the rat GCase residues Asp146, Trp198, Tyr263, Phe265, Tyr331, His329, Glu358, and Trp399 with binding energy (BE -8.92±0.68 Kcal/mol) and binding affinity (K_i: 0.64±0.12 μM). This study has provided valuable insights into the potential of the novel compound, GC466, to enhance GCase activity and exhibit neuroprotective effects in a cell line model of PD (Tripathi, Ganeshpurkar et al. 2022). However, its *in vivo* effectiveness against PD remains unknown. Before delving into the evaluation of GC466's anti-PD effects *in vivo*, it is essential to assess its toxicity profile. Surprisingly, the toxicity of GC466 has not yet been examined in an *in vivo* preclinical model. However, such an evaluation is crucial for determining the appropriate dosage range, assessing potential adverse effects, and ensuring the safety of orally administered novel drugs.

Therefore, there is a significant unmet need for focused research aimed at comprehensively understanding the safety profile of GC466 before progressing to the evaluation of its anti-PD effects *in vivo*. Thus, the present study was initiated to bridge this knowledge gap and evaluate the acute oral toxicity of GC466 using an experimental animal model.

In the current investigation, a comprehensive assessment was conducted encompassing alterations in behavioural pattern, body weight, food and water intake, organ coefficients, analysis of hematological parameters, estimation of various biochemical enzymes, and histological analysis of organs like liver, kidneys, brain, lung, and spleen. These preclinical oral toxicity studies can be serve a crucial role in establishing both the safety and efficacy of the identified chaperone.

4.1 Materials and methods

4.1.1 Materials

The top screened compound (Code, MolPort-029-998-466: GC466) was procured from the Mole-port database. Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) kits were procured from TARA Clinical Systems, India. CK MB and creatinine assay kits were obtained from Coral Clinical Systems, India. The ultrapure water was used to prepare the reagents and was generated using Direct-Q® 5 UV system (Merk KGaA, Darmstadt, Germany).

4.1.2 Animals and their ethical statement

In this study, adult female Wistar rats, weighing 210 ± 20 g, were obtained from the Central Animal House of the Department of Zoology, Banaras Hindu University. To ensure their well-being and accurate experimental conditions, the rats were housed in a controlled environment with a temperature of 25 ± 1 °C, relative humidity maintained between 45% - 55%, and a 12:12 h light-dark cycle. Throughout the study, the rats had

access to a balanced diet from Paramount Laboratory Animal feed (Lanka, India), as well as ad libitum access to water. Care was taken to treat the animals humanely, adhering strictly to the host institutional animal ethics guidelines. The study protocol underwent a comprehensive review by the institutional animal ethical committee of Banaras Hindu University, and approval for the study was granted under the reference number BHU/DoZ/IAEC/2021-2022/046. Furthermore, we followed internationally recognized standards for animal care and experimentation. Specifically, the National Institutes of Health's guide for the care and use of laboratory animals, as outlined in NIH Publications No. 8023 (revised in 1978), was strictly adhered to in the execution of every experiment. By maintaining these high standards, we aimed to ensure the welfare of the animals and the validity and reliability of the study results.

4.1.3 Experimental design for acute oral toxicity studies

The oral acute toxicity study of compound GC466 was evaluated according to Organization for Economic Cooperation and Development (OECD) guideline 423 (Acute Toxic Class Method) on the healthy young adult female rats (as they are the most sensitive between the sexes) to determine the range of median lethal dose (LD_{50}) (Jonsson, Jestoi et al. 2013, Bedi and Krishan 2020). In accordance with the established experimental protocol, the animal subjects were carefully and randomly assigned to distinct groups, with each group consisting of three animals. Considering the absence of any prior data regarding the toxicity profile of the test substance, specifically the compound GC466, and a prudent starting dose of 300 mg/kg body weight (b.w.) was thoughtfully chosen. In the event that none or only one animal exhibited signs of mortality or severe illness, an additional administration of 300 mg/kg was administered to another set of three rats. Subsequent doses were adjusted based on the obtained results, either increasing or

decreasing as necessary. For a comprehensive visual representation of the entire experimental procedure, refer to **Figure 4.1**.

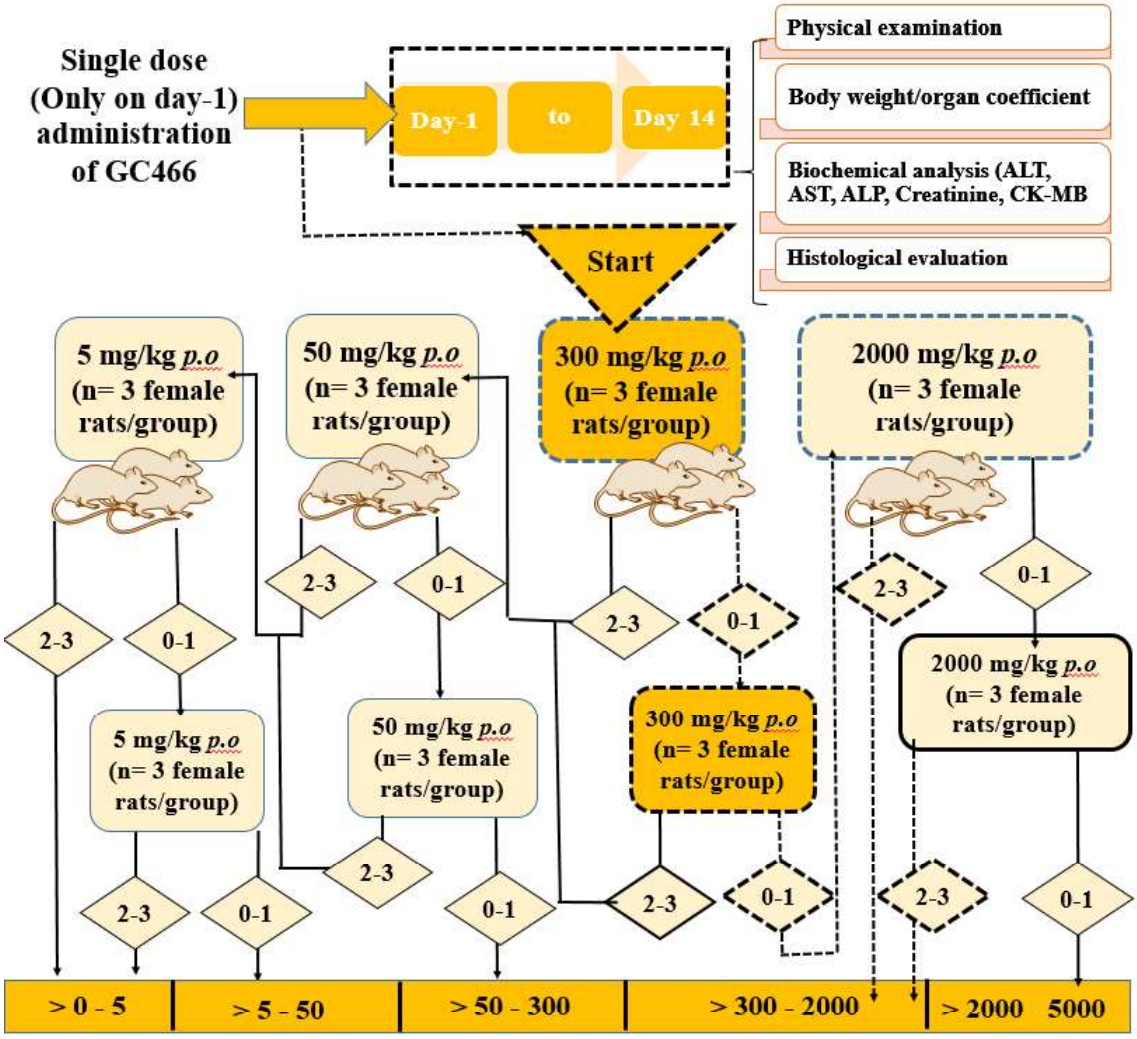

Figure 4.1 Experimental protocol for single-dose acute toxicity study (OECD 423, Organization for Economic Cooperation and Development). The starting dose of 300 mg/kg b.w. was selected. If 2–3 animals died, a lower test dose (i.e., 50 mg/kg) will be tested and if no or one animal died, the next higher dose (i.e., 2000 mg/kg) will be tested. Dash lines or arrows indicate the test procedure followed in our study.

The compound GC466 was freshly prepared in the water. In the experimental protocol, the test substances were administered to the overnight fasted rats through gavage at a single-dose of 25 mg/kg. The group without treatment of GC466 was served as a control group. Throughout the study, each rat was individually observed at least once during the initial 4-hour period and subsequently on a daily basis for a total of 14 days. The individual body weight of the rats in each experimental group was measured on a weekly basis. Moreover, during the observation period, careful attention was given to identifying various signs of toxicity, including changes in the skin, fur, eyes, and salivation. Additionally, the rats were closely monitored for any indications of changes in behavioural patterns (convulsions, moving around or inability to move, sleep), body shaking, lethargy, diarrhoea, and mortality. Throughout the 14-day testing period, food and water intake (volume) were also assessed daily. To calculate the food intake, the remaining feeds were weighed and subtracted from the initial weight. Water intake was also reported for each treatment group. At the end of the study, the animals were killed and blood samples were then collected for biochemical analysis. Additionally, various vital organs such as the brain, heart, liver, kidney, spleen, and lungs were carefully collected for a thorough necropsy to observe any potential indications of pathological changes caused by the test substance.

4.1.4 Hematological analysis

Hematological analyses were performed on blood samples collected in tubes coated with anticoagulant (EDTA) to evaluate the impact of GC466 on blood cells, employing the advanced Hematology Analyzer from Beckman Coulter Cell Counter Inc., CA, USA. The study focused on several crucial parameters, including Red Blood Cell count (RBC), White Blood Cell count (WBC), Hemoglobin (Hb) concentration, and Platelet Count (PLT) (Deng, Cao et al. 2013, Thangavelu, Balusamy et al. 2020).

4.1.5 Estimation of hepatic, renal and heart toxicity markers

The serum samples underwent meticulous biochemical analysis to thoroughly examine the impact of GC466 on the functioning of highly perfused organs. This evaluation was carried out using assay kits, following the precise guidelines provided by the manufacturer. Serum samples were collected from blood without the addition of any anticoagulant. The blood was allowed to clot at room temperature and subsequently centrifuged at 3000 rpm for 10 minutes. After centrifugation, the supernatant was carefully decanted and then stored at -20°C to preserve its integrity for further analysis. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) are essential enzymes that were meticulously measured to assess the liver's overall function. Additionally, the kidney and heart functionalities were carefully evaluated through the examination of creatinine (CRE) levels for the kidney, and creatine kinase-MB (CK-MB) for the heart.

4.1.6 Measurement of Organ coefficient

At the end of the study, the animals were killed, and their organs were dissected and precisely weighted. These measurements were then used to assess the potential toxic effects of the test compounds on the vital organs. To determine the organ coefficient of various organs, we employed the following formulae:

$$\text{Organ coefficient} = \frac{\text{Weight of the organ (g)}}{\text{Total body weight (g)}} \; X \; 100$$

4.1.7 Histopathological examination

To assess the histopathological alterations resulting from the administration of different doses of GC466 in rats, we conducted a comprehensive examination of various organs, including the brain, heart, lungs, liver, kidney, and spleen. The organs were carefully dissected and subsequently fixed in 4% paraformaldehyde. Thin tissue sections

measuring 10 µm were prepared and subjected to staining with hematoxylin for a duration of 3 minutes, followed by rinsing in running tap water. Then, the samples were then counterstained with eosin for 1 minute. Next, a series of graded alcohol solutions were employed to dehydrate the tissues, and finally, the slides were mounted using dibutyl phthalate xylene (DPX) as a medium. To observe any notable macroscopic pathological abnormalities, the prepared slides were diligently examined under a microscope (Nikon, Japan).

4.2 Statistical analysis

The data were presented as the mean ± standard deviation. Excel-20 and Power point-13 version used for data representation.

4.3 Results and discussion

4.3.1 Effect of GC466 on behavioural and physical changes in the acute oral toxicity study

After administering GC466 orally at doses of 300 and 2000 mg/kg *b.w.*, the animals were consistently monitored. Distinct patterns of toxicity emerged in the high dose group (2000 mg/kg b.w.), where five animals succumbed to mortality (**Table 4.1**). They died about within 64, 76, 86, and 94, and 122, minutes post-administration, respectively. The signs of toxicity were evident through various changes in behavioural patterns, including excessive salivation, convulsions, reduced activity (sluggish movements), clustering together as if experiencing chills, altered body positions, muscular weakness, and heightened sensitivity to noise. These observations provided a comprehensive understanding of the adverse effects of the high dosage (2000 mg/kg *p.o*) of GC466 on the subjects. A reduction in activity was noted approximately 20-30 minutes after exposure. During this time, rats lying in various positions with flaccid tails. Remarkably,

two of the rats exhibited spasmodic jumping just before experiencing sudden death. At higher doses, additional concerning symptoms were observed, including piloerection, diarrhoea, and lethargy. Notably, all the rats exposed to higher doses exhibited a bleached eye colour and faintly palpable heartbeats.

On the other hand, when a rat was administered a dose of 300 mg/kg *b.w.* of GC466, there were no noticeable severe alterations in their behavioral or physical condition. However, mild indications of toxicity were observed, such as lethargy, trembling, diarrhoea, and a slight decrease in activity (resting and lying down) for approximately 2.1 hours after administration. Fortunately, the rat fully recovered within 24 hours and resumed its normal activities after consuming food. Throughout the 14-day study period, the control rats remained in good health without experiencing any adverse effects. Based on the guidelines provided by the Globally Harmonized Classification System (GHS) and the OECD (Organization for Economic Cooperation and Development) Guideline for Acute Oral Toxicity - Acute Toxic Class Method 423, our findings suggest that the tested compound GC466 can be categorized as non-toxic (category 4), with an LD_{50} cut-off value of 500 mg/kg *b.w*.

Table 4.1 The results of acute oral toxicity study of the compound GC466 on mortality rate

Groups	Dosage (mg/kg)	Animal number	Death number	Mortality rate (%)
1	Control	6	0	0
2	300	6	0	0
3	2000	6	5	90 %

4.3.2 Effect of GC466 on the body weight in the acute oral toxicity study

The weight of the body plays a crucial role in determining the proper functioning of internal organs. Any involuntary changes in body weight caused by the test compounds can be seen as indicators of accumulation of fats, severe pain, decrease in appetite, lower the caloric intake by animals, distress, or even imminent mortality in the experimental animals. Monitoring these changes is essential for assessing their well-being during the study. Consequently, in the ongoing investigation on the acute toxicity of the chaperone GC466 through oral administration, close attention was paid to monitoring the bodyweight of the rats in each experimental group. This information is visually represented in **Table 4.2**.

Table 4.2 The results of acute oral toxicity study of the compound GC466 on body weight

Groups	Dosage (mg/kg)	Before (g)	After (g)
1	Control	232.76 ± 13.74 (n=6)	241.12 ± 17.48 (n=6)
2	300	235.22 ± 11.55 (n=6)	239.43 ± 15.37 (n=6)
3	2000	237.34 ± 16.04 (n=6)	182.56 ± 0[$] (n=1)

Effect of single-dose oral administration of chaperone GC466 on body weight (g=Gram) of rats at different days during the experimental protocol. All values are presented as mean ± SD; n = 6. Before" refers to Day-0, which signifies the initial stage before the experiments commence. "After" signifies the end of the experiments on Day-14. To represent the weight of a single rat that survived throughout the entire study, the symbol "$" is used.

The chaperone GC466, administered at a dosage of 300 mg/kg (GC466-300), exhibited not much changes in body weight compared to the control group. However, when observing **Table 4.2**, it becomes evident that the weight of rats in the GC466-2000 group (GC466 treated with 2000 mg/kg) showed a decline from D-7, indicating the presence of toxicity at this higher dose in compared to the control group. However, the changes in body weight may be due to either the decreases in the food and water consumption or the organ injuries caused by the test substance.

4.3.3 Effect of GC466 on the food and water intake in the acute oral toxicity Study

The toxic effects of chemicals and drugs can be influenced by changes in food and water intake, either decreases or increases (Travlos, Morris et al. 1996). As a result, it was crucial to monitor the daily food and water intake (volume) throughout the entire 14-day testing period. To determine the food intake, the remaining feed was carefully weighed and subtracted from the initial weight. Additionally, the water intake for each treatment group was also recorded and reported.

Table 4.3 Effect of single-dose oral administration of compound GC466 on average food consumption of rats at different weeks during the experimental protocol.

Groups	Dosage (mg/kg)	Average food consumptions (g/week)	
		1 week	2 week
1	Control	25.6 ± 1.84 (n=6)	30.2 ± 3.14 (n=6)
2	300	27.2 ± 3.26 (n=6)	29.24 ± 2.72 (n=6)

3	2000	2.34$ (n=1)	2.05$ (n=1)

All values are presented as mean ± SD; n = 6. "$" denotes the average weekly food consumption of a single rat out of the six which survived up to end of study.

The results depicted in **Table 4.3 and Table 4.4** demonstrate a noteworthy observation regarding the impact of GC466 on rats in the high dose group (2000 mg/kg b.*w.*). These rats exhibited a noticeable reduction in their food and water intakes, which serves as a strong indication of toxicity. However, it is worth mentioning that rats treated with a lower dosage of GC466 (300 mg/kg) experienced only a slight decrease in food and water consumption during the first week of the study. Remarkably, by the end of the study, their intake levels had fully recovered and returned to normal. Taking all findings into account, it is plausible to conclude that the decrease in food and water consumption could be attributed to the corresponding decrease in body weight, as evidenced by the aforementioned study.

Table 4.4 Effect of single-dose oral administration of compound GC466 on average water intake of rats at different weeks during the experimental protocol.

Groups	**Dosage (mg/kg)**	**Average water intake (ml/week)**	
		1 week	**2 week**
1	Control	78.5 ± 9.23 (n=6)	81.7 ± 6.52 (n=6)
2	300	69.2 ± 7.45 (n=6)	76.9 ± 8.87 (n=6)
3	2000	7.52	7.12

	(n=1)	(n=1)

All values are presented as mean ± SD (n=6). "$" denotes the average weekly water intake of a single rat out of the six which survived up to end of study.

4.3.4 Effect of GC466 on the Haematological analysis in the acute oral toxicity study

The hematopoietic system plays a vital role in the production of cellular blood components, making it a crucial system within the body. Notably, any changes in hematological parameters can serve as indicators of drug-induced toxicity in both humans and animals (Piao, Liu et al. 2013, Albrecht, Kappenberg et al. 2019, Vysakh, Jayesh et al. 2020). In the current study, we aimed to assess the impact of oral administration of chaperone GC466 on hematological parameters (including Hb, RBC, WBC, and PLT) in female rats, comparing them to a control group of rats **(Table 4.5**). Reduced hematological parameters such as Hb, RBC, WBC, and PLT were noticed by the rats treated with higher doses of GC466, as shown in **Table 4.5**. Among the animals subjected to the experimental protocol at a higher dose (2000 mg/kg), only a one rat survived until the completion of the study. The hematological parameters of this survivor can also be found in **Table 4.5**. However, it is noteworthy that the remaining animals succumbed within approximately two hours after the post-administration of GC466 2000 mg/kg. The hematological data of these animals can be found in **Table 4.1 of the appendices**.

These findings strongly suggest that the test compounds, particularly at higher doses, interfere with the process of haematopoiesis in the experimental rats. This implies that the chaperone GC466, when administered orally at higher doses, can have adverse effects on the production of blood cells, leading to disruptions in the hematopoietic system.

Table 4.5 Effect of single-dose oral administration of compound GC466 on hematological parameters of rats at the end of study during the experimental protocol

Dosage (mg/kg)	Hb (g/dL)	RBC (x 10^6 cells/mL)	WBC (x 10^3 Cell/mL)	PLT Platelets (x 10^5 Cells/mL)
Control	13.63 ± 1.28 (n=6)	6.48 ± 0.68 (n=6)	6.53 ± 1.24 (n=6)	8.52 ± 0.72 (n=6)
300	13.97 ± 2.12 (n=6)	7.57 ± 1.03 (n=6)	6.58 ± 1.56 (n=6)	7.40 ± 1.63 (n=6)
2000	9.08 ± 00$^{\$}$ (n=1)	4.86 ± 00$^{\$}$ (n=1)	4.57 ± 00$^{\$}$ (n=1)	3.90 ± 00$^{\$}$ (n=1)

All values are presented as mean ± SD; n = 6. "$" denotes hematological parameters of a single rat out of the six which survived up to end of study. Hb= hemoglobin, RBC = Red blood cells, WBC = White blood cells, and PLT= platelets.

4.3.5 Effect of GC466 on the organ coefficient in the acute oral toxicity study

The measurement of organ weight serves as a highly sensitive indicator for drug toxicity, often detecting changes prior to the emergence of morphological alterations. Therefore, it holds crucial importance to include organ weight assessment in the toxicological screening of any test compounds (Piao, Liu et al. 2013). In our investigation of the acute toxicity study, we examined the impact of GC466, administered orally in a single dose, on the organ coefficient of vital organs, namely the brain, heart, lung, kidneys, liver, and spleen. Among the animals subjected to the experimental protocol at a higher dose (2000mg/kg), only a one rat survived until the completion of the study. The organs coefficient of this survivor and with other group's animal (control and GC466 treated with 300 mg /kg) can be found in **Table 4.6**. The administration of compound GC466 in rats at a higher dosage (2000 mg/kg) resulted in an increase in the organ coefficient of the

lung, spleen, liver, and kidneys, compared to the control group. Additionally, slight changes were observed in the heart and brain at this dosage. Among the animals subjected to the experimental protocol at a higher dose (2000mg/kg), only a one rat survived until the completion of the study. However, it is noteworthy that the remaining animals succumbed within approximately two hours after the post-administration of GC466 2000 mg/kg. The organs coefficient of these animals can be found in **Table 4.2 and Table 4.3 of the appendices**. Conversely, rats treated with 300 mg/kg of GC466 did not exhibit any changes in organ coefficients when compared to the control group. The reason behind the observed increase in organ coefficients caused by GC466 could potentially be attributed to the inhibition of the glucocerebrosidase enzyme at higher doses. This study supports the earlier study that found that inhibiting GCase causes harmful quantities of certain lipids to build up throughout the body, particularly in the spleen, liver, and kidneys (Baccam, Xie et al. 2022) . These findings suggest that GC466 at higher doses may have a direct impact on lipid metabolism, potentially affecting organ function.

Table 4.6 Effect of single-dose oral administration of compound GC466 on the organs coefficient at the end of the experimental protocol

Dosage (mg/kg)	Liver	Kidneys	Heart	Lung	Brain	Spleen
Control	3.71 ± 0.27 (n=6)	0.75 ± 0.13 (n=6)	0.44 ± 0.03 (n=6)	0.82 ± 0.17 (n=6)	1.22 ± 0.057 (n=6)	0.32 ± 0.049 (n=6)
300	3.45 ± 0.33 (n=6)	0.785 ± 0.21 (n=6)	0.45 ± 0.14 (n=6)	0.93 ± 0.19 (n=6)	1.24 ± 0.062 (n=6)	0.34 ± 0.073 (n=6)

2000	5.11 ± 00$^{\$}$ (n=1)	0.52 ± 00$^{\$}$ (n=1)	0.42 ± 00$^{\$}$ (n=1)	1.31 ± 00$^{\$}$ (n=1)	1.02 ± 00$^{\$}$ (n=1)	0.48 ± 00$^{\$}$ (n=1)

All values are presented as mean ± SD; n = 6. "$" denotes the average organs coefficient (in grams) of a single rat out of the six which survived up to end of study.

4.3.6 Effect of GC466 on the serum biochemistry parameters in the acute oral toxicity study

Serum biochemical parameters play a crucial role in the identification of acute toxicity in various organs, including the liver, kidney, and heart. These parameters encompass a range of enzymes that provide valuable insights into organ toxicities. In normal circumstances, vital organs possess intricate mechanisms to mitigate their toxic load by efficiently eliminating metabolites, primarily through the liver and kidneys. Consequently, the presence of these enzymes in abnormal levels within the serum can serve as indicators of organ injury caused by toxicity (Deng, Cao et al. 2013, Wen, Dan et al. 2017) . Therefore, when evaluating the toxicity of chaperone GC466, assessing the liver and kidney enzyme functions becomes an indispensable factor. Among the clinically significant enzymes, ALT, AST, and ALP hold immense importance in identifying hepatic functioning. By examining the levels of these enzymes, valuable information can be gleaned regarding the overall health and integrity of the liver. According to reports, AST is found in both the mitochondria and cytosol of hepatocytes, whereas ALT is exclusively located in the cytosol (Ahmed, Hasona et al. 2008). Consequently, variations in AST levels are commonly regarded as a clinical indicator of hepatocellular necrosis. On the other hand, changes in serum ALT levels specifically signify hepatocyte hypertrophy or other liver diseases. Additionally, ALP is a significant enzyme present in

the body, and an elevation in its levels causes bile ducts obstruction (Deng, Cao et al. 2013, Vysakh, Jayesh et al. 2020). In our study, we administered GC466 to rats at a dosage of 2000 mg/kg and observed an increase in the levels of hepatic enzymes in one surviving rat throughout the duration of the study, compared to the control group **(Table 4.7).** However, at this dose, the remaining animals succumbed to the effects of GC466 within one day after a 2-hour post-administration period. The liver biochemical serum enzyme parameters corresponding to these observations can be found in **Table 4.4 in the appendices**. This suggests potential liver damage resulting from the administration of GC466. However, a minimal change in ALT levels compared to controls was found in a few rats treated with 300 mg/kg. No differences were observed between the control group and the GC466-300 group in terms of AST and ALP levels. In the context of liver function, GCase, an enzyme primarily abundant in the liver, plays a significant role in the breakdown of lipids, particularly glucocerebrosides (Murray, Oliver et al. 1995, Marí and Fernández-Checa 2007). However, the use of higher dosages of GC466, may be inhibits this enzyme and lead to hepatotoxicity, as evidenced by an elevation in the levels of various enzymes associated with liver damage. This suggests that the increased presence of higher dosage GCase chaperone GC466 interferes with the normal functioning of GCase, resulting in toxic effect on the liver.

Kidney, also known as the renal organ, is particularly susceptible to drug-induced toxicity due to its crucial role in excretion. As the primary eliminator of exogenous drugs and toxins, the kidneys are naturally exposed to a larger proportion of circulating drugs and chemical compounds (Pazhayattil, Shirali et al. 2014, Radi 2019). Monitoring kidney function becomes essential in assessing drug safety. One common indicator of renal function is the measurement of serum creatinine levels, which represents the accumulation of nitrogenous waste products in the blood (Kluwe and Pharmacology

1981, Perrone, Madias et al. 1992) . In our study, we investigated the impact of different dosages of GC466, a potential therapeutic agent, on kidney function. We measured the serum creatinine levels as an indicator of renal function. Notably, when rats were administered a higher dosage of GC466 (2000 mg/kg), an increase in creatinine levels was observed compared to the control group. This observation suggests the possibility of kidney damage in the experimental groups receiving higher dosages of GC466 (**refer to Table 4.7 and Table 4.5 in appendices**). However, no differences in creatinine levels were found between the control group and the group treated with a 300 mg/kg of GC466. The cardiovascular system (heart) is a vital muscular organ responsible for circulating blood throughout the entire body. Through its rhythmic contraction and relaxation of the involuntary cardiac muscle, it ensures a continuous flow of blood (Abbott 2001). However, when there is an obstruction in the blood flow, it can result in a severe condition known as myocardial infarction, which stands as one of the primary causes of death on a global scale. Creatine kinase-MB (CK-MB) is an enzyme predominantly found in the cardiac muscle cells, serving as a crucial cardiac marker for the diagnosis of acute myocardial infarction (Schneider, Dennehy et al. 1995). Extensive research has consistently demonstrated that following a heart attack, also known as myocardial infarction, certain enzymes, including CK-MB (creatine kinase-myocardial band), are released from the damaged heart tissue into the bloodstream approximately 4 to 6 hours after the event (Jacob and Khan 2018, Joseph, Builders et al. 2019).

Table 4.7 Effect of single-dose administration of compound GC466 on the serum biochemistry parameters at the end of experimental protocol

Dosage (mg/kg)	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	CRE (µmol/L)	CK-MB (ng/mL)

Control	108 ± 23.29 (n=6)	23.67 ± 14.95 (n=6)	70.98 ± 12.33 (n=6)	11.47 ± 1.29 (n=6)	12.45 ± 2.01 (n=6)
300	95.72 ± 15.52 (n=6)	35.13 ± 6.67 (n=6)	77.86 ± 18.28 (n=6)	10.16 ± 2.47 (n=6)	15 ± 0.062 (n=6)
2000	165 ± 00$ (n=1)	49.78 ± 00$ (n=1)	104.20 ± 00$ (n=1)	20.14 ± 00$ (n=1)	46.92 ± 00$ (n=1)

All values are presented as mean ± SD; n = 6. "$" denotes the serum biochemistry parameters of a single rat out of the six which survived up to end of study. AST = Aspartate aminotransferase, ALT = Alanine aminotransferase, and ALP = Alkaline phosphatase.

In addition to myocardial infarction, severe acute exposure to toxic substances can induce detrimental alterations in the physiology of heart tissue or even lead to cardiac cell death. To investigate the potential effects of a test substance, GC466, on heart tissue, the present study measured the levels of CK-MB in the serum. The findings are presented in **Table 4.7.** Notably, when rats were administered a higher dosage of GC466 (2000 mg/kg), an increase in CK-MB levels in one surviving rat throughout the duration of the study, compared to the control group **(Table 4.7).** However, at this dose, the remaining animals succumbed to the effects of GC466 within one day after a 2-hour post-administration period. The CK-MB serum enzyme level corresponding to these observations can be found in **Table 4.5 in the appendices**. This finding implies the possibility of heart damage in rats subjected to higher doses of GC466. However, no differences in CK-MB

levels were detected between the control group and the group treated with a dosage of 300 mg/kg of GC466.

Overall, based on the findings of the serum biochemistry parameter studies mentioned above, it can be deduced that the elevation of serum biomarkers observed with higher doses of GC466 may be attributed to the impaired functionality of the glucocerebrosidase enzyme. This particular enzyme is responsible for the normal breakdown of glucosylceramide/glucocerebrosides (GlcCer/GC) by directing it to lysosomes for degradation. Consequently, inhibiting this enzyme, as previously reported, can result in metabolic abnormalities of GlcCer/GC, leading to the accumulation of lipids and the occurrence of lipotoxicity in various organs (Atsumi, Nosaka et al. 1992, Schulze and Sandhoff 2011).

4.3.7 Effect of GC466 on the microstructural (histological) changes of organ in the acute oral toxicity study

The histopathology test is a crucial diagnostic procedure conducted worldwide to identify structural changes in organs associated with various toxicities. These changes primarily occur in organs such as the brain, heart, liver, kidney, lungs, and spleen following exposure to toxic exogenous compounds, due to their metabolic reactions (Jothy, Zakaria et al. 2011, Campion, Aubrecht et al. 2013). Therefore, it is essential to examine the microstructural alterations in these organs at the conclusion of a study.

4.3.7.1 Effect of GC466 on the histological changes in liver in the acute oral toxicity study

In the current study, the histopathological sections of the liver were analyzed in both the control group and each of the treated groups with GC466. In the control group, the cross-section of the liver **(Figure 4.2)**, exhibited a normal appearance, with the central vein (CV), portal tract (PT), sinusoids (Si), and hepatocytes clearly intact. However, in the experimental group treated with higher doses of GC466 (2000 mg/kg, **Figure 4.2c**), congestion was observed in the central vein, portal vein, and sinusoids within the liver lobule. Additionally, destruction of hepatocytes and binucleated cells (BN) were observed in this particular group. Conversely, the group treated with a dose of 300 mg/kg (**Figure 4.2b)** only showed slight damage to the hepatocytes. This finding aligns with the levels of liver enzymes (AST, ALT, and ALP) **(Table 4.7)** and the organ coefficient **(Table 4.6),** indicating elevated enzyme levels and organ weight in the higher dose group. The destruction of hepatocytes may due to the elevation of transaminases (AST and ALT), as reported previously. GC466 is a type of pH dependent GCase inhibitor that exhibits a mixed mode of action (Tripathi, Ganeshpurkar et al. 2023). However, it is important to note that at higher doses, GC466 may fail to maintain the optimal balance of overall enzyme activity in the hepatic area. Consequently, this imbalance has been observed to potentially lead to hepatic dysfunctions, similar to the toxic effects reported in previous studies involving other GCase inhibitors (Furbish, Oliver et al. 1984, Ayto, Hughes et al. 2010, Pastores and Hughes 2018, Zhang, Zheng et al. 2022). These findings are consistent with earlier research that established a connection between GCase dysfunctions and the occurrence of hepatic dysfunctions.

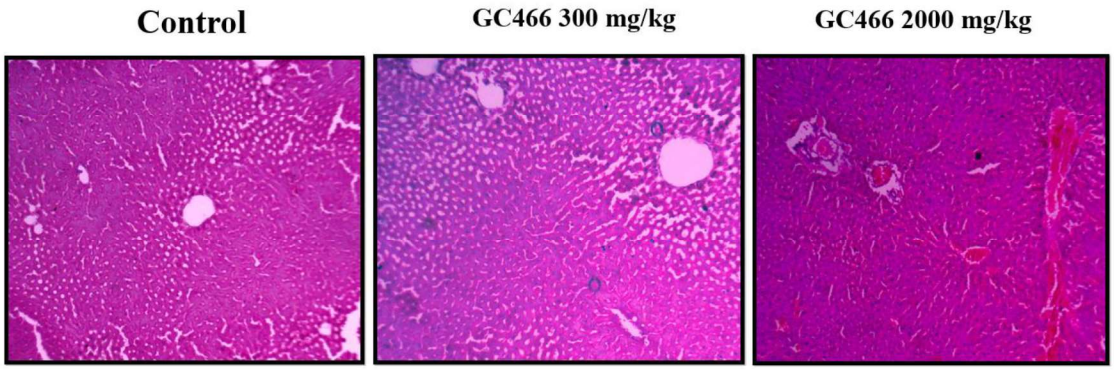

Figure 4.2 Effect of single-dose oral administration of GC466 (300 and 2000 mg/kg b.w.) on the liver tissue stained with haematoxylin-eosin.

4.3.7.2 Effect of GC466 on the histological changes in liver and lung in the acute oral toxicity study

In addition to assessing liver function, the study also included an examination of kidney and lung function upon its completion. The kidney (**Figure 4.3**) and lung (**Figure 4.4**) organ sections revealed that no significant damages were observed in the treatment group receiving lower doses (300 mg/kg) compared to the control group (without treatment). However, the administration of GC466 at a higher dose of 2000 mg/kg resulted in severe damages to the kidneys, including vascular congestion, glomerulus atrophy, necrosis in the proximal convoluted tubules (PCT) and distal convoluted tubules (DCT). Similarly, in the lungs, the higher doses, caused thickening of the alveolar capillary membrane, alveolar destruction, increased thickness of alveolar walls, and intense congestion in the alveolar region when compared to control group.

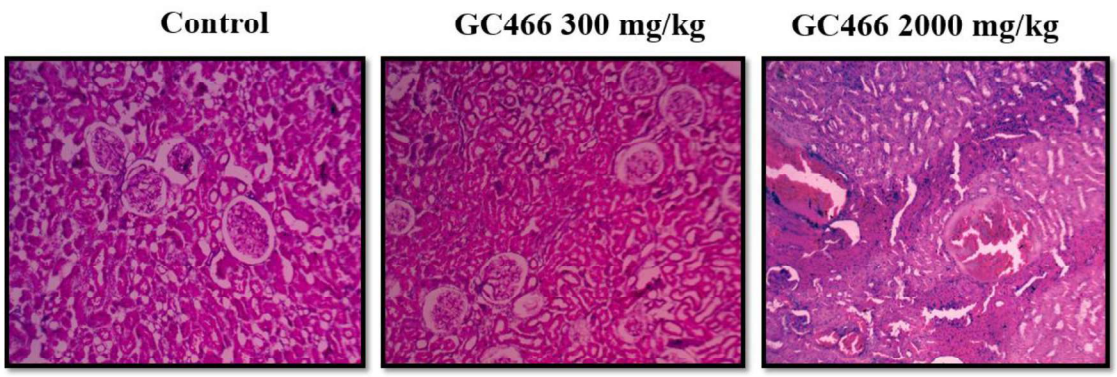

Figure 4.3 Effect of GC466 given orally in a single dose at 300 and 2000 mg/kg body weight on kidneys sections stained with haematoxylin and eosin.

These findings are consistent with the observed increase in the organ coefficient of the lungs and kidneys, as well as the elevated serum creatinine levels in the kidneys reported in the aforementioned study. The observed toxic effects may be attributed to the dysfunction of glucocerebrosidase activity induced by the higher doses of GCase chaperone GC466. This enzymatic dysfunction may disrupt the normal breakdown of glucocerebrosides, resulting in its accumulation within lysosomes. This accumulation leads to cellular dysfunction and tissue damage, particularly in organs that heavily rely on proper lysosomal function, such as the lungs and kidneys, as previously reported (Baccam, Xie et al. 2022).

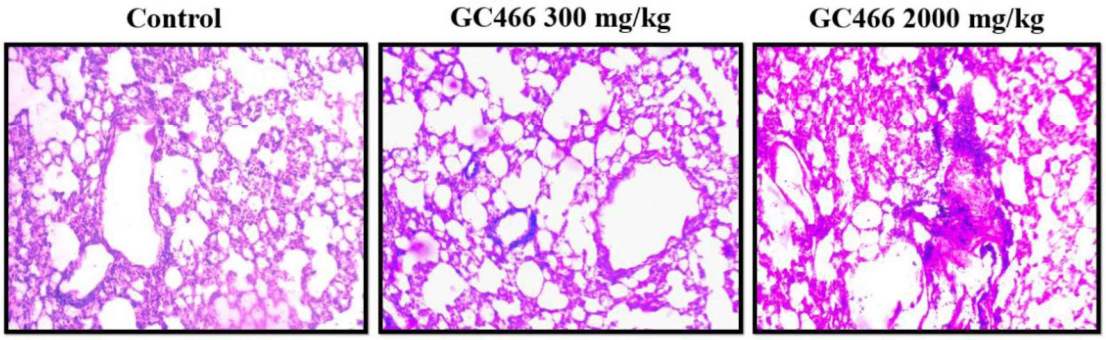

Figure 4.4 Effect of single-dose oral administration of GC466 (300 and 2000 mg/kg b.w.) on the lung sections stained with haematoxylin-eosin.

4.3.7.3 Effect of GC466 on the histological changes in spleen in the acute oral toxicity study

It's important to note that the liver is the primary organ responsible for metabolizing and eliminating toxins from the body. However, the spleen as part of the immune system and blood filtration system, indirectly supports the body's response to toxic substances by participating in immune defence and maintaining blood cell homeostasis. Spleen contains two important structures, i.e., red pulp and white pulp. The red pulp is a blood filter that stores iron and removes the foreign materials and older RBCs from the blood circulation. The white pulp (lymphocytes and macrophages) is reported to that help in the detection and destruction of pathogens (bacteria, viruses, etc.) and foreign particles that enter the bloodstream (help in immune responses). The spleen can also store platelets, which are important for blood clotting. When needed, the spleen can release these platelets into circulation to aid in the clotting process and prevent excessive bleeding (López-Guillermo, Cervantes et al. 1991, Mebius and Kraal 2005). Therefore, it is important to acknowledge that spleen damage can have significant implications on both the immune system and blood-filtering abilities, rendering the rat more vulnerable to infections and certain health issues. Thus, during toxicity screening, it is crucial to assess the toxic effects of the test substance as it enters the bloodstream and reaches the spleen either

through diffusion into the spleen's tissues *via* blood vessels or through the splenic artery, where it can be eliminated through phagocytosis. In the case of lower doses (300 mg/kg/day) the cross-section analysis revealed that the spleen (**Figure 4.5**) exhibited a normal appearance, including intact spleen trabecular, lymphatic nodules of white pulp, and splenic cords of red pulp, as compared to the control group. However, in the group of rats receiving 2000 mg/kg of GC466, various abnormalities were observed in the spleen, such as splenic haemorrhage, deposition of hemosiderin pigment in the red pulp, enlargement of the red pulp, destruction of the splenic parenchyma, and depletion of white pulp with moderate lymphocyte reduction.

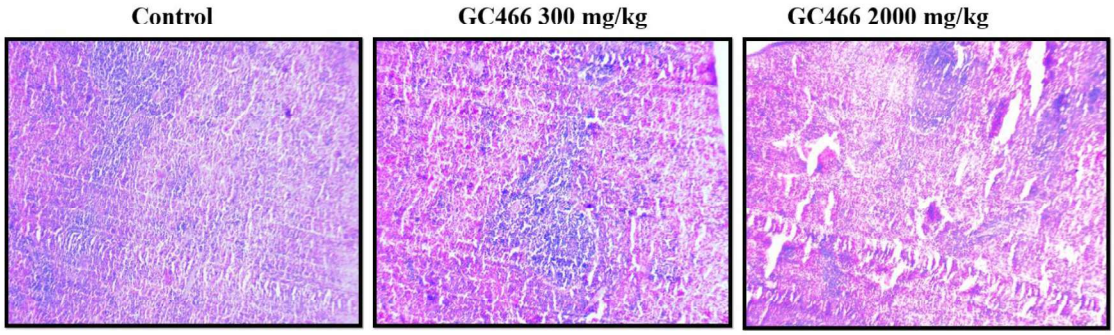

Figure 4.5 Effect of single-dose oral administration of GC466 (300 and 2000 mg/kg b.w.) on the spleen sections stained with haematoxylin-eosin.

GCase is an enzyme present not only in the liver but also in various tissues throughout the body, including the spleen. This enzyme plays a role in lipid metabolism, specifically in the breakdown of glucocerebrosides, as previously reported (Pastores and Hughes 2018, Baccam, Xie et al. 2022). However, it is important to note that at higher doses, GC466 higher doses may fail to maintain the optimal balance of overall enzyme activity in the spleen area and may lead to the accumulation of glucocerebrosides within these cells, particularly in macrophages, a type of immune cell found in the spleen. This

accumulation could be responsible for the disruption of the spleen's normal architecture and function, as observed in this study.

4.3.7.4 Effect of GC466 on the histological changes in heart and brain in the acute oral toxicity study

Heart, a muscular organ that pumps oxygenated blood to the body, and damage may lead to ischemic stroke due reduced blood pumping to the brain. Brain function refers to the various activities and processes performed by the brain, which is the control centre of the nervous system (Maynard 1960, Samuels 2007). It comprises various regions, with the brain's cortical regions being crucial components. These regions constitute the outermost layer of the brain, responsible for higher-order cognitive functions, sensory processing, and motor control (Fuster 1997, Fuster 2000). Consequently, it is vital to evaluate the impact of administered compounds (GC466) on both the cardiac muscle and cortical region. The examination of heart (**Figure 4.6)** and brain sections (**Figure 4.7)** revealed that the treatment group receiving lower doses (300 mg/kg) showed no damage compared to the control group. However, when administering GC466 at a higher dose of 2000 mg/kg, the heart suffered notable harm, including ruptured cardiomyocytes, vascular dilation with congestion, and destruction of myocardial muscle bundles. Additionally, the heart slice from this group displayed irregularly arranged cardiomyocytes with multiple, elongated nuclei, mostly not centrally located within the myofibers. Likewise, within the brain, higher doses, induced various toxic effects, including cellular infiltration, vascular congestion, the presence of vacuolated cells in the cerebral cortex, hyperchromatic cells, cellular atrophy, shrinkage, and cellular necrosis, as evidenced by the findings of this study. These outcomes strongly align with the observed changes in organ coefficient for both the heart and brain, which exhibited slight alterations when compared to the control group.

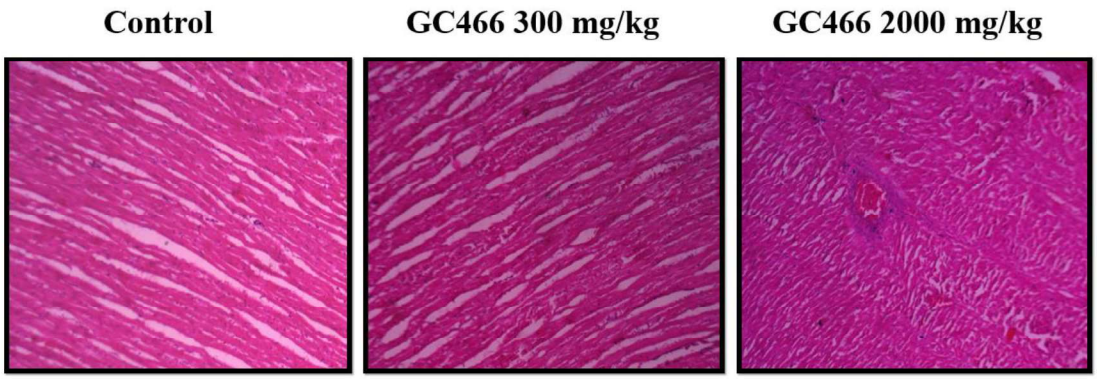

Figure 4.6 Effect of single-dose oral administration of GC466 (300 and 2000 mg/kg b.w.) on the heart sections stained with haematoxylin-eosin.

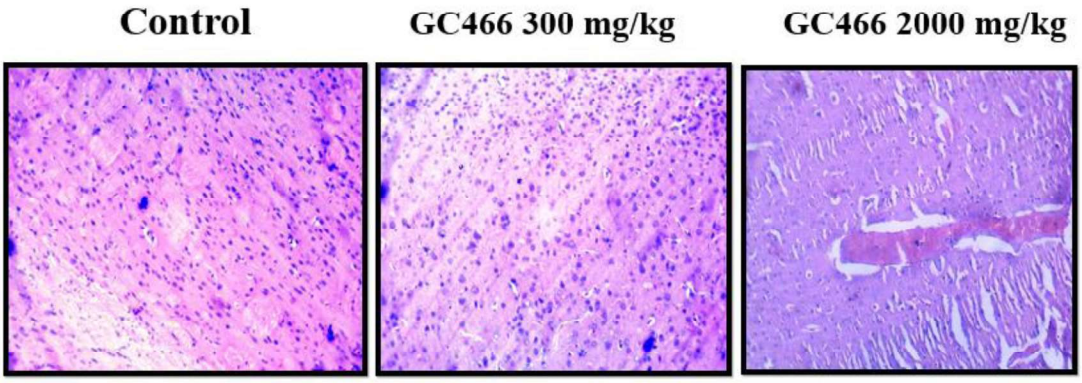

Figure 4.7 Effect of single-dose oral administration of GC466 (300 and 2000 mg/kg b.w.) on the brain sections stained with haematoxylin-eosin.

However, the damage to the heart and brain regions caused by the higher doses GCase chaperone (GC466) appears to be linked to the accumulation of a lipid called glucocerebrosides in these areas. This accumulation arises due to the defective activity of

the enzyme GCase. Previous research has provided substantial evidence for this association, as it demonstrated the build-up of glucocerebrosides in the hearts of gaucher disease patients, which arises from dysfunctions of the GCase enzyme (Pastores and Hughes 2018, Vieira, Schapira et al. 2021, Baccam, Xie et al. 2022). This accumulation led to cardiomyopathy, a condition characterized by the enlargement or thickening of the heart muscle, resulting in impaired heart function. Additionally, the occurrence of brain cortex destruction in gaucher disease patients further supports the findings of this study (Vieira, Schapira et al. 2021, Furderer, Hertz et al. 2022). It appears that the defective GCase activity leads to the accumulation of glucocerebrosides in both the heart and brain, contributing to the observed detrimental effects on these vital organs.

4.4 Conclusion

The present study provides compelling evidence regarding the safety profile of GC466 and its usage up to a dose of 300 mg/kg of body weight in experimental rats. To assess its safety, the study followed the OECD guidelines and conducted an oral acute toxicity examination of GC466 in rats, comparing them to control rats. Notably, no mortality or signs of toxicity were observed throughout the acute toxicity study, even at doses up to 300 mg/kg. Furthermore, administering GC466 at 300 mg/kg did not yield any significant changes in organ coefficients or hematological parameters. Similarly, there were no alterations observed in the activities of hepatotoxicity marker enzymes (ALT, AST, and ALP), renal marker (creatinine), and heart marker (CK-MB) levels, which indicate normal liver, renal, and heart functions. Moreover, histological examinations of the lungs, spleen, and brain in rats treated with GC466 (300 mg/kg) revealed normal findings. Based on these findings, it can be concluded that the novel GCase chaperone GC466, employed in this acute toxicity study, can be deemed safe for further preclinical investigations.

4.5 Summary

- Administration of a single oral dosage of GC466 up to 300 mg/kg demonstrated no significant changes in AST, ALT, ALP, creatinine, and Ck-MB levels.
- No hematological parameters toxicity was observed during the acute toxicity study up to 300 mg/kg.
- Histological analysis demonstrated the absence of any indications of organ toxicity in rats, even at doses as high as 300 mg/kg.
- GC466 up to 300 mg/kg is safe in acute oral toxicity study.
- LD_{50} was found to be a cut-off of 500 mg/kg b.w.

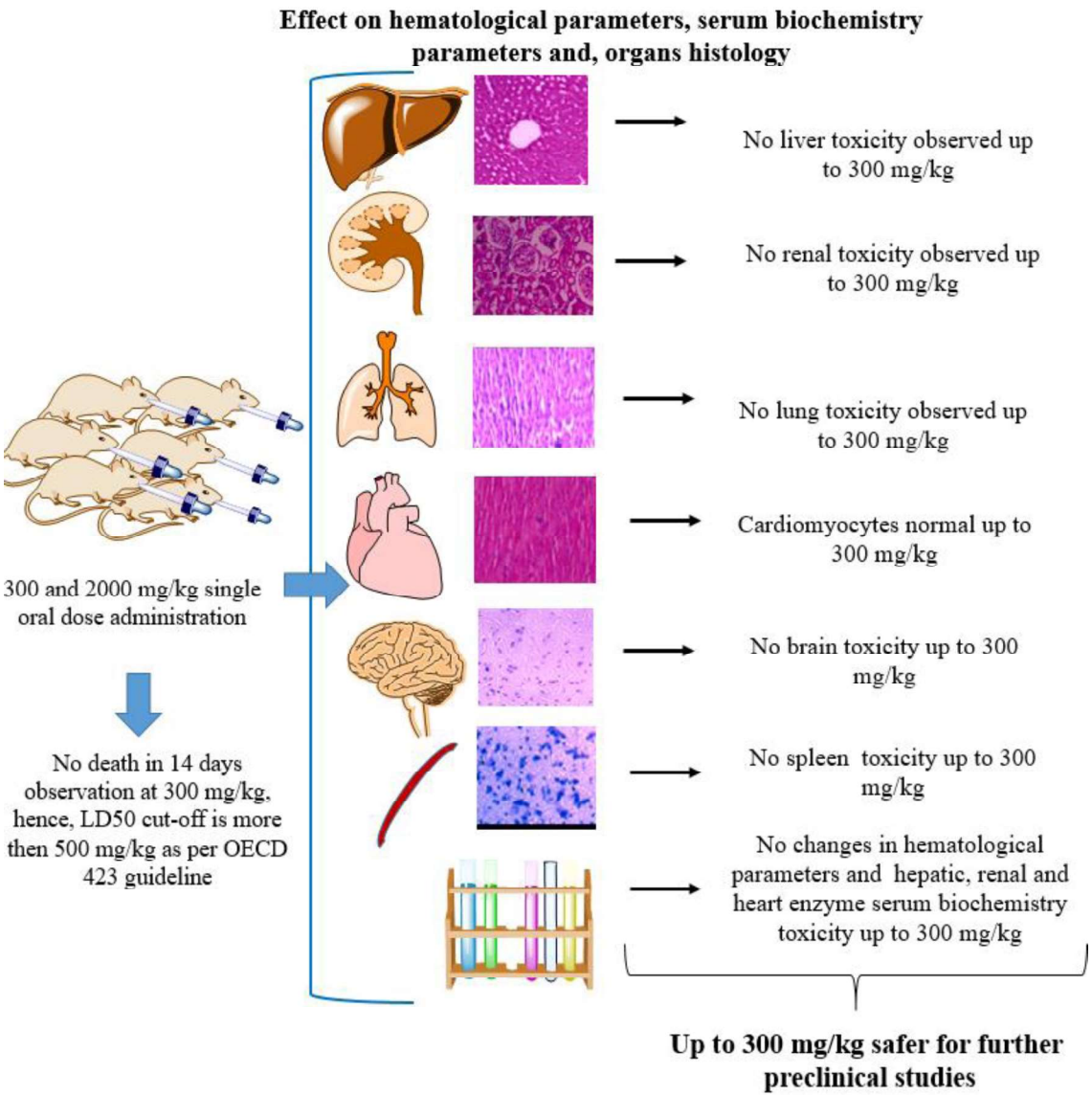

Figure 4.2 summarizes the experimental outcomes of the oral acute toxicity study.