Chapter 11

Hepatotoxicity Studies

11.1 INTRODUCTION

11.1.1 General

Drug-induced liver and kidney injury is a potential complication of nearly every medication that is prescribed, because the liver is the central organ for the metabolic disposition of virtually all drugs and foreign substances and kidney is the main organ related to excretion of metabolites generated by liver (352-354). Although drugs are usually metabolized excreted without injury to the liver and kidney, many fatal and near-fatal drug reactions are reported each year. A few compounds produce metabolites that cause liver and kidney injury in a uniform, dose-dependent fashion (355). Injury to hepatocytes and kidney cells result either directly from the disruption of intracellular function or membrane integrity or indirectly from immune-mediated membrane damage. Factors promoting the accumulation of hepatocytes and kidney toxins include genetic alterations in enzymes that allow the formation of the harmful metabolite, competition by other drugs, and depletion of the substrates required to detoxify the metabolite (356,357).

Flutamide, an oral antiandrogen agent, was marketed for patients with metastatic prostate cancer (stage D_2). Reported flutamide toxicity has included diarrhea (358) and hepatitis (359-363). 6-MP itself is biologically inactive as an inhibitor of purine synthesis (364). It must be anabolized by the enzyme hypoxanthine guanine phosphoribosyl transferase to thioguanine nucleotides to exert its cytotoxic effects (365,366). Incorporation of thioguanine nucleotides into cellular nucleic acids is the mechanism of 6-MP's cytotoxicity. Because the cytotoxic and immunosuppressive effects of 6-MP, it produces nonspecific, undesired effects such as hepatotoxicity, nephrotoxicity and an increased risk of neoplasia may occur with use of these drugs (367). Evidence of increased enzyme levels, associated clinical symptoms, and histopathological studies could be helpful to evaluate drug related hepatotoxicity and nephrotoxicity.

11.1.2 Drug related side effects

Main side effects associated with flutamide and 6-Mercaptopurine are:

Flutamide	**6-Mercaptopurine**
1. Hepatic Injury	1. Jaundice, Hepatic necrosis
2. Renal Impairment	2.Hyperuricemia, Hematuria and

3. Drowsiness, confusion, depression, anxiety, nervousness 4. Anorexia 5. Anemia, leukopenia and thrombocytopenia	crystalluria 3. Myelosuppression and Immunosuppression 4. Gastrointestinal ulceration

11.1.3 Laboratory Tests

Since transaminase abnormalities and rarely jaundice have been reported with the use of FLT and 6-MP, periodic liver function tests should be considered. Laboratory abnormalities including SGOT (serum glutamic oxaloacetic transaminase), SGPT (serum glutamic pyruvic transaminase)have been performed to evaluate hepatotoxicity.

11.2 EXPERIMENTAL

11.2.1 Selection of animals

Wistar-Albino Rats of both sex weighing 200-300 gm obtained from Zydus Research Center, Gujarat, India. The animals were housed in departmental animal house under natural light conditions, fed a standard pellet diet and water ad libidium. Their care and handling were in accordance with the provisions of Social Justice and Empowerment Committee as recognized and adopted by the Ministry of Government of India, New Delhi. Institute's Animal Ethics Committee approved the project. No diet restriction was enforced prior to studies. Three rats were taken for the study in each group.

11.2.2 Hepatotoxicity studies of FLT and its preparations

11.2.2.1 Histopathological studies and Biochemical analysis

Each animal was treated with 25 mg/kg/day intravenously with control, free drug, encapsulated drug in CL, SL and microbubbles for 7 days and was sacrificed by cervical dislocation and dissected to collect liver after one week of last injection. After each microbubbles dose everyday 30 sec ultrasound exposure was given at tail vein using 0.5 MHz ultrasound transducer. The liver was fixed in 10% neutral buffered formalin. Sections of 3-5 mm thickness were stained with hematoxylin and eosin (H&E) for microscopical examination under Olympus (BX 40F4, Tokyo, Japan) microscope. An alternative estimation method is the measurement of ALT

(Alanine aminotransferase) level to prove liver damage. After three days of injection of all formulations during histopathological studies, 1 ml of blood from each rat was collected, serum was separated by centrifugation at 1500 RPM for 10 min at 4°C and ALT level was measured using standard diagnostic kit. Statistical analysis of ALT estimation was performed by analysis of variance (ANOVA), difference was determined by applying student 't' test and a statistical probability of $p < 0.01$ was considered to be significant. All results were expressed as mean ± standard deviation.

11.2.3 Hepatotoxicity studies of 6-MP and its preparations

11.2.3.1 Histopathological studies and Biochemical analysis

Each animal was treated with 5 mg/kg/day intravenously with control, free drug and encapsulated drug in CL and SL for 7 days and was sacrificed by cervical dislocation and dissected to collect liver after one week of last injection. After each microbubbles dose everyday 30 sec ultrasound exposure was given at tail vein using 0.5 MHz ultrasound transducer. The liver was fixed in 10% neutral buffered formalin and treated the same as above. After one week of last injection of all formulations, 1 ml of blood from each rat was collected and serum was separated by centrifugation at 1500 RPM for 10 min at 4°C. Hepatic function abnormalities were detected by measuring the serum GOT (glutamic oxaloacetic transaminase) and GPT (glutamic pyruvic transaminase) level using established test kits (Span Diagnostics Ltd., India) in pathological laboratory. Statistical analysis for biochemical estimation was performed by applying student 't' test and a statistical probability of $p < 0.01$ was considered to be significant. All results were expressed as mean ±standard deviation.

11.3 RESULTS AND DISCUSSION

11.3.1 Histopathological studies and Biochemical analysis of FLT and its preparations

Each drug produces different morphological and functional alterations and therefore different clinical manifestations. Reported FLT toxicity includes diarrhoea and hepatotoxicity. Figure 11.1 shows microscopic examination of liver collected after treated animals as described above with (a) control, (b) free drug, (c) CL, (d) SL and (e) AALs.

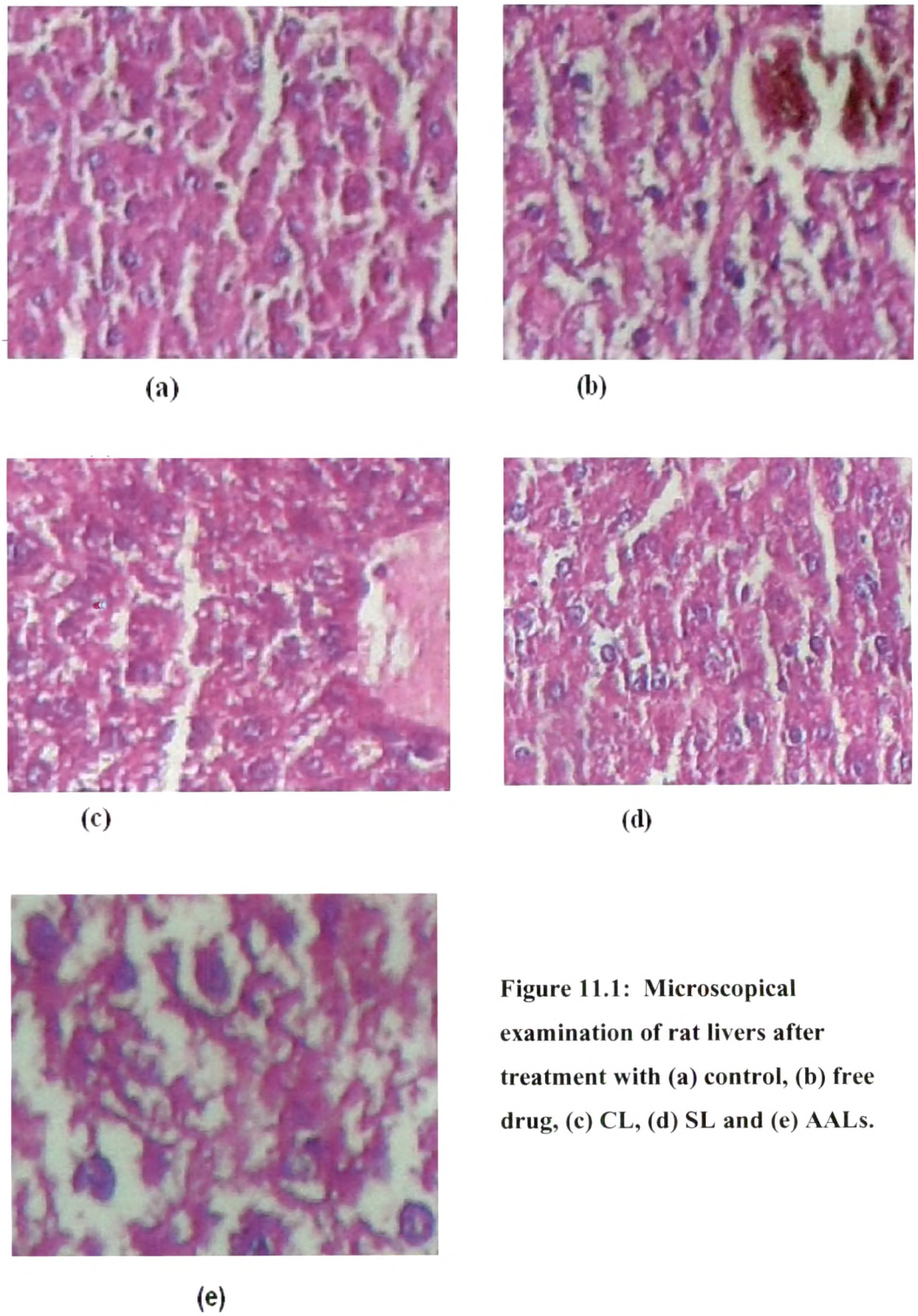


Figure 11.1: Microscopical examination of rat livers after treatment with (a) control, (b) free drug, (c) CL, (d) SL and (e) AALs.

It is evident that free drug showed patchy marked necrosis, fatty degeneration changes and eccentrically situated nuclei with bile duct proliferation. CL showed cloudy degeneration and patchy necrosis, while SL showed no changes in

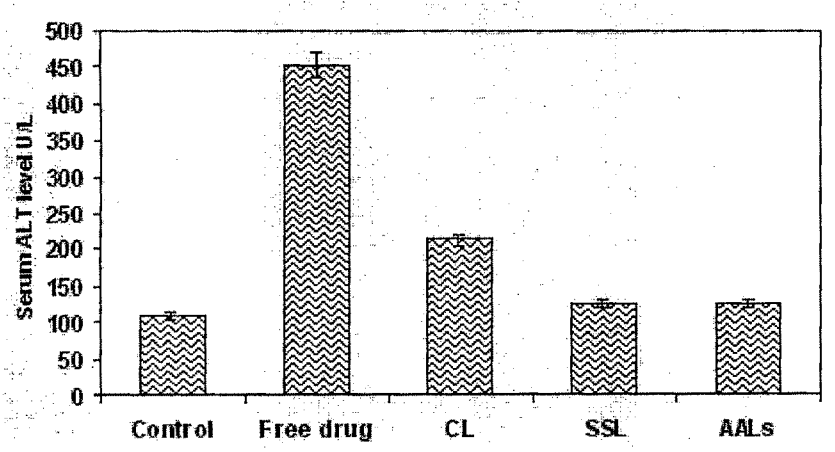
hepatocytes and any other liver structure and are same as control. The actual cause of cell death remains unclear. One result of the covalent binding of substrate or lipid peroxidation within cells is an increase in levels of cytosolic calcium. Calcium is important for the regulation of a number of cell functions, including maintenance of the cytoskeleton and membrane integrity. Actin depolymerization and polymerization are dependent on calcium ion fluxes within the cytosol. The results of studies using NAPQI (*N* -acetyl- *p* -benzoquinoneimine) in isolated hepatocytes suggest that alterations in calcium homeostasis occur with the influx of calcium ions into the cytosol. Whether this is the cause or the result of disordered membrane transport is unclear, but altered permeability may lead to blebs in the cell membrane and loss of membrane integrity. Other mechanisms may also be at play; in each instance, the covalent binding of reactive intermediates to cell proteins seems to be the initiating step.

Table 11.1: Serum ALT level of different formulations of FLT in rats at dose of 25 mg/kg/day (n=3)

Type of formulation	Control	FLT	FLT-CL	FLT-SL	FLT-AALs
Serum ALT level	110.33 ± 14.01	453±27.51	213.3 ±18.58	125 ±10.81	132±9.84
'p' cal	-	0.0048*	0.00117*	0.024[a]	0.0087[a]
Pearson correlation	-	-0.999	0.308	0.9797	0.99

[a]$p<0.01$, compared with control, *$p>0.01$, compared with control

Figure 11.2: Serum Alanine Transaminase level after i.v administration of control, free drug, CL, SL at dose of 25 mg/kg/day of FLT after 3 days



[a]$p<0.01$, ($p=0.0048$) indicates significantly different from control, [b] $p<0.01$, ($p=0.011$) indicates significantly different from control

Table 11.1 and Figure 11.2 show serum ALT level after treating animals as above with control, pure drug, CL, SL and AALs. ALT level in free drug (453±27.51U/L with p= 0.0048) was also higher than those in CL (213.3±18.58 U/L with p= 0.0011). ALT level in SL (125±10.81 U/L with p =0.0248) and AALs (132±9.34 U/L with p =0.0087) was insignificant different from control (110.33±14.01 U/L). Pearson correlation of SL (0.9797) and AALs (0.99) showed good correlation with control as compared to CL (0.308) and pure FLT (-0.999). The increase or decrease of enzyme activity is related to the intensity of cellular damage. Therefore, increase of transaminase activity may be the consequence of FLT induced pathological changes of the liver.

11.3.2 Histopathological studies and Biochemical analysis of 6-Mercaptopurine and its preparations

Since the hepatocytes are the main metabolic engine of the liver, most adverse drug reactions result in hepatocytes necrosis. The most common reaction leading to cell necrosis is the formation of covalent bonds between a reactive metabolite of the parent compound and cell proteins or DNA. Figure 11.3 indicated that free drug and CL showed injured bile ducts (bile duct proliferation) or canaliculi causing cholestasis without marked damage of hepatocytes as well as fatty degeneration changes, while SL showed no changes or injury to bile ducts or canaliculi hepatocytes and any other liver structure the same as control. A liver biopsy reveals engorgement of the canaliculi with bile and minimal hepatocellular injury. Eosinophils may be found in mildly inflamed portal tracts. The mechanism of cholestatic injury remains unclear. It may decrease bile flow and Na^+/K^+-ATPase, change tight junctions between cells, and alter the fluidity of the hepatocytes membrane.

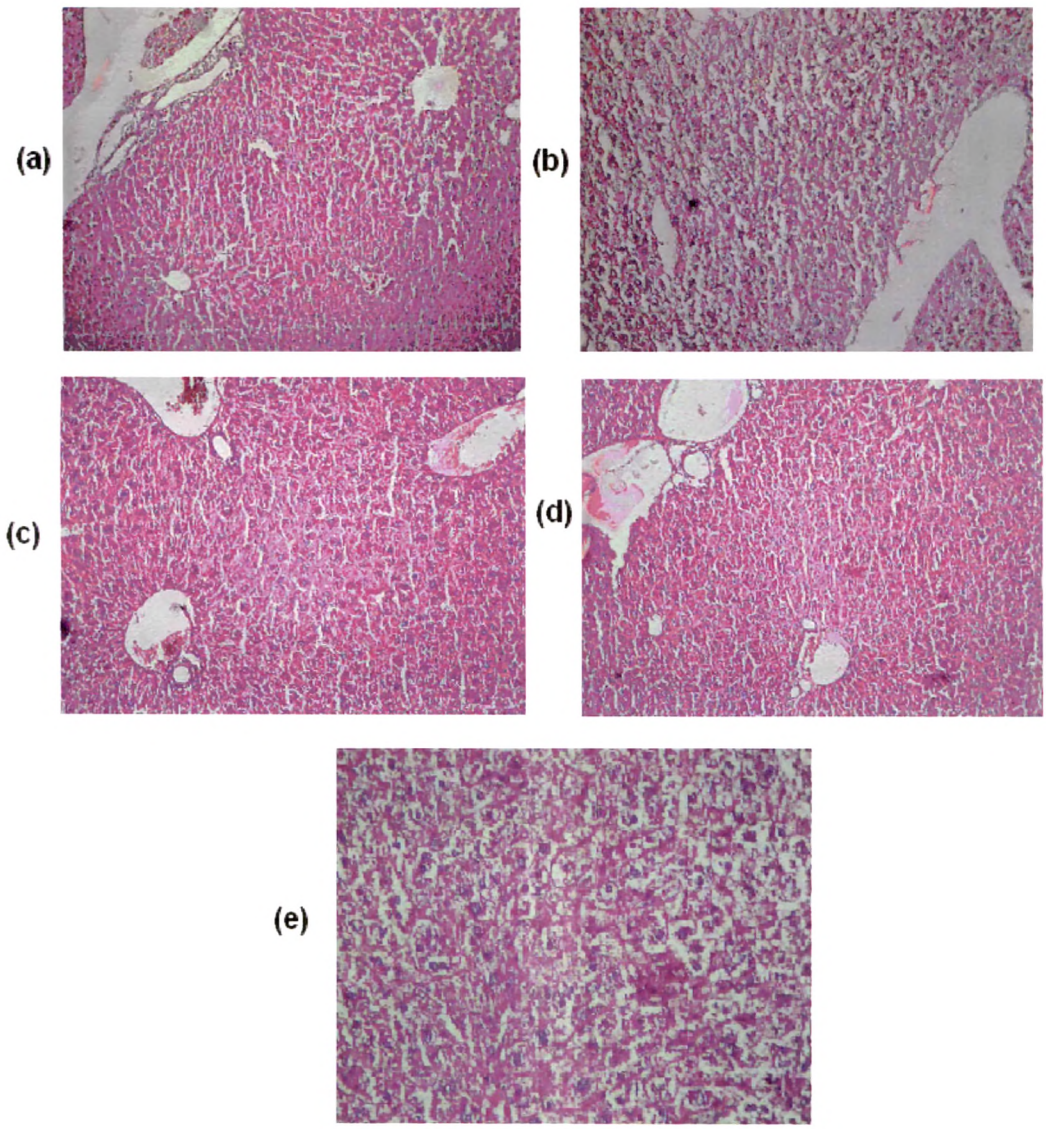


Figure 11.3 Microscopical examination of liver without treatment (a) control and after treated animals with (b) free drug, (c) CL, (d) SL and (e) AALs

When liver damage is suspected in rats, the amount of serum ALT, GOT, GPT level for liver abnormality are to be measured. There were no significant difference found in serum ALT level after giving treatment of all formulations to rats. Biochemical data (SGOT and SGPT) measured in rats treated with control, free drug, encapsulated drug in CL, SL and AALs is shown in Table 11.2.

Table 11.2: Serum biochemical data of different formulations of 6-MP in rats at dose of 5 mg/kg/day (n=3)

Type of formulation	Control	6-MP	CL	SL	AALs
SGOT (IU/L)	396±27	1961±122[a]	751±84[a]	401±26*	499± 31*
SGPT (IU/L)	80±16	726±68[a]	411±45[a]	91±22*	92±23*

[a]$p<0.01$, compared with control, *$p>0.01$, compared with control

In the present study, serum GOT and serum GPT level were higher in case of 6-MP (1961±122 IU/L, p=0.001 and 726±68 IU/L, p=0.0055) and conventional liposomes (751±84 IU/L, p=0.008 and 411±45 IU/L, p=0.002), whereas stealth liposomes and AALs showed 401±26 IU/L, p= 0.013 and 439± 31 IU/L, p=0.015 of serum GOT and 91±22 IU/L, p= 0.086 and 102±23 IU/L, p=0.018 of serum GPT level. It indicated that stealth liposomes and AALs could reduce hepatotoxicity associated with 6-MP.

11.4 CONCLUSION

Histopathological changes in structure of liver after injecting different formulations of both drugs indicated that SL and AALs showed no changes or injury to bile ducts or canaliculi hepatocytes and any other liver structure and are the same as control. Biochemical analysis showed that no significant difference was observed in serum ALT, GOT and GPT after SL and AALs were injected. It indicated that stealth liposomes could reduce hepatotoxicity associated with FLT and 6-MP.