Chapter 9

In vitro Cell Cytotoxicity Studies

9.1 INTRODUCTION

While cytotoxicity testing is currently conducted using animals, studies published in recent years have shown a correlation between in vitro and in vivo acute toxicity. These studies suggest that in vitro methods may be helpful in predicting in vivo cytotoxicity. The use of cell cultures in vitro as alternatives to predict acute lethality in vivo has been under study for almost 50 years (319-321).

Numerous demonstrations of correlations between cytotoxicity in vitro and animal lethality in vivo exist (322,323). Recently, several major international in vitro initiatives have been directed toward reducing the use of laboratory animals for acute toxicity testing (324-329).

9.1.1 Methods for measurement of cytotoxicity

The measurement of cell viability and growth is a valuable tool in a wide range of research areas. Many measurement endpoints for cytotoxicity are well established and have been used to assess basal cytotoxicity. For inclusion of inhibitory concentration, IC_{50} values in the registry of cytotoxicity (RC), the following endpoints were accepted as sufficiently characteristic of basal cytotoxicity (330).

1) Inhibition of cell proliferation

- Cell number
- Cell protein
- DNA content, DNAS synthesis
- Colony formation

2) Cell viability – metabolic markers

- Metabolic inhibition test (MIT 24)
- Mitochondrial reduction of tetrazolium salts into insoluble dye (MTT test), or, more recently, into soluble dye (MTS test or XTT test).

3) Decreased cell viability – membrane markers

- NRU into cell lysosomes
- Trypan Blue exclusion
- Cell attachment, cell detachment

4) Differentiation markers

- Functional differentiation within cell islets
- Morphological differentiation within cell islets.
- Intracellular morphology

The in vitro cytotoxicity of the anticancer drugs and their liposome formulations can be evaluated by using MTT (3-(4-5 dimethyulthiazol 2-yl) 2,5-diphenyl tetrazolium bromide) assay. MTT cell proliferation assay offers a quantitative convenient method for evaluating a cell population's response to external factors, whether it is an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis.

9.1.2 Principle of MTT assay

The antitumor activity of the drugs can be evaluated by MTT assay. MTT (3-4,5 dimethylthiazol-2-yl) s, 5 di-phenyl tetrazolium bromide) is water soluble chemical. Active mitrochondrial dehydrogenases of living cells convert the yellowish MTT to an insoluble purple formazan crystal (331). This conversion does not take place in dead cells. Tetrazolium salts are cleaved to foramen by the succinate tetrazolium reductase system. Which belong to the respiratory chain of the mitochondria, and is only active in metabolically intact cells. This water insoluble formazan can be solubilized using dimethyl sulphoxide (DMSO) and the dissolved material can be measured spectrophotometrically.

The reduction of tetrazolium salts is now recognized as a safe, accurate alternative to radiometric testing. For each cell type a linear relationship between cell number and absorbance is established, enabling accurate, straightforward quantification of changes in proliferation. Among the applications for the method are drug sensitivity, cytotoxicity, response to growth factors, and cell activation.

9.1.3 Advantages of MTT method

1. Proven technology: the utility of the MTT method has been documented in the literature for many different applications.
2. Accurate measurements: The spectrophotometric procedure can detect slight changes in cell metabolism, making it much more sensitive than trepan blue staining.
3. Easy to use: the procedure is relatively simple and uses equipment already available in most laboratories.
4. Rapid processing: Assays are run in a 96 well plate and read with a microtitre plate reader, allowing high-throughput handling of samples.
5. Convenient storage: The kit is stable for 18 months when stored under refrigeration in the dark.

9.2 EXPERIMENTAL

9.2.1 Cell lines

The HL-60 and PC-3 cell lines was established in National Center for Cell Sciences, Pune, India. The cells were maintained as monolayer cultures in plastic flasks (Tarsons, India) and passaged after every three by seeding in two T-25 flasks. Both cell lines was maintained on RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics.

9.2.2 Solutions

9.2.2.1 MTT solution

5mg/ml MTT (Hi-media Laboratories Pvt. Ltd., Mumbai) in phosphate buffer saline pH 7.4 (PBS). This solution was filtered through a 0.2 μm filter and stored at 2.8°C.

9.2.2.2 Solution of Flutamide (FLT) and its liposomal formulations

Stock solution of FLT (2 mM) was prepared by dissolving 11.7 mg of FLT in 1 ml of water for injection (WFI) containing 0.1 ml of PEG-400 for solubilizing FLT. The liposomal formulations containing FLT were also diluted to give a stock solution of final concentration of 4 mM solution. 50 μl of the stock solution of FLT and its liposome formulations were added into the well containing 50 μl RPMI 1640 medium and then applying 2-fold dilution 50 μl of mixture present in previous well was added to next well to give final concentrations of 1000, 500, 250, 125, 62.5 and 31.25 mM of FLT.

9.2.2.3 Solution of 6-Mercaptopurine (6-MP) and its liposomal formulations

Stock solution of (2 mM) was prepared by dissolving 13.68 mg of 6-MP in 1 ml of WFI containing 0.01 N 6-MP for solubilizing 6-MP. The liposomal formulations containing 6-MP were also diluted to give a stock solution of final concentration of 2 mM solution. 50 μl of the stock solution of 6-MP and its liposome formulations were added into the well containing 50 μl RPMI 1640 medium and then applying 2 fold dilution 50 μl of mixture present in previous well was added to next well to give final concentrations of 2000, 1000, 500, 250, 125, and 62.5 mM of 6-MP.

9.2.2.4 MTT Assay for liposomal formulations

HL-60 and PC-3 cancer cells were harvested for 6-MP and FLT, respectively and about 20,000 cells/well were added into 96 well flat bottom plate in 50 µl of RPMI 1640 containing 10% fetal bovine serum. The drug solution and liposomal formulations containing respective drug concentration was added in one well and serial dilution was made by adding 50 µl of mixture present in previous well was added to next well in a pattern shown in Figure 9.1.

Figure 9.1: Pattern of formulations added in 96 well flat bottom ELISA plate

1	2	3	4	5	6	7	8	9	10	11	12
	D6	D6	D6	L6	L6	L6	SL6	SL6	SL6	M1	M2
	D5	D5	D5	L5	L5	L5	SL5	SL5	SL5	M4	M3
	D4	D4	D4	L4	L4	L4	SL4	SL4	SL4		
	D3	D3	D3	L3	L3	L3	SL3	SL3	SL3		
	D2	D2	D2	L2	L2	L2	SL2	SL2	SL2	C1	C2
	D1	D1	D1	L1	L1	L1	SL1	SL1	SL1	C4	C3

D: Drug solution; L: Conventional liposomes; SL: Stealth liposomes; M: Medium and C: Cells with medium

The plates were incubated for 24 hr and 48 hr at 37°C in a humidified incubator (5% CO_2 and 95% air). 20 µl of MTT solution was added to each well and incubated at 37°C in a humidified incubator for 4 hr. 150 µl of DMSO was added to each well and further incubated for 16 hr. The plate was mechanically shaken for a few minutes to dissolve the purple colored formazan crystals. The optical density was measured at 570 nm by keeping the reference at 630 nm using an Elisa Plate Reader.

9.2.2.5 Microbubble formulations containing Flutamide (FLT) and 6-Mercaptopurine (6-MP)

The various types of microbubble formulations containing FLT and 6-MP were diluted to give a stock solution of final concentration of 1 mM solution. 50 µl of the stock solutions were added into the well containing 50 µl RPMI 1640 medium and

then applying 2-fold dilution 50 µl of mixture present in previous well was added to next well to give final concentrations of 500, 250 and 125 mM of FLT and 6-MP.

9.2.2.6 MTT Assay for microbubbles

HL-60 and PC-3 cancer cells were harvested for 6-MP and FLT, respectively and about 20,000 cells/well were added into 96 well flat bottom plate in 50 µl of RPMI 1640 containing 10% fetal bovine serum. The various formulations of microbubbles containing respective drug concentration was added in one well and serial dilution was made by adding 50 µl of mixture present in previous well was added to next well in a pattern shown in Figure 9.2.

Figure 9.2: Pattern of formulations added in 96 well flat bottom Elisa plate

1	2	3	4	5	6	7	8	9	10	11	12
										M1	M2
										M4	M3
	MB3	MB3	MB3	GFM3	GFM3	GFM3	AAL3	AAL3	AAL3		
	MB2	MB2	MB2	GFM2	GFM2	GFM2	AAL2	AAL2	AAL2		
	MB1	MB1	MB1	GFM1	GFM1	GFM1	AAL1	AAL1	AAL1	C1	C2
										C4	C3

MB: Microbubbles (Method 1); GFM: Gas-filled Microspheres; AAL: Acoustically Active Lipospheres; M: Medium and C: Cells with medium

To evaluate the types of ultrasound probe (2 MHz or 0.5 MHz) and time in second required to burst microbubbles, the wells were exposed to both types of ultrasound probes for different time intervals (15, 30 and 45 sec). The plates were incubated for 48 hr at 37°C in a humidified incubator (5% CO_2 and 95% air). 20 µl of MTT solution was added to each well and incubated at 37°C in a humidified incubator for 4 hr. 150 µl of DMSO was added to each well and further incubated for 16 hr. The plate was mechanically shaken for a few minutes to dissolve the purple colored formazan crystals. The optical density was measured at 570 nm by keeping the reference at 630 nm using an Elisa Plate Reader.

9.3 RESULTS AND DISCUSSION

The *in vitro* cytotoxicity study was carried out as a preliminary study before performing the *in vivo* biodistribution study of the liposomes encapsulated anticancer drugs (FLT and 6-MP). This was carried out to evaluate the cytotoxicity potential of the drugs when encapsulated in liposomes and microbubbles.

9.3.1 Flutamide and its liposomes

Various concentrations (31.25mM to 1000mM) of flutamide and its liposomes (CL and SL) were added to the 96 wells flat bottomed plate containing PC-3 cells grown in RPMI 1640 medium. The Figure 9.3 (a) and (b) explain the relationship between the various concentration of FLT and its liposomes and cytotoxicity (% cell viability) of FLT at 24 hr and 48 hr, respectively. The cytotoxicity effect of FLT was found after 24 hr, but higher amount of cytotoxicity was found after 48h of incubation. The cytotoxicity effect produced by FLT and its liposomes after 48h of incubation at 37°C in a humidified incubator (5% CO_2 and 95% air) is shown in Table 9.1 and Figure 9.3 (b). As the concentration of the drug (in free form as well as in liposomal formulations) increases there was a gradual reduction in the % cell viability, which shows the cytotoxicity effect produced by flutamide.

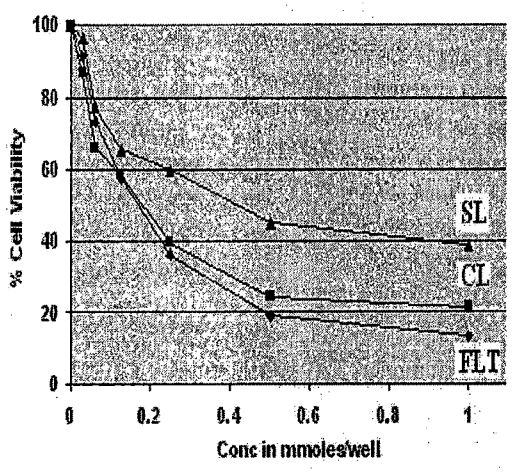

Figure 9.3 (a): % cell viability Vs. concentrations of FLT at 24 hr

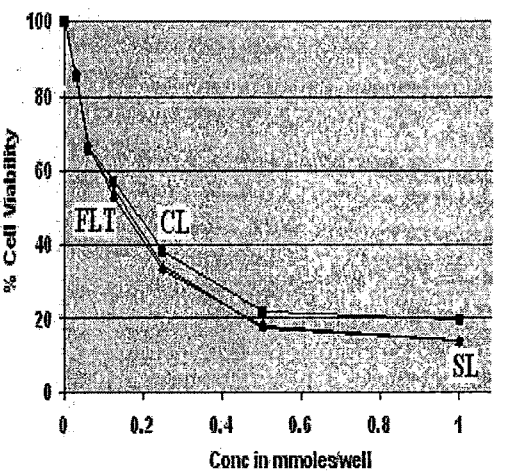

Figure 9.3 (b): % cell viability Vs. concentrations of FLT at 48 hr

Table 9.1: In vitro cytotoxicity study of flutamide and its liposomes at 48 hr of incubation

Concentration in mM	OD_{570}			% Cell Viability		
	FLT	CL	SL	FLT	CL	SL
0	1.464	1.464	1.464	100	100	100
0.03125	1.261	1.247	1.258	86.11	85.20	85.91
0.0625	0.975	0.965	0.962	66.62	65.89	65.73
0.125	0.774	0.832	0.799	52.87	56.81	54.55
0.25	0.488	0.561	0.503	33.36	38.34	34.34
0.5	0.266	0.324	0.262	18.19	22.15	17.92
1	0.200	0.290	0.206	13.64	19.79	14.09

The cytotoxicity produced by the FLT and by its liposomes was almost similar and no significant difference was found between them. After 48h of incubation, there was a reduction of almost 85% of cell viability at a concentration of 1000 mM of FLT. The reason for the similar, cytotoxicity effect may be due to the phospholipid used. The use of PC, which is having a transition temperature at around 37°C, may be attributed to the drug release from the liposomes when incubated at 37°C. The addition of the polymer ($mPEG_{2000}$-CC-PE) in the bilayer for producing steric stability to the liposomes did not cause any significant change in the cytotoxicity of the drug.

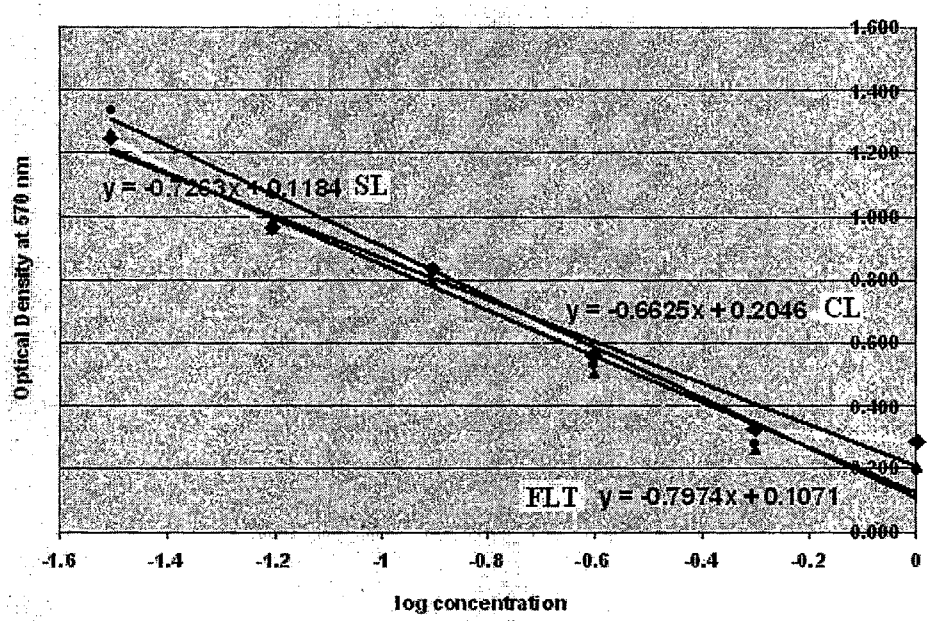

Figure 9.4: plot of Log conc. [mmoles/well] Vs OD_{570}

The cytotoxic potential of the empty liposomes was also tested and found to be non-toxic to the cells, which proves that the cytotoxicity caused by the liposomal FLT is due to the released drug and not due to any components of the liposome bilayer. The plot of Log conc. [mmoles/well] Vs OD_{570} was used to calculate IC_{50} (Figure 9.4). IC_{50} (concentration of drug required to kill 50% of cells) of FLT conventional liposomes (0.160 mM) and stealth liposomes (0.1438 mM) were found statistically insignificant different from pure FLT (0.1655).

9.3.2 6-Mercaptopurine and its liposomes

Various concentrations (62.5 mM to 1000mM) of 6-MP and its liposomes (CL and SL) were added to the 96 wells flat bottomed plate containing HL-60 cells grown in RPMI 1640 medium. The Figure 9.5 (a) and (b) explain the relationship between the various concentration of 6-MP and its liposomes and cytotoxicity (% cell viability) of 6-MP at 24 hr and 48 hr, respectively. The cytotoxicity effect of 6-MP was found after 24 hr, but higher amount of cytotoxicity was found after 48h of incubation. As the concentration of the drug (in free form as well as in liposomal formulations) increases there was a gradual reduction in the % cell viability, which shows the cytotoxicity effect produced by 6-MP.

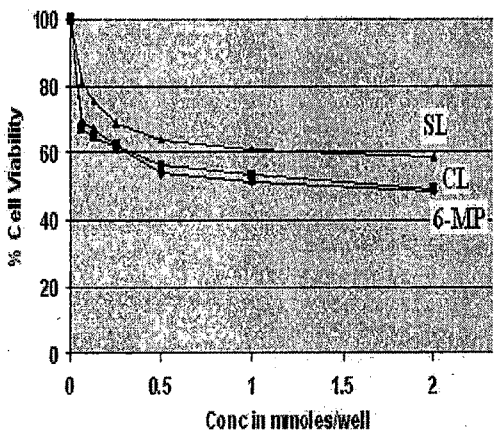

Figure 9.5 (a): % cell viability Vs. concentrations of 6-MP at 24 hr

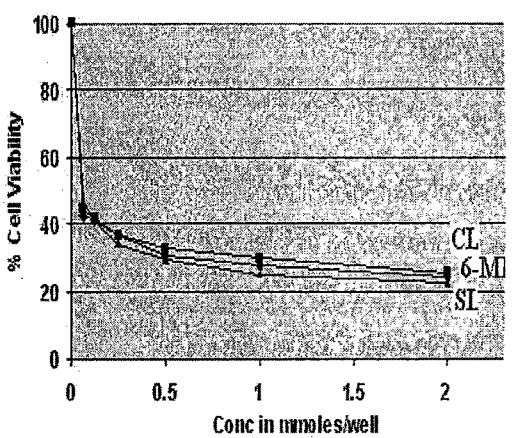

Figure 9.5 (b): % cell viability Vs. concentrations of 6-MP at 48 hr

Table 9.2: In vitro cytotoxicity study of 6-Mercaptopurine and its liposomes at 48 hr of incubation

Concentration	OD_{570}			% Cell Viability		
in mM	6-MP	CL	SL	6-MP	CL	SL
0	0.972	0.972	0.972	100	100	100
0.0625	0.414	0.434	0.450	42.56	44.65	46.26
0.125	0.398	0.402	0.398	40.98	41.36	40.98
0.25	0.355	0.355	0.335	36.52	36.49	34.43
0.5	0.294	0.320	0.287	30.28	32.96	29.53
1	0.265	0.291	0.242	27.30	29.97	24.90
2	0.234	0.250	0.219	24.11	25.69	22.50

The cytotoxicity produced by the 6-MP and by its liposomes was almost similar and no significant difference was found between them. After 48h of incubation, there was a reduction of almost 78 % of cell viability at a concentration of 2000 mM of 6-MP. The addition of the polymer ($mPEG_{2000}$-CC-PE) in the bilayer for producing steric stability to the liposomes did not cause any significant change in the cytotoxicity of the drug.

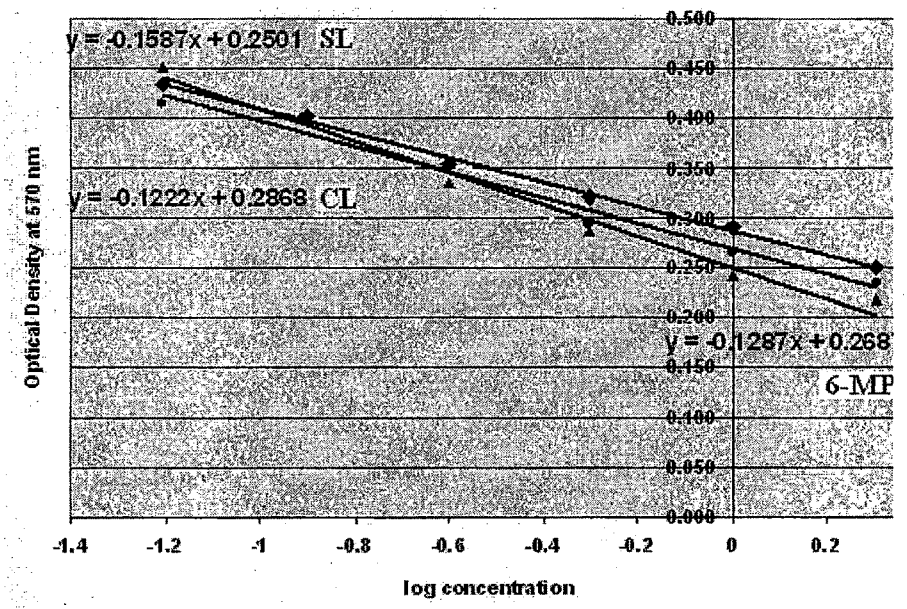

Figure 9.6: plot of Log conc. [mmoles/well] Vs

The cytotoxic potential of the empty liposomes was also tested and found to be non-toxic to the cells, which proves that the cytotoxicity caused by the liposomal 6-MP is due to the released drug and not due to any components of the liposome bilayer.
The plot of Log conc. [mmoles/well] Vs OD_{570} was used to calculate IC_{50} (Figure 9.6). IC_{50} of 6-MP conventional liposomes (0.025 mM) and stealth liposomes (0.030 mM) were found statistically insignificant different from pure 6-MP (0.022 mM).

9.3.3 Flutamide microbubbles

50 µl of various concentrations (0.125, 0.25 and 0.5 mM) of all types of microbubbles were added to the 96 wells flat bottomed plate containing PC-3 cells grown in RPMI 1640 medium. The Figure 9.7 (a, b and c) and Table 9.3 explain the relationship between the various concentrations of all types of FLT microbubbles exposed to ultrasound transducer with frequency of 2 MHz at different time intervals and cytotoxicity (% cell viability) of FLT at 48 hr. As the concentration of the drug (in free form as well as in liposomal formulations) increases there was a gradual reduction in the % cell viability, which shows the cytotoxicity effect produced by 6-MP. Figure 9.7 (a, b and c) and Table 9.3 data indicated that the exposure of 2 MHz ultrasound frequency for 45 sec showed 85-87% reduction in cell viability in all types of microbubbles.

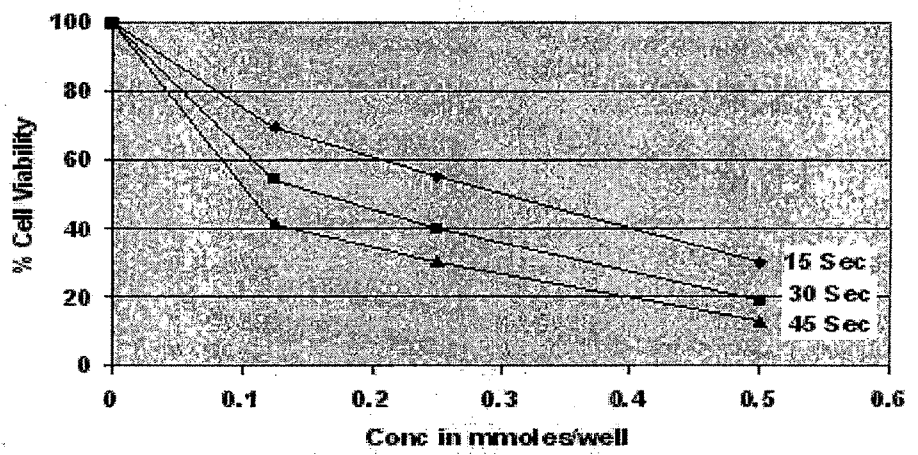

Figure 9.7 (a): % cell viability Vs. different concentrations of FLT microbubbles exposed to 2 MHz ultrasound frequency transducer at different time intervals for 48 hr of incubation

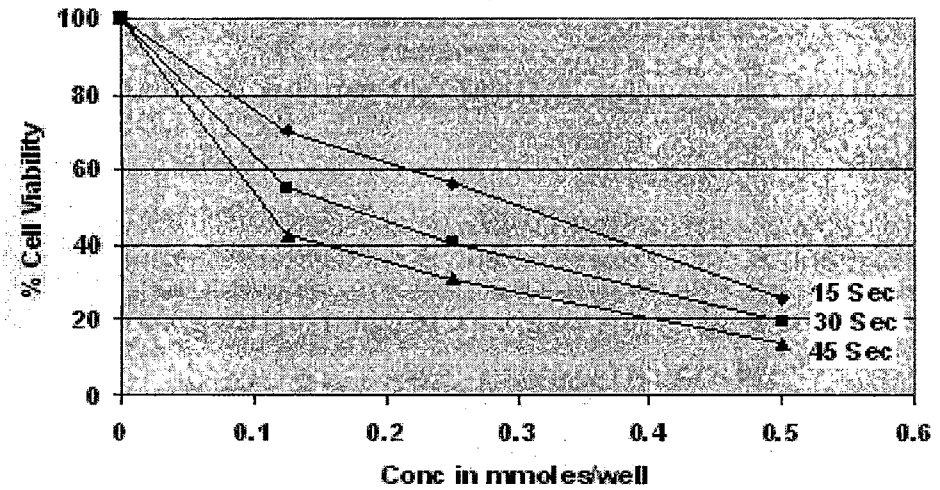

Figure 9.7 (b): % cell viability Vs. different concentrations of FLT gas-filled microspheres exposed to 2 MHz ultrasound frequency transducer at different time intervals for 48 hr of incubation

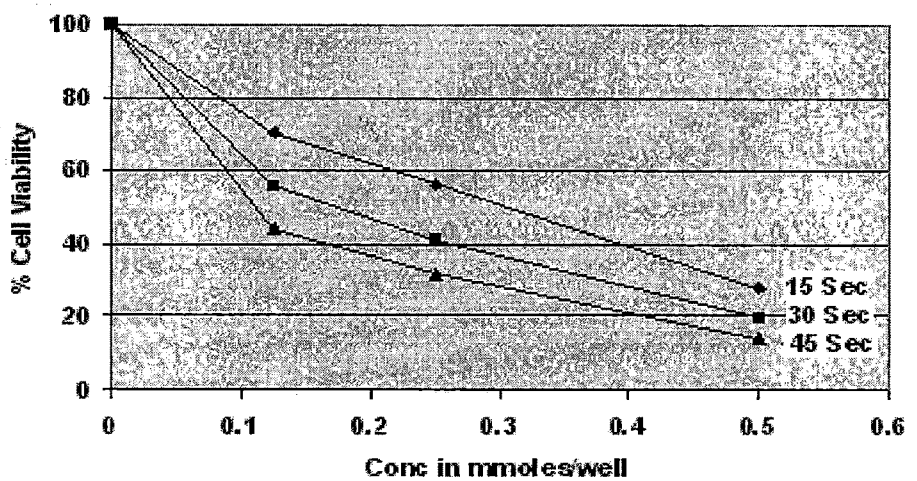

Figure 9.7 (c): % cell viability Vs. different concentrations of FLT acoustically active lipospheres exposed to 2 MHz ultrasound frequency transducer at different time intervals for 48 hr of incubation

There was no significant difference found between different types of microbubbles to reduce % cell viability. The AALs showed the maximum reduction in % cell viability (87%) as compared to MBs (85%) and GFMs (86%).

Table 9.3: In vitro cell cytotoxicity of various types of FLT microbubbles exposed to ultrasound transducer with frequency of 2 MHz at different time intervals after 48 hr of incubation

Con in mM	% Cell Viability								
	MBs			GFMs			AALs		
	15 sec	30 sec	45 sec	15 sec	30 sec	45 sec	15 sec	30 sec	45 sec
0	100	100	100	100	100	100	100	100	100
0.125	69.60	54.16	41.10	70.09	54.92	42.23	70.03	55.76	43.92
0.25	55.19	39.66	30.24	55.87	40.22	30.85	56.17	41.15	31.81
0.5	30.06	18.90	14.34	26.01	19.87	13.67	27.65	19.79	12.9

MBs: Microbubbles (Method 1); GFMs: Gas-filled Microspheres; AALs: Acoustically Active Lipospheres

Table 9.4: In vitro cell cytotoxicity of various types of FLT microbubbles exposed to ultrasound transducer with frequency of 0.5 MHz at different time intervals after 48 hr of incubation

Con in mM	% Cell Viability								
	MBs			GFMs			AALs		
	15 sec	30 sec	45 sec	15 sec	30 sec	45 sec	15 sec	30 sec	45 sec
0	100	100	100	100	100	100	100	100	100
0.125	62.34	40.89	40.67	61.48	42.06	42.81	59.01	40.07	44.95
0.25	51.22	30.43	30.75	49.02	32.12	31.57	45.02	30.24	32.01
0.5	34.71	13.22	13.4	29.81	13.80	14.02	24.63	13.26	12.7

The Figure 9.8 (a, b and c) and Table 9.4 explain the relationship between the various concentrations of all types of FLT microbubbles exposed to ultrasound transducer with frequency of 0.5 MHz at different time intervals and cytotoxicity (% cell viability) of FLT at 48 hr. The data indicated that the exposure of 0.5 MHz ultrasound frequency for 45 sec showed 86-87% reduction in cell viability in all types of microbubbles. There was no significant difference found between microbubbles to reduce % cell viability, when exposure time was increased after 30 sec. The AALs showed the maximum reduction in % cell viability (87%) as compared to MBs (86%) and GFMs (86%).

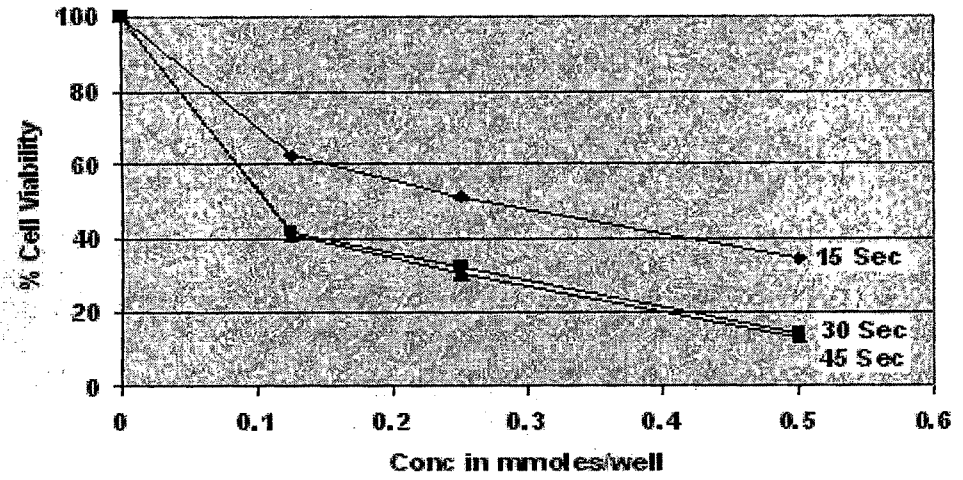

Figure 9.8 (a): % cell viability Vs. different concentrations of FLT microbubbles exposed to 0.5 MHz ultrasound frequency transducer at different time intervals for 48 hr of incubation

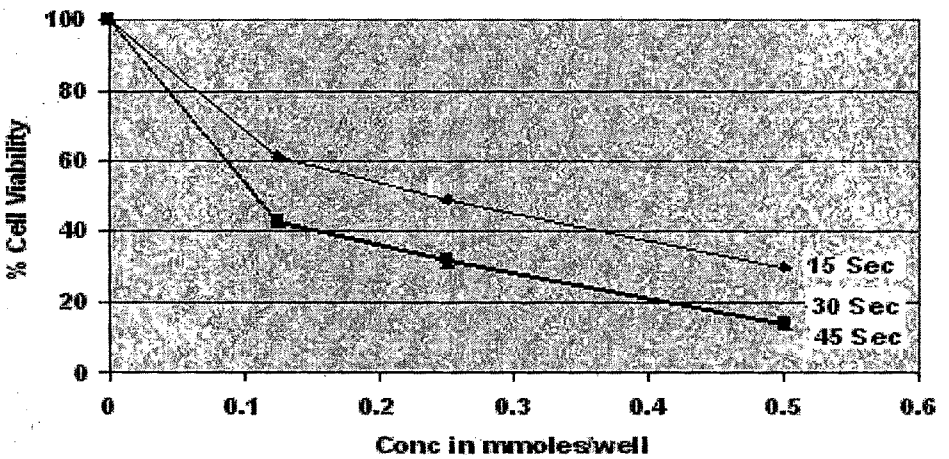

Figure 9.8 (b): % cell viability Vs. different concentrations of FLT gas-filled microspheres exposed to 0.5 MHz ultrasound frequency transducer at different time intervals for 48 hr of incubation

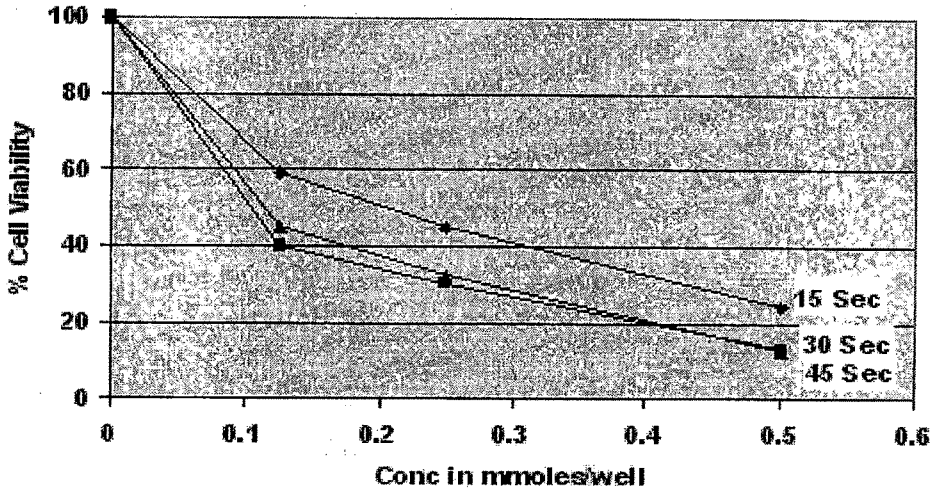

Figure 9.8 (c): % cell viability Vs. different concentrations of FLT acoustically active lipospheres exposed to 0.5 MHz ultrasound frequency transducer at different time intervals for 48 hr of incubation

9.3.4 6-Mercaptopurine microbubbles

Table 9.5: In vitro cell cytotoxicity of various types of 6-MP microbubbles exposed to ultrasound transducer with frequency of 2 MHz at different time intervals after 48 hr of incubation

Con in mM	% Cell Viability								
	MBs			GFMs			AALs		
	15 sec	30 sec	45 sec	15 sec	30 sec	45 sec	15 sec	30 sec	45 sec
2 MHz ultrasound transducer									
0	100	100	100	100	100	100	100	100	100
0.25	60.19	55.49	52.74	57.96	53.46	52.16	59.98	56.00	50.27
0.5	53.26	49.28	48.18	55.14	46.67	48.83	55.97	49.07	47.67
1	48.73	43.48	42.01	49.97	43.38	43.93	53.29	45.23	43.35
0.5 MHz ultrasound transducer									
0	100	100	100	100	100	100	100	100	100
0.25	52.64	43.11	42.56	51.65	43.55	42.32	54.70	42.26	41.27
0.5	50.62	40.60	40.67	49.28	40.57	39.37	53.12	33.98	32.27
1	47.60	36.15	35.97	47.09	36.28	36.08	48.66	26.43	25.02

50 µl of various concentrations (0.25, 0.5 and 1mM) of all types of microbubbles were added to the 96 wells flat bottomed plate containing HL-60 cells grown in

RPMI 1640 medium. Table 8.5 explains the relationship between the various concentrations of all types of 6-MP microbubbles exposed to ultrasound transducers with frequency of 2 MHz and 0.5 MHz at different time intervals and cytotoxicity (% cell viability) of 6-MP at 48 hr.

There was significant difference was found between different frequencies ultrasound transducers to reduce % cell viability. There was more reduction in % cell viability was found, when microbubbles were exposed to 0.5 MHz transducer as compared to 2 MHz transducer.

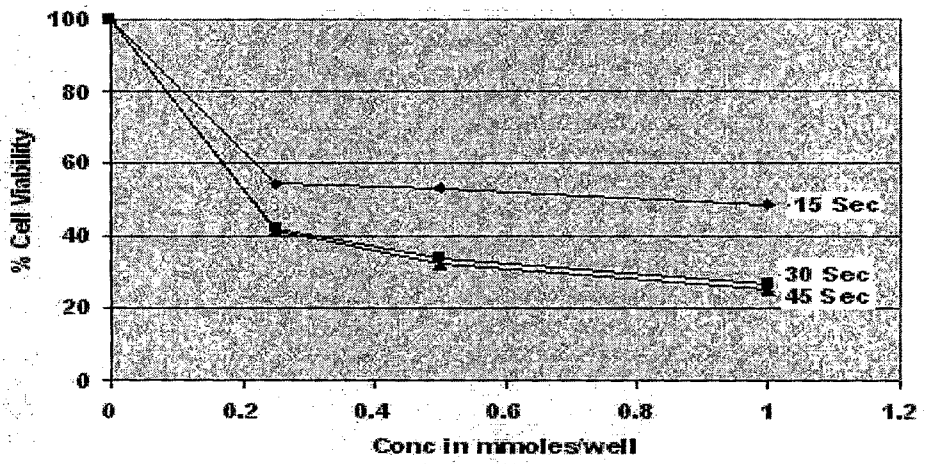

Figure 9.9: % cell viability Vs. different concentrations of 6-MP acoustically active lipospheres exposed to 0.5 MHz ultrasound frequency transducer at different time intervals for 48 hr of incubation

The exposure of 0.5 MHz ultrasound frequency for 45 sec showed the maximum reduction in % cell viability (66%) in AALs type of microbubbles (Figure 9.9). The AALs showed the maximum reduction in % cell viability (66%) as compared to MBs (64%) and GFMs (64%), when exposed to 0.5 MHz ultrasound transducer for 30 sec or more. Increase in exposure time of 2 MHz transducer in the range of 15 to 45 sec, progressive increase in cell viability was observed. There was no significant difference found between microbubbles to reduce % cell viability, when exposure time was increased after 30 sec, in case of 0.5 MHz ultrasound transducer.

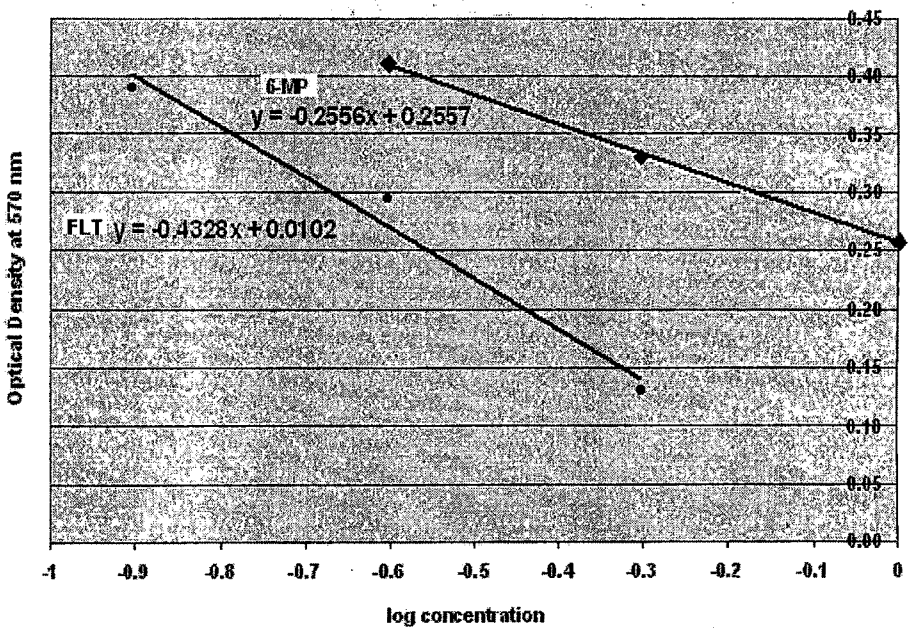

Figure 9.10: plot of Log conc. [mmoles/well] Vs OD_{570}

Figure 9.10 shows the plot of Log conc. [mmoles/well] Vs OD_{570} and was used to calculate IC_{50} of FLT and 6-MP AALs. IC_{50} of FLT and 6-MP AALs was found to be 0.021 mM and 0.012 mM, which were found to be statistically insignificant difference from pure FLT (0.016 mM) and pure 6-MP (0.022).

9.4 CONCLUSION

The cytotoxicity effect was found after 24 hr, but appreciable amount of cytotoxicity was found after 48 hr. IC_{50} (concentration of agent required to kill 50 % of cancer cells) was found 0.32, 0.38, and 0.35 mmoles against PC-3 cell line and 0.015, 0.018 and 0.016 mmoles of IC_{50} was found against HL-60 cell lines of pure drug, CL and SL, respectively and were not significantly different from each other. The addition of polymers in the bilayer to produce steric stability did not cause significant change in the cytotoxicity of the drug. It was found that 0.5 MHz ultrasound transducer is enabled to burst all microbubbles within 30 sec of exposure time, which may help during in-vivo studies. The AALs showed the cytotoxic effect similar to that of pure drugs. The in-vivo experiments were conducted using microbubbles prepared by method 3 (AALs) and 0.5 MHz ultrasound transducer.