Chapter 6

In-Vitro STUDIES ON NANOPARTICLES

6. *In-Vitro* STUDIES ON NANOPARTICLES

6.1Introduction:

This chapter will deal with the in vitro drug release and in vitro cell cytotoxicity studies.

In the present studies, the *in vitro* release of drug from ETN and QDN were studied by using dialysis bag technique as reported by LeRay et al. (1994).

The *in vitro* cytotoxic activity was studied by determining cell viabilities and IC50 values using MTT assay method on A549 cancer cell line(Human lung adenocarcinoma epithelial cell line – obtained from National Centre for Cell Science (NCCS), Pune).

6.2 In vitro drug release from ETN and QDN:

In vitro release studies of ET and QD from PLGA NPs were carried out at 37 ± 2°C by dialysis bag diffusion technique. Briefly 2.0 ml of the aqueous nanoparticulate dispersion was placed in cellulose dialysis bag (cutoff 12000, Hi-Media, India) and sealed at both ends. Then it was immersed in the 20.0 ml of pH 7.4 phosphate buffer solution in recipient compartment and stirred at about 100 rpm with magnetic stirrer. BSA (3mg/ml) was added to pH 7.4 phosphate buffer used for the QD release study to stabilize the drug in buffer. The receptor compartment was covered to prevent the evaporation of dissolution medium. The samples from the compartment were withdrawn at predetermined time intervals and replaced with the fresh release medium.The samples were analyzed for the drug content using UV-Vis Spectrophotometer (UV-Vis, Shimadzu 1701, Japan). A control experiment to determine the release behavior of the free drugs was also performed in the same way using the drug solutions. All experiments were performed in triplicate and average values were calculated.

The data for release study is presented in Table 6.1.

6.3 In vitro cell line studies: Cytotoxicity by MTT assay

The *in vitro* cytotoxic activities of the nanoparticle formulations (ETN and QDN) were investigated in comparison with free drug solutions of ET and QD on A549 cancer cell line. The comparison was also made with respect to cytotoxic activity of single drug and combination of drugs in the form of free drugs as well as nanoparticles. The IC 50 values were determined. The cell viabilities for predetermined time duration were found by MTT assay method reported by Mosmann (1983). In brief, the method is as under.

A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37°C with 5% CO2 in humidified air. Then cultures in the exponential growth phase were trypsinized and diluted in medium to give a total cell count of 5×10^4 cells/mL. The cell suspension was then transferred to 96-well plate to ensure 5000 cells/well and allowed to attach overnight. The medium was changed with 100-µl medium containing ET, QD, ETN and QDN of different concentrations (0.5, 5, 10, 50 µM). The combinations EQ and EQN were studied by using fixed concentration of QD (10 µM) and ET concentration Beeing varied at range mentioned above. The free drugs ET and QD were dissolved in dimethyl sulfoxide (DMSO), diluted with complete media. (The concentration of DMSO was different in the final serial diluted media but was less than 1%). Each formulation dilution was assessed in triplicate. Three wells containing only cells suspended in complete medium were used as controls for cell viability. After incubation for 24, 48, and 72h suspension was removed and the wells were washed with PBS. 10µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5mg/ml) and 90 µl of medium were added and incubated for around 3-4h. Then the media containing MTT was removed, leaving the precipitate. 100µl of DMSO was added to the wells. Plate was observed at 570nm using microplate reader. The results for IC 50 are presented in Table 6.4 and Figure 6.2. while that of cell viability studies are presented in Table 6.5, 6.6, 6.7 and Figure 6.3, 6.4, 6.5.

6.4 Result and Discussion:

6.4.1 In vitro drug release from ETN and QDN:

Table 6.1 and Figure 6.1 depict the release profiles of ET and QD from PLGA NPs (i.e. ETN and QDN). In contrast with the plain drug solutions (ET and QD), there was a pronounced time prolongation of drugs release from NPs.

Nearly 100% of drugs were released from plain drug solutions approximately in 6 h. But only about 40% of drugs released from NPs after 24 hrs (96.98±2.3% and 87.08±1.9% for ET and QD respectively from free drug solutions at 6 hrs, 46.69±2.7% and 38.65±2.0% for ET and QD from nanoparticles at 24 hrs). The reason behind this prolongation of release time is attributed to slow degradation of PLGA, therefore the release of ET and QD from nanoparticles depends on drug diffusion from PLGA surface and matrix as well as bulk erosion or swelling of polymer (Mu and Feng, 2003).

Moreover, drug release pattern from nanoparticles showed burst-release of drugs during the first 2 hrs of study, followed by a lag phase of relatively slow release, which is also reported earlier by various authors for PLGA NPs (Govender et al., 1999; Mainardes and Evangelista, 2005; Go´mez-Gaete et al., C 2007). The high initial burst can be attributed to the immediate dissolution and release of drugs adsorbed on the surface and diffusion of drugs located near the surface of the nanoparticles.

According to the release curves, it may be concluded that QD released slower than ET in both free drug solutions and their respective nanoparticles. This may be caused due to poor aqueous solubility of QD in comparison to ET in pH 7.4 Phosphate buffer. The cumulative release percentage of QD was found to be lower than that of ET at any time point from their respective nanoparticles.

The kinetic analysis of drug release was performed by subjecting the data to dissolution study models as represented by Table 6.2. The result of kinetic analysis reveals that the release of ET and QD from free drug solutions followed the first order kinetics and from ETN and QDN followed Higuchi model. This indicates the drug release from matrix type of systems.

The mechanism of drug release from the nanoparticles was determined using the Korsmeyer-Peppas model (Korsmeyer et al., 1983; Peppas, 1985 and 1989; Costa et al., 2001). The data obtained from the drug release from nanoparticles were analysed according to following equation of Korsmeyer-Peppas model.

$$M_t / M_\infty = kt^n$$

Where (M_t/ M_∞)is the fraction of drug released, k is release constant and n is release exponent The results for this analysis are given in Table 6.3.

The value of the release exponent 'n' indicates the release mechanism (Fickian diffusion, case II transport or anomalous transport). In the present study the limits considered were n = 0.45 (indicates a classical Fickian diffusion-controlled drug release) and n = 0.89 (indicates a case II relaxation release transport; non-Fickian, zero-order release). Values of n between 0.45 and 0.89 can be regarded as an indicator of both phenomena (drug diffusion in the hydrated matrix and the polymer relaxation) commonly called anomalous transport.

From the release exponent values in the Korsmeyer-Peppas model for the release of ET and QD from their respective nanoparticles , it could be concluded that the mechanism that led to the release of drugs was an anomalous transport.

Table 6.1 % Cumulative drug release in pH7.4 phosphate buffer

Time (hrs)	% Cumulative drug release			
	ET	ETN	QD	QDN
0.25	14.33±2.3	2.43±0.6	8.45±2.7	0.67±0.5
0.5	48.26±2.5	5.39±1.2	23.58±3.2	3.62±2.4
1	65.33±1.8	8.22±1.3	45.68±3.6	6.35±1.8
2	80.66±3.4	11.60±0.8	72.35±4.2	9.44±3.2
6	96.98±2.3	20.34±3.2	87.08±1.9	15.78±2.2
12		35.23±1.4		27.5±2.7
24		46.69±2.7		38.65±2.0
48		51.03±2.3		47.18±3.5

n = 3

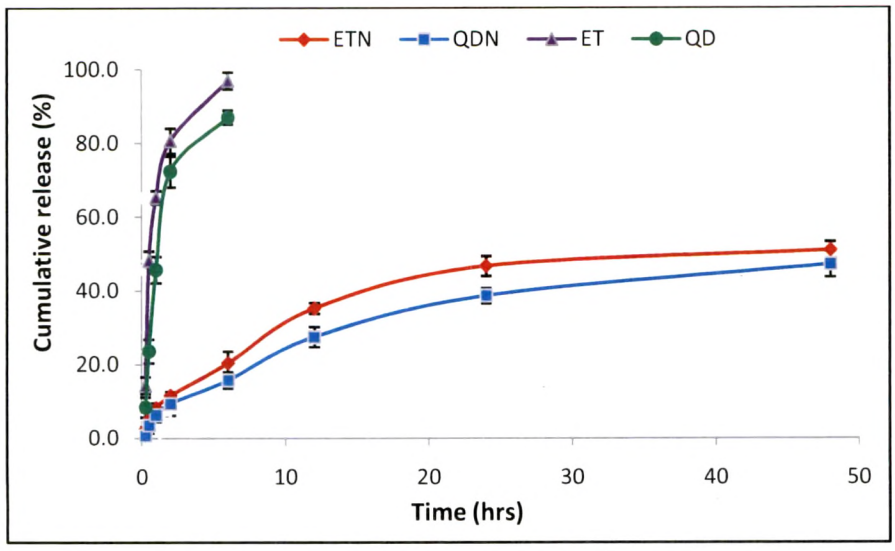

Figure 6.1 ***In vitro*** **release profile of drugs in pH 7.4 phosphate buffer from ETN and QDN and the plain drugs ET and QD. (Mean ± SD, n = 3)**

Table 6.2 Correlation Co-efficients for different dissolution models

Formulations	Correlation Co-efficient (R^2)			
	Zero Order Kinetics	First Order Kinetics	Higuchi Model	Hixon-Crowel Cube Root Law
ET	0.633	0.966	0.861	0.875
QD	0.743	0.910	0.911	0.858
ETN	0.808	0.856	0.959	0.840
QDN	0.873	0.918	0.984	0.904

Table 6.3 Disolution parameters for drug release from nanoparicles for Korsmeyer-Peppas Kinetics

Formulation	Correlaion Co-efficient (R^2)	Release Exponent (n)	Release constant (k)
ETN	0.974	0.571	7.194
QDN	0.911	0.714	4.187

6.4.2 In vitro cytotoxicity study:

IC 50 values at 72 hrs incubation for ETN and QDN were found significantly lower comparable to the reference drugs ET and QD respectively (Table 6.4 and Figure 6.2).

The comparison of the IC 50 values for various treatments also shows that combination treatment (EQ) showed higher activity when compared with individual drug treatments.

The increased cytotoxicity of etoposide by adding 50μM Quercetin on human Ewing's tumor cells is reported by Debes et al, (2003). The combination in the form of nanoparticles (EQN) exhibited even higher activity than the plain drug combination (EQ).

There was an increase in cytotoxicity in case of ETN and QDN at all time points (Fig. 6.3, 6.4, 6.5) in comparison to free drug but the effect was non-significant at 24 hrs ($p>0.05$). In the same case, significant ($p<0.01$) differences in cytotoxicities were found at 48 hrs and 72 hrs.

The combination treatment EQ exhibited significantly lower cell viabilities ($p<0.05$) as compared to individual drug treatment. The EQN treatment was found to produce significant improvement in cytotoxicity effect ($p<0.05$) compared with the free drug combination (EQ) except for the results at 24 hrs where the difference is insignificant.

When the comparison is made between the cytotoxic effect of ET with the EQN, extremely significant ($p < 0.001$) enhancement in cytotoxic effect due to EQN is observed at 48 hrs and highly significant ($p < 0.01$) enhancement is observed at 24 and 72hrs.

The greater antiproliferative activity of combination therapy (EQ) may be attributed to additive effect of single drug treatments. The significance of the difference in cytotoxicity effect of free drugs and nanoparticulate formulation was established at 72 hrs and not at 24 hrs. This may be due to the sustained release of drugs from the nanoparticles which can be well correlated with the results of in-vitro release studies in previous section.

The combination treatment EQN is found to produce far better results as compared to single chemotherapeutic drug treatment i.e. ET alone.

Table 6.4 IC 50 values for various treatments for cell line A549

IC 50 values (μM)					
ET	**QD**	**EQ**	**ETN**	**QDN**	**EQN**
5.58	45.15	1.99	2.3	21.75	1.19

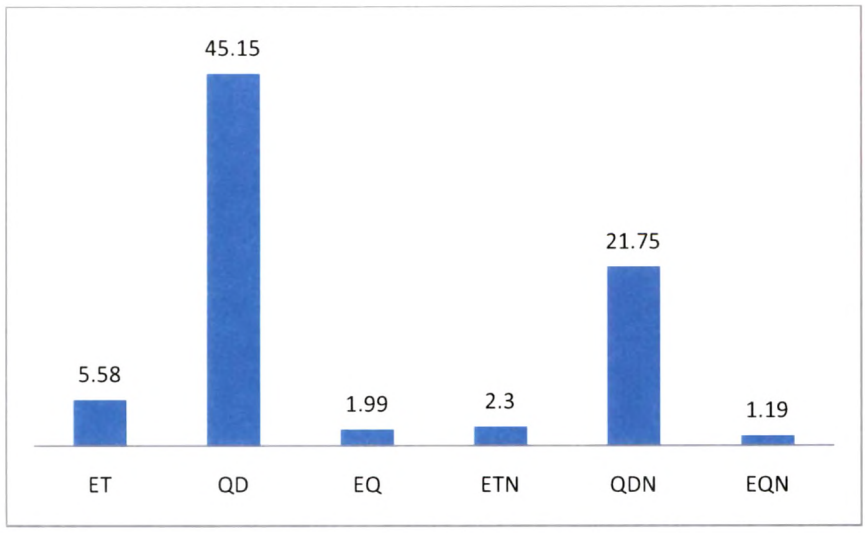

Figure 6.2 Comparison of IC 50 values for various treatments

Table 6.5 A549 Cell viabilities in % at 24 hrs for various treatments

Conc. (µM)	Mean Cell Viability (%)					
	ET	QD	EQ	ETN	QDN	EQN
0.5	84.37±1.2	89.92±2.0	82.54±0.46	90.65±1.5	94.52±2.2	72.89±2.15
5.0	70.63±0.65	78.78±1.44	56.35±1.22	76.26±1.06	85.62±1.5	58.68±2.4
10.0	65.08±2.5	70.25±3.08	42.87±0.94	66.91±1.24	80.82±0.78	46.30±1.68
50.0	54.76±1.8	65.85±2.10	38.33±3.2	60.43±0.65	70.55±2.2	41.56±0.44

n = 3

Table 6.6 A549 Cell viabilities in % at 48 hrs for various treatments

Conc. (µM)	Mean Cell Viability (%)					
	ET	QD	EQ	ETN	QDN	EQN
0.5	79.77±2.30	80.29±1.64	65.77±0.56	61.08±0.84	78.09±1.33	59.70±1.78
5.0	66.36±2.13	71.57±1.26	52.68±0.48	55.50±2.38	65.88±3.05	46.32±1.45
10.0	60.43±1.66	64.71±0.98	40.30±3.20	49.11±1.80	56.94±2.18	34.99±0.85
50.0	48.60±3.50	55.86±2.55	35.89±2.02	43.92±1.05	48.27±1.52	27.57±1.25

n = 3

Table 6.7 A549 Cell viabilities in % at 72 hrs for various treatments

Conc. (µM)	Mean Cell Viability (%)					
	ET	QD	EQ	ETN	QDN	EQN
0.5	63.28±1.75	74.60±1.44	55.72±2.10	58.96±2.20	72.09±1.75	52.78±1.15
5.0	52.70±1.58	66.78±1.63	48.69±1.58	46.73±1.56	61.87±1.04	38.12±0.88
10.0	45.48±2.34	58.82±0.88	41.10±2.34	39.12±0.78	54.27±0.85	32.25±1.42
50.0	37.25±2.72	47.49±1.78	31.25±1.93	33.88±2.08	43.82±1.56	18.02±0.75

n = 3

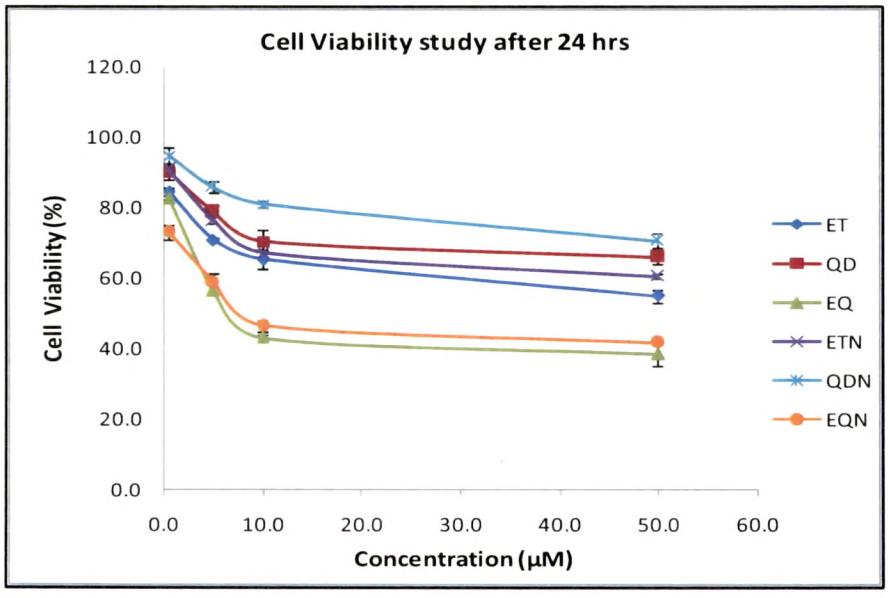

Figure 6.3 A549 Cell viabilities in % at 24 hrs for various treatments

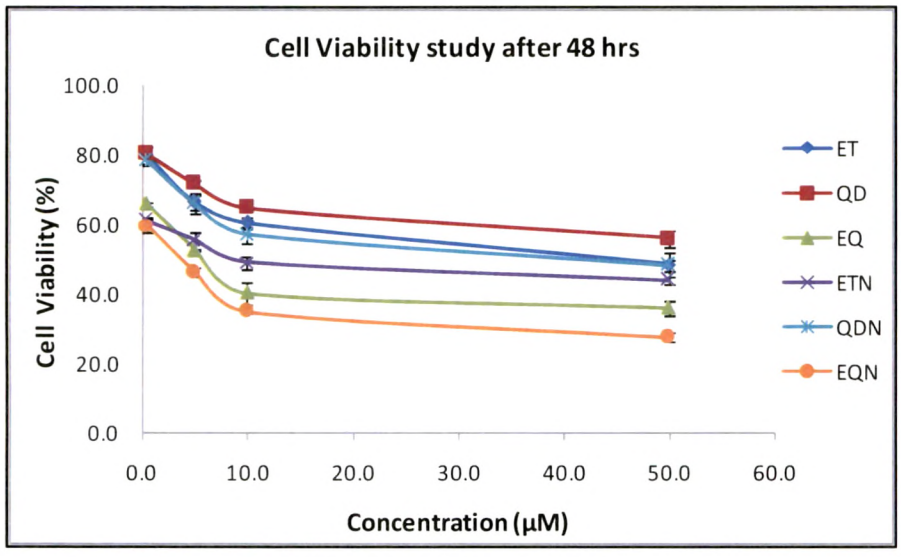

Figure 6.4 A549 Cell viabilities in % at 48 hrs for various treatments

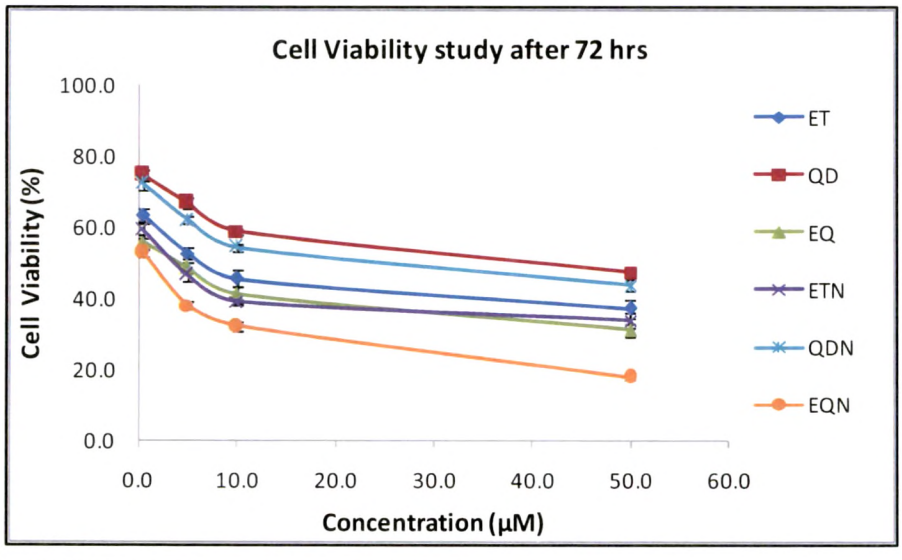

Figure 6.5 A549 Cell viabilities in % at 72 hrs for various treatments

6.5 Conclusions:

Thus the results of *in vitro* studies reveal that encapsulating the drugs in the form of PLGA nanoparticles led to the sustained release of the drugs as compared to faster release of free form of drugs.

The conclusion can be drawn from the *in vitro* cytotoxicity studies performed using MTT assay that combination treatment in the form of PLGA nanoparticles was found far better than any individual drug treatment. Thus these results justify the evaluation of combination treatment in the form of PLGA nanoparticles *in vivo.*

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