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### Research Article

## Ex-vivo and In-vivo Evaluation of Nanostructured Lipid Carrier Loaded with Gemcitabine and Paclitaxel in A549 Lung Cell for Management of Resistant Cancers

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### ABSTRACT

The combination of gemcitabine (GEM) and paclitaxel (PTX) are effective anti-cancer regimens due to their different mechanisms of action and partially non-overlapping toxicities. The present study aimed to elucidate a potential effect of folic acid-conjugated-Gemcitabine-Paclitaxel-loaded nanostructured lipid carriers (FA-GEM-PTX-NLCs) on the proliferation of cancer cell lines A549 for the management of resistant cancer. Cell uptake of NLCs in A549 cell lines was carried out with fluorescent dye, and Fluorescein isothiocyanate loaded NLCs (FITC-NLCs). Flow cytometry showed that FA-PTX-GEM-NLCs exhibited enhanced cellular uptake via folate receptor-mediated endocytosis. Cytotoxic effect (GI<sub>50</sub>) of formulations was measured by MTT assay method using A549 cells. Cytotoxicity results reveal that the GI<sub>50</sub> of FA-GEM-PTX-NLCs was 2.16 µg/mL, which is three times less than the GI<sub>50</sub> (6.48 µg/mL) GEM-PTX-NLCs.

Furthermore, data obtained from the pharmacokinetics study explored the extended half-life and higher serum concentration of GEM and PTX when delivered through FA-PTX-GEM-NLCs compared to PTX-GEM-NLCs and free drugs. Tumor growth inhibition results advocate for the higher anti-tumor activity of FA-GEM-PTX-NLCs in A549 cell line induced tumor-bearing Balb/c mice by estimating tumor burden. In conclusion, the novel folic acid conjugated NLCs loaded with GEM and PTX could be used as a potential chemotherapeutic formulation for cancer management.

### INTRODUCTION

The main challenge in the fight against cancer is multidrug resistance (MDR).<sup>[1]</sup> Cancer cells exhibited acquired and intrinsic resistance to chemotherapy treatment owing to its unique characteristics tumor microenvironment.<sup>[2]</sup> Gemcitabine (GEM), a deoxycytidine analog, is used as single or combination chemotherapy for solid tumors.<sup>[3]</sup> Combining its active phosphorylated metabolite, difluorodeoxycytidine triphosphate (dFdCTP), into DNA synthesis in the G<sub>0</sub>/G<sub>1</sub> and S phase of the cell cycle will induce cell apoptosis; it has a short half-life.<sup>[4]</sup> The combination of GEM and other anti-cancer drugs is recommended for systemic therapy in cancer. Paclitaxel (PTX) is a microtubule-interfering drug that promotes the polymerization of tubulin.<sup>[5]</sup> Disassembly of the

microtubules leads to dynamic changes in a cell, including mitotic block and cell apoptosis.<sup>[6]</sup> Targeting drugs to specific cellular pathways that effort cancer cells is a highly promising treatment modality; therefore, a PTX-based combination was needed to treat cancer.

The combination of GEM and PTX appealed for clinical exploration because these drugs exhibit different mechanisms of action and partially non-overlapping toxicities.<sup>[7]</sup> Interestingly, most previous studies investigating the interaction of GEM and PTX have been performed by focusing on the function of PTX as an agent that reinforces the action of GEM. Indeed, PTX was shown to enhance the anti-tumor activity of GEM by increasing levels of the GEM-metabolizing enzyme, deoxycytidine kinase (dCK), which eventually helps to concentrate GEM in cancer cells.<sup>[8]</sup> However, combination chemotherapy also

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has its own drawbacks due to different pharmacokinetic properties of the drugs, which increases the difficulty to obtain the optimal dose and further causes more adverse side effects.<sup>[9]</sup>

Besides potentiating effect in therapeutic efficacy, combination chemotherapy as a single nano-carrier minimizes drug resistance. In addition, multidrug chemotherapy doses can be minimized and prevent adverse effects associated with higher dosages of the toxic anti-cancer drugs.<sup>[10]</sup> General principles of development of combination chemotherapeutics include, (i) the use of combination drug must not have over-lapping dose-related toxicities; (ii) combination drug have a different mechanism of action; (iii) individual drug have proven activity.<sup>[11]</sup> To achieve an optimal efficacy-specific mechanism of action of both, the drug encapsulated within a nano-carrier needs to be fully elucidated. The result of combination therapy shows potentiation and synergism effect. In drug synergism, the therapeutic effect of the combined drug is greater than the total effect of the individual drugs, while in additive effect, it is equal to the summed effect of the individual drugs.

The nano-carrier may achieve modulation of a drug's desired pharmacokinetics and pharmacodynamics pattern with control over targeting ligand, size, and shape.<sup>[12],[13]</sup>

Nanoparticles (NPs) can prolong drug half-life, reduce nonspecific uptake, and preferentially accumulate tumors via the enhanced permeation and retention effect. Nanostructured lipid carriers (NLCs) contain solid lipids and liquid lipids, and present superiority over solid lipid NPs. The addition of spatially incompatible liquid lipids will change the high crystallization from solid lipids. Apart from improved bioavailability, loading capacity, and stability, NLCs can still load drugs with different physical and chemical properties and control release.<sup>[14]</sup>

In a previous study, Folic acid-conjugated-Gemcitabine-Paclitaxel-loaded NLCs (FA-conjugated-GEM-PTX-NLCs) and Gemcitabine-Paclitaxel-loaded NLCs (GEM-PTX-NLCs) were prepared by solvent emulsion-evaporation method reported by Di *et al.*<sup>[15]</sup> Physicochemical properties of FA-conjugated-GEM-PTX-NLCs and GEM-PTX-NLCs were characterized for particle size, size distribution, morphology<sup>[16]</sup> and entrapment efficiency.<sup>[17]</sup> Then *in vitro* drug release behavior was confirmed by dialysis.<sup>[18,19]</sup> Prepared FA-conjugated-GEM-PTX-NLCs formulations needs to be evaluated for the anti-cancer efficiency of receptor-targeted delivery of GEM and PTX through nano-carriers. In the current work, we quantified the percent cell uptake, cytotoxic response (GI<sub>50</sub>), and synergism study of free drug and drug-loaded NLCs. Furthermore, *in-vivo* pharmacokinetic study and anti-tumor activity were evaluated, suggesting that FA-conjugated-GEM-PTX-NLCs could be successfully internalized via receptor-mediated endocytosis.

## MATERIALS AND METHODS

### Materials

The adeno-carcinomic human alveolar basal epithelial cells line (A549 cells) was obtained from the National Centre for Cell Science (NCCS), Pune, Maharashtra, India. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) (Himedia, Mumbai, India), MTT cell proliferation kit was purchased from Sigma-Aldrich.

### Animals

Balb/c mice (4-5 weeks old, 20-25 g weight) were procured from the Institute of Animal Health and Veterinary Biologicals Rasalpara, Mhow (MP) and housed under standard laboratory conditions. All the animal protocols had been approved and complied with the IAEC of Veda College of B. Pharmacy, Bhopal. The animal use permission number is IAEC/VCP/2019/001/1.

### Ex-vivo Study

#### Cell Uptake study

Fluorescent dye fluorescein isothiocyanate-loaded NLCs (FITC-NLCs) were prepared following the same procedure as mentioned in the previous article in section preparation of folic acid-conjugated-Gemcitabine-Paclitaxel-loaded NLCs (FA-conjugated-GEM-PTX-NLCs), but replacing the drug with FITC dye (400 µg) to the solution before the formation of NLCs. The incorporated FITC acts as NLCs and is a sensitive method to determine qualitative and quantitative cellular binding. The cell uptake study was carried out as reported by Wang *et al.* 2016.<sup>[20]</sup> Concisely, T-25 flasks were seeded with A549 cells, were grown to attain 80% confluency, and were treated with folic acid conjugated as well as unconjugated NLCs at a particle concentration of 100 mg/mL. After incubation for 3 hours, the cells were washed twice with PBS, trypsinized, centrifuged, and resuspended in the PBS buffer. Analysis of particle uptake was estimated using the FAC-Scan flow cytometer system (Becton Dickinson, USA UK). At the same time, inhibition studies were carried out, and cells were preincubated for 2 hours with folic acid (1 mg/mL), notably a high-affinity ligand for the folic acid receptor, in a complete medium.

### Cytotoxicity and Synergism Study of Free Drugs and Drugs Loaded NLCs

MTT assay was used for studying the anti-proliferative effect of FA-PTX-GEM-NLCs using A549 cells.<sup>[21]</sup> A549 cells were stored in DMEM supplemented with 10% heat-inactivated FBS, 3 mM glutamine, 1% streptomycin in a 37°C humidified incubator, and 5% CO<sub>2</sub> atmosphere. Exponentially growing A549 cells were seeded at 3 × 10<sup>4</sup> cells/mL in 96-well plates (Sigma, Aachen, Germany). The cells were individually treated with FA-GEM-PTX-NLCs, GEM-PTX-NLCs, plain (without drug loading)-nanostructured lipid carrier(s) (X-NLCs), GEM. Moreover,

PTX mixed solution (GEM-PTX-sol), Gemcitabine solution (GEM-sol), paclitaxel solution (PTX-sol), and untreated A549 cells were used as positive control which is incubated under a controlled environment (37°C humidified incubator and 5% CO<sub>2</sub>) for 72 hours. Furthermore, the MTT solution (5 mg/mL) was added to every single well and incubated for 4 hours at 37°C to facilitate the reduction of MTT by viable cells with the formation of purple formazan crystals. The formazan crystals were dissolved in DMSO, and the absorbance of individual wells was read at 590 nm. The drug concentration causing 50% growth inhibition (GI<sub>50</sub>) was calculated using GraphPad Prism 9 software. CI<sub>50</sub> was measured according to Chou's method:

$$CI_x = \{D_1/(D_x)_1\} + \{D_2/(D_x)_2\}$$

where (D<sub>x</sub>)<sub>1</sub> and (D<sub>x</sub>)<sub>2</sub> denote the IC<sub>50</sub> value when drug 1 or 2 performances singly. D<sub>1</sub> and D<sub>2</sub> represent the concentrations of drug 1 and drug 2 when given as a pair at the GI<sub>50</sub> value. Using this method, CI<sub>50</sub><1 indicates synergism, CI<sub>50</sub>=1 indicates additive, while CI<sub>50</sub>>1 indicates antagonism.<sup>[22]</sup>

### **In-vivo Study**

#### *Pharmacokinetic Studies*

The pharmacokinetics study was conducted according to Nandini *et al.*<sup>[23]</sup> with slight modifications. Briefly, balb/c mice were incubated subcutaneously in the left armpit with 5.0×10<sup>6</sup> cells/100 μL suspension of A549 cell lines. When the tumor volume (TV) reached 100 mm<sup>3</sup>, mice were divided into four groups (n=6 per group). Group I was administered with plain PTX, Group II was administered with GEM, Group III was administered with PTX-GEM-NLCs, and Group IV was administered with FA-PTX-GEM-NLCs with a dose equivalent to 6 mg/kg of free drug through the lateral tail vein. Blood samples (200 μL) were collected at different time points through the retro-orbital plexus, and the plasma was separated by centrifuging the blood samples at 8000 g for 5 minutes. Plasma samples thus obtained were stored at 20°C until use. Glacial acetic acid (50 μL) was added to plasma samples to decrease hydrogen bonding between nucleosides and proteins. Acetonitrile (0.7 mL, HPLC grade) was added to precipitate the vortex-mixed plasma proteins and centrifuged at 9000 g for 15 minutes at 4°C. The supernatant was removed and collected in a glass tube, and acetonitrile (1 mL) was added to the pellet. Three cycles of vortex mixing and centrifugation procedure were carried out. The supernatants were pooled, evaporated to dryness under nitrogen flux at 42°C (thermo-stated water bath), and stored at -80°C. Before HPLC analysis, the residue was resuspended in water (1 mL, HPLC grade), incubated for 5 minutes at 37°C, and then centrifuged at 12,000 g for 15 minutes at 20°C. Further, supernatant was removed, filtered through a 0.22 μm syringe filter, and placed in 4 mL HPLC glass vials for analytical determination. Detection of

PTX and GEM in serum was carried out using a RP-HPLC method. Other pharmacokinetic parameters from drug serum level were determined by Kinetica software version 5.<sup>[24]</sup>

$$Tumor\ Volume\ (TV) = \frac{a \times b^2}{2}$$

Where a and b are longest and widest diameters, respectively.

### **Assessment of Anti-tumor Activity In-vivo**

Tumors were experimentally induced subcutaneously into the armpit of the mice. Once a substantially solid mass of tumors has been developed (an average volume of 290 mm<sup>3</sup>), mice were divided into five groups of three mice each. Group I served as control, and Groups II, III, IV, and V were injected with PTX, GEM, PTX-GEM-NLCs, and FA-PTX-GEM-NLCs. Negative control group treated with saline and positive group with pure drug. The pure drug group showed severe toxicity above the concentration of 5 mg/kg. The nano-carrier group exhibited better biocompatibility at the dose equivalent to 15 mg/kg because of the sustained release of drugs.<sup>[9]</sup> Therefore pure drug group and nano-carrier groups were treated through tail vein at a dose of 5 mg/kg and 15 mg/kg body weight respectively on days 0, 2, 6, and 12. Mice were monitored regularly for changes in tumor size. Subsequently, the mice were sacrificed, the solid tumors were separated. The size of tumor masses was measured with vernier calliper, and tumor volumes were calculated according to the following formula:  $V = 0.5 \times a \times b^2$ , where a and b are the long and the short diameter of tumor, respectively.

Tissue samples of tumors were kept on ice after the homogenization procedure; the homogenate was then centrifuged at 10,000 g for 10 minutes, ACN was added to the supernatant to precipitate unwanted proteins; samples were centrifuged (10,000 g, 10 minutes). The aliquots were assayed for PTX and GEM levels using HPLC to estimate the total amount of PTX and GEM.<sup>[25]</sup>

## **RESULT AND DISCUSSION**

### **Ex-vivo Study**

#### *Cell Uptake Study*

The cell uptake of NLCs is an important step in confirming the cytotoxic efficiency of drugs. The cellular uptake of NLCs was determined on A549 cancer cells. The cellular uptake was understood to show time dependence with all formulations. Cell uptake was found to be increased till 2 hours, which followed a not-so-significant (P > 0.05) trend of cell uptake. This might be possibly due to the saturation of cells (Table 1 and Fig. 1). The uptake of FITC-labeled FA-NLCs (FA-FITC-NLCs) was found significantly better as compared with that observed with FITC labeled plain NLCs (FITC-NLCs) and plain FITC (Fig. 2).

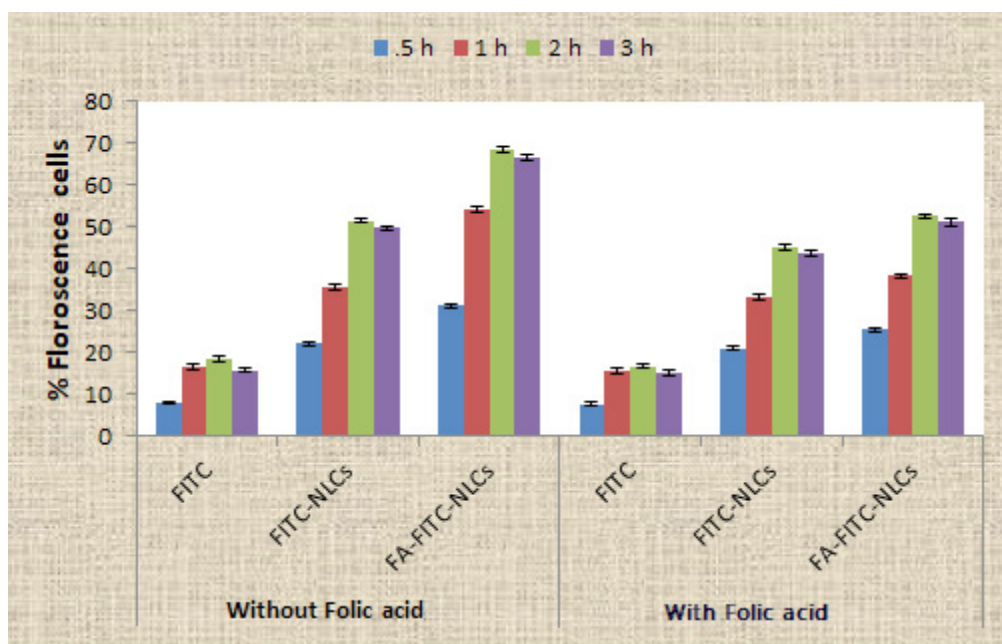




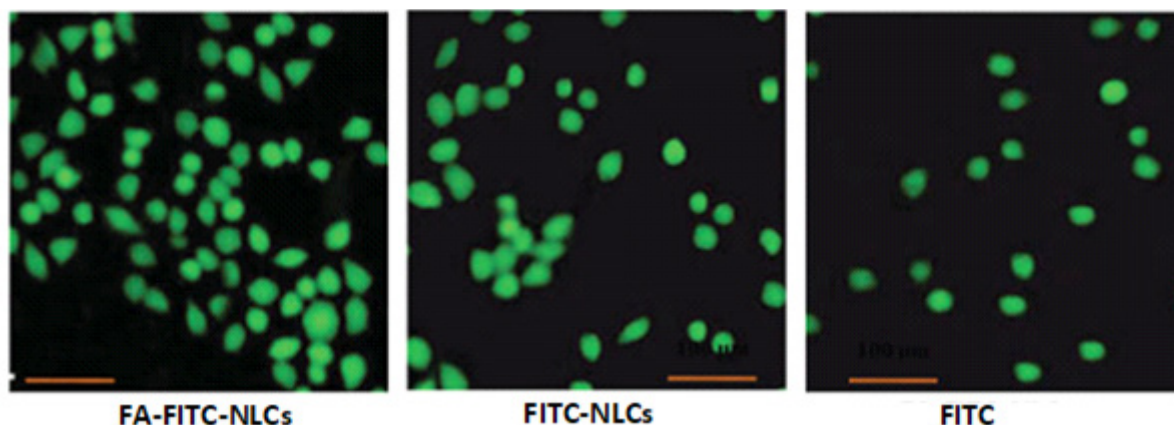
**Table 1:** Percentage fluorescent cells after 3 hours incubation with FITC-NLCs and FA-FITC-NLCs

FR Saturation	Formulation	% Florescence cells after Incubation for			
		.5 h	1 h	2h	3 h
With Folic Acid	FITC	8.15 ± 0.29	16.58 ± 0.7	18.35 ± 0.63	15.74 ± 0.57
	FITC-NLCs	22.24 ± 0.5	35.73 ± 0.65	51.4 ± 0.5	49.78 ± 0.52
	FA-FITC-NLCs	31.32 ± 0.5	54.18 ± 0.7	68.3 ± 0.71	66.68 ± 0.56
Without Folic Acid	FITC	7.6 ± 0.5	15.78 ± 0.63	16.73 ± 0.56	15.18 ± 0.65
	FITC-NLCs	21.03 ± 0.55	33.23 ± 0.65	45.23 ± 0.65	43.7 ± 0.55
	FA-FITC-NLCs	25.48 ± 0.37	38.29 ± 0.41	52.69 ± 0.47	51.23 ± 0.88

The data represent mean ± SD (n = 3).



**Fig. 1:** Graph showing %fluorescent cells after 3 hours incubation with FITC-NLCs and FA-FITC-NLCs. The data represent mean ± SD (n = 3).



**Fig. 2:** The fluorescence microscopy photomicrographs showing uptake of FITC-NLCs in A549 cell line.

Thus, greater cellular binding with subsequent uptake observed with FA-PTX-GEM-NLCs is presumably due to greater intracellular delivery of FA-PTX-GEM-NLCs by

receptor-mediated endocytosis. However, the cellular uptake of FA-PTX-GEM-NLCs was significantly ( $P < 0.05$ ) inhibited by pre-treatment with folic acid. On the contrary,

pre-treatment of folic acid with FA-PTX-GEM-NLCs leads to favored folic acid binding with folate receptors, thus saturating them and ruling out the entry of FA-PTX-GEM-NLCs through receptor-mediated endocytosis.<sup>[26]</sup> The reduced cellular entry upon adding folic acid and, in the case of unconjugated NLCs, clearly showed that amplified uptake is facilitated by receptors-mediated uptake of FA-PTX-GEM-NLCs. Further, cellular uptake of FA-PTX-GEM-NLCs is comparable with the cellular uptake of PTX-GEM-NLCs. In conclusion, our results suggest that the folic acid receptors specifically mediated enhanced cellular uptake of FA-PTX-GEM-NLCs.

#### *Cytotoxicity and Synergism Study of Free Drugs and Drugs Loaded NLCs*

The MTT assay was conducted for a comparative cytotoxic response on A549 cell lines (control and formulation treated) to explore the effectiveness of plain PTX, plain GEM, GEM-NLCs, PTX-NLCs, GEM-PTX-NLCs and FA-GEM-PTX-NLCs (Fig. 3). The  $GI_{50}$  of FA-GEM-PTX-NLCs for A549 cells was measured to be 2.16  $\mu\text{g/mL}$ , which is three times less than the  $GI_{50}$  value (6.48  $\mu\text{g/mL}$ ) of GEM-PTX-NLCs. The  $GI_{50}$  of GEM-sol, PTX-sol, and mixture of GEM-PTX-sol recorded was 16.58  $\mu\text{g/mL}$ , 4.94  $\mu\text{g/mL}$ , and 9.72  $\mu\text{g/mL}$ , respectively. In some cases, it was observed that dose ratio affects the combination effects and the combined effect of drug-induced synergistic effects.  $CI_{50}$  value lower than, equal to or higher than 1 represents synergism, additive or antagonism, respectively.<sup>[27]</sup> The maximum cell-specific cytotoxicity was thus noted in case of the system which carried and delivered the drugs in combination to the target cells, i.e., FA-GEM-PTX-NLCs. Further, the cytotoxicity exhibited by GEM-PTX-NLCs was comparatively more as matched to GEM-PTX mixed solution which could be endorsed to relatively higher drug accumulation that resulted due to cationic carrier(s) and negatively charged A549 cell line interaction as compared to plain drug(s) solution. Furthermore, the lowest  $CI_{50}$  value of 0.859 and 0.426 were observed in the GEM-PTX

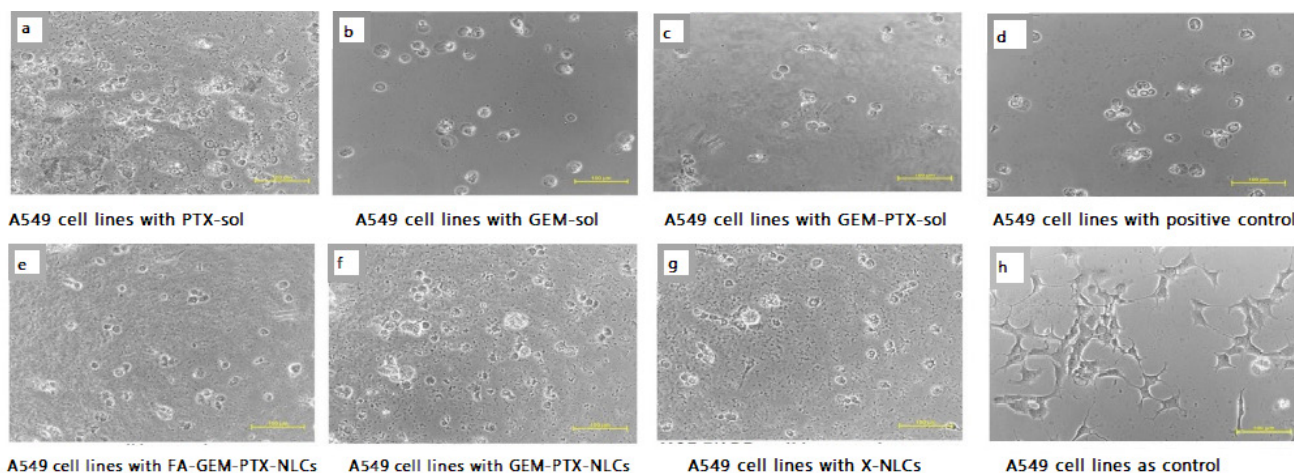
(3:1)-NLCs and FA-GEM-PTX (3:1)-NLCs, respectively. Therefore, GEM-PTX (3:1)-NLCs and FA-GEM-PTX (3:1)-NLCs report the highest cytotoxicity effect and the top-notch synergistic effects in all other formulations (Table 2).

However, the activity of GEM-PTX-NLCs was less than FA-GEM-PTX-NLCs. This differentiated activity profile may be ascribed to the targetability of FA-GEM-PTX-NLCs leading to cell-specific higher accumulation and, therefore, the activity. The results show that the amount of each drug could be reduced significantly ( $P < 0.05$ ) owing to the co-administration of two drugs by using target-oriented NLCs conjugated with folic acid. The targeting moiety helps localize both the drugs in the vicinity of the cancer cells, followed by internalization, resulting in a greater cytotoxic response with reduced possibility of systemic toxicity.<sup>[28]</sup>

#### *In-vivo Study*

##### *Pharmacokinetic Studies*

The *in-vivo* study suggests the reputation of NLCs because of alteration in pharmacokinetic of PTX and GEM when delivered through these nano-carriers. In particular, the half-life ( $t_{1/2}$ ) of GEM was increased from  $0.48 \pm 0.06$  hours (free drug) to that of  $3.96 \pm 0.15$  hours and  $4.16 \pm 0.34$  hours, respectively, for PTX-GEM-NLCs and FA-PTX-GEM-NLCs on the other hand, half-life ( $t_{1/2}$ ) of PTX was increased from  $7.12 \pm 2.05$  hours (free drug) to that of  $21.96 \pm 1.24$  hours and  $22.36 \pm 1.54$  hours, respectively, for PTX-GEM-NLCs and FA-PTX-GEM-NLCs (Table 3). These findings are suggestive of the ability of these NLCs to extend the half-life of PTX and GEM. In addition, other pharmacokinetic parameters, i.e., the maximum concentration of drug in plasma ( $C_{max}$ ), mean resident time (MRT; hr), and the area-under-the-curve ( $AUC_{0-\infty}$ ) (Table 2), prove the utility of this carrier in drug delivery. The plasma concentration results of FA-PTX-GEM-NLCs was comparable with that of PTX-GEM-NLCs and free drug. Other pharmacokinetic parameters of FA-PTX-GEM-NLCs seem to have higher distribution, lower clearance, lower elimination, and improved drug half-life compared to PTX-GEM-NLCs and



**Fig. 3:** Photomicrographs of A549 cell lines (control and formulation treated)



free drugs. PTX and GEM dissolved in lipid matrix releases slowly from the NLCs leading to slower elimination time, which may cause the lower clearance of the NLCs. Higher tissue distribution established from the higher volume of distribution while improved  $T_{1/2}$  signifies boosted half-life over other formulations. The drugs constricted and retained in the systemic circulation at the end decrease the amount of this anti-tumor agent eliminated from the bloodstream. The data obtained from pharmacokinetic studies show higher serum concentration of PTX and GEM when delivered through FA-PTX-GEM-NLCs compared to that seen with PTX-GEM-NLCs. Therefore, the *in-vivo* pharmacokinetic study was performed to achieve the fate of NLCs in the body.

### In-vivo Anti-tumor Activity

Anti-tumor activity was assessed with plain PTX + GEM, PTX-GEM-NLCs, and FA-PTX-GEM-NLCs (Fig. 4). We observed that free drugs failed to reduce the tumor burden/ volume. It may be probably due to its rapid clearance from circulation or its less tumor-targeting efficiency. However, to an extent, free drugs showed a rapid increase in tumor volume, marked by a constant rise in the curve of the animals treated with normal saline. The PTX-GEM-NLCs was shown to reduce the tumor volume. Fascinatingly, free drugs showed an improved and significantly higher ( $p < 0.05$ ) anti-tumor activity of PTX + GEM delivered through PTX-GEM-NLCs and FA-PTX-GEM-NLCs at all-time points from the second day onwards. It is believed that incorporation of PTX and GEM into NLCs will shield drugs from being metabolized in circulation, and sustained release from NLCs matrix might have improved the anti-cancer activity. The greater reduction in tumor volume was estimated with FA-PTX-GEM-NLCs and PTX-GEM-NLCs formulations (Fig. 5). This can be attributed to the active targeting of folic acid-conjugated PTX-GEM-NLCs towards the FA receptors overly expressed on the tumor surface. Also, it leads to better uptake of NLCs than that seen with unconjugated NLCs. This may be a characteristic feature of selective accumulation (Figs 1 and 2) of FA-PTX-GEM-NLCs in tumors followed by

receptor-mediated endocytosis,<sup>[29]</sup> which eventually leads to improved anti-tumor activity compared with PTX-GEM-NLCs. Therefore, optimum therapeutic responses, which improved therapeutic efficacy, were achieved.

### Statistical Analysis

The study data collected as an average of three readings were analyzed statistically. Data were expressed as means  $\pm$  SD. The student's *t*-test was used for statistical comparisons between two groups. One-way analysis

**Table 2:** GI50 and CI50 of different formulation in A549 cells

Formulation	GI <sub>50</sub> (µg/mL)	CI <sub>50</sub>
Free GEM	16.58 $\pm$ 0.78	-
Free PTX	4.94 $\pm$ 0.17	-
Free GEM:PTX (5:1)	16.05 $\pm$ 0.74	3.246
Free GEM:PTX (3:1)	9.72 $\pm$ 0.60	1.768
Free GEM:PTX (2:1)	11.51 $\pm$ 0.38	4.576
Free GEM:PTX (1:1)	10.76 $\pm$ 0.77	3.492
Free GEM:PTX (1:2)	10.89 $\pm$ 0.24	4.986
Free GEM:PTX (1:3)	7.67 $\pm$ 0.56	2.318
GEM-PTX (5:1)-NLCs	9.68 $\pm$ 0.45	2.26
GEM-PTX (3:1)-NLCs	6.48 $\pm$ 0.18	0.859
GEM-PTX (2:1)-NLCs	8.44 $\pm$ 0.41	1.786
GEM-PTX (1:1)-NLCs	6.74 $\pm$ 0.58	1.768
GEM-PTX (1:2)-NLCs	6.52 $\pm$ 0.43	1.943
GEM-PTX (1:3)-NLCs	5.21 $\pm$ 0.40	2.864
FA-GEM-PTX (5:1)-NLCs	6.98 $\pm$ 0.15*	1.12
FA-GEM-PTX (3:1)-NLCs	2.16 $\pm$ 0.31*	0.426
FA-GEM-PTX (2:1)-NLCs	5.78 $\pm$ 0.34*	0.994
FA-GEM-PTX (1:1)-NLCs	3.19 $\pm$ 0.48*	0.823
FA-GEM-PTX (1:2)-NLCs	3.59 $\pm$ 0.30*	0.94
FA-GEM-PTX (1:3)-NLCs	1.58 $\pm$ 0.29*	1.467

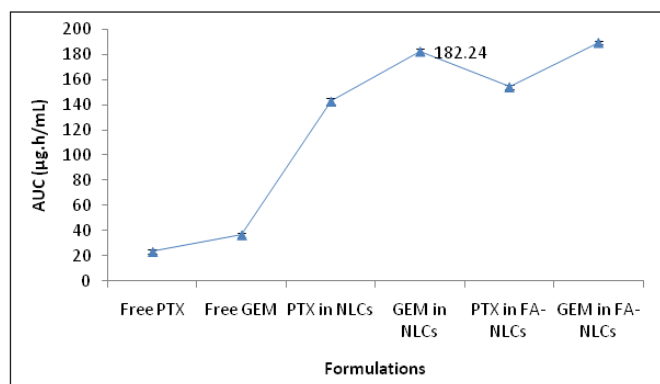
Data are expressed as mean  $\pm$  SD (n = 6). \* Significantly (P < 0.05) amount of each drug reduced when co-administration of two drugs by folic acid conjugated NLCs delivered.

**Table 3:** Pharmacokinetic parameters in serum of Balb/c mice

Parameters	Free PTX	Free GEM			PTX-GEM-NLCs		FA-PTX-GEM-NLCs	
		Free GEM	PTX	GEM	PTX	GEM		
C <sub>max</sub> (µg/mL)	11.24 $\pm$ 1.36	27.87 $\pm$ 2.48	9.62 $\pm$ 1.28	21.38 $\pm$ 2.64	10.47 $\pm$ 1.12*	23.04 $\pm$ 1.87*		
AUC <sub>0-t</sub> (µg hr /mL)	23.57 $\pm$ 1.45	36.84 $\pm$ 1.64	142.64 $\pm$ 2.64	182.24 $\pm$ 2.86	154.12 $\pm$ 1.57*	189.24 $\pm$ 1.23*		
T <sub>1/2</sub> (hr)	7.12 $\pm$ 2.05	0.48 $\pm$ 0.06	21.69 $\pm$ 1.24	3.96 $\pm$ 0.15	22.36 $\pm$ 1.54*	4.16 $\pm$ 0.34*		
MRT <sub>0-t</sub> (hr)	3.70 $\pm$ 0.58	6.19 $\pm$ 0.16	24.68 $\pm$ 0.89	31.24 $\pm$ 0.68	25.43 $\pm$ 0.59*	32.04 $\pm$ 0.27*		
Cl (L/hr)	9.64 $\pm$ 0.23	7.76 $\pm$ 0.54	6.48 $\pm$ 0.42	5.62 $\pm$ 0.34	5.86 $\pm$ 0.62*	4.56 $\pm$ 0.42*		
V <sub>d</sub> (l/kg)	12.32 $\pm$ 0.12	18.56 $\pm$ 0.36	36.81 $\pm$ 0.28	58.16 $\pm$ 0.32	21.28 $\pm$ 0.25*	48.62 $\pm$ 0.34*		
Ke (hr <sup>-1</sup> )	0.78 $\pm$ 0.024	0.43 $\pm$ 0.036	0.18 $\pm$ 0.054	0.10 $\pm$ 0.062	0.27 $\pm$ 0.028*	0.09 $\pm$ 0.024*		

The data represented as mean  $\pm$  SD (n = 6), \*p < 0.05





**Fig. 4:** Representation of AUC of different formulations in serum of Balb/c mice

of variance (ANOVA) was used to compare three or more groups.  $P < 0.05$  was considered significant. Pharmacokinetic and cytotoxic data were computed from Kinetica version 5 and GraphPad Prism 9 software, respectively.

## CONCLUSION

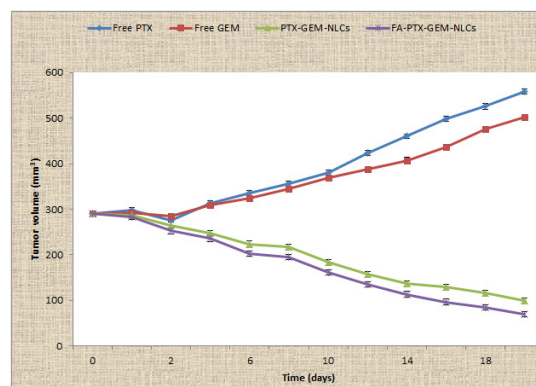
Nanostructured lipid carrier system offers a promising route for combination cancer therapy by concurrently loading two or more kinds of chemotherapeutics. *In vitro* targeting studies reveal that FA-GEM-PTX-NLCs exhibited higher uptake efficiency in A549 cells via folate receptor-mediated endocytosis. Cytotoxicity assays showed FA-GEM-PTX-NLCs have dramatic anti-cancer activity on A459 lung cells in comparison to other formulations. Furthermore, dual drug Gemcitabine and Paclitaxel-loaded folic acid conjugated NLCs exerted effective synergistic anti-cancer effects in the *in-vivo* A549 adenocarcinoma model because of the synergy between GEM and PTX and the improved pharmacokinetic of the nano-carriers. In conclusion, folic acid conjugated NLCs loaded with GEM and PTX shows the highest cytotoxicity effect and the top-notch synergistic effects in the area tested. Our interpretations also reveal that when NLCs conjugated with folic acid were done, anti-cancer drugs delivered facilitated targeted to the tumor site and reduced entrance to non-tumor sites. The forthcoming exploration should be refreshed to study a dose-dependent response and optimum dose with maximum anti-cancer efficacy but fewer side effects.

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**Fig.5:** Estimation of anti-tumor activity in A549 cell line induced tumor-bearing animal model by estimating tumor burden

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