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Orally Delivered Decitabine Loaded PLGA Nanoparticles Combined with Docetaxel Supress Solid Tumours: an in vitro investigation.

Prateek Jain[1], Mradul Tiwari[1], Nitesh Kumar[3], J Venkata Rao[1], and N Udupa[2]*.

[1]Cell and Molecular Biology Lab, Department of Pharmaceutical Biotechnology; Manipal College of Pharmaceutical Sciences, Manipal University, Karnataka, India.
[2]Directorate of Research, Manipal University, Manipal, Karnataka, India.
[3]Department of Pharmacology; Manipal College of Pharmaceutical Sciences, Manipal University, Karnataka, India.

ABSTRACT

Docetaxel is one of the greatest chemotherapeutic agent used in the treatment of solid tumours. However, resistance to docetaxel is a major clinical issue, which obstacle to achieve long time survival for patients with advanced disease. The inherent presence or emergence of drug-resistant tumour clones confines its efficiency in the treatment of cancers. The understanding that epigenetic changes are prevalent in cancer and play a causative role in its biology has led to the development of new therapeutic approaches that target the epigenetic machinery. Exploiting the gene reactivation by using epigenetically acting agents in combination with cytotoxic therapies, is a strategy of huge clinical relevance. Decitabine (DEC) is one such epigenetic drug, but its major disadvantage is its low oral bioavailability. There is no oral dosage form available for DEC. Since PLGA has an advantage of overcoming acidic and enzymatic degradation, the present investigation was aimed at fabricating PLGA 50:50 nanoparticles of decitabine (DEC-NPs); and thereafter, examine a combination treatment in vitro and in vivo with docetaxel (DTX). DEC-NPs were formulated by spontaneous emulsification solvent diffusion technique. The optimized formulation had PS of 124.3 ± 4.2 nm, ZP of -23.2 ± 1.2 mV, and EE of 41.8 ± 4.3%. A comparative study indicated that the cytotoxicity of DTX and DEC combination on MCF-7 cells was significantly higher (p <0.05) than DTX and DEC alone.

Keywords: Decitabine nanoparticles (DEC-NPs), MCF-7, A-549, solid tumours, oral chemotherapy

Corresponding author

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INTRODUCTION

The oral delivery of many anticancer drugs has been a significant focus of exploration in pharmaceutical research for years. Unfortunately, these anticancer drugs, do not perform to their expectations in the market due to reasons like low oral bioavailability because of GI barriers and many instabilities. Therefore, the chemotherapy drugs are delivered through i.v. injections or infusions in hospitals, leading to extra costs, non-compliance and ultimately the failure of the therapy. However, the oral route of drug delivery is the most ideal route due to ease of administration, cheaper costs, patient compliance and persistence, etc. [1]. In the recent years, drug delivery technology has advanced to the extent of delivering molecules to targeted organs, bypassing the GI tract and protecting the molecules from destructive enzymes in the body. The oral chemotherapy has an advantage of maintaining sustained moderate plasma concentration of the drug which leads to prolonged exposure of drug to the cancer cells.

Decitabine (DEC) or 5-aza-2′-deoxycytidine, is a distinct cytosine analog with a property to inhibit DNMTs. It chemically reverses gene silencing of tumour suppressor genes, and has become an exciting approach for cancer treatment [2]. DEC is active in MDS as well as both acute and chronic leukemias as well [3].The utility of DEC as a pharmaceutical, however, is restricted by its very low and variable bioavailability (3.9-14 %) [4]. DEC is sparingly soluble in aqueous media, unstable in acidic conditions and is metabolized by cytidine deaminase enzyme in the liver. Another major disadvantage of DEC is its instability. To overcome these limitations, a favourable strategy is to formulate DEC into a particulate carrier system that could protect the drug from acidic degradation in the stomach and allow it to reach the intestines.

Docetaxel is the second molecule of the cytotoxic class of taxanes to have reached its potential in clinical use. Docetaxel is an effective chemotherapy drug used to treat solid tumours with a wide spectrum of antitumour activity such as in breast cancers and lung cancers. It induces polymerization of monomers of tubulin and inhibits depolymerization. This leads to mitotic arrest in the G_2M phase of the cell cycle [5]. Docetaxel also induces cell death by apoptosis via stimulation of phosphorylation of bcl-2 protein [6].The underlying molecular mechanisms of docetaxel resistance are not fully understood.

This study investigated the potential involvement of DNA methylation machinery in the treatment of solid tumours, upon concurrent administration with docetaxel, in three cancer cell line models A549, MCF-7, and LNCaP-231. The effect of decitabine nanoparticles was investigated to propose that nanoparticles can be used in combination with docetaxel to overcome the drug resistance.

MATERIALS AND METHODS

Materials

TPGS, Trehalose, Poloxamer 188, PLGA 75:25, PLGA 85:15, PLGA 50:50, decitabine were purchased from Sigma Aldrich. Decitex, Sun Pharma was obtained from retail market in New Delhi. Dichloromethane (DCM), HPLC grade acetonitrile and Methanol were obtained from Fisher Scientific. All other chemicals were purchased from Himedia.

Preparation of Decitabine loaded PLGA NPsby Multiple emulsification technique

Decitabine loaded PLGA NPs were formulated by multiple emusification solvent evaporation method. Decitabine was dissolved in 1 mL acidified water. The resultant aqueous phase was dropwise added while sonicating for 5 min at 75 W, to a solution of PLGA (50:50) previously dissolved in DCM. This emulsion was added to aqueous solution Poloxamer 188 with constant homogenization for 15 min at 14,000 rpm under cool conditions. This was further kept on magnetic stirrer for 4 h. The nanoparticle suspension was centrifuged for 45 min at 22,000 × *g* at 4°C (Sigma, USA). DEC-NPs were lysophilised using mannitol as cryoprotectant.

Characterization of DEC-NPs

The above prepared nanoparticles were characterized for particle size and zeta potential using Malvern NanoZS (Malvern instruments Ltd., Worcestershire, UK) [7]. The surface morphology of NPs was determined by scanning electron microscopy. DSC thermograms of the pure drug, physical mixture and

lyophilized nanoparticles were recorded on a thermal analyzer (Shimadzu DT-60, Kyoto, Japan). DSC analysis was performed for DEC, PLGA (50:50), DEC and PLGA (50:50) physical mixture and DEC-NPs to determine the interaction between formulation excipients, stability and nature of nanoparticles. Powder XRD patterns of DEC powder and DEC-NPs were investigated using X-ray diffractometer.

Drug entrapment efficiency (EE)

The drug entrapment in nanoparticles was determined after separation of entrapped drug and free drug by centrifugation at 22,000 *g* at 4-8°C for 45 min (Sigma centrifuge, SciQuip Ltd, UK). The pellet was dissolved in DMSO and diluted sufficiently before analysis [8].

EE was calculated by the formula:

$$\% \text{ EE} = \{\text{Pellet}/ (\text{supernatant}+\text{pellet})\} *100$$

Analytical method for estimation of decitabine

Separation was achieved under optimized chromatographic condition on a Phenomenex Luna C-18 column, (250 x 4.6 mm diameter, 5 µ pore size) using Shimadzu HPLC (High-performance liquid chromatography) system. The mobile phase consisted of ammonium acetate buffer (10 mM pH 6.4), pumped through the chromatographic system at a flow rate of 1.5 ml min^{-1}. The retention time was 12.1 min and run time 20 min. Sodium bisulphite, pH 2.5, was used as a diluent to maintain stability of the drug [9]. The column temperature was 25ºC and UV detection done at 230 nm. Standard plot of decitabine was prepared within the concentration ranges of 0.5-25 µg mL^{-1}.

In vitro drug release studies

The drug release study was performed in a dialysis sac (Mw cut-off 12000, 43 mm flat width, Sigma Aldrich) with phosphate buffered saline pH 7.4 as release medium at 37°C. 2 mg equivalent of DEC-NPs dispersion was added to the dialysis tubing, clipped on both ends, immersed in a beaker with 100 mL of PBS 7.4, and stirred at 100 rpm. At different time intervals (0, 0.5, 1, 2, 4, 6, 12, 24, 48, 72, 96, 120 h), 1 mL aliquots were collected from the release medium outside the dialysis tubing [10] and replaced with fresh PBS. The DEC concentration was determined using HPLC method.

Stability studies

DEC-NPs were lyophilized using 5% mannitol. Stability testing was carried out as per the ICH guideline Q1A (R2). Formulation was stored at accelerated conditions (40ºC ± 2ºC/ 75% RH ± 5% RH) for a period of 6 months. Upon reconstitution, the samples were analyzed for particle size, PDI, entrapment efficiency, and drug release after 90 and 180 days.

Cytotoxicity studies

Cytotoxic effects of DTX, DTX+DEC, and DTX+DEC-NPs were assessed using MTT assay on MCF-7, LNCaP-231, and A-549 cells. Each well of 96 well plates was seeded with 1×10^4 cells and incubated at 37 °C for 24 h. After the formation of monolayer, different dilutions of DTX prepared in maintenance medium (2% FBS) were replaced with the spent medium in wells. After 48 h treatment, the medium was aspirated, and 50 µl of MTT (2 mg/ml in PBS) was added to each well [7]. The plate was incubated at 37 °C for 4 h. After incubation, contents in the plate were gently removed and the formazan crystals were dissolved by adding 50 µl of DMSO to each well followed by shaking plate on orbital shaker for 30 min. Absorbance was measured at 540 nm using micro-plate reader.

The percentage growth inhibition was calculated using the formula below:

$$\% \text{ growth inhibition} = \frac{\text{control absorbance} - \text{test absorbance}}{\text{control absorbance}} \times 100$$

The percentage cell viability was calculated by subtracting value of % growth inhibition from 100. The combination dose was calculated by estimating DTX at 50% below the IC_{50} and different dilutions of DEC and DEC-NPs.

RESULTS AND DISCUSSION

Optimization of decitabine loaded PLGA nanoparticles

Acidified water was chosen to dissolve the drug to form the primary emulsion and DCM was selected as the solvent for PLGA. To formulate the secondary emulsion, Poloxamer 188 was chosen as it formed particles below 150 nm. As the concentration of PLGA increased, the particle size also increased which indicated increased thickness of PLGA coating over the drug surface. Conversely, the decrease in PLGA concentration caused inconsistency in the mean particle size distribution. Therefore, the polymer to drug ratio was optimized at 3:1 **(table 1)**.

Table 1: Optimized batches of DEC-NPs

Sample code	Composition(DEC:PLGA)	Polox 188 (%)	TPGS (%)	Method (SD/DE)	PS (nm)	PDI	ZP (mV)	EE (%)
D34	1:2	1	-	SD	232.9 ± 5.4	0.120 ± 0.09	-23.2 ± 0.8	31.7±1.3
D39	**1:3**	**1**	**-**	**SD**	**143.4 ± 4.9**	**0.105 ± 0.09**	**-26.0 ± 1.1**	**45.2±2.8**
D47	1:5	1	-	SD	361.1 ± 7.9	0.111 ± 0.09	-20.0 ± 1.9	46.3±3.2
D58	1:2	1	-	DE	194.6 ± 5.32	0.089 ± 0.09	-22.3 ± 1.2	22.3±5.9
D61	**1:3**	**1**	**-**	**DE**	**192.6 ± 3.54**	**0.113 ± 0.09**	**-23.8 ± 0.8**	**52.8±3.47**
D63	1:5	1	-	DE	254.2 ± 4.91	0.198 ± 0.09	-26.7 ± 0.9	43.7±4.8
D89	1:3	0.90	0.10	SD	202.3 ± 3.96	0.131 ± 0.09	-14.1 ± 1.3	39.7±2.8
D94	1:3	0.75	0.25	DE	376.2 ± 5.29	0.342 ± 0.09	-15.9 ± 2.7	36.6±3.1
D102	**1:3**	**0.90**	**0.10**	**DE**	**269.7 ± 2.73**	**0.288 ± 0.09**	**-22.1 ± 1.5**	**44.8±4.3**

Characterization of DEC-NPs

The particle size and PDI of the optimized DEC-NP were found to be 124.3 ± 4.2 nm and 0.102 ± 0.021 respectively, sample code D61 **(table 1)**. The zeta potential was found to be -23.2 ± 1.2 mV (Jain et al., 2015). The negative value of zeta potential can be majorly attributed to PLGA and a zeta potential value above 20 infers that DEC-NPs had high stearic stability which prevented the agglomeration [11] and maintained dispersion quality among the nanoparticles. Moreover, the negatively charged particles have been stated to be uptaken by the Peyer's patches and further getting translocated to the systemic circulation [12]. Inclusion of TPGS as a surfactant along with poloxamer reduced the negativity of zeta potential compared to zeta potential of nanoparticles surface coated with only poloxamer; this may be attributed to additive effect of non-ionic characteristics of TPGS with poloxamer.

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The DSC thermogram of PLGA exhibited its melting endotherm at 53.2°C, which is the characteristic glass transition temperature of PLGA 50:50 (Tg 44-55°C). The DSC analysis of lyophilised nanoparticles, DEC-NPs, gave the characteristic peak of PLGA but there was a complete disappearance of the decitabine peak, which confirmed a complete incorporation of decitabine into the PLGA matrix **(Fig 1)**. In the XRD analysis, characteristic diffraction peaks were observed for pure drug. However, no sharp peaks were observed in the case of DEC-NPs, which indicated a complete incorporation of DEC in PLGA polymer **(Fig 2)**. The SEM images of the optimized nanoparticles showed spherical surface with uniform distribution **(Fig 3)** and particle size between 100-150 nm, in agreement with the Malvern NanoZS results. The entrapment efficiency of DEC-NPs was 41.82 ± 4.3 % [13], calculated using the HPLC method. The decitabine peak was obtained at the retention time of 12.1 min **(Fig 4)**.

Fig 1: DSC thermogram of decitabine, PLGA 50:50, decitabine and PLGA physical mixture and decitabine loaded PLGA nanoparticles.

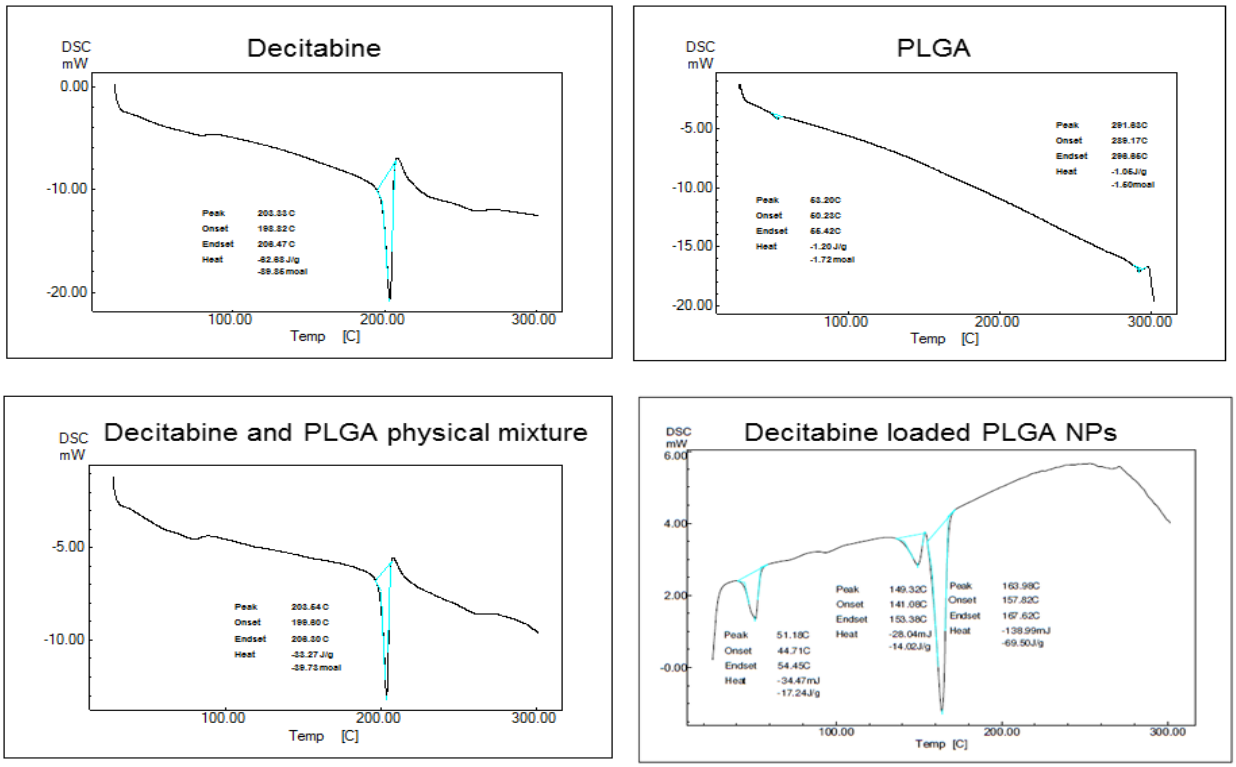

Fig 2: XRD analysis of decitabine and decitabine loaded PLGA nanoparticles

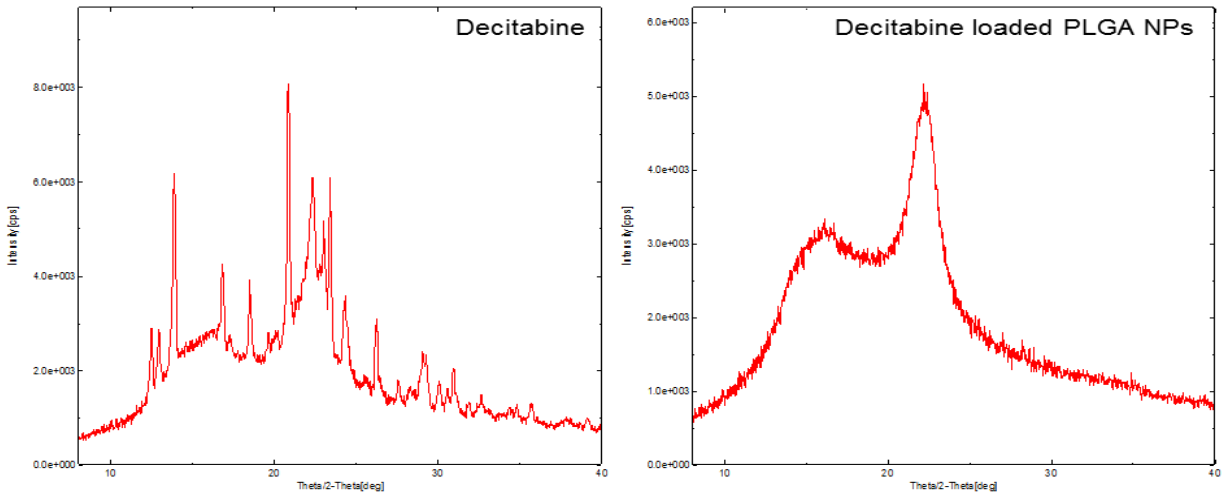

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Fig 3: SEM analysis of decitabine loaded PLGA nanoparticles showing spherical and discrete structures

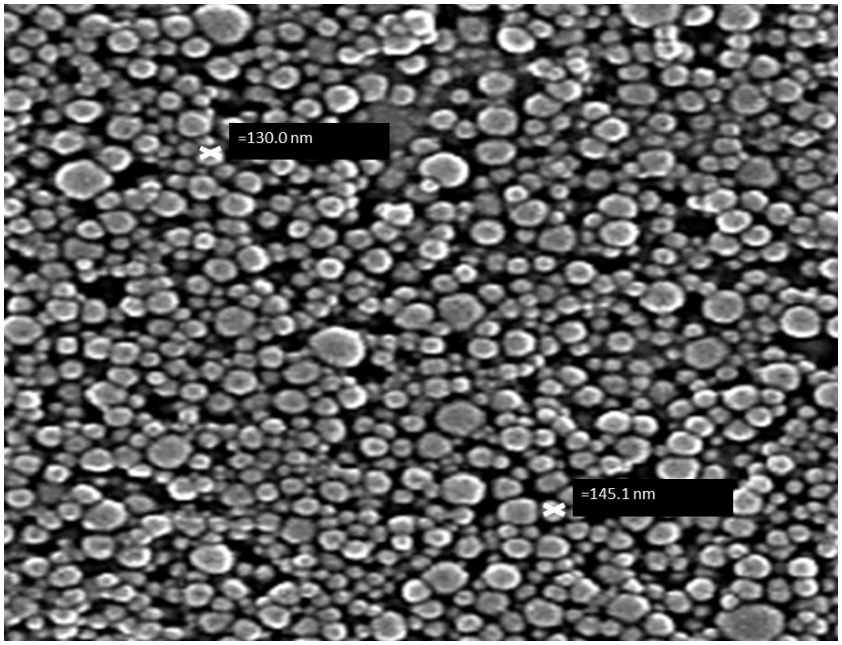

Fig 4: HPLC representative chromatograms with decitabine peak at 12.1 min

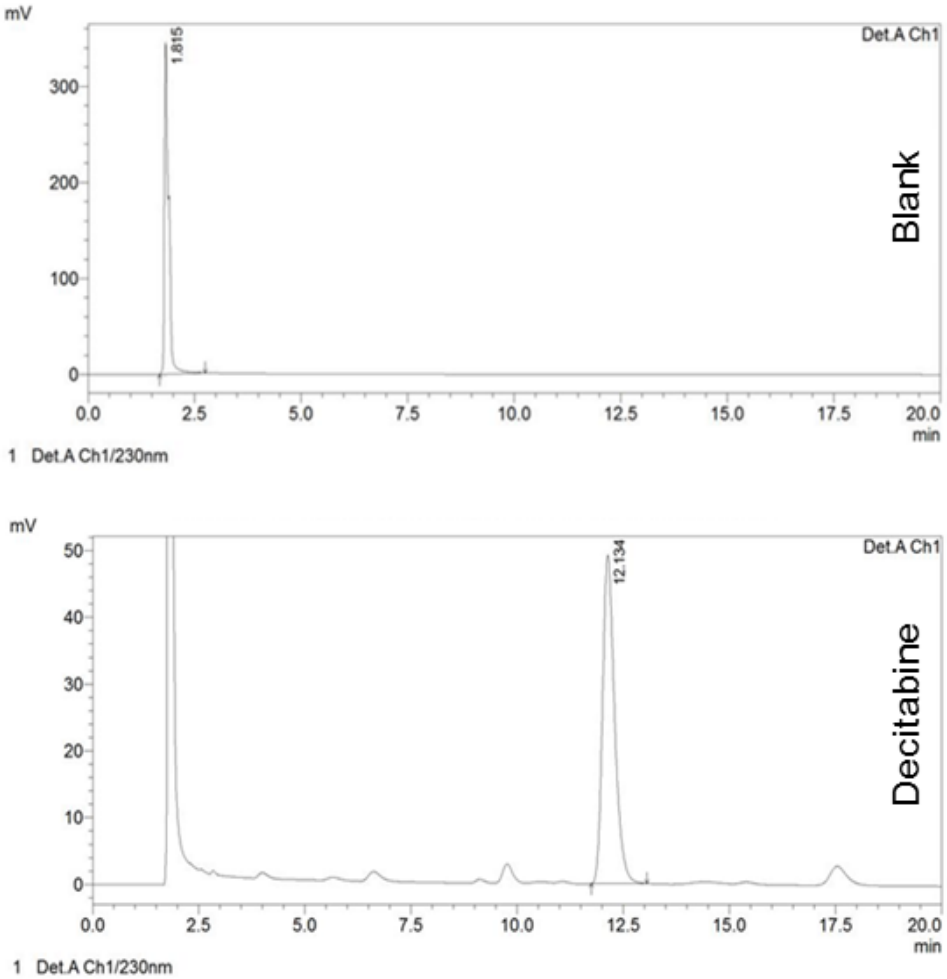

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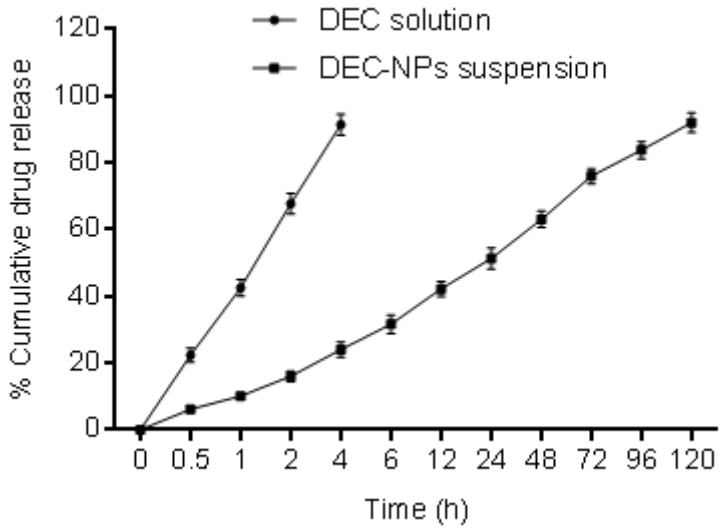
Fig 5: In vitro release pattern of decitabine from plain drug solution and decitabine loaded PLGA nanoparticles. The plain drug solution released 100% drug within 6 h, while approximately 92 % of drug was released after 120 h from DEC-NPs



In vitro release studies

In vitro drug release from DEC solution and DEC-NPs is shown in **Fig 5**. The DEC solution released 40% drug within an hour and complete 100% drug in 6 h. However, 24.03 ± 2.3%, 51.3 ± 3.12% and 92.03 ± 2.9% of drug was released after 4, 24 and 120 h, respectively from DEC-NPs. The drug release from NPs seems to be slower and sustained, since the drug was entrapped in PLGA polymeric matrix.

Stability studies

There was no significant difference in the particle size distribution, entrapment efficiency and in vitro release profile after 0 month, 3 months and 6 months for the tested DEC-NPs confirming their stability.

Antiproliferative activity

DEC-NPs showed higher anti-proliferative effect than plain decitabine solution in combination with docetaxel in all the cell cultures **(table 2)**. This could be owed to a greater internalization of PLGA NPs as incubation time increased while the DEC solution was unable to internalize due to its hydrophilicity. Another factor could be a higher uptake of NPs via endocytosis [14]. The results clearly indicated that the decitabine nanoparticles had greater penetration leading to higher cytotoxicity in cell cultures.

Table 2: Cytotoxicity on different cell lines

S. No.	Cell culture	Source	IC_{50}(µg mL^{-1})		
			DTX	DTX+DEC	DTX+DEC-NPs
1	MCF-7	Human breast epithelial adenocarcinoma	0.084	0.042+0.24	0.042+0.15
2	LNCaP-231	Human prostrate epithelialcarcinoma	0.128	0.064+0.39	0.064+0.22
3	A-549	Human lung epithelial adenocarcinoma	0.073	0.036+0.31	0.036+0.21

CONCLUSION

The polymer PLGA 50:50 used for development has advantages in this area and has been used previously for similar anticancer drugs. The particle size obtained was lower than 150 nm and entrapment efficiency of more than 50 % was achieved. The in vitro studies confirmed that the drug release was

significantly slower and sustained which could maintain the plasma concentration of decitabine for a longer time. In the present study, we investigated the effectiveness of decitabine and its nanoparticles in combination with docetaxel for the treatment of solid tumours. The combination was studied extensively on cell cultures. The MTT assay in all the cell lines showed that NPs were more effective than the plain drug.

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