

Oral Administration of Decitabine Nanoparticles Effectively Suppresses Nmu-Induced Leukaemia in Sprague-Dawley Rats and Arrests K562 Cells in S-Phase

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ABSTRACT

Oral delivery of anticancer drugs has been an important topic of research in medicine for a decade. Unfortunately, most of the anticancer drugs, either old or novel, with high therapeutic efficacy, do not realize their full potential in the market. The epigenetic drug, decitabine (DEC), is one such potent hypomethylating agent, but its effect is temporary. The main reason is low oral bioavailability due to the gastrointestinal (GI) drug barrier and other instabilities. Therefore, it is commercially available as i.v. infusion to be used in hospital settings. We aimed to design and fabricate decitabine loaded PLGA nanoparticles (DEC-NPs) for potential oral delivery of DEC. The DEC-NPs were characterized by particle size, zeta potential, differential scanning calorimetry (DSC), X-ray diffraction (XRD), scanning electron microscopy (SEM), and *in vitro*, ex-vivo and in vivo release studies. The efficacy of DEC-NPs was investigated in vitro in leukaemia cells, K562, and in vivo by NMU induced leukaemia model of male Sprague Dawley rats. The antiproliferative effect of DEC-NPs was significantly enhanced ($p < 0.05$) over DEC in K562 cells with a significantly prolonged ($p < 0.05$) cell cycle arrest in the S-phase. Upon oral administration, the WBC count was significantly reduced ($p < 0.05$) by DEC-NPs in rats compared to plain drug. The studies suggested that DEC-NPs have high potential for effective oral delivery of decitabine.

Keywords: Decitabine nanoparticles (DEC-NPs), NMU induced leukaemia, Caco-2, K562, oral chemotherapy

INTRODUCTION

Oral delivery of anticancer drugs has been an important topic of research in medicine for a decade. Unfortunately, most of the anticancer drugs, either old or novel, with high therapeutic efficacy, do not realize their full potential in the market. The main reason is low oral bioavailability of these molecules due to the gastrointestinal (GI) drug barrier and other instabilities. Therefore, in the present regimen of chemotherapy, the anticancer drugs are administered in a painful manner through i.v. injections or infusions in hospital settings, which leads to high costs, non-compliance and eventual failure of the therapy.

The solution to these challenges is oral chemotherapy, a dream of cancer patients, and the only strategic step towards achieving chemotherapy at home. The success of oral chemotherapy can profoundly transform the clinical practice of chemotherapy and significantly improve the quality of life of the cancer patients. The oral route is by far the most preferred route due to higher patient compliance, ease of administration, cheaper costs, etc.¹. Pharmaceutical nanotechnology and formulation sciences are the crucial stake holders in solving the problems of drug delivery that may provide solutions to change the way

of making and taking drugs. Fortunately, there have been a plethora of advances in understanding drug delivery of small molecules to targeted organs, bypassing the GI tract and allowing protection from destructive enzymes in the body.–The oral chemotherapy is has an advantage of maintaining sustained moderate plasma concentration of the drug which leads to prolonged exposure of drug to the cancer cells.

Leukaemia represents a set of malignant ailments characterized by an abnormal accumulation of blood cells, typically WBCs (white blood cells). On the basis of the progression of the disease and hematopoietic lineages involved, leukaemia is categorized as acute vs chronic and myeloid versus lymphoid². Although, the causes of leukaemia have been vaguely understood, yet it appears to encompass some rearrangements in DNA. In animals, leukaemia progresses either spontaneously or due to treatment with internal or external leukemogenic factors. The internal factors comprise chromosomal abnormalities while external factors comprise alkylating drugs, ionizing radiation, or chemical treatment. The treatments involving the cure of leukaemia entail intense chemotherapy, which

Table 1: NMU model study groups of male Sprague-Dawley rats (n = 6)

Group	Treatment
I	Sham control
II	Disease control
III	Decitabine (p.o.)
IV	DEC-NPs (p.o.)
V	Blank NPs (p.o.)
VI	Decitex (i.v.)

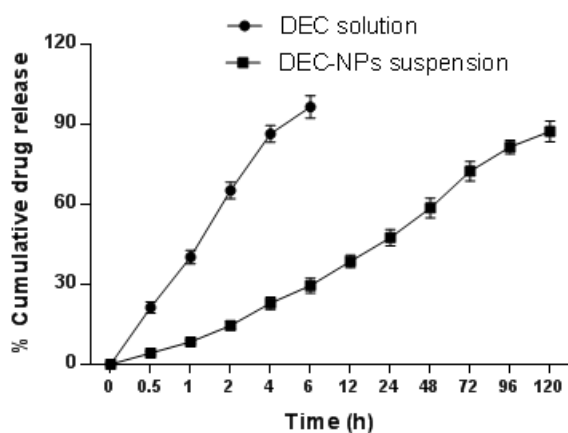


Figure 1: In vitro release pattern of decitabine from plain drug solution and decitabine loaded PLGA nanoparticles

Table 2: IC₅₀ values on K562 cell culture

Cell Line	Source	DEC (µM)	DEC-NPs (µM)
K562 (24 h)	Human Bone Marrow	0.137	0.113
K562 (48 h)	Human Bone Marrow	0.108	0.055

might lead to life-threatening worries in the medically fragile cases.

Aberrant expression of DNA methyltransferases (DNMTs) promotes methylation of DNA in certain genes. 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 treatment³. It is approved by the US FDA for use as a monotherapeutic agent against hematological cancers⁴. It has successfully shown therapeutic action in patients with myelodysplastic syndrome (MDS) in clinical trials at higher phases. DEC is also active in both acute and chronic leukaemias as well⁵. The utility of DEC as a pharmaceutical, however, is restricted by its very low and variable bioavailability (3.9-14%)⁶. 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. Its half-life in aqueous solution is nearly 4 h⁷ and in cell culture medium approximately 17 h⁸, while in vivo half-life varies from 10-35 min⁹. On oral administration to humans, decitabine may not be absorbed to any significant extent into the

bloodstream. Therefore, it is currently available as i.v. infusion, which has several drawbacks. It often leads to high peak of the drug in plasma, over the maximum tolerable concentration (MTC). This is trailed by a quick excretion causing inadequate area under the curve (AUC) and severe side effects.

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. The reduction in particle size to nano scale results into an enhanced oral bioavailability of the drug via enhanced permeability through intestinal epithelium. The particle transport through the intestinal epithelial barrier takes place either (a) paracellularly for particles that are 50 nm or less; (2) by endocytosis for particles smaller than 200 nm through intestinal enterocytes; and (3) by lymphatic uptake for particles smaller than 5 µm, across M-cells of Peyer's patches¹⁰.

Researchers have been working to improve the delivery of decitabine on different systems such as nanogels¹¹, nanostructured lipid carriers^{12,14}, liposomes¹³ and engineered erythrocyte (Erythro-Magneto-Hemagglutinin Virosomes, EMHVs) drug delivery system¹⁵. At all instances, the major tasks for researchers are to improve the entrapment of the drug in the delivery system and to enhance the bioavailability. Here, the nanoparticulate delivery systems aim to improve the stability of drug, its duration of therapeutic action, and bioavailability, while minimising its degradation, metabolism and cellular efflux^{16,17}. Owing to their smaller size and coating on the surface, nanoparticles have also shown the capability to dodge the P-glycoprotein efflux from intestinal epithelial cells, ultimately enhancing the intestinal absorption. They offer additional advantages over other colloidal systems with superior stability of nanoparticles and protection of entrapped drug in GIT^{18,19}.

In the present study, we selected PLGA 50:50 as the polymeric carrier for many reasons. PLGA is one of the most widely used polymers for pharmaceuticals, approved by FDA due to its biocompatibility and biodegradability²⁰. Since PLGA is resistant to acid degradation, we predicted that a nanoparticulate formulation would be able to protect DEC from stomach environment and deliver it efficiently to intestines, for absorption into the blood stream. The physicochemical characteristics, particle morphology and in vitro release behaviour of DEC loaded nanoparticles have also been elucidated. The standard i.v. infusion regimen for decitabine is 15 mg/m² over 3 h, every 8 h for three days and repeated every six weeks, i.e., 135 mg/m² per cycle. The present study design for the treatment of NMU induced leukaemia in Sprague Dawley rats was based on the same regimen. Instead of i.v. infusion, the DEC-NPs were administered orally and investigated for their efficacy.

MATERIALS AND METHODS

Materials

TPGS, Trehalose, Lucifer yellow, Poloxamer, PLGA 50:50, decitabine, N-nitroso-N-methyl urea (NMU),

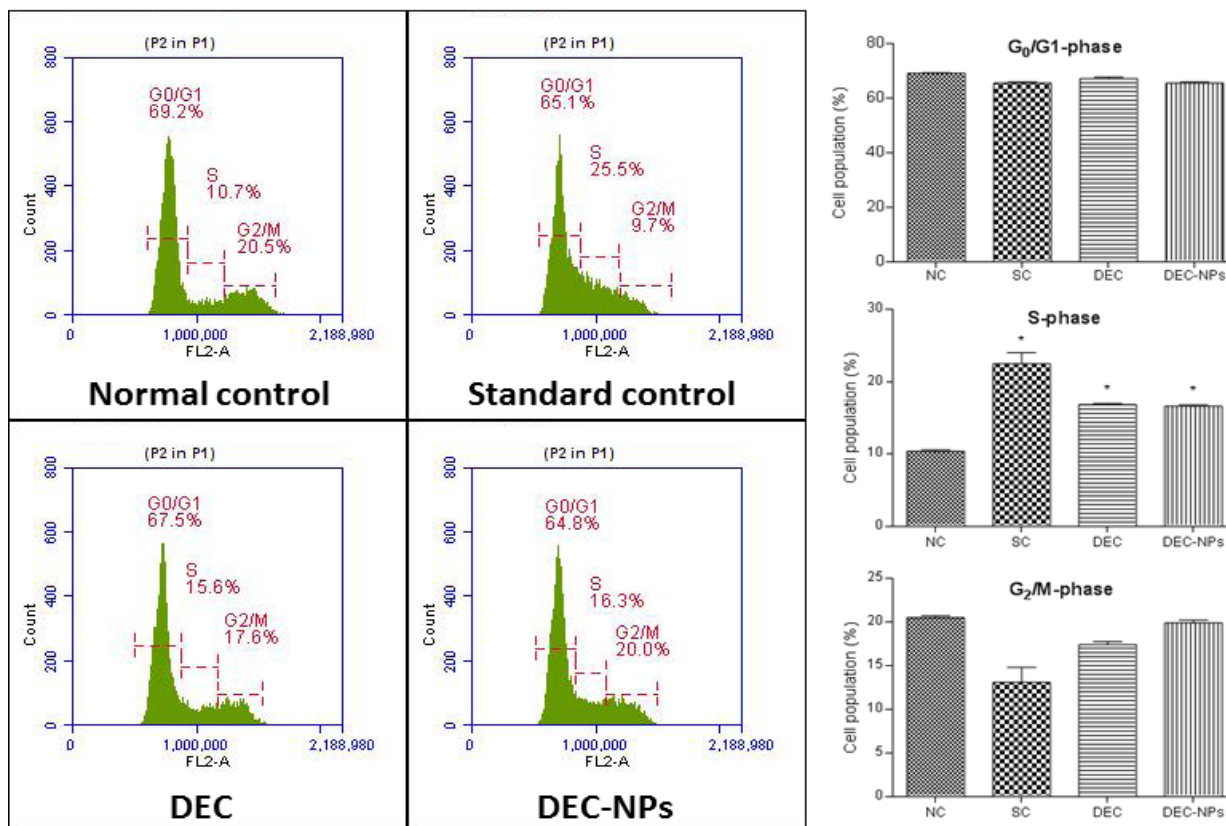


Figure 2: Cell cycle analysis in K562 cells showed S-phase arrest. The percentage of cells in S-phase were significantly higher ($p < 0.05$) upon treatment with DEC and DEC-NPs when compared to the untreated cells. Standard S-phase inhibitor cisplatin was used as positive control.

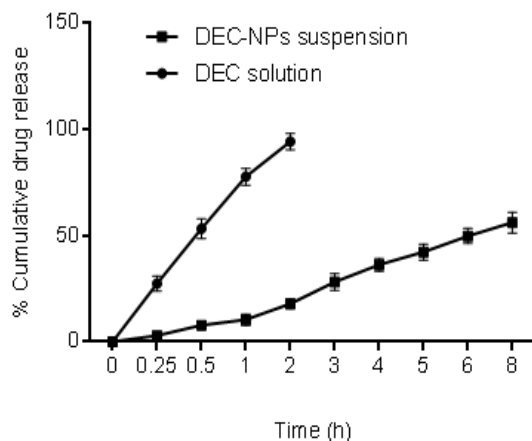


Figure 3: Ex-vivo permeation studies in PBS 6.8 for 8 h. The plain drug solution showed nearly complete drug release in the intestines in 2 h whereas at the end of 8 h study, approx. 50% of drug was released from NPs indicating slow and sustained release.

Costar transwell plates 6 well 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 NPs by Multiple emulsification technique

Decitabine loaded PLGA NPs were formulated by multiple emulsification solvent evaporation method. DEC (10 mg) was dissolved in 0.5 mL acidified water. The resultant aqueous phase was dropwise added to a solution of 30 mg PLGA (50:50) in 2 mL DCM to give a water in oil emulsion, with pulsed sonication for 2 min at 60 W (Ultrasonic Processor, Sonics and Materials Inc., Ct, USA)²¹. This emulsion was added to aqueous solution (1.0%) Poloxamer 188 with constant homogenization for 15 min at 14,000 rpm under cool conditions. This was further sonicated for 10 min with 2 sec pulse at 75 W to reduce the particle size and then kept on magnetic stirrer for 4 h to allow solvent evaporation and precipitation of polymer to form NPs. The nanoparticle suspension was centrifuged for 60 min at $22,000 \times g$ at 4°C (Sigma, USA). DEC-NPs were lyophilized using mannitol as cryoprotectant.

To remove the non-encapsulated drug, the resulting dispersion was centrifuged at $22,000 g$ for 1 h at 4°C and supernatant separated. The residue was washed twice with deionised water to remove any untrapped drug. The pellet was then re-suspended in 10 ml of ultra-purified water. The NP suspension was lyophilized using various cryoprotectants like mannitol, trehalose, etc. at different concentrations. The lyophilized NP were tested for stability and used for efficacy studies.

Characterization of DEC-NPs

The prepared nanoparticles were characterized for particle size and zeta potential using Malvern NanoZS (Malvern

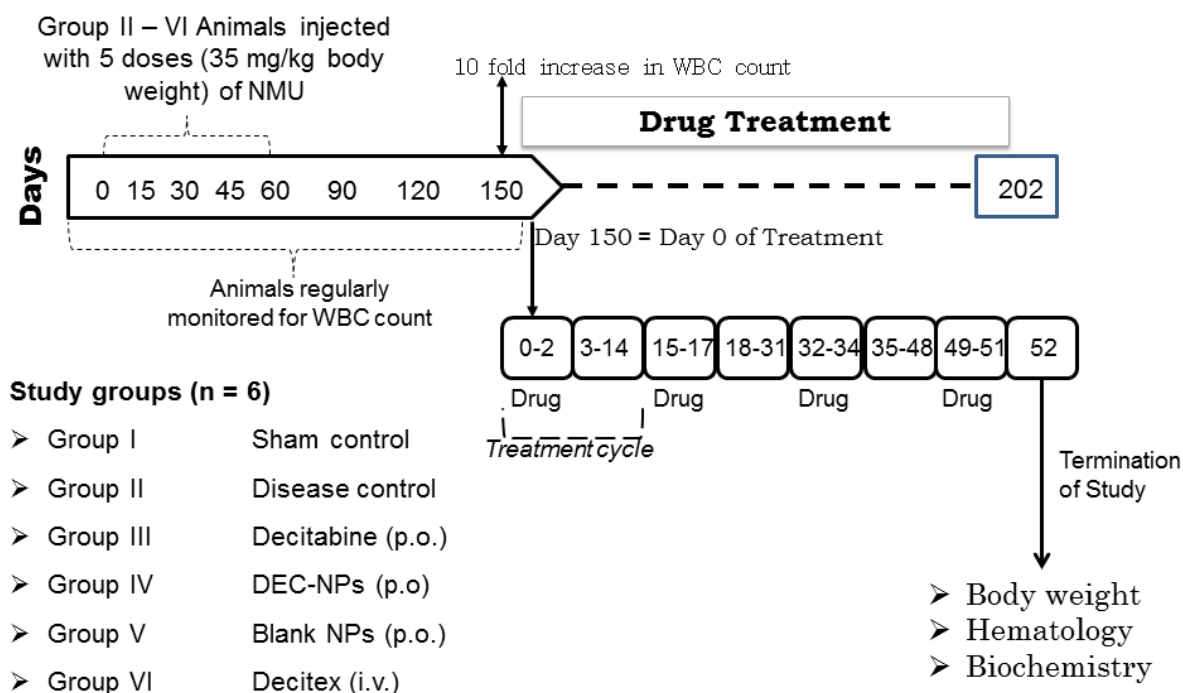


Figure 4: Experimental design of NMU induction in S-D rats. The initiation of NMU induction was designated as day 0. After 5 injections at 15 day intervals, the animals were observed until 10 fold increase in WBC count was recorded on day 150. The treatment was initiated for 52 days from that point, in four cycles of drug administration

instruments Ltd., Worcestershire, UK). The results were used to select the most competent batches with smaller sizes and higher zeta potential. DSC analysis was performed for DEC, PLGA (50:50), DEC and PLGA (50:50) physical mixture and DEC-NPs for to determine the interaction between formulation excipients, stability and nature of nanoparticles. X-ray diffraction patterns were obtained using Rigaku miniflex 600 X-ray diffractometer (Rigaku Co., Tokyo, Japan) using $\text{Cu-K}\alpha$ X-radiation^{22,24}. The drug entrapment in nanoparticles was determined by measuring the drug content in nanoparticles.

EE was calculated by the formula:

$$\% \text{ EE} = \left\{ \frac{\text{Pellet}}{\text{supernatant} + \text{pellet}} \right\} * 100^{23}$$

Shimadzu HPLC (High-performance liquid chromatography) system was used for chromatographic separation on a Phenomenex Luna C-18 column, (250 x 4.6 mm diameter, 5 μ pore size)²². The mobile phase consisted of ammonium acetate buffer: methanol (98:2), at a flow rate of 1.5 ml min^{-1} . The column temperature was 25°C and UV detection done at 230 nm. Standard plot of decitabine was prepared within the concentration ranges of 0.5-25 $\mu\text{g mL}^{-1}$. The retention time was 8.1 min.

In vitro drug release studies

The drug release study was performed as per the method described²².

Stability studies

DEC-NPs were lyophilized using 5% mannitol and stability testing was carried out as per the method described²².

In vitro cell culture studies

K562 cells were used as a model of in vitro anti-proliferative studies. The cells were cultured in RPMI1640

medium that was supplemented with 2 mM L-glutamine. 1% penicillin-streptomycin was added to the medium after filtration and 10% fetal bovine serum for cell growth. Exponentially growing cells were used for all experiments. *Cytotoxicity study by MTT assay*

The in vitro antiproliferative effect of DEC and DEC-NPs on K562 cells was evaluated for 24 and 48 h using the MTT assay. Briefly, 100 μL (6×10^4 cells/well) of a K562 cell suspension were seeded in a 96-well plate for 12 h. Thereafter, the cells were treated with varying concentrations of DEC and DEC-NPs (0.01, 0.1, 1, 10 μM) while an equal volume of DMSO was added to control cells. The cells were cultured for an additional 24 or 48 h, and then 50 μL MTT (2 mg/mL) in growth medium was added per well, and the plates incubated at 37°C for 4 h²⁵. Plates were then centrifuged at 1000 g for 10 min. Supernatant was removed from each well, and the reduced MTT dye was solubilized in 100 μL DMSO. Absorbance was measured using the microplate reader (Bio-Tek, ELX-800 MS) at 540 nm. The experiments were repeated five times. Viability was calculated as follows:

$$\text{Cell Viability (\%)} = \left(\frac{\text{OD}_{\text{test}}}{\text{OD}_{\text{control}}} \right) \times 100\%$$

Cytotoxicity of DEC-NPs on Caco-2 cells were executed for 6 h to evaluate the toxicity of NPs during the permeability studies. The cells were cultured in 96-well plates at a seeding density of 5×10^3 cells/well. DEC and freeze-dried DEC-NP were diluted in culture media and 100 μL of different concentrations added to wells. After 6 h, the supernatant was flicked off, 50 μL of MTT (2 mg/mL) added to each well and incubated for 4 h. The unreduced MTT and medium were then discarded. 100 μL of DMSO was added to each well to dissolve the MTT formazan crystals. Plates were shaken for 20 min and

absorbance was measured at 540 nm using the microplate reader (Bio-Tek, ELX-800 MS)²⁵. The IC₅₀ values were graphically calculated and the experiments were performed five times.

Cell cycle analysis

The effect of DEC solution and DEC-NPs on cell-cycle analysis was studied in K562 cells. Briefly, 1×10^6 cells mL⁻¹ were seeded in T-75 flasks and allowed to grow for 48 h. The cells were treated with a single dose of DEC solution (0.02 μ M) or DEC-NPs (equivalent drug dose) and incubated for 24 h without further treatment. Cells were collected by centrifugation at 1500 rpm for 3 min at 4 °C. The pellet was re-dispersed in PBS and centrifuged again to remove the culture medium. The cells were then fixed in 70% ice cold ethanol for 30 min at -20°C²³. Ethanol was removed after centrifugation at 1500 rpm for 3 min at 4 °C and cells were resuspended in 1mL of PI solution containing 25 μ g mL⁻¹ PI, 40 μ g mL⁻¹ RNAase A and 0.03% Triton-X solution in PBS²⁶. The cells were analyzed using Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) using excitation at 488 nm and emission at 575/540 nm. A minimum of 10,000 events was acquired and analysis of flow cytometric data was performed.

Transport of NPs across Caco-2 cells

Transwell inserts (0.4 μ m pore diameter, 1.13 cm² area) were used to culture Caco-2 cells for transport experiments. The inserts were equilibrated for 30 min with pre-warmed HBSS containing 25 mM of HEPES, pH 7.4. The cells were seeded at a density of 1×10^5 cells mL⁻¹ and left for after 21 days post seeding with regular growth medium replacement with 2% FBS. The permeability of Lucifer yellow, the paracellular leakage marker was used to monitor the integrity of the monolayers which were considered tight if Papp (apparent permeability coefficient) was less than 0.5×10^{-6} cm/s. DEC and DEC-NPs were added to the apical (0.5 ml) side. On the other hand, 1.5 ml of the transport buffer, HBSS was at the basolateral side of the inserts. At predetermined intervals of 30 min, 60 min, 120 min, 180 min, and 240 min of incubation, 100 μ l aliquot was pipetted from the receiver chamber which was instantaneously refilled with prewarmed HBSS. The concentration of DEC and DEC-NPs in the transport medium was analyzed by HPLC method described in section 2.3.6. The apical-to-basolateral permeability coefficient (Papp in cm/s) was calculated according to following equation: $Papp = (dQ/dt) / (A * C_0 * 60)$, where dQ/dt is the amount of nanoparticles/drug in basolateral compartment as a function of time (mg/min), A the monolayer area (cm²), and C₀ is the initial concentration of drug in apical compartment (mg/mL)¹⁸.

Ex-vivo intestinal release studies

The ex-vivo intestinal release method was carried out to simulate the in vivo conditions. The animals were fasted overnight, sacrificed by cervical dislocation and the abdomen was exposed quickly by a midline incision. The ileum was freed from mesentery by hand and isolated by cutting it at duodenal end and at the lower end of the ileum. The ileum was further flushed with normal saline using the

blunt end of a syringe to remove its contents and divided into pieces of around 5cm. The plain drug solution and DEC-NPs (2 mL) were filled in the mucosal side of two separate tissues¹². Thereafter, the sacs were ligated tightly from both the ends. The sacs were immersed in 100 mL Krebs solution in a glass beaker individually and kept on magnetic stirrer to maintain $37 \pm 2^\circ\text{C}$ and provided with continuous aeration for 8 h. At predetermined time intervals (30, 60, 120, 180, 240 min), 1 mL aliquots were withdrawn from the serosal compartment and equivalent volume was replenished. The samples collected were filtered, and the drug permeation through the sac was calculated using HPLC method in section 2.3.

Pharmacodynamic evaluation of DEC-NPs in male

Sprague-Dawley rats

Animals

36 male Sprague Dawley rats of forty days of age were procured from the Central Animal Research Facility, Manipal University, Manipal. The animals were approved by the Institutional Animal Ethical Committee under protocol no. IAEC/KMC/16/2013. The animals were housed at a maximum of three per cage in a controlled environment with a 12 hour light/dark cycle and allowed to acclimatize for 5 days. Water and feed were provided *ad libitum*.

Induction of leukaemia by N-nitroso-N-methylurea (NMU)

The induction of leukaemia by NMU was performed according to the procedure mentioned by Chang et al²⁷. The animals were bled from retro-orbital plexus under ether anaesthesia and around 200 μ L of blood was obtained in tubes coated with dipotassium EDTA. Detailed blood cell counts were determined using a fully automated veterinary blood cell counter. The animals were then randomized into six groups; a control group and others for NMU induction, maintaining a mean WBC count of 11.16 ± 0.94 to 12.26 ± 1.04 ($\times 10^3/\mu\text{L}$). All the animals, except for those belonging to the control group, were administered with NMU at a dose of 35 mg/kg body weight of the animal. The solution was freshly prepared before administration by dissolving 200 mg of NMU in 20 mL of acidified saline to obtain a concentration of 10 mg mL⁻¹. This was kept on bath sonication for 5 min. The rats received five i.v. injections of NMU, in a caudal vein with an interval of 15 days between injections, with the first injection designated as day 0 of the study model. Before each dose of NMU, the animals were bled and complete blood cell count was estimated. After the last dose of NMU, the blood cell count was recorded at 30 day intervals. The changes in WBC count were observed and treatment was started on 150th day, once there was 10 fold increase in the WBC count of animals.

Experimental design

Total of 3.375 mg/kg body weight of decitabine per cycle was given in four cycles. In each cycle, 1.125 mg/kg drug per day was administered for three consecutive days followed by a gap of two weeks. The standard drug was dissolved in KN buffer (1.2 gL⁻¹ sodium hydroxide, 7.08 gL⁻¹ potassium dihydrogen phosphate) and delivered orally to group III. A solution of 1 mg mL⁻¹ was prepared and kept on ice until administered. Group IV received DEC-

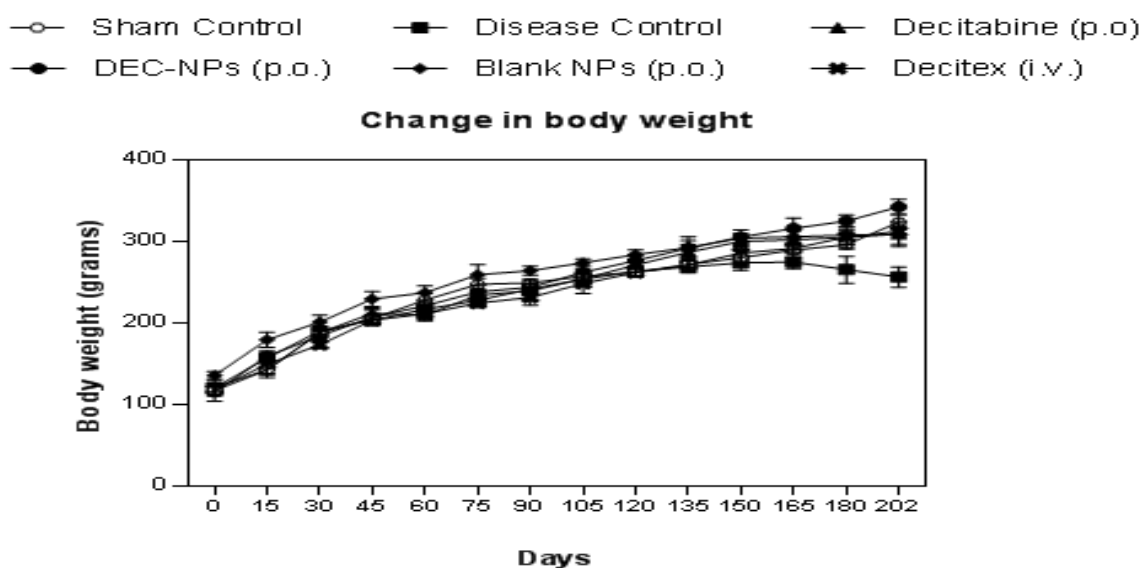


Figure 5: Body weight changes in male S-D rats. There was a steady weight gain in the first four months, followed by a plateau in the average body weight. The DEC-NPs treatment group were able to recover and gained more weight compared to the untreated animals, by the end of the study.

NPs orally while group V received blank NPs. Lyophilised DEC-NPs were reconstituted in KN buffer to a concentration of 1 mg mL^{-1} prior to administration. The market preparation Decitex (Sun Pharma Ltd.) was dissolved in the prescribed water for injection and administered intravenously to the group VI. Group I was administered with the same vehicle as group III (table 1). All the drug preparations were freshly made before administration.

Measurement of change in body weight

The body weight of the rats was measured from the first day of NMU induction until the termination of study, at 15 day intervals.

Measurement of haematological parameters

The haematological parameters were recorded fortnightly after the initiation of the treatment on day 150th till the end of the study period on day 202, with recording procedure as explained in section 2.8.2. The primary end point of the study was to investigate the effect of DEC-NPs on the WBC count of animals and compare with the oral DEC and i.v. decitex. The RBC and haemoglobin count were also considered to explore the secondary diseases if any.

Measurement of biochemical parameters

On day 202, before sacrificing the animal, 2 mL of blood was collected by retro-orbital puncture for biochemical investigation. The samples were quickly centrifuged at 10000 rpm for 5 min at -4°C , separated the serum and stored at -80°C . Serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT) levels were measured with enzymatic kinetic method by automatic biochemical analyzer (Cobas, Germany) using kits^{23,28}.

Statistical analysis

Data were represented as mean \pm SEM. Statistical analysis of the data was carried out by one way ANOVA (Graph PAD Prism Version 5.02, InStat Software La Jolla, CA,

USA) followed by Tukey's post hoc test. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Optimization of decitabine loaded PLGA nanoparticles

The multiple emulsification solvent evaporation method was preferred since decitabine is insoluble in commonly used solvents like DCM, ethyl acetate, etc. Therefore, acidified water (pH 3) 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, polyvinyl alcohol, poloxamer 188 and poloxamer 407 were tried as surfactants. Poloxamer 188 was chosen as it formed particles below 150 nm. The concentration of PLGA was varied from very low to very high in order to achieve the desirable particle size without hampering the entrapment of the drug. 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.

Characterization of DEC-NPs

The particle size and PDI of the optimized DEC-NP were optimised to be nearly 150 nm and 0.1 respectively. The zeta potential was -23.2 mV (Jain et al., 2015), which infers a good steric stability that prohibited the agglomeration and sustained distribution of size in the nanoparticles. The DSC and XRD analysis established a complete incorporation of the drug into the PLGA matrix²². The SEM images of the optimized nanoparticles showed spherical surface with uniform distribution. The entrapment efficiency of DEC-NPs was over 50.

In vitro release studies

In vitro drug release from DEC solution and DEC-NPs was performed thrice. The DEC solution released 40% drug within an hour and 95% drug within 6 hours. However,

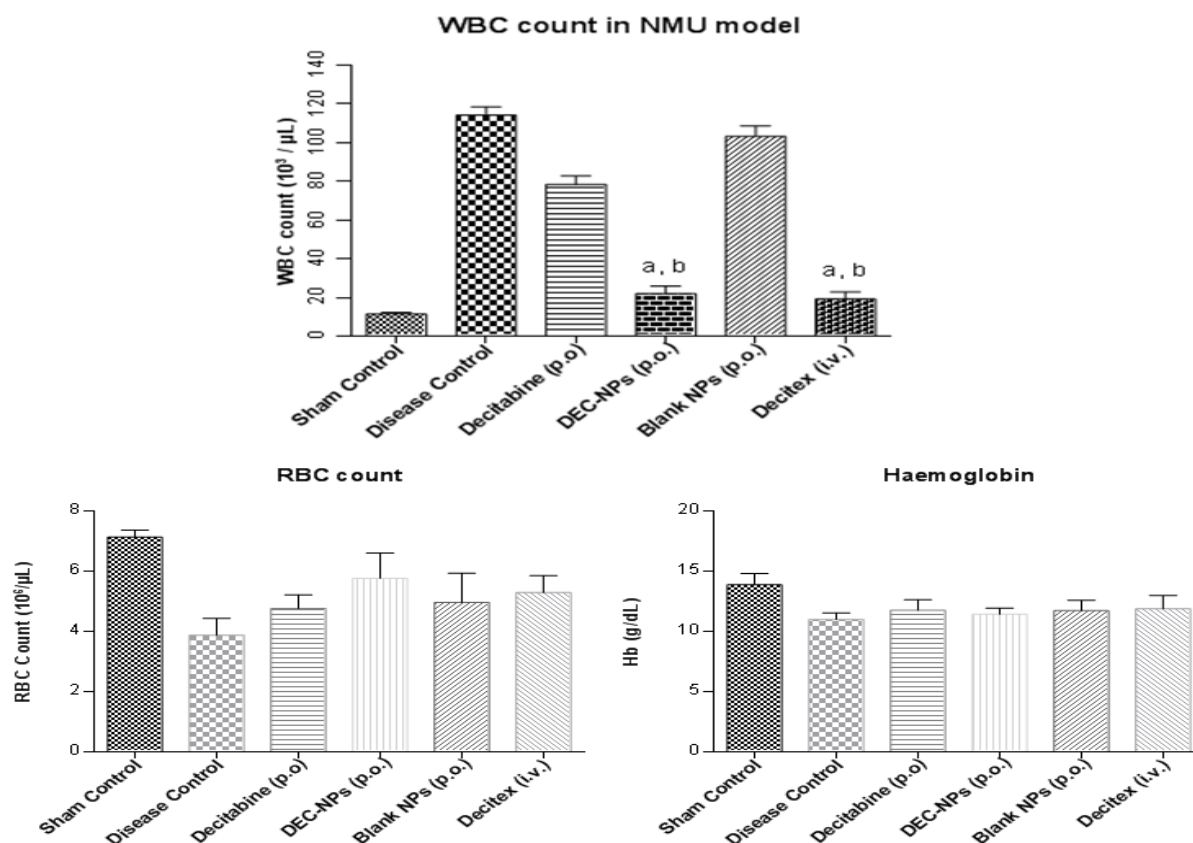


Figure 6: Haematological changes in male S-D rats. Mean WBC count increased in the untreated group II and was recorded at $110 \times 10^3 \mu\text{L}^{-1}$. After the treatment with DEC-NPs and decitex, there was a significant decrease ($p < 0.05$) in the mean WBC count compared to the sham control ($p^a < 0.05$) and plain drug solution ($p^b < 0.05$). Mean RBC count and Hb was not significantly affected by the drug treatment and remained unchanged.

approx. 50% and 90% of drug was released after 24 and 120 h, respectively from DEC-NPs (Fig. 1). The drug release from NPs seems to be slower and sustained, since the drug was entrapped in PLGA polymeric matrix.

Stability studies

The stability of the tested DEC-NPs was confirmed as 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.

Antiproliferative activity

Cytotoxicity studies

DEC-NPs showed higher anti-proliferative effect than decitabine solution at both the time points (table 2). The DEC-NPs were found to be 2-fold more cytotoxic on K562 cancer cells after 48 h incubation. 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 endocytosis²⁹.

Cell cycle analysis by flow cytometry

Flow cytometric results demonstrated that the cell cycle in K562 cells was arrested in S-phase (Fig. 2). The percentage of cells in S-phase were significantly higher ($p < 0.05$) upon treatment with DEC (15.6 %) and DEC-NPs (16.3 %) when compared to the untreated control cells (10.7 %). This S-phase specificity of DEC was a result of its incorporation into DNA to produce antineoplastic

action. Decitabine did not block cell cycle progression of G1 phase cells into the S phase. The standard S-phase specific drug cisplatin, confirmed the experimental accuracy by arresting 25.5 % cells in the S-phase.

Transport studies of NPs through intestinal absorption model (Caco-2 cells)

The Caco-2 cells model was studied to evaluate the gastrointestinal permeability of DEC-NPs by calculating permeability coefficients in the cells. Transepithelial permeability of DEC measured at concentration $100 \mu\text{g mL}^{-1}$ showed negligible toxicity on Caco-2 cells after 21 days culturing. The permeability coefficient for DEC solution was found to be $0.41 \times 10^5 \text{ ml min}^{-1} \text{ cm}^{-2}$ while that of DEC-NPs was found to be $3.2 \times 10^5 \text{ ml min}^{-1} \text{ cm}^{-2}$ which was 7.8 folds higher than the DEC solution. Permeability coefficient between 1 and 10×10^5 signifies moderate to good permeability³⁰.

Ex-vivo gut permeation study

The ex-vivo drug release studies in intestine were performed to find out the pattern of drug release from NPs in intestinal environment. The DEC solution exhibited practically complete drug release within 2 h. However, towards the end of the 8 h study, around 50% of drug was released from NPs. This clearly indicated a slow and sustained release of the drug from the NPs (Fig. 3).

In vivo treatment of leukaemia in NMU induced model

The rats induced with leukaemia by a series of NMU injections were subjected to treatment with decitabine, DEC-NPs and decitex (marketed decitabine formulation) (Fig. 4).

Effect on mean body weight

The mean body weight decreased in the disease group toward the peak phase of the study. This could be attributed to the weakening of the immune system of rats. The other groups showed a steady increase in the body weight from day 0 till the termination of the study (Fig. 5).

Effect on WBC count

The WBC count of all the NMU induced rats was above 110×10^3 before the initiation of decitabine therapy in the designated groups. A series of blood count estimations after the initiation of treatment revealed that the WBC count was significantly reduced ($p < 0.05$) in the group of animals treated with orally administered DEC-NPs and i.v. decitex. The NPs successfully treated the leukaemia condition of the animals and brought the WBC count to approximately 20×10^3 at the end of the study, Fig. 6. The RBC count and Hb remained unaffected and played no significant role in the findings.

Changes in liver, kidney and spleen weight

There was no significant difference in the liver and kidney weights between the groups. However, spleen weight was found to be significantly increased ($p < 0.05$) in the disease control group II. There was no significant difference in the mean spleen weight between the other groups.

Effect on biochemical parameters

The normal levels in sham control animals were found to be 92.3 ± 3.8 and 71.9 ± 4.1 for SGOT and SGPT, respectively. There was a 3 fold elevation in the SGOT and 4 fold increase in SGPT levels of animals of induced with leukaemia. However, the drug treatment was unable to lower these abnormal levels in any of the groups.

CONCLUSION

In the past few years there has been a global debate on whether to discover new molecules in oncology, or to unearth the full potential of the existing molecules and save at large dollars on drug development and clinical trials. Interestingly, the latter has been voted for and practiced for many reasons. Drug reformulation of existing molecules plays a fundamental role in improving patient compliance and refining clinical outcomes. It also empowers the full commercial potential of a molecule to be recognized and maximizing returns on investment. A potential oral particulate drug delivery system has been developed in our study, for effective delivery of decitabine. The study was powered to detect small but pre-clinically worthwhile benefits in disease-free survival, a step towards the improvement of quality of life. 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 was less than 150 nm and entrapment efficiency above 50 % was achieved. The in vitro, ex vivo and in vivo studies confirmed that the drug release was significantly slower and sustained which could maintain the plasma concentration for a longer time. The MTT assay in leukaemia cell lines showed that NPs were more

effective than the plain drug. The efficacy of the NPs was further assessed in the treatment of NMU induced leukaemia the rats. Thus, the orally delivered DEC-NPs can significantly improve its oral bioavailability, achieving a sustained therapeutic effect, and overcome the drawbacks associated with infusions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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