Decitabine Nanoparticles and Docetaxel Combination Shrinks Mammary Carcinoma Induced by DMBA in Female Sprague-Dawley Rats

Jain P¹, Kumar N², Tiwari M¹, Rao J V¹, Shilpee C², Udupa N³*

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

Available Online: 25th October, 2016

ABSTRACT

Docetaxel (DTX) is one of the most effective chemotherapeutic agents in the treatment of breast cancer. Unfortunately, there is altered response to DTX due to resistance. DNA methylation, an epigenetic event, plays a vital role in cancer and chemotherapy drug resistance. Exploiting the gene reactivation by using epigenetically acting agents in combination with cytototoxic therapies, is a strategy of huge clinical relevance. Decitabine (DEC) is one such epigenetic drug. It has low oral bioavailability (4-5%) due to rapid degradation in acidic conditions and metabolism by cytidine deaminase in the liver and is therefore administered in clinical settings as i.v. infusion. In spite of the numerous advantages of oral chemotherapy, there is no conveniently administrable 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. Furthermore, cell uptake studies in Caco-2 cells, evidenced enhanced uptake of DEC-NPs in comparison to DEC. For in vivo studies, 8week old female SD rats were used and induced mammary tumour with 2 oral doses of DMBA in olive oil; 40 and 20 mg/kg. The results demonstrated that the combination of DEC and DEC-NPs with DTX significantly improved (p <0.05) the tumour response rate, significantly reduced (p <0.05) the tumour weight and volume when compared to single agents DTX or DEC. Treatment groups with DEC-NPs also displayed better response, as compared to DEC. The study proves that DEC and DEC-NPs enhance the anticancer potential of DTX.

Keywords: epigenetic therapy, breast cancer treatment, decitabine nanoparticles,

INTRODUCTION

Breast cancer is the commonest forms of cancer in women worldwide and a leading cause of cancer related deaths. In 2014, the United States reported more nearly 240,000 cases of breast cancer diagnosed at any stage. The first evidence associated with breast cancer is often a lump or area of thickened breast tissue. The lump may be cancerous or no-cancerous, invasive or non-invasive. Most breast lumps are not cancerous and hence non-invasive. In the invasive form, cancer cells growing inside the milk ducts or lobules break out into nearby breast tissue. These cancer cells can even travel to other parts of the body through blood or the lymphatic system, called as metastasis. Although overall survival rates of the non-invasive breast cancer patients have improved tremendously, the metastatic advanced breast cancer is still life-threatening. Advanced breast cancer patients can be broadly divided into four histologically distinct subpopulations, based on expression of the human epidermal growth factor receptor-2 (HER2) and hormone receptors (HRs): HER2+/HR-; HER2+/HR+; HER2-/HR+; HER2-/HR- (triple-negative). Patients with HER2 overexpression are candidates for HER2-targeted therapy. Because HER2 overexpression is related with aggressive disease, most HER2-positive patients will receive HER2-targeted therapy with chemotherapy for initial treatment of advanced/metastatic disease. Triple-negative disease is considered the most aggressive breast cancer subtype and is treated with chemotherapy agents. The lack of therapies approved specifically for triple-negative disease and its poor prognosis make it an area of high unmet need. The treatment of breast cancer includes local and systemic strategies. The local therapy includes surgery and radiation, which treats a tumour at the particular site without affecting the rest of the body. Whereas, systemic therapy refers to agents that kill the

*Author for Correspondence: n.udupa@manipal.edu
cancer cells throughout the body and includes chemotherapy, hormonal therapy, and targeted therapy. Chemotherapy extends the survival of patients but a major challenge in breast cancer is relapse and resistance to chemotherapy. Host defence system is developed by the cancer cells and they become resistant to one or more chemotherapeutic agents. The resistance can develop before or during the drug treatment. Moreover, prolonged exposure to chemotherapeutic agents may lead to multi drug resistance (MDR) or cross resistance. Docetaxel (DTX) is currently the most effective agent in the treatment of patients with advanced breast cancer. It 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 G2/M phase of the cell cycle. Docetaxel also induces cell death by apoptosis via stimulation of phosphorylation of bcl-2 protein. The underlying molecular mechanisms of docetaxel resistance are not fully understood. The nucleoside deoxycytidine analog 5-aza-2'-deoxycytidine (decitabine), the most widely investigated demethylating agent, is approved by the US FDA for use as a monotherapeutic agent against hematological cancers. The major disadvantage of DAC is its instability, both in vitro (half-life in aqueous solution is ~4 h, in cell culture medium ~17 h) and in vivo (half-life 10-35 min). The main mechanism of action of DAC is via depletion of DNMT1. Because of its transient effect, DAC has not been effective in sustaining DNMT1 depletion; hence its efficacy in treating solid tumours has been limited. This study investigated the potential involvement of DNA methylation machinery in the treatment of breast cancer, upon concurrent administration with docetaxel. The effect of decitabine nanoparticles was investigated to propose that nanoparticles can be used in combination with docetaxel to overcome the drug resistance. An attempt has been made in the systemic chemotherapeutic treatment of breast cancer by oral route.

MATERIALS AND METHODS

Materials

Triton-X 100, trypsin, phosphate buffer saline (PBS) 7.4, and common laboratory chemicals were purchased from Himedia lab Pvt. Ltd. (Mumbai, India). DMSO, isopropanol and glacial acetic acid were purchased from Qualigens fine chemicals (Mumbai, India). Decitabine, DMBA, Dulbecco’s modified eagle’s medium (DMEM), 3-(4, 5 dimethyl thiazole-2 yl) - 2, 5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), agarose, Fetal bovine serum (FBS) was procured from Gibco. Trizol LS, RNase, Proteinase K and 12 kbp DNA ladder were purchased from Invitrogen Life Technologies Co, (Carlsbad, CA, USA). HPLC grade solvents were procured from Merck (Darmstadt, Germany). All other chemicals used in the study were of analytical grade.

Cell cultures

Three human breast cancer cell lines MCF-7 and T47D (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative), were obtained from NCCLS, Pune. Cells were cultured and maintained in DMEM, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (100,000 U/1 penicillin, 100 mg/l streptomycin), at 37°C in a humidified atmosphere containing 5% carbon dioxide.

Animals

Female Sprague-Dawley rats aged between 50 and 55 days, 120-140g in weight, were procured from the Central Animal Research Facility, Manipal University, Manipal. The animals were approved by the Institutional Animal Ethical Committee (IAEC) under protocol no. IAEC/KMC/16/2013. Animals were housed in plastic cages at a maximum of three per cage. The animals were maintained under controlled environmental condition on alternative 12-h dark/light cycle. Commercial pelleted feed and water ad libitum were provided to animals. The rats were acclimatized on pulverized for five days before initiating the experimental protocol. The health status of the rats was determined and no major lesions were found. Animal care and handling was done as per the guidelines issued by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Govt. of India.

Preparation and characterization of decitabine nanoparticles (DEC-NPs)

DEC-NPs were prepared and characterised as described in our publication.

In vitro cell culture studies

(a) Cytotoxicity studies

Cytotoxic effects of DTX, DEC, DEC-NPs were assessed using MTT assay on MCF-7, MDA-MB-231 and T47D cells (Mosmann, 1983). Each well of 96 plates was seeded with 1 x 10^4 cells and incubated at 37 °C for 24 h. After the formation of monolayer, different dilutions of DTX, DEC, DEC-NPs, and blank NPs 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. 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. Graph was plotted for % growth inhibition against concentration. From this plot, IC_{50} (Concentration of drug required to kill 50% of cells in exponentially growing cultures) values were calculated.

(b) DNA fragmentation study

24 h before the treatment, 1x 10^5 cells were seeded in each well of 6 good plates with culture medium
containing 10% FBS. After 24 h, cells were treated with DTX, DEC, DTX+DEC and DTX+DEC-NPs for 24 h. After the treatment, cells were harvested with a cell scraper and centrifuged at 10,000 x g at 4°C for 15 min. The pellet was washed with PBS pH 7.4 and treated with 1 mL of digestion buffer (Tris, pH 7.5, 10 mM, NaCl 100 mM, EDTA 1 mM, SDS 1%) for overnight at 37°C. 40 µg/mL of Proteinase K was added and incubated for 2 h at 37°C. 50 µg/mL of RNase A was added and incubated for 2 h at 55°C. Finally, the DNA was extracted with phenol/chloroform/isoamyl alcohol (50:49:1), ppeted with 2.5 vol of ice-cold ethanol and 0.1 mL of 3M sod acetate, washed with 75% ethanol, dried and resuspended in 1mL TE. The DNA content in each sample was calculated by ultraviolet spectrophotometry at 260 nm. 40 µg of DNA was run on a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide and visualised in Alpha Imager under ultraviolet light.

(c) Cell cycle analysis

The cell phase distribution in MCF-7, MDA-MB-231 and T470 cells was analysed by PI staining. Initially, 1× 10⁶ cells/mL were plated into 6 good Corning Costar plates and incubated at 37°C in 5% CO₂ atmosphere for 24 h. After monolayer formation, cells were treated with low concentrations (below IC₅₀) of DTX, DEC, DTX+DEC and DEC-NPs prepared in 2% maintenance medium for 24 h. After the treatment, cells were harvested using trysin-EbDTA solution, centrifuged at 3000 rpm for 5 min. The pellet was re-dispersed in PBS and centrifuged again. The cells were then fixed with 70% ice cold ethanol and kept in -20°C. Ethanol was removed after centrifugation at 3000 rpm for 5 min at 4°C and cells were suspended in 500 µL of PI solution containing 25 µg/mL PI, 40 µg/mL RNase A and 0.03% Triton-X solution in PBS. 20 µL of RNase A (10 mg/mL in PBS) solution was added to cell suspension and incubated for 1 h at 37°C. PI (20 µg/mL in PBS) was added in cell suspension and incubated at 4°C for 30 min followed by washing with PBS twice before flow cytometry analysis. The cells were analysed 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.

In vivo DMBA model in Female Sprague-Dawley rats

Based on the in vitro data generated, the nanoparticles were tested in a cohort of female Sprague Dawley rats.

Acute toxicity studies and dose selection

The OECD 425 guidelines were followed for acute oral toxicity studies, to calculate dose of decitabine and decitabine nanoparticles. A limiting dose of 20 mg/kg body weight of DEC was administered. The rats were observed for any signs of toxicity, such as morphological, neurological or behavioral, for the first 4h continuously and thereafter daily for 14 days. The observed toxicities were normal with all treatments. The final dose was 2.5 mg/kg body weight given orally (p.o.). Similar toxicity studies were performed for docetaxel and the final dose was 7.5 mg/kg body weight intraperitoneal (i.p.).

Carcinogen treatment and tumour induction

The animals were divided in to seven groups of six animals each. Group I animals served as control (c/c), Group II-VII animals were treated with two doses of 7, 12-dimethylbenz (a) anthracene (DMBA) in olive oil to induce breast cancer, i.e., 40 mg/kg body weight and repeated dose of 20 mg/kg body weight of DMBA were administered in olive oil with two months’ interval. Animals were observed and palpated to check the tumour development every week for the next 90 days. Once palpable tumours were visible in animals, they were separated and divided based on their weight and tumour size. Dimensions of the tumour were calculated using Vernier caliper and tumour burden was calculated using the formula 0.5 x (length)x (width)². At the conclusion of the study, the animals were sacrificed, blood was collected and the serum was separated for biochemical analysis.

Experimental groups and treatments

Group I was maintained as sham control with no DMBA administration and group II was maintained as tumour control without any drug treatment. Group distribution and treatment module are shown in table 1. The total duration of the treatment was three weeks. Group III was administered with 7.5 mg/kg body weight, intra peritoneal (i.p.) docetaxel, While group IV was given 2.5 mg/kg body weight dose of docetaxel alone via oral route, the same dose of DEC-NPs was given to group V orally. The dose of decitabine in the combination was maintained, however, docetaxel dose was reduced to half in the groups VI and VII. After three weeks, blood was collected by retro-orbital plexus puncture for measuring hematological parameters and serum were separated and stored at ~80°C for estimation of biochemical parameters. Rats were sacrificed by euthanasia and tumours were excised. Location, weight, and dimensions of excised mammary tumours were recorded. Tumours were processed for histopathological examination.

General observations

The total gain in body weight of the rats was recorded weekly, throughout the study. At the conclusion of the experiment, the rats were sacrificed by cervical dislocation and weight of different organs was recorded to observe any abnormalities, if any. Tumour weight and volume

Tumour weight was recorded by weighing the excised tumours on a weighing balance. The resulting solid tumour was deliberated to be prelate ellipsoid with 1 long axis and 2 short axes. The two short axes were measured with vernier caliper and the tumour volume was calculated by the formula: Tumour volume= (length x width)² x 2/3.

The length and width were measured in centimeters.

Hematological parameters

Samples of blood for hematological analyses were withdrawn under light ether anesthesia. The blood was collected into vacutainers (Becton Dickinson, BD Vacutainer) coated with diopotasium-EDTA, by retro-orbital plexus puncture for measuring hematological parameters. The sample were instantly analysed for
RESULTS AND DISCUSSION

Decitabine loaded PLGA nanoparticles (DEC-NPs)
The mean particle size and polydispersity index of decitabine nanoparticles were found to be 124.3 ± 4.2 nm and 0.102 ± 0.021 respectively, with entrapment efficiency more than 40%. The optimized formulations were characterized by DSC analysis, FTIR, and SEM.

In vitro cell culture studies
(a) MTT assay
The MTT assay was performed in triplicate for all three breast cancer cell cultures and average of the three values was taken. The combination of DTX and DEC was able to reduce the IC50 of DTX. This clearly led to the hypothesis that the use of DEC in the treatment of breast cancer can reduce the dose of DTX thereby reducing its side effects as well as multi drug resistance. The values are given in table 2.

(b) DNA fragmentation studies
DNA fragmentation in the treated cells is an indicator of apoptosis. There was clear pattern of laddering observed when the cell cultures were treated with the combination of DTX+DEC and DTX+DEC-NPs. This indicated that there was a synergy in the effect produced by the two drugs. The drug DTX is an apoptosis inducing drug which was visible in the results, and there was no interference by DEC. Lane I- DNA ladder (100-1000 bp); Lane II- DTX; Lane III- DEC; Lane IV- DTX+DEC; Lane V- DTX+DEC-NPs; Lane VI- Untreated cells (intact DNA); Lane VII- Cisplatin (positive control) The lanes IV and V clearly indicate the laddering pattern of the combination DTX+DEC and DTX+DEC-NPs. The untreated DNA was found to be intact, as on the lane VI. The lane VII shows the DNA damage caused due to cisplatin, used as a positive indicator of DNA fragmentation. Lane III treated with DEC alone, shows unresolved DNA on the gel.

c) Cell cycle analysis of MCF-7 cells
The cell cycle analysis was performed to study the synergy in the two drugs. The experiments were done with individual drugs as well as combination. The results showed the cells treated with DEC were blocked in the S-phase while those treated with DTX were blocked in G2/M-phase. As we observed the results of DTX and DEC combination, it was clear that both the drugs acted in synergy, i.e., the cells were blocked in the S-phase as well as G2/M-phase of the cell cycle, figure 6.5. This confirmed the hypothesis in vitro, that the apoptotic and epigenetic mechanisms of drug action can co-exist and shall be beneficial in the treatment of carcinomas. DEC is a S-phase specific inhibitor while DTX effects the cells in the G2/M-phase of the cell cycle. The combination of DEC and DTX affects cells in S-phase as well as G2/M-phase. This signifies their individual therapeutic potential at cellular level.

In vivo studies in female Sprague-Dawley rats
DMBA is a polycyclic aromatic hydrocarbon, and a powerful carcinogenic and mutagenic agent. It is a major provider of stress-induced cancer by oxidation that leads to production of highly toxic free radicals. (Costa et al., 2002) DMBA induces mammary tumour in rats in a similar way as breast cancer in humans14. Since DMBA is lipid-soluble, it accumulates in the mammary fat pads causing high threat of breast cancer15. The DMBA model parameters that were studied are as follows: tumour weight, tumour volume, spleen weight, kidney weight, liver weight, heart weight, body weight, haematological parameters, biochemical and anti-oxidant parameters in breast tissue.

Effect on organ and body weight
After sacrificing the rats, the weight of liver, spleen, heart and kidney was measured and analyzed (table 3). The mean spleen weight was significantly higher in the DMBA control rats compared to the normal control rats. The results showed that there was a significant decrease (p < 0.05) in the spleen weight of the animals treated with DTX and DEC combinations (groups VI and VII), compared to DMBA control groups. The weights of liver, heart and kidneys were found to be consistent in all the

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Sham control</td>
</tr>
<tr>
<td>II</td>
<td>Tumour control</td>
</tr>
<tr>
<td>III</td>
<td>Docetaxel (7.5 mg/kg, i.p.)</td>
</tr>
<tr>
<td>IV</td>
<td>Decitabine (2.5 mg/kg, p.o.)</td>
</tr>
<tr>
<td>V</td>
<td>DEC-NPs (2.5 mg/kg, p.o.)</td>
</tr>
<tr>
<td>VI</td>
<td>Docetaxel (3.725 mg/kg, i.p.) + Decitabine (2.5 mg/kg, p.o.)</td>
</tr>
<tr>
<td>VII</td>
<td>Docetaxel (3.725 mg/kg, i.p.) + DEC-NPs (2.5 mg/kg, p.o.)</td>
</tr>
</tbody>
</table>

Table 1: Animal groups for DMBA model
groups with no significant change. The body weight of the DMBA treated tumour induced animals was higher than the normal control animals. The increase in weight could be attributed to the weight of tumours formed in the animals. The results on the last day of the study showed that the mean body weight of animals was significantly decreased (p < 0.05) in the groups III, VI and VII, which showed apparent decrease in tumour mass in these groups.

Tumour growth and weight

The treatment effect on the growth of tumours was observed every week by measuring tumour volumes. After sacrificing the rats by cervical dislocation, the mean tumour weight was also determined in all the groups. The combination treatments in groups VI and VII significantly decreased (p < 0.05) the mean tumour volume as well as mean tumour weight in the animals compared to the DMBA control animals. It was also observed that the combination treatment was far better in decreasing the tumours when compared to DTX alone. The treatment with DEC or DEC-NPs alone did not show any significant decrease in tumour weight and volume compared to the DMBA control animals.

Effect on hematological parameters

The WBC count was higher in the DMBA control group compared to the normal c/c. There was no significant change in the RBC count in all the groups. The Hb count was slightly lower in the DMBA control and treatment groups compared to the normal control (Figure 6).

Effect on lipid peroxidation and catalase levels in breast tissue

The lipid peroxidation (LP) levels can point out the levels of oxidative stress/damage in the breast tissues. Malondialdehyde (MDA), an end product of LP was studied and evaluated in the breast tumours. The levels of MDA were comparably similar in the groups treated with combination dose. However, there was a significant increase in DMBA control group II and DEC alone treated group IV and V. The catalase activity was significantly decreased (p < 0.05) in the DMBA and DEC alone groups as compared to the normal control group. There were no significant changes in the catalase levels in the breast tissues of all other groups.

Effect on biochemical parameters

There was a tremendous increase in the AST, ALT and ALP levels in DMBA groups which might be due to the toxicity of DMBA on liver. The levels were significantly (p < 0.05) restored in all the treatment groups (Figure 7). The urea level was found too higher in the DMBA control group while there was no significant change observed in the creatinine levels throughout the groups.

Histopathological studies

The breast tissue sections of the DMBA c/c gp depicted infiltration in the lobules with comedo, cribriform and papillary cell arrangement, indicating ductal carcinoma. The breast tissues treated with DTX+DEC and DTX+DEC-NPs showed less necrosis and infiltration.

Table 2: IC50 Value on different breast cancer cell lines in µg mL−1

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Cell culture</th>
<th>Source</th>
<th>DTX</th>
<th>DTX+DEC</th>
<th>DTX+DEC-NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MCF-7</td>
<td>Human breast epithelial adenocarcinoma</td>
<td>0.0492</td>
<td>0.032+0.24</td>
<td>0.032+0.15</td>
</tr>
<tr>
<td>2</td>
<td>MDA-MB-231</td>
<td>Human Breast Adenocarcinoma</td>
<td>0.0587</td>
<td>0.039+0.39</td>
<td>0.039+0.22</td>
</tr>
<tr>
<td>3</td>
<td>T47D</td>
<td>Human Breast ductal carcinoma</td>
<td>0.0234</td>
<td>0.016+0.31</td>
<td>0.016+0.21</td>
</tr>
</tbody>
</table>

DEC, decitabine; DEC-NPs, decitabine loaded PLGA nanoparticles; DTX, docetaxel.

Table 3: Effect of drug treatment on organ weight and body weight in Sprague Dawley rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Liver</th>
<th>Kidneys</th>
<th>Spleen</th>
<th>Heart</th>
<th>% Increase in body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>I- Sham Control</td>
<td>5.42 ± 0.23</td>
<td>0.74 ± 0.02</td>
<td>0.61 ± 0.01</td>
<td>0.82 ± 0.02</td>
<td>93.9 ± 4.3</td>
</tr>
<tr>
<td>II- Tumour Control</td>
<td>5.56 ± 0.24</td>
<td>0.81 ± 0.04</td>
<td>0.76 ± 0.02</td>
<td>0.88 ± 0.04</td>
<td>147.6 ± 6.8</td>
</tr>
<tr>
<td>III- DTX (i.p.)</td>
<td>5.32 ± 0.29</td>
<td>0.77 ± 0.02</td>
<td>0.69 ± 0.02</td>
<td>0.79 ± 0.03</td>
<td>116.7 ± 4.6*</td>
</tr>
<tr>
<td>IV- DEC (p.o.)</td>
<td>5.89 ± 0.16</td>
<td>0.68 ± 0.03</td>
<td>0.79 ± 0.03</td>
<td>0.73 ± 0.05</td>
<td>126.4 ± 6.2</td>
</tr>
<tr>
<td>V- DEC-NPs (p.o.)</td>
<td>5.52 ± 0.27</td>
<td>0.71 ± 0.03</td>
<td>0.72 ± 0.03</td>
<td>0.76 ± 0.02</td>
<td>124.9 ± 5.3</td>
</tr>
<tr>
<td>VI- DTX+DEC</td>
<td>5.35 ± 0.19</td>
<td>0.74 ± 0.03</td>
<td>0.64 ± 0.03b</td>
<td>0.84 ± 0.03</td>
<td>106.2 ± 5.9b</td>
</tr>
<tr>
<td>VII- DTX+DEC-NPs</td>
<td>5.93 ± 0.21</td>
<td>0.76 ± 0.02</td>
<td>0.66 ± 0.02b</td>
<td>0.86 ± 0.04</td>
<td>102.6 ± 4.7b</td>
</tr>
</tbody>
</table>

Values represented as mean ± SEM; n=6. *p < 0.05 vs. normal control; b p < 0.05 vs. DMBA control.

Figure 1: DNA fragmentation study on MCF-7 cells.
Figure 2: Cell cycle analysis of MCF-7 cells.

Figure 3: (a) Tumour size was measured by vernier calipers. (b) Tumour weight was recorded after excision. (c) Tumour volume was calculated by measuring length and width of the tumour.

Figure 4: Effect of treatment on mean tumour weight. The dissected tumours were cleaned, blotted dry and weighed. The groups III, VI and VII showed significant decrease in the mean tumour weight compared to the DMBA control group II.
Figure 5: Tumour volume was calculated by measuring length and width of the tumour. The groups III, VI and VII showed significant decrease in the mean tumour volume compared to the DMBA control group II.

Figure 6: Haematological parameters. The WBC count was found to be higher in tumour control animals. There was a significant decrease ($p < 0.05$) in the WBC count of rats in group V and VII compared to the DMBA control group, which could be attributed to the activity of decitabine.

Figure 7: Biochemical investigations showed marked increase in the AST, ALT and ALP of the tumour induced animals. The levels were significantly reduced ($p < 0.05$) in all treatment groups.
CONCLUSION
In the present study, we investigated the effectiveness of decitabine and its nanoparticles in combination with docetaxel for the treatment of breast cancer. The combination was studied extensively on cell cultures and pre-clinical investigation was performed on female Sprague Dawley rats. In conclusion, the in vitro and in vivo studies showed that the combination docetaxel and decitabine appears to enhance the chemotherapeutic effect of both drugs in the reduction of mammary tumours. The combinations were able to lower the intensity of the DMBA induced mammary tumours in female Sprague Dawley rats. The biochemical, antioxidant and histopathological findings also suggested that the combinations were safer to the animals. The study proves that DEC and DEC-NPs enhanced the anticancer potential of DTX. The incorporation of decitabine as an adjuvant treatment for early breast cancer offers potential for further improvement of taxane based treatment.

ACKNOWLEDGMENT
We thank Manipal College of Pharmaceutical Sciences, Manipal University for providing necessary facilities to carry out the work and department of science and technology, government of India for INSPIRE fellowship to Mr Prateek Jain.

Conflict of interest
The authors declare no conflict of interest.

REFERENCES
12. Arivazhagan L, Pillai SS. Tangeretin, a citrus pentamethoxyflavone, exerts cytotoxic effect via p53/p21 up-regulation and suppresses metastasis in 7,12-dimethylbenz (a) anthracene-induced rat mammary
carcinoma. The Journal of nutritional biochemistry. 2014, 25(11), 1140-1153