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Research Paper

PLGA Nanoparticles Stabilized with Cationic Surfactant: Safety Studies and Application in Oral Delivery of Paclitaxel to Treat Chemical-Induced Breast Cancer in Rat

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Purpose. This study was carried out to formulate poly(lactide-co-glycolide) (PLGA) nanoparticles using a quaternary ammonium salt didodecyl dimethylammonium bromide (DMAB) and checking their utility to deliver paclitaxel by oral route.
Methods. Particles were prepared by emulsion solvent diffusion evaporation method. DMAB and particles stabilized with it were evaluated by MTT and LDH cytotoxicity assays. Paclitaxel was encapsulated in these nanoparticles and evaluated in a chemical carcinogenesis model in Sprague Dawley rats.
Results. MTT and LDH assays showed the surfactant to be safe to *in vitro* cell cultures at concentrations <33 μM. PLGA nanoparticles prepared using this stabilizer were also found to be non-toxic to cell lines for the duration of the study. When administered orally to rats bearing chemically induced breast cancer, nanoparticles were equally effective/better than intravenous paclitaxel in cremophor EL at 50% lower dose.
Conclusions. This study proves the safety and utility of DMAB in stabilizing preformed polymers like PLGA resulting in nanoparticles. This preliminary data provides a proof of concept of enabling oral chemotherapy by efficacy enhancement for paclitaxel.

KEY WORDS: breast cancer; EPR effect; nanoparticles; oral drug delivery; paclitaxel.

INTRODUCTION

The process of bringing new molecules to the market is becoming more expensive, time-consuming and increasingly challenging, and we are witnessing a parallel climbing interest in novel drug delivery systems (1). Pharmaceutical companies and researchers worldwide are exploring new strategies to increase the efficacy of existing drugs, reduce adverse effects, and achieve site specificity. Innovation is achieved by design (osmotic pumps, matrix or depots, gels), use of excipients (formulation design), and derivatization. However, the novelty that introduces functionality is also seen as potency that could go either way, and, hence, the regulators demand that all innovations be proved safe prior to use. Achieving drug delivery by the aid of nanotechnology is not new (2,3), but the last few years have seen an overwhelming research thrust in this area (4–7). Small particles have been shown to be taken up through and across the biomembranes by unique mechanisms, which can address one or more of the bioavailability problems (8,9). The particles are believed to be delivered to the circulatory system through the lymphatics, with the absorption more pronounced and rapid for smaller particles. Because of the facilitation by particle uptake mechanisms (9,10), the nano-sized drug delivery systems can cross the biological barriers.

The performance of nanotechnology-based drug delivery systems is influenced mostly by size (11,12) and surface properties (charge and hydrophilicity). The stabilizer used in the preparation of nanoparticles plays a significant role not only in the final product characteristics, but also in the uptake, biodistribution, fate, release profile of incorporated drug (13), and, hence, the therapeutic efficacy *in vivo*. Development of new pharmaceutical products often involves new material with no prior safety data. Such substances need to be screened for possible toxicity. In this study, we have used a quaternary ammonium salt didodecyldimethylammonium bromide (DMAB). Our interest in this surfactant/stabilizer is because of the small size particles that it produces (14,15). Recently, Peetla and Labhasetwar reported that DMAB enables nanoparticles to interact with a model cellular membrane, and this interaction was proportional to their cellular uptake *in vitro* (16).

Paclitaxel is one of the most promising drugs for treatment of solid tumours. The approved indications of paclitaxel are metastatic ovarian and breast carcinoma and non-small-cell lung cancer. However, paclitaxel has very low water solubility and poor intestinal permeability, which makes it a

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Class IV drug in the Biopharmaceutic Classification System (BCS). It has a high molecular weight (853.9) and is extensively effluxed out by the P-glycoprotein (Pgp) pump (17). As a result, the drug shows poor bioavailability when administered orally. Therefore, it is typically administered intravenously in a mixture of Cremophor EL™ and alcohol, but this vehicle can cause anaphylactoid reactions (18). Additionally, paclitaxel does not act specifically on cancer cells, and, thus, there is a strong need to decrease the body burden of the drug to reduce side-effects.

The gastrointestinal tract is the nature's intended mode of uptake of foreign matter (food) and is anatomically and physiologically adapted for it. Considering the advantages of oral administration and the incidence of adverse effects associated with parenteral delivery, the healthcare fraternity is continuously devising strategies to make these drugs more orally bioavailable. Nanoparticulate drug delivery systems are an interesting and promising prospect in this field. However, not much is known about the biodistribution and fate of these nanoparticles after absorption. Studies have shown particles releasing incorporated drug for over 11 days after oral administration (19). Even in absence of conclusive pharmacokinetic profiling of nanoparticulate formulations, therapeutic efficacy has been recorded (20). Besides, due to the enhanced permeation and retention (EPR) effect, pharmacokinetic studies may not offer significant insights into cancer therapeutics. Therefore, a pilot study was designed to establish the therapeutic efficacy of nanoparticles incorporating paclitaxel in a chemical induced mammary carcinogenesis model in rats.

MATERIAL AND METHODS

Material

PLGA 50:50 block copolymer (RH 503, Molecular weight 35–40 kDa) was procured from Boehringer Ingelheim KG (Ingelheim, Germany). Paclitaxel (Genexol®) was obtained from Samyang Genex Co. (Seoul, South Korea). Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids (NEAA) and fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen Corp., Carlsbad, CA, USA). The "cytotoxicity detection kit (LDH)" was from Roche Diagnostics Corp. (Indianapolis, IN, USA). DMAB was purchased from Fluka Buchs SG, Switzerland. 7,12-Dimethylbenz-anthracene was purchased from Sigma (St. Louis, MO, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as obtained. Radioactive paclitaxel (*[2-benzoyl ring-^{14}C(U)]*-specific activity 65 mCi/mol) was obtained from Moravek biochemicals (Brea, CA, USA). Soluene-350 was purchased from Perkin Elmer (Amsterdam, Netherlands).

Preparation of DMAB Stabilized Blank Nanoparticles

Nanoparticles were prepared by solvent emulsification-diffusion-evaporation method (21). Briefly, 50 mg of PLGA 50:50 block copolymer (35–40 kDa) solution in 2.5 ml ethyl acetate was poured in 5 ml of aqueous phase containing the stabilizer and stirred to get a primary emulsion. This primary emulsion was then subjected to high shear using a rotor shaft-casing type tissue homogenizer (T25 Basic, Ultra Turrax). The size-reduced emulsion was then diluted with water and stirred for 4 h to remove the organic solvent. The effect of variables like surfactant concentration, organic-to-aqueous phase ratio, and speed of the high shear homogenizer on the particle characteristics were studied. The prepared formulation was characterized for size, zeta potential, and polydispersity index (PDI) using Zetasizer (Nano-ZS, Malvern, UK).

Cell Culture

Caco-2 cells, clone C2BBe1, were purchased at passage 60 from American Type Culture Collection (ATCC, Manassas, VA) and used up to passage 90. MDCK II -mdr1 cells, which were a kind gift by Piet Borst (Netherlands Cancer Institute, Division of Molecular Biology, Amsterdam), were used from passage 11 to 32. Cells were grown to ~90% confluence in 75 cm^2 T-flasks with DMEM supplemented with 10% foetal bovine serum (FBS). Caco-2 cell culture was supplemented with 1% non-essential amino acids (NEAA) and MDCK cells were cultured in presence of 100–200 µg/ml of geneticin (G418). Culture medium was changed on alternate days, and cells were cultured at a temperature of ~37°C in an atmosphere of ~85% relative humidity and ~5% CO_2.

LDH Assay Using Different Concentrations of DMAB and the Respective Nanoparticles

Caco-2 cells were grown on 96-well tissue culture plates with a flat bottom (Greiner Bio-One GmbH, Frickenhausen, Germany) for 8 days. In fresh Hank's balanced salt solution (HBSS) (~37°C, pH 7.4), monolayers were incubated (4 h) with the analyte. The particles used for the study were washed twice with water to remove the unbound surfactant and were redispersed in buffer solution. After the incubation, lactate dehydrogenase (LDH) release into the supernatant was determined using the cytotoxicity LDH kit as described by Roche Diagnostics, estimating a formazan dye, which is colorimetrically detected at 490 nm. Fresh HBSS pH 7.4 and Triton X-100 (1% *w*/*v*) in HBSS pH 7.4 were used as negative and positive controls, respectively. LDH release has been expressed relative to control values. Experiments were performed with $n=4$ for each sample.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

The particles used for the study were washed twice with water to remove the unbound surfactant and were redispersed in buffer solution. After incubating the cells with the surfactant or particles for 6 h, 15 µl of MTT solution (5 mg/ml) was added to each well, followed by incubation at 37°C for 6 h. The cell culture medium including complex solution was carefully removed, and 150 µl of MTT solvent was added to dissolve the formed formazan crystals. After shaking the plate for 30 min, absorbance was measured at 570 nm using a microplate reader. Background absorbance at 690 nm was subtracted. Cells incubated with buffer were used as a control.

The cell viability was expressed as a percentage using the following equation:

$$\text{Cell viability}(\%) = \frac{OD^{sample}_{(570-690)}}{OD^{control}_{(570-690)}} \times 100\%$$

Preparation of Paclitaxel-Loaded Nanoparticles

Drug-loaded nanoparticles were prepared incorporating 5% (*w/w* of PLGA) paclitaxel. In brief, 2.5 mg paclitaxel was dissolved in 5 ml ethyl acetate, and 50 mg PLGA was added to this solution and stirred for 2 h. This solution was poured with stirring in 5 ml of aqueous 1.0% *w/v* DMAB solution. The primary emulsion so obtained was homogenized for 5 min by a shaft-type tissue homogenizer at 15,000 rpm (Polytron 4000, Kinematica, Switzerland). Finally, this emulsion was diluted six times and stirred at 800 rpm with a magnetic bar for 4–6 h to remove the ethyl acetate. Nanoparticle suspension obtained was washed twice by centrifugation to remove the unbound drug and surfactant.

Analysis of Paclitaxel

Analysis was carried out by high performance liquid chromatography (HPLC, Waters, USA) using UV detection at 227 nm on a 250×4.6 mm reverse phase C18 (Symmetry or Lichrocart) column. A mobile phase consisting of methanol, acetonitrile and 5 mM phosphate buffer (pH 2.5) in the ratio 70.0: 2.5: 27.5 eluted paclitaxel from a 20 µl injection in 7–8 min when pumped at 0.7 ml/min.

Characterization of Paclitaxel-Loaded Nanoparticles

Drug entrapment was calculated by dissolving an aliquot of the suspension of washed particles in acetonitrile and analyzing the paclitaxel by validated HPLC method. A drop of the nanoparticle suspension was placed on a silicon wafer and air dried, and the particles were imaged using an atomic force microscope (AFM) (Bioscope, Veeco Instruments, USA) to check the surface texture and shape and to cross-verify the size determined by zetasizer. Measurements were done with a scanning probe of force constant k ~40 N/m in Tapping Mode™.

Mammary Tumour Induction

The developed nanoparticulate formulations were evaluated in an animal model. 7,12-dimethylbenz-anthracene is an experimental carcinogen routinely used for induction of breast cancer in animals and is implicated in DNA-adduct formation (22). This model was originally reported long back in the 1950s (23) and modified later on (24). At 47 to 50 days of age, female Sprague Dawley (SD) rats were administered 7,12-dimethylbenz-anthracene dissolved in sunflower oil at a dose of 100 mg/kg body weight by oral gavage. The animals were regularly examined and breasts palpitated to examine the incidence and progression of cancerous lumps.

Tissue Distribution Study in Rats Bearing Mammary Tumours

Experiments on SD rats for the tissue distribution studies were carried out under a Project Licence issued under the U.K. Home Office Animals (Scientific Procedures) Act 1986. Normal paclitaxel was spiked with C-14 labelled paclitaxel for a dose of 7.5 mg/kg body weight (containing 0.24 µCi/mg of paclitaxel) for i.v. formulation and 3.75 mg/kg body weight (containing 0.67 µCi/mg of total paclitaxel) in nanoparticles administered orally. For i.v. formulation, 6 mg paclitaxel was dissolved in each ml of a mixture of 50:50 *v/v* cremophor EL and ethanol. The dose was administered to female SD rats ($n=3$) 13 weeks after administration of carcinogen. Animals were sacrificed 24 h after dosing, and their tissues were collected and frozen at −20°C until analysis. The estimation of radioactivity from C-14 labelled paclitaxel was carried out by liquid scintillation counting (Tri-Carb 1500, Packard, USA). Tissues were homogenized in phosphate buffered saline at 20,000 rpm for 1–2 min (Polytron 4000, Kinematica, Switzerland), mixed with two volumes of Soluene-350 tissue solubilizer and incubated for 4 h at 50°C. Hydrogen peroxide (50–200 µl) was added with constant vortexing to bleach coloured samples, 3.5 ml of scintillation cocktail (Ultima Gold, Perkin Elmer, Amsterdam, Netherlands) was added and samples read for 10 min. Radioactive counts were normalized on a C-14 quench curve, and background values were subtracted. Paclitaxel concentration was calculated from the known dilution of the labelled drug.

Treatment of Mammary Tumours

The animal studies were conducted after approval of the institutional animal ethics committee of NIPER, India.

The treatment was started on four groups of five animals each after the 22nd week of administration of carcinogen as follows: Group1 received no treatment; Group 2 received paclitaxel (7.5 mg/kg body weight) in cremophor EL (oral gavage); Group 3 received paclitaxel (7.5 mg/kg body weight) in cremophor EL (i.v.); Group 4 received drug-loaded nanoparticles (equivalent to 3.75 mg of paclitaxel/kg body weight) in the form of nanoparticles (oral gavage), frequency of administration being once in three weeks for all the three treatment groups (three doses). The dose was calculated based on conversion-to-weight basis from the human dose of 175 mg/m^2 given to breast cancer patients. The nanoparticles used in group 4 were suitably concentrated to contain the equivalent dose. Two weeks after the administration of the third dose (30th week from administration of the carcinogen), animals were sacrificed, the tumours were removed, and their weight was measured.

Statistics

The tumour masses were compared by one-way analysis of variance (ANOVA) followed by Tukey test; P value less than 0.05 was considered to denote a statistically significant difference.

RESULTS

Preparation of DMAB Stabilized Blank Nanoparticles

As seen in Table I, by increasing the surfactant concentration, a decrease in both particle size as well as PDI was observed. An increase in the speed (rpm) of the shaft tissue homogenizer used to reduce the globule size of the emulsions

Table I. Effect of Surfactant (DMAB) Concentration on Blank Particle Characteristics. Homogenization Speed 24,000 rpm (Maximum for the Instrument), Organic to Aqueous Phase Ratio 1:2

DMAB concentration (%*w*/*v* in aq. phase)	Size (nm) ± s.d.	Polydispersity index ± s.d.
0.1	163.2±3.6	0.15±0.01
0.33	122.7±3.1	0.13±0.01
1.0	87.1±5.5	0.09±0.01

Table III. Effect of Organic to Aqueous Phase Ratio on Blank Nanoparticle Characteristics. Homogenization Speed 24,000 rpm, Surfactant Concentration 1% *w*/*v*

Organic to aqueous phase ratio	Size (nm) ± s.d.	Polydispersity index ± s.d.
10:10	95.1±4.1	0.11±0.01
5:10	87.1±5.5	0.09±0.01
3:10	72.0±1.5	0.12±0.01

resulted in decrease of particle size (Table II). Table III shows that a higher ratio of aqueous-to-organic phase yields smaller particles. The particles so prepared had a zeta potential of +75 to +85 mV, which decreases by washing with water to +35 to +45 mV. Washing also slightly increased the particle size prepared with 0.1, 0.33 and 1.0% *w*/*v* DMAB to 180, 135 and 100 nm respectively.

Cytotoxicity Assays with Surfactant Solutions and Blank Nanoparticles

Cytotoxicity of blank PLGA nanoparticles (containing no drug) made with DMAB was studied by using LDH and MTT assays. Both MTT (Fig. 1) as well as LDH (Fig. 2) assays performed with aqueous solutions of DMAB showed concentration-dependent toxicity. The surfactant was found to be non-toxic after 4–6 h incubation for concentrations <33 μM. Plain medium and medium with 1% *w*/*v* Triton X were used as reference for 100 and 0% cell survival, respectively.

Nanoparticles prepared with 1.0, 0.33 and 0.1% *w*/*v* DMAB (of sizes 100, 135 and 180 nm respectively after washing step) were also found to be non-toxic to the cell lines in these two assays (Figs. 3 and 4).

Analysis of Paclitaxel

The HPLC method provided a linear analysis profile in the concentration range of 0.1–10 μg/ml. The regression correlation by least square method was found to be 0.9993.

Preparation of Paclitaxel-loaded Nanoparticles

Particles prepared with 1% DMAB with an initial load of 5% *w*/*w* of paclitaxel relative to the polymer weight had an average particle size of about 121 (± 6) nm (Fig. 5), with PDI of 0.09 (± 0.02), and a zeta potential of +75 mV (at pH 4.5). Paclitaxel concentrations higher than 5% resulted in precipitation, which might be due to limited drug holding capacity of the polymer (PLGA) used in this study. The encapsulation efficiency was approximately 47 (±6)%. The sample preparation method was found to avoid any interference in analysis from the surfactant(s) and/or polymer. The particles had a smooth spherical architecture (Fig. 6).

Table II. Effect of Shear (Speed of Tissue Homogenizer) on Blank Particle Characteristics. Surfactant Concentration 1% *w*/*v*, Organic to Aqueous Phase Ratio 1:2

Speed (rpm)	Size (nm) ± s.d.	Polydispersity index ± s.d.
15,000	154.5±6.1	0.06±0.02
20,000	115.0±2.3	0.07±0.01
22,000	105.1±1.9	0.08±0.01
24,000	87.1±5.5	0.09±0.01

Tissue Distribution Study in Rats Bearing Mammary Tumours

Within 24 h of oral administration of nanoparticles, paclitaxel was detected in tissues (Fig. 7). The levels achieved in liver, tumour and kidney were almost 10% of the those seen with the i.v. formulation; however, the oral dose used was half of the i.v. dose. The proportion of drug in lungs and spleen after oral administration was comparatively higher than that attained after i.v. injection.

In Vivo Tumour Treatment Study

After 22 weeks, the single oral dose of 7,12-dimethylbenzanthracene resulted in tumour induction in 100% of the rats taken up for the study. At the end of treatment schedule, tumour volumes and mass were recorded. As seen in Fig. 8, paclitaxel nanoparticles administered orally were equally effective as paclitaxel given intravenously with cremophor EL although at a 50% reduced dose and significantly better than the oral paclitaxel in cremophor EL group.

DISCUSSION

PLGA is a biodegradable and biocompatible polymer, and products based on this polymer are already approved by

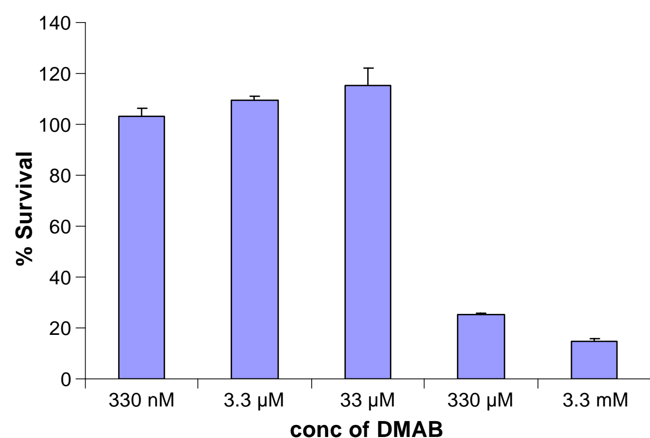

Fig. 1. MTT assay for different concentrations of DMAB in MDCK II mdr1 cell line.

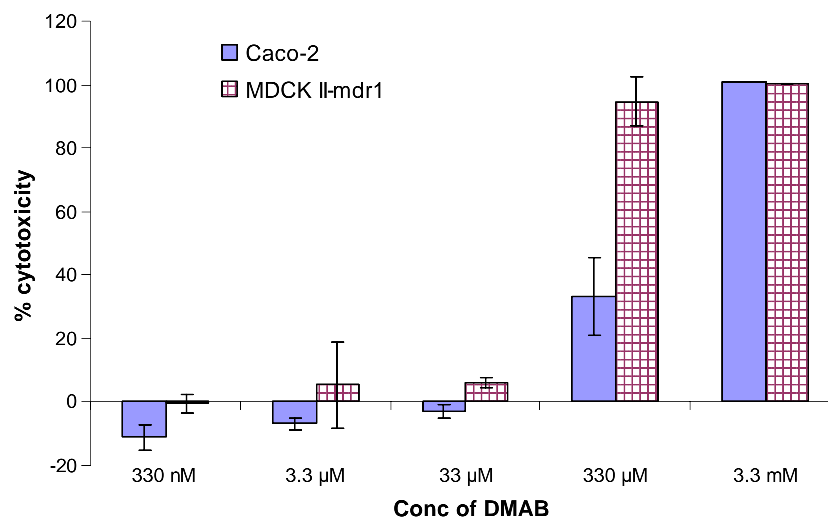

Fig. 2. Lactate dehydrogenase assay for different concentrations of DMAB.

the United States Food & Drug Administrations (US FDA) for human use. In 1999, the US FDA approved a PLGA microsphere formulation, Nutropin Depot, as a once-a-month alternative to daily injections of human growth hormone. To increase the physical stability of nanoparticles, surfactants or stabilizers are used. Reports on the positive surface charge of DMAB (25) provided the incentive to aid the delivery of paclitaxel, since it is expected to ensure better interaction with the negatively charged cell membrane. This can result in increased retention time at the cell surface, thus increasing the chances of particle uptake. DMAB is capable of producing small and highly stable nanoparticles at 1% *w*/*v* concentration (15). Due to the charged surface, the particle agglomeration is impeded.

The particle preparation process was studied using variables like surfactant concentration, phase ratio, and homogenizer speed to understand the influence of these parameters on defining the particle characteristics. The smallest particle size with 1% surfactant might be due to better stabilization of the nanoglobules by a more comprehensive presence of the stabilizer at the interface of the two phases. Increase in specific surface area increases the surface free energy, and the decreasing particle size with increasing speed of the homogenizer reflects the transfer of higher amount of energy to the colloidal system. Similarly, a direct correlation was seen between particle size and ratio of organic-to-aqueous phase with 3:10 ratio producing particles around 70 nm (Table III). This can be due to the surfactant effectively lining the interface between the globules and the aqueous phase when the internal phase volume is lower. In none of the parameters, we have seen a saturation effect implying that it is possible to increase or decrease the particle size beyond the obtained values, but the effect might not hold linear and would plateau outside a range. In our experience, the foremost criteria to be set is the desired particle size, and the particle preparation exercise should be carried out to then satisfy other product characteristics like drug-loading and residual surfactant concentration. Additional factors like the practical limits of available instruments/techniques sometimes limit the extent to which the desirables can be achieved. For example, if centrifugation is the only method available for washing the

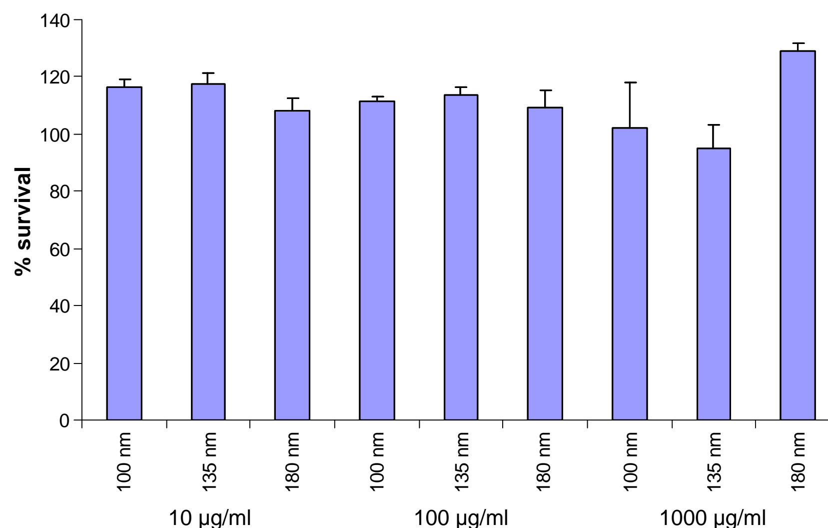

Fig. 3. MTT assay with different concentrations of nanoparticles of three different sizes stabilized with DMAB in MDCK II mdr1 cell line.

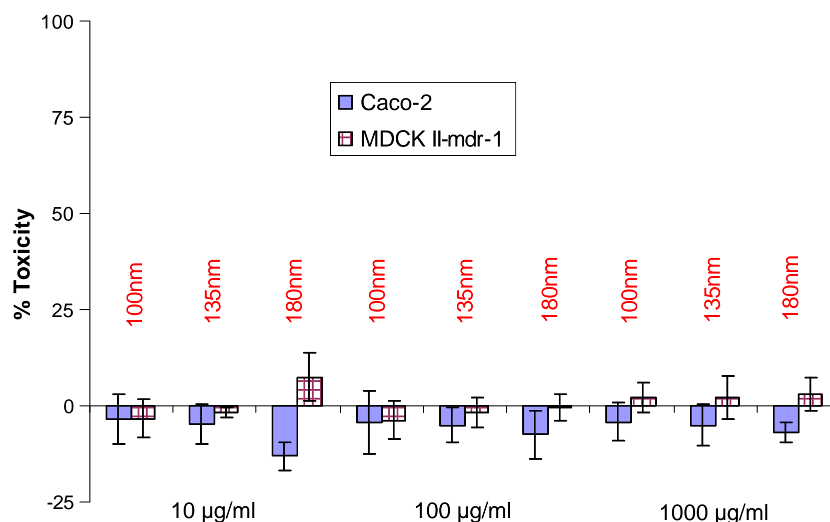

Fig. 4. LDH assay with different concentrations of nanoparticles of three different sizes.

particles, then the speeds of centrifuge create a bottleneck for the lowest particle size that can be recovered as sediment.

Due to the ethical and cost issues, *in vitro* cell-based assays are preferred over animal studies for toxicity screening. Both the cytotoxicity tests used in this study rely on the formation of a formazan dye (albeit using different mechanisms) that can be directly read on a plate reader with UV-Visible detector. In MTT assay, the yellow tetrazolium salt MTT is converted by mitochondrial dehydrogenases of metabolically active cells to an insoluble purple formazan product, which is then solubilized and read on a plate reader. LDH is a cytosolic enzyme that is readily released upon cell membrane damage; hence, LDH may be used as a tool to monitor cell membrane integrity. LDH released into the assay medium can be measured via a coupled enzymatic assay: conversion of the yellow tetrazolium salt (2-(4-iodophenyl)- 3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride) into a red formazan salt. Both LDH and MTT assays gave no indication that the DMAB solutions at and below the concentrations of 33 µM are toxic to cells. This concentration is lower than the maximum amount of bound surfactant measured (less than 20 µM) on the nanoparticles with even the highest concentration of surfactant (1% *w*/*v*) used. Although DMAB causes significant cell death in a 4–6 h exposure study with cells above these concentrations, the likelihood of such local high concentrations can be excluded. Thus, at the level used, DMAB appears to be a safe surfactant, at least at *in vitro* level. These results corroborate earlier studies done in animals where no inflammatory response was observed in liver, spleen and other body tissues (15,19). This makes the process of particle washing a very critical requirement, however, ensuring that the particles themselves, with low amounts of bound surfactant, are not toxic for exposure to various body tissues. Similarly, a study performed with three different sized (100, 135, and 180 nm) particles made using DMAB proved that particles at or below a concentration of 1,000 µg/ml cause no significant toxicity to the cells in terms of percentage survival.

It may be noted that the particle preparation process has not been optimized for the drug-loaded particles in the present study since the initial aim was to establish the proof of concept of oral delivery through nanoparticles. The particular concentration of the surfactant and homogenizer speed has been used to target particle size around 125 nm. This value was decided keeping in view the literature reports that smaller particles are taken up more and reside in the circulation longer, and also that there is probably a lower size limit to the EPR effect. The PDI, which is a mark of the heterogeneity of the particle size, was below 0.11, signifying acceptable uniformity. Also, the spherical shape as established by the AFM images corroborates the size estimation by the zetasizer, since the latter calculates the size assuming a regular geometry.

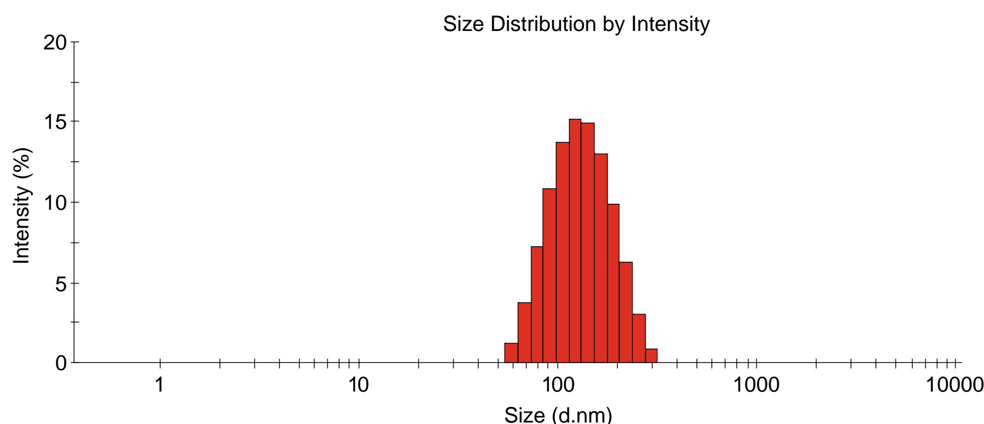

Fig. 5. Size distribution by intensity for 5% paclitaxel loaded nanoparticles.

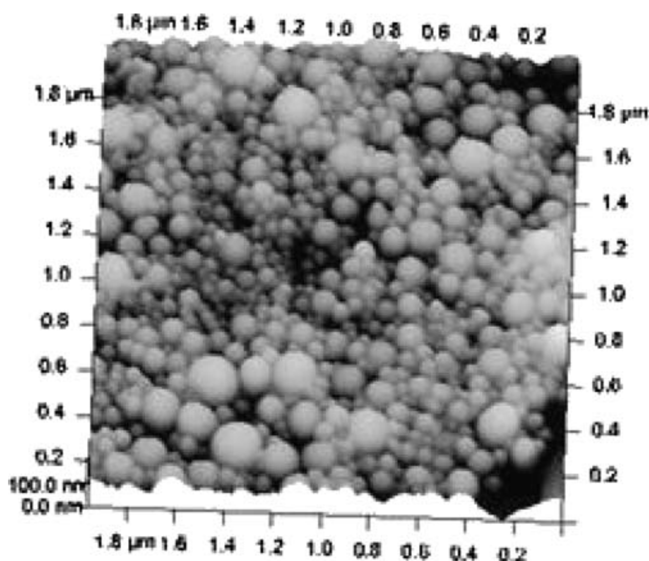

Fig. 6. AFM image of the 5% paclitaxel loaded nanoparticles.

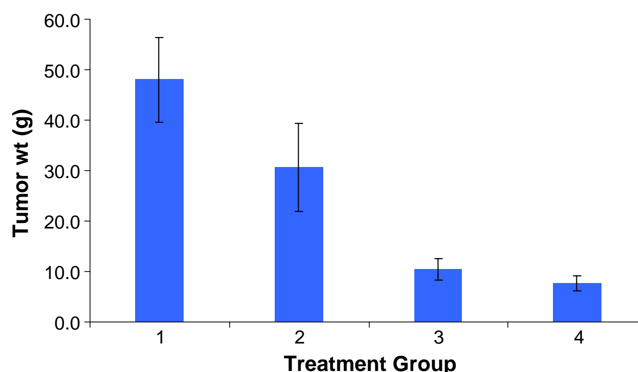

Fig. 8. Tumour burden in female SD rats (five animals per group). Groups: (*1*) no treatment; (*2*) Paclitaxel (7.5 mg/kg) in cremophor EL (oral), (*3*) Paclitaxel (7.5 mg/kg) in cremophor EL (i.v.), (*4*) Paclitaxel (3.75 mg/kg) in the form of nanoparticles (oral). *P* value for group1 *vs*. group4: 0.005; group1 *vs*. group3: 0.009; group1 *vs*. group2: 0.284; group2 *vs*. group4: 0.013; group2 *vs*. group3: 0.034; group3 *vs*. group4: 0.948. *P* value smaller than 0.05 signifies a statistically significant difference between the compared groups. *Error bars* denote standard error of mean.

By possessing smooth architecture and spherical shape, the particles are expected to be taken up, circulate and extravasate easily. However, recent reports claim that non-spherical shapes transport better and would be expected to have greater tendency of escaping phagocytosis (26,27). Shape inhibition of phagocytosis was shown to be achieved by minimizing the regions of high length-normalized curvature on the particle, with the effect highest for worm-like particles. However, these reports require further exploration, since they open up tremendous opportunities for particle engineering and understanding of the effects of geometrical features on drug delivery.

An ideal anticancer drug therapy should kill cancerous cells selectively and allow the drug to act on the neoplastic cells for a sufficient period of time. The disease models normally used for cancer studies are xenografts, genetically engineered animals and chemical mutagens. The most commonly used chemicals for inducing breast cancer are (7,12-dimethylbenz-anthracene) and alkylating agents like N-methylnitroso-urea. A single oral dose of the compound administered at the age of sexual development in female rats has shown high incidence of mammary tumours (28). It is worth noting that the human breast cancers bear many similarities to 7,12-dimethylbenz-anthracene-induced breast tumours in rodents, especially at the ultrastructural level (29).

In tissue distribution study, the oral dose was half of the i.v. dose. The tissue levels attained with oral nanoparticulate formulation denote a significant leap in the oral bioavailability of paclitaxel, signifying the utility of nanoparticles in increasing the absorption of the entrapped drug. Moreover, the higher proportion of nanoparticles seen in the lungs and spleen indicates interaction with the reticulo-endothelial system (RES) (30).

The *in vivo* study provides an encouraging proof of concept of utility of nanoparticulate anticancer formulations, highlighting the efficacy of paclitaxel incorporated in nanoparticles. The nanoparticle formulation was equally effective

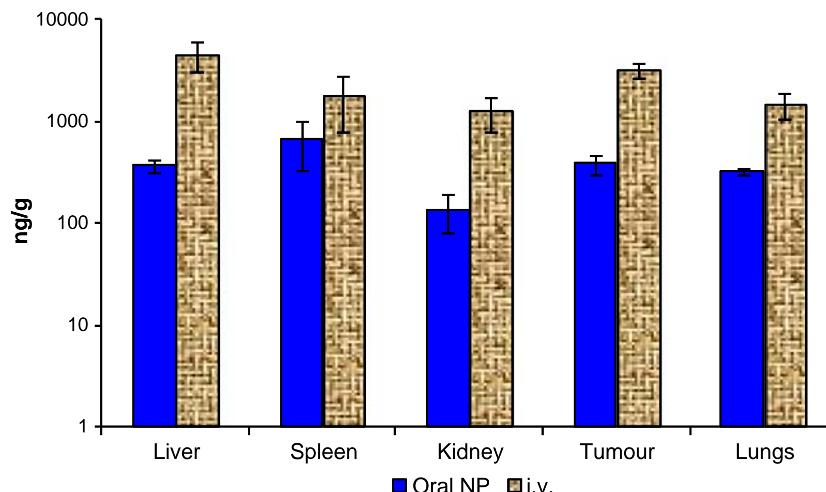

Fig. 7. Tissue distribution of paclitaxel in rats (n=3) bearing mammary tumours 24 h after dose. Oral NP group received 3.75 mg/kg and i.v. group received 7.5 mg/kg paclitaxel. Concentration of drug is denoted as nanogram of drug per gram of tissue. *Error bars* denote standard error of mean.

as the relevant treatment group but at half the dose. The average tumor weight measured after the orally administered paclitaxel in Cremophor was roughly 3-fold higher than nanoparticulate formulation.

Although increase in oral bioavailability seems to be the most probable explanation of these findings, there nevertheless exists another prospective explanation for the improved comparison with the intravenous paclitaxel. The particles of 120 nm used for the animal experiment have a potential to extravasate into the tumour from the vasculature and trapped by the EPR effect (31). Duncan and coworkers have reviewed that studies using liposomes, nano- and microparticles indicated that the capillary escape cut-off size can be as large as 200 nm–1.2 μm in some animal tumour models (32). The EPR effect might be the key to the efficacy of the nanoparticle formulation because of their preferential accumulation in the tumours (31). We have observed in other studies that size of the nanoparticles is the key for maintaining them in circulation and found that smaller particles stay in systemic circulation for longer times compared with the bigger ones (19,33). Therefore, longer circulation times can result in cumulative accumulation of the particles in the tumours. Although not proved by this study, it is an exciting prospect that needs further mechanistic investigation.

Furthermore, by administering the drug intravenously, smaller average tumour weights were seen. Although the effect was comparable for the intravenous paclitaxel and orally administered particles, the concentration of the drug in nanoparticulate formulation was only 50% of that administered intravenously with cremophor. This result indicates increased potency of the nanoparticulate formulation over the conventional treatment. In addition, this result suggests an increased oral bioavailability of paclitaxel when administered via the nanoparticles compared to the cremophor EL formulation. It is also worthwhile mentioning that it may be possible to see better effects with a higher dose of paclitaxel or altered dosing frequency. Also, since the size dependency is not clearly known for the EPR effect, both smaller or bigger particles may give different results. The pharmacodynamic efficacy may also be offset by a parallel change in the degree of uptake of particles from the gastrointestinal tract and subsequent circulation.

The oral administration of paclitaxel can significantly reduce the hospital expenditure associated with chemotherapy. This would allow their use to full therapeutic potential especially in high benefit-to-risk ratio indications. One of the factors that has limited the use of paclitaxel is the lack of effective formulations. Paclitaxel is also used as an anti-proliferative agent for the prevention of restenosis (recurrent narrowing) of coronary stents; locally delivered to the wall of the coronary artery, as paclitaxel coating limits the growth of neointima (scar tissue) within stents (USFDA). An oral formulation shall facilitate other and new uses of paclitaxel like in multiple sclerosis (34), Alzheimer's disease (35), rheumatoid arthritis (36,37), polycystic kidney syndrome (38), psoriasis (39) and Parkinson's disease (40).

CONCLUSION

DMAB is a versatile stabilizer capable of producing particles smaller than 100 nm, and the particle size can be controlled easily by varying the formulation parameters. The surfactant itself and particles made from it appear to be safe to cells *in vitro*. The nanoparticles carrying paclitaxel were successful in reducing the tumour burden in SD rats compared to control group. From the preliminary animal data it is evident that oral delivery of paclitaxel is possible and effective. The findings are certainly promising, suggesting possibilities of exploring these formulations for other cancers as well as other diseases mentioned above.

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