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RESEARCH PAPER

Nano-Co-Delivery of Berberine and Anticancer Drug Using PLGA Nanoparticles: Exploration of Better Anticancer Activity and *In Vivo* Kinetics

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ABSTRACT

Purpose Combinatorial approach can be beneficial for cancer treatment with better patient recovery. Co-delivery of natural and synthetic anticancer drug not only valuable to achieve better anticancer effectivity but also to ascertain toxicity. This study was aimed to co-deliver berberine (natural origin) and doxorubicin (synthetic origin) utilizing conjugation/encapsulation strategy through poly (lactic-co-glycolic acid) (PLGA) nanoparticles.

Methods Doxorubicin was efficiently conjugated to PLGA via carbodiimide chemistry and the PLGA-doxorubicin conjugate (PDC) was used for encapsulation of berberine (PDBNP).

Results Significant anti-proliferative against MDA-MB-231 and T47D breast cancer cell lines were observed with IC_{50} of 1.94 ± 0.22 and 1.02 ± 0.36 μM, which was significantly better than both the bio-actives ($p < 0.05$). The ROS study revealed that the PDBNP portrayed the slight increase in the reactive oxygen species (ROS) pattern in MDA-MB-231 cell line in a dose-dependent manner, while in T47D cells, no significant change in ROS was seen. PDBNP exhibits significant alteration (depolarization) in mitochondrial membrane permeability and arrest of cell cycle progression at sub G1 phase while the Annexin V/PI assay followed by confocal microscopy resulted into cell death mode to be because of necrosis against MDA-MB-231 cells. *In vivo* studies in Sprague Dawley rats revealed almost 14-fold increase in half life and a significant increase in plasma drug concentration.

Conclusion The overall approach of PLGA based co-delivery of doxorubicin and berberine witnessed synergetic effect and reduced toxicity as evidenced by preliminary toxicity studies.

KEY WORDS berberine · encapsulation · *in vivo* pharmacokinetics · mitochondrial pathway · necrosis · PLGA-doxorubicin conjugate

ABBREVIATIONS

AFM	Atomic force microscopy
DCM	Dichloromethane
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
hPBMCs	Human Peripheral Blood Mononuclear Cells
NHS	N-hydroxy succinimide
PDBNP	Berberine loaded PLGA-doxorubicin nanoparticles
PDC	PLGA-doxorubicin conjugate
PLGA	Poly lactide-co-glycolide
PNP	Blank nanoparticle
PVA	Polyvinyl alcohol
RBCs	Red blood cells
ROS	Reactive oxygen species
SEM	Scanning electron microscopy

INTRODUCTION

Mono-therapy in cancer treatment may not result in the better outcome as expected and therefore combination cancer therapy is always beneficial. However, selecting the mode of

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combination therapy may be crucial in attaining the objective of treating cancer. The therapeutic fate may be dependent on what kind of combinatorial delivery it is? Is it “simultaneous” or “sequential”? The simultaneous delivery as “cocktail” of two or more synthetic drugs is meritorious, as it may lead to enhanced efficacy due to synergism which help in overcoming drug resistance due to monotherapy. However, the cocktail delivery may not be beneficial always (1–3). This could be due to the possible drug-drug interactions, enhanced adverse effects (as the extend and frequency of adverse and side effects may be aggravated in combinatorial delivery) etc. In contrast, sequential delivery of drugs leads to several advantages over simultaneous delivery in terms of improved therapeutic efficacy and reduced side effects (4,5).

Nanocarrier based co-delivery approaches have become highly popular in the last two decades in cancer chemotherapy. The nanocarrier based co-delivery could target the different pathways at cellular level of the cell, which can be beneficial in terms of reducing the mutation and prolonged delayed in cancer adaption (6). Nanocarrier based co-delivery of drugs have advantages of long circulation time, enhanced target binding, controlled and sustained release and improved pharmacokinetics as well as pharmacodynamics (7). Nanocarrier based co-delivery has been majorly attempted following encapsulation strategy. In the present study, however, the attempts were made to combine the conjugation and encapsulation strategy for the co-delivery. The reason was to ascertain no leakage and higher drug pay load of the developed nanocarrier. The physical encapsulation may be challenging in terms of leakage of drugs and optimization. The hypothesis is that the conjugation of one drug followed by encapsulation of other can be beneficial and may result into better outcome.

Traditional breast cancer chemotherapy faces challenges of resistance and adverse effect of drugs. Most challenging is that, the majority of initially chemo-responsive tumors develops resistance to once-effective chemotherapeutic agents (8,9). The additional side effects such as cellular toxicity further necessitates to work on novel strategy which is better (10,11). In addition to nanotechnological approaches for the effective treatment of breast cancers, now-a-days a new area is opening, which encompasses “natural bio-active(s)” for better and safer management of cancers due to their very little propensity to produce adverse effects and better chemo-sensitivity towards cancer cells. Some of the natural anticancer bioactives which have recently been utilised in the treatment of different cancers includes but not limited to curcumin, quercetin, berberine etc. (12–14).

Doxorubicin is one of the most widely used anticancer drug against breast cancer in different combinations either with monoclonal antibodies as well as other chemotherapeutic agents. But due to development of resistance, it does not show its effectiveness in lower doses (15). In these circumstances, a holistic and biocompatible approach in combination with traditional drug can be beneficial for reducing resistance and to achieve the better synergetic effects (16). Poly (lactic-co-glycolic acid) (PLGA) has been widely used for the development of novel drug carriers due to its bio-degradable, biocompatible and versatile nature. Moreover, it is US-FDA approved and a GRAS category polymer for bio-medical applications. It has been extensively used for nanoparticulate delivery of anticancer drugs (17).

In the present study, it was attempted to co-deliver anticancer drug doxorubicin and natural anticancer-bioactive berberine using nanoparticulate carrier i.e. PLGA. One of the objectives, was to address the doxorubicin toxicity by combining them with an herbal origin anticancer bio-active. Another objective was to utilize advantages of polymeric nanocarriers for sequential delivery using conjugation and encapsulation strategy opting for nanoparticles. In our recent study, the importance of conjugation along with the encapsulation in PAMAM dendrimers (14). The study reached to a conclusion that the conjugation strategy works well compared to encapsulation. In the present attempt also, the conjugation of doxorubicin was intended to ensure the better drug entrapment in the final formulation and to reduce the overall exposure of synthetic anticancer drugs.

MATERIALS AND METHODS

Materials

PLGA (poly lactide-co-glycolide; 50:50), doxorubicin hydrochloride and berberine chloride was purchased from Sigma-Aldrich, Bangalore, India. Polyvinyl alcohol (PVA), HPLC grade water, acetonitrile, dichloromethane (DCM), [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxy succinimide (NHS) were purchased from CDH, New Delhi, India. Dialysis membrane (10-12 kDa, MWCO) was purchased from Hi-media laboratories Pvt. Ltd., Mumbai, India. All other chemicals reagents and solvents were used as received without any further purification.

Synthesis of PLGA-Doxorubicin Conjugate (PDC)

Briefly, poly(lactide-co-glycolide) (PLGA) was converted into its NHS (N-hydroxy succinimide) ester through synthetic pathway as reported earlier [18,19]. PLGA (200 mg, 0.029 mmol) was taken in 100 mL round bottom flask (RBF) and anhydrous dichloromethane (DCM, 4 mL) was added slowly. Thereafter, NHS (3.3 mg, 0.029 mmol) was added to PLGA solution and stirred for few minutes. In the last step, [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, 4.54 mg, 0.029 mmol) was mixed into anhydrous DCM (4 mL) and transferred to PLGA solution dropwise and the reaction mixture was stirred for 6–8 h. After completion of the

reaction, the synthesized compound (PLGA-NHS) was dried under vacuum using rotary vacuum evaporator (Buchi, Switzerland). PLGA-NHS was washed with cold mixture of methanol and ethyl ether to remove excess amount of NHS. PLGA-NHS conjugate was used as such for further conjugation with doxorubicin. In this step, PLGA-NHS (200 mg, 0.017 mmol) was taken in 100 mL RBF and anhydrous DCM (5 mL) was added in subsequent step through subsequent addition of doxorubicin (30 mg, 0.017 mmol) and DMAP (3.2 mg, 0.025 mmol). The reaction was performed in an inert atmosphere in dark place at room temperature for 18–24 h. The resulting PLGA-doxorubicin conjugate (PDC) (Fig. 1) was dried under vacuum and dialysed against water to remove excess doxorubicin and was used for further encapsulation of another bioactive, berberine.

Preparation of Nanoparticles

Berberine loaded PLGA-doxorubicin nanoparticles (PDBNP) were prepared as per previously reported method (18,19). Briefly, PDC (200 mg) was dissolved in DCM (5 mL) and stirred for few minutes until complete dissolution. PDC solution was added into methanolic solution of berberine (20 mg/5 mL) with continuous stirring. Further, the above solution was poured into poly vinyl alcohol (PVA) solution (8 mL, 1% *w/v*) and ultrasonicated (Qsonica 125, USA; 4–5 min). After sonication, sonicated emulsion was transferred into distilled water (15 mL) under constant stirring (800 ± 50 rpm, Remi, India) at ambient temperature for 18–24 h to evaporate efficiently, the present organic residue. The obtained nanoparticulate dispersion was separated using centrifugation (18,000 rpm speed, CPR-30 plus, Remi, India). The formed nanoparticles were washed with the help of distilled water to confiscate additional surfactants. Similar methodology was adopted for the preparation of blank PLGA nanoparticles (PNP) without adding the drug.

Characterization

NMR and FT-IR Spectroscopy

PLGA-doxorubicin conjugate (PDC) was well characterized through NMR and FT-IR spectroscopy. ^{1}H-NMR was performed at Central University of Rajasthan, India through Bruker Avance-500 NMR spectrophotometer using $CDCl_3$ as a deuterated solvent (Bruker Bio-Spin Corporation, Switzerland) and FT-IR (Perkin Elmer) was performed using KBr pellet method.

Particle Size, Zeta Potential, and PDI

The average particle size (nm) and the zeta potential (mV) of the PDC conjugate, formed PDBNP and blank PLGA nanoparticles were characterized through, Malvern Zetasizer (Nano ZS, UK). For the sample preparation, the analysed samples were dispersed in deionized water before analysis. The same procedure was adopted for analysing the zeta potential (mV). The analysis was performed in triplicate (20–22).

Scanning Electron Microscopy

The surface morphology of PDC was performed through scanning electron microscopy at 10,000X magnification (Nova Nano SEM 450, FEI) at Malaviya National Institute of Technology (MNIT), Jaipur, India. The analysed samples were attached to a double side tape on aluminium stub which were gold coated (Quorum, Q150T ES) and samples were examined under scanning electron microscope. The surface morphology of PDBNP was also performed through scanning electron microscopy at 26,000X magnification (23).

Atomic Force Microscopy

The surface morphology of the PDC and PDBNP was performed through atomic force microscopy (AFM, Bruker Tapping mode, Co., Germany) at Malaviya National Institute of Technology (MNIT), Jaipur, India. PDC sample was prepared in water with sonication up to 30 min. A thin film was prepared on a glass slide. The slides were dried and examined under atomic force microscope. Similar sample preparation steps were followed for the PDBNP. After sonication, the sample was poured on a glass slide to make a thin film. After making a thin film the glass slide was dried and examined under atomic force microscope (AFM, Bruker Tapping mode, Co., Germany) (23).

HPLC Analysis

Chromatography System and Conditions

Liquid chromatography (Shimadzu LC, Japan) equipped with 4.6 × 250 mm column RP-18, ODS (Merck, USA) with particle size 5 μm and detector PDA detector with fixed analytical wavelength was used in measurements.

Mobile Phase and Sample Preparation

For the preparation of mobile phase, the solvents were properly filtered and degassed through sonication. Acetonitrile and sodium acetate buffer (pH 4.10) in ratio 70:30 *v*/v were used. Wavelength of 487 and 346 nm was selected respectively for doxorubicin and berberine. Flow rate for mobile phase was 1.0 mL/min and column temperature were kept at 25°C (24,25).

Doxorubicin and berberine (each 1.0 mg) were dissolved in methanol (10 mL) in a volumetric flask, separately, and the

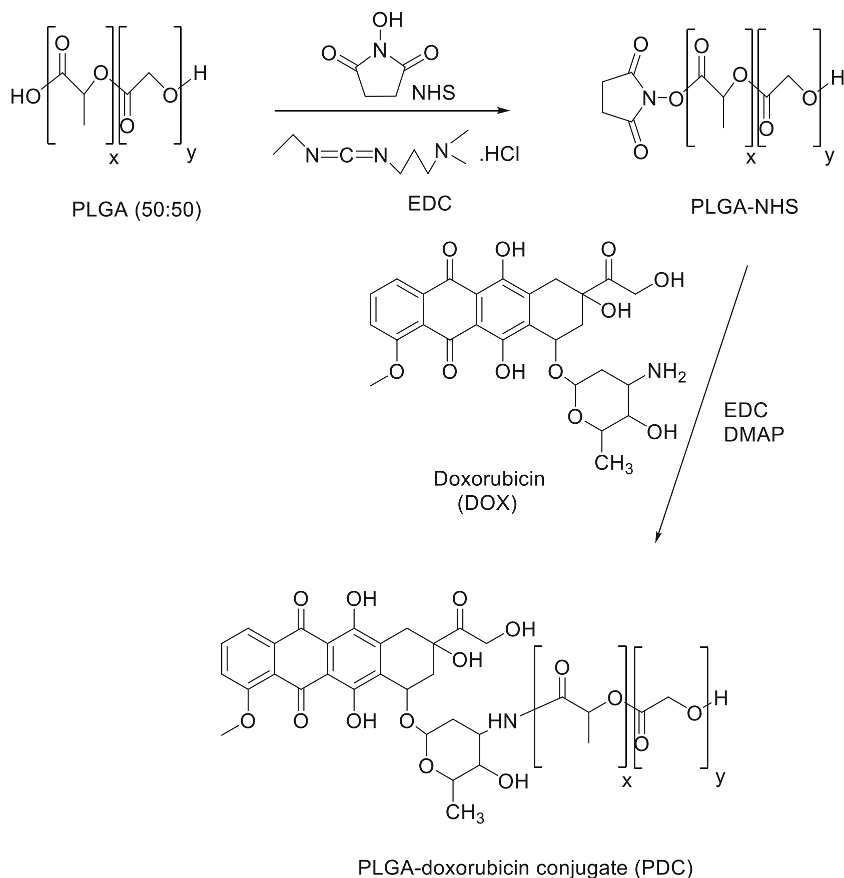


Fig. 1 Schematic representation of PLGA-doxorubicin conjugation.

concentration of final stock solution was made up to 100 μg/mL. Ten different aliquots of known concentration were prepared for HPLC in concentration range of 2 to 20 ppm. Appropriate amount was taken from stock solution and was diluted with acetonitrile. The sample mixtures were immediately injected in the HPLC system and analyzed for retention time and other parameters.

Entrapment Efficiency and Drug Loading

Entrapment efficiency and drug loading was determined using earlier reported method. Briefly, 10 mg of PDBNP was mixed in the solvent mixture (acetonitrile, sodium acetate buffer, 70:30). The solvent mixture was centrifuged (approx. 800 rpm, 50 G, for 10 min) and filtered with the help of syringe filter (pore size 0.22 μ, Moxcare, India). The filtrate was assessed with the help of HPLC (Shimadzu LC-2010 CHT, Japan) as per previously reported method (24,25). Doxorubicin entrapment in PDBNP was estimated based on reaction yield of PLGA and doxorubicin in percentage.

Ex Vivo Erythrocytic Membrane Toxicity

For red bold cells (RBCs) compatibility of the formulations, the *ex vivo* erythrocytic membrane interaction was performed. Briefly, the whole human blood was collected from healthy human volunteers and stored in anticoagulant vials (Hi-Media, India). Further, the whole blood sample was centrifuged (1500 ± 100 rpm, 154–201 G, Remi, India) and RBCs were separated. In the next step, RBCs were transferred into different solutions such as distilled water which was considered as 100% hemolysis. In other samples, the RBCs were mixed into doxorubicin, berberine, PDC and PDBNP (0.5 mL, 20 ppm) and added to normal saline (0.9%, 4.5 mL) in separate vials. For blank samples, the RBCs were mixed into normal saline (0.9%). The aforementioned samples were kept for interaction with RBCs and centrifuged (1800 ± 200 rpm, 201–314 G, Remi, India). The supernatant solution was collected and estimated with the help of UV-visible spectrophotometer (Labtronics Double Beam UV-Vis Spectrophotometer, LT-2800) at 540 nm. Percent hemolysis was calculated as per reported formula (14,26,27). The study was performed in triplicate.

In Vitro Release

In vitro release of doxorubicin and berberine from PDBNP was accomplished in phosphate buffer saline (PBS) at pH 7.4, using indirect dialysis method the used dialysis membrane was of MWCO 10–12 kDa; Hi-Media, India. Similar method

was followed for the release study of PDBNP nanoparticles. Briefly, doxorubicin, berberine and PDBNP (equivalent to 2 mg of pure drug) was suspended in dialysis membrane and dialysis membrane was immersed into PBS buffer (pH 7.4, 100 mL). The whole study was performed at ambient and 37°C temperature with constant stirring (100 ± 20 rpm). At predefined intervals aliquots of 2.0 mL were withdrawn and collected. For maintaining perfect sink conditions, fresh media was replaced each time the aliquot was withdrawn. All the collected samples were analysed for drug content using HPLC analysis as per earlier reported method (21,24,25,28–31).

In Vitro Cytotoxicity Study

Cell Culture and Treatment

MD-MBA-231 and T47D breast cancer cells were purchased from NCCS Pune and were grown in DMEM media with FBS (10%) and an antibiotic (penicillin and streptomycin 100 units/mL). The cells were incubated at 37°C in CO_2 (5%) using a CO_2 incubator. After the cells became confluent, the culture media was removed and washed with 1X PBS solution to inactivate the existing media in culture. The cells were further, treated with Trypsin-EDTA 0.25% (*w*/*v*) solution for trypsinization. Subsequently, trypsin was inactivated by adding DMEM media; cells were collected and centrifuged at 1200 rpm at 37°C for 5 min. The maintenance of cultured cell line was done in 25 cm^2 flasks (32). The same steps were repeated for maintaining the cells.

Similarly, human peripheral blood mononuclear cells (hPBMCs) culturing was done as per Protocol No. CUPB/cc/14/IEC/4483 approved by Institutional Ethics Committee of Central University of Punjab, Bathinda, India, according to guidelines issued by ICMR, New Delhi, India. The similar procedure for cancer cell culture was utilized, with slight modification whereby DMEM media was replaced by RPMI.

3-(4,5-Dimethythiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay

Anti-proliferative potential of the investigational compound was evaluated using MTT based assay. The assay was performed in 96-well plates in which each well consisted of approximately 8×10^3 cells (counted by trypan blue dye) suspended in 100 μL complete media. Treatment was given at three concentrations viz., 1, 5 and 25 μM and incubated for 48 h. After 48 h, the whole media was discarded, and cells were washed with PBS (1X). After washing with PBS, the cells were treated with MTT dye (0.5 mg/1.0 mL) and cells were incubated for 4 h, to allow formation of formazan crystals. The formazan crystals were dissolved using fixed quantity of DMSO. The resulting absorbance was spectrometrically read (570 nm) using microplate reader. The result was established by comparing the data in triplicate (33). The IC_{50} calculation was further done using Origin software. MTT assay was performed against MDA-MB-231 and T47D cells.

Human Peripheral Blood Mononuclear Cells (hPBMCs) Based MTT Assay

Briefly, fresh blood from healthy volunteer was drawn, which was added with RBC lysis buffer and was hold as such for 20 min at 4°C. After this the mixture was centrifuged and pellet was obtained by discarding the supernatant. The pellet was cultured using RPMI media under standard condition. MTT assay was performed as per the previous discussed methodology. The similar procedure for cancer cell culture was utilized, with slight modification whereby DMEM media was replaced by RPMI.

Reactive Oxygen Species (ROS) Detection

Cell Rox was employed for the detection of ROS levels. The assay was conducted using MD-MBA-231 and T47D breast cancer cells as per discussed in above section. After incubated time (48 h) Cell Rox red dye was added to the plates and analysis was done by measuring fluorescence at Em/Ex (665 and 644). The whole experiments were performed at 37°C followed by washing (PBS, 1X) to remove the unused dye.

Mitochondrial Permeability Assay

JC-1 dye was employed for the detection of alteration in mitochondrial permeability. The assay was conducted using MD-MBA-231 and T47D breast cancer cells as per above mentioned procedures. After incubated time (48 h), JC-1 dye was added to the plates separately. JC-1 assay analysis was done by measuring fluorescence at Em/Ex (527 and 590) using a microplate reader. The whole experiments were performed at 37°C followed by washing (PBS, 1X) to remove the unused dye.

Cell Cycle Analysis Using Propidium Iodide

Cellular samples treated with investigational compounds (PDC and PDBNP) at their sub IC_{50} concentration using MDA-MB-231 cancer cell and were incubated for 48 h. The assay as per the manufacturer protocol (Sigma Aldrich, Product No. P4170) The cells approximately 10^6 were trypsinised and transferred to each tube. The cells were further centrifuged (1200 rpm) for 5 min and washed with the help of 1x PBS buffer. After that cells were fixed using chilled methanol (70%) and incubated for 3 h at −20°C. Post 3 h cells were centrifuged (2500 rpm) and washed with 1x PBS buffer. Further, propidium iodide (40 μL) and Ribonuclease A (50 μL) was mixed into each well and incubated for 30 min

at room temperature in the dark. The DNA content was then measured using Flow cytometer (BD Accuri C6).

Cell Uptake Study

Flow cytometry (BD Accuri C6) was utilized to determine the fluorescence shift of the synthesized conjugate and nanoparticles (PDC and PDBNP). The compounds after confirmation of their fluorescence shift in flow cytometer were analysed via confocal microscopy (Model: Olympus FV 1200, Japan) to determine the actual cell uptake. Briefly, MDA-MB-231 breast cancer cells were cultured using cover slip. Cultured cells (MDA-MB-231 Cells) were treated with formulation PDC and PDBNP at their sub IC_{50} concentration for 12 and 24 h, respectively. After each time period cells were thoroughly washed with 1x PBS and were mounted in glass slide using mounting media, before analysing them in confocal microscopy. The green and red channel of confocal wavelength was used for the study.

Annexin V vs Propidium Iodide Assay

Annexin V *vs* PI assay was done in accordance with manufacturer protocol (SKU No. V13242; Thermo Fisher). Briefly, after 48 h treatment, cells were trypsinized, centrifuged and washed with 1x PBS buffer. Thereafter cells were prepared in Annexin Binding Buffer provided by manufacturer. Annexin V and PI was added separately and was analysed in BD C6 Flow Cytometer after 30 min staining with dyes.

In Vivo Pharmacokinetics

The *in vivo* pharmacokinetics protocol was approved by Institutional Animal Ethics committee (IAEC) Rungta College of Pharmaceutical Sciences and Research, Bhilai, C.G., India (Reg. No. 1189/Po/Re/S/08/CPCSEA/2017/04). Sprague Dawley rats (male) weighing 200–250 g were used for the evaluation of the pharmacokinetic parameters. The animals were divided into five groups; Group 1 received doxorubicin (positive control), Group 2 received berberine (positive control), Group 3 received PDC, Group 4 received PDBNP, and Group 5 was kept as the control (negative control). The respective sterile doses were carefully injected (0.3 mL; dose equivalent to 1 mg/kg of doxorubicin and berberine) (14,34) through the tail vein of each rat. After predefined time intervals, the blood samples (0.2 mL) were collected from retro-orbital plexus. To the collected samples, methyl tert-butyl ether (2 mL) was added and centrifuged (2000×g). The supernatant was collected, dried and mixed into acetonitrile. The whole collected samples were filtered through 0.22 μ syringe filters and analyzed for doxorubicin and berberine through HPLC as reported in earlier paragraphs (24,25).

Statistical Treatment

All experimentations were executed at least three, times. Simple Student's t test and one-way analysis of variance (ANOVA) was used with the help of GraphPad Prism software (5.0, CA, USA) for statistical treatment of data, with $p < 0.05$ considered to be a significant difference.

RESULTS AND DISCUSSION

Synthesis and Characterization of PLGA-Doxorubicin Conjugate (PDC)

NMR and FT-IR Spectroscopy

The PLGA-doxorubicin conjugate (PDC) was synthesized following the reported protocols (18,35) with slight modifications. The developed conjugation was a two-step reaction and the obtained conjugate was characterized by FT-IR and ^{1}H-NMR. A strong peak at 1641.33 cm^{-1} demonstrated the existence expected amide link between PLGA and doxorubicin. The broad peak of OH at 3318.65 cm^{-1} in PLGA-doxorubicin conjugates revealed the doxorubicin OH peak (Fig. 2). Another strong peak at 1085.58 cm^{-1} exposed C-O stretching band and aromatic peak (doxorubicin) at 1564.13 cm^{-1} confirmed the presence of aromatic peak of doxorubicin in PDC. All the observed FT-IR peaks in spectra established the formation of PLGA-doxorubicin conjugate (PDC). Similar to FT-IR spectroscopy, the conjugation was further inveterate with the help of ^{1}H-NMR.

In proton NMR spectra (Fig. 3), the peak at 7.54 and 7.70 ppm chemical shift indicated the anthracene ring of doxorubicin. The peak at 8.24 ppm showed the formation of the amide bond between the COOH terminal of PLGA molecules and the NH_2 terminal of doxorubicin. The PLGA molecules signified the typical shift at 5.2 and 4.82 which inveterate the existence of methine (m, CH) and methylene (m, CH_2) protons, respectively.

Preparation and Characterization of PDBNP

Particle Size and Zeta Potential

The average particle size of PDC conjugates was found to be 706.6 ± 11.25 with 0.838 ± 0.127 pdi and − 14.0 ± 1.46 mV zeta potential. The higher size of the PDC was due to conjugation between PLGA and doxorubicin molecule. Further, PDC conjugate was used for the preparation of nanoparticles (PDBNP). The average particle size (nm) and zeta potential (mV) of PDBNP were found to be 198.01 ± 3.25 nm and − 8.76 ± 1.58 mV, respectively. The polydispersity index (Pdi) of PDBNP was 0.140 ± 0.095 (Table I). The low Pdi of PDBNP

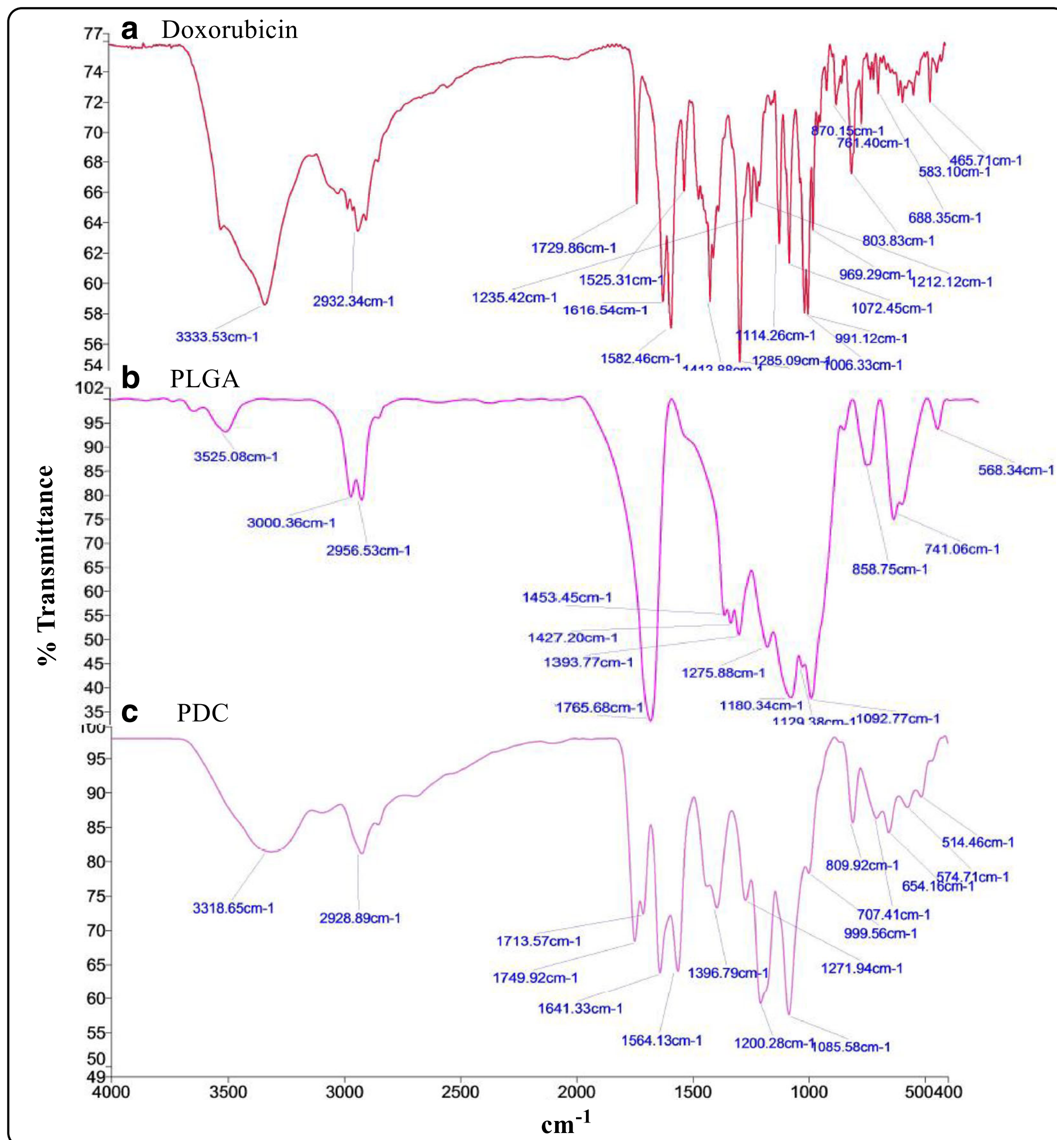


Fig. 2 FT-IR spectra of; (**a**) doxorubicin, (**b**) PLGA, and (**c**) PDC (PLGA-doxorubicin conjugate).

showed the uniform size distribution. The blank nanoparticles (PNP) were found to be of 75.07 ± 1.58 nm with −27.05 ± 2.47 mV and a Pdi of 0.102 ± 0.045 (Table I). The size of the PDBNP was higher than blank nanoparticles which might be due to the encapsulation of berberine in PDC, which would be resulting from the size enhancement than blank nanoparticle (PNP). The higher size of the PDC was due to conjugation between PLGA and doxorubicin molecule. Further, the PDC conjugate was fabricated into nanoparticles (PDBNP). Generally, the conjugates may have different size (usually larger) than the formulated nanoparticles. In nanoparticles, the conjugates acquire a definite shape due to high speed stirring and other conditions used for the formation of nanoparticles. So due to overall process the size of the nanoparticles might be less than the conjugates. The overall particle size characterization concludes that the size of the PDBNP nanoparticles was within limits of a standard nanoparticulate drug carrier and can be used further to explore its applications.

Scanning Electron Microscopy (SEM)

The prepared PLGA-doxorubicin conjugate (PDC) was rough and agglomerated in shape. Figure 4a, indicates that the PDC conjugates were not homogeneous in shape and were not with smooth morphology. The PDC was of irregular shape with uneven surface morphology which was due to conjugation of PLGA and doxorubicin. The conjugation leads to the formation of amide bond between PLGA and doxorubicin and owing to SEM image of this conjugate was not spherical.

At another side, the surface morphology of the prepared PDBNP was spherical in shape (Fig. 4b). Figure 4b, describes that the PDBNP nanoparticulate formulation was homogeneous with smooth surface morphology and of spherical in

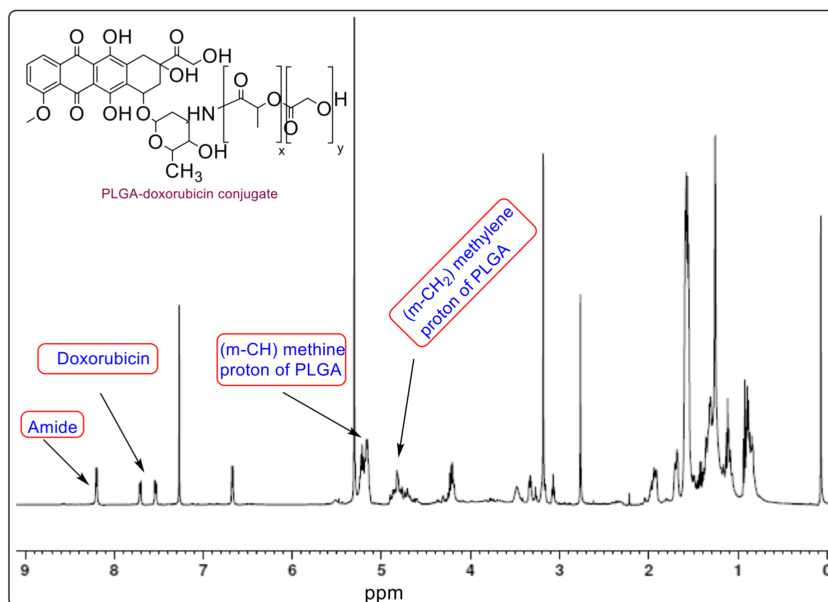


Fig. 3 ^{1}H-NMR spectra of PDC (PLGA-doxorubicin conjugate).

shape. The spherical shape of the formed nanoparticle was homogenous due to formation of nanoparticulate system.

Atomic Force Microscopy (AFM)

Atomic force microscopy provides more information about the surface morphology in terms of average roughness (Ra), and the root-mean-square roughness (Rq) of prepared PDC conjugates. Figure 4c and d, shows the 2 dimensional and 3-dimensional structural AFM images of PDC at 10 μM. These images also demonstrated morphology of the PDC conjugates with the Ra and Rq of 2.98 and 4.33 nm. The PLGA-doxorubicin conjugate (PDC) showed irregular surface morphology due to conjugation between PLGA and doxorubicin. Figure 4c and d indicated that the surface morphology of the PDC was not spherical in shape.

Atomic force microscopy of nanoparticles (PDBNP) delivers the more evidence for the surface texture and morphology. Figure 4e, f, g and h showed the 2 dimensional and 3 dimensional images of PDBNP at 10 and 5 μM, respectively. The average roughness (Ra), and the root-mean-square roughness (Rq) was observed 4.99 nm and 6.11 nm respectively. These images also demonstrate the smooth surface with spherical in shape and morphology which further confirmed the SEM results.

HPLC, Entrapment Efficiency and Drug Loading

Aliquots of different concentrations of doxorubicin were selected in the range of 2 to 20 μg/mL for analysis in HPLC system. There retention time (RT) showed in the chromatogram were approximately at 3.82 min as in Fig. S1 (supplementary data) and linearity (R^2) was 0.9994. Same protocol was adopted for berberine with different wavelength. For this, different concentrations of berberine were selected in the range of 2 to 20 μg/mL run into HPLC system with continuous mobile phase. The obtained RT in case of berberine was approximately 5.16 min. as shown in Fig. S2 (supplementary data) with a linearity (R^2) of 0.9986.

Entrapment efficiency and drug loading was determined with the help of HPLC analysis. The PDC was used to entrap berberine, which was further stretching the PLGA length resulting into increased entrapment efficiency. The entrapment efficiency and drug loading of PDBNP was found to be 52.98 ± 1.58 and 12.8 ± 1.35% (%w/w), respectively (Table I). The entrapment efficiency of PDBNP revealed that the berberine

Table I Particle Size, Zeta Potential, Polydispersity Index, Entrapment Efficiency and Drug Loading of Prepared Formulations

S. No.	Formulation code	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%w/w)	Drug loading (%w/w)
1.	PNP	75.07 ± 1.58	0.102 ± 0.045	−27.05 ± 2.47	–	–
2.	PDC	706.6 ± 11.25	0.838 ± 0.127	−14.0 ± 1.46	–	–
3.	PDBNP	198.01 ± 3.25	0.140 ± 0.095	−8.76 ± 1.58	52.98 ± 1.58	12.8 ± 1.35

Data represented as mean ± SD (n = 3)

PNP: Blank PLGA nanoparticles; PDC: PLGA-doxorubicin conjugate, PDBNP: Berberine loaded PLGA-doxorubicin nanoparticles

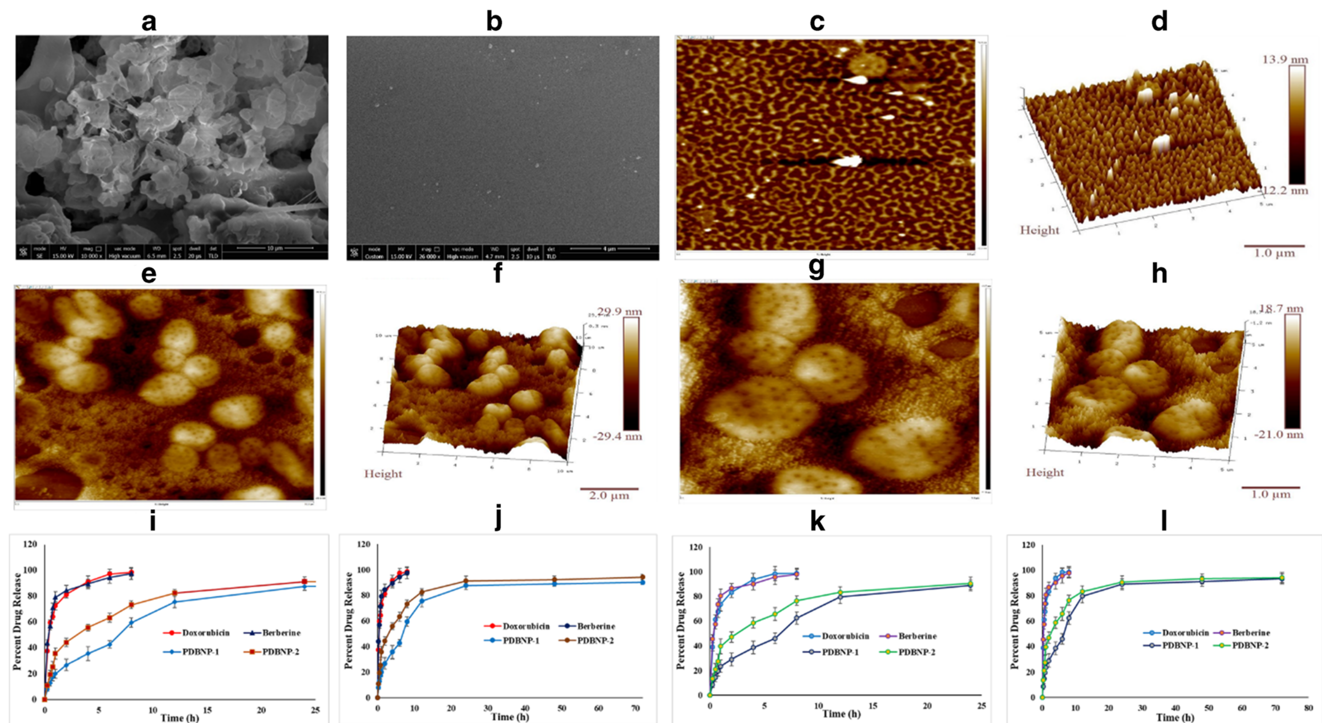


Fig. 4 (**a**) SEM microphotograph of PDC at 10,000x, and (**b**) PDBNP at 26,000x, (**c**) two and (**d**) three dimensional AFM images of PDC at 10 μM, (**e**) Two and (**f**) three dimensional AFM images of PDBNP at 10 μM, (**g**) Two and (**h**) three dimensional AFM images of PDBNP at 5 μM, (**i**) and (**j**) *In vitro* drug release pattern of doxorubicin, berberine and PDBNPs from PBS buffer at pH 7.4 at ambient temperature and (**k**) and (**l**) at 37°C. (PDBNP-1: Doxorubicin release from PDBNP and PDBNP-2: Berberine release from PDBNP).

was entrapped into the hydrophobic core of PLGA molecule. The drug was, probably entrapped into polymeric core due to the capacity of PLGA. The percentage of conjugated doxorubicin in PDBNP was 6.55% w/w which was calculated based on moles of doxorubicin conjugated to PLGA.

In Vitro Release

The *in vitro* release study for both berberine and doxorubicin was performed in PBS buffer at pH 7.4, as release media. The media was selected as PBS to mimic the physiological conditions. The results concluded that the PDBNP showed an initial slow and sustained release pattern followed by constant release during initial 24 h (Fig. 4i, j, k and l). The preliminary sluggish release of PDBNP-1 (Doxorubicin release from PDBNP) was due to conjugation of doxorubicin with PLGA. The conjugate displayed the slow pattern rather than PDBNP-2 (Berberine release from PDBNP). PDBNP-2 showed a fast release for initial 2 to 4 h, which may be due to surface adhered drug (berberine) and afterward it showed a constant release due to encapsulation in nanoparticles. The pure doxorubicin and berberine were released completely less than 10 h. On the other hand, the sluggish and continuous release was mainly dependent on the dispersal of the drug from the matrix of the nanoparticles and conjugation of the drug. The overall results showed that drug release from PDBNP followed a sustained pattern. The release study was conducted up to 24 h in which both drugs behaved in a sustained manner, however, the release of berberine was quite faster initially compared to doxorubicin. The possible reason could be the conjugation of doxorubicin to PLGA (Fig. 4i, j, k and l). The in vitro release study was reported till 72 h time period and found to be similar and sustained release of drugs thorough PDBNP.

Ex Vivo Erythrocytic Membrane Toxicity and Microscopy

Ex vivo erythrocytic membrane toxicity exhibited the extent of RBC rupturing as percentage for doxorubicin and berberine, which was observed to be 26.47 and 4.88%, respectively (Fig. 5a). In this data, the results concluded that the doxorubicin showed higher RBCs toxicity compared to berberine. However, when PLGA-doxorubicin conjugates (PDC) were used then hemolytic toxicity was reduced compared to the pure doxorubicin, which was approx. 11.08% ($p < 0.0001$). Similarly, PLGA-doxorubicin loaded berberine NPs (PDBNP) exhibited 5.23% (Fig. 5a) hemolysis which was approximately half of erythrocyte toxicity compared to PDC conjugates ($p < 0.005$) and nearly 5 times less than pure doxorubicin ($p < 0.0001$). Hemolytic toxicity was significantly reduced, which is essential for a safe formulation. The incorporation of drug in PLGA might have hindered the drug associated toxicity leading to less hemolysis. The results were favourable to develop a safer nanoparticulate formulation.

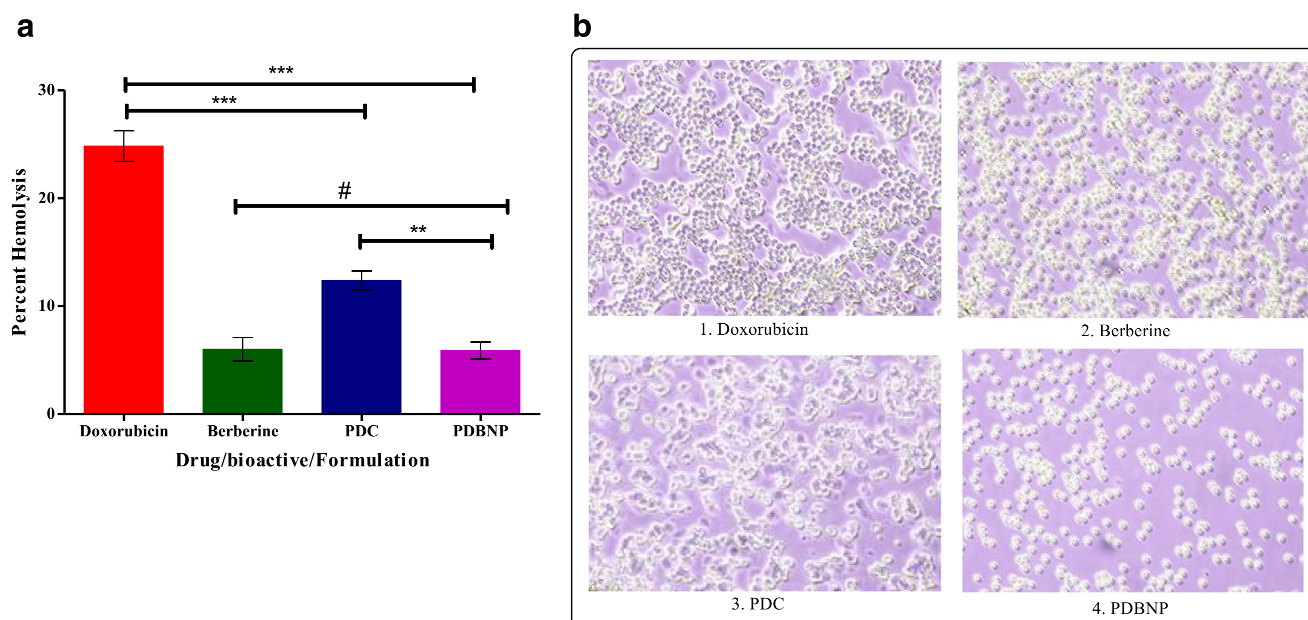


Fig. 5 (**a**) Percent hemolysis of naïve doxorubicin, berberine, PDC and PDBNP. Values represents mean ± SD ($n = 3$). *** indicates the extremely significant differentiate with p value <0.0001). ** indicates significant differentiate with p value <0.005 and # indicates no significant difference), (**b**) Microscopic images of drugs as well as formulation effected RBC's (1) doxorubicin effected RBC's, (2) berberine affected RBC's, (3) PDC affected RBC's, (4) PDBNP affected RBC's. (PDC: PLGA-doxorubicin conjugate, PDBNP: Berberine loaded PLGA-doxorubicin nanoparticles).

Microscopic analysis of hemolysed RBCs was performed with the help of fluorescence microscope at 40x (Olympus Inverted Fluorescence Microscope CKX53, Japan). Naïve doxorubicin ruptured the RBCs and changed the shape which might be due to its high toxicity (Fig. 5b) and can be seen clearly, and to another side less RBCs were ruptured due to berberine, which is a natural bioactive (Fig. 5b). But when doxorubicin was conjugated with PLGA, it showed almost half hemolysis compared to naïve doxorubicin indicating less toxicity (Fig. 5b). Further, PDBNP showed very less hemolysis compared to naïve doxorubicin approximately 5.23% and less ruptured RBC than the naïve drug alone. PDBNP showed very less toxicity probably because of incorporation of PLGA in the nanoparticulate system (Fig. 5b). This is also evident from the microscope images. It seems that there is definitely some role which is being played by berberine in reducing hemolysis due to PDC. It might be that the conjugated doxorubicin is interacting with encapsulated berberine molecules rendering it less available for interaction with RBC membrane.

In Vitro Cytotoxicity

3-(4,5-Dimethythiazol-2-Yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay

The MTT assay was performed in MDA-MB-231 and T47D breast cancer cell lines. In case of MDA-MB-231 breast cancer cells, the IC_{50} of pure doxorubicin was found to be 3.89 ± 0.72 μM and for berberine was 4.93 ± 0.34 μM respectively at 48 h period. To our delight our formulations were found to have better antiproliferative potential, where PDC showed half maximal inhibition of 2.05 ± 0.36 μM, which was less than doxorubicin ($p < 0.005$) and PDBNP showed IC_{50} of 1.94 ± 0.22 μM, which was significant ($p < 0.05$) and more effective than naïve doxorubicin and or all above results (Fig. 6a).

Whereas, in T47D breast cancer cells treatment with doxorubicin and berberine exhibited IC_{50} of 3.12 ± 0.42 and 6.12 ± 0.77 μM, respectively. The antiproliferative potential of the formulations as traced by half maximal inhibitory concentration was in alignment to results of MDA-MB-231, where PDC and PDBNP showed IC_{50} of 2.88 ± 0.63 μM and 1.02 ± 0.36 μM respectively ($p < 0.05$) (Fig. 6b).

Further we were also interested in evaluating whether the formulations are selective toward breast cancer cells only, we performed MTT assay utilising human peripheral blood mononuclear cells (hPBMCs). To our delight we found formulations to be significantly non-cytotoxic as compared to naïve drugs toward hPBMCs thus establishing their selectivity toward breast cancer cells (Fig. 6c). The idea behind the experiment was to assess the toxicity of nanoparticles formulation towards normal cells. Since the toxicity towards normal cell is a major drawback of cancer cells, we wanted to ascertain whether or not our formulation exhibits any sort of toxicity at

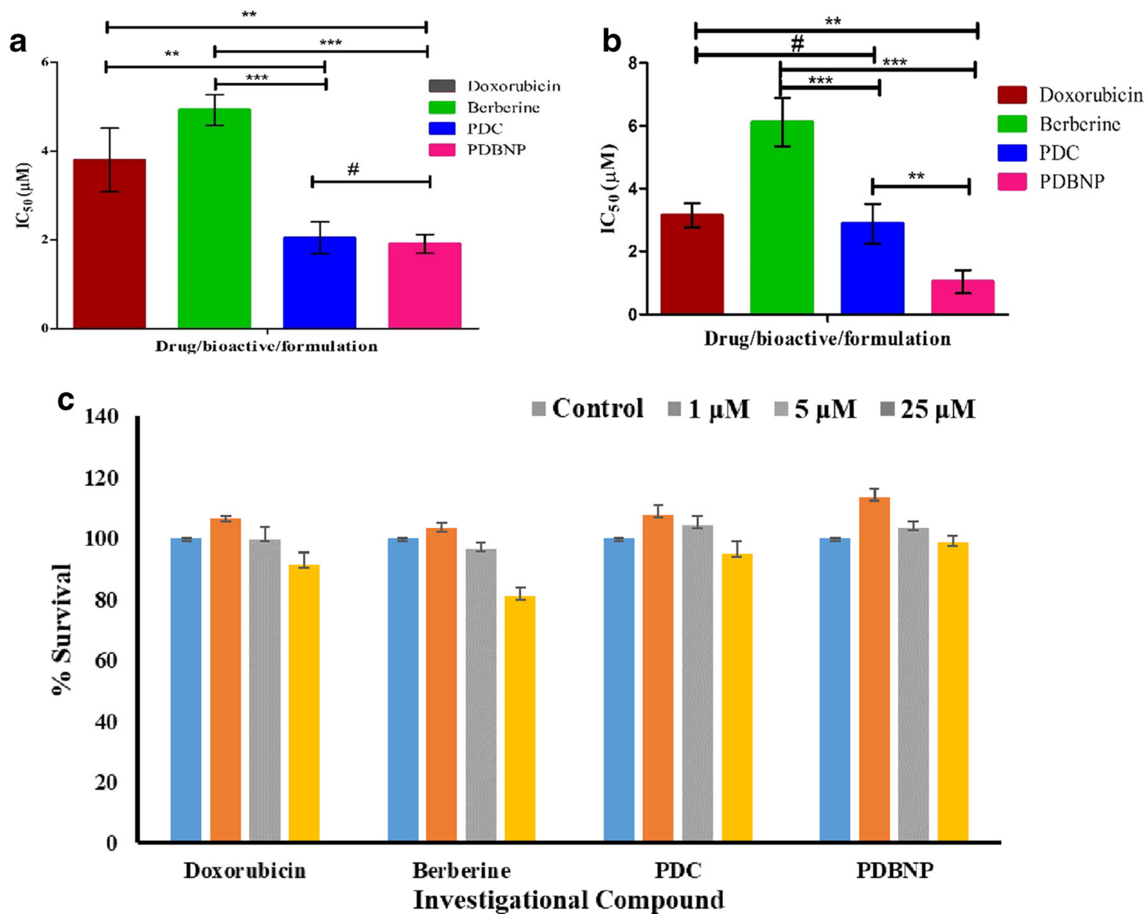


Fig. 6 (**a**) Antiproliferative activity against MDA-MB-231 and (**b**) T47D breast cancer cells, (**c**) Human peripheral blood mononuclear cell (hPBMCs) based MTT assay. [*** indicates the extremely significant differentiate with $p < 0.005$, ** indicates significant differentiate with $p < 0.05$ and # indicates no significant difference. One-way ANOVA analysis was used with post Newman-Keuls Multiple Comparison Test].

high concentration (above IC_{50}) as compared to their parent drug. We were only focused with the effect of berberine loaded PLGA-doxorubicin nanoparticles (PDBNP) on cytotoxicity no other toxicity in general.

Reactive Oxygen Species (ROS)

Majority of anticancer drugs are associated with the upsurge in ROS level beyond a threshold to induce cancer cell death as secondary mechanism. We were interested to find out the effect of our formulations (PDC and PDBNP) and pure drugs (doxorubicin, berberine) on ROS levels in breast cancer cells. To quantify same, we utilized Cell ROX Red based ROS assay. The study was conducted at three concentrations viz., 1, 5 and 25 μM in MDA-MB-231 and T47D cells. Doxorubicin, berberine, PDC and PDBNP portrayed slight increase in the ROS pattern in the MDA-MB-231 cell line in a dose-dependent pattern (Fig. 7a). Another side, in case of T47D cells, no major and significant ROS rise was seen even up to the highest concentration of 25 μM of drug concentration (Fig. 7b). The data, therefore, suggest that the PDC and PDBNP may be exhibiting their anticancer effect via some other mechanism excluding ROS gain (Fig. 7).

Mitochondrial Permeability Assay

During the event of extrinsic cell death induced by anticancer agents, the mitochondrial potential of a cell is known to be significantly altered. To evaluate the changes in mitochondrial potential, we performed JC-1 dye-based assay against MDA-MB-231 and T47D cell lines breast cancer cells by treating them with our formulations (PDC and PDBNP) and pure drugs (doxorubicin, berberine) at three concentrations viz., 1, 5 and 25 μM. The results portrayed that doxorubicin, berberine alone possess significantly less mitochondrial potential alteration as compared to the formulations in both cell lines employed for assay (Fig. 8a and b). PDBNP exposed to the MDA-MB-231 cells at 25 μM exhibited the sudden depolarization in mitochondrial membrane potential that may be attributed to cell death at this highest concentration used. The overall results suggest

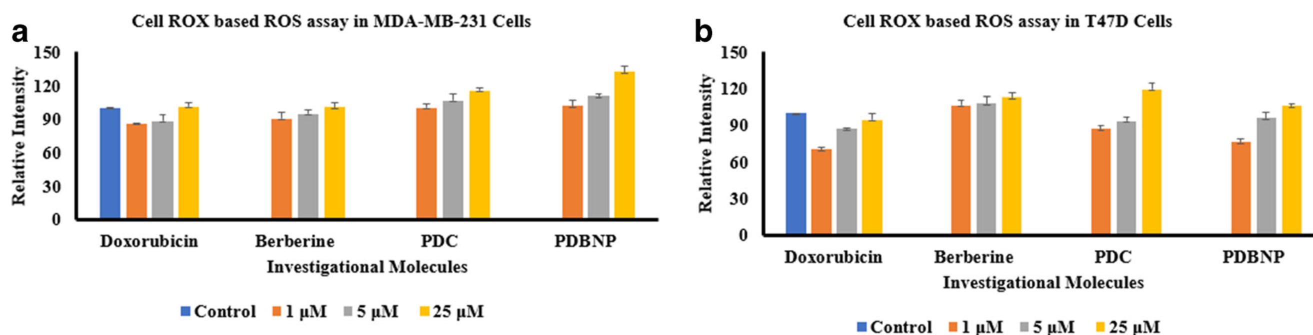


Fig. 7 Cell ROX based assay to measure intracellular reactive oxygen species (ROS) induced in, (**a**) MDA-MB-231 and (**b**) T47D cancer cells. The Investigational compounds portrayed the slight increase in the ROS pattern in each cell line in a dose dependent pattern. The study was conducted in triplicate at three concentrations viz., 1, 5 and 25 μM.

that the mode of cancer cell death by formulations PDC and PDBNP could be via mitochondrial-dependent pathway. The past reports also suggest that the mechanism of berberine induced apoptosis is via alterations in the Bcl-2/Bax ratio, production of ROS, and due to decrease in mitochondrial membrane potential (36). Berberine acts on mitochondria of tumor cells and results into destabilization, membrane permeabilization and subsequently apoptosis (37–39). The outcome of this study could be correlated that the developed nano-therapeutic might be responsible for better mitochondrial uptake of berberine as well as doxorubicin leading to better effectivity.

Cell Cycle Analysis Using Propidium Iodide

To check the exact phase of cell cycle affected by the formulations and naïve compounds, we performed the propidium iodide-based cell cycle analysis. The assay was conducted on MDA-MB-231 cells at 5 µM concentration with selected investigational compounds. The result suggested that the doxorubicin arrested the cell cycle at sub G1 phase (along with G2/M inhibition) and PDC arrested the cell cycle at G2/M phase, whereas berberine was able to halt the cell cycle progression at G1, and PDBNP was able to cause profound death and halted the cell cycle progression at Sub G1 phase (along with G2/M inhibition). The doxorubicin and PDBNP caused profound cell death of 24.38 and 44.95%, respectively (Fig. 9). The difference may be attributed to the fact that nanoparticles alter the pharmacokinetics parameters of the drug encapsulated. Owing to which there is slow and sustained release of both the drugs and may possess a combinatorial effect on cytotoxicity, cell cycle and apoptotic cell death (40,41).

Annexin V vs Propidium Iodide Assay

In order to investigate the exact mode of cell death propagated upon treatment with investigational compounds, we performed Annexin V *vs* Propidium Iodide (PI) based assay. The assay relies upon the binding of Annexin V with phosphatidyl serine, which is a major protein that is leaks from cytosol to cell surface during the process of apoptosis. Once the membrane gets premetallized the PI enters and intercalates the DNA signifying the necrosis. The assay was performed against human breast cancer MDA-MB-231 cells and treatment was given at sub IC_{50} concentration for 48 h. After this time point, cells were harvested and processed as per the manufacturer protocol and our previously published methodology. The result analysis (Fig. 10a and b) portrayed that nanoparticle formulation (PDBNP) exhibited necrosis (26.2%) mode of cell death, in comparison

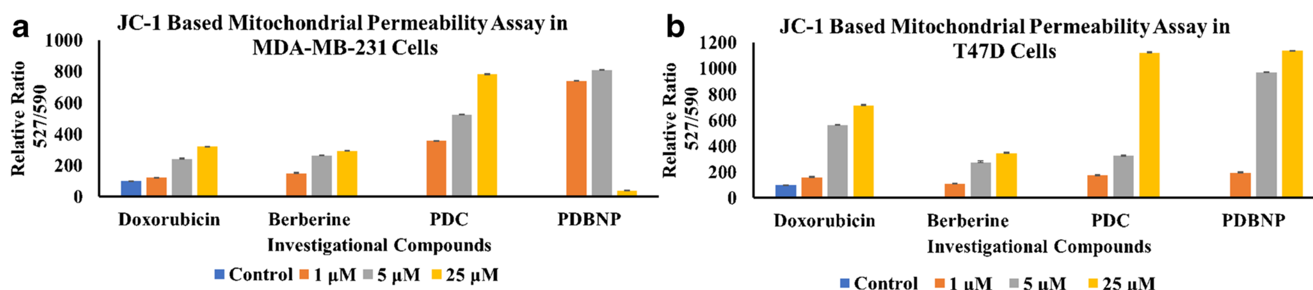


Fig. 8 Investigational compounds altered the mitochondrial potential drastically in, (**a**) MDA-MB-231 and (**b**) T47D cancer cells. The high upsurge in mitochondrial potential by compounds under investigation suggest the trigger of mitochondrial dependent pathway as possible explanation of anticancer effect. The study was conducted in triplicate at three concentrations viz., 1, 5 and 25 μM.

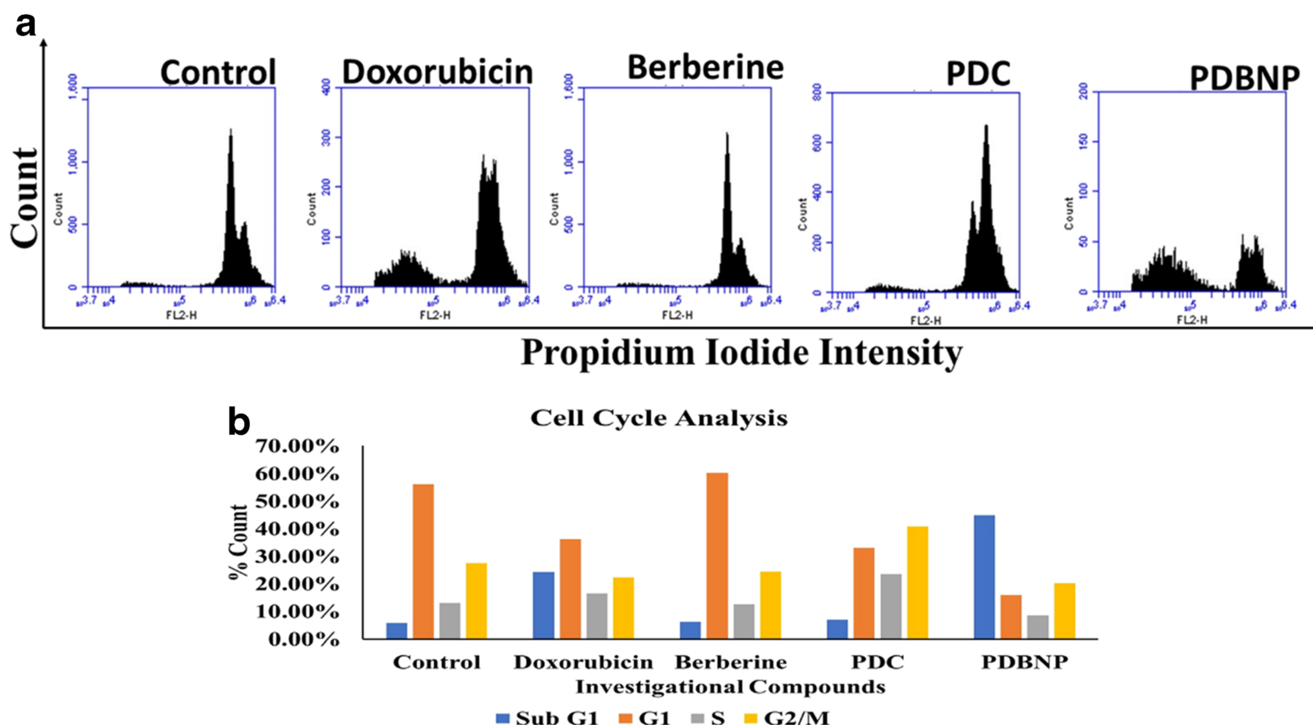
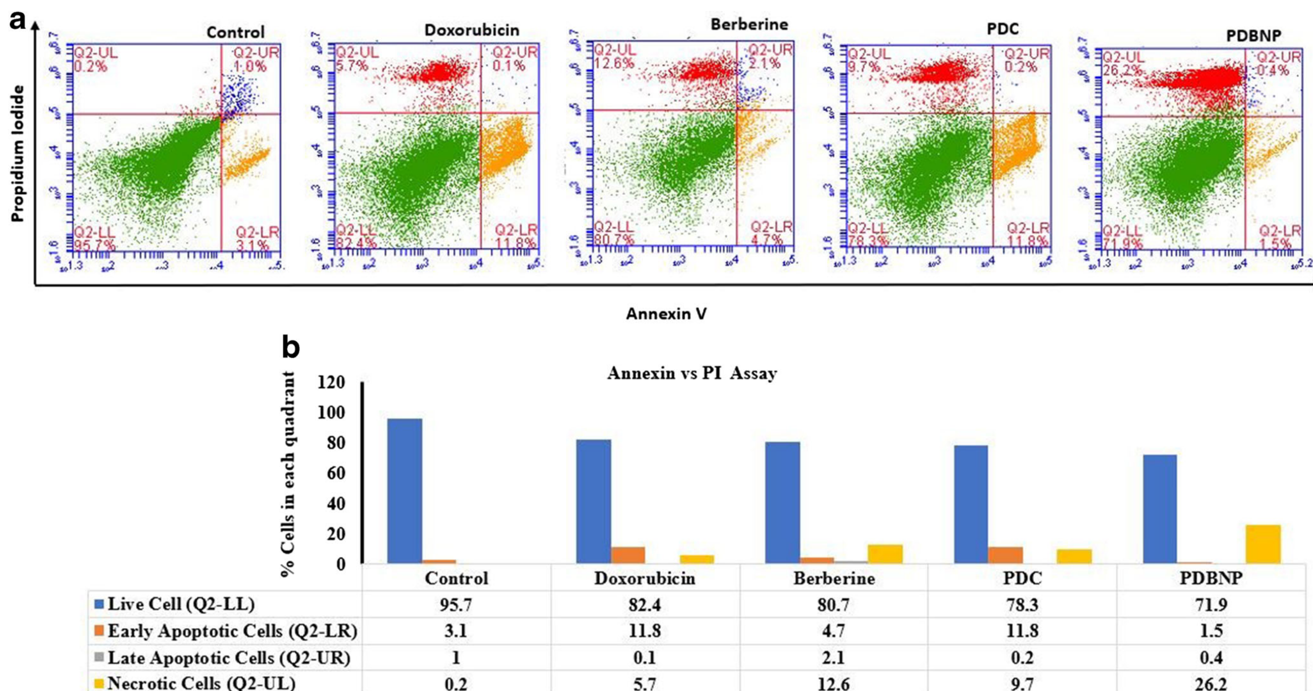


Fig. 9 The effect of investigational compounds on specific cell line was studied using MD-MBA-231 cells, (**a**) represents the histogram representing specific cell cycle phase, (**b**) represents the quantitatively cells at each phase as compared to control. The assay was conducted at concentration of 5 μM.

to its counterpart doxorubicin, berberine and PDC, showing 5.7, 12.6 and 9.7% death via necrosis mode. Another side, PDC conjugate caused majority of cell to undergo early apoptosis (11.8%). All results were found to be significant as compared to normal control and individual drugs employed for formulation preparation. The analysis was done using BD Accuri C6 flow cytometer. The rupturing of cell by treatment with



	Control	Doxorubicin	Berberine	PDC	PDBNP
Live Cell (Q2-LL)	95.7	82.4	80.7	78.3	71.9
Early Apoptotic Cells (Q2-LR)	3.1	11.8	4.7	11.8	1.5
Late Apoptotic Cells (Q2-UR)	1	0.1	2.1	0.2	0.4
Necrotic Cells (Q2-UL)	0.2	5.7	12.6	9.7	26.2

Fig. 10 (**a**) Effect of investigational compound treatment on cell mode death (apoptosis and necrosis) performed on MDA-MB-231 cells. Apoptosis was measured using Annexin V and necrosis by PI stain, (**b**) The bar graph represents the quantitative analysis suggesting the percentage of each type of cell in each quadrant.

PDBNP at 24 h was also illustrated using confocal microscopy (Fig. S3, Supplementary data).

Cell Uptake Study

The PDC and PDBNP were found to be auto-fluorescent as deduced by flow cytometry experiments (Fig. S4, Supplementary data). The compounds were therefore used for their cell uptake studies in absence of any fluorescent dyes. The study was conducted for 12 and 24 h, respectively, using MDA-MB-231 breast cancer cells at IC_{50} concentrations. The study demonstrated that PDC and PDBNP was able to enter the cell membrane at 24 h. The PDBNP caused cell rupturing at 12 h interval suggesting it cells necrotic potency (Fig. 11). At 24 h time period PDC caused profound cell rupturing while localizing itself inside the cellular compartment (Fig. 11). Formulation PDBNP portrayed higher cell death and we were able to locate only few ruptured cells containing the formulation based upon the fluorescence. Thus, the study concluded that there was significant drug uptake of formulations by the breast cancer cells and were able to cause profound cell damage at higher time interval by releasing the active drug(s).

***In Vivo* Pharmacokinetics**

Pharmacokinetic parameters were determined for the prepared nanoparticulate formulation in Sprague Dawley rats model using plasma-drug profile standard protocol. The bioavailability of prepared PDBNP was calculated from the area under the plasma concentration *vs* time curve, and it was compared to doxorubicin and berberine. After administration of PDBNP, $AUC_{0\text{-}t}$ of doxorubicin in plasma was 3.9 times and approximately 2 times higher than pure berberine. The $AUC_{0\text{-}\infty}$ was 699.51 ± 8.14 μg mL^{-1}/h for pure doxorubicin and 1171.60 ± 14.55 μg mL^{-1}/h for berberine, respectively. In case of PDBNP administration the $AUC_{0\text{-}\infty}$ was increased to $13{,}120.82 \pm 158.05$ and 3473.77 ± 85.55 μg mL^{-1}/h, respectively for doxorubicin and berberine. The AUC in both instances for PDBNP was higher compared to pure bio-actives. In parallel, the urinary excretion of doxorubicin and berberine was reduced in case of PDBNP. The half-life of PDBNP was 42.51 ± 3.47 and 25.29 ± 4.01 h (Fig. 12 and Table II), for doxorubicin and berberine, respectively. The $t_{1/2}$ was approximately 14.65 and 5.52 times higher than pure doxorubicin and berberine which indicated the higher bioavailability of these drugs. In case of PDC also, the different parameters were found to be favourable for better effectivity. For instance, the half-life of PDC was around 15 h which is itself indicating a sustained nature of release and behaviour.

CONCLUSIONS

The objective of co-delivery was attempted to address in the present study. The co-delivery of doxorubicin and berberine utilizing conjugation and encapsulation strategy was found to be meritorious. The synthetic as well as natural bioactive together established synergistic effect against breast cancer cells *in vitro* as well as *in vivo*. PLGA is a biodegradable polymer which is non-toxic to normal cells, that's why doxorubicin was conjugated to PLGA using carbodiimide chemistry and further this conjugate was used to encapsulate berberine to prepare the optimized nanoparticles (PDBNP). The study resulted in positive outcome as per as in vitro anticancer studies against MDA-MB-231, and T47D breast cancer cells are concerned. The duo combination resulted into

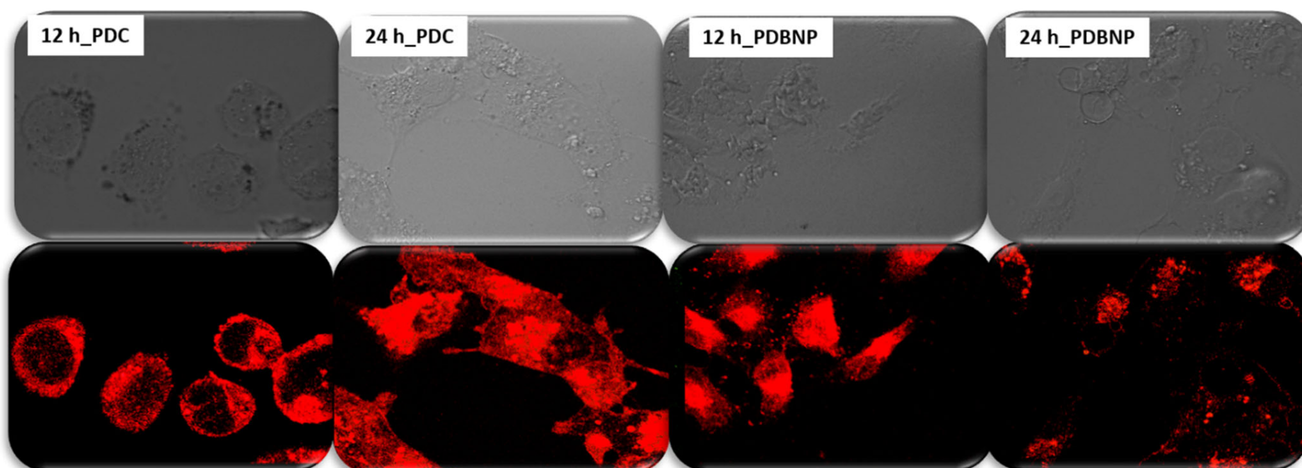


Fig. 11 Confocal microscopy images (along with contrast images) obtained at time interval of 12 h and 24 h, respectively, upon treatment with ions PDC and PDBNP nanoparticles. The data suggest efficient drug uptake by the cellular compartment during the period of study.

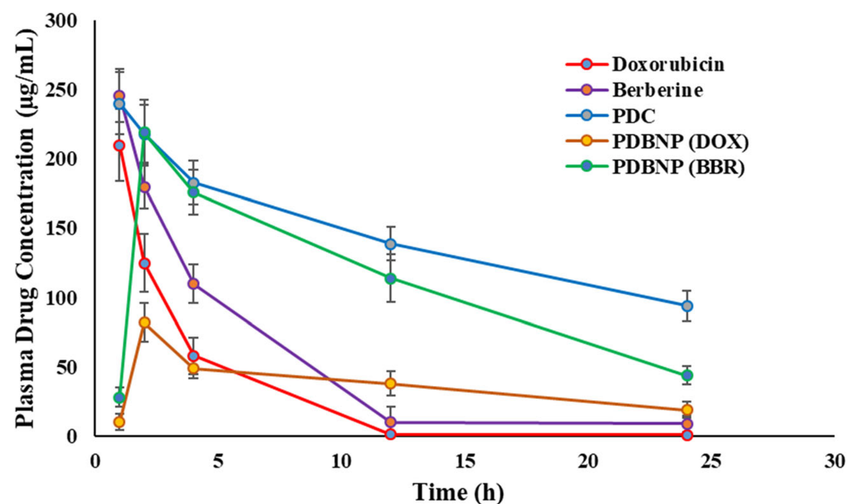


Fig. 12 Plasma drug concentration-time curve for doxorubicin, berberine, PDC and PDBNP. Values represent mean ± SD (*n* = 3). PDC: PLGA-doxorubicin conjugate; PDBNP: Berberine loaded PLGA-doxorubicin nanoparticles.

favourable results as per as *in vitro* studies are concerned.

The formed nanoparticles were found to exhibit excellent anti-proliferative potential as compared to individual drugs/bio-actives against MDA-MB-231, and T47D breast cancer cell lines. The formulations also portrayed selective toxicity towards cancer cells as compared to normal hPBMCs. PDBNP exhibited the slight increase in the ROS pattern in the MDA-MB-231 cell line in a dose-dependent manner but in case of T47D cells, no major and significant ROS rise was seen even up to the highest concentration. The PDBNP formulation was found to alter the mitochondrial potential significantly thus proving their mode of cell death via mitochondrial dependent pathway beside causing cell cycle arrest at Sub G1 phase (along with G2/M inhibition) and inducing necrosis as mechanism of cell death as inferred by Annexin V/propidium iodide assay, respectively. Cell uptake study further confirmed the uptake of drug formulation by breast cancer cells initially and inducing organelle burst (necrosis) as revealed by confocal microscopy. The *in vivo* pharmacokinetics revealed the significant increase in bioavailability as observed in plasma-concentration drug profile study in rat model *in vivo* leading to significant improvement in overall half-life. The overall approach via co-delivery of doxorubicin and berberine, using PLGA will improve the synergetic effects and reduced the toxicity associated with drugs.

Usually co-delivery of drugs has been reported with the physical encapsulation approaches in the past using drug carriers such as liposomes, nanoparticles, dendrimers etc. In the current approach, however, it was attempted to conjugate one drug followed by encapsulation of another natural bio-active which resulted into improved activity *in vitro* and improved kinetic parameters *in vivo*. Conjugation of both drugs together can be challenging and can be another aspect which can be explored in future. The future protocol would also include the assessment of the nanoparticles in tumor induced animal model.

Table II Different Pharmacokinetic Parameters of the Doxorubicin, Berberine, PDC and PDBNP Obtained from *In-Vivo* Studies

Parameter	Doxorubicin	Berberine	PDC	PDBNP	
				Doxorubicin	Berberine
$AUC_{0\text{-}t}$ (μg mL^{-1}/h)	694.9 ± 7.28	1112 ± 68.05	2478.5 ± 154.05	2765 ± 51.01	1218.3 ± 25.04
$AUC_{0\text{-}\infty}$ (μg mL^{-1}/h)	699.51 ± 8.14	1171.60 ± 14.55	5878.5 ± 29.34	13,120.82 ± 158.05	3473.77 ± 85.55
K (h^{-1})	0.2385 ± 0.089	0.151 ± 0.047	0.046 ± 0.003	0.0163 ± 0.0071	0.0274 ± 0.0069
$t_{1/2}$ (h)	2.90 ± 0.284	4.58 ± 0.39	15.06 ± 1.98	42.51 ± 3.47	25.29 ± 4.01
V_d (L)	1.29 ± 0.047	1.20 ± 0.079	1.19 ± 0.059	1.15 ± 0.073	1.386 ± 0.048
Cl (L/h)	0.307 ± 0.054	0.181 ± 0.084	0.024 ± 0.005	0.0188 ± 0.0015	0.0379 ± 0.0024

PDC: PLGA-doxorubicin conjugate; PDBNP: Berberine loaded PLGA-doxorubicin nanoparticles

Here, $AUC_{0\text{-}\infty}$ denotes the area under curve (bioavailability), K denotes elimination rate constant, $t_{1/2}$ denotes the half-life, V_d denotes volume of distribution and Cl denotes clearance

[a] data represented as mean ± SD (n = 3)

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REFERENCES

1. Shim G, Kim MG, Kim D, Park JY, Oh YK. Nanoformulation-based sequential combination cancer therapy. Adv Drug Deliv Rev. 2017;115:57–81.
2. Hu Q, Sun W, Wang C, Gu Z. Recent advances of cocktail chemotherapy by combination drug delivery systems. Adv Drug Deliv Rev. 2016;98:19–34.
3. Gautam CS, Saha L. Fixed dose drug combinations (FDCs): rational or irrational: a view point. Br J Clin Pharmacol. 2008;65:795–6.
4. Ducreux M, Malka D, Mendiboure J, Etienne PL, Texereau P, Auby D, et al. Sequential versus combination chemotherapy for the treatment of advanced colorectal cancer (FFCD 2000-05): an open-label, randomised, phase 3 trial. Lancet Oncol. 2011;12: 1032–44.
5. Parhi P, Mohanty C, Sahoo SK. Nanotechnology-based combinational drug delivery: an emerging approach for cancer therapy. Drug Discov Today. 2012;17:1044–52.
6. Qi SS, Sun JH, Yu HH, Yu SQ. Co-delivery nanoparticles of anticancer drugs for improving chemotherapy efficacy. Drug Delivery. 2017;24:1909–26.
7. Kemp JA, Shim MS, Heo CY, Kwon YJ. "Combo" nanomedicine: co-delivery of multi-modal therapeutics for efficient, targeted, and safe cancer therapy. Adv Drug Deliv Rev. 2016;98:3–18.
8. Kleibl Z, Kristensen VN. Women at high risk of breast cancer: molecular characteristics, clinical presentation and management. Breast. 2016;28:136–44.
9. Nabholtz JM, Falkson C, Campos D, Szanto J, Martin M, Chan S, et al. Docetaxel and doxorubicin compared with doxorubicin and cyclophosphamide as first-line chemotherapy for metastatic breast cancer: results of a randomized, multicenter, phase III trial. J Clin Oncol. 2003;21:968–75.
10. Henderson IC, Berry DA, Demetri GD, Goldstein LJ, Martino S, Ingle JN, et al. Improved outcomes from adding sequential paclitaxel but not from escalating doxorubicin dose in an adjuvant chemotherapy regimen for patients with node-positive primary breast cancer. J Clin Oncol. 2003;21:976–83.
11. Burger H, Foekens JA, Look MP, Meijer-van Gelder ME, Klijn JG, Wiemer EA, et al. RNA expression of breast cancer resistance protein, lung resistance related protein, multi-drug resistance gene-1 in breast cancer: correlation with chemotherapeutic response. Clin Cancer Res. 2003;9:827–36.
12. Anitha A, Maya S, Deepa N, Chennazhi KP, Naira SV, Tamura H, et al. Efficient water-soluble O-carboxymethyl chitosan nanocarrier for the delivery of curcumin to cancer cells. Carbohydr Polym. 2011;83:452–61.
13. Murugan C, Rayappan K, Thangam R, Bhanumathi R, Shanthi K, Vivek R, et al. Combinatorial nanocarrier based drug delivery approach for amalgamation of anti-tumor agents in breast cancer cells: an improved nanomedicine strategy. Sci Rep. 2016;6:34053.
14. Gupta L, Sharma AK, Gothwal A, Khan MS, Khinchi MP, Qayum A, et al. Dendrimer encapsulated and conjugated delivery of berberine: a novel approach mitigating toxicity and improving in vivo pharmacokinetics. Int J Pharm. 2017;528:88–99.
15. Ebrahimian M, Taghavi S, Ghoreishi M, Sedghi S, Farzad SA, Ramezani M, et al. Evaluation of efficiency of modified polypropylenimine (PPI) with alkyl chains as non-viral vectors used in co-delivery of doxorubicin and TRAILplasmid. AAPS Pharm Sci Tech. 2017;19:31029–36.
16. Han Y, Zhang P, Chen Y, Sun J, Kong F. Co-delivery of plasmid DNA and doxorubicin by solid lipid nanoparticles for lung cancer therapy. Int J Mol Med. 2014;34(1):191–6.
17. Khan I, Gothwal A, Sharma AK, Kesharwani P, Gupta L, Iyer AK, et al. PLGA nanoparticles and their versatile role in anticancer drug delivery. Cri Rev Ther Drug Carr Sys. 2016;33(2):159–93.
18. Khan I, Gothwal A, Sharma AK, Qayum A, Singh SK, Gupta U. Biodegradable nano-architectural PEGylated approach for the improved stability and anticancer efficacy of bendamustine. Int J Bio Macromol. 2016;92:1242–51.
19. Desgouilles S, Vauthier C, Bazile D, Vacus J, Grossiord JL, Veillard M, et al. The design of nanoparticles obtained by solvent evaporation: a comprehensive study. Langmuir. 2003;19(22): 9504–10.
20. Malvern. ISO13320 2009. Particle Size Analysis - Laser Diffraction Methods, Part 1: General Principles.
21. Pamunuwa G, Karunaratne V, Karunaratne DN. Effect of lipid composition on in vitro release and skin deposition of curcumin encapsulated liposomes. J Nanomaterials. 2016:1–9.
22. Ahmad A, Fauzia E, Kumar M, Kumar R. Gelatin-coated Polycaprolactone nanoparticle-mediated Naringenin delivery rescue human mesenchymal stem cells from oxygen glucose deprivation-induced inflammatory stress. ACS Biomater Sci Eng. 2019;5(2):683–95.
23. Kumar H, Gothwal A, Khan I, Nakhate KT, Alexander A. Ajazuddin, et al. galactose anchored gelatin nanoparticles for primaquine delivery and improved pharmacokinetics: a biodegradable and safe approach for effective anti-plasmodial activity against *P. falciparum* 3D7 and in vivo hepatocytes targeting. Mol Pharm. 2017;14:3356–69.
24. Chin DL, Lum BL, Sikic BI, et al. Rapid determination of PEGylated liposomal doxorubicin and its major metabolite in human plasma by ultraviolet-visible high-performance liquid chromatography. J Chromato B. 2002;779:259–69.
25. Tsai PL, Tsai TH. HPLC determination of berberine in medicinal herbs and a related traditional chinese medicine. Analytical Lett. 2002;35(15):2459–70.
26. Agarwal GU, Jain NK. Glycoconjugated peptide dendrimers-based nanoparticulate system for the delivery of chloroquine phosphate. Biomaterials. 2007;28:3349–59.
27. Singhai AK, Jain S, Jain NK. Evaluation of an aqueous injection of Ketoprofen. Pharmazie. 1997;52:149–51.
28. Jain AK, Thanki K, Jain S. Co-encapsulation of tamoxifen and quercetin in polymeric nanoparticles: implications on oral bioavailability, antitumor efficacy, and drug-induced toxicity. Mol Pharm. 2013;10:3459–74.
29. Yoo HS, Lee KH, Oh JE, Park TG. In vitro and in vivo anti-tumor activities of nanoparticles based on doxorubicin-PLGA conjugates. J Control Release. 2000;68:419–31.

30. Wanga X, Wanga Q, Liua Z, Zheng X. Preparation, pharmacokinetics and tumour-suppressive activity of berberine liposomes. J Pharm Pharmacol. 2017;69:625–32.
31. Fan X, Wang X, Cao M, Wang C, Hu Z, Wu Y-L, et al. "Y"-shape armed amphiphilic star-like copolymers: design, synthesis and dual-responsive unimolecular micelle formation for controlled drug delivery. Polym Chem. 2017;8:5611–20.
32. Langdon SP. Cancer cell culture, methods and protocols, methods in molecular medicine, Humana Press, 2004.
33. Meerloo JV, Kaspers GL, Cloos J. Cell sensitivity assays: the MTT assay. Cancer Cell Culture. 2011:237–45.
34. Maksimenkoa A, Dosiob F, Mougina J, Ferrerob A, Wacka S, Reddya LH, et al. A unique squalenoylated and nonpegylated doxorubicin nanomedicine with systemic long-circulating properties. Proc Natl Acad Sci. 2014;111(2):E217–26.
35. Cheng J, Teply BA, Sherifi I, Sung J, Luther G, Gu FX. Formulation of functionalized PLGA-PEG nanoparticles for in vivo targeted drug delivery. Biomaterials. 2007;28:869–76.
36. Diogo CV, Machado NG, Barbosa IA, Serafim TL, Burgeiro A, Paulo J. Berberine as a promising safe anti-Cancer agent-is there a role for mitochondria? Oliveira. Curr Drug Targets. 2011;12:850–9.
37. Schneckenburger H, Stock K, Lyttek M, Strauss WS, Sailer R. Fluorescence lifetime imaging (FLIM) of rhodamine 123 in living cells. Photochem Photobiol Sci. 2004;3:127–31.
38. Borodina VM, Zelenin AV. Fluorescence microscopy demonstration of mitochondria in tissue culture cells using berberine. Tsitologiia. 1977;19:1067–8.
39. Pereira CV, Machado NG, Oliveira PO. Mechanisms of Berberine (natural yellow 18)-induced mitochondrial dysfunction: interaction with the adenine nucleotide translocator. Toxicological Sci. 2008;105(2):408–17.
40. Lüpertz R, Wätjen W, Kahl R, Chovolou Y. Dose and time-dependent effects of doxorubicin on cytotoxicity, cell cycle and apoptotic cell death in human colon cancer cells. Toxicology. 2010;271:115–21.
41. Mahmoudi M, Azadmanesh K, Shokrgozar MA, Journeay WS, Laurent S. Effect of nanoparticles on the cell life cycle. Chem Rev. 2011;111:3407–32.

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