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RESEARCH PAPER

Drug Delivery Using Platelet Cancer Cell Interaction

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ABSTRACT

Purpose To develop an efficient biocompatible and targeted drug delivery system in which platelets, an essential blood component having a natural affinity for cancer cells, are used as carrier of anticancer drug as delivery of drug to the targeted site is crucial for cancer treatment.

Methods Doxorubicin hydrochloride, a potent anti cancer drug, was delivered in lung adenocarcinoma cell line (A549) using platelet as a delivery agent. This delivery mode was also tested in Ehrlich ascites carcinoma (EAC) bearing mice in presence and absence of platelets.

Results The results show that platelets can uptake the drug and release the same upon activation. The efficiency of drug loaded platelets in inducing cytotoxicity was significantly higher in both *in vitro* and *in vivo* model, as compared to the free drug.

Conclusions The proposed drug delivery strategy may lead to clinical improvement in the management of cancer treatment as lower drug concentration can be used in a targeted mode. Additionally the method can be personalized as patient's own platelet can be used for deliver various drugs.

KEY WORDS cancer cell line · doxorubicin · drug delivery · EAC · platelets

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ABBREVIATIONS

Dox	Doxorubicin hydrochloride
EAC	Ehrlich ascites carcinoma
PPP	Platelet poor plasma
PRP	Platelet rich plasma
WP	Washed platelet

INTRODUCTION

Drug delivery system in a controlled manner is as important as discovery of new drugs (1). For the sake of improved health care, newer approaches are employed to make drug delivery more efficient and targeted. There has been a three decade long popularity of the liposome mediated techniques (2–4). Some of liposome based, lipid based and even nanoparticle based formulations have been approved or selected for final phase clinical trial (5). Though the anti tumor efficacy as well as targeting is increased for some drugs using liposomal encapsulation (3,6), there are some problems with side effects of this liposomal drug (7). The major drawbacks of this delivery system are as follows: liposome mostly carries hydrophilic drugs, as hydrophobicity of hydrophobic drug interferes with the release of the drug from liposome (8,9). Secondly, liposome is eliminated from the blood rapidly (3). Many such limitations can be overcome by the nanoparticle mediated drug delivery. With the advent of nano-biotechnology, it is felt that the liposome era is going to end leaving future scope for nano-biotechnology (10,11). However the metallic (or even polymeric) nanoparticle systems have the problem of limited biodegradability (12). Toxicity and adverse immune-response constitutes the other limitation for many of these approaches (13–15).

Any smart and controlled delivery system that can efficiently target tissues or organs should retain its (drug) bioavailability without causing any adverse physiological response. Secondly, release of a given drug in the target site in a controlled manner would be of great therapeutic interest.

Our choice for platelet as a smart delivery systems stems from the following facts. Firstly, the platelet target is well defined, namely injury sites or sites with higher density of proliferating cells (16–20). Secondly, the perspective from which we can attribute the "smart" nature is that platelets can be made to release the drug by artificially exposing them to agonists. Thirdly, the most important aspect of the choice stems from the personalized nature of the delivery system, as one can in principle use the platelet of a given diseased subject as carrier of a drug he/she is treated with.

The main function of platelet is prevention of blood loss at sites of vascular injury. During this time platelets first adhere to the vessel wall, undergo a release reaction and then aggregate, called normal hemostasis and thereby preventing blood loss (16,17). Beside this, platelets also have a role in tumor prognosis and it reportedly takes place during the time of metastasis of tumor cell. Interaction with circulating platelets actually helps the tumor to assume metastasis and to invade the host defense (18–20). Platelets form aggregates with tumor cells in circulation facilitating their adhesion to the vascular endothelium (18). Activation of platelets by tumor cells is considered as an important step. Activation of platelets leads to release of the granular matter (dense granules). The concept that inspires the present work is that a drug loaded platelet would also be similarly activated, and consequently would release the drug along with the granules to the target site (for example circulating tumor cells). The concept has been verified using *in vitro* and *in vivo* cancer models. We believe that this study will provide new insights into the drug delivery field.

MATERIALS AND METHODS

Animals

Male Swiss albino mice weighing 20±2 g and male white rabbit (age 8–10 weeks, weighing 2.0±0.5 kg) were purchased from approved animal breeders. All mice (housed five/cages) and rabbits (housed individually) were fed standard laboratory diet and water *ad libitum* with 12-h dark/light cycles and constant temperature of 25±2°C. The animals were allowed 1 week to adapt to their environment. All animal experiments were performed following 'Principles of laboratory animal care' (NIH publication No. 85–23, revised in 1985) and specific Indian laws on 'Protection of Animals' under the prevision of authorized investigators.

Ehrlich Ascites Carcinoma

Ehrlich ascites carcinoma (EAC) cells were collected from the ascitic fluid of Swiss albino mice harbouring 10–15 days old ascitic tumor. 1×10^6 EAC cells were injected intra peritoneally (*i.p*) in 25 male Swiss albino mice selected for the experiment on day 0. The next day, animals were randomized and divided into five groups and treatment started. After 10 days of successive treatment, the animals were kept in fasting for next day and then sacrificed. The details of treatment groups are presented in Table I.

Platelet Isolation and Washing

From Human Blood

9 ml blood was drawn from normal volunteers with permission of the institutional ethical committee and the written consents of the individual were taken prior to testing. A total of 9 ml of blood was mixed in 1 ml of 3.2% sodium citrate anticoagulant (final ratio 9:1 whole blood/citrate). PRP (platelet rich plasma) was obtained after centrifuging blood at 200×g for 12 min. PPP (platelet poor plasma) was obtained by centrifugation of blood at 1,500×g for 10 min (14) and served as blank for aggregometric study.

Isolated PRP was incubated for 15 min at 37°C. Washed platelet (WP) from PRP was isolated by sepharose 2B column. Cells were eluted in slightly modified complete HEPES buffer (137 mM NaCl, 11.9 mM $NaHCO_3$, 2.6 mM KCl, 10 mM HEPES, 5.5 mM dextrose, glucose- 0.1% W/V, pH 7.4) (21). All steps were carried out under sterile conditions, and precautions were taken to prevent undesirable activation of the platelets. All *in vitro* experiments were done with isolated human platelets within 4 h of blood draw.

From Rabbit Blood

5 ml blood was drawn very carefully from marginal ear vein of rabbit. 9 volume of blood was mixed with 1 volume of 3.2% tri-sodium citrate. The mixture was centrifuged at 200×g for 20 min to obtain PRP.

Platelet was washed by centrifuging PRP to 400×g for 5 min and resuspended the pellet in HEPES buffer. Rabbit platelet was used to treat the mice groups. All experiments with platelets were completed within 4 h of blood collection (30). After drug loading to these platelets (mentioned in "Drug Loading into Platelets" section), platelet count was adjusted to $2.5–3.0\times10^5$ per µl and injected into the peritoneal cavity of mice.

Drug Loading into Platelets

Drug loading procedure to platelet was same for both human and rabbit source. Fluorescence enabled Doxorubicin hydrochloride (Dox) (from Sigma), a potent anticancer drug, dissolved in filtered Mili-Q water, was added in the washed

Table I Detailed Animal Groups and Treatment Schedule

Group	Name	Description	Number of mice
I	HEPES Control	0.1 mL of HEPES injected, i.p, in EAC bearing mice from day 1 to 10	5
II	EAC Control	0.1 mL of MiliQ water injected in EAC bearing mice, i.p. from day 1 to 10	5
III	Platelet treated	0.1 mL of platelet in HEPES injected, i.p., in EAC mice from day 1 to 10	5
IV	Dox treated	0.1 mL of Dox in HEPES injected, i.p., in EAC mice from day 1 to 10	5
V	Dox-Platelet treated	0.1 mL of Dox loaded platelet in HEPES injected, i.p., in EAC Mice from day 1 to 10	5

platelet (platelets suspended in HEPES buffer). Final concentration of Dox used was 0.58 nM to carry out *in vitro* experiments and that of 2.32 nM for *in vivo* experiments. This drug mixed platelet suspension was incubated at 37°C for 1 h at dark. Further experiments were done after that. For loading of drug into platelets in PRP, same procedure was followed. The possible changes in the platelet size and morphology are studied using FSC profiles of the platelets before and after drug loading (see Figure S1 of the Supplementary Material). While the relative size information is obtained in Figure S1, the Figure S2 provides the size details using atomic force microscopy. Tapping mode was used and the study was undertaken using Bruker (model Innova).

Isolation of EAC from Mice Peritoneal Cavity

EAC cells from both control and treated were isolated from the peritoneal cavity of tumor-bearing mice. 3 ml of sterile normal saline was injected into the peritoneal cavity of the mice and the peritoneal fluid containing the tumor cells was withdrawn, collected in sterile measuring cylinder, to measure the volume of the peritoneal fluid. Then they were transferred to sterile glass Petri dishes and incubated at 37°C for 1 h. The cells of macrophage lineage adhered to the bottom of the Petri dishes. The non-adherent population was aspirated out gently and washed repeatedly with PBS. The viability of these cells was assessed to be 97% by Trypan Blue dye exclusion. The viable EAC cells were processed for further experiments (27,28).

Cell Culture

A549, human lung carcinoma cell line, was procured from NCCS, Pune, India and was maintained in Ham's F12 media (Gibco-BRL, NY) supplemented with 10% heat-inactivated foetal bovine serum (Gibco-BRL, USA), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco-BRL, USA). The cell line was treated with Dox loaded platelet (isolated from human). Procedure for drug loading into platelets has already been described (in "Materials and Methods" section "Drug Loading into Platelets"). Prior treatment, Dox incubated washed platelet was centrifuged at 1,500×g and supernatant was discarded carefully so the chance of presence of significant concentration of free Dox is minimal. The pellet was then resuspended in same amount of HEPES buffer. The cultured A549 cells plated as 1×10^6 cells per ml were treated with the resuspended pellet aseptically. Control for this was prepared by lysing same amount of drug loaded platelets by SDS. Fluorescence was measured by HITACHI (F-7000) fluoremeter and concentration of Dox was obtained by a fluorescence standard curve (Supplementary Materials Fig 2) of Dox. That amount of Dox was used as control and treated on A549 cell line. All experiments with the treated cell line were done after 24 h.

Confocal Microscopic Study

Slides were prepared for microscopic examination of platelets for their drug uptake property. In brief, 10 µl of drug incubated platelet suspension was taken in a clean and grease free glass slide. A cover slip was then placed on the slide and edges were sealed. The slides were then seen under a laser confocal microscope of Olympus model no IX 81 FV 1,000 with 20× and 60× objective lenses.

Flow Cytometry

Drug loaded WP and ADP were used to understand the efficacy of platelets for releasing drug upon activation by agonist like ADP (final concentration 10 µM). In short, sepharose 2B column washed platelet was incubated with drug (final concentration 0.58 nM) for 1 h at 37°C. Then this drug loaded platelet was subjected to activation by ADP in a chrono-log aggregometer (model 700) with stirring speed 1,000 rpm for 5 min. Finally the samples were analyzed in a Becton Dickinson FACS Calibur flow cytometer. Drug loaded platelet without ADP treatment play the role of control here.

Inverted Phase Contrast Microscopy

A549 cell after treatment was investigated under a phase contrast microscope (Nikon, eclipse-Ti-U, Japan) for their change in morphology. In brief, 1×10^6/ml A549 cells were plated in 35 mm dish (from nunc) and treated with Dox loaded platelet. Only Dox and only platelet were also used

as Dox control and platelet control respectively. Untreated A549 cell was used as control. After 24 h of incubation, the plates were washed by PBS (Gibco-BRL, USA) and seen under microscope using 10× objective lens.

Apoptosis Study

AO- EtBr Double Staining

Fluorescence microscope was used to study the nuclear integrity and membrane permeability of the EAC cells. Both treated and untreated EAC cells were collected from different animals and centrifuged at 1,000 rpm for 5 min. The pellet was rinsed twice and re-suspended in PBS. It was then treated with acridine orange (AO) and ethidium bromide (EtBr) solution (4 μg/ml) and observed under an Olympus (Model no. IX81) fluorescence microscope for the qualitative determination of apoptotic cells.

Single Cell Gel Electrophoresis (SCGE) Assay

Single Cell Gel Electrophoresis or Comet assay is a standard technique to detect deterioration of DNA in cells. The EAC cells isolated from Swiss albino mice were mixed with 1% low melting agarose (LMA) and layered over pre-coated glass slides and incubated at 0°C for 5°mins. Then a third layer of 1% LMA was applied on it and incubated at 0°C for 5°mins. Then slides were dipped in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, 1% Triton X-100) and incubated at 4°C for overnight in the dark. After lysis, slides were kept in alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, ph > 13) for 20 min and then run at a voltage of 24 V/300 mA. After that slides were neutralised. For neutralisation, slides were incubated with neutralising buffer (0.4 M Tris, pH 7.4) and incubated for 5 min at room temperature. This step is repeated twice. The slides were then stained with 1X EtBr and incubated for 5 min and then washed with chilled milliQ water and blotted by tissue paper and covered with a glass cover slip and observed under fluorescence microscope (29). Image of comets were captured with 10× objective and analyzed by the freely available comet program COMET SCORE.

Cell Cycle Assay

Cell-cycle assay for the amount of DNA distribution in different phase was determined by staining DNA with PI (Propidium iodide) (Sigma). In brief, nearly 1×10^6 EAC cells, after isolation and washing, were fixed in 70% ethanol and kept for overnight at −20°C for 24 h. The fixed cells were washed twice with PBS and then subjected to cell cycle analysis kit from BD Bioscience, USA. Then flow cytometry was done to determine the percentage of cells in different phases of cell cycle using a FACS Calibur (BD Biosciences, USA) instrument.

RESULTS

Drug Uptake by Platelets

The Confocal microscopic analysis (Fig. 1) revealed that platelets can efficiently uptake drug (fluorescent enabled Doxorubicin hydrochloride). Figure 1a and b respectively represent the bright field and the fluorescent image of platelets at 20× magnification. Figure 1c shows the overlap of Figs. 1a and b. Figure 1d shows the fluorescent image at 60× magnification with 2× optical zoom.

Drug Release by Platelet

Figure 2 represents the drug releasing capacity of Dox loaded WP when activated by ADP. The FACS analysis revealed that the upper right population decreases from 65 ± 7% to 36 ± 8% when ADP is added to drug loaded platelet (Fig. 2b). Figure 2a is the representative dot plot that clearly states that the upper right population decreases from 61.58% to 32.27% (Panels a (ii) and a (iii) of Fig. 2). The fluorescence intensity peak shown in Fig. 2c (Fl2-distribution), shows a left shift of the histogram peak upon activation by 10 μM ADP. This implies release of Dox from loaded platelets (as the free drug will not contribute any signal to the FACS detector).

During ADP activation of drug loaded platelet the gated population decreases significantly (Fig. 2d). This figure clearly states that, the M2 population (with count higher than the gated value) of loaded platelet decreases from 62.18% to 37.46% after activation by ADP.

In Vitro Morphology Change of A549

Change in the morphology of adherent cells is a characteristic of cytotoxic response. The morphology change of A549 cells after treatment with drug loaded platelets is seen clearly in the phase contrast microscope in lower (10×) magnification (Fig. 3). Figure 3a, b, c and d respectively represents the phase contrast images of untreated A549 cell, Dox control (cells treated with Dox solution, corresponding to the concentration of Dox loaded platelet), platelet control (cells treated with platelets without Dox) and drug loaded platelet treated cells. Notably, the morphology of drug loaded platelet treated A549 cells in Fig. 3d is different from other controls (Fig. 3a, b and c).

Effect on EAC Cells

Fluid Accumulation in Peritoneal Cavity

EAC cells increased in the peritoneal cavity of mice via rapid cell division during proliferating phase. This proliferation of the

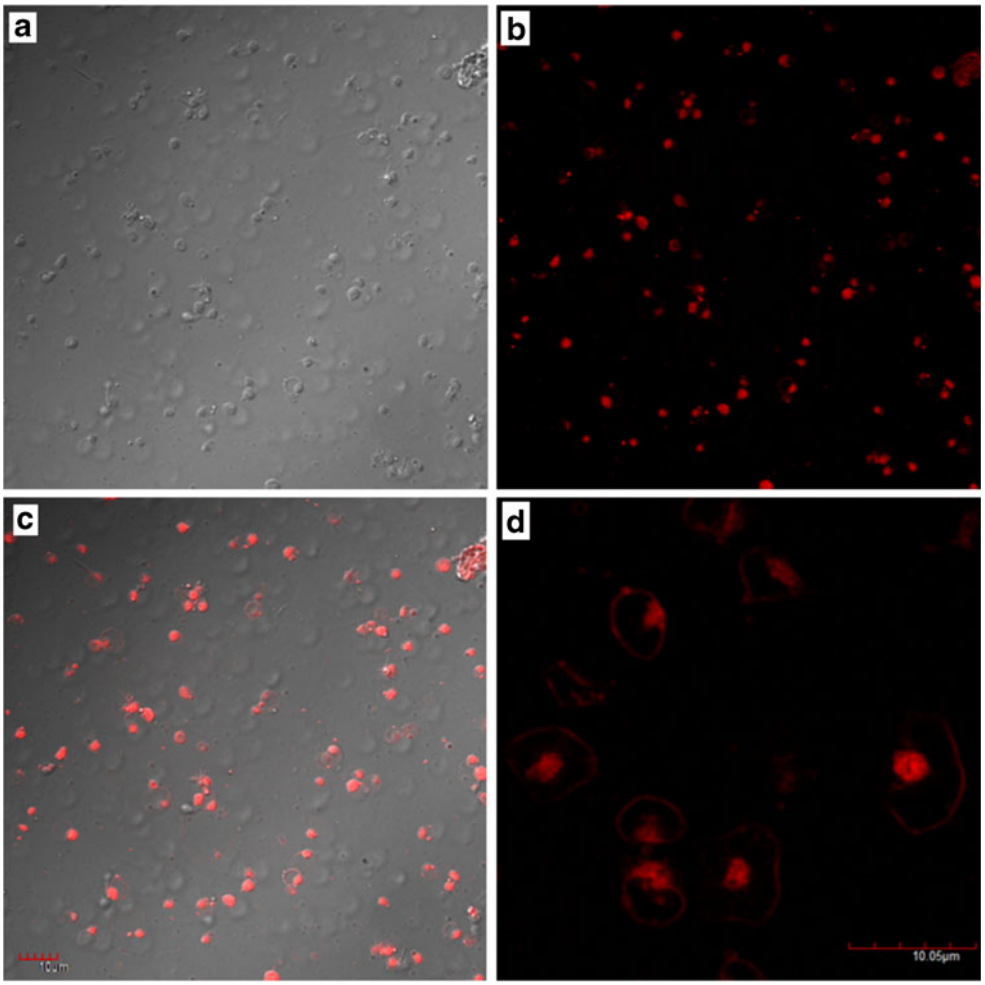

Fig. 1 Confocal microscopic image of Dox loaded platelet. Bright field (**a**), fluorescent (**b**), and marged image (**c**) of (**a**) and (**b**) were captured at 20× magnification. (**d**) Image of Dox loaded platelet captured at 60× magnifications with 2× optical zoom.

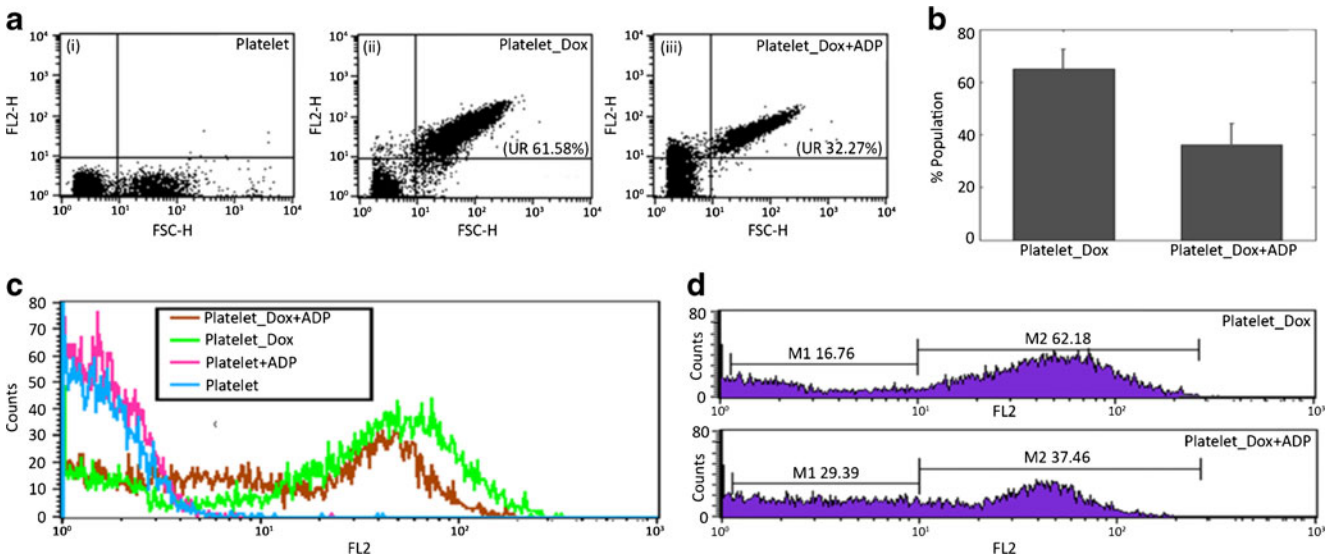

Fig. 2 Release of drug from platelets upon ADP activation. (i), (ii) and (iii) of (**a**) shows the FSC *vs.* FL2 dot plot and histogram plot (**c**) of washed platelet, Dox loaded WP and ADP activated Dox loaded WP respectively. (**b**) Average of upper right quadrants of dot plots of Dox loaded platelets and ADP activated of the same. Sample number is five. (**d**) The gated population (M2) becomes lower in case of ADP activated Dox loaded platelet.

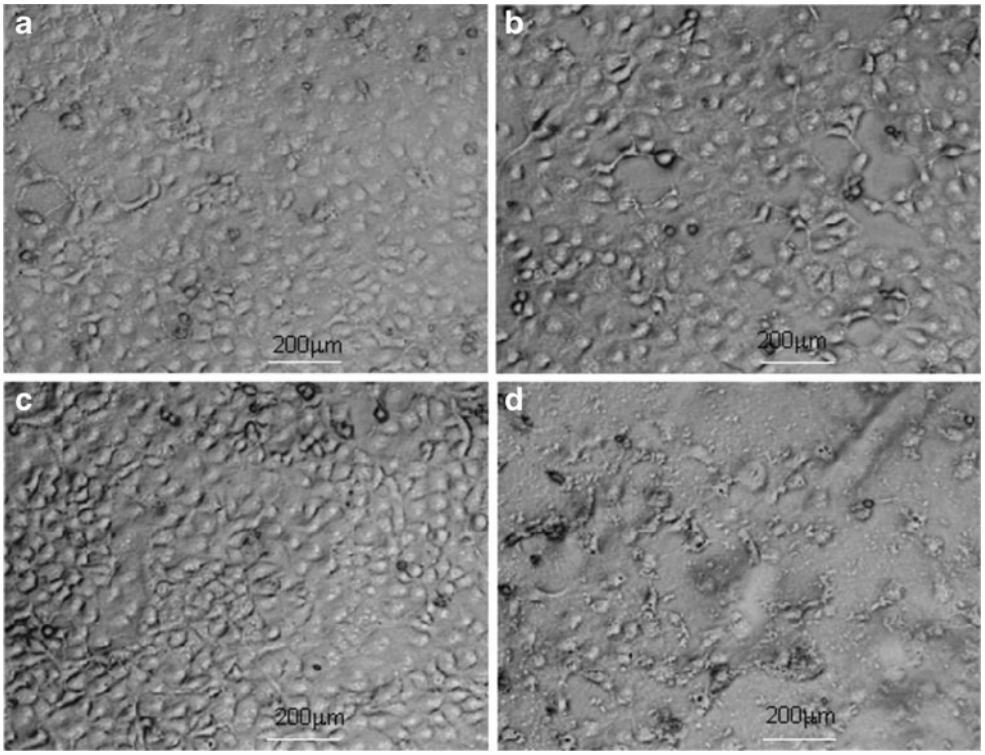

Fig. 3 Phase contrast inverted microscopic image of control (**a**), free Dox (**b**), only platelet (**c**) and Dox loaded platelet treated (**d**) cells A549 cell at 10X magnification.

EAC cells accumulates ascites fluid in parallel. So the volume of collected fluid from treated and untreated group is a reflection of the growth inhibition of EAC cells in the peritoneal cavity. Figure 4 shows how the EAC cell growth is modulated when platelet is used as a delivery agent for Dox. The volume of ascitic fluid (V_{EAC}) showing the minimal value in this case implying the maximal growth inhibition of the EAC cells

Double Staining

Double staining by AO and EtBr is widely used to differentiate dead cells from live cells. Live cells appear green for AO and dead cells look orange for EtBr. Figure 5 is the merged microscopic image of green and red filter of double stained EAC cells isolated from treated and untreated mice. From left to right, the images represent EAC control, platelet treated, Dox loaded platelet treated, Dox treated and HEPES treated cells respectively. From Fig. 5 it is clear that the number of live cells markedly decreases in Dox loaded platelet treated (platelet_Dox treated) EAC cells compared to any other set. Presence of apoptotic body in this group suggests that cell death occurs via apoptosis.

Comet Assay

Fragmentation of DNA is another characteristic of apoptosis. Comet assay or SCGE is one such assay in which detection of DNA fragmentation in a single cell level is possible. From Fig. 6, it is clear that fragmentation of DNA and thus comet formation occurs in case of Dox loaded platelet treated EAC cells compared to any other group. The lower panel of Fig. 6 represents from left to right the comet image of EAC cells isolated from platelet treated, Dox loaded platelet treated, Dox treated, HEPES treated and EAC control group respectively. The upper panel is the comparison of the tail moments (with arbitrary unit) in Y-axis of the above

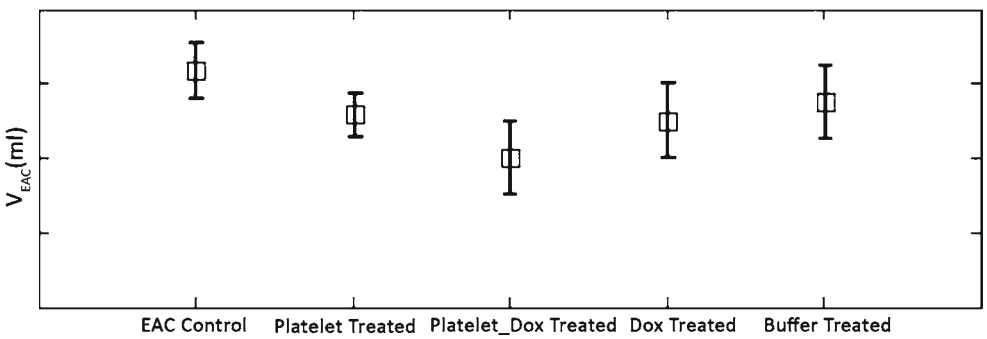

Fig. 4 Volume of ascitic fluids collected from treated and untreated groups of mice. Y axis, denoted as V_{EAC}, represents the volume of fluids in millilitre (ml). In X axis, the name of the groups are indicated.

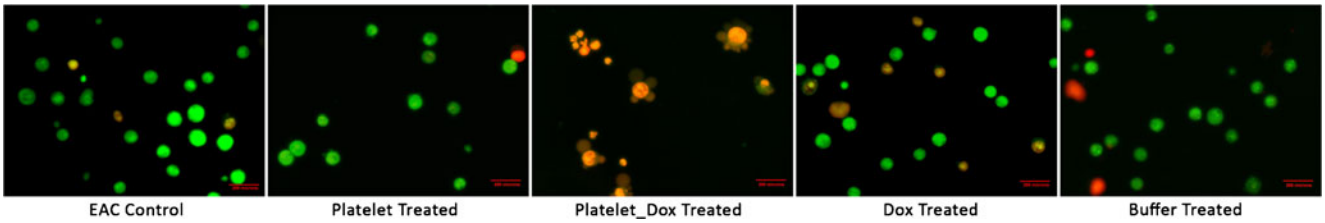

Fig. 5 Acridine orange/EtBr double staining of EAC cells. From left to right, the images represents EAC control, platelet treated, Dox loaded platelet treated, Dox treated and HEPES buffer treated groups respectively.

mentioned groups with same sequence. Amount of DNA fragmentation is proportional to tail moments and are calculated by comet score software taking spot of more than 100 single cells of each sample. This is clear from Fig. 6 that comet formation is significantly higher when EAC bearing mice were treated with Dox loaded platelets as compared to other controls.

Cell Cycle

As Dox mediated treatment primarily acts on cell cycle, so cell cycle analysis was performed with isolated EAC cells from treated and untreated sets of mice. The population distribution of cells at different phases of cell cycle was assessed by their DNA content. Figure 7a shows the histogram plots of 5 different sets of treated and untreated mice. M1, M2 and M3 denote the population distribution of G0/G1, S and G2/M phase of cell cycle respectively. Figure 7b is the comparison of population distribution of EAC cells in 3 different phases of cell cycle which indicates a significant increase in G2/M population for platelet_Dox treated cells. Figure 7c describes the population distribution of EAC cells from different treated and untreated groups according to their DNA content.

DISCUSSION

Platelets play a pivotal role in metastasis. In the pathological conditions, ADP is secreted from metastatic body, released into circulation and subsequently activates neighbouring platelets. Upon activation, platelets release their granular content leading to aggregation on tumor cells. This confers a protection on the tumor cells to invade host immune system and also helps them to dock in a distal place from the source (17–19).

Platelet release reactions (both alpha and dense granules) also play a crucial role in normal haemostasis (15,16). Circulating platelets can sense damage in the vessel wall in response to the exposed agonists in the wound site. On the ruptured vascular bed, platelets get a chance to interact with collagen and vWf (sub-endothelium layer) which are otherwise covered by endothelium. Binding of collagen and vWf to their corresponding platelet receptors trigger downstream signalling cascade. One of the major outcomes of this

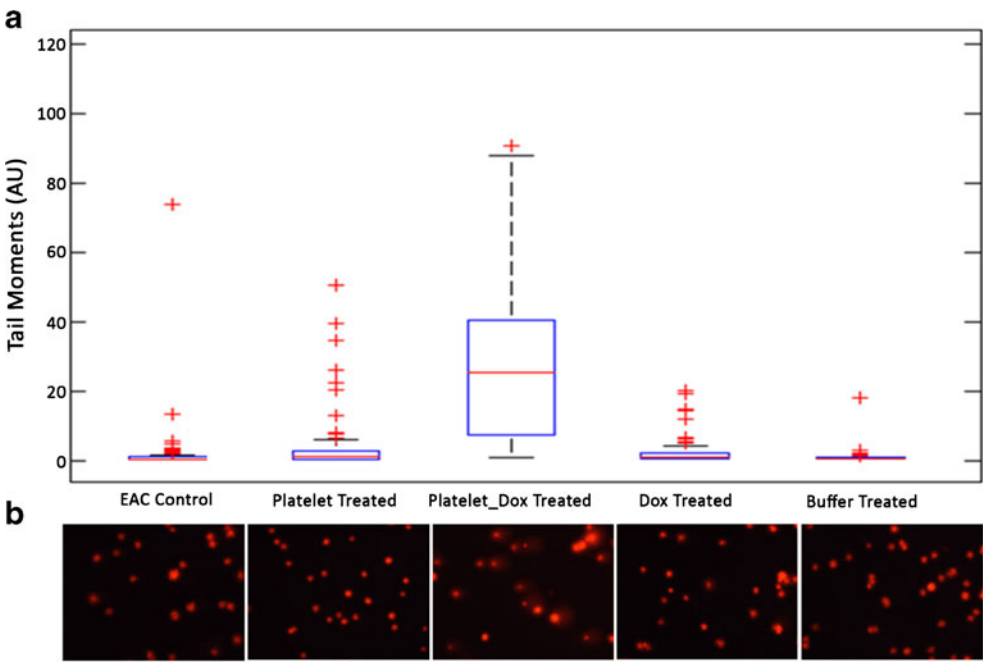

Fig. 6 Comet images of treated and untreated EAC cells. The lower panel of the image represents EAC control, platelet treated, Dox loaded platelet treated, Dox treated and HEPES buffer treated groups respectively from left to right side. The upper panel of the image represents intensity of DNA fragmentation in terms of tail moments (arbitrary unit) of treated and untreated EAC cells with same sequence. Tail moments are calculated by COMET SCORE software counting more than 100 cells from each group.

signalling is the release of platelet granules. These granules contain varieties of platelet agonists. Thus the released content of granules furthermore activates resting platelet population, therefore leads to the formation of stable platelet aggregate i.e., haemostatic plug (22,23).

Platelets uptake small neighbouring molecules or particulate matters (24). Such scavenging property differs from conventional phagocytosis. In phagocytosis the engulfed entity is ultimately metabolised inside the phagocytic cells (25) where, in case of platelet, engulfed material remains intact inside the cell. It thus acts as a covercytes (26). Uptake of soluble drugs and small particulate matter by platelets is already reported (21,26). However, to the best of our knowledge, role of platelets in drug delivery to desired destination is reported for the first time, the advantage clearly being presence of an inbuilt target (cancer cells).

The drug loaded platelet can thus be compared with a logical gate with two states '0' and '1', the '0' state representing the drug loaded covercytes and the '1' state, representing the release of the drug and destruction of the said gate after the platelet encounters a target.

As tumor cells are known targets for platelets, the transition from '0' to '1' will follow leading to drug release at the target sites

Firstly, we have shown that platelets can indeed be loaded with an anticancer drug (Fig. 1). Figure 1c shows that a substantial fraction of the fluorescent drug has been taken by the resting platelets. The number of drug molecules taken by a single platelet can be calculated using a simple argument. As seen from the Supplementary Material (Figure 1a, b) there is a monotonic relation between loading dose of drug and the bulk concentration of drug obtained after disrupting the platelet membrane. Assuming platelet volume to be of the order of 9–15 femtoL and assuming approximately 25:1 ratio of the loading and pellet contained bulk concentration of drug one may find that approximately 10^{13} molecules of drugs are

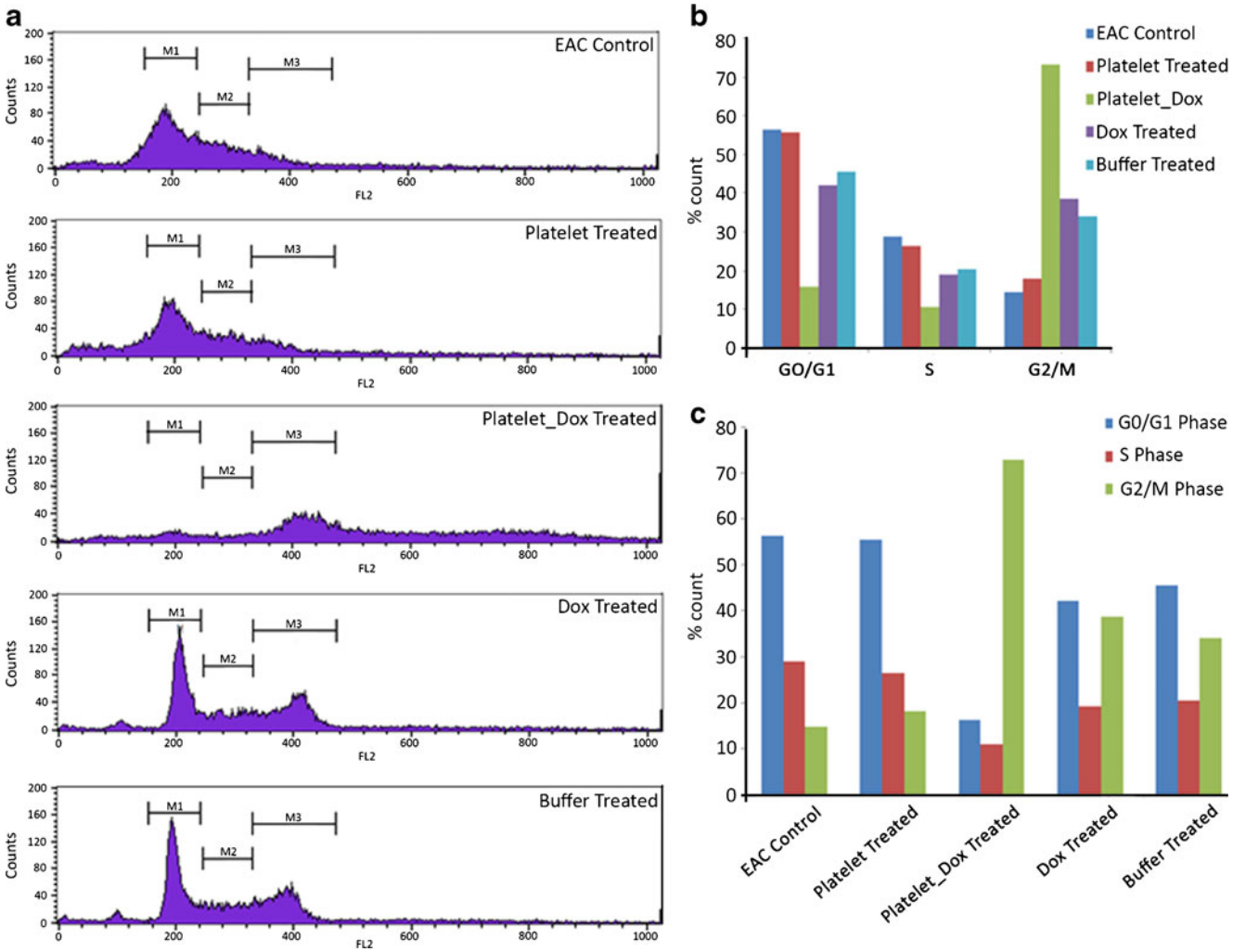

Fig. 7 Cell cycle analysis of EAC cells. (**a**) Showing histogram plot of different set of treated and untreated EAC cells. (**b**) Comparison of percentage of population distribution of cells from different groups in three different phase of cell cycle (G0/G1, S and G2/M phase). (**c**) Comparison of percentage of population distribution in three different phases of cell cycle of individual set.

contained per platelet (i.e. in 9–15 fL). The high local concentration of platelet loaded drug explains why even for a very low bulk concentration (order of μM), one obtains such effective local concentration of drug resulting in a target specific cytotoxic effect. Though drug uptake property of platelet is proportional to applied drug concentration (see Supplementary Materials 1, Fig. 1a), we have used a very low concentration of drug (0.58 nm final concentrations) as that was enough to perform *in vitro* experiments. The fact that the platelet loaded drug can be released is shown in Fig. 2, where, panel B clearly highlights the difference of % population of loaded platelet (about 65±7%) with ADP activated of same (about 36±8%). ADP induced activation reduces the fluorescent platelet population as shown by the upper right quadrant in panel A (II–III) of Fig. 2. The activated platelets (platelet_Dox_ADP) release the fluorescent drug and therefore show lesser population than the control (platelet_Dox). A tuneable delivery system must not be leaky one, before reaching to its target site. It was found that there is no spontaneous release of drug (see Supplementary Material 4).

Platelet mediated release of Dox is effective in killing cancer cells. The most striking aspect of this release is the lower requirement of drug. This in turn suggests lower exposure of normal (untargeted) cells from the drug in question, a typical hazard encountered in any chemotherapy. This is supported by the morphological evidence (see Fig. 3), in which it is clear that the dose (0.58 nM final concentration) at which virtually there is no appreciable killing of cells by the free drug (Fig. 3b) leads to significant growth inhibition with (Fig. 3d) platelet loaded drugs. This amplification may be further realizable in *in vivo* situation due to inherent targeting action by the platelets. It is always better to use same species platelets for drug loading. But we used rabbit's platelet for treatment because we needed large volume of blood to get desired amount of platelets to treat all 25 mice for 10 days. As the platelets devoid of nucleus, genomic DNA and they do not express Rh antigen, so there is less chance of cross reactivity. Moreover, to confirm this, we have injected (i.p) 1 ml of platelet solution to the mice and monitored for about 24 h. Initially, mice developed itching after 5 min of platelet injection which gradually subsides within 1 h. But we have used only 0.1 ml of platelets for treatment purpose.

Secondly, whether Dox itself alters platelet function is another important question to be answered. Our experimental results suggest that Dox does not have any adverse effect on platelets at the given dose Moreover the platelets remain stable and active in the applied drug concentration (Supplementary Material 2 and 3). Usually platelets can be stored for 5–7 days in active condition. As the applied drug concentration does not hamper platelet activity, so it is expected that loaded platelets will be active for 5–7 days. The cells grown in media (A549) and also in animal peritoneal fluid (EAC) respond to the platelet loaded drug more vigorously. In all aspects, in terms of live/dead cell staining (Fig. 5) or comet assay (Fig. 6), damage of EAC cells occurs with significant higher rate for Dox loaded platelet treatment. The tumor load in terms of volume of collected ascitic fluid from treated and untreated mice (Fig. 4) indicates the growth inhibition of EAC cells when platelets are used as vehicles of Dox. Dox acts as a chemotherapeutic drug by arresting G2/M phase of cell cycle for most cell type. The higher population of EAC cells in the G2/M region of cell cycle when treated with Dox loaded platelets (Fig. 7) strongly recommends the role of platelets as drug delivery vehicle.

Lastly, unlike nanoparticle mediated drug delivery system, the proposed method has similar if not higher dose amplifying power but is free from the undesirable aspects like toxicity (31) or lack of excretion routes. The proposed system is aided with an inherent targeting capacity and is completely integrable with the metabolic network.

CONCLUSION

An efficient biocompatible drug delivery system is developed and is tested in both *in vitro* and *in vivo* cancer models. The method is effective in killing cancer cell at a dose that is significantly lower than the prescribed dose and the chance of normal cells being exposed is also less. Two attributes of platelets, namely its tendency to uptake variety of drugs, and to release the same when activated are used. The most noteworthy point is the target specificity of platelets towards cancer cells, and flexibility in choice of platelet sources, (e.g. a person's own platelet can be used as a vehicle for drug delivery).

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