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A Simple Way to Enhance Doxil® Therapy: Drug Release from Liposomes at the Tumor Site by Amphiphilic Block Copolymer

Yi Zhao^{1,2,&}, Daria Y. Alakhova^{1,2,&§}, Jong Oh Kim^{1,2,#}, Tatiana K. Bronich^{1,2}, and Alexander V. Kabanov^{1,2,3,§,*}

¹Center for Drug Delivery and Nanomedicine, College of Pharmacy, University of Nebraska Medical Center, 985850 Nebraska Medical Center, Omaha, NE 68198-5830, USA

²Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, 985850 Nebraska Medical Center, Omaha, NE 68198-5830, USA

³Laboratory of Chemical Design of Bionanomaterials, Faculty of Chemistry, M.V. Lomonosov Moscow State University, Moscow 119899, Russia

Abstract

The antitumor efficacy of Doxil® is hindered by the poor release of the active drug from the liposome at the tumor sites. This study investigates a possibility to enhance drug release from the liposomes and increase therapeutic efficacy of Doxil® by administering Pluronic block copolymers once the liposomal drug accumulates in the tumor sites. In our study, the fluorescence de-quenching experiments were designed to investigate the drug release from liposome by Pluronic P85. MTT cytotoxicity assay and confocal microscopy images were carried out to determine whether Pluronic P85 could facilitate release of Dox from Doxil®. Anti-tumor growth and distribution of drug were evaluated when Pluronic P85 was injected 1 hr, 48 hrs, or 96 hrs after the Doxil® administration in A2780 human ovarian cancer xenografts. Addition of Pluronic P85 resulted in release of Dox from the liposomes accompanied with significant increases of Dox delivery and cytotoxic effect in cancer cells. The greatest anti-tumor effect of single injection of Doxil® was achieved when Pluronic P85 was administered 48 hrs after Doxil®. The Confocal tile scanning images of tumor section showed that copolymer treatment induced the release of the drug in the tumors from the vessels regions to the bulk of the tumor. No release of the drug remaining in circulation was observed. Our study has demonstrated a simple approach for localized release of Dox from liposome by Pluronic P85 at the tumor site, which was therapeutically beneficial.

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*Corresponding author: Center for Nanotechnology in Drug Delivery and Division of Molecular Therapeutics, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Genetic Medicine Building, Room 1094, Campus Box 7362, Chapel Hill, NC 27599-7362 (kabanov@email.unc.edu).

&Authors contributed equally to this work

§Current address: Center for Nanotechnology in Drug Delivery and Division of Molecular Therapeutics, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7362, USA

#Current address: College of Pharmacy, Yeungnam University, Gyeongsan, South Korea

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Keywords

Liposome; Doxorubicin; copolymer; combination; cancer

Introduction

Liposomal drug delivery systems have been instrumental in developing the first generation of nanomedicines. These have already reached clinical stage providing a considerable benefit to patients, such as Doxil[®], a liposomal form of Doxorubicin (Dox) for the treatment of ovarian and some other forms of cancers [1–4]. After systemic injection the Doxil[®] liposomes drain through the leaky tumor blood vessels and retain at the tumor sites, resulting in increased tumor accumulation of the liposomal drug compared to the free Dox. This general phenomenon is often referred to as “Enhanced Permeability and Retention” effect (EPR) [5]. However, further penetration of liposomal drug from the vascular regions to the distal tumor cells is hindered [6, 7], particularly in the case of fibrotic tumors [4, 8]. The latter produce viscoelastic gel-like extracellular matrix (ECM) that is rich in fibronectin and collagen, and is associated with poor survival [9, 10]. Thus, methods that are able to improve the permeability of the matrix, for example collagen synthesis inhibitors [11], collagenase and hyaluronidase [12], or tumor-penetrating peptides [13] have been used to facilitate the distribution and enhance the efficacy of liposomal drugs [14]. However, these methods may lead to increased toxicity of the drug to normal tissues and/or enhance the risk of the tumor progression and metastasis [11]. Other studies have also explored impacts of physical fields such as ultrasound [15], temperature (hyperthermia) [16] or high energy radiation [17] to enhance permeability of tumor microvasculature and/or microenvironment [18, 19]. However, these methods are technically complex, and have limited use for distal and diffuse tumor sites. Moreover, several studies suggested that the cellular uptake of the intact liposome is restricted at the tumor site [20] and the drug trapped in liposomes in interstitial space remains inactive until it is released in the free form [21, 22]. Therefore, it would be ideal to enable the liposomes to release their low molecular weight cargo at the right site and the right time once the liposomal drug accumulates in the tumor vessels. This would allow for diffusion of the low molecular mass drug from the vascular sites into the distal tumor areas and could boost the cytotoxic effect of the drug upon the cancer cells. Thus, the strategies that could facilitate drug delivery to tumors followed with efficient release of the drug at the tumor sites with minimum side effects are urgently needed [23–25].

Here we propose a new simple strategy to promote the drug release from the liposomal carriers at the tumor sites. This strategy is based on ability of amphiphilic triblock copolymers known as “poloxamer” or “Pluronic” (poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide), PEO-PPO-PEO) to incorporate in liposomal membranes and increase permeability of these membranes to liposome-encapsulated compounds. Importantly this approach can be used with already approved liposomal drugs such as Doxil[®]. In the present work, we provide the proof-of-principle by showing a possibility to trigger the release of Dox from liposomes and enhance therapeutic effect of Doxil[®] *in vitro* and in animal tumor model.

Materials and Methods

Chemicals and materials

Doxil[®], (Doxorubicin HCl liposome injection) were purchased from ALZA Corp (Mountain View, CA). Thiazolyl Blue Tetrazolium Bromide (MTT, #M5655-1G), Dulbecco's phosphate buffered saline solution (PBS) were purchased from Sigma-Aldrich (St. Louis,

MO). Pluronic P85 (lot # WPYE537B) was kindly provided by BASF Corporation (North Mount Olive, NJ). A PEO/PPO/PEO monomer unit ratio is 26/40/26 in P85.

Cells and culture conditions

A2780, MCF7 were purchased from ATCC, and drug resistant cell line, A2780/Dox was kindly provided by Dr. Natalya Y. Rapoport (University of Utah, Salt Lake City, UT). MCF7/Adr, was kindly presented by Y.L. Lee (William Beaumont Hospital, Royal Oak, MI). A2780 and A2780/Dox were cultured in RPMI 1640 media, MCF7 and MCF7/Adr were cultured in DMEM media, with 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, 100 µg/mL streptomycin; MCF7/Adr, A2780/Dox were cultured in the presence of 1 µg/mL Dox. Cells were grown at 37 °C in a humidified atmosphere of 5% CO₂ (v/v) in air. All experiments were performed on cells in the exponential growth phase.

Cytotoxicity Assay

Cells were seeded in 96 well plates at an initial density of 8×10^3 cells/well 24 hrs prior to treatment. On the following day, cells were treated with Dox or Doxil[®] with or without 0.1 wt.% P85 for 2 or 24 hrs at 37°C in a humidified, 5% CO₂ atmosphere. Following treatment, the medium was removed and the cells were rinsed three times by PBS and cultured for three days in fresh media. The cytotoxicity was evaluated using a standard MTT assay and the absorbance was determined at 562 nm using Spectra MX M5[®]. Each concentration point was determined from samples from eight separate wells. IC₅₀ values were calculated based on the percentage ratio between treated cells and untreated control by using GraphPad Prism 5 Software (GraphPad Software, San Diego, CA).

Cellular uptake assay

Cells were seeded on 24 well plates with the initial density of 8×10^3 cells/well, on the day of treatment, the cells were 70–80% confluent. After 2 or 24 hrs incubation with Doxil[®] and with or without 0.1 wt.% P85, cells were rinsed three times with PBS, trypsinized, supplemented with 1 mL of complete media, and collected by centrifugation at 1500 rpm for 3 min. The cell pellet was re-suspended in 1mL of PBS containing 1% BSA and analyzed for Dox fluorescence by Fluorescence Activated Cell Sorting at University of Nebraska Medical Center (UNMC) cell analysis core facility. After the treatment cells were washed three times with PBS, suspended by trypsinization and analyzed by flow cytometry.

Confocal images

Cells were seeded 48 hrs prior treatments in 8 well chamber slides with an initial cell density of 1.0×10^4 . Confluent cells were exposed to Doxil[®] (200 or 400 µg/mL) for 24 or 48 hrs to identify the intracellular localization of drug. Cell nuclei were additionally stained by Hoechst 33342 (Sigma, St. Louis, MO). P85 was covalently labeled with Atto 647. Briefly, at the first stage the mono-amine P85 was prepared as reported previously [26]. At the second stage mono-amine P85 (3.1 mg) was reacted with 2-fold molar (1 mg) of Atto 647N N-hydroxyssuccinimide ester (Catalog number: 18373, Sigma, St. Louis, MO) in N,N-Dimethylformamide (DMF) (0.5 mL) supplemented with N,N-Diisopropylethylamine (2 µL). The reaction mixture was incubated at room temperature for 5 days and then subjected to LH-20 size exclusion column upon methanol elution. The labeled polymer was then collected and weight. The estimated labeling ratio was about 20%. After the synthesis, the stability of Atto 647-P85 was closely monitored and confirmed by thin layer chromatography (TLC) before it was used in cellular experiments. The 0.007% Atto 647-P85 was mixed with 0.1% unlabeled P85 and used to investigate the co-localization of Pluronic and cytoplasm. Cells were visualized by live cell confocal imaging (Carl Zeiss

LSM 510 Meta, Peabody, MA). Previous work has shown stability of mono-amine P85 labeled with fluorescent dyes via amide bond in cells [27].

Drug release studies

Effect of P85 on the release of Dox from Doxil[®] was studied by dialysis method using a membrane (MW cut-off 3,500 Da). Stock solution of Doxil[®] (with Dox 2 mg/mL) was diluted 10 times in the presence or absence of P85 in either PBS, pH 7.4 or 50 mM acetate buffer, pH 5.5. The resulting solutions of Doxil[®] with/without P85 (1mL) were placed into a dialysis bags and dialyzed against 25 mL of the corresponding buffer under continuous shaking at 37°C in dark. Samples (1 mL) of the dialysate solution were withdrawn at a definite time points (1, 2, 4, 8 and 24 hrs) and replaced with an equal volume of fresh buffer. The concentration of Dox in the dialysate samples was determined by measuring absorbance at 485 nm using Lambda 25 UV/VIS spectrophotometer (PerkinElmer). The amount of Dox released from Doxil[®] was expressed as a percentage of the total Dox and plotted as a function of time.

Fluorescence emission spectra

Fluorescence emission spectra of free Dox and Doxil[®] liposome in the presence of 0.1% P85 were recorded using a spectrofluorometer system (Fluorolog[®], Horiba Jobin Yvon Inc., NJ) at wavelength of 480 nm with the bandwidth of 5 nm of excitation and emission. Solutions of free Dox and Doxil[®] used for these studies were at 50 μ M (29 μ g/mL) equivalent concentrations of Dox.

Animals

All experiments were carried out with approval of the UNMC Institutional Animal Care and Use Committee (IACUC) and in accordance with the NIH Guide for Laboratory Animal Use. Athymic nu/nu mice (6- to 8-weeks-old females, National Cancer Institute, Frederick, MD) were used to generate the tumor model throughout this study. BALB/c mice (6- to 8-weeks-old females, Charles River Breeding Laboratories, Raleigh, NC) were used to perform the pharmacokinetics study. The animals were kept in groups of five and fed *ad libitum*.

Animal tumor model and antitumor activity

Human ovarian carcinoma xenografts were used as previously described [28, 29]. A2780 human ovarian cancer cells (4.0×10^6) were subcutaneously (*s.c.*) injected into the right flanks of female athymic nu/nu mice. When the tumors reached a size of about 0.1 cm³ (10–15 days after transplantation), the mice were given intravenous (*i.v.*) injections via the tail vein with Doxil[®] (12 mg Dox/kg for the single injection). In the post treatment groups, 1 hr, 48, or 96 hrs later, the mice were treated with 100 μ L 0.02 wt.% P85. Animal weight and tumor volumes were measured every other day. The tumor length (L) and width (W) were calculated by equation: $WR = 1/2 \times L \times W^2$.

Plasma drug distribution

Six-week old female BALB/c mice were purchased from Charles River Breeding Laboratories (Raleigh, NC, USA). Mice were treated with a single *i.v.* injection of 12 mg/kg of Doxil[®]. P85 (0.02%, 100 μ L/mice) was administered *i.v.* 48 hrs after the drug, animals were sacrificed and blood samples were collected every 12 hrs after the treatments, followed by the HPLC and hematologic analysis.

Immunohistochemistry

Immunohistochemical analyses were performed on tumor sections derived from A2780-bearing nude mice. Tumors derived from untreated mice or from those treated with Doxil[®], administered either alone or in combination with P85. Paraffin-embedded formalin fixed tissue sections were either stained with Mayer's H&E (Sigma Chemical Co., St. Louis, MO) or FITC-labeled CD31 antibody (BD Biosciences, diluted 1:100). All staining procedures ended with a 5 min application of Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) to counterstain for cellular nuclei. The slides were mounted and pictures were taken under a fluorescence microscope (Zeiss Axioplan 2 imaging microscope).

HPLC analysis

Dox concentration in tumor tissue samples and plasma were determined using HPLC method. Before analysis, the tumor tissues were firstly homogenized using a tissue TEAROR[®] equipped with a 7 mm probe (Biospec Products, Inc.). The tissues were diluted 1:1 (w/v) with HPLC grade water, and homogenized. For 40 μ L tissue homogenate or plasma, 8 μ L daunomycin (0.2 mg/mL) was used as an internal standard (IS). The frozen sample was lyophilized using a vacuum freeze drier (FreeZone 2.5 liter, Labconco Inc., Kansas City, MO). For each sample, Trichloroacetic acid (TCA, 10%) 100 μ L were added prior being vortex-mixed for 30 s. Samples were centrifuged at 15,000 g for 10 min at 4 °C. The supernatant (80 μ L) was transferred into a tube and evaporated to dryness for 2 hrs under a stream of gas in a 40 °C heating pad. The residue was reconstituted in 80 μ L of the mobile phase, and a 60 μ L aliquot was used for HPLC analysis. The tissue was determined using a reversed-phase C18 column (Agilent Eclipse XDB, 150 $\times$ 4.6 mm i.d., 5 μ m particle size) and Agilent 1200 HPLC system (G1353B UV detector, G1321A fluorescence detector, G1311A pump, G1329A Autosampler, G1316A column oven) at flow rate of 1.2 mL/min. The mobile phase was: 1.0% acetic acid: acetonitrile solution = 48% : 52%. Fluorescence detection of Dox was carried out at λ_{ex} = 480 nm and λ_{em} = 590 nm.

Confocal tile scan image of the paraffin-embedded formalin fixed tumor tissue

Confocal tile scan images were performed on tumor sections derived from A2780-bearing nude mice, which were administered with either Doxil[®] alone or in combination with P85. Serial 5 μ m tissue sections from paraffin-embedded formalin fixed tissue sections were cut and placed on glass slides. Tile-scan mode was used to obtain a 8 $\times$ 8 or 10 $\times$ 10 phase contrast tile image that covered the whole area of the tumor tissue on a coverslip. The drug distribution in the tumor tissue was imaged with an LSM 710 confocal microscope (Carl Zeiss GmbH, Jena, Germany) equipped with 458/488/514 nm Argon/2 laser, using a 10 $\times$ objective.

Statistical analysis

The differences between treatment groups were analyzed by using Student's t-test for pairs of groups and one-way analysis of variance (ANOVA) for multiple groups. The p-value less than 0.05 were considered statistically significant. All statistical analyses were carried out using GraphPad Prism Software (Version 5.0, GraphPad Software, San Diego, CA).

Results

Drug release from liposomes by Pluronic

The release of the drug from the liposomes triggered by Pluronic was evident based on the fluorescence de-quenching experiment. When Dox was crystallized inside Doxil[®], particles the drug fluorescence was quenched compared to the free drug. As seen in Fig. 1A addition of P85 to Doxil[®] dispersion resulted in a rapid (30 min) increase of fluorescence. In the

absence of the copolymer no drug release was observed for at least 60 min (Fig. 1B). Studying the drug release by the method of equilibrium dialysis reinforced this result suggesting that addition of P85 to Doxil[®] dispersion at either pH5.5 or pH7.4 substantially increased the release rate of Dox (Fig. 1C). In this case about 50% of the drug was released during 24 hrs while the release of the drug from Doxil[®] alone was less than 20%. Notably, the ability of P85 to release the drug from the liposomes was dependent on the concentration of the copolymer. Specifically, the release enhancement was relatively strong with 0.02% to 0.5% P85, considerably less with 0.001% P85 and virtually non-existing with 0.0001% P85 (Supplementary data, Fig. S1). Notably, the size and polydispersity of the liposomes in the presence of even highest concentration of P85 did not change for the same time period (Supplementary data, Table S1), suggesting that the increased drug release was not associated with the disruption of the liposomes. At the same time, when Tritc-labeled P85 (Tritc-P85) was mixed with empty PEGylated liposomes, the fluorescence of Tritc was immediately quenched (ca. 50%), suggesting that the copolymer incorporated into the liposomal membrane (Supplementary data, Fig. S2). The Atomic Force Microscopy (AFM) experiment suggested that addition of P85 to liposomal membranes produces transient holes in the membranes that are nearly 5 nm deep and heal within minutes after their formation (Fig. S3 and Fig. S4).

Effect of Pluronic on *in vitro* cytotoxicity of Doxil[®] to cancer cells

To further characterize the effects of the drug release from the liposomes by Pluronic the cytotoxicity experiments were carried out in ovarian and breast cancer cells. As shown in Doxil[®] alone at concentrations up to 200 µg/mL (counting per Dox contained in the liposomal formulation) did not induce cytotoxicity in either cell line (Table 1, Supplementary Table S2). However, co-treatment of the cells with 0.1 wt.% P85 and Doxil[®] increased the cytotoxicity of the liposomal drug in both cell lines. Consistent with our previous results [30] 2 hrs exposures to 0.1% P85 alone did not induce cytotoxicity in the same cells (data not shown). Next we examined effects of P85 on Doxil[®] cytotoxicity upon 24 hrs exposure. In this study Doxil[®] displayed toxicity in sensitive but not resistant cell lines (Tables 1, Supplementary Table S2). To avoid P85 toxicity upon long-term exposure, the copolymer was added to cells for 2 hrs either immediately before or after exposure to Doxil[®]. In this experiment the media was replaced each time between switching to either Doxil[®] or P85, which excluded direct interaction between P85 and Doxil[®] in the media. As shown in Table 1 and Table S2 (supplementary) the pretreatment and post treatment with P85 increased cytotoxicity of Doxil[®] in sensitive cell lines (A2780 and MCF7), compared to Doxil[®] alone groups. However, in multidrug resistant (MDR) cells (A2780/Dox and MCF7/adr) we were unable to reach toxic concentrations of drug in any treatment group other than the co-culture of the drug with the copolymer that is discussed above.

Effect of Pluronic on drug uptake in cancer cells

Flow cytometry analysis was carried out to determine whether facilitated release of Dox from Doxil[®] triggered by Pluronic also results in greater drug accumulation in cancer cells. This method provided a good measure of total Dox fluorescence although did not distinguish between liposome-bound and free Dox forms. Cells were exposed to Doxil[®] for 2 hrs or 24 hrs. P85 was added either concurrently with Doxil[®] (2 hrs exposures), or for 2 hrs before or after Doxil[®] (24 hrs exposures). Addition of 0.1% P85 in the media resulted in significant increases in cell fluorescence (Fig. 2A, Supplementary Fig. S5). The effect of the copolymer was most pronounced when it was added simultaneously with Doxil[®]. This trend was seen in all cell lines. Notably, the drug uptake in MDR cells (A2780/Dox and MCF7/adr) that overexpress drug efflux pump, P-glycoprotein (Pgp) was greatly decreased compared to their sensitive counterparts (A2780 and MCF7) (Fig. 2A, Supplementary Fig. S5). The P85 shown to be capable of inhibiting this efflux pump [31] also reduced these differences in the

uptake in resistant and sensitive cells, which may provide additional potential benefits for cancer therapy. In a separate study the drug fluorescence was measured in cell lysates and normalized to cell protein. The overall picture was similar to the flow cytometry analysis (Supplementary Fig. S6). Taken together this suggests that P85 facilitates drug release from liposomes and in the case of MDR cells inhibits the Dox efflux by Pgp. Combined these effects result in increased drug uptake.

Effect of Pluronic on intracellular localization of the drug

The liposomal drug uptake was very slow compared to free Dox (Fig. 2B, left and middle panels). Little if any drug was seen in the intracellular vesicles and nucleus even after 24 hrs exposures of the cells to the highest dose of Doxil[®] (200 µg/mL). However, by adding 0.1% P85 simultaneously with Doxil[®] the nuclear delivery was greatly increased in both drug sensitive and resistant cells (Fig. 2B, right panels). In this case the drug was taken up as early as at 15 min, followed by its rapid translocation to the nucleus (Supplementary data, Fig. S7). Interestingly, the fluorescently labeled copolymer was internalized in the cells within the same timeframe although did not co-localize with the drug (Fig. 2B, right panels). This could indicate that upon simultaneous treatment with Doxil[®] and P85, Dox was released in extracellular media rather than within the cells. This prompted us to examine effects of post-treatment, with P85 added for 1 hr after 24 hrs exposure of cells to Doxil[®]. Although some co-localization of the drug and copolymer was observed at later time points, the liposomal drug remained mainly localized in the intracellular compartments but not the nucleus while the labeled copolymer remained in the cytoplasmic compartments (Fig. 2C). Therefore, we extended the incubation time of the cells with Doxil[®] to 48 hrs. Even in this case no co-localization was observed with P85 added after Doxil[®] (Fig. 2D, bottom row panels). However, some co-localization was observed both in resistant and sensitive cells when the copolymer was added to cells before the Doxil[®].

Effect of Pluronic on anti-tumor efficacy of Doxil[®] *in vivo*

Antitumor effects of single injection of Doxil[®] were evaluated to determine whether the copolymer could enhance tumor suppression by the liposomal drug (Fig. 3A). To avoid instantaneous drug release from the liposomes the copolymer was injected at different time points after the drug: 1 hr, 48 hrs, or 96 hrs. The tumors were quite aggressive and the size reached 2 cm³ by day 9 (requiring euthanasia of the animal) if not treated (saline control). Surprisingly, the greatest enhancement of the antitumor effect was observed when P85 was administered 48 hrs after Doxil[®]. In this case by day 21 the size was decreased almost 3 fold compared to Doxil[®]. When the copolymer was administered 1 hr or 96 hrs after the drug, the tumor growth rates did not differ significantly compared to Doxil[®] alone group. To evaluate the systemic toxicity of different treatments we isolated heart, liver, spleen and kidneys from tumor bearing animals at the end point of the experiment. Histological analysis (Supplementary Fig. S8) and hematologic toxicity analysis (Supplementary Table S3) did not show changes in Doxil[®] toxicity upon copolymer treatment. For example, increase in glucose and decrease in hemoglobin levels in blood were previously associated with cardiotoxicity of free Dox, but not observed in the liposomal formulation [32]. Indeed, our data suggest that free Dox increased glucose levels by nearly 38% compared to the control group. In contrast no such increase was observed for either Doxil[®] or Doxil[®] + P85 treatment groups. Moreover, Dox alone group displayed a tendency (albeit not significant) for the decrease of the globins by nearly 30% compared to the control. No such changes were observed in either Doxil[®] or Doxil[®] + P85 treatment groups.

Effect of Pluronic on drug distribution in the tumor and blood levels

To better understand the synergistic anti-tumor effect of Doxil[®] and P85, we examined the effect of the copolymer on the distribution of the drug in the tumors. In this study P85 was

administered either 48 or 96 hrs after Doxil[®], which corresponded to the greatest increase or no change of anti-tumor activity, respectively. The animals were sacrificed 1 or 6 hrs after administration of the copolymer and the drug fluorescence in the tumor sections was analyzed. The blood vessels (CD31) and nucleus (DAPI) were also stained for reference. As seen from fluorescence analysis, following administration of P85 at the 48 hrs time point the overall fluorescence of Dox in tumor sections greatly increased compared to Doxil[®] alone (Fig. 3B). This effect was most pronounced 6 hrs after the copolymer administration. Notably, in the absence of P85 the drug fluorescence was mainly co-localized with blood vessels, while after addition of P85 the drug became spread throughout the tumor reaching distal areas. This suggested that at 48 hrs Doxil[®] particles were deposited mainly in the blood vessels, while P85 promoted the release of Dox from the liposomes in the tumor tissue. Analysis of drug disposition to the tumor by HPLC suggests that P85 had induced small albeit significant increase in the drug levels 6 hrs after the copolymer administration (Fig. 3C). The whole tumor images clearly reinforced increased drug fluorescence in the tumors after administration of the copolymer (Fig. 3D, upper panel). However, a strikingly different picture was observed when the copolymer was injected 96 hrs after Doxil[®]. In this case the drug appeared to be already released from the liposomes and its fluorescence in the tumors did not change after injection of the copolymer (Fig. 3D, lower panel). Finally, since a considerable amount of Doxil[®] may be still circulating in the blood at 48 hrs [33], we examined whether injection of the copolymer at this time point can change the levels of the drug in circulation. There is no direct method for measuring separately the free and liposome-incorporated drug in the plasma as the HPLC assay provides the total amount of the extracted drug. Administration of P85 at 48 hrs practically did not change the subsequent drug pharmacokinetics profile, which remained similar to that of Doxil[®] alone (Supplementary Fig. S9). This indirectly suggests that post-injection of Pluronic did not release the drug from circulating Doxil[®] liposomes since free Dox would have been rapidly cleared from the blood.

Discussion

This work proposes a simple way to enhance Doxil[®]-based chemotherapy of tumors comprising 1) administration of the liposomal drug to tumor-bearing species, 2) waiting for an optimal period of time to ensure disposition of the liposomal drug to the tumors, and 3) then administering a solution of Pluronic block copolymer to induce release of the active drug from the liposomes at the tumor sites. The optimal regimen of administration of the Doxil[®] liposomes and the copolymer produces synergistic antitumor effect resulting in the decrease in the tumor growth.

Doxil[®], the first nanoformulated drug approved by the US Food and Drug Administration [20], was designed to reduce cardiotoxicity of Dox by encapsulating it in PEGylated (“stealth”) liposome. The liposomal drug has prolonged circulation time, which aims to increase exposure of the drug to the tumor. The antitumor efficacy of Doxil[®] is dependent on both efficient delivery of liposomes to the tumor sites and release of the active drug molecules from the liposomes at these sites. The delivery of Doxil[®] or other polymeric drugs to solid tumors commonly rely on EPR effect [5].

Up to date the mechanism of internalization of Doxil[®] liposomes by the tumor cells and the release of the active drug from the liposomes remain poorly understood [20, 34]. It has been recognized that the therapeutic effectiveness of Doxil[®] is limited by its penetration to the tumor and transport in cancer cells which are hindered, especially in the fibrotic tumors [11]. A most recent review by Dr. Barenholz [20] supports the notion that the free Dox is released in the tumor interstitial fluid and this provides for the anticancer effect of Doxil[®]. In clinical studies, Doxil[®] exhibited limited cardiotoxicity but did not show significant improvement of

progression-free survival for breast cancer patients [35]. Increasing evidence has shown that combination of Doxil[®] therapy with the regimens that enhance permeability of tumor vasculature can improve the response of tumors to chemotherapy. These data led us to rethink the Doxil[®] combination principles. Hereby, we proposed that instead of choosing an agent to enhance the permeability of the biological vessels, one should use a safe regimen to enhance the permeability of the liposomal membranes.

Herein, we propose to use Pluronic block copolymers as such enhancing agents. These copolymers have been studied extensively as excipients in a variety of pharmaceutical formulations to enhance delivery and activity of low molecular mass drugs and polypeptides in the body [36–38]. Most relevant to this study is the anticancer drug SP1049C, which comprises a mixture of Dox and micelle composition of hydrophobic Pluronic L61 and hydrophilic Pluronic F127. This drug has shown considerable activity and favorable safety profile in clinical trials [39, 40]. However, in contrast to simultaneous administration of drug and copolymer in one formulation (as done in SP1049C) we propose to separate the liposomal drug and copolymer to ensure that the drug is released once the liposomal particles accumulate at the tumor sites. In this study we use a single copolymer P85 as an experimental enhancing agent. Similarly to a mixture of L61 and F127 employed in SP1049C P85 has shown ability to sensitize MDR cancer cells [38]. Extensive in vitro and in vivo studies of P85 have been previously reported as overviewed elsewhere [41, 42], including pharmacokinetics and biodistribution accumulation in tumors [42, 43] and toxicity [44]. Following i.v. administration of 1% P85 in mouse there was no evidence of nephrotoxicity (acute tubular necrosis, glomerular changes or edema/fibrosis of interstitium) or liver toxicity (hepatocyte necrosis, micro- or macrosteatosis, inflammatory changes in bile ducts or microvessels) [45].

The ability of P85 to increase the rate of Dox diffusion across planar lipid and liposomal membranes without affecting the overall integrity of these membranes was previously described [46]. The studies presented in this work also demonstrated that the block copolymer greatly increased the rate of the release of Dox from the Doxil[®] liposomes. Moreover, the copolymer was shown here to have no effect on the liposome size. Yet it was capable of incorporating into the membranes, which presumably involves penetration of hydrophobic PPO chains in the nonpolar regions of the lipid bilayers. A recent study also suggested that hydrophobic Pluronics insert in the lipid membranes and loosen the lipid packing during initial embedding below the lipid headgroups, thereby acting as membrane permeabilizers [47, 48]. Moreover, we have observed formation of transient holes in the membranes upon addition P85, which were cured within minutes after their formation and did not lead to membrane disintegration. Such holes could serve as “pores” that facilitate both anti-transport of hydroxyl vs. chloride ions across liposomal membranes to equilibrate external and internal pH, as well as the diffusion of Dox from the liposome interior into external solution. Altogether the molecular mechanisms of the increased permeability of liposomal membranes with respect to Dox require further investigation. But there is clear evidence that this copolymer can serve as a potent permeabilizer allowing the release of the drug in the external media.

Our findings also demonstrate that the release of the drug from Doxil[®] liposomes by adding P85 enhances the drug uptake in ovarian and breast cancer cells compared to the liposomes alone. The uptake of the Doxil[®] liposomes in the cells proceeds via endocytosis [49], which is a relatively slow process compared to transmembrane diffusion of the free Dox. The live cell imaging data clearly show that the entry of Doxil[®] alone even after 24 and 48 hrs was minimal compared to the free drug. However, when Doxil[®] was combined with the copolymer, the drug rapidly entered cells (15 min) and then accumulated in the nucleus (60 min). This effect was most pronounced when the copolymer was administered in the media

concurrently with Doxil[®]. The latter was probably explained by extracellular release of free Dox from the liposomes followed by the transport of the free drug into the cells. This was accompanied by an increased cytotoxic effect of the drug. Interestingly in the non-MDR cells the cytotoxic effect was increased not only upon co-exposure of P85 and Doxil[®] but also upon pre- or post-exposure of the copolymer (i.e. when P85 was added to cells before or after the liposomes). This is probably explained by the ability of the copolymer to accumulate in cells and release Dox intracellularly while coming in contact with the liposomes that are internalized after the copolymer was added (pre-exposure) or before the copolymer was added (post-exposure). The uptake of P85 in cells has been documented elsewhere suggesting that the copolymer micelles are internalized through the clathrin-mediated endocytosis (CME) while the single chains (“unimers”) are internalized through caveolae as well as non-CME and non-caveolae dependent pathway [27]. Under the conditions of the experiment the concentration of P85 (0.1%) was above its critical micelle concentration (CMC, ca. 0.03% [50]). Hence, about 1/3 of the copolymer was in the form of unimers while about 2/3 was in the form of micelles. The P85 unimers and micelles could rapidly internalize in cells through multiple pathways and reach various intracellular compartments [27].

Furthermore, the copolymer not only facilitated the release of the drug from the liposomes but also helped overcoming the drug resistance in the MDR cancer cells. This phenomenon has been very well documented in the literature [51]. It includes inhibition of the Pgp drug efflux pump by P85 resulting in increased drug accumulation in MDR cells. This is coupled with the inhibitory effects of P85 on the respiratory function in the MDR cell mitochondria, ATP depletion and enhanced pro-apoptotic signaling. All together combined with increased drug accumulation these effects lead to the increase in the cytotoxic effect of the drug in MDR cells [41]. It is noteworthy that in MDR cells incubated with Doxil[®] (without P85) the levels of the drug accumulation were considerably decreased compared to the non-MDR cells. Evidently as the free drug was released from the liposomes inside of the cells it was rapidly transported out of the cells by the efflux pump. However, as demonstrated in the present study co-administration of Doxil[®] with P85 rectified the decreased accumulation of the liposomal drug and led to a considerable increase in cytotoxicity of Doxil[®] in the MDR cells. Therefore, successful cell culture studies demonstrated that there can be multiple beneficial effects of Doxil[®] and P85 combination treatments including facilitated drug release from the liposomal carriers as well as sensitization effect in MDR cells. This led us investigating whether the copolymer can also be used to improve the therapeutic effect of Doxil[®] in solid tumors.

The in vivo study has reinforced the validity of our approach. Indeed, using a mouse subcutaneous model of human ovarian cancer, we demonstrated that the combination of Doxil[®] with sequential injection of Pluronic exerts better antitumor activity, compared to administration of Doxil[®] alone, with little if any tissue toxicity. The choice of the concentration of the copolymer used in the in vivo experiment (0.02 wt.% P85) was guided on the one hand by our previous studies on the pharmacokinetics and tumor accumulation of the P85 in mice and on the other hand by the results reported herein of the in vitro study, which related the drug release from Doxil[®] to P85 concentration. Specifically, it was shown that 0.02% following i.v. administration of the copolymer bolus it is rapidly diluted in the bloodstream to less than 1 µg/mL or 0.0001% [43], a concentration which according to our data does not facilitate the drug release. At the same time the copolymer was shown to rapidly (min) concentrate in the tumor and remain there for dozens of hours at approx. 0.1 µg/mg [42]. This amount of the copolymer in the case of the MDR tumors was shown to inhibit the membrane drug efflux transporter, Pgp, resulting in increased accumulation of the drugs in the MDR tumors [42]. Hence we hypothesized that using the same dose of P85 will minimize the copolymer interactions and drug release from the liposome circulating in the

blood, but allow its interaction with the membrane of the liposomes entrapped in the tumor, resulting in tumor-specific drug release. Indeed, the antitumor effect was the greatest when P85 was administered 48 hrs after Doxil[®] administration. In this case the tumor sizes were reduced by 60% compared to Doxil[®] alone treatment group. The administration of Pluronic did not appear to facilitate the fast release of the free Dox from Doxil[®] in the blood stream. However, at this time point according to our data as well as literature [33] Doxil[®] particles are already disposed to tumors, but the free drug remained bound to the liposomes. Using our discovered protocol, addition of Pluronic facilitated drug release at the right time and the right site. At the comparable doses of Doxil[®] (9 mg/kg to 16 mg/kg) the maximal accumulation of drug in the solid tumors in mouse are observed between 24 and 48 hrs [33].

This effect was attenuated when the copolymer was administered 1 hr or 96 hrs after the drug. In the first case the copolymer was administered perhaps too prematurely and before substantial accumulation of the Doxil[®] particles in the tumor sites. Notably, only ca. 10% of the free drug distributes into tumors after *i.v.* injection compared to injection of Doxil[®] [33, 52]. In the second case the drug was already leaked from the liposome accumulated in the tumors prior to administration of the copolymer. However, according to the EPR the free drug is more rapidly removed by lymphatic drainage compared to liposomes [53]. Therefore its slow leakage from intact liposomes may not create sufficiently high concentration at the tumors sites compared to the rapid release achieved by the copolymer post administration. It should be pointed out that based on the available literature the time point of the maximal accumulation of Doxil[®] in the tumor, t_{max} , is dependent on both the tumor model and the dose of the drug (see, Supplementary Table S4). Therefore, one should expect that the regimen for treatment, especially the time period and the doses, should be optimized in a clinical trial for use in humans.

In conclusion, our data show that Pluronic block copolymer, P85, administered at the optimal time point after Doxil[®], when the liposomal drug is already distributed to the tumor sites enhances drug release and distribution to the tumor tissue and concomitantly contributes to an increased drug activity inhibiting the tumor growth. Therefore, systemic application of the copolymer may be a useful adjuvant to the Doxil[®]-based chemotherapy regimens. It could also be helpful in improving efficacy of other liposomal drug formulations. Thorough examination of the toxic effect of the proposed regimen, especially cardiotoxicity studies in rabbits would be further required in the future studies to substantiate potential lack of heart muscle poisoning. Such studies can be further undertaken if warranted by the needs of translational product development. Therefore the results of this work are of significance and potential use for combination therapy using liposomal drug formats and may ultimately assist physicians in developing next therapeutic regimens for cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Song G, Wu H, Yoshino K, Zamboni WC. Factors affecting the pharmacokinetics and pharmacodynamics of liposomal drugs. *J Liposome Res.* 2012
2. Schwendener RA. Liposomes in biology and medicine. *Adv Exp Med Biol.* 2007; 620:117–128. [PubMed: 18217339]
3. Maurer N, Fenske DB, Cullis PR. Developments in liposomal drug delivery systems. *Expert Opin Biol Ther.* 2001; 1:923–947. [PubMed: 11728226]
4. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov.* 2005; 4:145–160. [PubMed: 15688077]
5. Maeda H, Sawa T, Konno T. Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. *J Control Release.* 2001; 74:47–61. [PubMed: 11489482]
6. Kostarelos K, Emfietzoglou D, Papakostas A, Yang WH, Ballangrud A, Sgouros G. Binding and interstitial penetration of liposomes within avascular tumor spheroids. *Int J Cancer.* 2004; 112:713–721. [PubMed: 15382056]
7. Chauhan VP, Stylianopoulos T, Boucher Y, Jain RK. Delivery of molecular and nanoscale medicine to tumors: transport barriers and strategies. *Annu Rev Chem Biomol Eng.* 2011; 2:281–298. [PubMed: 22432620]
8. Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res.* 1994; 54:3352–3356. [PubMed: 8012948]
9. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer.* 2006; 6:392–401. [PubMed: 16572188]
10. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature.* 2004; 432:332–337. [PubMed: 15549095]
11. Diop-Frimpong B, Chauhan VP, Krane S, Boucher Y, Jain RK. Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. *Proc Natl Acad Sci U S A.* 2011; 108:2909–2914. [PubMed: 21282607]
12. Eikenes L, Tufto I, Schnell EA, Bjorkoy A, De Lange Davies C. Effect of collagenase and hyaluronidase on free and anomalous diffusion in multicellular spheroids and xenografts. *Anticancer Res.* 2010; 30:359–368. [PubMed: 20332440]
13. Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Greenwald DR, Ruoslahti E. Coadministration of a tumor-penetrating peptide enhances the efficacy of cancer drugs. *Science.* 2010; 328:1031–1035. [PubMed: 20378772]
14. Jain RK, Stylianopoulos T. Delivering nanomedicine to solid tumors. *Nat Rev Clin Oncol.* 2010; 7:653–664. [PubMed: 20838415]
15. Schroeder A, Honen R, Turjeman K, Gabizon A, Kost J, Barenholz Y. Ultrasound triggered release of cisplatin from liposomes in murine tumors. *J Control Release.* 2009; 137:63–68. [PubMed: 19303426]
16. Kong G, Anyambhatla G, Petros WP, Braun RD, Colvin OM, Needham D, Dewhirst MW. Efficacy of liposomes and hyperthermia in a human tumor xenograft model: importance of triggered drug release. *Cancer Res.* 2000; 60:6950–6957. [PubMed: 11156395]
17. Hagtvet E, Roe K, Olsen DR. Liposomal doxorubicin improves radiotherapy response in hypoxic prostate cancer xenografts. *Radiat Oncol.* 2011; 6:135. [PubMed: 21981945]
18. Harrington KJ, Rowlinson-Busza G, Syrigos KN, Vile RG, Uster PS, Peters AM, Stewart JS. Pegylated liposome-encapsulated doxorubicin and cisplatin enhance the effect of radiotherapy in a tumor xenograft model. *Clin Cancer Res.* 2000; 6:4939–4949. [PubMed: 11156255]

19. Khaibullina A, Jang BS, Sun H, Le N, Yu S, Frenkel V, Carrasquillo JA, Pastan I, Li KC, Paik CH. Pulsed high-intensity focused ultrasound enhances uptake of radiolabeled monoclonal antibody to human epidermoid tumor in nude mice. *J Nucl Med.* 2008; 49:295–302. [PubMed: 18199622]
20. Barenholz YC. Doxil(R) - The first FDA-approved nano-drug: Lessons learned. *J Control Release.* 2012
21. Zamboni WC. Concept and clinical evaluation of carrier-mediated anticancer agents. *Oncologist.* 2008; 13:248–260. [PubMed: 18378535]
22. Zamboni WC. Liposomal, nanoparticle, and conjugated formulations of anticancer agents. *Clin Cancer Res.* 2005; 11:8230–8234. [PubMed: 16322279]
23. Kwon IK, Lee SC, Han B, Park K. Analysis on the current status of targeted drug delivery to tumors. *J Control Release.* 2012; 164:108–114. [PubMed: 22800574]
24. Bae YH, Park K. Targeted drug delivery to tumors: myths, reality and possibility. *J Control Release.* 2011; 153:198–205. [PubMed: 21663778]
25. Florence AT. Reductionism and complexity in nanoparticle-vectored drug targeting. *J Control Release.* 2012; 161:399–402. [PubMed: 22100439]
26. Yi X, Zimmerman MC, Yang R, Tong J, Vinogradov S, Kabanov AV. Pluronic-modified superoxide dismutase 1 attenuates angiotensin II-induced increase in intracellular superoxide in neurons. *Free Radic Biol Med.* 2010; 49:548–558. [PubMed: 20493251]
27. Sahay G, Batrakova EV, Kabanov AV. Different internalization pathways of polymeric micelles and unimers and their effects on vesicular transport. *Bioconjugate chemistry.* 2008; 19:2023–2029. [PubMed: 18729494]
28. Pakunlu RI, Wang Y, Saad M, Khandare JJ, Starovoytov V, Minko T. In vitro and in vivo intracellular liposomal delivery of antisense oligonucleotides and anticancer drug. *J Control Release.* 2006; 114:153–162. [PubMed: 16889867]
29. Wang Y, Saad M, Pakunlu RI, Khandare JJ, Garbuzenko OB, Vetcher AA, Soldatenkov VA, Pozharov VP, Minko T. Nonviral nanoscale-based delivery of antisense oligonucleotides targeted to hypoxia-inducible factor 1 alpha enhances the efficacy of chemotherapy in drug-resistant tumor. *Clin Cancer Res.* 2008; 14:3607–3616. [PubMed: 18519795]
30. Rapoport N, Marin A, Luo Y, Pres twich GD, Muniruzzaman MD. Intracellular uptake and trafficking of Pluronic micelles in drug-sensitive and MDR cells: effect on the intracellular drug localization. *J Pharm Sci.* 2002; 91:157–170. [PubMed: 11782905]
31. Batrakova EV, Li S, Li Y, Alakhov VY, Kabanov AV. Effect of pluronic P85 on ATPase activity of drug efflux transporters. *Pharm Res.* 2004; 21:2226–2233. [PubMed: 15648254]
32. Herman EH, Rahman A, Ferrans VJ, Vick JA, Schein PS. Prevention of chronic doxorubicin cardiotoxicity in beagles by liposomal encapsulation. *Cancer Res.* 1983; 43:5427–5432. [PubMed: 6616474]
33. Laginha KM, Verwoert S, Charrois GJ, Allen TM. Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors. *Clin Cancer Res.* 2005; 11:6944–6949. [PubMed: 16203786]
34. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *Int J Nanomedicine.* 2006; 1:297–315. [PubMed: 17717971]
35. Harris L, Batist G, Belt R, Rovira D, Navari R, Azarnia N, Welles L, Winer E. Liposome-encapsulated doxorubicin compared with conventional doxorubicin in a randomized multicenter trial as first-line therapy of metastatic breast carcinoma. *Cancer.* 2002; 94:25–36. [PubMed: 11815957]
36. Price TO, Farr SA, Yi X, Vinogradov S, Batrakova E, Banks WA, Kabanov AV. Transport across the blood-brain barrier of pluronic leptin. *J Pharmacol Exp Ther.* 2010; 333:253–263. [PubMed: 20053933]
37. Kabanov AV, Batrakova EV, Alakhov VY. Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery. *J Control Release.* 2002; 82:189–212. [PubMed: 12175737]

38. Batrakova EV, Kabanov AV. Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers. *J Control Release*. 2008; 130:98–106. [PubMed: 18534704]
39. Valle JW, Armstrong A, Newman C, Alakhov V, Pietrzynski G, Brewer J, Campbell S, Corrie P, Rowinsky EK, Ranson M. A phase 2 study of SP1049C, doxorubicin in P-glycoprotein-targeting pluronics, in patients with advanced adenocarcinoma of the esophagus and gastroesophageal junction. *Investigational new drugs*. 2011; 29:1029–1037. [PubMed: 20179989]
40. Danson S, Ferry D, Alakhov V, Margison J, Kerr D, Jowle D, Brampton M, Halbert G, Ranson M. Phase I dose escalation and pharmacokinetic study of pluronic polymer-bound doxorubicin (SP1049C) in patients with advanced cancer. *Br J Cancer*. 2004; 90:2085–2091. [PubMed: 15150584]
41. Alakhova DY, Rapoport NY, Batrakova EV, Timoshin AA, Li S, Nicholls D, Alakhov VY, Kabanov AV. Differential metabolic responses to pluronic in MDR and non-MDR cells: a novel pathway for chemosensitization of drug resistant cancers. *J Control Release*. 2010; 142:89–100. [PubMed: 19815037]
42. Batrakova EV, Li S, Brynskikh AM, Sharma AK, Li Y, Boska M, Gong N, Mosley RL, Alakhov VY, Gendelman HE, Kabanov AV. Effects of pluronic and doxorubicin on drug uptake, cellular metabolism, apoptosis and tumor inhibition in animal models of MDR cancers. *J Control Release*. 2010; 143:290–301. [PubMed: 20074598]
43. Batrakova EV, Li S, Li Y, Alakhov VY, Elmquist WF, Kabanov AV. Distribution kinetics of a micelle-forming block copolymer Pluronic P85. *J Control Release*. 2004; 100:389–397. [PubMed: 15567504]
44. Batrakova EV, Li S, Alakhov VY, Elmquist WF, Miller DW, Kabanov AV. Sensitization of cells overexpressing multidrug-resistant proteins by pluronic P85. *Pharm Res*. 2003; 20:1581–1590. [PubMed: 14620511]
45. Batrakova EV, Zhang Y, Li Y, Li S, Vinogradov SV, Persidsky Y, Alakhov VY, Miller DW, Kabanov AV. Effects of pluronic P85 on GLUT1 and MCT1 transporters in the blood-brain barrier. *Pharm Res*. 2004; 21:1993–2000. [PubMed: 15587920]
46. Erukova VY, Krylova OO, Antonenko YN, Melik-Nubarov NS. Effect of ethylene oxide and propylene oxide block copolymers on the permeability of bilayer lipid membranes to small solutes including doxorubicin. *Biochim Biophys Acta*. 2000; 1468:73–86. [PubMed: 11018653]
47. Wang JY, Marks J, Lee KY. Nature of interactions between PEO-PPO-PEO triblock copolymers and lipid membranes: (I) effect of polymer hydrophobicity on its ability to protect liposomes from peroxidation. *Biomacromolecules*. 2012; 13:2616–2623. [PubMed: 22808900]
48. Cheng CY, Wang JY, Kausik R, Lee KY, Han S. Nature of interactions between PEO-PPO-PEO triblock copolymers and lipid membranes: (II) role of hydration dynamics revealed by dynamic nuclear polarization. *Biomacromolecules*. 2012; 13:2624–2633. [PubMed: 22808941]
49. Sahay G, Kim JO, Kabanov AV, Bronich TK. The exploitation of differential endocytic pathways in normal and tumor cells in the selective targeting of nanoparticulate chemotherapeutic agents. *Biomaterials*. 2010; 31:923–933. [PubMed: 19853293]
50. Batrakova E, Lee S, Li S, Venne A, Alakhov V, Kabanov A. Fundamental relationships between the composition of pluronic block copolymers and their hypersensitization effect in MDR cancer cells. *Pharm Res*. 1999; 16:1373–1379. [PubMed: 10496652]
51. Kabanov AV, Batrakova EV, Alakhov VY. Pluronic block copolymers for overcoming drug resistance in cancer. *Adv Drug Deliv Rev*. 2002; 54:759–779. [PubMed: 12204601]
52. Gabizon A, Tzemach D, Mak L, Bronstein M, Horowitz AT. Dose dependency of pharmacokinetics and therapeutic efficacy of pegylated liposomal doxorubicin (DOXIL) in murine models. *J Drug Target*. 2002; 10:539–548. [PubMed: 12683721]
53. Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. *Adv Enzyme Regul*. 2001; 41:189–207. [PubMed: 11384745]

Figure 1.

P85 triggered release of Dox from Doxil® liposomes. Fluorescence emission spectra of dispersion of Doxil® (A) with or (B) without 0.1% P85 at different time points. (C) Doxil® dispersed at 37°C in either PBS (pH 7.4, ▲ or △) or acetate buffer (pH 5.5, ■ or □) with (□ or △) or without (■ or ▲) 0.1% P85. The release of the free Dox from the dialysis bags at 37°C in either PBS (pH 7.4 ◆, dashed line) or acetate buffer (pH 5.5 ●, dashed line) is shown for comparison. Data are mean ± SD (n = 4).

Figure 2.

Uptake of the drug in ovarian cancer cells after their exposure to Doxil[®] determined by – flow cytometry and Confocal analysis. **(A)** Cells were treated with 1) Doxil[®] alone for 2 hrs (white bars); 2) Doxil[®] and 0.1% P85 mixture for 2 hrs (light grey bars), 3) Doxil[®] alone for 24 hrs (grey bars); 4) 0.1% P85 for 2 hrs, followed by 3× wash and Doxil[®] for 24 hrs (dark grey bars); 5) Doxil[®] for 24 hrs, followed by 3× wash and 0.1% P85 for 2 hrs (black bars). Data are mean ± SD (n = 6), *** p < 0.001, n.s. - no significance (Student's t-test). **(B,C,D)** Confocal micrographs showing localization of drug (red) in the A2780 and A2780/Dox ovarian cancer cells in the presence or absence of P85 (labeled with Atto647, digitally

converted to green color to facilitate image analysis). **(B)** Cells were incubated with Dox for 2 hrs, Doxil[®] for 24 hrs, or Doxil[®] and 0.1% P85 for 2 hrs. **(C)** Cells were treated with Doxil[®] for 24 hrs, washed and then treated with 0.1% P85 for 1 hr. **(D)** Cells were treated with Doxil[®] for 48 hrs, and 0.1% P85 was added for 2 hrs either before or after treatment with Doxil[®]. Magnification $\times 63$.

Figure 3.

Effect of P85 on anti-tumor efficacy and drug distribution in A2780 xenografts. (A) Two weeks after the tumor implantation mice were treated by a single *i.v.* injection of 12 mg/kg of Doxil[®]. 1 hr (▲), 48 hrs (▼), 96 hrs (■) after Doxil[®] administration, mice received 0.02% P85. The data represent mean $\pm$ SEM ($n = 8$), ** $p < 0.01$, n.s. - no significance. The p values were obtained using the Mann – Whitney test by comparing tumor volume in Doxil[®] alone group (○) and other Doxil[®] and P85 groups. Control group (◆) was injected with saline. (B, C and D) Two weeks after the tumor implantation mice were treated by a single *i.v.* injection of 12 mg/kg of Doxil[®]. P85 (0.02%) was administered *i.v.* either 48 hrs or 96 hrs after the drug, animals were sacrificed and tumor samples were collected 1 or 6 hrs

later. **(B)** Fluorescent micrographs ($\times 10$) of distribution of drug (red) in tumor sections stained for CD31 (green) and nucleus (blue). **(C)** HPLC analysis of Dox in the tumor homogenates of tumors presented in **(B)**. **(D)** Confocal images ($\times 10$) of drug distribution in tumor sections. **(B, D)** Images from paraffin-embedded tissue sections ($0.5 \mu\text{m}$) were performed with **(B)** Zeiss Axioplan 2 imaging microscope or **(D)** Zeiss 710 Confocal Laser Scanning Microscope. **(C)** Data are mean $\pm$ SEM ($n=5$), $*p<0.05$, n.s. - no significance (unpaired t test with Welch's correction).

Table 1

IC₅₀ values (µg/mL) of Doxil® in ovarian cancer cells upon different treatments.

Treatment	Doxil® IC ₅₀ , µg/mL ^a	
	A2780	A2780/Dox
Doxil® (2 hrs)	N.D. [§]	N.D.
Doxil® + 0.1% P85 (2 hrs, co-exposure) ^b	7.55 ± 0.72	8.32 ± 1.12
Doxil® (24 hrs)	48.02 ± 9.70	N.D.
Doxil® (24 hrs) + 0.1% P85 (2 hrs, pre-exposure) ^c	17.48 ± 2.28 ([*])	N.D.
Doxil® (24 hrs) + 0.1% P85 (2 hrs, post-exposure) ^d	14.76 ± 6.48 ([*])	N.D.

^aExperiments were performed in quadruplicate and data is expressed as means ± SD of at least 4 independent experiments.

^bCells were co-incubated for 2 hrs with Doxil® and 0.1% P85, washed 3 times with PBS, and grown in fresh media for 72 hrs before measuring cytotoxicity.

^cCells were first treated with 0.1% P85 for 2 hrs, washed 3 times with PBS, further incubated with Doxil® for 24 hrs, washed 3 times with PBS, and grown in fresh media for 72 hrs before measuring cytotoxicity.

^dCells were first treated with Doxil® for 24 hrs, washed 3 times with PBS, followed by 2 hrs treatment with 0.1% P85, washed 3 times with PBS, and grown in fresh media for 72 hrs before measuring cytotoxicity.

[§]N.D. non-detectable up to 200 µg/mL.

Statistical comparisons were made by Student's t-test between Doxil® (24 hrs) and either one of the pre-or post-exposure groups:

^{*}p < 0.05.