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Lipophilic prodrug conjugates allow facile and rapid synthesis of high loading capacity liposomes without the need for post-assembly purification

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Abstract

Dihydropyridopyrazoles are simplified synthetic analogues of podophyllotoxin that can effectively mimic its molecular scaffold and act as potent mitotic spindle poisons in dividing cancer cells. However, despite nanomolar potencies and ease of synthetic preparation, further clinical development of these promising anticancer agents is hampered due to their poor aqueous solubility. In this paper, we developed a prodrug strategy that enables incorporation of dihydropyridopyrazoles into liposome bilayers to overcome the solubility issues. The active drug was covalently connected to either myristic or palmitic acid anchor via carboxylesterase hydrolyzable linkage. The resulting prodrugs were self assembled into liposome bilayers from hydrated lipid films using ultrasound without the need for post-assembly purification. The average particle size of the prodrug-loaded liposomes was about 90 nm. The prodrug incorporation was verified by differential scanning calorimetry, spectrophotometry and gel filtration reaching maximum at 0.3 and 0.35 prodrug/lipid molar ratios for myristic and palmitic conjugates, respectively. However, the ratio of 0.2 was used in the particle size and biological activity experiments to maintain long-term stability of the prodrug-loaded liposomes against phase separation during storage. Antiproliferative activity was tested against HeLa and Jurkat cancer cell lines *in vitro* showing that the liposomal prodrug retained antitubulin activity of the parent drug and induced apoptosis mediated cancer cell death. Overall, the established data provide a powerful platform for further clinical development of dihydropyridopyrazoles using liposomes as the drug delivery system.

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Keywords

Podophyllotoxin analogue; dihydropyridopyrazole; prodrug-loaded liposome

INTRODUCTION

It is common that many promising natural and synthetic chemotherapy agents fail laboratory and clinical trials due to poor aqueous solubility, instability, insufficient site specificity, and severe general toxicity or formulation issues. (Silverman, 2004, Bildstein *et al.*, 2011) Therefore, it is critically important to address these diverse challenges by developing efficient and reliable drug delivery systems that can greatly enhance the therapeutic efficacy of otherwise unusable drugs. Significant improvement in bioavailability and site specificity of anticancer therapeutics can be achieved through liposome-mediated drug delivery. (Gao *et al.*, 2013, Gulati *et al.*, 1998, Coimbra *et al.*, 2011, Gabizon *et al.*, 2004, Barenholz, 2001) Among other delivery systems, liposomes represent an advanced clinical technology for selective delivery of chemotherapeutics to a desired site of action providing higher intracellular drug concentrations in comparison to systemic administration of the respective drug or its molecular conjugate. (Mahato *et al.*, 2011, Schrama *et al.*, 2006, Singh *et al.*, 2008, Allen and Cullis, 2004, Huang, 2008, Cao *et al.*, 2009) The therapeutic advantages of liposomes for delivering drugs to cancerous tissues include, but are not limited to, prolonged blood circulation to achieve increased tumor accumulation, active targeting with targeting ligands, controlled drug release with pH or temperature, and efficient uptake by cancer cells. (Andresen *et al.*, 2005), (Immordino *et al.*, 2006, Maeda, 2001, Heidel and Davis, 2011) Liposomes consist of an aqueous core surrounded by a lipid bilayer and can be considered universal therapeutic carriers in terms of their ability to accommodate delivery of both hydrophilic and hydrophobic substances. In liposome-mediated cancer therapy, it is important to make sure that the drug-carrying vesicles are stable enough to prevent leakage of the liposomal cargo into the bloodstream. Vesicles that carry hydrophilic drugs in their aqueous compartment must have a composition with a phase transition temperature above body temperature to avoid leakage through liposomal membrane packing defects associated with the fluid phase. (Drummond *et al.*, 1999) In contrast, liposomal formulations of highly hydrophobic or lipophilic anticancer agents, which can be intercalated in the liposome bilayers, produce non-leaky vesicles as it has been demonstrated for liposomal gemcitabine and retinoids. (Pili *et al.*, 2010, Pedersen *et al.*, 2010)

Previously, Magedov *et al.* demonstrated a straightforward one-step multi-component synthesis producing mimetic analogues of podophyllotoxin with low micro-molar antiproliferative activities. (Magedov *et al.*, 2007) More recently, the replacement of methylenedioxybenzene subunit with a pyrazole moiety yielded the second-generation tetracyclic dihydropyridopyrazoles (DPPs) with enhanced nano-molar potencies. (Magedov *et al.*, 2011) It was shown that DPPs exerted anticancer effects by disrupting microtubule dynamics in cancer cells through binding to the colchicine site of β -tubulin. However, despite their nanomolar antiproliferative potencies and potential advantages commonly associated with the “colchinoids,” such as the ability to act as selective vascular disrupting agents and overcome the efflux pump/mutant tubulin/class III β -tubulin overexpression-

mediated multidrug resistance, (Lu, 2012) further development of these agents was hampered due to poor water solubility. The problems associated with these physicochemical characteristics are not unique to DPPs, they are responsible for the failure of dozens of colchinoids that have entered human cancer clinical trials. The poor aqueous solubility of colchinoids has been generally addressed by the preparation of water soluble prodrugs, such as phosphates, (Edsall, 2007) or incorporation of protonatable amino residues. (Ohsumi, 1998) However, the encapsulation of colchinoids into liposomes has not been a popular drug delivery strategy due to the challenge of formulating long-circulating liposomes that retain these fairly low lipophilic agents, while enabling appropriate release kinetics once accumulated in the target site. (Crielaard, 2012)

In this article, we demonstrate that the lipophilic prodrugs of DPP can be used to prepare high loading capacity liposomes using simple self-assembly technique without the need for post assembly purification. Dihydropyridopyrazoles can be readily prepared using a one-step multi-component reaction (MCR) from commercially available starting materials. Here, we synthesized a novel analogue of podophyllotoxin with the dihydropyridopyrazole (DPP) molecular scaffold using inexpensive 3-aminopyrazole, 5-bromovanillin and tetronic acid. In addition, DPP was conjugated to either myristic or palmitic acid anchor producing two lipophilic prodrug derivatives of DPP, DPP-C14 and DPP-C16, for incorporation into liposome bilayers. To this end, synthesis strategy was devised to obtain prodrugs of DPP that incorporate a covalently-linked carboxylesterase-hydrolyzable lipophilic promoiety (fatty acid). Consequently, the prodrug activation was attributed to the action of carboxylesterase, which catalyzes cleavage of an ester bond producing the active drug and inactive promoiety. (Rooseboom *et al.*, 2004, Xu *et al.*, 2008)

MATERIALS AND METHODS

Tetronic acid, myristic acid, palmitic acid, 3-aminopyrazole, 5-bromovanillin, triethylamine (Et_3N), thionyl chloride (SOCl_2), 4-dimethylaminopyridine (DMAP), ethyl acetate (EtOAc), benzene, hexanes, ethanol (EtOH), methanol (MeOH), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), methylene chloride (CH_2Cl_2), sephadex G75, phosphate buffered saline (PBS), RPMI-1640, DMEM, fetal bovine serum (FBS), MTT reagent, porcine pancreas lipase and porcine liver esterase were purchased from Sigma-Aldrich or Fisher Scientific, USA. Chloroform solutions of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)2000] ammonium salt (DSPE-PEG2000) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, USA). Lipophilic tracers DiO and DiI were purchased from Life Technologies, USA. Annexin V-FITC conjugate was ordered from Southern Biotech, USA.

Synthetic methods

General procedure for the synthesis of dihydropyridopyrazoles, including ^1H and ^{13}C NMR and HRMS spectra, is provided in the appendix for this article.

Preparation of the prodrug-enriched lipid films

To prepare prodrug-enriched lipid films the chloroform solutions of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)2000] ammonium salt (DSPE-PEG2000) were mixed with the prodrug solution in ethanol. A solution of a lipophilic fluorescent dye DiO was added to the resulting solutions to enable fluorescence microscopic examination of the mixed components. (Torchilin, 2003) The solvent was evaporated with a nitrogen flow and the resulting films were further dried in the vacuum oven at 50°C for 2 hours. Large lipid vesicles were obtained after hydration of the dry lipid films in DI water at 50°C for 5 minutes followed by slight manual agitation of the hydrated films. The surfaces of dry films and large lipid vesicles were examined using fluorescent microscopy to detect inhomogeneities in the molecular distribution of DiO.

Preparation of liposome suspensions

Lipids composition of empty liposomes (without the prodrug) consisted of DPPC (95 mol%) and DSPE-PEG2000 (5 mol%). The empty liposomes were prepared by consecutive mixing of lipids in chloroform, solvent removal under a stream of nitrogen, vacuum drying at 50°C for 2 hours to obtain dry lipid films, hydration of the lipid film (swelling) in PBS for 2-5 minutes at 50°C and ultrasonic agitation in a laboratory sonicator (Branson, USA) for 15 minutes at 50°C using 1 mg of lipids per 1 mL of PBS. The resulting liposome suspensions were extruded through a 200 nm pore membrane for total of 11 passes. The prodrug-loaded liposomes were produced using the same procedure except that the prodrug solution (10 mM in EtOH) was added while mixing solutions of lipids, so that the prodrug/lipid molar ratio in the initial formulation varied from 0.05 to 0.5. To differentiate the effect of conjugation, DPP-loaded liposome suspensions were prepared by adding DPP solution (100 mM in DMSO) to lipids in chloroform. The DPP/lipids molar ratio varied from 0.05 to 0.2 using 0.05 increments. DMSO was removed from DPP-enriched lipid films by freeze-drying. The DPP-loaded liposome suspensions contained 20 mg of lipids per 1 mL of DI water. A fluorescent dye, DiO, was used in the lipid formulations to acquire images of dry and hydrated prodrug-enriched lipid films on a Nikon Eclipse LV100 fluorescence microscope with a 60X objective lens using a Hamamatsu ORCA-ER digital camera.

Prodrug concentration in the prodrug-loaded liposomes

Prodrug concentrations in the prodrug-loaded liposome suspensions were determined by UV-Vis spectrophotometry using a NanoDrop 2000 spectrophotometer (Cole-Parmer, USA) at 296 nm. Before the spectrophotometry measurements, 10 µL of the prodrug-loaded liposome suspension was dried in a vacuum oven at 50°C and the resulting pellet was dissolved in 10 µL of DMSO. Liposome suspensions with known amount of the prodrug were used as standards for the calibration curve. Empty liposomes were used as the base line reference. The prodrug loading capacity (LC) in the liposomes was defined as the molar percentage of the prodrug intercalated in the liposome membrane and calculated using the following formula: $LC = C_{pro} / (C_{pro} + C_{lip}) \times 100$, where LC is the prodrug loading capacity in the liposome bilayer (mol%), C_{pro} is the prodrug concentration in the prodrug loaded liposome (PLL) suspension (mM), C_{lip} is the total lipids concentration (mM). Solubilized

prodrug concentrations and corresponding prodrug intercalation efficiencies were calculated as averages of three prodrug concentration measurements using independently prepared samples.

Gel-filtration chromatography

Sephadex G75 was hydrated in PBS or DMSO for 24 hours at room temperature. Typical gel suspension had 1.0 g of Sephadex G75 per 20 mL of PBS or 30 mL of DMSO. Mini spin columns of 0.85 mL capacity were packed with the hydrated gel using a laboratory centrifuge (VWR, USA) at 3000 rpm for 30 seconds. After packing, the spin columns were equilibrated with 100 μ L of PBS or DMSO by centrifuging at 2000 rpm for 30 seconds. The column equilibration was repeated three times. Gel-filtration was performed with 100 μ L of an analyte at 2000 rpm for 30 seconds. Inverted fluorescence images of the gel columns and corresponding eluted fractions were acquired using UV transilluminator equipped with a digital camera.

Differential scanning calorimetry (DSC)

The DSC measurements were conducted using a Q2000 differential scanning calorimeter (Thermal Analysis Instruments, USA) and TA Universal Analysis 2000 software. The liposome suspensions for the DSC analysis were prepared without extrusion using the concentration of lipids at 20 mg per 1 mL of DI water. The prodrug/lipid molar ratio varied in the prodrug-loaded liposomes from 0.05 to 0.5 using 0.05 increments. In each DSC measurement, 10 μ L of a liposome suspension was loaded and sealed in an aluminum DSC pan for volatile liquid or solid samples. Either empty aluminum pan or aluminum pan containing 10 μ L of DI water were used as the reference. The heating from 15°C to 55°C was conducted under nitrogen flow at 50 mL/min at the scanning rate of 5 °C/min.

Particle size distribution analysis

Particle size distribution of liposomes was determined on a Zetasizer Nano ZS90 instrument (Malvern Instruments Ltd., UK) equipped with the Zetasizer software (6.20) based on the dynamic light scattering (DLS) analysis. All size measurements were taken in disposable sizing vials using 1 mL of a liposome suspension. Intensity size distributions based on the cumulant DLS data analysis were used to obtain information about the average size of particles in the distribution. Volume size distributions were used to determine relative amount of the material for multiple peaks. The sizes of particles were reported as a mean diameter of three DLS measurements using independently prepared liposome samples.

SEM analysis

Prodrug-loaded liposome sample was prepared using prodrug-enriched lipid film at the prodrug/lipid molar ratio of 0.2. The sample contained 20 mg of lipids per 1 mL of PBS. Before analysis, a small sample quantity was dropped on a Si substrate and let dry in air. The SEM imaging was performed at the Center for Integrated Nanotechnologies (CINT) (Albuquerque, NM) on a FEI Nova Nanolab 600 dual beam system (USA). The electron beam accelerating voltage was set at 2.5 kV and the beam current at 13 pA. A high resolution through-lens-detector was used for secondary electron imaging at 5.0 mm

working distance. No metal coating of the dried liposomes was used before the SEM imaging.

Cell culture

Jurkat cells (non-adherent human T-cell line) were cultured in RPMI-1640 supplemented with 10% FBS, 100 mg/L penicillin G, 100 mg/L streptomycin, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose. HeLa cells (adherent human cervical cancer cell line) were cultured in DMEM supplemented with 10% FBS and 1% of antibiotic solution. The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂. To estimate the number of cells in cell suspensions, Jurkat and HeLa cells were stained with Trypan Blue and counted using a hemocytometer. In all experiments, the initial number of Jurkat cells in cell culture medium was 5×10^5 cells/mL; and the number of HeLa cells was 5×10^4 cells/mL. Live-cell microscopy images were acquired on a phase contrast Fischer Scientific inverted microscope with a 40X objective lens using a digital camera and Micron software (1.08).

MTT assay

Jurkat and HeLa cells were transferred into a 96-well microtiter plate in the volume of 100 µL per well. Prior to treatment, HeLa cells were incubated for 12 hours to allow for proper adhesion and the media were refreshed. Next, cells were treated with the PLL suspensions in two-fold dilution series starting with 1 vol% of the PLLs suspension (prodrug/lipid, 0.2 mol) in the first well. Empty liposomes (1 vol%) and phenylarsine oxide (PAO) at a final concentration of 10 µM were used as positive controls for cell death. For MTT assay without liposomes, cells were treated with DPP or podophyllotoxin using DMSO and PAO as positive controls. After 48 hours of incubation, 20 µL of MTT reagent (5 mg/mL) was added into each well. The plate was incubated for 2 hours at 37°C. Plates containing Jurkat cells were centrifuged at 5000 rpm and 37°C for 15 minutes allowing formation of cell pellets. The media were removed and the resulting formazan crystals were dissolved in 100 µL of DMSO per well. Optical density (OD) at 595 nm was measured using a 96 well microplate reader (Thermomax, USA). The experiments were performed in four replicates and repeated three times for each compound per cell line.

Annexin V/Propidium iodide apoptosis assay

To prepare apoptosis dual staining buffer, 200 µL of a standard Annexin V-FITC conjugate solution (Southern Biotech, USA), 20 µL of propidium iodide (PI) solution (1 mg/mL) and 15 µL of CaCl₂ solution (1.5 mM) were added to 10 mL of HHB buffer. Jurkat cells were treated with the PLLs and incubated at 37°C. After 3, 6, 9, 12, 18, 24, 36, and 48 hours, 200 µL of the cell suspension was centrifuged to form a cell pellet, and the media were replaced with 200 µL of the apoptosis staining buffer. Cells were resuspended and placed in incubator at 37°C for 10 min. The FL1 and FL2 fluorescence were measured using a Becton Dickinson FACScan flow cytometer (BD Biosciences, USA) equipped with Cell Quest analysis software.

Agarose gel electrophoresis of DNA

Jurkat cells were treated with podophyllotoxin, DPP or prodrug-loaded liposomes (prodrug/lipid, 0.2 mol) in a 12 well plate using 1 mL of cell suspension. Cells were harvested for DNA extraction after 24 hours of incubation at 37°C. After centrifugation at 13000 rpm for 15 seconds, the media were removed and 30 µL of a cell lysis buffer containing protease and RNase was added to the cell pellets. The pellets were then resuspended and heated at 55°C for at least 8 hours returning water condensate back to the cell pellets every 2 hours. Next, 15 µL of a gel loading buffer solution was added to each pellet. The resulting solutions, and a 1 kb DNA marker solution in the volume of 20 µL per lane, were loaded on a 1.4% agarose gel stained with ethidium bromide. Gel electrophoresis was carried out in Tris/Borate/EDTA (TBE) buffer at 150 V. The gel images were taken under UV light using a transilluminator equipped with a digital camera.

RESULTS

The synthesis of tetracyclic dihydropyridopyrazole-containing prodrugs DPP-C14 and DPP-C16 is described in the appendix A for this article. Briefly, a one-step multi-component reaction using commercially available and inexpensive reagents was used to obtain these prodrugs. The chemical structures of the synthesized prodrugs were confirmed with ¹H and ¹³C NMR and HRMS analysis (Fig.A1-A13). Both prodrugs contain a hydrophobic fatty acid anchor to incorporate them into the membrane of liposomes. In DPP-C14 the prodrug is composed of a myristic acid and dihydropyridopyrazole (DPP, a fluorophore with two excitation peaks at 280 nm and 305 nm, and one emission peak at 425 nm, Fig.A14), whereas in DPP-C16 the prodrug is composed of a palmitic acid and DPP. The DPP molecule is connected to its hydrophobic anchor via a carboxylic ester linkage to enable enzymatic activation of the prodrug by carboxylesterase, once taken up intracellularly and digested in lysosomes (Fig.1). The design of the developed prodrugs is intended to enable the hydrocarbon chains of the prodrug to intercalate in the phospholipid bilayer simply by preparing the prodrug-enriched lipid films followed by hydration and ultrasound agitation in phosphate buffered saline (PBS) at a temperature above the phase transition temperature of the liposome.

The prodrug-loaded liposome (PLL) suspensions were made with the prodrug-enriched lipid films by consecutive hydration and ultrasonic agitation at 50°C in PBS for 15 minutes followed by extrusion through a polycarbonate membrane with 200 nm pores. The prodrug/lipid molar ratios in the dry lipid films varied from 0.05 to 0.5 to establish maximal prodrug loading capacities in liposomes. It should be noted that the prodrug solubility in the lipid films was limited. It can be seen from Fig.2C that the prodrug formed aggregates when the dry film became oversaturated with the prodrug at the prodrug/lipid molar ratio of 0.5. As shown in Fig.2A, the dry film had uneven DiO distribution at the prodrug/lipid molar ratio of 0.2 showing domains in which the DiO content seemed depleted. This observation suggests deviations from an ideal mixing behavior, where homogenous molecular distribution is expected. (Borden, 2006), (Pagano, 1972) In comparison to the dry films the DiO molecular distribution on the surfaces of large vesicles seemed more homogenous and without distinct domains as it can be seen from Fig. 2B. As a consequence of vesicles self-assembly during

the hydration, the lipophilic prodrug molecules became intercalated in between lipid molecules constituting the lipid bilayers suggesting more homogenous molecular distribution of mixed components in comparison to the bulk lipid films. (Gulati *et al.*, 1998, Kuznetsova *et al.*, 2013, Arouri and Mouritsen, 2012) The prodrug association with liposomes was verified by the gel filtration chromatography using Sephadex G75 (Fig.3). The prodrug-loaded liposomes and empty liposomes containing a fluorescent dye DiI were used to confirm presence of liposomes in the eluted volume and to show base line retention of liposomes on the gel. A true solution of prodrug in DMSO was used as a positive control to demonstrate that free prodrug was trapped in the gel and it was not present in the eluted volume. Since the liposomal prodrug may exchange with serum lipoproteins or other recipients present in biological fluids the prodrug-loaded liposomes were also mixed with 3 volumes of fetal bovine serum (FBS) and placed in incubator at 37°C for 48 hours. A high concentration of prodrug-loaded liposomes in serum was used for better visualization of weakly fluorescent prodrug-loaded liposomes on the gel and in eluted volume under UV light. However, it should be noted that 1:3 volume ratio between liposome suspension and serum is not realistically close to an *in vivo* liposomal drug administration scenario. The resulting liposome suspensions and all other control samples were filtered through the gel using mini spin columns and laboratory centrifuge. UV transilluminator was used to acquire UV images of gel columns and corresponding eluted volumes. It can be seen from Fig.3 that the free prodrug accumulated at the top of column 4, whereas fluorescently labeled and prodrug-loaded liposomes were collected in the eluted fractions labeled 1'-3'. Hence, the prodrug remained associated with liposomes even after incubation in FBS at 37°C for at least 48 hours. It may be suspected that the exchange of liposomal prodrug with lipoprotein constituents was inhibited by PEG2000 that sterically protects the liposomal membrane from collisions. (Song *et al.*, 2002, Jahnig, 1984) In the gel columns labeled 1-3, the gel acquired fluorescence due to the retention of liposomes on the gel surfaces. (Grabielle-Madelmont *et al.*, 2003, Ruyschaert *et al.*, 2005)

It is reasonable to assume that the membrane of PLLs became fully saturated with the prodrug when the liposome suspensions were made with the lipid films that were oversaturated with the prodrug. Cloudy PLL suspensions indicative of full saturation of the liposome bilayer with the prodrug were made with DPP-C14 at the prodrug/lipid molar ratio of 0.3, whereas DPP-C16-loaded liposome suspensions became cloudy at the prodrug/lipid molar ratio of 0.4. These cloudy suspensions were filtered through a 200 nm pores polycarbonate membrane to remove the unincorporated prodrug particles and determine the highest solubilized prodrug concentrations in the resulting liposome suspensions. The corresponding highest solubilized DPP-C14 and DPP-C16 concentrations, which were determined by UV-Vis spectrophotometry, were 0.36 ± 0.04 mM and 0.45 ± 0.02 mM, respectively. The corresponding maximal prodrug loading capacities were 23.4 ± 1.8 mol% and 27.7 ± 1.0 mol%. Consequently, the loading capacity of palmitic prodrug (DPP-C16) was 18.3% higher than that of myristic (DPP-C14) as shown in Fig.4.

The phase transition behavior of the prodrug-loaded liposomes was investigated by differential scanning calorimetry (DSC). (Ikeda *et al.*, 2011, van Wezel *et al.*, 1996, Demetzos, 2008) The choice of a reference sample for the DSC measurements (an empty pan or a water-filled pan) resulted in similar parameters derived from the parallel DSC

analysis, such as the onset temperature of the transition (T_o) and the temperature at the peak (T_m). The scanning rate of 0.5 °C/min resulted in the inability to identify the phase transition peak in the PLL suspensions with the prodrug/lipid molar ratio of 0.1 and higher because of the wide broadening of the phase transition peak that became indistinguishable from the base line at these ratios. Changing the scanning rate to 5 °C/min increased the DSC instrument sensitivity towards the phase transition signal such that all phase transition peaks were visible within the investigated range of prodrug/lipid molar ratios (Fig.5 and Fig.6). The DSC studies showed that the prodrug incorporation produced a decrease in both the onset temperature and the main phase transition temperature as the DSC endotherm broadened and the enthalpy of transition decreased (Fig.B1) indicating that the bilayer of the prodrug-loaded liposomes became less rigid and therefore less stable against phase separation. It can be seen from Fig.B2 and Fig.B3 that the minimal values of T_o and T_m indicative of saturation of the liposome bilayer with the prodrug occurred at 0.3 and 0.35 prodrug/lipid molar ratios for DPP-C14- and DPP-C16-loaded liposomes, respectively, whereas the half-height width (HHW) of the endotherms increased reaching maximal values at those ratios (Fig.B4). Hence, the DSC data on the maximal prodrug incorporation correlated fairly well with the maximal loading capacities that were determined spectrophotometrically. To differentiate the effect of conjugation, the DSC thermograms were also obtained for DPP-loaded liposome suspensions. It can be seen from Fig.7 that DPP did not lower the phase transition temperature as both prodrugs did. Moreover, DPP formed a precipitate when it was added to lipids in chloroform whereas the prodrugs were fully soluble. Overall, DPP loading capacity in liposomes seemed much smaller than that of the prodrug conjugates, suggesting the prodrug incorporation in liposome bilayers occurred via the fatty acid anchor. It should be noted that in all further experiments the prodrug-loaded liposomes were prepared using the prodrug/lipid molar ratio of 0.2 to determine the particle size distribution, the particle size stability and biological activity. The prodrug-loading capacity in liposomes was kept at this level to maintain a biologically relevant solubilized prodrug concentration in prodrug-loaded liposome suspensions and, at the same time, minimize the chances of phase separation in the prodrug-loaded liposome suspensions during storage.

Particle size distribution of prodrug-loaded liposomes at the 0.2 ratio was analyzed by dynamic light scattering (DLS). Typically, the DLS measured diameters of sonicated prodrug-loaded liposomes were in the 20-400 nm range with a polydispersity index of 0.3 or below indicating that the obtained data met quality criteria for the DLS measurements. The particle size and spherical morphology of prodrug-loaded liposomes were also confirmed by scanning electron microscope imaging (Fig.B5). The absence of micelles in the PLLs suspensions was supported by lack of a separate micelle peak in the particle size distribution (Fig.B6). Consequently, there is no need for the post-assembly purification from prodrug-containing micelles because the solubilized prodrug was incorporated entirely in liposomes. The average liposome diameter was not affected by the extrusion through a 200 nm pore membrane because the size of particles was typically smaller than 200 nm. However, the average diameter of liposomes that were filtered through Sephadex G75 gel slightly increased indicating that smaller liposomes remained on the gel after the gel filtration, whereas the mode diameter remained unaffected as shown in Table 1. After extrusion, the

average particle diameters of DPP-C14 and DPP-C16 liposomes were 85 ± 3 nm and 87 ± 4 nm, respectively, indicating that the size of the PLLs could be easily fitted within the acceptable range for clinical applications based on the enhanced permeability and retention (EPR) effect. (Maeda, 2001, Maeda, 2010) It can be seen from Fig.8 that the average particle sizes of both DPP-C14- and DPP-C16-loaded liposomes increased approximately by 15% during the first three weeks of storage at 4°C with no apparent change in the liposome suspensions that remained clear (*i.e.*, no phase separation occurred). However, long-term stability of the DPP-C16-loaded liposomes was greater than that of the DPP-C14-loaded liposomes. The formation of visible aggregates in the DPP-C14-loaded liposome suspensions was noticed after about 3 months of storage at 4°C. The DPP-C16-loaded liposome suspensions remained mostly clear after 6 months at 4°C.

To demonstrate that the liposomal prodrug can be activated by carboxylesterase, the prodrug-loaded liposomes and the empty liposomes were treated with the pig liver carboxylesterase using 1 mg of carboxylesterase per 1 mL of the liposome suspension. The presence of liberated DPP in the resulting suspensions was verified by the thin layer chromatography using DPP as the reference. All carboxylesterase experiments were carried out at 37°C for 24 hours. DLS measurements were used to monitor changes in the particle size distribution of liposomes during the prodrug activation reaction. The results of a set of DLS measurements are shown in Fig.B7. It can be seen from Fig.B7 that the prodrug-loaded liposome samples initially have the average diameter of about 100 nm, consistent with a transparent suspension of unilamellar liposomes. After 1 hour, samples that were treated with carboxylesterase demonstrated a quick increase of the average particle size. It should be indicated that the average particle size obtained after 2 hours varies from sample to sample and it is difficult to gain quantitatively reliable data by the DLS measurements due to the low transparency of the samples as the liberated DPP formed cloudy suspensions. After 24 hours of incubation, three peaks at 15 nm, 65 nm, and 304 nm as shown in Fig.B8 represented the size distribution in the prodrug-loaded samples that were treated with carboxylesterase. The corresponding size distribution by volume was used to determine the relative amounts of the material among these three peaks that were 88.4 % volume, 7.5 % volume, and 4.1 % volume, respectively, indicating that carboxylesterase induced the size degradation in the prodrug-loaded samples. In contrast to the prodrug-loaded liposomes, the particle size distribution of the empty liposomes that were treated with carboxylesterase remained unaffected over a period of 24 hours suggesting that the enzyme did not degrade empty liposomes as shown in Fig.B9. In addition, hydrolysis of the prodrug-loaded liposomes was not detected in FBS nor in FBS that was enriched with the pig pancreas lipase (2.5 mg of lipase per 1 mL of liposomes in FBS) for at least 7 days, underscoring that major serum esterases and lipases are unlikely to hydrolyze the liposomal prodrug.

Activation of the liposomal prodrug was also tested in Jurkat and HeLa cells *in vitro*. Cellular uptake of both empty and prodrug-loaded liposomes was confirmed by flow cytometry measurements using DiO as a fluorescent marker (Fig.C1). It was found that the equimolar concentrations of the liposomal DPP-C14 or DPP-C16 produced the same cytotoxic effect. The half-maximal inhibitory concentrations (IC_{50}) for the liposomal prodrug, dihydropyridopyrazole (parent drug), and podophyllotoxin were determined by the MTT assay. In the case of the liposomal prodrug, the IC_{50} value against Jurkat and HeLa

cells were 120 ± 10 nM and 780 ± 150 nM, respectively, whereas the IC_{50} values for the parent drug were 90 ± 10 nM and 1650 ± 610 nM. Hence, the liposomal prodrug retained the activity level of the parent drug against the Jurkat cells whereas it was about 2 times more potent than DPP against the HeLa cell line. The IC_{50} values were also determined for podophyllotoxin and compared to those found elsewhere. It was found that the IC_{50} value for podophyllotoxin against the Jurkat cells was 12 ± 1 nM, which was also determined by Chernysheva *et al.* at 10 nM (Chernysheva *et al.*, 2012); and the IC_{50} value against the HeLa cells was 25 ± 5 nM, whereas Jordan *et al.* reported the IC_{50} of 20 nM (Jordan *et al.*, 1992). In both cell lines, the treatment with the prodrug-loaded liposomes resulted in apoptosis mediated cancer cell death. Apoptosis was confirmed by the flowcytometry using an annexin V-FITC probe (Fig.C2). After the treatment with the prodrug-loaded liposomes, Jurkat cells were also positive for the nucleosomal DNA fragmentation (Fig.9). The mechanism of action of the liposomal prodrug was correlated from observing characteristic morphological changes in Jurkat cells that were treated with podophyllotoxin, dihydropyridopyrazole or prodrug-loaded liposomes (Fig.10). In all cases, dramatic distortion of cellular shape was observed as early as 3 hours post-treatment suggesting that the liposomal prodrug had a short activation time, and it retained the microtubule destabilizing activity of the parent DPP.

DISCUSSION

In this multidisciplinary work, a prototype of a nanoscale anticancer drug delivery system was developed. A second-generation dihydropyridopyrazole (DPP) with improved antiproliferative properties was chosen for conjugation with myristic or palmitic acid. The prodrug synthesis was carried out via a one-step multi-component reaction (MCR) using commercially available reagents, unveiling the potential to make the prodrug-loaded liposomes readily available for broader distribution. The obtained lipophilic prodrug was successfully incorporated into liposome bilayers from hydrated prodrug-enriched lipid films using ultrasound agitation. As a result, the prodrug was incorporated entirely in liposomes and was not present in a free form nor incorporated in micelles. These non-leaky prodrug-loaded liposomes were rapidly generated within 15 minutes using a simple self-assembly technique that did not require post-assembly purification, indicating that the PLL production on a larger scale could be cost effective and less time consuming. The prodrug association with liposomes was verified by spectrophotometry, gel filtration, and the DSC measurements. Although the maximal prodrug-loading capacities were determined at 0.3 and 0.35 prodrug/lipid molar ratios for DPP-C14 and DPP-C16, respectively, the prodrug/lipid molar ratio of 0.2 was used in the particle size and biological evaluation experiments. This ratio was preferred to avoid the prodrug precipitation in the lipid films and provide a midrange prodrug loading capacity in liposomes for better stability of the PLL suspensions against phase separation during storage. Resistance of the prodrug-loaded liposomes to the long-term aggregation underscores stability of the prodrug-loaded membrane against phase separation. The DPP-C16-loaded liposomes were more stable against phase separation in the PLL suspensions due to stronger Van der Waals interaction of the palmitic acid moiety with the DPPC acyl components. (Petrache, 2000) The liposomal prodrug was activated by carboxylesterase to release the parent DPP from the liposomes into the solution. Such mechanism of the drug release from the prodrug-loaded liposome may be relevant for the

accumulation of the liposomes in certain tumor tissues with elevated lysosomal esterase levels (Charles W. Young, 1973) and requires additional *in vivo* studies. It should be noted that it is unlikely that the liposomal prodrug can be a substrate candidate for major esterases found in human serum, such as Paraoxonase-1, which is involved in the hydrolysis of organophosphates, and Butyrylcholinesterase which hydrolyses many different choline esters, suggesting that the liposome-loaded prodrug should be resistant to hydrolysis upon injection and circulation in blood. (Hernandez, 2009) To support this notion, the PLLs demonstrated stability against prodrug hydrolysis in FBS for at least 7 days at 37°C. In addition, PLLs were also stable against hydrolysis in FBS that was enriched with pig pancreas lipase (2.5 mg/mL) for at least one week at 37°C. Interestingly, the pig liver carboxylesterase, that was used in this study for activation of the liposomal prodrug, is closely related to human carboxylesterase 1 (hCE-1) (Bencharit, 2003). hCE-1 has a broad range of hydrolytic activity towards ester- and amide-bond-containing drugs including long-chain fatty acid esters and thioesters, aromatic and aliphatic esters indicating that hCE-1 would be responsible for the hydrolysis of the DPP prodrugs. Importantly, hCE-1 is mostly found in the liver tissues and may only be present in plasma in negligible amounts unable to substantially affect the hydrolysis of the liposomal prodrug. (Na *et al.*, 2009) Thus, we suspect that the obtained prodrug-loaded liposomes will be stable against prodrug hydrolysis in the bloodstream. Alternatively, considering that endocytosis is often described as the privileged pathway for liposomes to enter the cellular interior, the prodrug activation is likely to occur in a hydrolytic environment of lysosomes, where acid hydrolase enzymes break down macromolecules, cellular debris and waste materials, while allowing the intact prodrug to take advantage of liposome targeting strategies. (Pollock *et al.*, 2010, Huth *et al.*, 2006) Hence, treatment with the prodrug-loaded liposomes that have greater therapeutic loading capacity is likely to provide higher intracellular therapeutic concentrations. Liposome recognition by cancer cells, as well as kinetic efficiency of endocytosis, can be improved by decorating the liposome surface with cancer-specific ligands or antibodies. If applicable, this approach is expected to improve not only the targetability of PLLs against cancer cells but also promote better liposome uptake rates by cancer cells and, therefore, enhance overall therapeutic efficacy of treatment with the PLLs. It should be indicated that the size of these liposomes is within the safe therapeutic range of 10-200 nm. (Yallapu *et al.*, 2012) Particles smaller than 10 nm are rapidly cleared by the kidneys or through extravasation, and larger entities (~100-200 nm) are cleared by the mononuclear phagocyte system (MPS). (Petros, 2004, Ruiz *et al.*, 2013) The average particle size of the prodrug-loaded liposomes was about 90 nm, which is essential for clinical applications based on the EPR effect. It should be noted that when the size of liposomes becomes smaller, the particle surface-area-to-volume ratio is increased. At the nanolevel, such an increase becomes significant and the number of therapeutic lipophilic molecules intercalated in the liposome bilayer may exceed that of the conventional liposome-encapsulated hydrophilic anticancer drugs, such as doxorubicin hydrochloride. (Niu *et al.*, 2010) Assuming that the prodrug molecule is interchangeable with DPPC in terms of the intermolecular space occupied in the bilayer, and liposomes are unilamellar and monodisperse (100 nm in diameter) with the bilayer thickness of 5 nm, the surface area of the DPPC head group of 0.71 nm², it can be estimated that the total number of molecules in one PLL is 80047. It can also be calculated that the number of liposomes in 1 mL of the PLL suspension is 1.2×10^{16} presuming that the

total phospholipids and solubilized prodrug concentrations are 1.20 mM and 0.45 mM, respectively. Given the maximal prodrug loading capacity of 27.7 mol%, each prodrug-loaded liposome will contain approximately 22160 prodrug molecules. Taking into account that the volume of the aqueous core of such liposomes is 3.8×10^{-19} L, the hypothetical prodrug concentration in this volume will be approximately 97mM. In comparison, the maximal concentration of liposome-encapsulated doxorubicin is expected not to exceed 17 mM based on the aqueous solubility of doxorubicin hydrochloride (10 mg/mL). (Fritze *et al.*, 2006) Consequently, the maximal therapeutic loading capacity of the obtained prodrug-loaded liposomes was estimated to be about 5.7 times higher than that of liposome-encapsulated doxorubicin systems. In addition, considerable amounts of doxorubicin may not be encapsulated in liposomes and therefore will have to be isolated from liposome suspensions during post-assembly purification (*i.e.*, dialysis), (Niu *et al.*, 2010) whereas the obtained lipophilic prodrugs can be fully incorporated in liposomes. Furthermore, the developed lipophilic prodrugs were fairly miscible with lipids and might find use in theranostic systems such as lipid-stabilized microbubbles further expanding possible future applications of these promising anticancer agents. (Tartis *et al.*, 2006, Tartis *et al.*, 2008) The rationale behind the use of microbubbles for drug delivery is based on the premise that the microbubble can act as both a drug carrier and a local drug delivery activator in conjunction with ultrasound, adding active targeting to this strategy. (Pitt *et al.*, 2004, Caskey *et al.*, 2011)

CONCLUSIONS

Poor water solubility of dihydropyridopyrazoles was resolved by designing lipophilic carboxylesterase-hydrolyzable prodrugs for liposomal incorporation. High therapeutic loading capacity of the prodrug-loaded liposomes was achieved due to prodrug incorporation in liposome bilayers compared to conventional hydrophilic drugs encapsulation. Prodrug-loaded liposomes were efficiently generated from prodrug-enriched lipid films using ultrasound without the need for post-assembly purification. The palmitic prodrug (DPP-C16) performed better than the myristic (DPP-C14) because it showed higher therapeutic loading capacity and better long-term stability of the resulting prodrug-loaded liposomes against phase separation during storage at 4°C. Prodrug activation was demonstrated in both the carboxylesterase activation assay and in Jurkat and HeLa cell lines *in vitro* confirming that the prodrug retained antitubulin activity of the parent DPP and induced apoptosis-mediated cancer cell death. Overall, the combined experimental results suggest that the obtained lipophilic prodrugs can be used as cytotoxic cargo of non-leaky liposomes. However, additional *in vivo* studies are required to determine feasibility of the developed prodrug-loaded liposomes in clinical implications for cancer.

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Appendix A. Chemical methods

General synthetic methods

All reactions were monitored by thin layer chromatography (TLC) on pre-coated (250 μ m) silica gel 60F254 glass-backed plates. Visualization was accomplished with UV light. Flash column chromatography was performed using Kieselgel 60 (230-400 mesh). Melting points were reported uncorrected. ^1H and ^{13}C NMR spectra were recorded on Jeol Eclipse 300 or Bruker Avance III 400 spectrometers. Chemical shifts (δ) were reported in ppm relative to the TMS internal standard. Abbreviations are as follows: s (singlet), d (doublet), t (triplet), m (multiplet). HRMS analyses were performed at the Mass Spectrometry Facility, University of New Mexico. Samples were run on LCT Premier TOF mass spectrometer.

Coupling reaction

The solution of myristic acid (2.2 mmol) or palmitic acid (2.2 mmol) in 7.0 mL of benzene was heated to reflux, and 2 mL of SOCl_2 was added in one portion. After stirring for 2 h, the solvent and remaining SOCl_2 were removed under reduced pressure. The obtained yellow oil was mixed with 3 mL of dried CH_2Cl_2 and added dropwise into the solution of 5-bromovanillin (2.0 mmol) and DMAP (2.0 mmol) in dried CH_2Cl_2 (7.0 mL). The reaction mixture was stirred at 0°C for 1 h. Solvent was removed under reduced pressure, and the final product was purified with a flash column (EtOAc/hexanes, 1/10).

Intermediate 1 (myristate-conjugated 5-bromovanillin)

80% as white solid, ^1H NMR (CDCl_3) δ 9.87 (s, 1H), 7.68 (s, 1H), 7.41 (s, 1H), 3.88 (s, 3H), 2.64 (t, $J = 7.4$ Hz, 2H), 1.78 (m, 2H), 1.25 (s, 20H), 0.87 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR (CDCl_3) δ 189.9, 170.2, 153.4, 143.2, 135.3, 127.9, 118.2, 109.9, 56.5, 33.9, 32.0, 29.7-29.1, 24.9, 22.8, 14.2.

Intermediate 2 (palmitate-conjugated 5-bromovanillin)

80% as white solid, ^1H NMR (CDCl_3) δ 9.87 (s, 1H), 7.68 (s, 1H), 7.41 (s, 1H), 3.88 (s, 3H), 2.64 (t, $J = 7.4$ Hz, 2H), 1.78 (m, 2H), 1.25 (s, 24H), 0.87 (t, $J = 6.9$ Hz, 3H); ^{13}C NMR (CDCl_3) δ 189.9, 170.2, 153.4, 143.2, 135.3, 127.9, 118.2, 109.9, 56.5, 33.9, 32.0, 29.7-29.1, 24.9, 22.8, 14.2.

Procedure for dihydropyridopyrazole synthesis

A mixture of 3-aminopyrazole (1 mmol), tetronic acid (1 mmol), triethylamine (0.05 mL), and corresponding aldehyde (5-bromovanillin or intermediate **1** or **2**) (1 mmol) in EtOH (4 mL) was refluxed for 1-2 h. The reaction mixture was allowed to cool to room temperature. DPP formed a white precipitate and was collected by vacuum filtration and recrystallized from DMF/ H_2O . Neither DPP-C14 nor DPP-C16 formed precipitates. These products were isolated and purified by silica gel flash column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 95/5).

DPP (active drug)

34% as white solid, MW = 378.17 g/mol, mp > 280°C decomp., ^1H NMR (DMSO- d_6) δ 12.27 (s, 1H), 9.19 (s, 1H), 7.37 (s, 1H), 6.87 (s, 1H), 6.73 (s, 1H), 4.97 (d, J = 15.3 Hz, 1H), 4.88 (s, 1H), 4.84 (d, J = 15.3 Hz, 1H), 3.79 (s, 3H); ^{13}C NMR (DMSO- d_6) δ 172.7, 161.1, 148.8, 147.0, 142.6, 138.8, 127.8, 122.9, 111.2, 109.7, 105.9, 95.2, 56.8, 31.4; HRMS m/z (ESI) calculated for $\text{C}_{15}\text{H}_{12}\text{BrN}_3\text{O}_4\text{Na}$ ($M + \text{Na}$) $^+$ 399.9909, found 399.9911.

DPP-C14

38% as white solid, MW = 588.53 g/mol, mp = 185-188°C, ^1H NMR (Aceton- d_6) δ 11.58 (s, 1H), 9.32 (s, 1H), 7.46 (s, 1H), 7.13 (s, 1H), 7.01 (s, 1H), 5.09 (s, 1H), 4.96 (d, J = 15.7 Hz, 1H), 4.86 (d, J = 15.4 Hz, 1H), 3.79 (s, 3H), 2.57 (t, J = 7.4 Hz, 2H), 1.72 (m, 2H), 1.28 (s, 20H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR (Acetone- d_6) δ 171.8, 170.0, 159.9, 152.5, 147.4, 145.9, 136.3, 127.5, 122.7, 116.5, 111.58, 105.3, 96.3, 65.1, 55.8, 33.3-13.6; HRMS m/z (ESI) calculated for $\text{C}_{29}\text{H}_{38}\text{BrN}_3\text{O}_5\text{Na}$ ($M + \text{Na}$) $^+$ 610.1893, found 610.1896.

mar2DPP-C16

37% as white solid, MW = 616.59 g/mol, mp = 175-178°C, ^1H NMR (Acetone- d_6) δ 11.58 (s, 1H), 9.32 (s, 1H), 7.46 (s, 1H), 7.13 (s, 1H), 7.01 (s, 1H), 5.09 (s, 1H), 4.96 (d, J = 15.7 Hz, 1H), 4.86 (d, J = 15.4 Hz, 1H), 3.79 (s, 3H), 2.57 (t, J = 7.4 Hz, 2H), 1.72 (m, 2H), 1.28 (s, 24H), 0.87 (t, J = 6.9 Hz, 3H); ^{13}C NMR (Acetone- d_6) δ 171.8, 170.0, 159.9, 152.5, 147.4, 145.9, 136.3, 127.5, 122.7, 116.5, 111.58, 105.3, 96.3, 65.1, 55.8, 33.3-13.6; HRMS m/z (ESI) calculated for $\text{C}_{31}\text{H}_{42}\text{BrN}_3\text{O}_5\text{Na}$ ($M + \text{Na}$) $^+$ 638.2206, found 638.2217.

Figure A1.

^1H NMR spectrum of the palmitate-conjugated 5-bromovanillin (intermediate 2) in CDCl_3 .

Figure A2.

^{13}C NMR spectrum of the palmitate-conjugated 5-bromovanillin (intermediate 2) in CDCl_3 .

Figure A4.
¹³C NMR spectrum of the myristate-conjugated 5-bromovanillin (intermediate 2) in CDCl₃.

Figure A5.

^1H NMR spectrum of the palmitate-conjugated dihydropyridopyrazole (DPP-C16) in acetone- d_6 .

Figure A6.

^{13}C NMR spectrum of the palmitate-conjugated dihydropyridopyrazole (DPP-C16) in acetone- d_6 .

Figure A7.
¹H NMR spectrum of the myristate-conjugated dihydropyridopyrazole (**DPP-C14**) in acetone-d₆.

Figure A8.
 ^{13}C NMR spectrum of the myristate-conjugated dihydropyridopyrazole (DPP-C14) in acetone- d_6 .

Figure A9.

^1H NMR spectrum of the dihydropyridopyrazole (DPP) in DMSO-d_6 .

Figure A10.
 ^{13}C NMR spectrum of the dihydropyridopyrazole (DPP) in DMSO- d_6 .

Figure A11.
HRMS spectrum of the palmitate-conjugated dihydropyridopyrazole (DPP-C16).

Figure A12.
HRMS spectrum of the myristate-conjugated dihydropyridopyrazole (DPP-C14).

Figure A13.
HRMS spectrum of the dihydropyridopyrazole (DPP).

Figure A14.
Excitation/emission spectrum of DPP in ethanol.

Appendix B. Characterization of liposomes

Figure B1.
The change in the enthalpy of the phase transition for DPP-C14- and DPP-C16-loaded liposomes.

Figure B2.

Comparison of the onset temperatures of the phase transition between DPP-C14- and DPP-C16-loaded liposomes.

Figure B3.

Comparison of the peak temperatures of the phase transition between DPP-C14- and DPP-C16-loaded liposomes.

Figure B4.

Comparison of the half-height width (HHW) of the endotherms between DPP-C14- and DPP-C16-loaded liposomes.

Figure B5.

SEM image of the prodrug-loaded liposomes (prodrug/lipid, 0.2 mol). The SEM sample was prepared without metal coating to avoid possible modification of the liposomal structure. Nonetheless, these liposomes were intact and retained their spherical shape even after they were dried and exposed to the electron beam during the SEM imaging analysis.

Figure B6.

Typical PSD diagram of the prodrug-loaded liposomes (prodrug/lipid, 0.2 mol). Absence of micelles in liposome suspensions indicates that the prodrug is solely associated with liposomes.

Figure B7.

Effect of addition of carboxylesterase on the average particle size distribution of the prodrug-loaded (DPP-C16) liposomes.

Figure B8.

Size distribution by intensity and the corresponding size distribution by volume for the PLLs (prodrug/lipid, 0.2 mol) after the 24 hours treatment with carboxylesterase at 37°C.

Figure B9.

Size stability of the empty liposomes in esterase enriched solutions. Black line represents the size distribution of empty liposomes before the treatment with carboxylesterase. Grey line represents the size distribution of empty liposomes after 24 hours of incubation with carboxylesterase at 37°C.

Appendix C. Biological characterization

Figure C1.

Uptake of liposomal DiO by Jurkat cells observed by flowcytometry. Definitions: No treatment – cells were not treated with liposomes; Empty liposomes – cells were treated with empty liposomes; DiO liposomes – cells were treated with empty liposomes labeled with DiO; DiO+DPP-C16 liposomes – cells were treated with prodrug-loaded liposomes labeled with DiO.

Figure C2.

Primary induction of apoptosis in Jurkat cells treated with the PLLs (prodrug/lipid, 0.2 mol). Definitions: Living (%) – percentage of live cells in the cell sample that was treated with the prodrug-loaded liposomes; Apoptotic (%) – percentage of apoptotic cells in the cell sample that was treated with the prodrug-loaded liposomes; Necrotic (%) – percentage of necrotic cells in the cell sample that was treated with the prodrug-loaded liposomes; Control living (%) – percentage of live cells in the untreated cells sample; Control apoptotic – percentage of apoptotic cells in the untreated cells sample. It can be seen that apoptosis is induced before appearance of necrotic cells in the cells sample that was treated with the prodrug-loaded liposomes.

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Figure 1.

The prodrug design strategy for intracellular enzymatic activation in cancer cells. A one-step multi-component reaction (MCR) was used to synthesize dihydropyridopyrazole (DPP). Two carboxylesterase-hydrolyzable lipophilic prodrugs DPP-C14 and DPP-C16 were obtained for incorporation in liposome bilayers. Enzymatic bioactivation of the liposomal prodrug resulted in dramatic morphological changes in Jurkat cells confirming antitubulin activity of the parent drug.

Figure 2.

Distribution of a lipophilic tracer DiO in dry (A and C) and hydrated (B) prodrug-enriched lipid films. A. Dry prodrug-enriched lipid film (prodrug/lipid, 0.2 mol); B. Hydrated prodrug-enriched lipid film (prodrug/lipid, 0.2 mol). This lipid film was hydrated in DI water at 50°C for 5-10 minutes without sonication. C. Dry oversaturated prodrug-enriched lipid film (prodrug/lipid, 0.5 mol) after the prodrug precipitation. In this image, the prodrug aggregates appear as black dots.

Figure 3.

Inverted fluorescence images of mini gel columns packed with Sephadex G75 and corresponding eluted fractions. The gel gained fluoresces in columns 1-3 due to the retention of liposomes containing fluorescent molecules (prodrug or DiI). Upper row demonstrates inverted fluoresce due to: 1.Retention of DiI-loaded liposomes as a positive control to show baseline retention of liposomes on gel and in eluted sample, 2.Retention of the prodrug-loaded liposomes, 3.Retention of the prodrug-loaded liposomes mixed with FBS, 4.Retention of the free prodrug. Lower row shows inverted fluorescence of the corresponding eluted fractions: 1'.DiI-loaded liposomes, 2'.Prodrug-loaded liposomes, 3'.Prodrug-loaded liposomes mixed with FBS, 4'.Clear DMSO (the free prodrug was trapped by the gel).

Figure 4. Prodrug loading capacity in liposomes. The loading capacity is expressed as the molar percentage of the prodrug intercalated within the liposome membrane (mol%).

Figure 5. DSC curves for the DPP-C14-loaded liposomes. Prodrug/lipid molar ratio in the prodrug-enriched lipid films varied from zero (empty liposomes) to 0.45.

Figure 6. DSC curves for the DPP-C16-loaded liposomes. Prodrug/lipid molar ratio in the prodrug-enriched lipid films varied from zero (empty liposomes) to 0.45.

Figure 7.
DSC curves for the DPP-loaded liposomes. DPP/lipid molar ratio in initial formulation varied from 0.05 to 0.2.

Figure 8.
Average particle size stability of DPP-C14- and DPP-C16-loaded liposomes at 4°C.

Figure 9.

Agarose gel electrophoresis of DNA from treated Jurkat cells. 1. DNA marker, 2. Untreated control, 3. DPP, 4. Podophyllotoxin, 5, 6. Prodrug-loaded liposomes containing DPP-C14 and DPP-C16, respectively.

Figure 10. Morphological changes in Jurkat cells indicative of microtubule destabilizing activity of the liposomal prodrug. A. Untreated control, B. Prodrug-loaded liposomes, C. DPP, D. Podophyllotoxin.

Table 1

Particle size distribution of the prodrug-loaded liposomes.

Sample condition	Average diameter (nm)	Mode diameter (nm)	PDI
DPP-C14-loaded liposomes			
After extrusion	85±3	128±18	0.265
After gel filtration	93±3	126±10	0.325
DPP-C16-loaded liposomes			
After extrusion	87±4	120±5	0.256
After gel filtration	94±1	130±6	0.314