Prodrug Strategy to Achieve Lyophilizable, High Drug Loading Micelle Formulations Through Diester Derivatives of β-Lapachone

*Xinpeng Ma, Xiumei Huang, Gang Huang, Longshan Li, Yiguang Wang, Xiuquan Luo, David A. Boothman, and Jinming Gao**

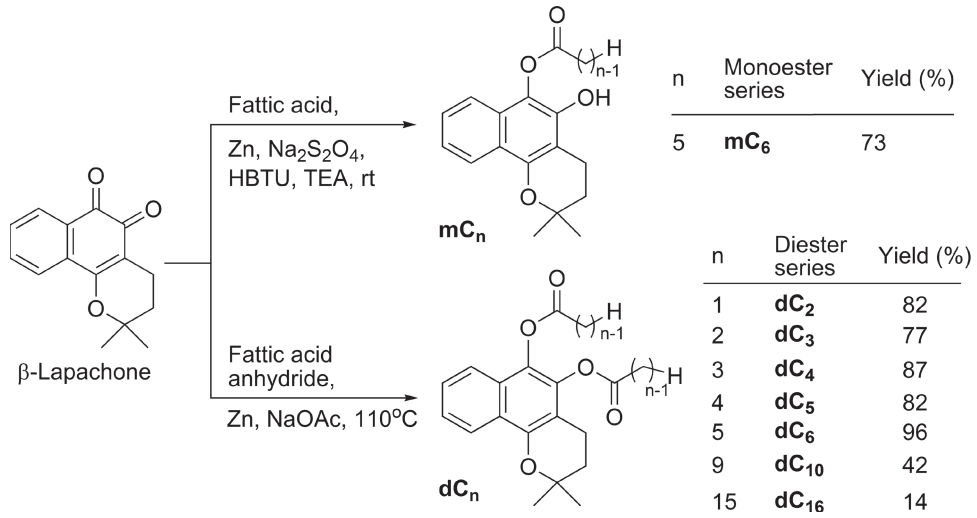
β-Lapachone (β-lap) is a natural product isolated from the Lapacho tree in the rainforest of South America.[1] It kills a broad spectrum of cancer cells in an NAD(P)H:quinone oxidoreductase-1 (NQO1, a.k.a. DT-diaphorase, E.C.1.6.5.2)-dependent manner.[2] In cancer cells overexpressing NQO1, β-lap undergoes a futile redox cycle, resulting in massive production of reactive oxygen species.[3] For every mole of β-lap, >60 moles of NAD(P)H are consumed and >120 moles of H_2O_2 are generated in ≈2 min.[4] Cell death occurs specifically in cancer cells, while normal cells with endogenous, low levels of the enzyme are spared.[5] Despite its therapeutic promise, clinical use of β-lap is greatly hampered by its low water solubility (0.038 mg mL^{-1}) and poor pharmacokinetics. Previous and current formulations using hydroxylpropyl β-cyclodextrin (HP•β-CD) (ARQ501, ARQ761, respectively) showed a 400-fold increase in solubility.[6] However, rapid drug clearance from the blood ($t_{1/2,}\beta$ = 24 min), hemolysis due to HP•β-CD carrier and drug-induced methemoglobinemia were also observed.[7] Recently, our laboratory reported the development of polymeric micelles for the delivery of β-lap.[7b,8] Previous results show that micelles composed of poly(ethylene glycol)-*b*-poly(D,L-lactic acid) (PEG-*b*-PLA), a copolymer that is considered safe by the FDA for drug delivery, significantly improved the safety and antitumor efficacy over ARQ501. However, the major limitation of this micellar formulation was the low drug loading density (2.2 wt%) and efficiency (<40%), resulting from the fast crystallization of β-lap (yellow needle crystals).[8] In this study, we investigated a prodrug strategy to improve the formulation properties of β-lap. Prodrugs have been widely used in pharmaceutical industry to improve the physicochemical and biopharmaceutical properties of parent drugs.[9] Among these, ester groups are most commonly used to improve lipophilicity and membrane permeability of drugs containing carboxylate or phosphate groups. Ester groups are readily hydrolyzed by many types of esterase and readily convert inactive prodrugs into active parental drugs in the body.[10] In this study, we investigated the use of carbonic ester prodrugs of b-lap to improve drug compatibility with the PEG-*b*-PLA carrier while reducing their crystallization propensity. Results showed greatly improved drug loading density (15 wt%) and efficiency (>90%), high apparent drug solubility (>7 mg mL^{-1}), storage stability, efficient esterase-mediated conversion to β-lap, and the ready ability of reconstitution after lyophilization.

Figure 1 shows the synthetic scheme of β-lap prodrug derivatives. We first examined the monoester derivative of β-lap (mC_6 was used as an example). At room temperature, in the presence of zinc powder and sodium dithionite, β-lap was reduced to the hydroquinone intermediate, which then reacted with hexanoic acid (activated by HBTU) to produce mC_6 (73% yield). Although mC_6 formed micelles with relatively high drug loading efficiency (≈70%, data not shown), it is hydrolytically active (aided by the neighboring hydroxyl group) resulting in unstable micelle composition during storage in the phosphate buffered saline (PBS) buffer (50% conversion after 2 d at 4 °C, data not shown). Consequently, we decided to focus on diester derivatives of b-lap for micelle formulation. Diester prodrugs were synthesized at higher temperature (110 °C) from fattic acid anhydrides using zinc powder as the reducing agent.[11] For anhydrides with shorter chain lengths (i.e., C_2 to C_6), over 80% yields were obtained (Figure 1). For β-lap-dC_{10} and β-lap-dC_{16} prodrugs (abbreviated to dC_n in subsequent names), yields decreased to 42% and 14%, respectively. All diester prodrugs were hydrolytically stable in PBS.

After prodrug syntheses, we performed drug loading studies in PEG-*b*-PLA micelles ($\overline{M}_n$ = 10 kDa with 5 kDa for the PEG and PLA blocks). We compared micelle properties from two formulation methods, solvent evaporation versus film hydration (**Figure 2**). In the solvent evaporation method, prodrugs were first dissolved in an organic solvent (e.g., tetrahydrfuran, or THF) and then added dropwise in water under sonication.[12] THF solvent was allowed to evaporate during magnetic stirring. For the film hydration method, prodrugs and PEG-*b*-PLA copolymers were first dissolved in acetonitrile. A solid film was formed after acetonitrile evaporation, and hot water (60 °C) was added to form micelles.[13] For b-lap-dC_2, neither method allowed formation of stable, high drug loading micelles because of its fast crystallization rate in water (similar to β-lap). Drug loading density was ≈2 wt% (theoretical loading density

Dr. X. Ma, Dr. X. Huang, Dr. G. Huang, Dr. Y. Wang, Dr. X. Luo, Prof. D. A. Boothman, Prof. J. Gao
Departments of Pharmacology
Simmons Comprehensive Cancer Center
University of Texas Southwestern Medical Center
6001 Forest Park Road, Dallas, TX 75390-8807, USA
E-mail: jinming.gao@utsouthwestern.edu
Dr. X. Huang, Dr. L. Li, Dr. X. Luo, Prof. D. A. Boothman
Departments of Radiation Oncology
Simmons Comprehensive Cancer Center
University of Texas Southwestern Medical Center
6001 Forest Park Road, Dallas, TX 75390-8807, USA

DOI: 10.1002/adhm.201300590



n	Monoester series	Yield (%)
5	**mC$_6$**	73

n	Diester series	Yield (%)
1	**dC$_2$**	82
2	**dC$_3$**	77
3	**dC$_4$**	87
4	**dC$_5$**	82
5	**dC$_6$**	96
9	**dC$_{10}$**	42
15	**dC$_{16}$**	14

Figure 1. Syntheses of monoester and diester derivatives of *β*-lap.

at 10 wt%). Other diester derivatives were able to form stable micelles with high drug loading. We chose dC_3 and dC_6 for detailed analyses (**Table 1**). The solvent evaporation method was able to load dC_3 and dC_6 in micelles at 79% and 100% loading efficiency, respectively. We measured the apparent solubility (maximum solubility where no micelle aggregation/drug precipitation was found) of *β*-lap (converted from prodrug) at 4.1 and 4.9 mg mL^{-1} for dC_3 and dC_6 micelles, respectively. At these concentrations, micelle sizes (40–130 nm range) appeared larger than those fabricated using the film hydration method (30–70 nm) and moreover, the dC_3 micelles from solvent evaporation were stable for only 12 h at 4 °C. In comparison, the film hydration method allowed for a more efficient drug loading (>95% loading efficiency), larger apparent solubility (>7 mg mL^{-1}) and higher stability (>48 h) for both prodrugs. Close comparison between dC_3 and dC_6 micelles showed that dC_3 micelles had smaller average diameters (30–40 nm) and a narrower size distribution compared with dC_6 micelles (40–70 nm) by dynamic light scattering (DLS) analyses (Table 1). This was further corroborated by transmission electron microscopy that illustrated spherical morphology for both micelle formulations (Figure 2). dC_3 micelles were selected for further characterization and formulation studies.

To investigate the conversion efficiency of dC_3 prodrugs to *β*-lap, we chose porcine liver esterase (PLE) as a model esterase for proof of concept studies. In the absence of PLE, dC_3 alone was stable in PBS buffer (pH 7.4, 1% methanol was added to solubilize dC_3) and no hydrolysis was observed in 7 d. In the presence of 0.2 U mL^{-1} PLE, conversion of dC_3 to *β*-lap was rapid, evident by UV–vis spectroscopy illustrated by decreased dC_3 maximum absorbance peak (240 nm) with concomitant *β*-lap peak (257 nm, **Figure 3**a) increases. For dC_3 micelle conversion studies, we used 10 U mL^{-1} PLE, where this enzyme activity would be comparable to levels found in mouse serum.[14] Visual inspection showed that in the presence of PLE, the colorless emulsion of dC_3 micelles turned to a distinctive yellow color corresponding to the parental drug (i.e., *β*-lap) after 1 h (Figure 3b). Quantitative analysis (Equations 1–3, experimental section) showed that conversion of free dC_3 was completed within 10 min, with a half-life of 5 min. Micelle-encapsulated dC_3 had a slower conversion with a half-life of 15 min. After 50 min, ≈95% dC_3 was converted to *β*-lap (Figure 3c). Comparison of dC_3 conversion with *β*-lap release kinetics from the micelles indicated that the majority of prodrug hydrolysis occurred inside polymeric micelles in the first hour. More than 85% of dC_3 was converted to *β*-lap in the first 30 min, while only 4% of *β*-lap was released from micelles. The release profile of converted *β*-lap had an initial burst release (40% total dose), followed by a more sustained release (Figure 3d), which is consistent with our previously reported *β*-lap release kinetics from PEG-*b*-PLA micelles.[15] This core-based enzyme prodrug conversion also agrees with studies by Wooley et al., who reported the hydrolysis of micelle cores by proteinase K in cross-linked micelles.[16]

To achieve a solid formulation of dC_3 micelles, we investigated a series of lyoprotectants and examined their impact on the lyophilization-reconstitution properties (Table S1, Supporting Information). These lyoprotectants consist of sugar molecules (e.g., glucose, mannose, trehalose), sugar derivatives (e.g., mannitol, sorbitol), or macromolecules (e.g., dextran, PEG) and are either currently used in clinical formulations or are considered safe by the FDA in drug formulation applications.[17] After lyophilization, the dC_3 micelle powder was reconstituted by adding a saline solution to an intended concentration of 5 mg mL^{-1} (converted to b-lap concentration). The reconstituted solution was filtered through a 0.45-mm membrane before analysis. We measured the particle size and polydispersity index before and after lyophilization reconstitution, apparent drug solubility after filtration, and recovery yield (Table S1, Supporting Information). Results show that most of the sugar molecules and derivatives were not effective at protecting dC_3 micelle integrity during the lyophilization-reconstitution process as indicated by the low recovery yield (25%–80%), larger particle size, and elevated polydispersity index. Among these, 10 wt% of mannitol and trehalose (relative to dC_3 micelles) allowed for a relatively high recovery yield (80%–85%) and apparent solubility (4.0–4.2 mg mL^{-1} *β*-lap). For the macromolecular lyoprotectants, dextran did not

Adv. Healthcare Mater. **2014**,
DOI: 10.1002/adhm.201300590

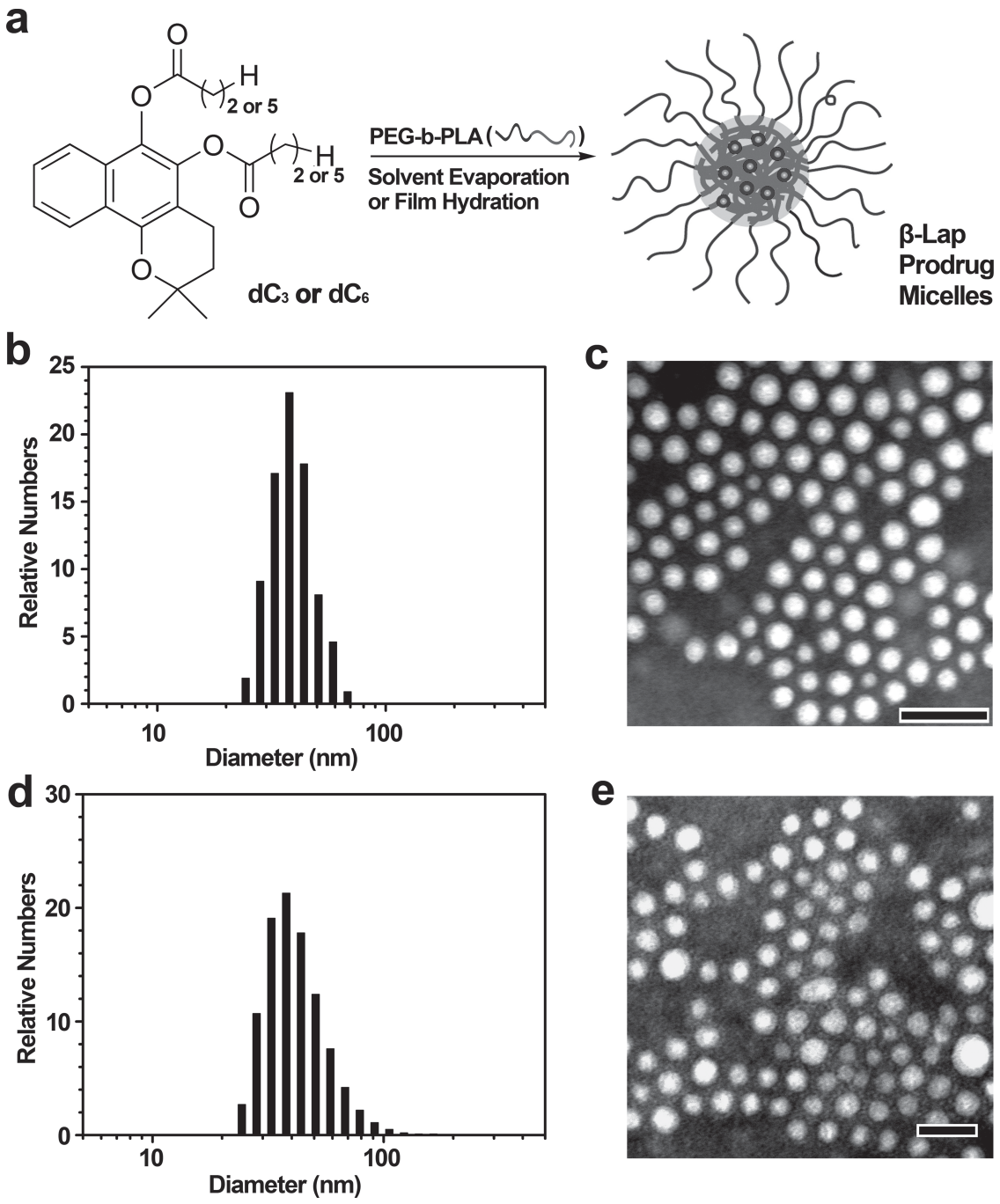


Figure 2. a) Formation of prodrug-loaded micelles by solvent evaporation or film hydration methods. Size distribution histograms of b) dC_3 or d) dC_6 micellesfrom dynamic light scattering (DLS) analyses. TEM images of c) dC_3 or e) dC_6 micelles showing spherical morphologies of prodrug micelles (scale bars = 100 nm). All micelles in (b–e) were made from the film hydration method.

yield satisfactory protection as indicated by low recovery yield (20%–40%). Among all the lyoprotectants, 10 wt% PEG2k or PEG5k allowed for the most optimal outcome with quantitative recovery yield and small changes in particle size and polydispersity (Table S1, Supporting Information).

To examine whether dC_3-converted drug maintains NQO1 specificity, we performed cytotoxicity studies of dC_3 micelles using A549 and H596 human lung cancer cell lines.[18] A549 cells endogenously express high level of NQO1 and we used dicoumarol, a competitive inhibitor of NQO1, to compete with dC_3 micelles to examine the NQO1 specificity.[19] On the other hand, native H596 cells do not express NQO1 due to homozygous *2 polymorphism, and these cells were stably transfected with a CMV-NQO1 plasmid to create a genetically matched cell line expressing NQO1.[2] **Figure** 4a,b depicts relative survival of A549 and H596 cells treated with dC_3 micelles at different drug

ADVANCED HEALTHCARE MATERIALS
www.advhealthmat.de

www.MaterialsViews.com

Table 1. Comparison of dC_3 and dC_6 micelle formulations by solvent evaporation versus film hydration methods.

Method	Prodrug	Theoretical loading density [wt%]	Experimental loading density [wt%]	Drug loading efficiency [%]	size [nm][a)]	Apparent β-lap solubility [mg mL^{-1}][b)]	Stability [h][c)]
Solvent evaporation	dC_3	15	11.9 ± 0.4	79.0 ± 2.7	45 ± 9	4.1	≈12
	dC_6	15	15.0 ± 0.5	100.0 ± 3.3	133 ± 11	4.9	>48
Film hydration	dC_3	10	9.7 ± 0.4	97.8 ± 4.2	35 ± 4	>7	>48
		15	14.5 ± 0.3	95.6 ± 3.2	38 ± 7	>7	>48
	dC_6	10	10.0 ± 0.3	100.0 ± 3.1	42 ± 13	>7	>48
		15	15.0 ± 0.9	99.3 ± 6.1	69 ± 17	>7	>48

[a)]Micelle sizes were measured by dynamic light scattering ($n = 5$); [b)]Apparent drug solubilities were calculated and converted to b-lap concentrations in mg mL^{-1}; [c)]Stability experiments were performed at 4 °C in PBS buffer (pH 7.4).

doses. After 2 h incubation without PLE addition, almost no cytotoxicity was observed at 10×10^{-6} M dC_3 micelles in NQO1+ and NQO1– H596 cells (Figure 4b). Addition of 10 U mL^{-1} PLE to the cell culture medium, led to a significant increase in cytotoxicity in NQO1+ H596 (8% survival) versus NQO1– H596 cells (95% survival). Similarly, dC_3 micelle toxicity in A549 cells was abrogated by addition of 50×10^{-3} M dicoumarol to inhibit NQO1 (Figure 4a). Cytotoxic responses for dC_3 micelles in A549 and NQO1+ H596 cells were slightly less than noted for β-lap alone (in dimethyl sulfoxide (DMSO), Figure S1a,b, Supporting Information), which might attribute to a delay in drug release from micelles. Figure 4c,d summarized the LD_{50} values (drug dose at which 50% of the cells are killed) for dC_3 micelles versus β-lap in A549 and H596 cells. With or without

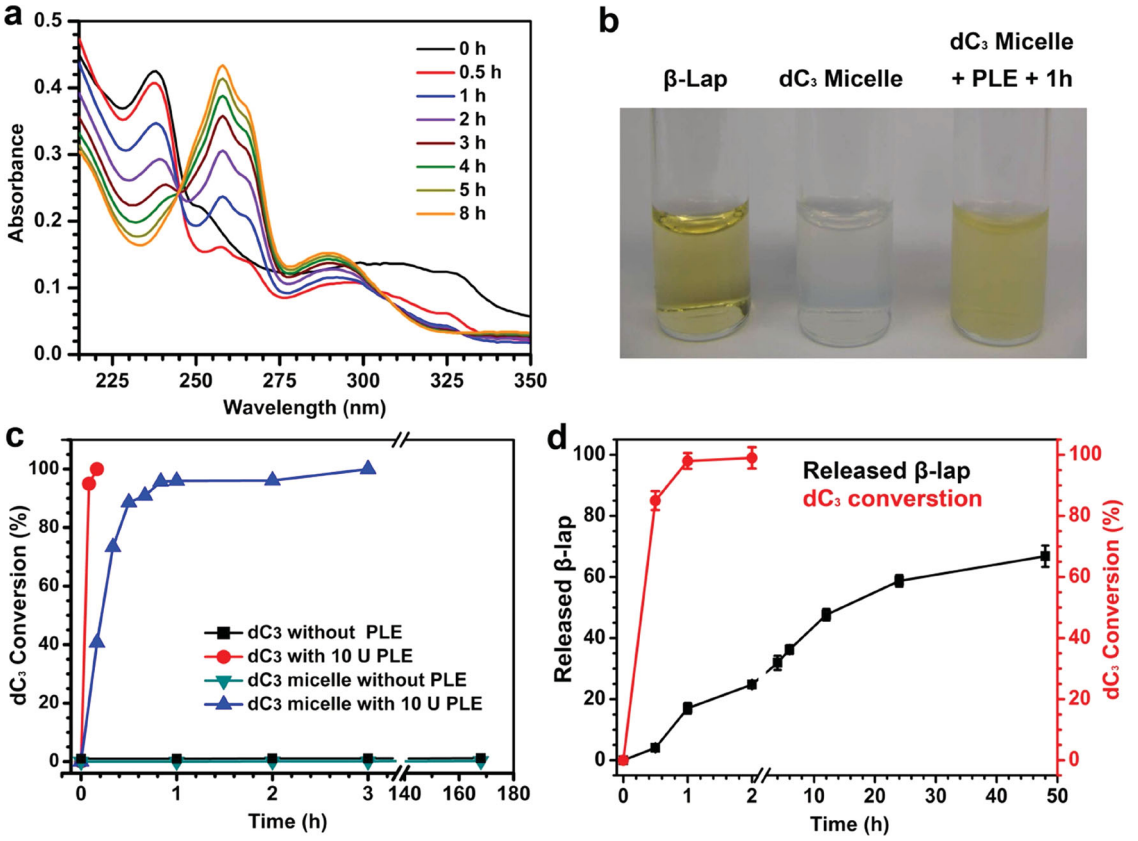


Figure 3. a) UV–vis analyses of conversion of free dC_3 (λ_{max} = 240 nm) to β-lap (λ_{max} = 257 nm) in the presence of porcine liver esterase (PLE, 0.2 U mL^{-1}) in PBS buffer. Methanol (1%) was added to help solubilizing dC_3 (10 μg mL^{-1}). b) Visual examination of dC_3 micelle solutions showing prodrug conversion to parent drug in the presence of PLE (10 U mL^{-1}) as indicated by the distinctive yellow color of soluble β-lap. dC_3 micelle concentration was at 0.1 mg mL^{-1}. c) Quantitative analyses of dC_3 conversion with and without 10 U mL^{-1} PLE. dC_3 and dC_3 micelle concentration was at 10 μg mL^{-1}. d) Comparison of dC_3 conversion versus β-lap release kinetics ($n = 3$) from polymeric micelles in the presence of PLE (10 U mL^{-1}), which indicates that prodrugs were converted to β-lap in the first hour before release from micelles. dC_3 micelle concentration was at 10 μg mL^{-1}. In b–d), dC_3 micelles with 9.7 wt% loading density were used.

DOI: 10.1002/adhm.201300590

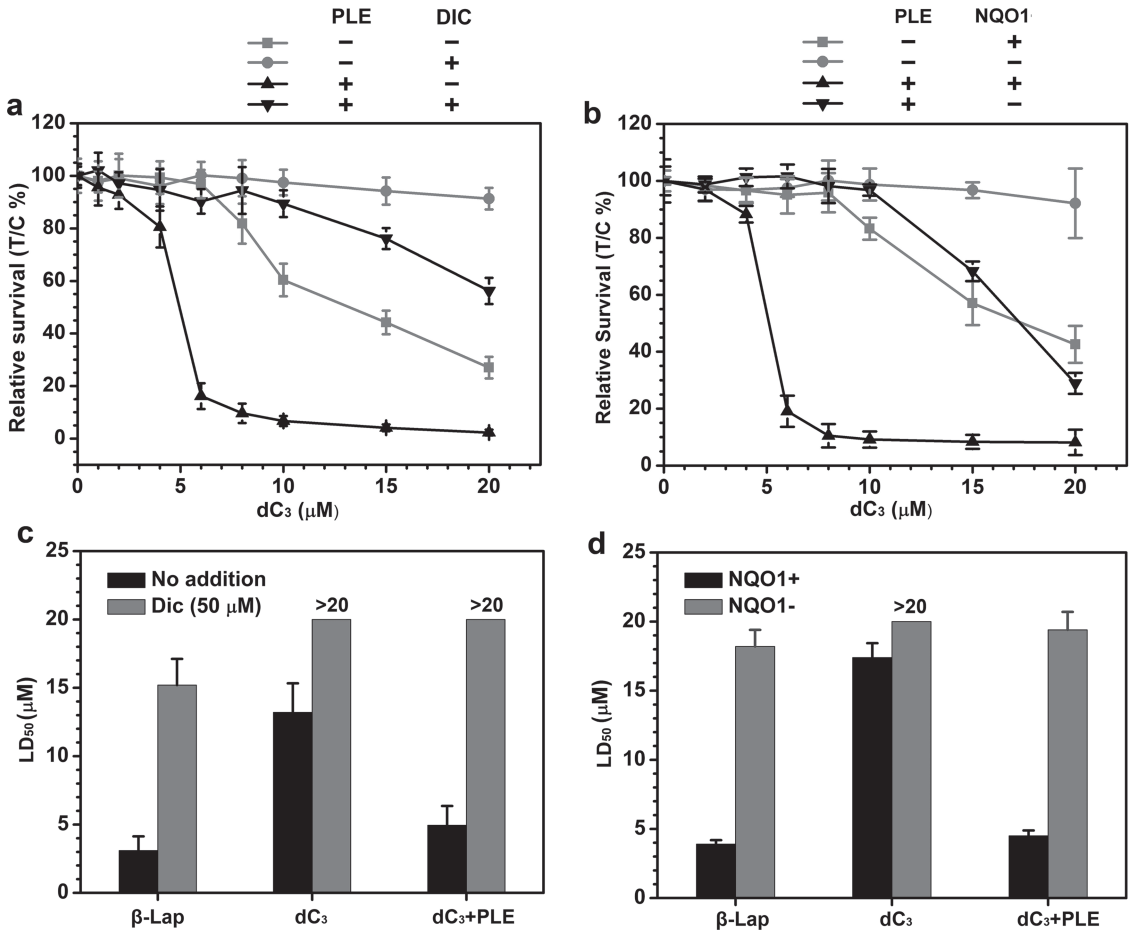


Figure 4. a,b) Long-term relative survival assays of dC_3 micelles in A549 and H596 nonsmall cell lung cancer cells, respectively, at indicated doses after micelle incubation for 2 h under indicated conditions as follows. Dicoumarol (50×10^{-6} M, 2 h) was used as a competitive NQO1 inhibitor in A549 cells, or in genetically matched NQO1 ± H596 NSCLC cells to examine NQO1 specificity. c,d) LD_{50} values of dC_3 micelles and β-lap in A549 or H596 NSCLC cells, respectively, with or without PLE.

addition of PLE, the LD_{50} values of dC_3 micelles to NQO1-deficient H596 and dicoumarol-protected A549 cells were $>10 \times 10^{-6}$ M, the highest doses tested. Conversely, a dramatic increase in cytotoxicity was observed in NQO1-expressed cells after adding 10 U mL^{-1} of PLE to the cell culture medium. The LD_{50} values of dC_3 micelles in A549 or NQO1+ H596 cells decreased to 4.5 or 3.1×10^{-6} M, respectively, highlighting the NQO1-dependent cytotoxicity of dC_3 micelles.

In conclusion, we report a prodrug strategy through the synthesis of diester derivatives of β-lap to increase compatibility with the PEG-*b*-PLA copolymer using for micelle inclusion, while reducing drug crystallization for improved formulation of NQO1-targeted nanotherapeutics. In this study, our data showed that diester prodrugs of β-lap (except for the diacetyl derivative) have greatly improved drug loading density and efficiency in PEG-*b*-PLA micelles, which leads to high apparent drug solubility (>7 mg mL^{-1}), physical stability, and ability for reconstitution after lyophilization. In the presence of esterase, β-lap prodrugs (i.e., dC_3) were efficiently converted into β-lap within the micelles. Cell culture experiments in vitro demonstrated NQO1-specific toxicity in nonsmall cell lung cancer (NSCLC) cells, similar to results previously published by our laboratories in NQO1-overexpressing solid cancers.[2,4,19b] These results establish β-lap prodrug micelle formulation for further evaluation of safety and antitumor efficacy in vivo in NQO1-targeted therapy of NSCLC.

Experimental Section

Typical Procedure for the Syntheses of dC_n (dC_3 as an Example): β-Lap (242 mg, 1 mmol), zinc powder (320 mg, 4.9 mmol), 40 mg sodium acetate (0.49 mmol), and 1 mL anhydrous propionic anhydride were mixed and stirred at 110 °C for 1 h. After reaction, the mixture was cooled to room temperature, filtered, and washed with 10 mL ethyl acetate. The filtrate was distilled under reduced pressure to remove propionic anhydride and ethyl acetate. The residue was dissolved in 20 mL CH_2Cl_2 and washed with water. The organic extract was dried over sodium sulfate and concentrated. The residue was recrystallized from isopropanol. Yield: 92%. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.24 (d, J = 8.0 Hz, 1H; Ar H), 7.69 (d, J = 8.0 Hz, 1H; Ar H), 7.49 (m, 2H; Ar H), 2.70 (t, J = 7.0 Hz, 2H; CH_2), 2.62 (t, J = 6.5 Hz, 4H; CH_2), 1.87 (t, J = 6.8 Hz, 2H; CH_2), 1.43 (s, 6H; CH_3), 1.33 (t, J = 7.0 Hz, 6H; CH_3); ^{13}C NMR (400 MHz, $CDCl_3$, δ): 171.50, 170.85, 147.79, 138.52, 130.00, 126.65, 126.40, 125.04, 124.26, 122.09, 120.66, 109.50, 74.77, 35.84, 31.89, 26.73, 18.71, 18.62, 18.03, 13.87, 13.83; MALDI-TOF MS m/z: $[M]^+$ calcd for $C_{21}H_{24}O_5$, 356.1624; found: 356.1702, 379.2693 ($M + Na^+$).

β-Lap Prodrug Micelle Fabrication by the Film Hydration Method: Both dC_3 and dC_6 micelles were prepared by the film hydration method following the same protocol. Here, we use dC_3 with 10 wt% theoretical loading density as an example. dC_3 (10 mg) and PEG-*b*-PLA (90 mg) were dissolved in 5 mL acetonitrile and solvent was removed using a rotary evaporator to form a solid thin film. Normal saline (1 mL) was added to the film at 60 °C and vortexed for 5 min. The resulting micelle solution was stored at 4 °C for 1 h and filtered through 0.45 μm membrane filters to remove non-encapsulated drug aggregates in solution. The resulting micelles were further analyzed by DLS (Malvern MicroV model DLS, He–Ne laser, $\lambda = 632$ nm, for hydrodynamic diameter, all the diameters were read as number average diameters) and transmission electron microscopy (TEM, JEOL 1200 EX model).

Esterase-Mediated Hydrolysis of Free dC_3 and dC_3 Micelles: In a typical procedure, free dC_3 (dissolved in methanol then dispersed in buffer) or dC_3 micelle solution was dispersed in 1 mL PBS buffer (pH 7.4) at a concentration of 10 μg mL^{-1} in a quartz cuvette. PLE was added to the solution and cuvettes were capped. Solution were kept at 37 °C with shaking. The absorbance spectrum of the solution was measured using a Hitachi UV–vis Spectrophotometer (Fremont, CA) at different times. β-Lap concentrations were determined by Equations (1,2) and prodrug conversion was then determined utilizing Equation 3:

$$A_1 = \varepsilon_1 c_1 L + \varepsilon_2 c_2 L \quad (1)$$

$$A_2 = \varepsilon_3 c_1 L + \varepsilon_4 c_2 L \quad (2)$$

$$dC_3\,\%\ \text{conversion} = \frac{\varepsilon_1 A_2 - \varepsilon_3 A1}{(\varepsilon_1 \varepsilon_4 - \varepsilon_2 \varepsilon_3) c_o L} \times 100 \quad (3)$$

where A_1 and A_2 are absorbances at 240 nm and 257 nm, respectively; ε_1 and ε_2 are extinction coefficients of dC_3 and β-lap at 240 nm ($\varepsilon_1 = 2.0 \times 10^4$ M^{-1} cm^{-1}, $\varepsilon_2 = 9.0 \times 10^3$ M^{-1} cm^{-1}), respectively; ε_3 and ε_4 are extinction coefficients of dC_3 and β-lap at 257 nm ($\varepsilon_3 = 1.1 \times 10^3$ M^{-1} cm^{-1}, $\varepsilon_4 = 1.9 \times 10^4$ M^{-1} cm^{-1}), respectively; L is the path length (1 cm), c_1 and c_2 are the concentration of dC_3 and β-lap. c_0 is the initial concentration of dC_3.

Lyoprotectant Screen for the Lyophilization-Reconstitution of dC_3 Micelles: dC_3 micelle formulations (9.7 wt%) were prepared using the film hydration method. The micelle solutions were then mixed with different amounts of lyoprotectant to reach a final lyoprotectant concentration of 5 or 10% w/v. The resulting solutions were transferred into glass vials and adjusted to 0.5 mL for all samples. Lyophilization was performed on a Labconco freeze-dryer (Kansas City, MO). The samples were frozen at –80 °C for 1 h, and primary drying was achieved at –80 °C and 0.006 mbar for 24 h. After lyophilization was finished, samples were reconstituted with 0.5 mL saline and analyzed by DLS measurements. After size measurement, reconstituted solutions were filtered through 0.45 μm membranes, and filtrates were analyzed by UV–vis to determine drug content and recovery.

Cytotoxicity Analyses In Vitro of β-Lap Prodrug Micelles: Cell survival assays based on DNA content were performed in A549 and H596 NSCLC cells as described.[18] Original H596 cells contain a homozygous *2 NQO1 polymorphism and thereby lack NQO1 expression. Genetically matched NQO1+ counterparts were generated and characterized for responses to β-lap alone as described.[20] A549 cells endogenously over-express NQO1, and its enzyme activity can be blocked by coadministration of dicoumarol, simulating an NQO1-deficient cell. Briefly, NQO1+ or NQO1– H596 or A549 NSCLC cells were seeded (10 000 cells/well) into each well of 48-well plates. A549 cells were seeded similarly. On the following day, media were removed, and replaced with that containing predetermined doses of free β-lap drug (dissolved in DMSO) or dC_3 micelles with or without PLE for 2 h. For A549 cells, dicoumarol at a concentration of 50×10^{-6} M was coadministered to inhibit NQO1. After 2 h exposures, media were replaced with control growth media and cells were allowed to grow for an additional 7 d. DNA content was determined by Hoescht dye 33258, using an adaptation of the method of Labarca and Paigen.[21] Samples were read in a PerkinElmer HTS 7000 Bio Assay Reader (Waltham, MA), and data were expressed as means±SE relative growth and graphed as treated/control (T/C) values from six wells per treatment.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work is supported by grants from the Cancer Prevention Research Institute of Texas (RP120897) and National Institutes of Health (5 R01 CA102792) to D.A.B. and J.G.

Received: October 28, 2013
Revised: January 23, 2014
Published online:

[1] a) R. Docampo, J. N. Lopes, F. S. Cruz, W. D. Souza, *Exp. Parasitol.* **1977**, *42*, 142; b) A. Boveris, R. Docampo, J. F. Turrens, A. O. M. Stoppani, *Biochem. J.* **1978**, *175*, 431; c) A. R. Schuerch, W. Wehrli, *Eur. J. Biochem.* **1978**, *84*, 197.
[2] E. A. Bey, M. S. Bentle, K. E. Reinicke, Y. Dong, C. R. Yang, L. Girard, J. D. Minna, W. G. Bornmann, J. M. Gao, D. A. Boothman, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 11832.
[3] K. E. Reinicke, E. A. Bey, M. S. Bentle, J. J. Pink, S. T. Ingalls, C. L. Hoppel, R. I. Misico, G. M. Arzac, G. Burton, W. G. Bornmann, D. Sutton, J. M. Gao, D. A. Boothman, *Clin. Cancer Res.* **2005**, *11*, 3055.
[4] J. J. Pink, S. M. Planchon, C. Tagliarino, S. M. Wuerzberger-Davis, M. E. Varnes, D. Siegel, D. A. Boothman, *J. Biol. Chem.* **2000**, *275*, 5416.
[5] Y. Dong, E. A. Bey, L. S. Li, W. Kabbani, J. Yan, X. J. Xie, J. T. Hsieh, J. Gao, D. A. Boothman, *Cancer Res.* **2010**, *70*, 8088.
[6] N. Nasongkla, A. Wiedmann, A. Bruening, M. Beman, D. Ray, W. Bornmann, D. Boothman, J. Gao, *Pharm. Res.* **2003**, *20*, 1626.
[7] a) H. T. Khong, L. Dreisbach, H. L. Kindler, D. F. Trent, K. G. Jeziorski, I. Bonderenko, T. Popiela, D. M. Yagovane, G. Dombal, *J. Clin. Oncol.* **2007**, *25*, 15017; b) E. Blanco, E. A. Bey, C. Khemtong, S. G. Yang, J. Setti-Guthi, H. Chen, C. W. Kessinger, K. A. Carnevale, W. G. Bornmann, D. A. Boothman, J. Gao, *Cancer Res.* **2010**, *70*, 3896.
[8] E. Blanco, E. A. Bey, Y. Dong, B. D. Weinberg, D. M. Sutton, D. A. Boothman, J. Gao, *J. Controlled Release* **2007**, *122*, 365.
[9] a) J. Rautio, H. Kumpulainen, T. Heimbach, R. Oliyai, D. Oh, T. Jarvinen, J. Savolainen, *Nat. Rev. Drug Discovery* **2008**, *7*, 255; b) F. Kratz, I. A. Muller, C. Ryppa, A. Warnecke, *ChemMedChem* **2008**, *3*, 20.
[10] C. Perez, K. B. Daniel, S. M. Cohen, *ChemMedChem* **2013**.
[11] S. C. Hooker, *J. Am. Chem. Soc.* **1936**, *58*, 1168.
[12] A. Lavasanifar, J. Samuel, G. S. Kwon, *J. Controlled Release* **2001**, *77*, 155.
[13] F. Ahmed, D. E. Discher, *J. Controlled Release* **2004**, *96*, 37.
[14] B. M. Liederer, R. T. Borchardt, *J. Pharm. Sci.* **2006**, *95*, 1177.
[15] D. Sutton, S. Wang, N. Nasongkla, J. Gao, E. E. Dormidontova, *Exp. Biol. Med.* **2007**, *232*, 1090.
[16] S. Samarajeewa, R. Shrestha, Y. Li, K. L. Wooley, *J. Am. Chem. Soc.* **2011**, *134*, 1235.
[17] S. Hirsjarvi, L. Peltonen, L. Kainu, J. Hirvonen, *J. Nanosci. Nanotechnol.* **2006**, *6*, 3110.

[18] M. S. Bentle, K. E. Reinicke, E. A. Bey, D. R. Spitz, D. A. Boothman, *J. Biol. Chem.* **2006**, *281*, 33684.
[19] a) F. Liu, G. Yu, G. J. Wang, H. Y. Liu, X. L. Wu, Q. Wang, M. Liu, K. Liao, M. Q. Wu, X. F. Cheng, H. P. Hao, *PLoS One* **2012**, *7*, *e42138*; b) X. M. Huang, Y. Dong, E. A. Bey, J. A. Kilgore, J. S. Bair, L. S. Li, M. Patel, E. I. Parkinson, Y. G. Wang, N. S. Williams, J. M. Gao, P. J. Hergenrother, D. A. Boothman, *Cancer Res.* **2012**, *72*, 3038.
[20] P. L. Olive, J. P. Banath, R. E. Durand, *Radiat. Res.* **1990**, *122*, 86.
[21] C. Labarca, K. Paigen, *Anal. Biochem.* **1980**, *102*, 344.