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

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Abstract

β -Lap prodrug micelle strategy improve the formulation properties of β -lap therapeutics. The resulting micelles yielded apparent high β -lap solubility (>7 mg/mL), physical stability, and ability to reconstitute after lyophilization. In the presence of esterase, β -lap prodrugs were efficiently converted into parent drug (i.e., β -lap), resulting in NQO1-dependent lethality of NSCLC cells.

Keywords

β -Lapachone; diester prodrug; polymeric micelles; enzyme activation; cancer nanomedicine

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 (ROS).^[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) 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 lab reported the development of polymeric micelles for the delivery of β -lap.^[7b, 8] Previous results show that

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Supporting Information

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

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 β -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), 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 (mC₆ 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 mC₆ (73% yield). Although mC₆ 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 PBS buffer (50% conversion after 2 days at 4 °C, data not shown). Consequently, we decided to focus on diester derivatives of β -lap for micelle formulation. Diester prodrugs were synthesized at higher temperature (110 °C) from fatty acid anhydrides using zinc powder as the reducing agent.^[11] For anhydrides with shorter chain lengths (i.e. C₂ to C₆), over 80% yields were obtained (Fig. 1). For β -lap-dC₁₀ and β -lap-dC₁₆ 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 (M_n = 10 kD with 5kD for the PEG and PLA blocks). We compared micelle properties from two formulation methods, solvent evaporation vs. film hydration (Fig. 2). In the solvent evaporation method, prodrugs were first dissolved in an organic solvent (e.g. tetrahydrofuran, 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 β -lap-dC₂, 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 at 10 wt%). Other diester derivatives were able to form stable micelles with high drug loading. We chose dC₃ and dC₆ for detailed analyses (Table 1). The solvent evaporation method was able to load dC₃ and dC₆ 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 for dC₃ and dC₆ 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 dC₃ 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) and higher stability (>48 h) for both prodrugs. Close comparison between dC₃ and dC₆ micelles showed that dC₃ micelles had smaller average diameters (30–40 nm) and a narrower size distribution compared to dC₆ 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 (Fig. 2). dC₃ micelles were selected for further characterization and formulation studies.

To investigate the conversion efficiency of dC₃ prodrugs to β -lap, we chose porcine liver esterase (PLE) as a model esterase for proof of concept studies. In the absence of PLE, dC₃ alone was stable in PBS buffer (pH 7.4, 1% methanol was added to solubilize dC₃) and no hydrolysis was observed in seven days. In the presence of 0.2 U/mL PLE, conversion of dC₃ to β -lap was rapid, evident by UV-Vis spectroscopy illustrated by decreased dC₃ maximum absorbance peak (240 nm) with concomitant β -lap peak (257 nm, Fig. 3a) increases. For dC₃ micelle conversion studies, we used 10 U/mL 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 dC₃ micelles turned to a distinct yellow color corresponding to the parental drug (i.e., β -lap) after one hour (Fig. 3b). Quantitative analysis (Eqs. 1–3, experimental section) showed that conversion of free dC₃ was completed within 10 min, with a half-life of 5 min. Micelle-encapsulated dC₃ had a slower conversion with a half-life of 15 min. After 50 mins, ~95% dC₃ was converted to β -lap (Fig. 3c). Comparison of dC₃ 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 dC₃ 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 (Fig. 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 crosslinked micelles.^[16]

To achieve a solid formulation of dC₃ 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 dC₃ micelle powder was reconstituted by adding a saline solution to an intended concentration of 5 mg/mL (converted to β -lap concentration). The reconstituted solution was filtered through a 0.45 μ m 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). Results show that most of the sugar molecules and derivatives were not

effective at protecting dC₃ 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 dC₃ micelles) allowed for a relatively high recovery yield (80–85%) and apparent solubility (4.0–4.2 mg/mL β -lap). For the macromolecular lyoprotectants, dextran did not 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).

To examine whether dC₃-converted drug maintains NQO1 specificity, we performed cytotoxicity studies of dC₃ 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 dC₃ 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] Figures 4a and 4b depict relative survival of A549 and H596 cells treated with dC₃ micelles at different drug doses. After 2 h incubation without PLE addition, almost no cytotoxicity was observed at 10 μ M dC₃ micelles in NQO1+ and NQO1– H596 cells (Fig. 4b). Addition of 10 U/mL 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, dC₃ micelle toxicity in A549 cells was abrogated by addition of 50 μ M dicoumarol to inhibit NQO1 (Fig. 4a). Cytotoxic responses for dC₃ micelles in A549 and NQO1+ H596 cells were slightly less than noted for β -lap alone (in DMSO, Figs. S1a–b), which might attribute to a delay in drug release from micelles. Figures 4c and 4d summarized the LD₅₀ values (drug dose at which 50% of the cells are killed) for dC₃ micelles vs. β -lap in A549 and H596 cells. With or without addition of PLE, the LD₅₀ values of dC₃ micelles to NQO1-deficient H596 and dicoumarol-protected A549 cells were >10 μ M, the highest doses tested. Conversely, a dramatic increase in cytotoxicity was observed in NQO1-expressed cells after adding 10 U/mL of PLE to the cell culture medium. The LD₅₀ values of dC₃ micelles in A549 or NQO1+ H596 cells decreased to 4.5 or 3.1 μ M, respectively, highlighting the NQO1-dependent cytotoxicity of dC₃ 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), physical stability, and ability for reconstitution after lyophilization. In the presence of esterase, β -lap prodrugs (i.e., dC₃) 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 (dC₃ 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 CH₂Cl₂ and washed with water. The organic extract was dried over sodium sulfate and concentrated. The residue was recrystallized from isopropanol. Yield: 92%. ¹H NMR (400 MHz, CDCl₃, 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; CH₂), 2.62 (t, *J* = 6.5 Hz, 4H; CH₂), 1.87 (t, *J* = 6.8 Hz, 2H; CH₂), 1.43 (s, 6H; CH₃), 1.33 (t, *J* = 7.0 Hz, 6H; CH₃); ¹³C NMR (400 MHz, CDCl₃, 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 C₂₁H₂₄O₅, 356.1624; found: 356.1702, 379.2693 (M + Na⁺).

β-Lap prodrug micelle fabrication by the film hydration method

Both dC₃ and dC₆ micelles were prepared by the film hydration method following the same protocol. Here, we use dC₃ with 10wt% theoretical loading density as an example. dC₃ (10 mg) and PEG-b-PLA (90 mg) were dissolved in 5 mL acetonitrile and solvent 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, λ = 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 dC₃ and dC₃ micelles

In a typical procedure, free dC₃ (dissolved in methanol then dispersed in buffer) or dC₃ micelle solution was dispersed in 1 mL PBS buffer (pH 7.4) at a concentration of 10 μg/mL 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:

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

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

$$dC_3\%conversion = \frac{\varepsilon_1 A_2 - \varepsilon_3 A_1}{\varepsilon_1 \varepsilon_4 - \varepsilon_2 \varepsilon_3} c_0 L \times 100 \quad (3)$$

where A_1 and A_2 are absorbances at 240 nm and 257 nm, respectively; ε_1 and ε_2 are extinction coefficients of dC₃ and β -lap at 240 nm ($\varepsilon_1 = 2.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, $\varepsilon_2 = 9.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$), respectively; ε_3 and ε_4 are extinction coefficients of dC₃ and β -lap at 257 nm ($\varepsilon_3 = 1.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, $\varepsilon_4 = 1.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), respectively; L is the path length (1 cm), c_1 and c_2 are the concentration of dC₃ and β -lap. c_0 is the initial concentration of dC₃.

Lyoprotectant screen for the lyophilization-reconstitution of dC₃ micelles

dC₃ 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 -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 co-administration 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 dC₃ micelles with or without PLE for 2 h. For A549 cells, dicoumarol at a concentration of 50 μM 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 days. DNA content was determined by Hoescht dye 33258, using an adaptation of the method of Labarca and Paigen.^[21] Samples were read in a Perkin Elmer HTS 7000 Bio Assay Reader (Waltham, MA) and data were expressed as means $\pm$ SE relative growth and graphed as treated/control (T/C) values from six wells per treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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

Figure 2.

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

Figure 3.

(a) UV-Vis analyses of conversion of free dC₃ ($\lambda_{\text{max}} = 240 \text{ nm}$) to β-lap ($\lambda_{\text{max}} = 257 \text{ nm}$) in the presence of porcine liver esterase (PLE, 0.2 U/mL) in PBS buffer. Methanol (1%) was added to help solubilizing dC₃ (10 μg/mL). (b) Visual examination of dC₃ micelle solutions showing prodrug conversion to parent drug in the presence of PLE (10 U/mL) as indicated by the distinctive yellow color of soluble β-lap. dC₃ micelle concentration was at 0.1 mg/mL. (c) Quantitative analyses of dC₃ conversion with and without 10 U/mL PLE. dC₃ and dC₃ micelle concentration was at 10 μg/mL. (d) Comparison of dC₃ conversion versus β-lap release kinetics (n=3) from polymeric micelles in the presence of PLE (10 U/mL), which indicates prodrugs were converted to β-lap in the first hour before release from micelles. dC₃ micelle concentration was at 10 μg/mL. In (b-d), dC₃ micelles with 9.7 wt% loading density were used.

Figure 4.

(a, b) Long-term relative survival assays of dC₃ 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 μM, 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) LD₅₀ values of dC₃ micelles and β-lap in A549 or H596 NSCLC cells, respectively, with or without PLE.

Table 1
Comparison of dC₃ and dC₆ 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) ^b	Stability (h) ^c
Solvent evaporation	dC ₃	15	11.9 $\pm$ 0.4	79.0 $\pm$ 2.7	45 $\pm$ 9	4.1	~12
	dC ₆	15	15.0 $\pm$ 0.5	100.0 $\pm$ 3.3	133 $\pm$ 11	4.9	> 48
Film hydration	dC ₃	10	9.7 $\pm$ 0.4	97.8 $\pm$ 4.2	35 $\pm$ 4	>7	> 48
		15	14.5 $\pm$ 0.3	95.6 $\pm$ 3.2	38 $\pm$ 7	>7	> 48
	dC ₆	10	10.0 $\pm$ 0.3	100.0 $\pm$ 3.1	42 $\pm$ 13	>7	> 48
		15	15.0 $\pm$ 0.9	99.3 $\pm$ 6.1	69 $\pm$ 17	>7	> 48

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