Intratumoral Delivery of β-Lapachone via Polymer Implants for Prostate Cancer Therapy

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Abstract

Purpose: β-Lapachone (ARQ 501, a formulation of β-lapachone complexed with hydroxypropyl-β-cyclodextrin) is a novel anticancer agent with selectivity against prostate cancer cells overexpressing the NAD(P)H:quinone oxidoreductase-1 enzyme. Lack of solubility and an efficient drug delivery strategy limits this compound in clinical applications. In this study, we aimed to develop β-lapachone–containing polymer implants (millirods) for direct implantation into prostate tumors to test the hypothesis that the combination of a tumor-specific anticancer agent with site-specific release of the agent will lead to significant antitumor efficacy.

Experimental Design: Survival assays *in vitro* were used to test the killing effect of β-lapachone in different prostate cancer cells. β-Lapachone release kinetics from millirods was determined *in vitro* and *in vivo*. PC-3 prostate tumor xenografts in athymic nude mice were used for antitumor efficacy studies *in vivo*.

Results: β-Lapachone killed three different prostate cancer cell lines in an NAD(P)H:quinone oxidoreductase-1–dependent manner. Upon incorporation of solid-state inclusion complexes of β-lapachone with hydroxypropyl-β-cyclodextrin into poly(D,L-lactide-co-glycolide) millirods, β-lapachone release kinetics *in vivo* showed a burst release of ~0.5 mg within 12 hours and a subsequently sustained release of the drug (~0.4 mg/kg/d) comparable with that observed *in vitro*. Antitumor efficacy studies showed significant tumor growth inhibition by β-lapachone millirods compared with controls ($P < 0.0001$; $n = 10$ per group). Kaplan-Meier survival curves showed that tumor-bearing mice treated with β-lapachone millirods survived nearly 2-fold longer than controls, without observable systemic toxicity.

Conclusions: Intratumoral delivery of β-lapachone using polymer millirods showed the promising therapeutic potential for human prostate tumors.

The incidence of prostate cancer is the highest among all estimated new cancer cases in American males, nearly doubling that of lung cancer. Prostate cancer was the second leading cause of cancer-related deaths in men in 2007 (1). With improvements in therapeutic technologies in early-stage prostate cancer treatment, especially less invasive therapies such as brachytherapy, cryotherapy, and high-intensity focused ultrasound, the morbidity associated with prostate cancer treatment has been substantially reduced. However, concerns remain over incomplete and inadequate treatment given the high rate of recurrence after primary salvage therapy (2, 3). Moreover, most procedures have notable side effects, including high rates of incontinence and impotence. Therefore, alternate strategies have been explored to treat the disease. The development of targeted strategies that exclusively kill prostate cancer cells in a tumor- and site-specific manner should yield increased antitumor efficacy, reduced normal tissue toxicity, and improved quality of life.

β-Lapachone (ARQ 501, a formulation of β-lapachone complexed with hydroxypropyl-β-cyclodextrin) is a novel 1,2-orthonaphthoquinone originally derived from the bark of the Lapacho tree in South America. It possesses a wide range of activities, including antifungal, antiviral, antitrypanosomal, and antitumor properties (4). Recent studies have shown that the mechanism of action of β-lapachone is highly specific and depends on the expression of NAD(P)H:quinone oxidoreductase-1 (NQO1), a two-electron oxidoreductase. NQO1 is a flavoprotein found overexpressed up to 20-fold compared with normal adjacent tissue in a variety of tumors, including those of the pancreas (5), lung (6), breast (7), and prostate (8). Elevated

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Translational Relevance

Prostate cancer is the second leading cause of cancer-related death for American men. Enormous side effects, including incontinence and impotence after salvage therapy, have severely limited the quality of life of prostate cancer patients. β-Lapachone is a novel anticancer agent and an effective radiosensitizer with specific selectivity against cancer cells that overexpress the two-electron oxidoreductase, NAD(P)H:quinone oxidoreductase-1. However, lack of solubility, inactivation of the drug in human serum, and the lack of an efficient drug delivery strategy limit the use of this compound in the clinics. Combined with a local polymer delivery device capable of controlled release, we show significant antitumor efficacy with minimal systemic toxicity. Thus, implantation of β-lapachone–containing millirods after salvage surgery or co-implantation of low-dose irradiation materials (such as radioactive seeds during brachytherapy) together with β-lapachone millirods should greatly increase efficacy of treating prostate cancer as well as bring a new era of synergistic therapy for prostate cancer.

endogenous levels of NQO1 have been noted in 70% of early- and late-stage human prostate cancers. NQO1 induces a futile cycling of drug that exhausts NAD(P)H from the cell, leading to a substantial amount of reactive oxygen species that causes DNA damage (Fig. 1A; ref. 6). A concomitant increase in endoplasmic reticulum–derived cytosolic Ca^{2+} results in the hyperactivation of poly(ADP-ribose) polymerase 1 (PARP-1); NAD^+/ATP depletion; and stimulation of a unique apoptotic response involving substrate proteolysis, DNA fragmentation, and μ-calpain–mediated cell death (9). This unique mechanism of action of β-lapachone sets it apart from all other traditional chemotherapeutic agents. The compound kills cells independent of caspases, p53 status, cell cycle stage, and all known antiapoptotic factors (10). In light of its heightened antitumor activity, specifically in cells overexpressing NQO1, β-lapachone is a powerful agent capable of offering tumor-selective killing.

Despite this advantage, the low solubility of β-lapachone in water (0.038 mg/mL or 0.16 mmol/L; ref. 11) hinders its clinical translation by traditional routes, such as i.v. administration (12). Moreover, the lack of site specificity following i.v. injection may result not only in low drug concentrations in the tumor, but also possible toxicity in normal cells lacking NQO1 activity because of high doses and prolonged exposure (6). Although ARQ 501 is currently in phase II clinical trial as systemic treatment for cancers, there is no report for local application of this drug, which may be more suitable for treating solid tumors in early stages. Recently, several biodegradable polymer depot systems were developed with the hope of achieving intratumoral controlled release of anticancer drugs. This strategy proves advantageous because therapeutic levels of a desired anticancer agent are maintained for prolonged periods while reducing systemic side effects (13). Given these potential advantages, several implantable devices were established for use in a wide variety of cancers. Gliadel wafer, a carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea]–containing polymer implant, was approved by the Food and Drug Administration in 1996 for treatment of glioblastoma multiforme (14–17). He and coworkers (18) developed 5-fluorouracil–releasing silicone implants capable of zero-order drug release for 24 weeks *in vitro*. Work by our own laboratory resulted in the fabrication of cylindrical poly(D,L-lactide-co-glycolide) polymer implants (millirods) releasing carboplatin or doxorubicin for intratumoral treatment of liver cancers (19–21). Upon incorporation of the latter drug within millirods, the antitumor efficacy *in vivo* was noted by decreased tumor volumes 4 and 8 days after millirod implantation in VX2 liver tumors in rabbits (22).

Based on this concept, we produced several polymer millirod formulations containing β-lapachone complexed with different cyclodextrins to achieve variable release kinetics (23). Depending on the cyclodextrin used, as well as the presence or lack of excipient molecules in the millirod, release of drug was modulated between rapid burst and sustained release kinetics. For example, when complexed with hydroxypropyl-β-cyclodextrin, release of β-lapachone showed burst kinetics (~80% release after 2 days), whereas drug alone released in a more sustained manner (~9% after 22 days). Previously, we reported that the therapeutic window of β-lapachone in lung (6), prostate (4), and breast (7) cancer cells was provided by a short high-dose pulse of β-lapachone to exploit elevated basal levels of NQO1 for cell death responses. Extended treatment with high doses of β-lapachone would lead to normal tissue

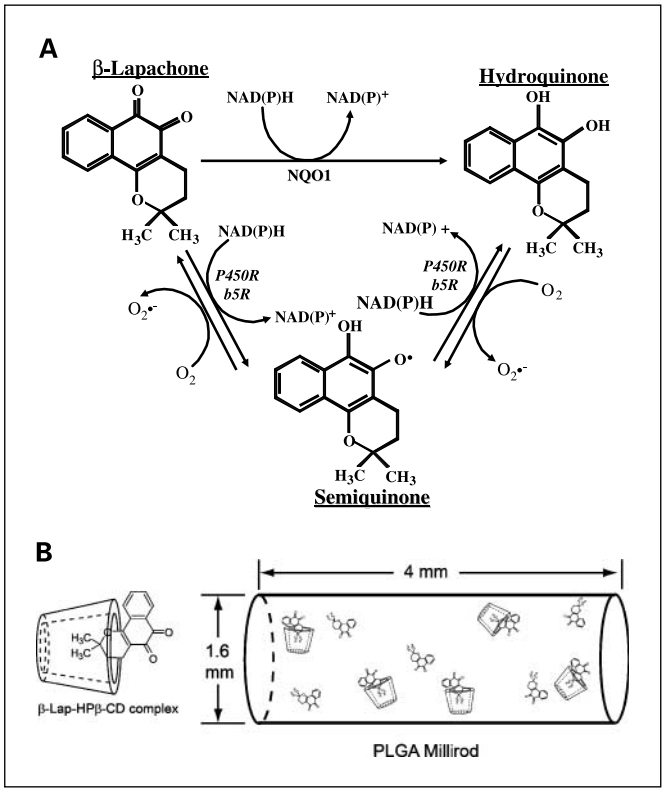

Fig. 1. Mechanism of β-lapachone redox cycling. *A,* β-lapachone undergoes a futile redox cycle in an NQO1-dependent manner. The hydroquinone form is unstable and through two one-electron oxidation steps converts back to the parent β-lapachone form. This cycling bioactivates β-lapachone and results in the generation of reactive oxygen species. *B,* schematic of complexation and incorporation of β-lapachone and β-lapachone·hydroxypropyl-β-cyclodextrin complexes into poly(D,L-lactide-co-glycolide) millirods.

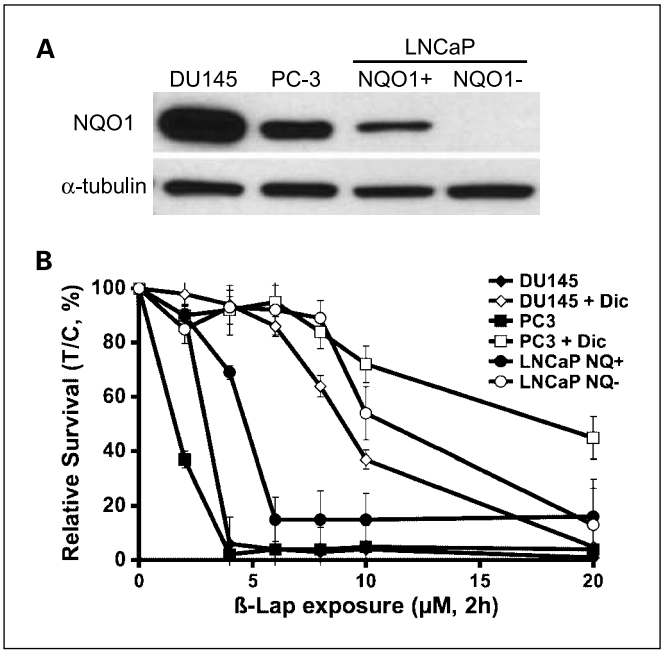

Fig. 2. β-Lapachone killed human prostate cancer cells in an NQO1-dependent manner. *A,* NQO1 protein expression levels in various human prostate cancer cell lines. LNCaP cells are DNA mismatch repair deficient (25), and one clone was isolated with an NQO1 deficiency. Cells were transfected with NQO1, creating an isogenic NQO1+/- system (4). *B,* relative survival assays of three different cell lines exposed to either β-lapachone alone at the indicated doses or β-lapachone + concomitant dicoumarol (40 μmol/L) for 2 h. Error bars, SEs from six replicates done each in duplicate. Dic, dicoumarol.

complications because of the two one-electron reductions by the b5 and P450 oxidoreductases present in normal tissues (Fig. 1A). Therefore, we optimized a formulation that can give a burst release in a short period to reach the highest killing efficiency in tumor while allowing a prolonged low dose of β-lapachone release to kill the residual cancer cells while sparing normal cells. The ultimate goal was to examine the antitumor efficacy *in vivo* of β-lapachone–containing polymer millirods in prostate tumor–bearing mice. Hydroxypropyl-β-cyclodextrin was used to form inclusion complexes with β-lapachone to achieve initial burst release of drug from poly(D,L-lactide-co-glycolide) millirods, whereas free noncomplexed drug was incorporated to provide prolonged release. Poly(D,L-lactide-co-glycolide) millirods were directly implanted inside PC-3 tumor xenografts followed by examination of drug release, antitumor efficacy, and systemic toxicity over time. The significant antitumor response highlights the unique advantage of integrating a novel and tumor-selective therapeutic agent (β-lapachone) while enabling drug delivery technology (polymer millirods) to achieve tumor- and site-specific therapy of human prostate cancers.

Materials and Methods

Materials. Poly(D,L-lactide-co-glycolide) (lactide-glycolide, 50:50; molecular weight, 50,000 Da; inherent viscosity, 0.65 dL/g) was purchased from Birmingham Polymers, Inc. β-Lapachone was synthesized following a previously published procedure (24) and prepared as described (8). Hydroxypropyl-β-cyclodextrin was obtained from Cyclodextrin Technologies Development, Inc., with >98% purity. Glucose anhydrous was obtained from Fisher Scientific. Dicoumarol, an NQO1 inhibitor, was purchased from the Sigma Chemical Co.

Cell culture. DU145, PC-3, and LNCaP human prostate cancer cells were originally obtained from Dr. George Wilding (University of Wisconsin-Madison, Madison, WI). DU145 and PC-3 cells were grown in GIBCO RPMI 1640 (1×; Invitrogen) with 5% fetal bovine serum, and LNCaP cells were grown in DMEM (Invitrogen) with 10% fetal bovine serum. All media contained 2 mmol/L L-glutamine, penicillin (100 units/mL), and streptomycin (100 mg/mL). Cells were cultured at 37°C in a 5% CO_2, 95% air humidified atmosphere and were free from *Mycoplasma* contamination.

NQO1 expression analyses. Western blots were prepared using standard methods. Briefly, cultured cells or xenograft tissues were harvested for assessment of NQO1 expression. Cells were washed in ice-cold PBS, lysed, sonicated, and stored at -20°C for future analyses. Western blots were first incubated with PBS containing 0.2% Tween 20 and 5% milk for 1 h to prevent nonspecific binding, then developed as previously described using SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific) and exposed using Autoradiography Film (Denville Scientific, Inc.; ref. 8). An antihuman NQO1 antibody was kindly provided by Dr. David Ross (University of Colorado Health Science Center, Denver, CO) and used at a 1:5,000 dilution overnight at 4°C. Glyceraldehyde-3-phosphate dehydrogenase or α-tubulin levels were monitored for loading control.

NQO1 enzyme assays. Enzymatic reactions from S9 cell preparations were done in reactions containing 77 μmol/L cytochrome *c* (Sigma) and 0.14% bovine serum albumin in Tris-HCl buffer (50 mmol/L; pH 7.5). NQO1 activity was measured using NADH (200 μmol/L) as an immediate electron donor and menadione (10 μmol/L) as an intermediate electron acceptor as described (8). Dicoumarol (40 μmol/L) was used to block NQO1 activity, and each

Table 1. NQO1 enzymatic activity and LD_{50} of β-lapachone ± dicoumarol in three different prostate cancer cell lines

Cell lines	NQO1 level (nmol cytochrome *c* reduced/min/μg protein)*	LD_{50} values of β-lap (μmol/L, 2 h)	
		Dicoumarol (-)	Dicoumarol (+)†
DU145	556 ± 18	3.0 ± 0.1	9.1 ± 0.6
PC-3	107 ± 17	1.5 ± 0.1	20.0 ± 4.4
LNCaP NQO1+	108 ± 21	2.7 ± 0.9	>20
LNCaP NQO1-	2.4 ± 0.1	11.8 ± 1.7	ND

Abbreviation: β-lap, β-lapachone. ND, not determined.
*Values are mean ± SEM; *n* = 6 per group.
† Dicoumarol was administered at 40 μmol/L concomitant with 2 h β-lap treatments.

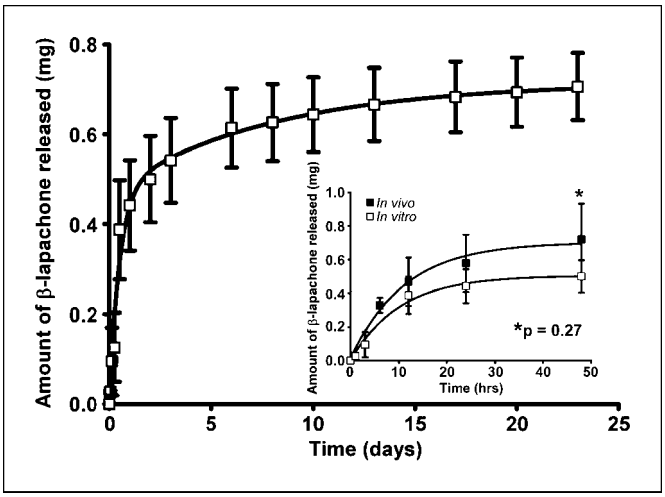

Fig. 3. Cumulative levels of β-lapachone released from poly(D,L-lactide-co-glycolide) millirods were plotted as a function of time (h). Inset, cumulative β-lapachone released *in vitro* compared with *in vivo* following millirod implantation into PC-3 tumor xenografts within 48 h. Release levels were calculated as the differences between initial amount of β-lapachone loaded and amount remaining in millirods over time. Points, mean (n = 3); bars, SE.

sample was measured in triplicate. Enzymatic activities were calculated as nanomole cytochrome *c* reduced per minute per microgram protein, based on initial rate of change in absorbance at 550 nm.

Relative survival assays. Cell survival was examined using a DNA assay as described (8). Briefly, cells were seeded at 5×10^3 per well in a 48-well plate and allowed to attach overnight. Cells were then treated for 2 h with various β-lapachone concentrations, alone or with 40 μmol/L dicoumarol. Drug-free medium was then added and cells were allowed to grow for 5 to 7 days until control cells reached 100% confluence. DNA content was then determined by Hoescht dye staining and fluorescence detection using a Perkin-Elmer plate reader. Results were reported as mean ± SE from at least three independent experiments done six times.

Preparation of β-lapachone·hydroxypropyl-β-cyclodextrin inclusion complexes. Following a previously published procedure (11), 25 g of hydroxypropyl-β-cyclodextrin were weighed and dissolved in 50 mL PBS (pH 7.4). After complete dissolution, ~1 g of β-lapachone was added to the hydroxypropyl-β-cyclodextrin solution. The solution was covered and allowed to stir at room temperature for 3 d, after which the contents were filtered using a 0.45-μm nylon filter, yielding a solution of β-lapachone·hydroxypropyl-β-cyclodextrin complex. The solution was then lyophilized and the resulting solid powder was grounded using a mortar and pestle.

Fabrication of polymer millirods. Polymer millirods were produced using a previously established compression-heat molding procedure (20). Millirod composition was modified to achieve the desired drug release profile. Briefly, millirod components [31% β-lapachone·hydroxypropyl-β-cyclodextrin complex, 19% free β-lapachone, and 50% poly(D,L-lactide-co-glycolide)] were weighed separately, placed in a mortar, and mixed well using a pestle. Control millirods were composed of 26% hydroxypropyl-β-cyclodextrin and 74% poly(D,L-lactide-co-glycolide). The contents were then placed into a Teflon tube (1.6 mm I.D.) within a stainless steel mold. The mold was then placed in an oven (Fisher Model 282A) at 90°C for 2 h with a compression pressure of 4.6 MPa. The resulting cylindrical millirods, with a diameter of 1.6 mm, were cut into lengths of 4 mm for subsequent biology studies *in vitro* and *in vivo* (Fig. 1B).

***Drug release studies* in vitro.** β-Lapachone–loaded poly(D,L-lactide-co-glycolide) millirods (n = 3) were placed in glass scintillation vials containing PBS (pH 7.4) at 37°C. Sample vials were placed in an orbital shaker (C24 model, New Brunswick Scientific) with a rotating speed of 150 rpm. At various times, millirods were removed and placed into new scintillation vials containing fresh buffer at 37°C. The concentration of released β-lapachone was measured using UV-Vis spectrophotometry (Perkin-Elmer Lambda 20 model) at the maximum absorption wavelength of drug (λ_{max} = 257.2 nm).

Characterization of β-lapachone release* in vivo *from polymer millirods. Drug release studies *in vivo* were conducted by intratumorally implanting β-lapachone millirods into PC-3 xenograft-bearing athymic nude mice. All animal procedures were approved by the University of Texas Southwestern Institutional Animal Care and Use Committee. PC-3 cells were grown to 80% to 90% confluence, harvested, prepared at 5×10^6/50 μL cell suspensions, and inoculated on both flanks athymic nude mice (6-8 wk old) with an average weight of 25 ± 1.6 g (Charles River Labs). Tumor dimensions were measured regularly with calipers and volumes were calculated as follows: volume (mm^3) = length × width × width / 2. The mean tumor size used to characterize β-lapachone release *in vivo* was ~300 mm^3, similar to mean tumor sizes in randomized studies used to determine antitumor efficacy. At different time points

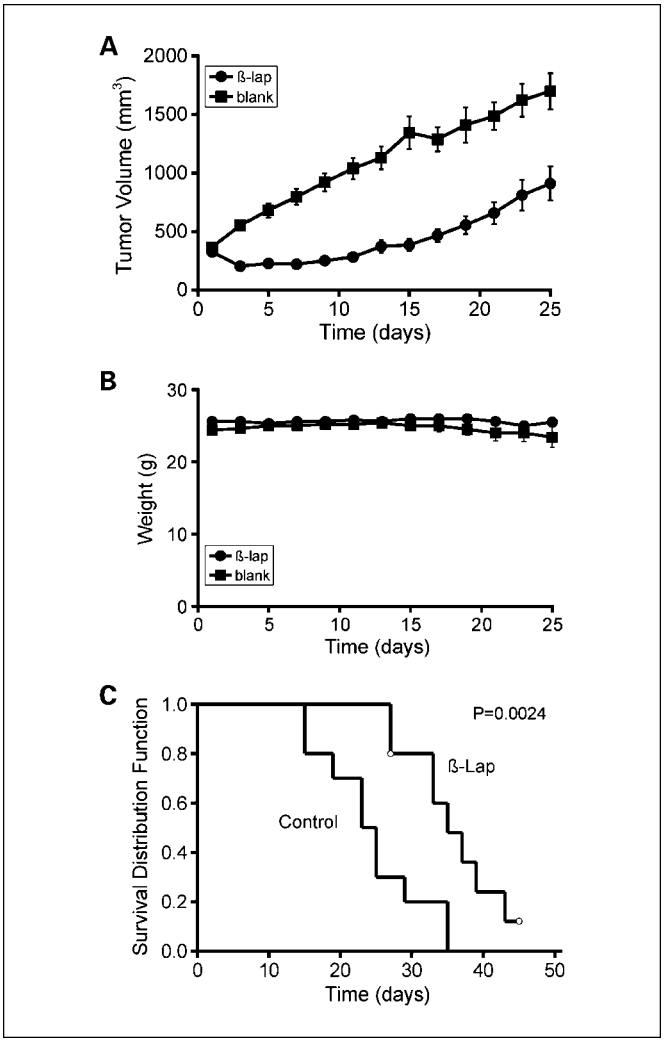

Fig. 4. Intratumoral delivery of β-lapachone using polymer millirods as a vehicle showed significant antitumor efficacy of β-lapachone in PC-3 tumor xenograft models. *A,* comparison of tumor volume after intratumoral implantation of either β-lapachone – loaded millirods or control millirods containing hydroxypropyl-β-cyclodextrin alone. Points, mean (n = 10); bars, SE. *B,* animal weight data over time (d) in control and treatment groups. Points, mean (n = 10); bars, SE. *C,* Kaplan-Meier survival curves reflecting the antitumor efficacies of two different types of millirods as shown in *A*. Open circles, either accidental animal loss because of skin infection at day 27 or animal survival at the end of the experiment.

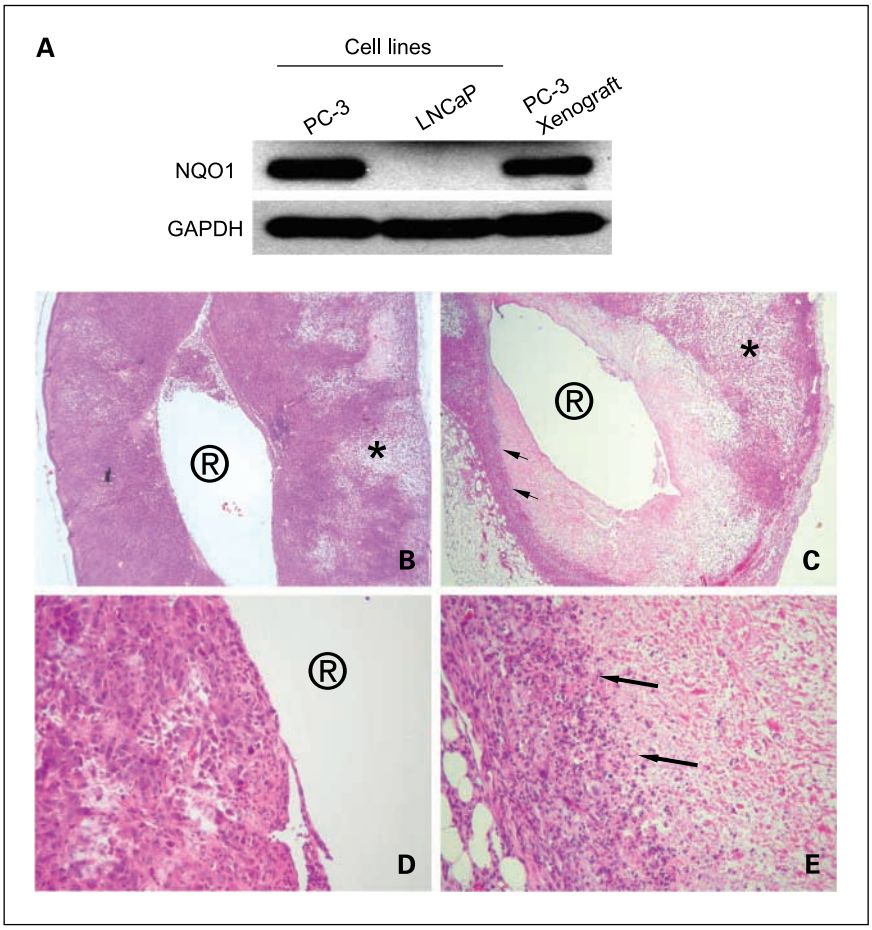

Fig. 5. Histologic examination (H&E) of PC-3 tumor xenograft sections after millirod implantation confirmed significant antitumor effects of β-lapachone *in vivo*. *A,* Western analyses showing NQO1 expression in explanted PC-3 xenografts. Cell lysate of parental LNCaP cells (NQO1-) was used as negative control. *B,* a cross-section of tumors treated with hydroxypropyl-β-cyclodextrin – loaded millirod. Original magnification, ×4. Circled R, the millirod implantation site; *, areas of patchy necrosis. *C,* a cross-section of a tumor xenografts 6 d after implantation of β-lapachone – loaded millirods. Original magnification, ×4. *D* and *E,* increased magnifications of (*B* and *C*), respectively. Original magnification, ×40.

(6, 12, and 24 h and days 2, 3, 4, 6, 8, 10, 13, 17, 20, and 24), mice were sacrificed and millirods were retrieved. Three animals were used at each time point. Experiments were repeated at least three times. Millirods were dissolved in acetonitrile to recover and quantify the remaining drug by high-performance liquid chromatography using a ZORBAX C-18 column (150 × 4.6 mm; 5.0 mm) with a mobile phase consisting of 70% acetonitrile and 30% of 25 mmol/L ammonium formate buffer (pH 7.0). Three millirods from three individual mice were measured for statistical analysis at each time point.

Antitumor efficacy. Subcutaneous tumors were inoculated in athymic nude mice as described above. In general, mice were randomly distributed into two groups. The mean sizes of tumors used at the start of these experiments were 325.1 ± 7.4 mm^3 for the experimental group and 354.6 ± 10.1 mm^3 for the control group. Statistically, these two groups were not different. β-Lapachone loaded (2.6 mg) or control millirods measuring 4 mm in length were intratumorally implanted using a 13-gauge trochar. Tumor volumes were calculated every other day after implantation. Mice were sacrificed when tumors reached 2 cm^3 or 10% total body weight. Ten mice per group were used in this study, which were repeated at least twice. Tumor, liver, and kidney tissues were removed and sent for histologic examination at the University of Texas Southwestern Medical Center histologic core. Portions of tumor tissues were also frozen in liquid nitrogen for Western analyses.

Histologic staining. Tumor, liver, and kidney tissues were harvested at day 6, when the drug release reached the maximum, and at all the end points when the mice were sacrificed. Tissues were fixed in 10% formalin overnight, paraffin embedded, and processed by the Department of Pathology, University of Texas Southwestern Medical Center. Briefly, 5-μm-thick sections were prepared from paraffin-embedded tissues and baked overnight at 37°C. H&E staining was conducted, and histology images were taken using a Nikon E400 microscope with a Nikon Coolpix 4500 camera.

Statistical analyses. Tumor growth profiles in two tested groups were analyzed using a mixed model approach. Log-rank tests were applied to survival analyses (Kaplan-Meier curves). *P* values of ≤0.05 were considered significant. All statistical analyses were done using SAS 9.1.3 Service Pack 3.

Results

NQO1-dependent cell killing by β-lapachone. NQO1 is considerably elevated in multiple tumor types, including human prostate cancers (4). Immunoblotting and enzymatic methods were used to confirm and quantify NQO1 expression levels in three different human prostate cancer cell lines: DU145, PC-3, and LNCaP (Fig. 2A; Table 1). DU145 cells have the highest NQO1 expression and activity (556 ± 18 nmol cytochrome *c* reduced/min/μg protein), with PC-3 cells having elevated but lower levels (108 ± 17 nmol cytochrome *c* reduced/min/μg protein). A particular clone of LNCaP cells lacks NQO1 expression, although they have a wild-type *NQO1* gene (25). We previously isolated an isogenic LNCaP clone transfected with cytomegalovirus-directed NQO1 (4). LNCaP NQO1+ cells showed similar NQO1 activity (108 ± 21 nmol cytochrome *c* reduced/min/μg protein) as PC-3 cells.

We then assessed the survival of the three prostate cancer cells after transient exposure to β-lapachone (Fig. 2B). The LD_{50}

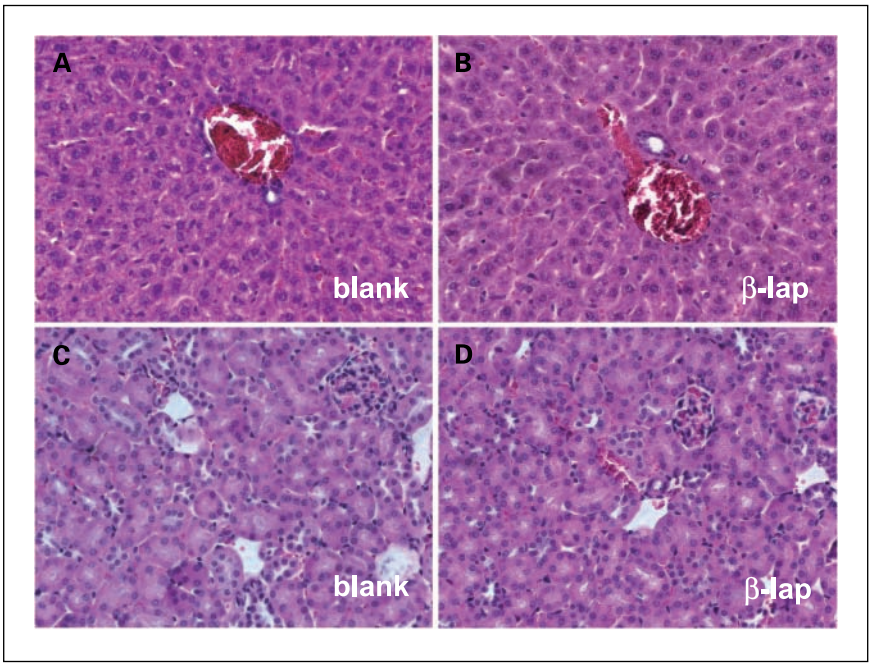

Fig. 6. Histologic examination (H&E) of liver and kidney tissues further corroborated negligible systemic toxicity observed with implant-delivered β-lapachone. Original magnification, ×40. *A* and *B*, liver tissues. *C* and *D*, kidney tissue. *A* and *C*, labeled as blank and refer to samples from control groups. *B* and *D*, tissues from experiments involving β-lapachone – loaded millirods.

values of the three cell lines following a 2-h exposure of β-lapachone can be found in Table 1. Dose-response data showed that, after a 2-h exposure, all three cell lines were killed by β-lapachone at minimum concentrations of 4 μmol/L (DU145 and PC-3 cells) and 6 μmol/L (LNCaP NQO1+ cells), respectively. These and other data in a variety of other breast, pancreatic, non–small cell lung, and colon cancer cell lines suggest that a minimum NQO1 activity of ~100 nmol cytochrome *c* reduced/min/μg protein was required for β-lapachone to irreversibly kill cells when exposed at 4 to 6 μmol/L for 2 h. Dicoumarol, an NQO1 inhibitor, rescued DU145, LNCaP NQO1+, and PC-3 cells from β-lapachone–induced cell death, confirming the NQO1-dependent cell killing mechanism of the drug. In contrast, LNCaP NQO1- cells were able to survive an 8 μmol/L dose of β-lapachone, with increasing doses (e.g., 10 and 20 μmol/L) resulting in lethality by NQO1-independent processes as described (7, 9, 10). Administration of dicoumarol failed to protect NQO1- cells at high doses of β-lapachone at >20 μmol/L, suggesting a role of other low-affinity reductases, such as cytochrome P450 and b5 oxidoreductases, in NQO1-independent β-lapachone cytotoxicity at high concentrations.

Enhanced delivery of β-lapachone via incorporation of free β-lapachone and β-lapachone·hydroxypropyl-β-cyclodextrin complexes into millirods. Polymer millirods for the intratumoral delivery of β-lapachone to prostate tumors were produced as described in Materials and Methods, and release kinetics of the drug *in vitro* were examined. Because a 2-hour exposure of β-lapachone resulted in irreversible cell death, an initial burst of the drug was desired to rapidly achieve therapeutic levels. Optimal millirod delivery would then provide a sustained release over a prolonged period. A burst release of ~0.5 mg occurred within 12 hours (Fig. 3) predominantly because of the complexation of the drug with hydroxypropyl-β-cyclodextrin that dramatically enhanced its solubility and drug release (23). Thereafter, release of drug decreased over time, with a release rate of ~0.05 mg/d for the next 3 days. By day 6, 0.6 mg of drug was released from the millirod, and the release rate for the subsequent 4 days was ~0.01 mg β-lapachone per day. By day 23, total release of drug from the millirod was ~0.7 mg, highlighting the sustained release of drug most likely because of the fraction of free drug remaining as a molecular dissolution in the poly(D,L-lactide-co-glycolide) polymer (23).

β-Lapachone release kinetics *in vitro* was similar to that in PC-3 tumor xenografts *in vivo* within 48 h after millirod implantation (Fig. 3, *inset*). After 6 h *in vivo*, a burst dose of 0.33 ± 0.04 mg of the drug was released from the millirod compared with 0.21 ± 0.03 mg released *in vitro*. At the transition point between burst and sustained release (12 h), 0.47 ± 0.14 mg of drug was released *in vivo* compared with 0.39 ± 0.11 mg *in vitro*. After 2 days, 0.72 ± 0.21 and 0.50 ± 0.10 mg were released *in vivo* and *in vitro*, respectively. Although release of drug *in vivo* was slightly greater than release *in vitro*, the difference was not statistically significant at all times examined ($P = 0.27$).

Antitumor efficacy of intratumoral delivery of β-lapachone–loaded millirods. To examine the antitumor efficacy of β-lapachone–containing millirods, implantation into subcutaneous PC-3 xenografts located on the flanks of female athymic nude mice were done. Tumor volumes were calculated every other day and compared to tumors containing control (vehicle alone) millirods. Three days after implantation, significant tumor regression was noted in the treatment group (204 ± 12 mm^3) compared with control tumors that nearly doubled in volume to an average of 551 ± 39 mm^3 (Fig. 4A). Delayed tumor growth lasted for nearly 2 weeks before β-lapachone–treated tumors grew to their original volume of 371 ± 54 mm^3 at day 13, whereas the average volume of control tumors surpassed 1,000 mm^3 (1,130 ± 98 mm^3). It took ~25 days for tumors in the treatment group to surpass 1,000 mm^3 mark. It is important to note that, after day 17, tumors treated with β-lapachone millirods began to grow at a similar rate as

control tumors, as evidenced by comparable growth rates (51.1 mm^3/d for control tumors and 55.6 mm^3/d for treatment tumors). Despite tumor regrowth, statistical analyses at each time point showed significant tumor volume differences ($P \leq 0.01$) between treated and control groups. Mixed model statistical analyses showed a more pronounced difference ($P \leq 0.0001$) in total tumor growth behavior between these two groups.

To monitor possible toxicity to normal tissue resulting from the proposed treatment regimen, animal weights were recorded over the duration of the study (Fig. 4B). Weights in control and treated groups remained similar throughout the course of the experiment, with the average weight being 25 ± 1.6 g. No statistical difference was found between the average weights of the two groups, suggesting no apparent adverse toxicity in mice treated with β-lapachone millirods. Although there seemed to be no difference in systemic toxicity, the two groups possessed significant differences in survival (see Kaplan-Meier survival curve; Fig. 4C). An ~50% loss in animals was observed after 25 days in the control group, whereas 50% loss in survival of β-lapachone–treated animals did not occur until after 35 days, a time when all animals in the control group were sacrificed. A fraction (2 of 10) of mice in the treatment group survived >40 days. A log-rank test of the Kaplan-Meier survival curve indicated that there was a significant delay in tumor-related death in β-lapachone–treated mice ($P = 0.0024$).

Histologic examination of tumor tissues following β-lapachone millirod treatment confirmed significant antitumor response. To determine whether PC-3 tumor xenografts maintained NQO1 expression, a key target for achieving cell killing specificity by β-lapachone–loaded poly(D,L-lactide-co-glycolide) millirods *in vivo*, xenograft tissues were harvested and Western analyses were conducted to monitor NQO1 levels over time (Fig. 5A). Results indicated that high NQO1 expression levels were preserved following s.c. implantation and growth in the flanks of mice, and that these levels were comparable with PC-3 cells harvested *in vitro*.

Histologic examination of tumor tissues explanted from mice 6 days after millirod implantation showed that the area immediately adjacent to control millirods contained numerous viable tumor cells in H&E-stained sections, easily identifiable by clusters of dark staining nuclei (Fig. 5B). Magnification of this implant boundary region (Fig. 5D) showed tumor cells that have large nuclei, are irregular in shape and size, and seem mitotic. These observations corroborate the presence of viable tumor cells in this area. At regions farther away from the implant, a typical patchy necrotic area (region labeled *) because of fast proliferation rates in high-grade tumors was observed. In contrast, β-lapachone–treated tumors (Fig. 5C) showed pronounced areas of coagulative necrosis in the region surrounding the millirod implant. A sharp demarcation line between necrotic and darkly stained cells at distances away from the implant was easily discernible. A higher magnification of this zone (Fig. 5E) revealed the presence of a large number of inflammatory cells, mostly neutrophils and lymphocytes. Immediately adjacent to the inflammatory cells were viable tumor cells noted by their large nuclei, irregular shape, and mitotic state that was more and more pronounced at distances farther away from the immediate treatment radius. As in control tumors, an area of patchy necrotic tissue at distances away from the implant was observed, interspersed with viable and apoptotic tumor cells, as well as inflammatory cells. Examining the tissues from the end points, we were encouraged by observing typical coagulative necrosis with sharp tissue demarcation after β-lapachone·hydroxypropyl-β-cyclodextrin–encoded millirod implantation on day 26, although clear tumor regrowth was noted in surrounding areas (not shown), which will be further discussed.

To evaluate possible systemic side effects of β-lapachone–loaded millirods, we did histologic examination of liver and kidney tissues 1 month after millirod implantation (Fig. 6). It has been reported that mice express abundant NQO1 levels in their livers, and high activity of NQO1 was detected in kidneys (26). These tissues were also chosen because β-lapachone is likely cleared through these organs *in vivo*. H&E staining of these tissues did not show any pathologic changes after β-lapachone treatment compared with those in animals receiving control millirods. The absence of tissue damage or cell abnormalities in the liver and kidney tissues strongly suggested that there were no noticeable signs of systemic toxicity brought about by millirod-delivered β-lapachone·hydroxypropyl-β-cyclodextrin.

Discussion

The objective of the present study was to evaluate the antitumor efficacy of β-lapachone *in vivo*, a novel and unique anticancer drug delivered intratumorally via a polymer depot. This drug, complexed as β-lapachone·hydroxypropyl-β-cyclodextrin (ARQ 501), is currently used in clinical trials against pancreatic cancers. Currently, 10- to 15-year survival rates for patients with primary prostate cancer are 65% after surgery (2) and 67% to 87% after brachytherapy (27). Invasiveness of the surgical procedure and high local recurrence rates provide motivation for the search for efficacious alternatives and supplemental therapies. Docetaxel is a frequently used drug for chemotherapy of prostate cancer that offers moderate success but results in high systemic toxicity (28, 29). Moreover, human prostate tumors are inherently resistant to many clinically used drugs against other tumors, given the fact that loss of androgen dependence and tumor suppressor genes (e.g., *p53*) are important factors in prostate cancer progression (30–32). In addition, these tumors are commonly deregulated with regard to calcium homeostasis (33) and lack caspases that mediate apoptosis (34). To overcome the shortcomings of current treatments, a novel agent is required that can offer an effective prostate tumor-specific treatment while sparing normal cells. The unique mechanism of action of β-lapachone, involving its bioactivation through the NQO1 enzyme, makes it an ideal agent for the treatment of tumors that overexpress this enzyme (Fig. 1A). Results from cell culture studies showed that a short exposure of β-lapachone for 2 h caused irreversible cell death, contrasting tremendously with other known anticancer drugs, wherein prolonged drug exposures might be required to elicit significant antitumor activity.

Matrix-based drug delivery devices represent an increasing trend in cancer chemotherapy, yielding the following specific advantages: (*a*) exposure of tumors to therapeutic levels of the drug for a prolonged time; (*b*) reduction of toxicity to healthy cells; and (*c*) potential tailoring of release kinetics for the design of most efficacious delivery regimen. Several implant strategies have been explored for intratumoral treatment of

cancers. Besides Gliadel wafers, as we have mentioned before (35), Ortiz et al. (36) developed a doxorubicin-releasing poly(lactide-co-glycolide) implant for the treatment of prostate tumors, showing feasibility for *in vivo* use, as well as low systemic concentrations of the drug after implantation into the prostate of beagle dogs. Recently, we reported β-lapachone–loaded millirods with tailorable release kinetics depending on inclusion of excipient molecules (e.g., glucose) or complexation with different cyclodextrin complexes (e.g., α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin; ref. 23). We showed that β-lapachone complexation with hydroxypropyl-β-cyclodextrin allowed for faster release of β-lapachone from millirods, in part because of the increase in drug solubility from 0.038 to 16 mg/mL. On the other hand, free drug showed the slowest release because of formation of a molecular-level mixture with the poly(D,L-lactide-co-glycolide) polymer and the lack of excipient molecules in the polymer matrix.

Based on the fact that a 2-h exposure of β-lapachone led to irreversible cell death, we designed a polymer millirod that featured a burst release of drug within a short period to achieve an elevated therapeutic dose of the drug (6), followed by a sustained release of the drug over a prolonged period. This was achieved by incorporating complexed and free drug within the millirod, with the complexed form of β-lapachone providing a burst release of drug (~0.5 mg released within the first 12 h) and the free form providing a sustained release of β-lapachone (release rate of 0.01 mg of drug/d over 22 days). The kinetics of drug release in the tumor is concluded from the drug release study *in vivo* as shown in Fig. 3. The burst release led to elevated drug concentrations within tumors that quickly exerted antitumor effects, whereas the sustained release maintained drug concentrations inside tumors for a prolonged period. Data showed that *in vivo* burst release kinetics of β-lapachone correlated very closely with release *in vitro*, with no statistical difference between the two conditions.

To evaluate antitumor efficacy and systemic toxicity, β-lapachone–loaded polymer millirods were implanted into PC-3 prostate tumor xenografts. Results from this study showed significant tumor regression and delayed growth after intratumoral implantation of the β-lapachone millirods. Moreover, survival of mice treated with β-lapachone millirods was significantly increased compared with untreated mice. Minimal, if any, systemic toxicity was observed, as evidenced by the lack of weight loss and normal seeming histology of major organs (e.g., liver, kidney). No complications were observed in the surrounding normal tissues. Taken together, these data highlight the potential of using intratumoral delivery of β-lapachone via polymer millirods as a viable treatment option for prostate cancer.

Results from this study showed tumor recurrence as a potential limitation of the current treatment. Tumor regrowth was found 2 weeks after millirod implantation (Fig. 4A), that was likely because of viable cancer cells in the region adjacent to the exposed area resulted from the limited drug penetration (Fig. 5B-E). To overcome this limitation, it may be necessary to combine the current treatment with another therapeutic modality, in an adjuvant or neoadjuvant manner. For example, patients may undergo radiation therapy or a minimally invasive ablative technique, such as cryotherapy or radiofrequency ablation, followed by implantation of β-lapachone–containing polymer millirods to maximize therapeutic potential. Previously, our laboratory showed that the combination of radiofrequency ablation of VX2 liver tumors followed by the intratumoral implantation of a doxorubicin-containing millirod resulted in increased efficacy compared with millirod implants alone (37). The selective killing feature of β-lapachone makes it more appealing than other drugs because of its tumor selectivity and reduced toxicity in tissues that do not express NQO1. Furthermore, previous studies have shown that β-lapachone functions as a radiosensitizer, with *in vitro* studies showing that a low dose of β-lapachone together with a low dose of ionizing radiation led to synergistic cell death (38). Therefore, β-lapachone millirods may be implanted initially, followed by low doses of ionizing radiation to provide synergy between the two treatment modalities and enhance antitumor efficacy. The combination can also be achieved by applying either external beam or co-implantation of low-dose irradiation materials, such as radioactive seeds during brachytherapy together with β-lapachone millirods. Thus, several viable combination strategies exist that can potentially maximize the therapeutic outcome of intratumoral β-lapachone implants.

Conclusion. β-Lapachone–containing polymer millirods represent an exciting therapeutic option for the targeted treatment of prostate tumors that overexpress the enzyme NQO1. By combining a novel drug, β-lapachone, whose mechanism of action is enhanced by an enzyme overexpressed in prostate tumors with a local polymer delivery device capable of controlled release, we were able to show significantly improved antitumor efficacy. Moreover, results from this study suggested minimal systemic toxicity and prolonged animal survival. Work is currently in progress to further maximize treatment efficacy by combining the therapy with existing radiotherapy strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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