[Cancer Biology & Therapy 4:1, 95-102, January 2005]; ©2005 Landes Bioscience

Research Paper

Efficacy of β-Lapachone in Pancreatic Cancer Treatment

Exploiting the Novel, Therapeutic Target NQO1

Matthew Ough[4]
Anne Lewis[4]
Erik A. Bey[6]
Jinming Gao[6]
Justine M. Ritchie[3]
William Bornmann[7]
David A. Boothman[6]
Larry W. Oberley[2,3,4]
Joseph J. Cullen[1,2,3,4,5,*]

Departments of [1]Surgery, [2]Radiation Oncology and the [3]Holden Comprehensive Cancer Center; [4]University of Iowa College of Medicine; Iowa City, Iowa USA

[5]Veterans Affairs Medical Center; Iowa City, Iowa USA

[6]Departments of Radiation Oncology and Pharmacology; Case Western Reserve University; Cleveland, Ohio USA

[7]Preparative Syntheses Core Facility; Memorial Sloan Kettering Cancer Center; New York, New York USA

*Correspondence to: Joseph J. Cullen; 4605 JCP; University of Iowa Hospitals and Clinics; Iowa City, Iowa 52242 USA; Tel.: 319.353.8297; Fax: 319.356.8378; Email: joseph-cullen@uiowa.edu

Received 09/24/04; Accepted 11/15/04

Previously published online as a *Cancer Biology & Therapy* E-publication: http://www.landesbioscience.com/journals/cbt/abstract.php?id=1382

KEY WORDS

pancreatic cancer, B-lapachone, NAD(P)H: quinone oxidoreductase, reactive oxygen species, quinones, cyclodextrins

ABBREVIATIONS

NQO1	NAD(P):quinone oxidoreductase
HPB-CD	hydroxypropyl-B-cyclodextrin

ACKNOWLEDGEMENTS

This work was supported by NIH grants DK 60618, CA 66081, HL07485-24 and the Medical Research Service, Department of Veterans Affairs.

ABSTRACT

NAD(P)H:quinone oxidoreductase (NQO1) is elevated in human pancreatic cancers. We hypothesized that β-lapachone, a novel 1,2-naphthoquinone with potential antitumor activity in cancer cells expressing elevated levels of NQO1, would induce cytotoxicity in pancreatic cancer cells, wherein this two-electron reductase was recently found elevated. β-lapachone decreased clonogenic cell survival, metabolic cell viability, and anchorage-independent growth in soft agar. The cytotoxic in vitro effects of β-lapachone were inhibited with coadministration of dicumarol, a specific inhibitor of NQO1. In preestablished human pancreatic tumor xenografts in nude mice, β-lapachone demonstrated greater tumor growth inhibition when given intratumorally compared to when complexed with cyclodextrin to increase its bioavailability. Due to the poor prognosis of patients with pancreatic cancer and the limited effectiveness of surgery, chemotherapy, and radiation therapy, treatment regimens based on sound, tumor-specific rationales are desperately need for this disease.

The prognosis of patients diagnosed with pancreatic adenocarcinoma is dismal. Pancreatic adenocarcinoma is now the fourth leading cause of cancer death in the United States with an overall 5-year survival rate of less than 5%.[1] Even after curative resection, the 5-year survival rates achieved at specialized centers are less than 20% and the majority of patients die of metastatic cancer recurrence.[2] Other adjuvant treatments such as radiation therapy and chemotherapy have not improved long-term survival after resection.

β-lapachone is a naturally occurring compound present in the bark of the South American Lapacho (*Tabebuia avellanedae*) tree. The compound has a number of antitumor, antiviral, and antitrypanosomal activities in vivo[3-5] β-lapachone has significant antineoplastic activity against a variety of human cancer cells, including breast, prostate, and lung cancers as well as promyelocytic leukemia cells.[6] Previous studies demonstrated that the cytotoxic response of breast and prostate cancer cells to β-lapachone is significantly enhanced by the expression of NAD(P)H:quinone oxidoreductase (NQO1, E.C. 1.6.99.2),[7] an enzyme that detoxifies quinones (e.g., β-lapachone, menadione), as well as serving as a protective mechanism against reactive oxygen species (ROS).[6-9] The NQO1-specific antitumor activity of β-lapachone in other cancer cells than breast or prostate have not been examined, and the universality of β-lapachone-mediated antitumor activity in other cancer cells has not been examined. ROS is initiated when reactive semiquinones generate a redox cycle, resulting in superoxide (O_2^-) formation. NQO1 utilizes either NADH or NADPH as electron donors to catalyze the two-electron reduction of various quinones to hydroquinones. It is a direct reduction, and therefore, the unstable semiquinone intermediate is not formed, preventing reactive oxygen species formation. Reduction of β-lapachone by NQO1 leads to a futile cycling of the compound, wherein the quinone and hydroquinone form a redox cycle with a net concomitant loss of reduced NAD(P)H.[7]

Recent studies by Logsdon et al. demonstrate that NQO1 is elevated in human pancreatic cancers.[10] Microarrays to profile gene expression in pancreatic adenocarcinoma, pancreatic cancer cell lines and normal pancreas, demonstrated a 12-fold increase of NQO1 expression compared to normal pancreatic tissue.[10] Recent studies from our laboratory suggest enzymatic overexpression of NQO1 in human pancreatic cancer cell lines.[11] RT-PCR, western blots, and activity assays demonstrated that NQO1 was elevated in the human pancreatic cancer cell lines tested but present in very low amounts in the normal human pancreas. The findings that NQO1 is elevated in pancreatic cancer cell lines is consistent with previous studies that have shown that expression of NQO1 is upregulated in tumors of the liver, lung (nonsmall cell only), colon, and breast compared to normal tissues of the same origin.[8] Additionally, in cells that lack mitochondria, activity of this

mostly cytosolic oxidoreductase system is greatly upregulated to support cell growth.[12] Previous investigations have hypothesized that NQO1 is elevated in various tumors to accommodate the needs of rapidly metabolizing cells to regenerate NAD+.[13] Combined, these studies suggest that NQO1 may be useful as a specific target for therapeutic purposes in pancreatic cancer.

Although β-lapachone should hypothetically kill NQO1-containing pancreatic cancer cells, the low water solubility of β-lapachone (0.04 mg/ml or 0.16 mM) limits its systemic administration and clinical applications in vivo. Recently, cyclodextrins, well known classes of host molecules that can form inclusion complexes with a variety of drugs, have been used to enhance solublilization, increase stability, and bioavailability by forming inclusion complexes.[14-17] Specifically, hydroxypropyl-β-cyclodextrin (HPβ-CD) is a modified β-CD obtained by treating a base-solubilized solution of β-CD with propylene oxide. This chemical modification further increased the solubility of HPβ-CD over β-CD. Most importantly, HPβ-CD in clinical trials has been demonstrated to be safe and well tolerated without the observable renal toxicity seen with β-CD.[17]

We hypothesized that β-lapachone would induce cytotoxicity in pancreatic cancer cells that have upregulation of NQO1. Our present study demonstrates that β-lapachone has in vitro cytotoxicity to pancreatic cancer cells that are known to overexpress NQO1. The β-lapachone-induced cytotoxicity can be reversed, by inhibiting NQO1 with dicumarol. In addition, β-lapachone appears to have efficacy in reducing in vivo tumor growth when given via an intratumoral route and with binding β-lapachone inside the hydrophobic cavity of cyclodextrin thereby increasing the drug solubility.

MATERIALS AND METHODS

Cell culture. MIA PaCa-2 cells were purchased from American Type Culture Collection (Manassas, VA) and are human primary pancreatic adenocarcinoma cells derived from tumor tissue of the pancreas obtained from a 65-year old male. The cell cultures were maintained at 37°C in Dulbecco modified Eagle medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum and 2.5% horse serum.

Cell growth. Cells (1×10^4) were plated in triplicate in 1.5 ml complete media in 24-well plates. Cells were trypsinized and then counted on alternate days for 2 weeks using a hemocytometer. Cell population doubling time in hours (*DT*) was determined in triplicate using the following equation:

$$DT \text{ (hours)} = 0.693(t - to)/\ln(Nt/No)$$

where *to* = time at which exponential growth began, *t* = time in hours, *Nt* = cell number at time *t*, and *No* = initial cell number.[11] β-lapachone (0, 2.5, 5, 10 μM) was added to MIA PaCa-2 cell cultures.

Anchorage-independent growth in soft agar. MIA PaCa-2 cells (5×10^3) were suspended in 3 ml of complete media containing a solution of 6% agar in double distilled H_2O so that the final concentration of the agar was 0.3%. This suspension was then plated over 3 ml of complete media made using a 6% agar solution in double distilled H_2O so that the final concentration of the bottom agar was 0.5% and the final concentration of β-lapachone was 5 or 10 μM. After 16 days, colonies of greater than 0.1 mm in diameter were scored. The clonogenic fraction was determined using the following equation:

Soft Agar Plating Efficiency (P.E.) = (colonies formed/cells seeded) x 100.

Clonogenic assay. MIA PaCa-2 cells (5×10^3) were treated with β-lapachone (0, 2.5, 5, 10 μM) for 4 hours with and without dicumarol (50 μM) or menadione (10 and 20 μM). For survival determination, cells were trypsinized and plated for clonogenic cell survival assay. After one week of incubation at 37°C, colonies greater than 50 cells were stained and counted. The clonogenic fraction was determined using the following equation:

Survival = (colonies formed/cells seeded) x 100.

Cell viability. As an indicator of cell metabolic viability, the MTT assay was used. Cells were seeded at 1×10^4 in a 96 well plate in full media. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) 5 mg/ml was added to the wells and incubated at 37°C for 3 hours. Lysing buffer, consisting of 20% SDS in a 1:1 solution of DMF (N,N-dimethyl formamide), was added and incubated at 37°C for 16 hours. The plate was read at 590 nm on an Ultramark microplate imaging system (Biorad, Hercules, CA).

Transient NQO1 siRNA transfection. MIA-PaCa-2 cells were seeded in 6 well dishes (50,000 cells /well) for 24 h. The cells were washed and resuspended in serum free media. 100 nM of Smartpool NQO1 siRNA duplexes (Dharmacon RNA Technologies) were complexed with Oligofectamine Reagent (Invitrogen) in OPTI-MEM-I media (Gibco) according to the Oligofectamine Reagent protocol. The NQO1 siRNA oligfectamine complexes in OPTI-MEM-I media were added to NQO1 siRNA treated wells. Mock-transfected cells were treated with transfection media only. After four hours, growth media containing 3x serum was added to each well. After 48 h, cells were harvested for Western blot analyses or trypsinized, counted and reseeded for relative survival assays.

Relative survival assay. Following transfection, cells were detached by trypsinization, washed in fresh growth media and reseeded in 48 well dishes at a density of 10,000 cells/well in 500 μl. Cells were treated with 10 μM β-lapachone ± 50 μM Dicumarol for 4 h or the NQO1 siRNA. Drug was removed and fresh growth media was added, and cells were allowed to grow for an additional four days. DNA content (relative survival) was determined by fluorescence of the DNA dye Hoescht 33258 (Sigma), according to the method previously described.[7] The data are expressed as mean ± S.E for three independent wells.

Nude mice. Thirty-day-old athymic nude mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). The nude mice protocol was reviewed and approved by the Animal Care and Use Committee of the University of Iowa on January 9, 2003. The animals were housed four to a cage and fed a sterile commercial stock diet and tap water, ad libitum. Animals were allowed to acclimate in the unit for one week before any manipulations were performed. In the first study, each experimental group consisted of four to five mice. In the second study, each experimental group consisted of 9–10 mice. In the third study, each group consisted of eight mice.

Treatment of established pancreatic tumor heterotopic xenografts with β-lapachone. MIA PaCa-2 tumor cells (2×10^6) were delivered subcutaneously into the flank region of nude mice from a 1-cc tuberculin syringe equipped with a 25-gauge needle. The tumors were allowed to grow for two weeks before treatment was initiated. β-lapachone was delivered through one or two injection sites in the tumor, depending on tumor size at the time of injection. In the first in vivo studies, β-lapachone (50 μM) dissolved in DMSO and sterile PBS (100 μL total volume) was delivered to the tumor by means of a 25-gauge needle attached to a 1-cc tuberculin syringe. This was defined as day 1 of the experiment. Control tumors received 100 μL of sterile DMSO and PBS at the same time points. Despite the potency and selectivity of β-lapachone in killing NQO1$^+$ cancer cells in vitro, the low water solubility of β-lapachone (0.04 mg/ml or 0.16 mM) limits its systemic administration and clinical applications in vivo. Thus, in the second study, the aqueous solubility of β-lapachone was achieved with the previously described technique of binding β-lapachone inside the hydrophobic cavity of cyclodextrin thereby increasing the drug solubility. The compound formed, HPβ-CD, has been demonstrated to have in vitro antitumor effects and a LD_{50} value of β-lapachone in HPβ-CD between 50–60 mg/kg (unpublished data). In the second series of studies three groups of mice were used consisting of 9–10 mice/group. Animals received HPβ-CD alone (50 mg/kg IP/d x two weeks), β-lapachone (50 mg/kg IP every day for two weeks), or β-lapachone (50 mg/kg IP twice a day, every day for two weeks).

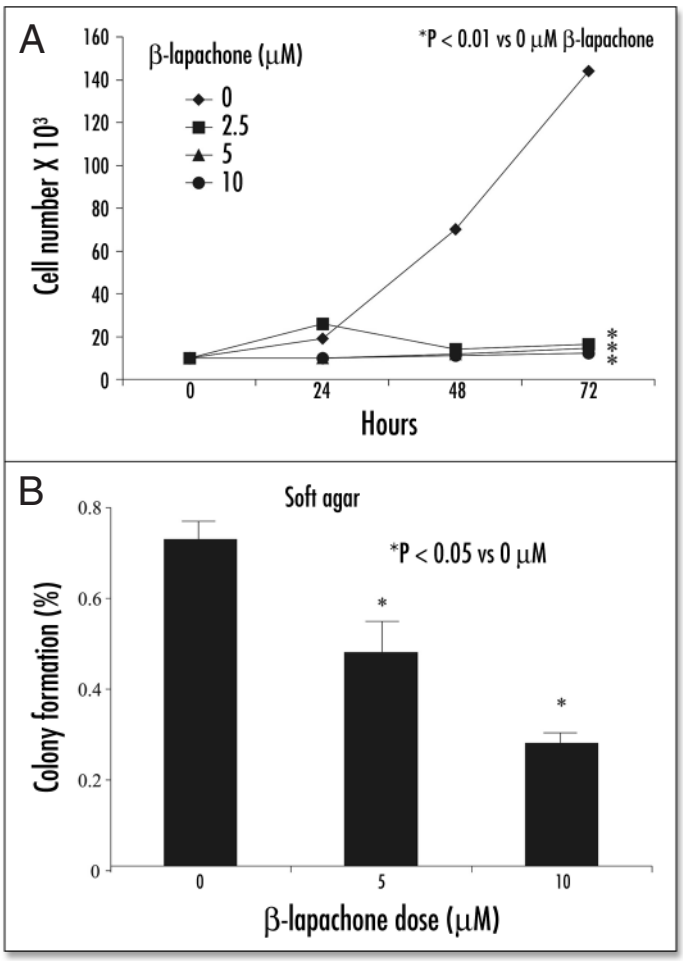

Figure 1. β-lapachone inhibits the in vitro pancreatic cancer malignant phenotype. (A) Cell growth. MIA PaCa-2 cells treated with β-lapachone (2.5, 5, and 10 μM) demonstrate reductions in cell growth. Each point was determined in triplicate. * P< 0.001 vs MIA PaCa-2 cells that received vehicle (controls). (B) Growth in soft agar. MIA PaCa-2 cells treated with β-lapachone (5 and 10 μM) demonstrate reductions in soft agar plating efficiency. Mean plating efficiency in soft agar of β-lapachone (0–10 μM) MIA PaCa-2 cells are shown. Each determination was performed in triplicate. *P < 0.05 vs 0 μM β-lapachone.

After determining the results from the first two studies, a third separate study was performed. Once again HPβ-CD was given in the controls but injected intratumorally in 100 μL total volume. In the second and third groups, β-lapachone (250 or 500 μM) dissolved in DMSO and sterile PBS (100 μL total volume) was also given intratumoral. In the fourth and fifth groups, β-lapachone complexed with HPβ-CD was given as a dose of 50 mg/kg or 75 mg/kg as an intratumoral dose. All injections in this group of animals were given as a one-time dose.

Tumor size was measured every three days by means of a vernier caliper, and tumor volume was estimated according to the following formula: tumor volume = $\pi/6 \times L \times W^2$, where L is the greatest dimension of the tumor, and W is the dimension of the tumor in the perpendicular direction.[18] When the tumors reached a predetermined size of 10 x 10 mm, the animal was sacrificed by CO_2 asphyxiation.

RESULTS

Tumor biological characteristics of β-lapachone treated cells. The in vitro growth of MIA PaCa-2 pancreatic cancer cells was significantly slowed after exposure to β-lapachone (Fig. 1A). MIA PaCa-2 cell doubling time significantly increased with β-lapachone (2.5, 5, and 10 μM, four-hour pulse), when compared to the parental cells. For example, 48 h after four-hour pulses of β-lapachone treatments, cell numbers decreased approximately 80% with 2.5 μM β-lapachone and 85% with 10 μM β-lapachone compared with the MIA PaCa-2 cells with no treatment (Fig. 1A).

To determine the effects of β-lapachone on malignant MIA PaCa-2 cancer cells in vitro, we examined anchorage-dependent growth, clonogenic cell survival, and metabolic cell viability. To examine anchorage-dependent growth, we performed a soft agar assay. Whereas malignant cells form colonies in soft agar, normal cells do so infrequently. MIA PaCa-2 pancreatic cancer cells treated with β-lapachone significantly reduced colony formation (Fig. 1B). Soft agar plating efficiency was 0.73% ± 0.04 in the parental cells. Colony formation was reduced with four-hour pulses of 5 μM β-lapachone where the plating efficiency decreased to 0.48% ± 0.06, and maximally reduced to 0.28% ± 0.03 with β-lapachone 10 μM (Means ± SEM, $p < 0.05$ β-lapachone treated vs. parental cells).

β-lapachone, a member of the naphthoquinone family, has been shown to be a substrate for NQO1, entering a futile redox cycling of the compound, wherein the quinone and hydroquinone form a redox cycle with a net concomitant loss of reduced NAD(P)H.[7] Thus, β-lapachone toxicity is influenced by NQO1 expression and suppressing (using dicumarol) or eliminating (using deficient cells) the activity of this enzyme dramatically reduces killing by this agent. NQO1 has been demonstrated to be elevated in both pancreatic cancer specimens (10) and in pancreatic cancer cell lines.[11] Treatment of MIA PaCa-2 pancreatic cancer cells with β-lapachone (2.5, 5, 10 μM) for four hours demonstrated a dose-dependent increase in cytotoxicity as measured by the clonogenic cell survival assay (Fig. 2A). In fact, at 5 μM and 10 μM, there were no colonies formed when 5,000 cells were plated. Loss of clonogenic activity in breast, prostate and leukemic cells, was previously demonstrated to be directly related to apoptotic-inducing ability of this compound.[6]

We then determined the effects of β-lapachone when administered with dicumarol, a compound that primarily inhibits NQO1, by competing with NADH for the binding site of the oxidized NQO_1 form.[13] Coadministration of 50 μM dicumarol during a four hour pulse of β-lapachone caused a significant enhancement in clonogenic cell survival in MIA PaCa-2 cells (Fig. 2A). The cytoprotective effect of inhibiting NQO1 was most dramatic at 10 μM β-lapachone, where dicumarol (50 μM) completely reversed the cytotoxic effects of the drug (Fig. 2B). To confirm the effects of dicumarol in reversing the cytotoxicity of β-lapachone seen in the clonogenic cell survival assay, we determined cell metabolic viability using the MTT assay. β-lapachone (at 5 and 10 μM, four-hour) significantly decreased metabolic cell viability (Fig. 2C) compared to untreated cells. As with survival, decreased metabolic cell viability induced by 10 μM, four-hour β-lapachone exposure was reversed with coadministration of dicumarol 50 μM.

We then extended these studies to compare the relative toxicity of menadione (2-methyl-1,4-naphthoquinone) to β-lapachone. Menadione has previously been demonstrated to have cytotoxic effects to pancreatic cancer cells in vitro when combined with dicumarol.[19] MIA PaCa-2 cells were treated with a four-hour pulse of drugs, and a clonogenic cell survival assay was repeated. In contrast to what was observed with β-lapachone, dicumarol enhanced the cytotoxic effects in MIA PaCa-2 cells treated with menadione (10 and 20 μM) (Fig. 2D).

MIA-PaCa-2 cells were then transfected for four hours with 100 nM siRNA- NQO1 or mock transfected (transfection media only). Cells were cultured for an additional 48 h before harvesting and immunoblotting demonstrated that NQO1 siRNA knocked-down NQO1 expression by 83% (Fig. 3A). Cells that were mock transfected or transfected with 100 nM siRNA-NQO1 and cells treated with 10 μM β-lapachone ± 50 μM dicumarol for 4 h were reseeded (Fig. 3B). Once again, β-lapachone (10 μM) decreased relative percent survival, while the combination of β-lapachone (10 μM) + dicumarol (50 μM) reversed the effects of β-lapachone alone. Transfections with siRNA-NQO1 significantly reversed the effects of β-lapachone alone, however not to the effect seen with dicumarol.

Efficacy of β-lapachone against established pancreatic tumor heterotopic xenografts. In our initial examination of antitumor activity, β-lapachone was

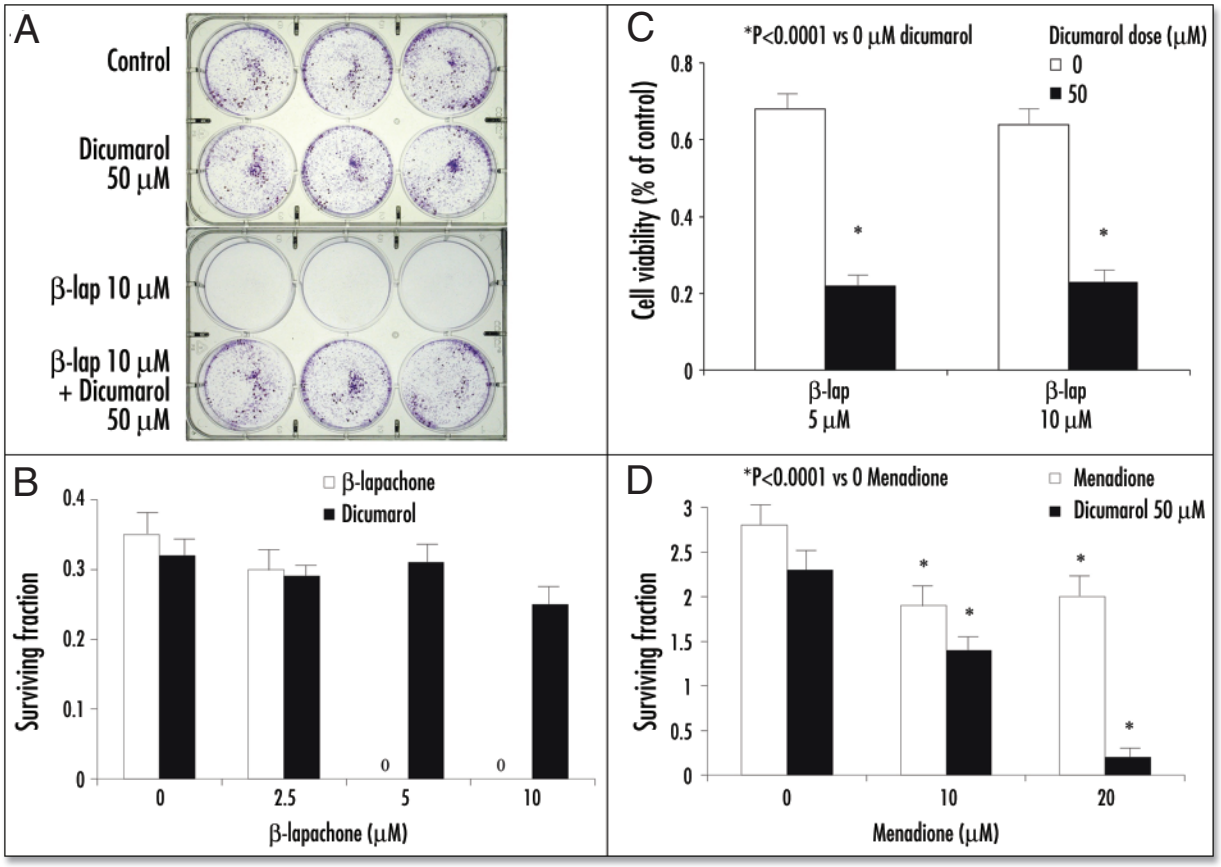

Figure 2. β-lapachone decreases clonogenic cell survival. (A) Clonogenic cell survival plating efficiency. MIA PaCa-2 cells treated with β-lapachone (0, 2.5, 5 and 10 μM) demonstrate reductions in plating efficiency. Mean plating efficiency of β-lapachone (0–10 μM) MIA PaCa-2 cells are shown. Each determination was performed in triplicate. Coadministration of 50 μM dicumarol during a 4-hour pulse of β-lapachone caused a significant enhancement in clonogenic cell survival in MIA PaCa-2 cells Cotreatment of the cells with dicumarol 50 μM during β-lapachone pulse caused an increase in clonogenic cell survival in MIA PaCa-2 cells, which was most notable at the 5 and 10 μM β-lapachone dose. The zero on the β-lapachone 5 and 10 μM + dicumarol 50 μM group indicates that no colonies were formed when 5,000 cells were plated in the dishes. Means, N = 3. (B) Clonogenic cell survival. Dicumarol (50 μM) completely reversed the cytotoxic effects of β-lapachone 10 μM. No colonies were formed when 5,000 cells were plated in dishes. (C) β-lapachone decreases metabolic cell viability as measured by monotetrazolium (MTT) assay. The absorbance of MIA PaCa-2 cells treated with β-lapachone (5 and 10 μM) with and without dicumarol 50 μM for four hours. β-lapachone treatment significantly decreases metabolic cell viability compared to untreated cells, while the decrease in metabolic cell viability is reversed with coadministration of dicumarol 50 μM as determined by a microplate reader at 590 nm. Means ± SEM, *P < 0.0001 vs 0 μM dicumarol, N = 6. (D) Dicumarol potentiates menadione-induced clonogenic cell death. A clonogenic assay was performed on MIA-PaCa-2 cells treated with menadione (10–20 μM) for four hrs with and without dicumarol (50 μM). *P < 0.05 vs 0 μM menadione, means, N = 3.

dissolved in DMSO and given intratumorally (50 μM in 100 μL volume x 1 dose) in preestablished pancreatic tumors in the flanks of nude mice. Control mice were given equal volumes of saline in DMSO. Tumor volume was compared amongst two groups using the data from days 1 thru 18 only. For animals that died prior to day 18, the last tumor volume was carried forward. To compare the treatment groups over time for tumor volume, the linear mixed model analysis,[20] assuming a compound symmetry covariance structure for within subjects, was used. In the linear mixed model analysis, a group was considered a fixed effect, and the day was considered a continuous covariate. An interaction term between day and group was also included in the model. Using day as a continuous covariate assumes that the mean tumor volume is a linear function of days. In these analyses the linear assumption seemed acceptable since the adjusted R^2s were all at least 0.62 (controls: adjusted $R^2 = 0.94$; β-lapachone 50 μM: adjusted $R^2 = 0.62$). The linear mixed model suggested that the interaction between day and group was statistically significant (p = 0.0002), that is, there was an overall difference among the groups in the slopes for tumor volume over time (Fig. 4A). The estimates for the slopes by group are provided in Table 1. For example, tumor volumes were significantly reduced from 517 ± 248 mm^3 in controls to 296 ± 271 mm^3 in tumors after a single injection of β-lapachone 50 μM (p < 0.001 vs controls, Means ± S.D.). Kaplan-Meier plots for survival of animals with tumors receiving β-lapachone 50 μM indicated that there were no significant differences in survival (Fig. 4B). Single injections of β-lapachone (50 μM) in tumors had no effect on time to sacrifice when compared with the control group (P = 0.10). These data suggested that β-lapachone dissolved in DMSO had effects on tumor volumes initially, but did not influence overall survival of tumor-bearing animals.

Table 1 **In vivo tumor volume slopes over time for the data shown in Figure 4A**

Group	Slope (95% CI)
Control	21.8 (16.9–26.8)
β-lapachone 50 μM in 100 μl	7.4 (2.5–12.3)

In the second study, β-lapachone was complexed with hydroxypropyl-β-cyclodextrin (HPβ-CD) to increase the bioavailability of the drug as

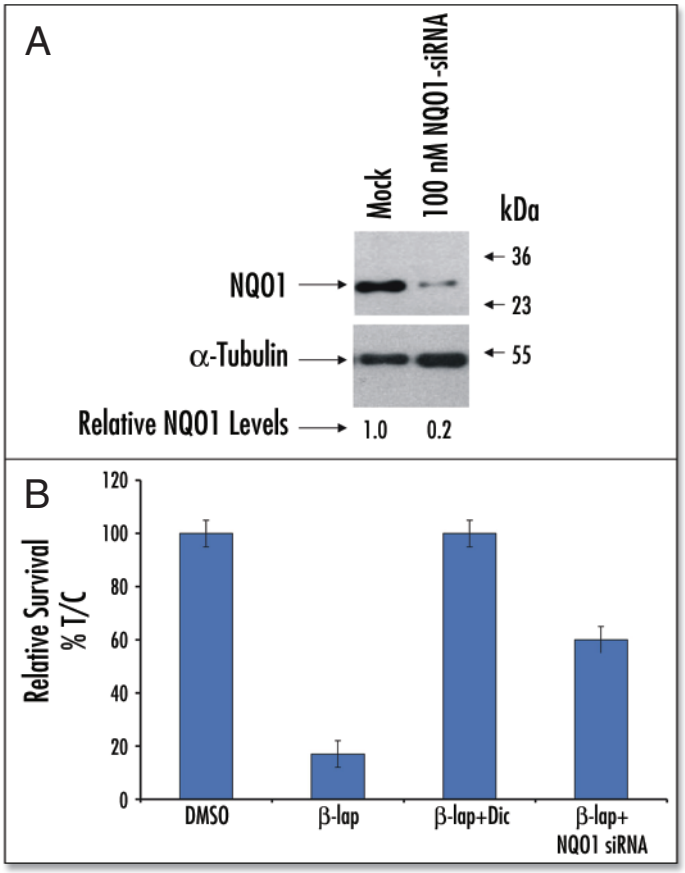

Figure 3. siRNA-NQO1 inhibits NQO1 protein expression and NQO1 mediated toxicity in Mia-PaCa pancreatic cancer cells. (A) MIA-PaCa-2 cells were transfected for four hours with 100 nM siRNA-NQO1 or mock transfected (transfection media only). Cells were cultured for an additonal 48 h before harvesting. Immunoblotting was performed using an anti-Human NQO1 antibody. Integrated densities of NQO1 siRNA and mock transfected bands were determine by NIH Image software. NQO1 siRNA knocked-down NQO1 expression by 83%. (B) MIA-PaCa-2 cells were mock transfected or transfected with 100 nM siRNA-NQO1 for four hours and reseeded after 48 h in 48 well dishes (20,000 cells/ml). Cells were treated with 10 μM β-lap ± 50 μM dicoumarol for four hours. Drug media were removed and fresh drug free media were added, and cells were allowed to grow for an additional four days. Relative DNA per well was assessed by Hoescht 33258 fluorescence, and relative percent survival (treated/control) was graphed. Each bar represents the mean of three independent wells ± S.E.

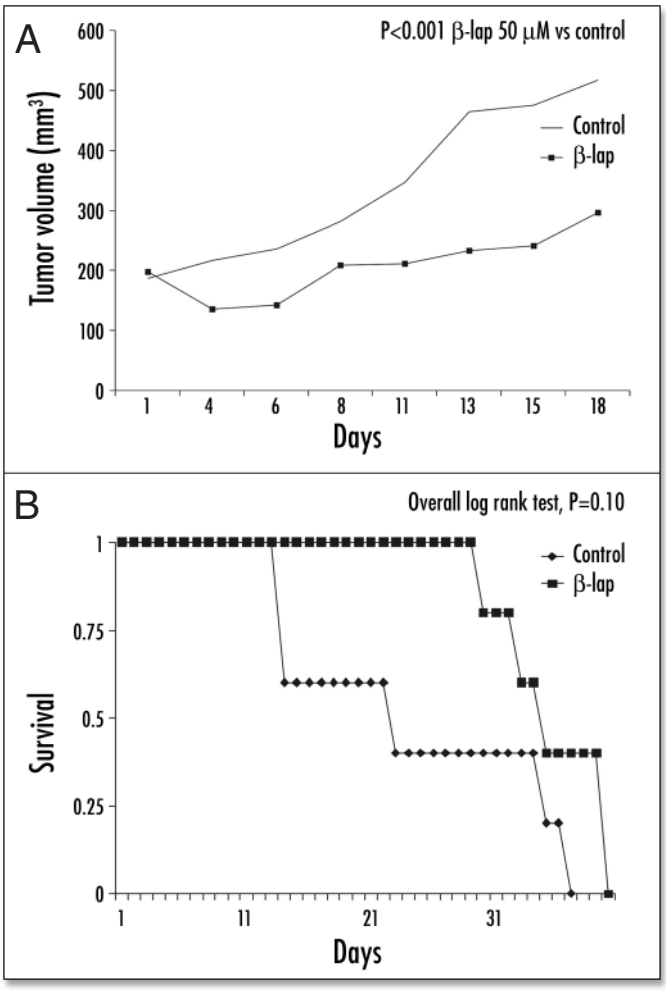

Figure 4. (A) Single intratumoral injection of β-lapachone (50 μM dissolved in DMSO injected in 100 μl volume) decreased MIA-PaCa-2 tumor growth in nude mice when studied out to 28 days postinjection. The β-lapachone treated group (50 μM) exhibited a 1.7-fold decrease in tumor growth over no treatment (0 μM). Day 18: median tumor volume 517 mm^3 in control tumors vs 296 mm^3 in tumors with one intratumoral injection of β-lapachone (50 μM). Means, N = 4–6/group. (B) Intratumoral injections of β-lapachone did not increase survival in pancreatic tumor xenografts. Kaplan-Meier plots of estimated survival after injection of MIA PaCa-2 tumors in nude mice. Single dose intratumor injections of β-lapachone (50 μM) did not result in increased time to sacrifice when compared with control group (P = 0.10).

Table 2 **In vivo tumor volume slopes over time for the data shown in Figure 5A**

Group	Slope (95% CI)
HPβCD 50 mg/kg IP x 2 weeks	14.3 (9.1–19.4)
β-lapachone 50 mg/kg IP x 2 weeks	7.3 (1.9–12.8)

described in reference 22. Controls received HPβ-CD I.P. alone. Treatment with β-lapachone 50 mg/kg intraperitoneal B.I.D. proved to be extremely toxic. In the first week after the intratumoral dose of 100 mg/kg was given, 55% of the mice were dead. In the first 19 days of 50 mg/kg I.P. B.I.D. treatment, only 33% of these mice were alive. None of these mice were sacrificed due to large tumor size. Tumor volume was compared among the three groups using the data from days 1 thru 19 only. For two mice from the HPβCD control group, the tumor volume from day 8 was carried through to days 15 and 19. To compare these treatment groups over time for tumor volume, the linear mixed model analysis[15] assuming an auto-regressive order one covariance structure for within subjects was used. Based on Akaike's Information Criterion (AIC),[21] the auto-regressive order one covariance structure (AIC = 1325.1) was selected instead of compound symmetry (AIC = 1349.1).

In the linear mixed model analysis, group was considered a fixed effect and day was considered a continuous covariate. An interaction term between day and group was also included in the model. Using day as a continuous covariate assumes that the mean tumor volume is a linear function of days. It turned out that the linear assumption was acceptable given that the adjusted R^2s were 0.80 or greater (HPβ-CD alone: R^2 = 0.98; β-lapachone 50 mg/kg IP x 2 weeks: adjusted R^2 = 0.83, animals that received the BID dose were excluded due to the toxicity and high mortality). The linear mixed model suggested that the interaction between day and group was not statistically

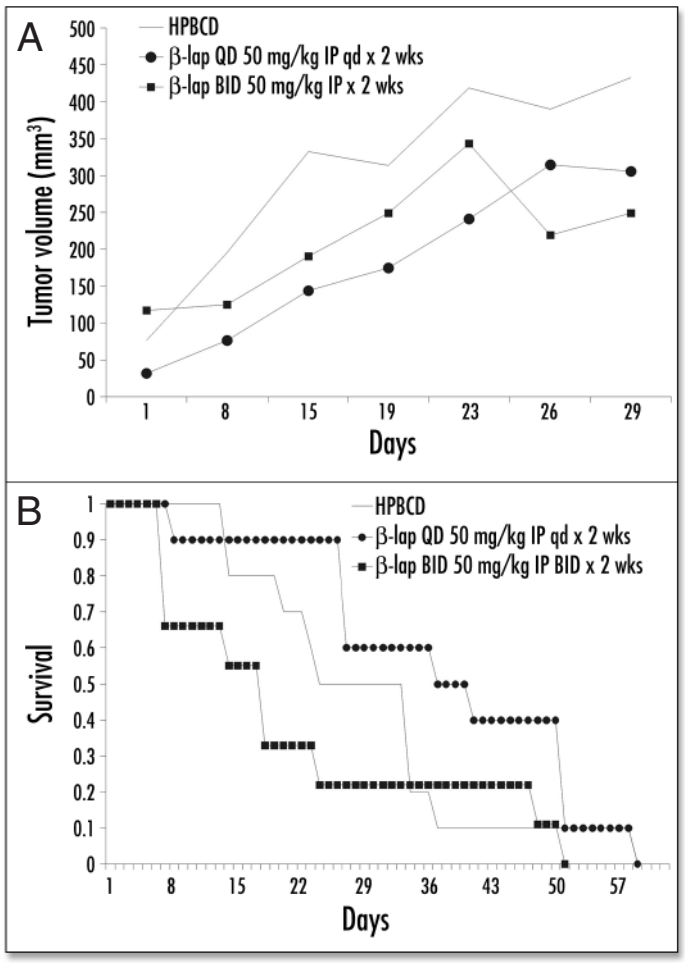

Figure 5. (A) Systemic treatment of pancreatic tumors with β-lapachone dissolved in HPβCD (50 mg/kg IP x two weeks) did not decrease MIA-PaCa-2 tumor growth in nude mice when studied out to 29 days postinjection. The β-lapachone treated group (50 mg/kg IP every day for two weeks) exhibited a 1.4-fold decrease in tumor growth over treatment with HPβCD alone. Day 29: median tumor volume 433 mm^3 in tumors treated with HPβCD alone vs 306 mm^3 in tumors treated with β-lapachone complexed with HPβCD. P = 0.07, Means, N = 8/group. (B) Intraperitoneal injections of β-lapachone did not increase survival in pancreatic tumor xenografts. Kaplan-Meier plots of estimated survival after injection of MIA PaCa-2 tumors in nude mice. β-lapachone (50 mg/kg I.P., every day for two weeks or 50 mg/kg I.P., twice a day for two weeks) did not result in increased time to sacrifice when compared with control group (P = 0.10).

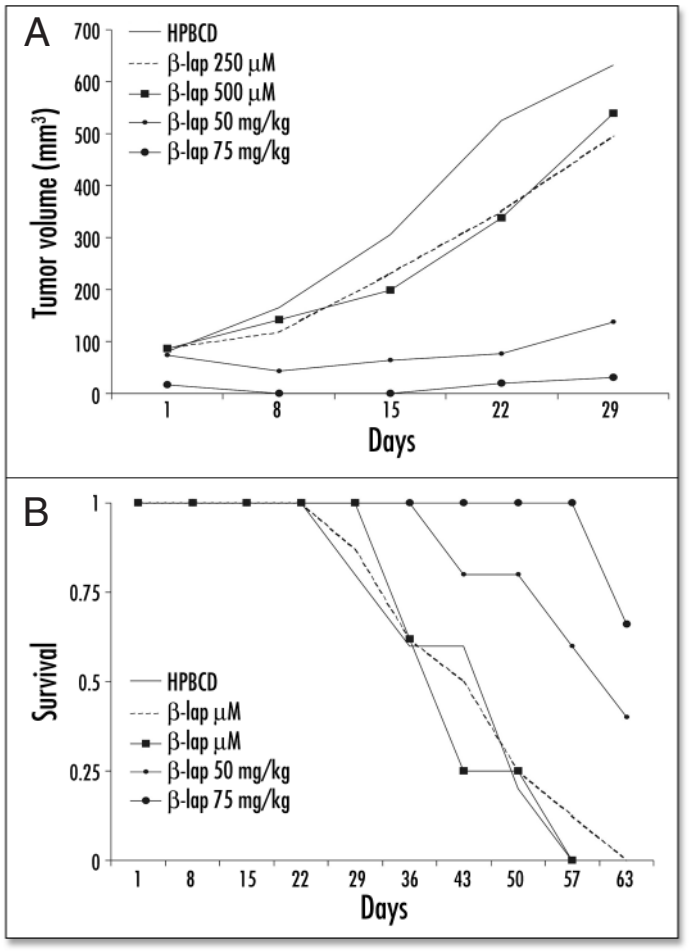

Figure 6. (A) Single intratumoral injections of β-lapachone (250 μM dissolved in DMSO, 50 mg/kg complexed with HPβCD, and 75 mg/kg complexed with HP βCD, all injected in 100 μl volume) decreased MIA-PaCa-2 tumor growth in nude mice when studied out to 29 days postinjection. The β-lapachone treated group (250 μM and 500 μM groups) exhibited a 1.3- and 1.2-fold decrease respectively in tumor growth over treatment with HPβCD alone (P < 0.05 HPβCD vs β-lapachone 250 μM; P = 0.08 HPβCD vs β-lapachone 500 μM). In the groups that received β-lapachone complexed with HPβCD (50 mg/kg and 75 mg/kg) there was a 4.6- and 21-fold decrease respectively in tumor growth over treatment with HPβCD alone (P < 0.0001 HPβCD vs β-lapachone 50 mg/kg or 75 mg/kg). Means, N = 8/group. (B) Single intratumoral injections of β-lapachone complexed with HPβCD (50 mg/kg and 75 mg/kg) increased survival in pancreatic tumor xenografts. Kaplan-Meier plots of estimated survival after injection of MIA PaCa-2 tumors in nude mice. Single dose intratumor injections of β-lapachone (50 mg/kg) or β-lapachone (75 mg/kg) result in increased time to sacrifice when compared with animals receiving HPβCD (HPβCD vs β-lapachone 50 mg/kg, P = 0.01; HPβCD vs β-lapachone 75 mg/kg, P = 0.006).

significant (p = 0.19), that is, there was not an overall difference among these groups in the slopes for tumor volume over time (Table 2). As demonstrated in Figure 5A when comparing differences among groups for tumor volumes, the group of mice that received β-lapachone 50 mg/kg IP every day for 2 weeks had a slight, but not significant decrease in tumor volume when studied out to day 19 (Means, N = 9 - 10/group, p = 0.07). For example on day 19 of treatment, animals that received HPβ-CD alone had a mean tumor volume of 327 ± 248 mm^3, compared to animals that received 50 mg/kg IP every day for two weeks 210 ± 158 mm^3 (Means ± S.D., p = 0.07). In this set of studies, Kaplan-Meier plots to determine survival demonstrated two major findings. First, β-lapachone given for a dose of 50 mg/kg I.P. twice a day for two weeks was extremely toxic with nearly one-half of animals dying during the treatment. Secondly, there were no differences among the groups (Fig. 5B).

In the third study, β-lapachone was given as an intratumoral dose using two different preparations. β-lapachone was dissolved in DMSO and given as a 250 μM or 500 μM dose in 100 μl. In the other groups, β-lapachone was complexed with HPβ-CD and given as an intratumoral dose of 50 mg/kg, or 75 mg/kg. Controls once again received intratumoral injections of HPβ-CD. Tumor volume was compared among the five groups using the data from days one thru 29 only among animals that survived beyond the first week of treatment. For the animals that died prior to day 29, the last tumor volume was carried forward. This was done for two animals in the control group 1 and one animal from the β-lapachone 250 μM. To compare the treatment groups over time for tumor volume, the linear mixed model analysis[20] was used assuming a compound symmetry covariance structure for within subjects was used. Due to the number of time points and the small number of subjects per group, the possible covariance structure was limited.

Table 3 **In vivo tumor volume slopes over time for the data shown in Figure 6A**

Group	Slope (95% CI)
HPβCD 50 mg/kg IT	20.9 (17.0–24.1)
β-lapachone 250 μM IT x 1	15.0 (10.6–19.3)
β-lapachone 500 μM IT x 1	15.8 (11.4–20.1)
β-lapachone 50 mg/kg IT x 1	2.9 (-2.6–8.4)
β-lapachone 75 mg/kg IT x 1	0.7 (-6.4–7.8)

In the linear mixed model analysis, group was considered a fixed effect and day was considered a continuous covariate. An interaction term between day and group was also included in the model. Using day as a continuous covariate assumes that the mean tumor volume is a linear function of days. The adjusted R^2s were as follows: HPβ-CD: $R^2 = 0.97$; β-lapachone 250 μM: $R^2 = 0.95$; β-lapachone 500 μM: $R^2 = 0.90$; β-lapachone 50 mg/kg: $R^2 = 0.65$; β-lapachone 75 mg/kg: $R^2 = 0.08$. The R^2 was low for the β-lapachone 50 mg/kg group and the β-lapachone 75 mg/kg group suggesting the linearity assumption may not be appropriate for these groups. The linear mixed model suggested that the interaction between day and group was statistical significant ($p < 0.0001$), that is, there appears to be a difference among the groups in the slopes for tumor volume over time (Fig. 6A). The estimates for the slopes by group are provided in Table 3. When compared to controls, the animals that received β-lapachone 250 μM had decreased tumor volume ($p < 0.05$) and a trend towards decreased tumor volume in the higher dose of β-lapachone 500 μM ($p = 0.08$). When β-lapachone was complexed with HPβ-CD, there were profound decreases in tumor size in both the 50 mg/kg IT dose ($p<0.0001$) and the 75 mg/kg IT dose ($p < 0.0001$) when compared to controls. For example, on Day 22, animals that received HPβ-CD alone had a tumor volume of 525 ± 390 mm^3, compared to tumor sizes of 77 ± 140 mm^3 and 18 ± 32 mm^3 in the β-lapachone 50 mg/kg and 75 mg/kg groups, respectively (Means ± S.D.).

To determine survival, animals that died during the first week of treatment were omitted from the analysis. This included three animals in the group that received β-lapachone 50 mg/kg IT and five animals in the group that received β-lapachone 75 mg/kg IT. Kaplan-Meier plots demonstrated that animals receiving the β-lapachone dissolved in DMSO had no significant changes in survival. However, animals with pancreatic tumor xenografts that had intratumoral injections of β-lapachone complexed with HPβ-CD treatment, had increased survival compared to controls in both the 50 mg/kg IT dose ($P = 0.01$) and the 75 mg/kg IT dose ($p = 0.006$) (Fig. 6B). For example, on day 50 of treatment, only 25% of animals that received HPβ-CD were still alive, compared to 75% of the animals that received β-lapachone 50 mg/kg IT and 100% of the animals that received β-lapachone 75 mg/kg IT.

DISCUSSION

NAD(P)H:quinone oxidoreductase (NQO1) is an enzyme that detoxifies quinones, protects against reactive oxygen species, and is upregulated in both human pancreatic cancers and human pancreatic cancer cell lines.[10,11] Our study demonstrates that β-lapachone has antitumor activity both in vitro and in vivo against human pancreatic cancer. In the presence of dicumarol, an inhibitor of NQO1, β-lapachone demonstrated little cytotoxicity. When cells were treated with menadione alone or without dicumarol the opposite was found: MIA PaCa-2 cells were sensitized to the coadministration of dicumarol with menadione. In human pancreatic tumor xenografts, β-lapachone demonstrated greater tumor growth inhibition when given intratumoral vs systemically; and greater tumor growth inhibition when complexed with hydroxypropyl- β-cyclodextrin (HPβ-CD) vs. dissolved in DMSO.

Our study correlates with other studies investigating the antitumor effect of β-lapachone. Pink et al demonstrated that NQO1 enhanced the toxicity of β-lapachone in breast cancer cells that overexpress NQO1,[7] while dicumarol also protected NQO1-expressing cells from the toxic effects of β-lapachone. Biochemical studies suggested that reduction of β-lapachone by NQO1 leads to a futile cycling between the quinone and hydroquinone forms, with a loss of reduced NAD(P)H. In addition, the activation of a cysteine protease, which has characteristics consistent with the calcium-dependent protease calpain, is observed after β-lapachone treatment.

Others have also found that β-lapachone has significant antitumor activity. Li et al. demonstrated that the combination of taxol and β-lapachone provided a synergistic induction of apoptosis in vitro, while tumor growth in vivo was inhibited with β-lapachone combined with taxol.[23] For the in vivo study, β-lapachone was formulated in Lipiodol to increase its solubility and then given systemically via intraperitoneal injections.

Despite the efficacy of β-lapachone on in vitro cytotoxicity of pancreatic cancer cells that we have demonstrated in our present study, effective delivery of this drug has been a barrier in evaluating its clinical efficacy. Since β-lapachone is unstable in the blood and gut of animals[22] and the fact that the drug is soluble in water at a maximum concentration of 16 μM, makes β-lapachone difficult to use by conventional means of drug administration. Our study demonstrates that when dissolved in DMSO and then given as an intratumoral injection, β-lapachone inhibits tumor growth. However, when β-lapachone is complexed with HPβ-CD, tumor growth is dramatically decreased when given intratumorally. HPβ-CD is formed by covalent modification of the external hydroxyl groups on β-CD by hydroxylpropyl groups. The modification significantly increases the solubility limit of HPβ-CD by a factor of 22.[22] When given systemically via intraperitoneal injections, β-lapachone had a slight, but not significant effect in inhibiting tumor growth. However, the toxicity was increased when doses were given twice a day resulting in 2/3rds of the mice dying during or soon after the treatment. Clearly complexation of β-lapachone with HPβ-CD increases the bioavailability, efficacy, and toxicity of this active antipancreatic cancer agent. Future studies to improve solubility with decreased toxicity are warranted.

Adenocarincoma of the pancreas is resistant to almost all classes of chemotherapeutic drugs. Currently, the only active agent appears to be the DNA chain terminator gemcitabine (2',2'-diflurodeoxycytidine), which results in an response rate of less than 20%.[24] Even after curative resection, the 5-year survival rates achieved at specialized centers are less than 20% and the majority of patients die of metastatic cancer recurrence.[2] Other adjuvant treatments such as radiation therapy and chemotherapy have not improved long-term survival after resection. Thus, novel treatment strategies directed against this devastating malignancy are greatly needed.

References:

1. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics 2002. CA: Cancer J Clin 2002; 52:23-47.
2. Yeo CJ, Cameron JL. Pancreatic cancer. Cur Probl Surg 1999; 36:59-152.
3. Cruz FS, Docampo R, deSouza W. Effect of β-lapachone on hydrogen peroxide production in Trypanosoma cruzi. Acta Trop 1978; 35:35-40.
4. Docampo R, Lopes JN, Cruz FS, Souza W. Trypanosoma cruzi: Ultrastructual and metabolic alterations of epimastigotes by β-lapachone. Exp Parasitol 1977; 42:142-9.
5. Schmidt TJ, Miller-Diener A, Litwack G. β-lapachone, a specific competitive inhibitor of ligand binding to the glucocorticoid receptor. J Biol Chem 1984; 259:9536-4.

6. Planchon SM, Wuerzberger SM, Frydman B, Witiak DT, Hutson P, Church DR, Wilding G, Boothman DA. β-lapachone-mediated apoptosis inhuman promyelocytic leukemia (HL-60) and human prostate cancer cells: A p53-independent response. Cancer Res 1995; 55:3706-11.
7. Pink JJ, Planchon SM, Tagliarino C, Varnes ME, Siegel D, Boothman DA. NAD(P)H: Quinone oxidoreductase activity is the principal determinant of β-lapachone cytotoxicity. J Biol Chem 2000; 275:5416-24.
8. Trush MA, Twerdok LE, Rembish SJ, Zhu H, Li YB. Analysis of target cell susceptibility as a basis for the development of a chemoprotective strategy against benzene-induced hematotoxicities. Environ Health Perspect 1996; 104:1227-34.
9. Siegel D, Ross D. Immunodetection of NQO1 in human tissues. Free Radic Biol Med 2000; 29:246-53.
10. Logsdon CD, Simeone DM, Binkley C, Arumugan T, Greenson JK, Giordano TJ, Misek DE, Hanash S. Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. Cancer Res 2003; 63:2649-57.
11. Cullen JJ, Hinkhouse MM, Grady M, Gaut AW, Liu J, Zhang Y, et al. Dicumarol inhibition of NAD(P)H:Quinone oxidoreductase (NQO_1) induces growth inhibition of pancreatic cancer via a superoxide-mediated mechanism. Cancer Res 2003; 63:5513-20.
12. Larm JA, Vaillant F, Linnane AW, Lawen A. Upregulation of the plasma membrane oxidoreductase as a prerequisite for the viability of human Namalwa ρ° cells. J Biol Chem 1994; 269:30097-100.
13. Brar SS, Kennedy TP, Whorton AR, Sturrock AB, Huecksteadt TP, Ghio AJ, Hoidal JR. Reactive oxygen species from NAD(P)H: Quinone oxidoreductase constitutively activate NF-κB in malignant melanoma cells. Am J Physiol 2001; 280:C659-76.
14. Stella VJ, Rajewski. Cyclodextrins: Their future drug formulation and delivery. Pharm Res 1997; 14:556-67.
15. Loftsson T, Brewster ME. Pharmaceutical applications of cyclodextrins. 1. Drug solubilization and stabilization. J Pharm Sci 1996; 85:1017-25.
16. Rajewski RA, Stella VJ. Pharmaceutical applications of cyclodextrins. 2. In vivo drug deliver. J Pharm Sci 1996; 85:1142-69.
17. Irie T, Uekama K. Pharmaceutical applications of cyclodextrins. 3. Toxicological issues and safety evaluation. J Pharm Sci 1997; 86:147-62.
18. Wydert C, Roling B, Liu J, Ritchie JM, Oberley LW, Cullen JJ. Suppression of the malignant phenotype in human pancreatic cancer cells by the overexpression of manganese superoxide dismutase. Molecular Cancer Therapeutics 2003; 2:361-9.
19. Lewis A, Ough M, Li L, Hinkhouse MM, Ritchie JM, Spitz DR, Cullen JJ. Treatment of pancreatic cancer cells with dicumarol induces cytotoxicity and oxidative stress. Clinical Cancer Research 2004; 10:4550-8.
20. Littell RC, Milliken GA, Stroup WW, Wolfinger RD. SAS system for mixed models. Cary, NC: SAS Institute Inc., 1996.
21. Akaike H. Information theory and an extension of the maximum likelihood principle. In: Petrov BN, Csaki F, eds. Second International Symposium on Information Theory. Budapest: Akademiai Kiado, 1973:267-81.
22. Nasongkla N, Wiedmann AF, Bruening A, Beman M, Ray D, Bornmann WG, Boothman DA, Guo J. Enhancement of solubility and bioavailability of β-lapachone using cyclodextrin inclusion complexes. Pharmaceutical Research 2003; 20:1626-33.
23. Li CJ, Li YZ, Pinto AV, Pardee AB. Potent inhibition of tumor survival in vivo by β-lapachone plus taxol: Combining drugs imposes different artificial checkpoints. Proc Nat Acad Sci 1999; 96:13369-74.
24. Burris HA, Moore MJ, Andersen J, Greem MR, Rothenber MI, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarrassoff P, Nelson R, Dorr FA, Stephens CD, von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreatic cancer: A randomized trial. J Clin Oncol 1997; 15:2403-13.