Modulating Endogenous NQO1 Levels Identifies Key Regulatory Mechanisms of Action of β-Lapachone for Pancreatic Cancer Therapy

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Abstract

Purpose: Pancreatic cancer is the fourth leading cause of cancer-related deaths, in which the 5-year survival rate is less than 5%. Current standard of care therapies offer little selectivity and high toxicity. Novel, tumor-selective approaches are desperately needed. Although prior work suggested that β-lapachone (β-lap) could be used for the treatment of pancreatic cancers, the lack of knowledge of the compound’s mechanism of action prevented optimal use of this agent.

Experimental Design: We examined the role of NAD(P)H:quinone oxidoreductase-1 (NQO1) in β-lap-mediated antitumor activity, using a series of Mia PaCa-2 pancreatic cancer clones varying in NQO1 levels by stable shRNA knockdown. The antitumor efficacy of β-lap was determined using an optimal hydroxypropyl-β-cyclodextran (HPβ-CD) vehicle formulation in metastatic pancreatic cancer models.

Results: β-Lap-mediated cell death required ~90 enzymatic units of NQO1. Essential downstream mediators of lethality were as follows: (i) reactive oxygen species (ROS); (ii) single-strand DNA breaks induced by ROS; (iii) poly(ADP-ribose)polymerase-1 (PARP1) hyperactivation; (iv) dramatic NAD+/ATP depletion; and (v) programmed necrosis. We showed that 1 regimen of β-lap therapy (5 treatments every other day) efficaciously regressed and reduced human pancreatic tumor burden and dramatically extended the survival of athymic mice, using metastatic pancreatic cancer models.

Conclusions: Because NQO1 enzyme activities are easily measured and commonly overexpressed (i.e., >70%) in pancreatic cancers 5- to 10-fold above normal tissue, strategies using β-lap to efficaciously treat pancreatic cancers are indicated. On the basis of optimal drug formulation and efficacious antitumor efficacy, such a therapy should be extremely safe and not accompanied with normal tissue toxicity or hemolytic anemia. Clin Cancer Res; 17(2): 275–85. ©2011 AACR.

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States. (1). Current standard therapies for these patients include surgery, often in combination with radiotherapy and/or chemotherapy, and offer a 5-year survival rate of less than 5%, due to the aggressive and invasive nature of this disease and considerable normal tissue toxicity in patients. Most pancreatic cancer patients are not candidates for surgical intervention. They present with locally advanced or metastatic disease and have a median survival of 6–10 or 3–6 months, respectively (2, 3). Thus, new approaches based on tumor-selective targets are desperately needed to efficaciously treat pancreatic cancer. As a result, considerable resources have been invested in the development of novel therapies that target molecular aberrations in pancreatic cancers (3–5), with the hope of exploiting specific markers that are elevated in pancreatic cancer.

One "marker" commonly elevated in various human cancers (6, 7), and particularly in pancreatic tumors (8–11), is NAD(P)H:quinone oxidoreductase-1 (NQO1; E.C. 1.6.99.2). NQO1 was overexpressed greater than 10-fold versus associated normal tissue in more than 70% of patients (8–11). It detoxifies most quinones by catalyzing a 2-electron reduction using NADH or NADPH (12),...
Translational Relevance

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States. Current standard therapies for these patients include surgery, often in combination with adjuvant radiotherapy and/or chemotherapy, and offer a 5-year survival rate of less than 5%. β-Lapachone (β-lap; clinically formulated as ARQ 501) has completed phase I trials in pancreatic cancer patients. Although tumor responses were noted, the studies were limited by hemolysis caused by incorrect regimen application and hydroxylpropyl-β-cyclodextran (HPβ-CD) vehicle formulation. Therefore, our in vitro as well as in vivo data clearly show that β-lap, in an optimal formulation, is ideally suited for treating pancreatic cancer.

converting them to hydroquinones, bypassing the normally unstable and highly reactive semiquinone intermediates. Hydroquinones are then typically and readily conjugated with glutathione (GSH) via glutathione-S-transferase and excreted, constituting a protective mechanism against quinone-mediated toxicity (13). Dicoumarol [3-3'-methylen-bis(4-hydroxy-coumarin); DIC] is a fairly specific NQO1 inhibitor that competes with NADH/NADPH substrate binding.

NQO1 can, however, catalyze certain quinones to more reactive DNA-damaging agents. These "bio-activation" reactions result in cytotoxic alkylating and/or intercalating quinones and include mitomycin C (MMC), streptonigrin (STN), and 3-hydroxy-5-aziridinyl-1-methyl-2-[1H-indole-4,7-dione]prop-β-en-α-ol (EO9; ref. 14). Exposure to these 3 agents results in DNA lesions in direct linear proportion to NQO1 levels in tumor and normal tissue (6, 15). Unfortunately, the clinical efficacy of these agents was greatly limited due to resistance caused by tumor-related DNA repair processes (16) and normal tissue toxicity (17).

β-Lapachone (β-lap, 3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5-6-dione) also requires NQO1 activity for effective killing of NQO1-overexpressing cancer cells, while causing minimal effects to neighboring "normal" cells that lack, or have low levels of, the enzyme (18–21). However, the mechanism of action of β-lap in vitro was significantly different from that of MMC, STN, or EO9. β-Lap undergoes an NQO1-dependent "futile cycle," wherein ~60 moles of NAD(P)H are used per mole drug in 5 minutes (18, 22). As a result, dramatic elevation of reactive oxygen species (ROS) and released Ca²⁺ from endoplasmic reticulum (ER) stores were noted and are required for cell death (19–21). Unlike EO9, STN, or MMC, β-lap does not seem to cause alkyllyation of DNA. β-lap (i.e., clinically formulated as ARQ 501) has completed phase I trials in pancreatic cancer patients (23). Although tumor responses were noted, the studies were limited by hemolysis caused by incorrect regimen application and hydroxylpropyl-β-cyclodextran (HPβ-CD) vehicle formulation.

We previously showed antitumor activities and mechanism of action of β-lap--mediated cell death in vitro in endogenous NQO1-expressing pancreatic (24, 25), and forced NQO1-overexpressing prostate, breast, and non–small cell lung cancer (NSCLC) cells (18, 19, 21, 26). A relationship between NQO1 levels and lethality in vitro using endogenous knockdown has not been established for this drug. Here, pancreatic cancer clones, whose endogenous NQO1 levels were stably knocked down in a gradient and ordered manner, were used to explore NQO1 levels required for β-lap--induced lethality. We show that a threshold level of NQO1 enzyme activity is required for β-lap--induced cell death responses and define key reactions essential for β-lap--induced cytotoxicity. We show that β-lap is an extremely efficacious agent against pancreatic cancers with endogenously elevated NQO1 levels, with extensive reductions in tumor burden, dramatic tumor growth delays, and extended survival in a metastatic pancreatic cancer animal model. Minimal to no toxicity to normal tissue or hemolytic anemia was noted using an optimal regimen.

Our current studies show that pancreatic tumors expressing NQO1 levels of at least 90 U would be responsive to β-lap therapy with minimal to no side-effects. Development of novel delivery vehicles, for example, using nanoparticles, to increase tumor-selective delivery should further augment the antitumor efficacy of β-lap.

Materials and Methods

Reagents and chemicals

β-Lap was synthesized and purified by us (21). DIC, hydrogen peroxide (H₂O₂), staurosporine, Hoechst 33258, bovine serum albumin, cytochrome c, and propidium iodide were from Sigma-Aldrich. 5-(and 6-)Chloro-methyl-2,7-dichlorodihydrofluorescein diacetate (DCFDA) was purchased from Invitrogen Life Technologies. HPβ-CD (>98% purity) was obtained from Cyclodextrin Technologies Development, Inc. β-Lap–HPβ-CD was prepared as previously described (27).

Cell culture and transfections

MIA PaCa-2 cells were obtained from Dr. Joseph J. Cullen (University of Iowa). Human pancreatic cancer cell lines ASPC1, BXPC3, CFPAC-1, HS766T, Capan 1, and Capan 2 were obtained from the ATCC. All cells were grown in Dulbecco’s modified Eagle medium (Invitrogen) as described (25) and were mycoplasma free. The human shRNA-NQO1 (RHS1764-9691437: 5′-TGCCTGTTCAGCAGGTCGCGGCAGGATCAGACACACT GTATTTAGTAAACCCACACACCACT TGCCCTCGGA-3′) retroviral vector was purchased from Open Biosystems. Stable shRNA knockdown clones (KD17-1, KD17-3, and KD17-7) were generated by infecting MIA PaCa-2 cells with polybren-supplemented medium obtained from Phoenix packaging cells transfected with the human retrovirus vector targeting NQO1 as described earlier (28). Cells containing stable
scrambled controls (shRNA-SCR) were also generated and were referred to as nonsilencing (NS) cells. Individual clones were isolated by limiting dilution in media containing puromycin (1 μg/mL) and screened for NQO1 expression levels individually as indicated. All experiments were carried out without antibiotics. For bioluminescence (BLI) analyses, MIA PaCa-2 cells were infected with a lentiviral construct that expresses the luciferase (Luc) gene under the control of a strong promoter, the cytomegalovirus (CMV-Luc) promoter, as described (29).

**NQO1 enzyme assays**

NQO1 enzyme levels (Fig. 1F Table; Supplementary Table 1) were determined from triplicate S9 whole-cell extracts, using NADH (200 μmol/L) as an immediate electron donor and menadione (10 μmol/L) as an intermediate electron acceptor as described (18, 30). Enzyme activity was measured as the formation of semiquinone (NQO1) activity (U) using NADH (200 μmol/L, 2 hours)-exposed, NS- or NQO1-knockdown MIA PaCa-2 cells titrated with increasing doses of DIC. Relative survival of β-Lap (6 μmol/L, 2 h)-exposed, NS- or NQO1-knockdown MIA PaCa-2 cells titrated with increasing doses of DIC. Relative survival of MIA PaCa-2 knockdown clones and pancreatic cancer cell lines with varying NQO1 activity (U) were determined from triplicate S9 whole-cell extracts, using NADH (200 μmol/L, 2 hours)-exposed, NS- or NQO1-knockdown MIA PaCa-2 cells titrated with increasing doses of DIC. Relative survival of MIA PaCa-2 knockdown clones and pancreatic cancer cell lines with varying NQO1 activity (U) were determined from triplicate S9 whole-cell extracts, using NADH (200 μmol/L, 2 hours)-exposed, NS- or NQO1-knockdown MIA PaCa-2 cells titrated with increasing doses of DIC.
units (U) of NQO1 were calculated as nmol of cytochrome c reduced/min/μg of protein, based on initial rate of change in absorbance at 550 nm.

Relative survival assays
Relative cell survival levels from pancreatic cancer cells seeded at 5 × 10⁴ per well of 48-well plates were determined using 7-day DNA assays as previously described (18). Results were reported as means ± standard error (SE) from at least 3 independent experiments done in sextuplicate.

ROS analyses
Disulfide glutathione (GSSG) and total GSH levels using spectrophotometric recycling assays and % GSSG normalized to protein content measured by Lowry assays (31) were assessed as previously described (32). ROS formation was further quantified by assessing digital images of the conversion of nonfluorescent 5 μmol/L of DCFDA to its fluorescent derivative at indicated times with a fluorescent microscope (Leica Microsystems) as described (33). All data were graphed as means ± SE for experiments performed 3 times, each in triplicate.

Alkaline comet assays
DNA single- and double-strand breaks (SSB and DSB, respectively) and base damage were assessed using alkaline comet assays (TREVIGEN). Digital photomicrographs of comet tail lengths were quantified from experiments done 3 or more times, each in duplicate (21).

Nucleotide analyses
Changes in intracellular NAD⁺ levels were measured and expressed as percentage treated divided by control (%T/C, ± SE) from at least 3 individual experiments, each in duplicate (21). ATP levels were analyzed from whole-cell extracts by using CellTier-Glo luminescent cell viability assays (Promega). Data were graphed as means ± SE of experiments done 3 or more times in triplicate for ATP, or 3 or more independent experiments for NAD⁺.

Flow cytometry
Cell-cycle distribution and apoptotic populations using TUNEL staining were analyzed as previously described (28, 34) using a FACS Calibur flow cytometer (BD Biosciences) and CellQuest software. Results from experiments repeated at least 3 times, each in duplicate, are presented. Apoptosis was observed from all phases of the cell cycle as described (35).

Immunoblot analyses
Whole-cell extracts and Western immunoblots were prepared and developed as described (28). For Western blots, primary antibodies and dilutions were as follows: poly (ADP-ribose) or PAR (BD Pharmingen), 1:1,000; human MutS homolog-2 (hMSH2, Ab-1; Oncogene), 1:500; p53 (DO-1; Santa Cruz Biotechnology), 1:5,000; and β-actin (Santa Cruz Biotechnology), 1:20,000. An α-human NQO1 antibody (1:5,000 dilution) was kindly provided by Dr. David Ross (University of Colorado Health Science Center). Results shown are representative of experiments performed at least 3 times.

Antitumor efficacy
A metastatic (spleen to liver tumor burden) pancreatic tumor model was used to evaluate β-lap efficacy. Pancreas tumors were generated by inoculating 2.5 × 10⁵ Luc-tagged MIA PaCa-2 cells into the spleens of female mice weighing 20 to 25 g as described (36). Mice were randomly distributed so that average group (5 mice/group) body weights were not statistically different. Four weeks after implantation, and after random imaging, mice were treated with tail-vein injections of β-lap–HPβ-CD or HPβ-CD at various doses every other day for 5 treatments (i.e., 1 regimen). Mouse body weights were measured thrice weekly. Mice were sacrificed when metastatic tumor burden reached 20% of initial body weight. Survival and body weight data were graphed from 3 separate studies. Separately, ex vivo Luc-tumor volumes (luciferase levels) of excised spleens were evaluated using a bioluminescent imager (Xenogen Vivolision IVIS Lumina) for tumor burden and reported as means ± SE from 3 separate experiments. Spleen, liver, and pancreatic tissues were removed for histologic examination; tissues were snap-frozen in liquid nitrogen for Western blot analyses to confirm NQO1 expression. In another series of studies, the spleens of athymic mice were inoculated with 2.5 × 10⁵ Luc-tagged Pan-GFP murine pancreatic tumor cells expressing human NQO1 (hNQ2) by lentiviral infection and similar spleen to liver tumor burden metastatic analyses performed (Supplementary Figs. 2 and 3).

Statistical analyses
Log-rank tests were applied to survival analyses (Kaplan–Meier curves). All in vitro statistical analyses were conducted using GraphPad Prism software. Statistical analyses of survival data in vitro were conducted using linear regression analyses. Analyses of covariance (ANCOVA) and nonlinear regression models and overall P values were simultaneously calculated, comparing intercepts and slopes for different treatments. In nonlinear regression models, β-lap LD₅₀ doses (denoted as Y) were fitted with NQO1 activities (denoted as X) by Y = a exp(−bX) + c, where a, b, and c were fitting constants. P values were 2-sided, and statistical calculations were carried out by SAS (Service Pack 4) for Windows and SigmaPlot.

Results
NQO1 was a key determinant of β-lap cytotoxicity in human pancreatic cancer cells
Prior studies from our laboratory used forced NQO1 overexpression via CMV-directed cDNA transfection into NQO1*2 polymorphic breast, prostate, or NSCLC cells to elucidate the mechanism of action of β-lap. Because forced NQO1 overexpression could result in the

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localization of the enzyme into compartments that are not physiologic, we knocked down NQO1 levels in pancreatic cancer cells that have naturally elevated levels of this 2-electron oxidoreductase. This allowed us to determine the threshold of NQO1 level (in enzymatic units) at which β-lap treatments were no longer effective, especially given the mechanism of action of this drug (Fig. 1A). MIA PaCa-2 cells that have ~400 NQO1 enzymatic units (U) were infected with retroviral shRNA specific to the 5′-untranslated region (UTR) of NQO1 mRNA or with an identical shRNA lentiviral vector driving nonspecific (NS) siRNA expression as defined in "Materials and Methods." Cell lines containing between 400 (clone NS) to 50 (clone 17-7) NQO1 units were isolated (Fig. 1B). Exposure of parental (410 U), NS (400 U), and clone 17-3 (~90 U) cells with varying doses of β-lap resulted in statistically similar lethalties (indicating LD₅₀ values of ~4.5 µmol/L; Fig. 1C) that were blocked with DIC (Fig. 1C). Interestingly, clones 17-1 and 17-7, with 70 and 50 U, respectively, resulted in significant resistance to β-lap under the same treatment conditions (Fig. 1C), with LD₅₀ values of ~10 to 12 µmol/L, respectively. To dissect the responses of the clones further, cells were treated with a fixed dose of β-lap (6 µmol/L, 2 hours), while varying DIC concentrations. Unlike the NS clone, in which ~5 µmol/L DIC was required for complete protection from β-lap, all 3 knockout clones required significantly less DIC (~1–2 µmol/L) for complete protection against β-lap (Fig. 1D). When LD₅₀ values of NS and knockdown clones (closed squares), and a series of available pancreatic cancer cells [Fig. 1E (open circles) and Fig. 1F Table], were graphed against expressed NQO1 levels (U), a clear biphasic curve was noted with a lethality inflection response to β-lap noted between 90 and 100 enzymatic units of NQO1 (Fig. 1E). These data strongly suggested that NQO1 levels were critical to β-lap lethality but that a mere 90 to 100 enzymatic units were required for lethality (Fig. 1E and F Table) due to NQO1 recycling of β-lap. In cells with higher NQO1 enzymatic activity, NAD (P)H (electron donor) most likely became rate-limiting, in which case higher NQO1 levels did not confer enhanced lethality and lowered LD₅₀ values for β-lap treatments in pancreatic cancer cells (Fig. 1E).

β-Lap lethality required released calcium (Ca²⁺) from ER stores

We previously showed in human breast, NSCLC, and prostate cancer cells that NQO1-dependent lethality caused by β-lap depended on Ca²⁺ release from ER stores (19–21, 37). Pretreatment of such NQO1 endogenously overexpressed cancer cells with BAPTA-AM, a specific intracellular Ca²⁺ chelator, spared β-lap lethality without affecting DNA damage caused by this agent (19–21, 37). In a similar manner, 60-minute pretreatment of various pancreatic cancer cells with nontoxic doses of 5 µmol/L of BAPTA-AM prevented cell death, showing the universal requirement of released intracellular ER Ca²⁺ in β-lap lethality (Fig 1F Table).

β-Lap–induced ROS formation, DNA damage, poly (ADP-ribose)polymerase-1 hyperactivation, and nucleotide depletions were NQO1-dependent

NQO1-knockdown MIA PaCa-2 clones were then used to delineate the mechanism by which β-lap induced cell death. The futile cycling of β-lap by NQO1, specifically its back-reaction, in which the hydroquinone is converted to a semiquinone and then back to β-lap (Fig. 1A), caused dramatic formation of ROS. Significantly reduced ROS formation in β-lap–treated NQO1-knockdown MIA PaCa-2 clones were noted by lowered % GSSG (Fig. 2A) and DCFDA fluorescence (Fig. 2B) in direct proportion to NQO1 enzymatic activities. Concomitant decreased DNA lesion formation monitored by alkaline comet assays (Fig. 2C and D) was also noted in the knockdown clones versus NS cells; interestingly, no DNA damage was apparent when cells were analyzed by neutral comet assays, as previously reported in endogenously NQO1-overexpressed breast, prostate, and NSCLC cells (19, 21). Thus, DNA lesions other than DSBs are formed in response to β-lap exposures. DIC coaddition completely blocked NQO1-mediated, β-lap–induced SSBs and base damage.

We previously reported that β-lap induced programmed necrosis involving poly(ADP-ribose)polymerase-1 (PARP1) hyperactivation as a result of released ER Ca²⁺ and extensive, threshold-reaching DNA damage events, particularly SSBs and base damages (19, 21, 38). Exposure of MIA PaCa-2 cells that endogenously overexpressed NQO1 with β-lap resulted in extensive PARP1 hyperactivation as measured by PAR formation within 10 to 90 minutes, which was completely blocked by DIC (Fig. 3A). As a positive control, exposure of cells to supra-lethal H₂O₂ doses caused extensive SSBs and base damages and hyperactivation of PARP1. Decreased PARP1 hyperactivation was noted in direct correlation to loss of NQO1 enzymatic activity in β-lap–exposed MIA PaCa-2 knockdown clones (compare NS clone with ordered NQO1-knockdown MIA PaCa-2 clones; Fig. 3B and C).

β-lap is one of only 3 agents (others are supra-lethal doses of MNNG or H₂O₂) known to cause hyperactivation of PARP1 and dramatic losses of total intracellular NAD⁺ levels, ultimately resulting in corresponding dramatic losses of ATP in exposed cells (19, 21, 39). However, β-lap is the only known agent that induces PARP1 hyperactivation with concomitant NAD⁺/ATP losses at doses achievable under preclinical and clinical conditions (19, 21). β-Lap is the only known tumor-selective agent acting in an NQO1-dependent manner, whereby DIC prevented these responses (Fig. 4A and C). Consistent with decreased PARP1 hyperactivation in NQO1-knockdown MIA PaCa-2 clones (Fig. 3B and C), ordered loss of NAD⁺ (Fig. 4B) and ATP (Fig. 4D) was inversely noted with NQO1 activities. The more extensive NQO1 levels were knocked down in MIA PaCa-2 clones, the weaker the losses of NAD⁺ and ATP, consistent with spared lethality of these clones after β-lap treatment (Fig. 1E, closed squares represent genetically matched MIA PaCa-2 clones). We then examined a series of pancreatic cancer cell lines from various sources whose NQO1 levels ranged from ~390 to 1,200 U, yet their
LD<sub>50</sub> values were all fairly similar, ranging from 1.8 to 4.2 μmol/L (Fig. 1E, open circles).

**β-Lap induced programmed necrosis in pancreatic cancer cells with endogenous NQO1 overexpression**

Cell death induced by β-lap occurred via a unique programmed necrotic mechanism, in which cells lost energy (NAD<sup>+</sup>/ATP) but activated a default cysteine proteolytic apoptotic response. This cell death response is mediated by β-calpain and exposed cells undergo dramatic nuclear condensation and apoptotic responses (20, 37). Importantly, cells undergoing this response showed diagnostic β-calpain–dependent p53 proteolytic cleavage events (20, 37). MIA PaCa-2 cells responded in an identical manner to β-lap as breast, prostate, and NSCLC cancers that exhibit endogenous NQO1 overexpression (19, 21, 26), inducing dramatic apoptotic (TUNEL<sup>+</sup> cells, Fig. 5A and B) responses and atypical proteolysis of p53 (see ~43kDa p53 cleavage fragment, Fig. 5C) at doses corresponding to β-lap cytotoxicity (Fig. 1). Significant decreases in apoptotic responses (TUNEL<sup>+</sup> cells, Fig. 5B) and downstream atypical p53 cleavage stimulated by activated β-calpain (Fig. 5C; refs. 20, 37) were noted in NQO1-knockdown clones that correlated well with their resistance to β-lap cytotoxicity (Fig. 1).

**β-Lap was an efficacious agent for the treatment of pancreatic cancer**

The overexpression of NQO1 in pancreatic cancers compared with associated normal tissues (8–11), and the ability of β-lap to kill cells expressing this enzyme, strongly suggested its use for the treatment of pancreatic cancer. Although β-lap can kill pancreatic cancers in vitro, the lack of an adequate delivery vehicle to make the drug bioavailable for therapy in vivo limited use of this agent until recently. A version of HPβ-CD complexed with β-lap (β-lap-HPβ-CD) that increased solubility and bioavailability of β-lap 400-fold (27) was used in clinical trials as ARQ 501 (23). In our study, β-lap–HPβ-CD was used in a spleen to liver metastatic pancreatic cancer model to test the...
PaCa-2-containing mice treated with 20 or 30 mg/kg β-lap–HPβ-CD correlated well with overall survival (P < 0.0001) versus mice treated with HPβ-CD alone (Fig. 6B). In fact, 100% (10/10) of mice treated with β-lap–HPβ-CD were alive at 11 weeks posttreatment and were "apparently cured" compared with 100% tumor-driven lethality of mice bearing metastatic MIA PaCa-2 tumors exposed to HPβ-CD. These findings were corroborated in parallel studies evaluating tumor volumes using BLI, in which significant tumor regression without regrowth was noted after drug treatment in metastatic pancreatic human/murine tumor model (Fig. 6C and D; Supplementary Figs. 2 and 3). Morbidity, monitored by weight loss, was not noted at these optimal doses of β-lap–HPβ-CD.

Discussion

Numerous studies reported elevated levels of NQO1 in various human cancers, linking overexpression of this enzyme for prognostic and predictive values (12, 40). We previously reported the NQO1-mediated antitumor activity in vitro of β-lap, using endogenous and exogenous NQO1-overexpressing pancreatic, breast, prostate, and NSCLC models (19, 21, 25, 26). Prior to this work, genetic demonstration of the role of NQO1 in β-lap–induced lethality was confined to forced overexpression of NQO1 in rare polymorphic cancer cells. These studies had the limitation of using potentially nonphysiologic NQO1 levels (due to forced overexpression), in which the enzyme may be improperly localized and/or overexpressed in specific compartments at nonphysiologic levels. We show for the first time that the antitumor activity of β-lap can be progressively abrogated in MIA PaCa-2 clones ordered for NQO1 knockdown. More importantly, these knockdown clones were used to delineate specific nodal points of metabolism critical for β-lap–induced lethality. These β-lap–induced events included: (i) ROS formation and intracellular Ca2+ release from ER stores, where preloading cells with BAPTA-AM significantly spared lethality (Fig. 1F Table); (ii) SSBs and base damage induced by ROS by alkaline comet assays (Fig. 2); (iii) PARP1 hyperactivation and PAR formation (Fig. 3); (iv) NAD+/ATP depletion (Fig. 4); and (v) programmed necrosis (Fig. 5). Further analyses showed that an optimal threshold level of NQO1 (~90 U, Fig. 1E) was necessary to trigger β-lap–induced cell death.

Our detailed understanding of metabolic changes occurring in NQO1-overexpressing compared with knockdown cells during β-lap–induced cytotoxicity will further improve the development of an efficacious therapeutic regimen. Sound, exploitable, tumor-selective approaches based on druggable, tumor-specific protein/enzyme expression, such as NQO1, are desperately needed for clinical trials against pancreatic cancer. Our studies highlight that NQO1 is more than a "biomarker," but an exploitable, tumor-selective target, whose expression is not cell-cycle regulated, nor affected by alterations in common tumor suppressors, such as p53 or Rb. Importantly, we show that
p53 cleavage fragments). hMSH2 levels served for loading. PaCa-2 cells were treated with $\beta$-lap (6 or 8 $\mu$mol/L) with or without DIC (50 $\mu$mol/L) for 2 hours. Cells were collected at 96 hours, and apoptosis was monitored by apoptotic reactions. Apoptotic (% TUNEL+ cells) data are means $\pm$ SE from 3 independent experiments. Student’s t tests were done (*$P < 0.01$, B, NS- and shRNA-NQO1−

2.0 (40). Gemcitabine (10 nmol/L, 24 hours) was reported to enhance human colon cancer cell lethality in a unique cell death termed “programmed necrosis” or “necroptosis.” $\beta$-Lap–HPβ-CD (ARQ 501) is the only clinically used drug that exploits this pathway of cell death. Our current studies show that cells from patients with pancreatic tumors expressing NQO1 levels of at least 90 U would be indicated for $\beta$-lap therapy. Development of novel delivery vehicles, for example, using nanoparticles (29) to increase tumor-selective delivery, should further augment the antitumor efficacy of $\beta$-lap. In addition to gemcitabine monotherapy, radiotherapy as a single agent, or in combination with gemcitabine, is often used to treat advanced stage pancreatic cancer patients (44). Gemcitabine has been reported to be a potent radiosensitizer with dose enhancement ratios of 1.6 to 2.0 (40). Gemcitabine (10 nmol/L, 24 hours) was reported to enhance human colon cancer cell lethality when administered immediately following ionizing radiation (IR) treatments (45). The mechanism of this combination therapy involves depletion of dATP, as well as dTTP pools. Our laboratory also reported that the mechanism of $\beta$-lap toxicity involves nucleotide depletion. However, in contrast to gemcitabine, $\beta$-lap depletion of nucleotide pools (NAD$^+$/ATP) is tumor specific, because it occurs in NQO1-expressing tumor tissue and not in cells with low enzymatic levels (e.g., normal tissue). Thus, an added

Figure 5. NQO1 expression knockdown protects cells from $\beta$-lap-induced apoptosis. A, MIA PaCa-2 cells were treated with $\beta$-lap (6 or 8 $\mu$mol/L) with or without DIC (50 $\mu$mol/L) for 2 hours. Cells were collected at 96 hours, and apoptosis was monitored by apoptotic reactions. Apoptotic (% TUNEL+ cells) data are means $\pm$ SE from 3 independent experiments. Student’s t tests were done (*$P < 0.01$, B, NS- and shRNA-NQO1−

NQO1 is exploitable using $\beta$-lap, whose unique futile cycle metabolism and broad metabolic changes induced downstream make the drug extremely efficacious for treatment of pancreatic cancer.

The current “standard of care” for treating advanced pancreatic cancer is gemcitabine (Gemzar), dideoxyfluorocytidine (ddFC, ref. 41). However, gemcitabine has proven palliative at best, offering only modest extension of long-term survival for patients with advanced disease (2). A major problem is that ddFC, a prodrug that requires phosphorylation by deoxycytidine kinase for activation, does not target a tumor-specific enzyme that distinguishes pancreatic cancers from normal pancreatic tissue (42). ddFC phosphorylation results in the accumulation of ddFC-triphosphate that competes with dCTP for DNA incorporation, leading to chain termination by DNA polymerase-alpha and replication stalling. These events cause unrecognized DNA lesions, preventing immediate DNA break detection by repair enzymes (42). Thus, the antitumor activity of gemcitabine is S-phase–dependent, whose efficacy is limited by the growth state of tumors (43). Importantly, ddFC antitumor activity has not been shown to be tumor selective, occurring in both normal and tumor tissues alike. Thus, nonselective cytotoxicity is a major disadvantage of this therapy. In contrast to gemcitabine, $\beta$-lap kills in a tumor-selective manner, eliciting essential oxidative stress and intracellular Ca$^{2+}$ release from ER stores generated in an NQO1-dependent manner (19, 21). These intracellular events cause irreversible DNA damage, as a consequence of the unique hyperactivation of PARP1, which leads to a cascade of events culminating in a unique cell death termed “programmed necrosis” or “necroptosis.” $\beta$-Lap–HPβ-CD (ARQ 501) is the only clinically used drug that exploits this pathway of cell death. Our current studies show that cells from patients with pancreatic tumors expressing NQO1 levels of at least 90 U would be indicated for $\beta$-lap therapy. Development of novel delivery vehicles, for example, using nanoparticles (29) to increase tumor-selective delivery, should further augment the antitumor efficacy of $\beta$-lap.

In addition to gemcitabine monotherapy, radiotherapy as a single agent, or in combination with gemcitabine, is often used to treat advanced stage pancreatic cancer patients (44). Gemcitabine has been reported to be a potent radiosensitizer with dose enhancement ratios of 1.6 to 2.0 (40). Gemcitabine (10 nmol/L, 24 hours) was reported to enhance human colon cancer cell lethality when administered immediately following ionizing radiation (IR) treatments (45). The mechanism of this combination therapy involves depletion of dATP, as well as dTTP pools. Our laboratory also reported that the mechanism of $\beta$-lap toxicity involves nucleotide depletion. However, in contrast to gemcitabine, $\beta$-lap depletion of nucleotide pools (NAD$^+$/ATP) is tumor specific, because it occurs in NQO1-expressing tumor tissue and not in cells with low enzymatic levels (e.g., normal tissue). Thus, an added
therapeutic advantage with β-lap therapy when treating pancreatic cancer patients is anticipated to be the ability to avoid systemic toxicity due to nonselective normal tissue cell death. β-Lap synergizes with IR (46, 47), and we are currently exploring its ability to enhance the radiation lethality responses of pancreatic cancers. Our group found currently exploring its ability to enhance the radiation lethality responses of pancreatic cancers. Our group found in vivo, as reported on April 20, 2017. © 2011 American Association for Cancer Research.

In summary, we theorize that β-lap, in an optimal formulation (and/or possibly enhanced when combined with DNA damaging agents), is ideally suited for treating pancreatic cancer patients. Our studies suggest that the antitumor efficacy of β-lap could be further enhanced with knowledge of key principal determinants of its tumor selectivity identified in our NQO1-knockdown clones. Our data specifically define the mechanism of PARP1 hyperactivation, strongly suggesting that agents that cause ROS or SSBs, such as IR, should be potentiated by β-lap because of a lowering of threshold of DNA lesions required for PARP1 hyperactivation. β-Lap therapy alone or in combination with other DNA damaging agents, therefore, represents an exploitable and clinically available therapy for pancreatic cancer, as well as other

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**Figure 6.** β-Lap has significant antitumor efficacy in MIA PaCa-2 tumor xenograft models. A, body weight changes of MIA PaCa-2 tumor-bearing mice. Mice bearing spleen-implanted, NQO1-overexpressing MIA PaCa-2 cells were intravenously (iv) treated by tail-vein injections with vehicle alone (HPβ-CD) or β-lap–HPβ-CD at 20 or 30 mg/kg every other day for 5 treatments (Methods and Materials). Results (means ± SE) are representative of 3 similar experiments. Student’s t tests (**P < 0.001) were conducted comparing treated versus control groups. B, Kaplan–Meier survival for pancreatic antitumor efficacy experiments described in [A]; log-rank analyses were conducted comparing survival curves (**P < 0.0001) for HPβ-CD versus β-lap–HPβ-CD at 20 or 30 mg/kg iv. Results are means ± SE (n = 5). Student’s t tests (* P < 0.05) were conducted comparing HPβ-CD versus [β-lap–HPβ-CD at 20 or 30 mg/kg iv. V, vehicle (HPβ-CD).
specific cancers (e.g., breast, prostate, and NSCLC) in which NQO1 levels are endogenously elevated.

Disclosure of Potential Conflicts of Interest:

No potential conflicts of interest were disclosed.

Acknowledgment

We are grateful to Dr. James K.V. Willson for his helpful discussions.

References

16. Grant Support

This research was supported by NIH/NCI grant, CA102792-01, and from the Robert A. and Virginia Payne endowment to D.A.B.

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Received July 23, 2010; revised October 11, 2010; accepted October 24, 2010; published OnlineFirst January 11, 2011.


Correction: Modulating Endogenous NQO1 Levels Identifies Key Regulatory Mechanisms of Action of β-Lapachone for Pancreatic Cancer Therapy

In this article (Clin Cancer Res 2011;17:275–85), which was published in the January 15, 2011 issue of Clinical Cancer Research (1), there are errors in Figures 1B, 1E, and 3A. In Figure 1B, the NQO1 enzymatic levels do not align with the Western blot lanes for NQO1 levels. In Figure 1E, all circles should be open symbols. In Figure 3A, the line under “β-Lap (6 μmol/L) + DIC (50 μmol/L)” should be the same as the line under “β-Lap (6 μmol/L).” Corrected versions of these figures appear below.

Figure 1
Reference


published OnlineFirst February 22, 2011.
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doi: 10.1158/1078-0432.CCR-11-0159
Modulating Endogenous NQO1 Levels Identifies Key Regulatory Mechanisms of Action of β-Lapachone for Pancreatic Cancer Therapy

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