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The orally active pterocarpanquinone LQB-118 exhibits cytotoxicity in prostate cancer cell and tumor models through cellular redox stress

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Abstract

Background—The targeted induction of reactive oxygen species (ROS) is a developing mechanism for cancer therapy. LQB-118 is a pterocarpanquinone and ROS-inducing agent with proven antineoplastic activity. Here, LQB-118 efficacy and mechanism of activity, were examined in Prostate Cancer (PCa) cell and tumor models.

Methods—PC3, LNCaP, and LAPC4 PCa cells were applied. Dicoumarol treatment was used to inhibit quinone reductase activity. N-acetylcysteine (NAC) was applied as a ROS scavenger. ROS production was quantified by H₂DCFDA flow cytometry. LQB-118 treated cells were evaluated for changes in lipid peroxidation, viability, and apoptosis. Treatment-induced gene expression was measured by RT-qPCR and Western Blot. SOD1 knockdown was achieved with siRNA or miRNA mimic transfection. MicroRNA specificity was determined by 3' UTR reporter assay. Oral LQB-118 treatment (10 mg/kg/day) efficacy was determined in athymic male nude mice bearing subcutaneous PC3 xenograft tumors.

Results—LQB-118 treatment triggered PCa cell death and apoptosis. Therapeutic activity was at least partially dependent upon quinone reduction and ROS generation. LQB-118 treatment caused an increase in cellular ROS and lipid peroxidation. Treated cells exhibited elevated levels of NQO1, Nrf2, and SOD1. The miRNAs miR-206, miR-1, and miR-101 targeted and reduced SOD1 expression. The knockdown of SOD1, by siRNA or miRNA, enhanced LQB-118 cytotoxicity.

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Orally administered LQB-118 treatment significantly reduced the growth of established PCa xenograft tumors.

Conclusion—LQB-118 is a developing and orally active pterocarpanquinone agent that effectively kills PCa cells through quinone reduction and ROS generation. The inhibition SOD1 expression enhances LQB-118 activity, presumably by impairing the cellular antioxidant response.

Keywords

Pterocarpanquinone; NQO1; SOD1; Nrf2; siRNA; microRNA

Introduction

Prostate cancer (PCa) is a leading cause of cancer death in American men [1]. The standard treatment for metastatic PCa is androgen deprivation therapy (ADT). While the majority of patients respond to ADT, most tumors will eventually progress to incurable castration resistant PCa (CRPC) [2]. Current approaches to manage CRPC include next-generation ADT, such as enzalutamide and abiraterone, as well as docetaxel, targeted radiotherapeutics, and immunotherapies [3]. There is a critical need to consider additional therapeutic targets, and therapeutic compounds, which may provide alternative mechanisms for CRPC management. The pterocarpanquinone, LQB-118, is a synthetic compound capable of inducing apoptosis in leukemia cell lines and leukemic samples from patients [4, 5]. LQB-118 demonstrated low cytotoxicity toward human peripheral blood mononuclear cells from healthy donors, as well as in *Swiss webster* mice [6, 7]. Initial studies of LQB-118 in PCa cell models have been positive, where treatment induced significant cytotoxicity and apoptosis in the androgen-independent PCa cell model, PC3. Treatment was associated with cell cycle arrest, decreased expression of c-Myc, cyclin D1 and cyclin B1 mRNA, and up-regulation of the cell cycle inhibitor p21 [8].

The mechanisms of LQB-118 activation and cytotoxicity have yet to be fully elucidated. It has been suggested that LQB-118, with a structure-related to naphthoquinone, might be reduced by the NQO1 enzyme [9]. Naphthoquinone reduction can generate elevated intracellular levels of ROS, causing significant redox stress [9]. A variety of drugs exert their anti-cancer function through increasing ROS production [10]. Nonetheless, some tumor cells can overcome drug-induced oxidative stress through the over-expression of redox-active factors such as nuclear factor erythroid-2 related factor 2 (Nrf2), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [11, 12]. This rapid adjustment to altered redox environments, so called ‘Redox Resetting’, can provide additional mechanisms toward therapeutic resistance [13].

Here we provide new analyses of LQB-118 therapy in PCa cell and tumor models, and we characterize mechanisms of drug activation, activity, and resistance. Our results correlate LQB-118 activity with quinone reduction, induced NQO1 expression, and ROS generation. LQB-118 treatment induced the redox responsive genes Nrf2 and SOD1, and transient knock-down of SOD1 through siRNA or miRNA caused an increase in PCa cell sensitivity to LQB-118. Oral LQB-118 therapy safely and effectively attenuated the growth of established PCa tumors, *in vivo*, in athymic nude mouse xenograft models. These results

provide further support for LQB-118 as a potential anti-cancer therapeutic, and they offer new insights into the mechanisms of LQB-118 activity in human cancer cells.

Methods

Cell lines

Androgen-dependent (LAPC4 and LNCaP) and androgen-independent (PC3) human PCa cells were cultured as follows: LNCaP and PC3 in RPMI 1640 media, and LAPC4 in IMDM plus 1 nM R1881, each supplemented with 10% fetal bovine serum and 5 μ M ciprofloxacin or 100 mg/l penicillin and 70 mg/l streptomycin. Cells were maintained at 37°C in a 5% CO₂ humidified atmosphere. All cell lines were authenticated by the DNA Diagnostics Center (Farifield, OH, USA) or BCRJ (Rio de Janeiro, RJ, Brazil) and confirmed mycoplasma free.

Chemicals and reagents

The pterocarpanquinone LQB-118 was synthesized in the Bioorganic Chemistry Laboratory, NPPN, Federal University of Rio de Janeiro (Brazil), as described elsewhere [6], and was kindly donated by Dr. Chaquip Netto and Dr. Paulo Costa. 3,3'-metileno-bis(4-hydroxycoumarin) (Dicumarol), N-acetylcystein (NAC) was purchased from Sigma. All compounds were dissolved in 100% DMSO (Merck, Germany), except for NAC, dissolved in culture medium. Maximal DMSO concentration in culture was 0.1%, without cytotoxic effects. For *in vivo* experiments, LQB-118 was diluted in 15% ethanol containing 1.5% Tween 20. Anti-human SOD1 (SAB1409700) and anti-human ACTB (AC-15) were purchased from Sigma-Aldrich (St. Louis, MO, USA). SiRNA FlexiTube GeneSolution (GS6647) was purchased from Qiagen (Germantown, MD, USA) and miRNA mimics were purchased from Dharmacon GE (Lafayette, CO, USA).

Cytotoxicity assays

After adhesion in 96 flat-bottom-well microplates, cells (2×10^4 cells/ml) were incubated in the absence (control) or presence of different LQB-118 concentrations at 37°C in a 5% CO₂ humidified atmosphere, to calculate the concentration of LQB-118 that inhibits 50% of relative cell viability (IC₅₀). Cells were either pretreated or not for 2 h with NAC (5 mM) followed by treatment with LQB-118 at concentrations equal to or moderately lower than the determined IC₅₀ (Table 1) and/or Dicoumarol (50 μ M) for 12 h. Cells were also transfected or without siRNA or miRNA mimics, after 48 h, cell lines were seeded and treated or not with LQB-118 for an additional 12 h. Cell survival/cytotoxicity was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, Sigma Chemical Co., USA) assay after 12 h by incubation at 37°C (last 2 h of culture) with 10 μ l/well of 5 mg/ml MTT stock solution [14]. Afterwards, 100 μ l/well of SDS 10% solution, containing 0.01 N HCl was added in the MTT wells and the absorbance was measured at 570 nm for MTT assay (microplate reader μ Quant, Bio-Tek Instruments, Inc.). The results are expressed as relative cell viability, using the control culture (without drug) as 100%.

Intracellular ROS measurement

A H₂DCFDA probe was used to detect ROS in cells. Cells (1×10^5) were pretreated or not with NAC (5 mM) for 30 min, followed by LQB-118 exposure and/or Dicoumarol (50 μ M)

for additional 10 min, totaling 40 min of treatment. Afterwards, cells were harvested, washed with Phosphate buffered saline-PBS (140 mM NaCl, 2,7 mM KCl, 8 mM Na₂HPO₄, 1,5 mM KH₂PO₄, pH 7.4), and labeled with H₂DCFDA (5 µM) probe according to manufacturer's instructions (Invitrogen, Brazil). A total of fifty thousand events were acquired in the Gallios flow cytometer (Beckman Coulter, USA) for fluorescence detection by the FL1 channel. Data were analyzed using Summit v4.3 software.

Lipid peroxidation measurement

Lipid peroxidation was evaluated by malondialdehyde (MDA)-TBA complex (TBARS), as previously described [15]. It was determined in cellular supernatant with LQB-118 (8 µM) or H₂O₂ (100 µM). Briefly, trichloroacetic acid 30% (600 µl) was added onto cell culture supernatant (300 µl), vigorously mixed and centrifuged at 7500 × g for 10 min at room temperature. The supernatant (200 µl) was transferred to a new tube, mixed with thiobarbituric acid (TBA), incubated at 95°C for 20 min and cooled to room temperature. A standard curve (0.5 to 20 nM) of 1, 1, 3, 3-tetraethoxypropane (malondialdehyde-MDA) was also determined. The absorbance was read at 532 nm and the results of lipid peroxidation were expressed as nmol of MDA equivalents.

Apoptosis assays

Cells (2×10^4 /ml) were cultured in the absence or presence of LQB-118 with Dicoumarol (50 µM) and/or NAC (5 mM) for 12 h at 37°C in a 5% CO₂ humidified atmosphere. Apoptosis was evaluated by Annexin-V labeling, according to the manufacturer's instructions (Annexin-V-FITC and propidium iodide apoptosis kit, BD Pharmingen, CA, USA). The cells (1×10^5) were washed with PBS, suspended in binding buffer and labeled with Annexin-V-FITC. After addition of the binding buffer (400 µl), cells were acquired (fifty thousand events) in the Gallios flow cytometer (Beckman Coulter, USA) and Annexin V and PI fluorescences detected by the FL1 and FL3 channels, respectively. Data were analyzed using Summit v4.3 software.

RNA isolation and Reverse Transcription (RT)-Quantitative PCR (qPCR)

RNA was isolated by TRIzol reagent (Invitrogen, Brazil). Complementary DNA (cDNA) synthesis was carried out by Moloney Murine Leukemia Virus Reverse Transcriptase enzyme (M-MLV RT) and qPCR reactions were performed with Fast SYBR Green Master Mix (Life Technologies, Brazil), following manufacturers' protocols. Alterations in mRNA levels were calculated by the 2^{-Ct} method [16]. All data were normalized using as reference the GAPDH mRNA levels. Each PCR efficiency was evaluated by standard curve and primers specificity were checked by melting curve analysis. The primer sequences were as follows: Nrf2 5'-TGCCAACTACTCCCAGGTT-3' and 5'-CAAACGGGAAATGTCTGCG-3'; NQO1 5'-AAGGATGGAAGAAACGCCTGGAGA-3' and 5'-GGCCACAGAAAGGCCAAATTTCT-3'; SOD1 5'-TGGCCGATGTGTCTATTGAA-3' and 5'-CACCTTTGCCCAAGTCATCT-3'; CAT 5'-GCCATTGCCACAGGAAAGTA-3' and 5'-CCTTGGTGAGATCGAATGGA-3'; GPx 5'-GGTTCGAGCCCAACTTCATG-3' and 5'-GGAGACCAGGTGATGAGCTT-3'.

miRNA Target Prediction Analysis

MicroRNAs target prediction for SOD1 was determined by TargetScan and Tarbase and candidates were considered with miRSVR scores equal to or below than -0.5, and miTG scores equal or higher than 0.5 [17, 18].

siRNA and miRNA Sensitivity Analysis

Two days before LQB-118 or H₂O₂ treatment, 1×10⁶ PCa cells were separately transfected with 20 nM of siRNA or miRNA mimics using Lipofectamine 2000 (Invitrogen, Grand Island, NY). Negative control of both miRNA and siRNA were included in the experiments, also siRNA#3 was used as a positive control. Cells (2.0 × 10⁵/ml) from each condition were either treated or not with LQB-118 (5 or 8 μM) for 12 h, followed by the MTT assay. Similarly, non-transfected cells were also either treated or not with LQB-118 (as an experimental control). The LQB-118 concentration was previously determined (IC₅₀) for optimum treatment-sensitization by siRNA or microRNAs.

Western Blot analysis

Cells were transfected or not with miRNA mimics or siRNA (20 nM) and extracts were harvested 48 h later in RIPA lysis buffer with protease inhibitor. Protein samples were quantified using BSA, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes were blocked with Odyssey blocking buffer and incubated overnight at 4°C with anti-SOD1 (1:1000) or anti-ACTB (1:10000). After washing, membranes were incubated with IRDye secondary antibody (LICOR, Lincoln, NE, USA) at room temperature for 1 h. Images were detected by Odyssey infrared imaging system (*Li-COR*). Signal for each protein were normalized to ACTB.

3'UTR dual luciferase enzyme assay

A region (352 bp) of the SOD 3'UTR containing putative miRNA binding sites was amplified from 293T genomic DNA (human embryonic kidney cells) by polymerase chain reaction (PCR). Primers were designed to incorporate HindII and SacI restriction enzyme sites; forward – 5'-GGTAGTGATTACTTGACAGC-3' and reverse – 5'-TTCCACTGAAGCTGTTTA-3'. PCR products were subcloned downstream of luciferase in the pMiR luciferase expression reporter vector (Ambion, Grand Island, NY), generating the pMiR-3'UTR-SOD1 reporter. 293T cells were transfected with 20 nM of control or miRNA mimic, 80 ng of pMiR-3'UTR-SOD1 reporter vector and 20 ng of reference pRL-CMV Renilla reporter vector. After 48 h, the reporter activity was assessed using a dual luciferase enzyme assay as previously described [19, 20]. Firefly luciferase signal was normalized to the internal control Renilla luciferase.

In vivo assay: Tumor xenograft studies

Mouse care and treatment was approved by and performed in accordance with the guidelines of the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Cells were placed in a 50% mixture of Matrigel Matrix (BD Biosciences, San Jose, CA) in RPMI 1640 at a concentration of 2.0 × 10⁶ cells per 100 μL of solution and

injected subcutaneously into the right dorsal area of 6 week old male nude mice ($n = 6$ /group). Using the therapeutic dose, according to preliminary tests, including toxicological test [21], LQB-118 was daily and orally administrated (10 mg/kg/dose), using oral gavage needle. Controls were treated with aqueous solution containing 15% ethanol and 1.5% Tween 20. Tumor growth rate was measured by determining tumor volume using digital calipers every other day and the tumor volume (cm^3) was calculated ($\text{volume} = 0.52 \times L \times W \times H$). The body weight was also measured along with tumor volume. Tumors were harvested after 20 days or when they reached maximum volume (2 cm in any diameter).

Statistical analysis

The results are reported as mean $\pm$ standard deviation (SD) of at least three independent experiments. Significant differences between pairs of groups were accessed using Student's t test, with a significance level set at $p < 0.05$. Kaplan-Meier graphs were statistically analyzed by Gehan-Breslow-Wilcoxon and Mantel-Cox tests. The IC₅₀ was determined by linear regression. All statistics analysis was performed using GraphPad Prism 7.0 software.

Results

LQB-118 cytotoxicity is associated with quinone reduction and ROS production

Previous studies have demonstrated that the pterocarpanquinone, LQB-118, is potently cytotoxic to PC3 PCa cells after 48 h of culture, when cell cycle arrest and apoptosis induction were demonstrated [8]. To study mechanism of LQB-118 action related to oxidative stress, we first improved its cytotoxicity assays in PCa cells. Three cell lines were treated with increasing LQB-118 concentrations for different time of culture, when the IC₅₀ was determined (MTT assay). The results demonstrate effective IC₅₀ concentrations of 2.8 μM to 8.7 μM (Table 1). According to the respective IC₅₀ concentration for each cell line, LQB-118 concentrations of 5 μM or 8 μM were applied for the mechanistic studies, using 12 h of culture.

To determine whether LQB-118 cytotoxicity is dependent upon the metabolism of the quinone moiety, PC3 cells were treated with LQB-118 (8 μM) in the presence or absence of the quinone-reductase inhibitor Dicoumarol (50 μM). As shown in Fig. 1A, Dicoumarol treatment alone had no effect on PC3 cell viability, while LQB-118 treatment decreased cell viability to $41.5 \pm 9.1\%$, when compared to control cells (100%). The addition of Dicoumarol partially blocked LQB-118 mediated toxicity, leaving $63.4 \pm 7.3\%$ of cells viable (Fig. 1A). LQB-118 treatment has also been previously shown to induce PC3 cell apoptosis [8]. LQB-118 treatment alone was confirmed to induce PC3 cell apoptosis (61.5%), when compared to control culture (15.9%) (Fig. 1B). The addition of Dicoumarol significantly protected PC3 cells from LQB-118-induced apoptosis, with only 25.8% of cells undergoing apoptosis, exhibiting results similar ($P > 0.05$) to control culture. These results implicate cellular quinone reduction in LQB-118 activation and activity.

To further investigate the mechanisms of LQB-118 cytotoxicity, NQO1 quinone reductase gene expression was quantified following treatment. LQB-118 treatment markedly increased (127%) NQO1 gene expression (Fig. 1C). This induction was not attenuated by Dicoumarol

treatment, indicating that Dicoumarol impacted LQB-118 metabolism and not NQO1 gene induction. Because quinone metabolism also generates superoxide anions, we evaluated whether LQB-118 treated PC3 cells produced ROS by using the fluorescent probe, DFC. Treatment with LQB-118 significantly increased DFC probe fluorescence by 58.2% in PC3 cells, when compared to untreated cells, at 40 min (Fig. 1D). Notably, ROS generation was blocked by Dicoumarol.

Cells have a number of natural responses to reactive oxygen stress. We reasoned that the supplementation of cell culture medium with NAC, a natural ROS scavenger and GSH precursor, might reduce LQB-118 mediated cytotoxicity. Indeed, the addition NAC significantly reduced LQB-118 cytotoxic effects (Fig. 2A), and it also protected PC3 cells against LQB-118-induced apoptosis (Fig. 2B), reducing the percentage of apoptotic cells from 65.5% (LQB-118) to 21.5% (LQB-118 with NAC). Treatment of PC3 cells with 5 mM NAC alone had no effect on cell viability. To further investigate the effects of LQB-118-induced ROS generation, we evaluated whether treatment caused cellular lipid peroxidation. LQB-118 treated PC3 cells showed increased levels of malondialdehyde (MDA), a lipid peroxidation product, at a level similar to cells treated with the positive control, H₂O₂ (Fig. 2C). These results implicate quinone reduction and ROS generation as mechanisms of LQB-118 activation and cytotoxicity.

LQB-118 treatment induces Nrf2 and SOD1 gene expression

To investigate the possible responses to LQB-118-induced ROS generation, the expression of cellular catalase, glutathione peroxidase (GPx), Nrf2, and SOD1 were quantified in PC3 cells (Figure 3). Surprisingly, LQB-118 treatment did not induce catalase (Fig. 3A) or glutathione peroxidase (Fig. 3B) gene expression. However, LQB-118 treatment did significantly induce the expression of Nrf2 by 80%, when compared to control treatment (Fig. 3C). Similarly, LQB-118 treatment induced SOD1 expression by 105%, when compared to control culture. As expected, the addition of Dicoumarol significantly blocked SOD1 induced expression. These results implicate Nrf2 and SOD1 as potential intrinsic mechanisms of LQB-118 treatment response and resistance in PCa cells.

SOD1 induction is protective against LQB-118 cellular cytotoxicity

Expanding upon LQB-118 cytotoxicity and ROS production in PC3 cells, we evaluated treatment responses in additional PCa cell lines. First, SOD1 protein expression was quantified, to complement the previous mRNA analyses. As expected, in PC3 cells, LQB-118 (8 μ M) treatment increased SOD1 protein levels by 29.1%, when compared to untreated cells (Fig. 4A). LQB-118 treatment also induced SOD1 protein levels by 93.5% in AR-positive LNCaP cells (8 μ M) (Fig. 4B) and by 17.5% in AR-positive LAPC4 cells (5 μ M) (Fig. 4C), when compared to their respective controls.

To investigate the role of SOD1 in LQB-118 treatment response, we sought to knockdown its expression in multiple cell lines. Initial studies evaluated multiple siRNAs (termed siRNA #1 to siRNA #4) in LAPC4, LNCaP and PC3. As illustrated in Fig. 5A, only siRNA #3 was capable of significant SOD1 knockdown in all three cell lines. Therefore, only one siRNA was utilized in LQB-118 cytotoxicity analyses. Strikingly, the knockdown of SOD1

expression significantly enhanced LQB-118 mediated cytotoxicity in all three cell lines, when compared to non-transfected cells or cells transfected with a negative control siRNA (siRNA-NTC). In PC3 cells, SOD1 knockdown increased LQB-118-induced cytotoxicity from 47.3% ($p<0.05$) in control siRNA transfected cells, to 69.9% ($p<0.01$) in siRNA #3 transfected cells (Fig. 5B). LNCaP cell viability was only moderately impacted by treatment with LQB-118 (Fig. 5C). However, this cytotoxicity was markedly enhanced to 50.1% ($p<0.01$) with SOD1 knockdown. SOD1 knockdown in LAPC4 cells similarly caused a significant increase in LQB-118 cytotoxicity, from 30.9% ($p<0.05$) to 56.6% ($p<0.05$) (Fig. 5D).

With only one functional siRNA, we sought additional means to regulate SOD1 gene expression in PCa cells. MicroRNAs (miRNA) offer an alternative means to regulate gene expression through direct interactions between the miRNA and the 3' Untranslated Regions (3'UTR) of the targeted mRNA, leading to loss of mRNA stability and inhibition of protein translation through the RNA-induced Silencing Complex (RISC). SOD1-targeting miRNAs were identified through publicly available miRNA target prediction algorithms. Three candidate SOD1-targeting miRNAs with favorable miRSVR and miTG scores were chosen [17,18]: miR-206, miR-1, and miR-101. Mimics of each miRNA were found to significantly reduce the activity of a SOD1 3'UTR luciferase reporter, when co-transfected into 293HEK cells, indicating direct miRNA-mediated gene suppression through the SOD1 3'UTR (Fig. 6A). Each miRNA was then evaluated for the ability to reduce endogenous SOD1 protein levels in PC3, LNCaP, and LAPC4 PCa cell lines, when compared to a non-targeting miRNA mimic (miRNA-NTC). In PC3 cells, only miR-101 was found to significantly reduce SOD1 protein levels (Fig. 6B). In LNCaP and LAPC4 cells, all three miRNAs significantly reduced SOD1 protein expression. Specifically, in LNCaP cells, miR-206 decreased SOD1 levels by 7.7% ($p<0.05$), miR-1 decreased SOD1 levels by 38.4% ($p<0.01$), and miR-101 reduced SOD1 levels by 44.2% ($p<0.01$) (Fig. 6C). In LAPC4 cells (Fig. 6D), miR-206 reduced SOD1 protein expression by 67.6% ($p<0.001$), miR-1 decreased SOD1 levels by 92.3% ($p<0.001$), and miR-101 down-regulated SOD1 levels by 64.1% ($p<0.01$). Therefore, only miR-101 was capable of targeted SOD1 reduction in all three cell lines.

Each miRNA mimic was then transfected into PCa cells to determine their influence on cell sensitivity to LQB-118. Surprisingly, in PC3 cells, none of the miRNAs demonstrated enhanced LQB-118 sensitivity, when compared to the control miRNA, including miR-101 (Fig. 7A). However, miRNA-101 was able to enhance LQB-118-induced cytotoxicity in LNCaP (Fig. 7B) and LAPC4 cells (Fig. 7C), when compared to control mimic transfected cells ($p<0.05$). Interestingly, the other SOD1-targeting miRNAs had mixed effects on LQB-118 treatment potency. These results may be due to the ability of each miRNA to target multiple gene transcripts, when compared to siRNAs, which are designed to target only a single gene.

Oral LQB-118 treatment significantly attenuates prostate cancer xenograft progression

With a new understanding of the mechanisms and efficacy of LQB-118 in PCa cell models, we finally evaluated the ability of LQB-118 to treat established PCa tumors *in vivo*. For this experiment, we selected PC3 cells due to their efficiency and reproducibility in

subcutaneous tumor xenograft development. We considered previous studies that had established the safety of LQB-118 oral treatment, where a dose of 10 mg/kg did not produce any detectable tissue toxicities by histopathology analysis [19 – 21]. PC3 xenografts were allowed to develop to approximately 100 mm³ prior to LQB-118 treatment. Mice then received daily vehicle (Control) or 10 mg/kg of LQB-118 vial oral gavage. The relative tumor progression of LQB-118 treated animals was significantly reduced, when compared to the untreated mice ($p < 0.0001$, Two-Way ANOVA) (Fig. 8A). By the twelfth day of treatment, LQB-118 treated tumors were 44.6% ($p < 0.01$) smaller than untreated tumors, with treatment peaking at the endpoint of 16 days, with a relative tumor reduction of 67.9% ($p < 0.001$). We also analyzed these data by considering the percent of animals with tumors below 800 mm³, a volume eight-fold greater than the initial tumor volume, throughout the time course of the experiment. Within thirteen days, the tumors of all untreated animals reached 800 mm³, when compared to only 60% of LQB-118 treated animals (Fig. 8B). Notably, LQB-118 treatment did not significantly change the body weight of the animals throughout the full time course of the experiment (Fig. 8C).

Discussion

There is a critical need to consider new therapeutic targets and agents in the treatment of CRPC. Such new strategies will benefit from a clear understanding of drug targets, drug metabolism, and the mechanisms underlying drug resistance and cell sensitivity. Here we examined the pterocarpanquinone, LQB-118, and its mechanisms of activation, cytotoxicity, and resistance in PCa cells, as well as its *in vivo* efficacy through oral treatment. Our results demonstrate that LQB-118 cytotoxicity is at least partially dependent upon cellular quinone reduction capability. This activity is associated with the induced expression of NQO1, a well-characterized quinone reductase [22]. Among intracellular quinone metabolism enzymes, NQO1 has emerged as a promising target for cancer therapy because of its elevated expression in most solid tumors [23, 24]. The association between cytotoxicity and increased NQO1 expression has been described for other naphthoquinones in many types of cancer cells [22, 23, 25, 26].

In general, naphthoquinone is reduced to an unstable hydroquinone or semiquinone, which rapidly undergoes one or two-steps of oxidation to return to the parental form, perpetuating a futile redox cycle and resulting in the generation of ROS, mainly O₂⁻ [25, 26]. Accordingly, LQB-118 induced ROS in PCa cells, and this activity was inhibited by the quinone reductase inhibitor, Dicoumarol. ROS can oxidize the thiol groups of several proteins and cause damage to lipids, organelles and nucleic acids, resulting in widespread cellular damage and cell death [27, 28]. Notably, the level of oxidative damage and lipid peroxidation from LQB-118 treatment was comparable to that of hydrogen peroxide treatment. Consequently, LQB-118 treatment significantly reduced PCa cell viability and induced apoptosis. This cytotoxicity was markedly reduced by the free radical scavenger NAC, which acts through diffusion across cell membranes, where its metabolism results in an intracellular accumulation of cysteines and thereby reduced ROS level [29].

Cells under oxidative stress activate Nrf2, a transcription factor and orchestrator of several antioxidant genes [30]. Nrf2 activation is also related to drug-resistance and enhanced cancer

cell proliferation [31, 32]. Herein, LQB-118 treatment increased the expression of Nrf2, which suggests a cellular antioxidant response. LQB-118 treatment also induced NQO1 gene expression, a known Nrf2 responsive gene. These effects are consistent with LQB-118 metabolism by a quinone reductase. However, we must consider that additional quinone reductase enzymes may play a role in LQB-118 activation. Another Nrf2 target gene is SOD1 [30], which dismutates O_2^- into H_2O_2 , along with others SOD isoforms. Notably, LQB-118 treatment significantly induced SOD1 expression in all three PCa cell lines, indicating a potential protective response of PCa cells to LQB-118 effects. Interestingly, we did not observe LQB-118 induction of the CAT or GPx redox enzymes.

SOD1 is a key regulator of ROS homeostasis in cancer cells and its elevated expression may contribute to pathways of proliferation and survival [33]. In light of this, SOD1 has been considered to be a potential therapeutic target for cancer [34]. Since LQB-118 treatment resulted in induced SOD1 protein expression levels, we considered whether SOD1 expression could drive resistance to LQB-118-induced cell death and apoptosis. Indeed, loss of SOD1 expression by siRNA knockdown significantly increased PCa cell sensitivity to LQB-118, indicating that SOD1 is a key regulator of cellular response and resistance to LQB-118 cytotoxicity. We further sought a physiological strategy to suppress SOD1 activity in PCa cells and found that SOD1 was a possible target of miR-206, miR-1, and miR-101, three miRNAs with frequently decreased expression in PCa [35]. All three miRNAs were confirmed to target and suppress SOD1 protein expression and SOD1 3'UTR reporter activity. However, the potency of each miRNA to target SOD1 and to enhance LQB-118 sensitivity varied significantly between cell lines. In general, miRNA-mediated gene regulation is consistent among different cell types. However, there is potential for altered miRNA targeting landscapes due to differences in gene expression or usage of alternative 3'UTR isoforms [36]. Nonetheless, miR-101 consistently reduced SOD1 protein expression in all three PCa cell lines and enhanced LQB-118 cytotoxicity in two of three PCa cell lines. In addition to SOD1, miR-101 has been reported to suppress EZH2 and FOXM1, two drivers of PCa cell viability, proliferation, migration, and invasion [37, 38].

Previous studies have demonstrated the promise of LQB-118 as a therapeutic agent in PCa cells [8]. Our data further establish LQB-118 activity in multiple PCa cell models. Here, for the first time, we also demonstrate the anti-cancer activity of LQB-118 *in vivo*, following oral administration (10 mg/kg). Treatment with LQB-118 at this dose appeared to have no measurable adverse effects, which are consistent with previous promising pre-clinical toxicological studies, even at doses ten times higher than the therapeutic dose [19]. Further studies are needed to evaluate the anti-cancer potential of LQB-118 at higher doses, while also balancing the potential for drug-related toxicities. In addition, new studies are required to evaluate the potential interactions between LQB-118 and current CRPC treatment strategies.

Conclusions

This work characterizes the mechanisms of LQB-118 induced cytotoxicity and resistance in PCa cell and tumor models. LQB-118-mediated cytotoxicity is at least partially mediated through quinone reduction and subsequent ROS generation, leading to PCa cell apoptosis.

The activity is associated with induced NQO1, Nrf2, and SOD1 gene expression. The induced SOD1 expression protects cells from LQB-118 induced ROS, and SOD1 depletion by siRNA or miRNA is sufficient to enhance LQB-118 cytotoxicity. Oral treatment with 10 mg/kg LQB-118 effectively reduces the growth of PCa tumors *in vivo*. These early pre-clinical studies characterize LQB-118 as a potential new tool for PC treatment.

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Fig. 1.

LQB-118 cytotoxicity requires quinone reduction activity and results in elevated NQO1 expression and ROS generation. (A) Cytotoxicity, (B) Apoptosis induction, (C) NQO1 gene expression and (D) Intracellular ROS level were determined in PC3 cells following 12 h of incubation in either LQB-118 (8 μ M), Dicoumarol (50 μ M), or LQB-118 plus Dicoumarol. Relative cell viability was determined by MTT assay, NQO1 gene expression by qPCR, ROS levels by DCF-DA probe fluorescence, and apoptosis through Annexin-V-PI labeling and flow cytometry. Results represent the mean $\pm$ SD of three independent experiments. ** P < 0.01, *** P < 0.001, relative to control (with no drug). # P < 0.05; ### P < 0.001, relative to LQB-118 treated cells (Student's t test).

Fig. 2.

LQB-118 cytotoxic activity is attenuated with ROS scavengers, and is associated with lipid peroxidation. (A) Cytotoxicity and (B) Apoptosis were determined in PC3 cells after 12 h of incubation in LQB-118 (8 μ M), or LQB-118 plus N-acetyl cysteine (NAC) (5 mM). Relative cell viability was determined by MTT assay and apoptosis by Annexin-V-PI labeling. (C) Lipid peroxidation was determined in PC3 cells by TBARS assay after incubation in LQB-118 or hydrogen peroxide. Results represent the mean $\pm$ SD of three independent

experiments. $**P < 0.01$, $***P < 0.001$, relative to control. $^{\#}P < 0.05$ and $^{###}P < 0.001$, relative to LQB-118 treated cells (Student's t test).

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Fig. 3.

LQB-118 treatment induces Nrf2 and SOD1 gene expression. (A) Catalase, (B) Glutathione peroxidase (C) Nrf2 and (D) SOD1 gene expression was quantified in PC3 cells by RT-qPCR following 12 h of treatment with LQB-118 or LQB-118 plus Dicoumarol. Results represent the mean $\pm$ SD of three independent experiments. * P < 0.05 and *** P < 0.001, relative to control (with no drug) and ## P < 0.01, relative to LQB-118 treated cells (Student's t test).

Fig. 4.

LQB-118 treatment induces SOD1 protein expression in AR-positive and AR-negative PCa cells. LQB-118 induced SOD1 expression as measured by western blotting in (A) PC3, (B) LNCaP, (C) LAPC4 cells following 12 h of treatment with LQB-118. SOD1 protein levels is quantified relative to β -actin (mean of arbitrary units -a.u). Graphs show mean $\pm$ SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, relative to control (Student's t test).

Fig. 5.

SOD1 knockdown increases PCa cell sensitivity to LQB-118 treatment. (A) SOD1 knockdown using different siRNAs and assessed by Western Blotting. The LQB-118 cytotoxic effects after SOD1 knockdown by siRNA#3 are shown on (B) PC3 cells, (C) LNCaP cells and (D) LAPC4 cells. Cells were transfected by siRNAs for 48 h, accompanied by LQB-118 treatment (8 μ M for PC3 and LNCaP cells and 5 μ M for LAPC4 cells) for 12 h, and relative cell viability determined by MTT assay. Results represent the mean $\pm$ SD of three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001, relative to control (with no drug). ^a P < 0.05, relative to siRNA-NTC transfected cells and treated with LQB-118. # P < 0.05 and ## P < 0.01, relative to treatment only with LQB-118 (Student's t test).

Fig. 6.

Targeted reduction of SOD1 protein expression by miRNA mimics. (A) SOD1 3'UTR reporter activity in 293T cell lines following transfection with control, miR-206, miR-1, or miR-101 mimics. SOD1 protein expression by western blot in (B) PC3 cells, (C) LNCaP cells, or (D) LAPC4 cells following transfection with control (miR-NTC), miR-206, miR-1, or miR-101 (20 nM) mimics for 48 h. siRNA#3 was used as a positive control for SOD1 knockdown. Results show mean $\pm$ SD of three independent experiments. ** P < 0.01, *** P < 0.001, relative to miR-NTC (Student's t test).

Fig. 7. miR-101 increases PCa cell sensitivity to LQB-118. (A) PC3 cells, (B) LNCaP cells, and (C) LAPC4 cells were transfected with miR-206, miR-1, or miR-101 (20 nM) mimics for 48 h, followed by LQB-118 treatment (8 μ M for PC3 and LNCaP cells and 5 μ M for LAPC4 cells) for 12 h. Relative cell viability was evaluated by MTT assay. Cell sensitivity to LQB-118 after miRNA transfection (black) was compared to cells treated only with LQB-118 (grey) and normalized to cells only transfected with respective miRNA mimics (striped). Results show mean $\pm$ SD of three independent experiments. * P < 0.05 and ** P <

0.01 and *** $P < 0.001$, relative to transfected cells with the respective miRNA, and # $P < 0.05$, relative to LQB-118 treated, but non-transfected cells (Student's t test).

Fig. 8. Orally administered LQB-118 reduced the growth of established subcutaneous PCa tumors. (A) Subcutaneous PC3 tumor volume measured over time in LQB-118 treated and non-treated animals. (B) Time for tumor progression to 8X initial volume in LQB-118 treated and non-treated animals. (C) Body weight of LQB-118 treated and non-treated animals. Mice (n = 6/group) were daily and orally treated with LQB-118 (10 mg/kg) or vehicle

(control group). $*P < 0.05$ and $**P < 0.01$, compared to control group (Student's t test). $^{\#}P < 0.05$ (Gehan-Breslow-Wilcoxon and Mantel-Cox tests).

Table 1

The cytotoxicity of LQB-118 in different prostate cancer cell lines.

Cell lines	IC50 (μ M)		
	12 h	24 h	48 h
LAPC4	6.1	5.5	5.5
LNCaP	ND	5	2.8
PC3	8.7	8	4.6

PCa cell lines were incubated in presence or absence of different concentration of LQB-118 (1 – 10 μ M) to determine IC50 (by linear regression) through different times. The cytotoxicity was evaluated by MTT assay. ND – Not determined.