**β-Lapachone Induces NAD(P)H:Quinone Oxidoreductase-1– and Oxidative Stress–Dependent Heat Shock Protein 90 Cleavage and Inhibits Tumor Growth and Angiogenesis**

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**ABSTRACT**

β-Lapachone [β-lap; 3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione] is a novel anticancer drug currently under investigation in phase I/II clinical trials. However, the mechanism underlying its clinical efficacy remains unclear. In this study, we found that β-lap provoked the cleavage of heat shock protein 90 (Hsp90) in NAD(P)H:quinone oxidoreductase-1 (NQO1)–expressing lung and prostate cancer cells as well as in primary human umbilical vein endothelial cells (HUVECs). These actions of β-lap were different from that of the conventional Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin. As a consequence of Hsp90 cleavage, Hsp90-associated oncoproteins, such as receptor-interacting protein, Raf-1, AKT, and CDK4, were degraded in treated cancer cells, and key receptor tyrosine kinases such as vascular endothelial cell growth factor receptor-2 and Her-2 were degraded in treated HUVECs through a proteasomal system. Further results revealed that specific inhibitors of NQO1 and reactive oxygen species could dramatically reduce β-lap–mediated Hsp90 cleavage. In addition to its cytotoxicity, β-lap effectively inhibited angiogenesis by suppressing tube formation and the invasion of HUVECs in vitro, rat aortic microvascular sprouts ex vivo, and mouse corneal neovascularization in vivo. Furthermore, β-lap markedly suppressed the growth and angiogenesis of human lung cancer xenografts in nude mice and decreased the levels of receptor-interacting protein, AKT, CDK4, and CD31 in the solid tumors. Unlike other NQO1-dependent cytotoxic quinones, such as streptonigrin, menadione, mitomycin, and 17-allylamino-17-demethoxygeldanamycin, β-lap was the only agent that could cause Hsp90 cleavage. Taken together, our results suggest a crucial mechanism underlying the antitumor efficacy of β-lap.

**Introduction**

Lung and prostate cancers still accounted for approximately one-third of total cancer deaths and total new cancer cases in 2015 (Siegel et al., 2015). Current standard therapies for these cancers include surgery followed by the combination of radiotherapy and/or chemotherapy. However, because of the aggressiveness and invasiveness of these cancers and the normal tissue toxicity of the radiotherapy and chemotherapy, the 5-year survival rate has not improved significantly over the past decades. Thus, developing novel drugs that target tumor-specific molecules is urgently needed for the effective treatment of these solid tumors.

One protein molecule that is highly expressed in various human cancers, especially lung and prostate cancers, is heat shock protein 90 (Hsp90). Hsp90 is an evolutionarily conserved molecular chaperone that guides the folding, intracellular disposition, and proteolytic turnover of many key oncoproteins for the regulation of cell growth, differentiation, and survival (Bagatell and Whitesell, 2004). Because Hsp90 is highly active in cancer cells, it is an ideal target for developing inhibitors in comparison with the largely uncomplexed and less-active form of Hsp90 in normal cells (Workman, 2004a). Often, cancer cells are “addicted” to Hsp90 (Isaacs et al., 2003; Trepel et al., 2010). A number of oncoproteins, including tyrosine kinase receptors [e.g., Her-2, vascular endothelial growth

**ABBREVIATIONS**: β-Lapachone, 3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; EG9, 3-hydroxy-5-aziridinyl-1-methyl-2-[1H-indole-4,7-dione]prop-β-en-α-dl; RH1, 2,5-diaziridinyl-3-hydroxymethyl]-6-methyl-1,4-benzoquinone, HP-β-CD, 2-hydroxypropyl-β-cyclodextrin; Hsp90, heat shock protein 90; HUVEC, human umbilical vein endothelial cell; MG132, N-(benzyloxycarbonyl)leucinylleucinylleucinal Z-Leu-Leu-Leu-al; NAC, N-acetyl-L-cysteine; Z-VAD-FMK, benzoxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone, E64d, ethyl (−)-[2S,3S]-3-[[S]-methyl-1-[3-methyl-butylcarbamoyl]-butylcarbamoyl]-2-oxiranecarboxylate; NIH, National Institutes of Health; NQO1, NAD(P)H: quinone oxidoreductase-1; RIP, receptor-interacting protein; ROS, reactive oxygen species; VEGF, vascular endothelial cell growth factor; VEGFR, vascular endothelial cell growth factor receptor.
growth factor receptor (VEGFR), platelet-derived growth factor receptor], cytosolic serine-threonine and tyrosine kinases [e.g., Akt, Raf-1, CDK4, and CDK6], death domain kinase [e.g., receptor-interacting protein (RIP)], transcription factors [e.g., hypoxia-inducible factor 1], and structural proteins and other enzymes [e.g., telomerase reverse transcriptase and Apa1] (Shames and Minna, 2008) require Hsp90 for appropriate folding. These so-called “client” proteins of Hsp90 play important roles in signal transduction, angiogenesis, antiapoptosis, and the metastasis of cancer cells. Therefore, pharmacologic interference of Hsp90 functions would have a strong combinatorial effect on the negation of multiple oncogenes (Workman, 2004a; Porter et al., 2010).

There are currently several Hsp90 inhibitors under development in various stages. Most of the inhibitors were developed to target the ATP binding site at the N terminus of Hsp90. New approaches have emerged, including inhibition of the C-terminal nucleotide binding pocket of Hsp90, disruption of the interaction between Hsp90 and co-chaperones (Li et al., 2009), antagonism of client/Hsp90 associations, interference with post-translational modifications of Hsp90, and Hsp90 deactivation by oxidative stress (Beck et al., 2009; 2011a, 2012). In this study, we explored the possible deactivation of Hsp90 by a redox-modulating agent as a likely therapy of human cancers. This agent is β-lapachone [β-lap; 3,4-dihydro-2,2-dimethyl-2H-naphthal[1,2-b]pyran-5,6-dione] a tricyclic pyrano-orthonaphthoquinone). β-Lap elicits an unique cytotoxicity depending on the expression and activity of NAD(P)H:quinone oxidoreductase-1 (NQO1) (Pardee et al., 2002), which is a two-electron oxidoreductase that is overexpressed up to 20-fold in cancers compared with adjacent normal tissues in more than 70% of patients (Belinsky and Jaiswal, 1993; Siegel and Ross, 2000; Ross and Siegel, 2004). β-Lap relies on NQO1-dependent “futile” redox cycling between the parent β-lap molecule and its hydroquinone form that consumes oxygen and generates extensive reactive oxygen species (ROS) (Pink et al., 2000; Planchon et al., 2001). Elevated ROS levels cause extensive DNA lesions, poly(ADP-ribose) polymerase-1 hyperactivation, and severe NAD+/ATP depletion and Ca2+-dependent μ-calpain activation (Bey et al., 2007; Dong et al., 2009). By contrast, exposure to other NQO1-dependent quinones including mitomycin C, streptonigrin, 3-hydroxy-5-aziridinyl-1-methyl-2-[1H-indole-4,7-dione]prop-β-en-α-ol (E09), and 2,5-diazaaziridinyl-3-(hydroxyethyl)-6-methyl-1,4-benzoquinone (RH1) often results in DNA lesions in both tumor and normal tissues (Belinsky and Jaiswal, 1993). The clinical benefits of these latter cytotoxic alkylating agents were greatly limited due to resistance caused by tumor-related DNA repair processes and normal tissue toxicity (Cheung et al., 2005). Although a more tumor-specific response of β-lap is well recognized in the clinical setting, the mechanism underlying its therapeutic effect remains unknown. It has been proposed that cancer cells are often deficient in antioxidant enzymes, and Hsp90 could be deactivated by oxidative stress (Beck et al., 2011a). Thus, we hypothesize that Hsp90 might be a specific target of the redox-modulating agent β-lap. In addition, angiogenesis is an essential process for tumor growth and metastasis and hence is a generic target for therapeutic intervention in cancer (Bicknell and Harris, 2004), but the antiangiogenic activity of β-lap has not been fully elucidated in vivo. Hence, in this study, we investigated the antiangiogenic activity of β-lap as well.

Materials and Methods

Reagents. β-Lap (98% purity) was purchased from Sigma-Aldrich (St. Louis, MO). A 50 mM stock solution was prepared in dimethylsulfoxide (Sigma-Aldrich), stored at ~80°C, and then diluted as needed in cell culture medium. N-acetylated-cysteine (NAC; Sigma-Aldrich) was dissolved in water, adjusted to pH 7.4, and stored at 4°C. The Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG), proteasome inhibitor MG132 [N-(benzoylcarboxyl)leucinylleucylleucinal Z-Leu-Leu-Leu-al], protease inhibitor calpeptin, and granzyme B inhibitor Ac-IEPD-CHO were obtained from Invitrogen (Carlsbad, CA). Bacteria-derived recombinant human vascular endothelial growth factor (VEGF165) was obtained from the National Institutes of Health (NIH) Experimental Branch (Bethesda, MD). Growth factor-reduced Matrigel was purchased from BD Biosciences (San Jose, CA). Antibody against Hsp90 (anti-Hsp90) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies anti-RIP, anti-ART, anti-CDK4, anti–Her-2, anti-VEGFR2, and anti–β-actin were purchased from Cell Signaling Technology (Danvers, MA). Anti–Raf-1 and anti-Hsp70 antibodies were purchased from Epitomics (Burlingame, CA). 2-Hydroxymethyl-1β-cyclodextrin (HP-β-CD) solution (>98% purity) was obtained from Sigma-Aldrich. β-Lap–HP–β-CD was prepared as described (Nasonkla et al., 2003). All other chemicals used in this study met purity standards set by American Chemical Society.

Cell Lines and Cell Culture. Human prostate cancer cell lines (LNCap, PC-3, and DU145), lung cancer cell lines (A549, NCI-H460, and Calu-1), and immortalized human normal adult prostatic epithelial cells (PNT1A) were purchased from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 or Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). Human fetal lung fibroblast cells MRC5 and WI-38 were obtained from the National Platform of Experimental Cell Resource for Sci-Tech (Shanghai, China). Primary human umbilical vein endothelial cells (HUVECs) were a kind gift from Dr. Xiini Wang (Cardiothoracic Surgery Division, Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, TX) in 2008 and cultured in endothelial cell culture medium as described previously (Pang et al., 2009). All cells were cultured at 37°C in a 5% CO2 and 95% air humidified atmosphere and were free from mycoplasma contamination.

Analysis of ROS. Intracellular ROS production was determined using the oxidation-sensitive 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate fluorescent probes (Invitrogen) as described previously (Fukuyo et al., 2008). A549 cells were plated at a density of 5 × 104 in six-well plates and allowed to attach overnight. Sequentially, cells were incubated with 5 mM of antioxidant N-acetylcysteine for 1 hour before being treated with β-lap (6 μM) for 8 hours. Cells were then loaded with the probe (10 μM) and incubated at 37°C for 30 minutes in the dark. The cells were then subjected to fluorescence microscopy at ×100 magnification (Olympus, Tokyo, Japan).

Cell Survival Assay. Cellular sensitivity to β-lap was measured by sulforhodamine B assay (Kelland et al., 1999). HUVECs (2–5 × 104 cells/well) were pretreated with 50 μM dicoumarol for 30 minutes and then exposed to β-lap for 7 hours, after which cells were rinsed free of drug and incubated in fresh medium for an additional 24 hours. The inhibition percentage was expressed by designating the untreated wells as 100%. Each experiment was repeated at least three times.

Capillary-Like Tube Formation Assay. Tube formation was assessed as described previously (Pang et al., 2010). Briefly, HUVECs (2 × 104 per well) were pretreated with various dilutions of β-lap with or without 40 μM dicoumarol for 1 hour before seeding them in the Matrigel-coated plate. The tubular structure of endothelial cells was quantified by calculating the tube length of high-power fields with an Olympus inverted microscope (Olympus; ×200 magnification), and the inhibition percentage was expressed in reference to the untreated wells designated as 100%. Each data point was assayed at least three times.
Transwell Migration Assay. The transwell migration assay was performed as described previously (Pang et al., 2010). Briefly, HUVECs (4 × 10^4 per transwell) were pretreated with various concentrations of β-lap with or without 40 μM dicoumarol for 2 hours and then seeded into the upper chamber of a transwell (BD Biosciences, San Jose, CA). The bottom chambers were filled with 500 μL endothelial cell culture medium. After 6–8 hours of incubation, the migrated cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an Olympus inverted microscope. Migrated cells in eight random fields were quantified, and the percentage of inhibition in the treated groups was calculated. The untreated group served as the control group.

Animal Studies. Sprague-Dawley rats, C57BL/6 mice, and BALB/cA nude mice were purchased from National Rodent Laboratory Animal Resources (Shanghai, China) and maintained according to the NIH standards established in the Guidelines for the Care and Use of Experimental Animals. All of the experimental protocols were approved by the Animal Investigation Committee of East China Normal University.

Rat Aortic Ring Assay. Rat aortic ring assays were performed as described previously (Pang et al., 2011). In brief, 48-well plates were coated with 120 μL Matrigel per well and polymerized in an incubator. Aortas isolated from Sprague-Dawley rats were cleaned and cut into rings. The aortic rings were randomly distributed into Matrigel-coated wells and sealed with a 100-μL overlay of Matrigel. VEGF, with or without different concentrations of β-lap, was added to the medium. Fresh medium with the respective additives was exchanged every 2 days. After 6 days, microvessel sprouts were fixed and photographed by using an Olympus inverted microscope (×100 magnification). Two independent experiments were performed with triplicates.

Mouse Corneal Micropocket Assay. To evaluate the antiangiogenic properties of β-lap in vivo, the corneal micropocket angiogenesis assay was performed (Benny et al., 2008). Before surgery, 4-week-old C57BL/6 mice were randomly divided into three groups: the control group, the VEGF-alone group, and the β-lap (VEGF plus β-lap) group (n = 10 per group). A micropellet of sucrose aluminum sulfate coated with hydron polymer containing 100 ng VEGF with or without 0.5 μg β-lap per pellet was implanted into micropockets of the mouse cornea. The eyes were photographed with a digital camera on day 6 after pellet implantation. The vascular growth area was measured using a slit lamp. The area of neovascularization was calculated as the vessel area by the product of vessel length measured from the limbus and clock hours around the cornea, using the following equation: vessel area (mm^2) = 3.14 × clock hours × vessel length (mm) × 0.2 (mm).

Xenograft Human Lung Tumor Mouse Model. The xenograft mouse model assay was performed as previously described (Li et al., 2011). Five-week-old male BALB/cA nude mice were randomly divided into separate groups (n = 6 to 7 mice per group). A549 cells (1 × 10^6 per mouse) were subcutaneously injected into the left flank of mice. After tumors grew to approximately 100 mm^3, tumor-bearing mice were treated with or without β-lap–HP-β-CD (25 mg/kg per day) or HP-β-CD via tail-vein injection every other day. The body weight of the mice and the tumor size were recorded. The tumor volume was determined by Vernier caliper measurements and calculated as length × width^2 × 0.52. Mice were euthanized when the tumor burden reached 20% of initial body weight. The images of solid tumors and angiogenesis were photographed with a digital camera. Tumor tissues were removed for histologic examination and snap-frozen in liquid nitrogen for Western blot analysis.

Histology and Immunohistochemistry. All solid tumors were removed, fixed with 10% formaldehyde, and embedded in paraffin for histology and immunohistochemistry (Lifespan Biosciences, Seattle, WA). The anti-CD31, AKT, and CDK4 antibodies were applied to 5-μm tumor sections. Sections were also stained with hematoxylin and eosin. Images were taken using a Leica DM 4000B photomicroscope (Leica, Wetzlar, Germany) at ×400 magnification, and typical images are shown.

Western Blot Analysis. Western blot analysis was performed as described previously (Fukuyo et al., 2008; Chen et al., 2012). Briefly, the whole-cell extracts of treated cells were prepared in radioimmunoprecipitation assay buffer (20 mM Tris, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mM NaF, 10 mM Na_2P_2O_7, and 1 mM phenylmethylsulfonyl fluoride) supplemented with proteinase inhibitor cocktail (EMD Millipore, Billerica, MA). Equivalent amounts of protein measured with Micro BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) were resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Immunoblots were incubated with primary antibodies overnight at 4°C. Detection was performed using goat anti-mouse or goat anti-rabbit IgG-horseradish peroxidase secondary antibody for 1 hour at room temperature and an enhanced chemiluminescence detection system. The relative expression levels were analyzed by Image J software (NIH). The figures are representative of experiments performed at least thrice.

Statistical Analysis. The statistical tests were carried out using Microsoft Excel (Microsoft, Redmond, WA) and GraphPad Prism software (version 5.0; GraphPad Software Inc., La Jolla, CA). Comparisons between groups were performed using one-way analysis of variance followed by the t test. Data are presented as means ± S.D. P values less than 0.05 were considered statistically significant.

Results

β-Lap Induces Hsp90 Cleavage. It has been reported that NQO1 is the principle determinant for β-lap–mediated action (Pink et al., 2000). In our study, we first examined the expression of NQO1 in a heterogeneous panel of cells. Our results showed that most of the cells used in our study expressed a certain amount of NQO1 (Supplemental Fig. 1). PC-3 cells did not express NQO1 protein, which was consistent with a previous report (Planchon et al., 2001). HUVECs, a popular cell model for angiogenesis studies, also expressed NQO1 (Supplemental Fig. 1). When coadministrated with dicoumarol, an NQO1 inhibitor, or partial depletion of NQO1 expression by small interfering RNA, PC-3 cells, and A549 cells showed evident resistance to the treatments (Supplemental Fig. 2, A and B). The microscope images confirmed the results (Supplemental Fig. 2C). These data suggest that NQO1 is critical to β-lap–mediated cytotoxicity in cancer cells.

Given recent findings that highlighted the potential interest of using oxidative stress to target Hsp90 (Beck et al., 2009, 2011a,b, 2012), we asked whether a NQO1-mediated futile redox cycle induced by β-lap could lead to Hsp90 inhibition. In an attempt to address this issue, we examined the effects of β-lap on Hsp90 by Western blotting assays. We found that β-lap apparently triggers the cleavage of Hsp90 in human lung and prostate cancer cells that expressed NQO1, as demonstrated by the appearance of a low molecular mass (approximately 70 kDa) species of the Hsp90 band (Fig. 1A). In contrast, this effect did not occur in PNT1A prostate epithelial cells or WI38 and MRC-5 lung fibroblast cells. Further results showed that β-lap provoked Hsp90 cleavage in a concentration-dependent manner (Fig. 1B, i) and time-dependent manner (Fig. 1B, ii) in treated A549 and PC-3 cells.

It is well known that most of the conventional Hsp90 inhibitors result in Hsp70 overexpression. We asked whether the mechanisms of β-lap in Hsp90 inhibition differed. Western blotting results revealed that β-lap had little effect on the Hsp70 protein level, whereas the conventional Hsp90 inhibitor 17-AAG led to the significantly increased expression.
of Hsp70 (Fig. 1C). These results suggest that the mechanism of \( \beta \)-lap in Hsp90 inhibition is different from that of inhibitors targeting the ATP binding site at the N terminus of Hsp90.

Previous studies demonstrated that \( \beta \)-lap causes NQO1- and Ca\(^{2+} \)-dependent \( \mu \)-calpain activation (Tagliarino et al., 2003; Dong et al., 2010). Calpain inhibitors were also reported to abolish the cleavage of Hsp90 (Bellocq et al., 1999). An independent work additionally reported that Hsp90 is the substrate of granzyme B (Hostetter et al., 2007). These published studies provided the notion that \( \beta \)-lap–mediated Hsp90 cleavage might be caused by the proteases calpain or granzyme B. However, when we treated the cells with the calpain inhibitor calpeptin or the granzyme B inhibitor Ac-IEPD-CHO along with \( \beta \)-lap, we found that neither of them could attenuate the Hsp90 cleavage induced by \( \beta \)-lap. A549 cells were treated with calpeptin (a calpain inhibitor) or Ac-IEPD-CHO (a granzyme B inhibitor) along with \( \beta \)-lap. Hsp90 was probed by Western blot assays. The representative bands from three independent experiments (left) and the relative expression levels analyzed by ImageJ software (right) are shown.

### \( \beta \)-Lap Induces the Proteasomal Degradation of Hsp90-Associated Oncoproteins.

We then investigated whether Hsp90 cleavage by \( \beta \)-lap would lead to the loss of Hsp90 client proteins. We treated A549 and PC-3 cancer cells with the indicated concentrations of \( \beta \)-lap. The results showed that the levels of several oncoproteins such as RIP, Raf-1, AKT, and CDK4 were dramatically decreased (Fig. 2A, i) in lung and prostate cancer cells. Because HUVECs are a unique cell model for angiogenesis studies, we further tested whether \( \beta \)-lap led to the deactivation of Hsp90 and its key client proteins in HUVECs. Similarly, our results showed that \( \beta \)-lap decreased the levels of Hsp90 client proteins such as VEGFR2, Her-2, and CDK4.

**Fig. 1.** \( \beta \)-Lap induces Hsp90 cleavage in a concentration- and time-dependent manner. (A) \( \beta \)-Lap provoked Hsp90 cleavage in NQO1-expressing cancer cells. PNT1A, WI-38, MRC5, PC-3, DU145, A549, NCI-H460, and Calu-1 cells were incubated with 6 \( \mu \)M \( \beta \)-lap for 8 hours. Cell extracts were probed for Hsp90 by Western blot analysis. (B) \( \beta \)-Lap provoked Hsp90 cleavage in a concentration- and time-dependent manner. (i) A549 and PC-3 cells were treated with the indicated concentrations of \( \beta \)-lap (1, 2, 4, and 6 \( \mu \)M) for 8 hours. (ii) A549 and PC-3 cells were exposed to 4 \( \mu \)M \( \beta \)-lap for the indicated time periods (0.5, 1, 2, 4, and 6 hours). Cell extracts were probed for Hsp90 by Western blot analysis. (C) \( \beta \)-Lap did not increase the Hsp70 expression level. A549 cells, PC-3 cells, and HUVECs were treated with 6 \( \mu \)M \( \beta \)-lap for 8 hours or 1 \( \mu \)M 17-AAG for 24 hours. The Hsp70 protein level was examined by Western blot analysis. (D) Inhibition of calpain and granzyme B did not attenuate the Hsp90 cleavage induced by \( \beta \)-lap. A549 cells were treated with calpeptin (a calpain inhibitor) or Ac-IEPD-CHO (a granzyme B inhibitor) along with \( \beta \)-lap. Hsp90 was probed by Western blot assays. The representative bands from three independent experiments (left) and the relative expression levels analyzed by ImageJ software (right) are shown.
in endothelial cells (Fig. 2A, ii). However, at the same treatment condition (β-lap at 6 μM for 8 hours), the mRNA levels of these client oncoproteins in treated cells were not affected (data not shown), indicating that the loss of Hsp90 client proteins by β-lap was instead due to protein degradation.

We thus explored the role of the proteasomal system in the β-lap–induced repression of Hsp90 client proteins. Our results showed that MG132, a specific proteasome inhibitor, evidently prevented the β-lap–mediated decrease of RIP, Raf-1, AKT, CDK4, VEGFR2, and Her-2 in treated cells (Fig. 2B, i and ii), suggesting that the proteasomal pathway was critical in this process. As expected, MG132 could not rescue the cleavage of Hsp90 triggered by β-lap (Supplemental Fig. 3). When comparing the pattern of Hsp90 cleavage with the degradation of Hsp90 client proteins mediated by β-lap, we deemed that both processes may be linked in a cause-effect manner.

Oxidative Stress by β-Lap Induces Hsp90 Cleavage and Destabilizes Hsp90 Client Proteins. Given that β-lap relied on the NQO1-dependent futile redox cycling between the parent β-lap molecule and its hydroquinone form (Planchon et al., 2001), we asked whether oxidative stress is involved in β-lap–mediated cleavage of Hsp90. To address this question, we first monitored intracellular ROS formation in β-lap–treated cancer cells. We found that β-lap markedly induced ROS generation, as indicated by the fluorescence microscopy data (Fig. 3A), whereas NAC, an antioxidant drug, could attenuate β-lap–induced oxidative stress. Next, we examined the levels of Hsp90 and its client proteins in NAC- or dicoumarol-treated A549 cells and HUVECs. We found that NAC or dicoumarol could apparently reduce the cleavage of Hsp90 (Fig. 3B) and restore its client protein degradation (Fig. 3C), suggesting that β-lap–induced Hsp90 cleavage was dependent on redox cycling, and oxidative stress played a principal role in the action of β-lap.

β-Lap Suppresses Angiogenesis In Vitro, Ex Vivo, and In Vivo. Angiogenesis is a critical process for tumor growth and metastasis, but the antiangiogenic activity of β-lap has not yet been clearly reported. Endothelial cells expressed a certain amount of NQO1; therefore, we speculated that β-lap might have the ability to modulate endothelial cell functions. To assess the antiangiogenic property of β-lap in vitro, we first examined its inhibitory effects on cell viability and the capillary-like structure formation of HUVECs. Our results showed that β-lap dose-dependently inhibited the viability of HUVECs (Fig. 4A). The similarity of angiogenesis inhibition was observed in the transwell migration assay (Fig. 4B), wherein β-lap significantly suppressed the invasion of endothelial cells. When HUVECs were seeded on Matrigel-coated plates, a tubule-like network formed, whereas the network organization was easily disrupted by β-lap and most of the endothelial cells became isolated (Fig. 4C). However, in all of these processes, dicoumarol cotreatment evidently reduced the antiangiogenic effect of β-lap, suggesting that NQO1 plays an important role in β-lap’s antiangiogenic properties.

To determine whether β-lap inhibited VEGF-induced angiogenesis ex vivo and in vivo, we examined the sprouting of vessels from aortic rings and the corneal micropocket neovascularization in the presence or absence of β-lap. We found that 2 μM β-lap drastically blocked VEGF-induced microvessel sprouts of rat aortic rings (Fig. 4D). As shown in
the control group of the mouse corneal assay, VEGF alone remarkably stimulated microvessel sprouting and corneal angiogenesis, whereas this effect was completely antagonized by β-lap (Fig. 4E). The quantification results showed that β-lap led to an 81% (0.13 ± 0.14 mm versus 0.70 ± 0.16 mm; P < 0.001) and 92% (0.07 ± 0.09 mm² versus 0.89 ± 0.59 mm²; P < 0.01) inhibition of vessel length and angiogenesis area, respectively, compared with the vehicle group. These results indicate that β-lap functions as an angiogenesis inhibitor and inhibits multiple biologic steps of endothelial cells.

β-Lap Suppresses Tumor Angiogenesis and the Growth of Human Lung Cancer Xenografts. To evaluate the efficacy of β-lap on tumor growth and tumor angiogenesis in vivo, we further transplanted A549 cells into mice and established a human lung cancer xenograft mouse model. Tumor-bearing mice were treated with or without β-lap–HP-β-CD (35 mg/kg per day) or HP-β-CD via tail-vein injection every other day for 22 days. The results showed that the administration of β-lap led to a significant inhibition of tumor volume (Fig. 5A) and tumor weight (Fig. 5B, i), without a noticeable reduction in body weight (data not shown). The average weight of tumors from the control group was 0.668 ± 0.195 g, whereas that of the β-lap–HP-β-CD–treated group was 0.270 ± 0.04 g. Interestingly, we found that tumor growth suppression mediated by β-lap was well correlated with angiogenesis inhibition (Fig. 5B, ii), which was further validated by the decreased CD31 staining in solid tumors (Fig. 5C).

To examine whether the in vivo molecular targets of β-lap were consistent with the in vitro targets, we further performed histochemistry and Western blots in randomly selected tumors of the two groups. As shown in Fig. 5, C and D, β-lap reduced the expression of RIP, AKT, and CDK4. Together, our findings demonstrate that β-lap effectively suppresses both tumor growth and tumor angiogenesis by interfering with Hsp90 chaperone functions in vivo.

β-Lap Provokes Structure-Specific Hsp90 Cleavage. In our study, we systematically evaluated the cytotoxic and antiangiogenic properties of β-lap in vitro and in vivo. Because of the quinone dependence of the NQO1-mediated redox reaction, we evaluated the effect of quinone inhibition on Hsp90 as well. Interestingly, unlike other NQO1-dependent cytotoxic quinones, such as streptonigrin, menadione, mitomycin, and 17-AAG, β-lap was the only agent that caused Hsp90 cleavage. This effect of β-lap was similar to that of the free radical–generating agent H2O2 (Fig. 6), indicating that the extensive ROS generated by the futile redox cycling of β-lap is fairly potent. These results indicated that the function of β-lap to trigger Hsp90 cleavage is strictly dependent on its chemical structure.

Discussion

In this study, we demonstrated that β-lap, a clinical anticancer drug, markedly inhibited tumor growth and angiogenesis in vitro and in vivo. We observed no significant body weight loss or other gross toxicity in the cohorts receiving
β-lap, suggesting few on- or off-target toxic effects. Mechanistically, β-lap led to the cleavage of Hsp90 in cancer cells and endothelial cells that expressed NQO1. This effect was well correlated with the decrease of Hsp90 client proteins, including RIP, Raf-1, CDK4, Akt, VEGFR2, and Her-2. Our findings offer a novel anticancer mechanism of β-lap.

β-Lap is bioactivated by NQO1, creating a "futile redox" cycle. By generating high levels of superoxide, β-lap causes programmed necrosis (called "necroptosis") in cancer cells (Bey et al., 2007; Dong et al., 2010). In this study, we showed for the first time that β-lap provokes the cleavage of Hsp90, leading to the degradation of various Hsp90 client oncoproteins in vitro and in vivo. Unlike other NQO1-dependent quinones, the efficacy of β-lap’s "futile cycle" was relatively potent, wherein approximately 60 mol NAD(P)H was used per mole of drug in 3 minutes with dramatic release of ROS. In an intact cell, this futile cycle will be expected to continue until one of the critical components of the reaction is exhausted, which is why β-lap was the only agent that caused Hsp90 cleavage in our experimental system (Fig. 6). There are two molecular characteristics of β-lap–mediated cleavage of Hsp90. First, the stimulation of β-lap is a rapid process that leads to more potent anticancer activity than the conventional Hsp90 inhibitors. In a heterogeneous panel of prostate cancer (Dong et al., 2010) and lung cancer cells that express high levels of endogenous NQO1, exposure to β-lap (6 μM) for 2 hours was sufficient to achieve maximal cytotoxicity. By contrast, a 24-hour treatment of 17-AAG (10 μM) was required to reduce cell viability by 50%–60% in various tumor cells (Plescia et al., 2005). Second and importantly, β-lap–induced Hsp90 cleavage is not accompanied by an increase of Hsp70 protein. A similar mechanism could also be observed in the anticancer action of other agents (Gyurkocza et al., 2006; Beck et al., 2009). Although increased Hsp70 expression was considered a surrogate biomarker of Hsp90 inhibition in clinical trials (Grem et al., 2005), increased Hsp70 expression...
might be detrimental to the anticancer activity of 17-AAG because Hsp70 can inhibit death receptor– and mitochondria-initiated signaling for apoptosis (Guo et al., 2005; Cervantes-Gomez et al., 2009). The fact that β-lap does not affect the Hsp70 level may be due to the extremely rapid induction of cell death in cells expressing elevated NQO1. Together with other

![Image of A549 Xenografts](image1)

**Fig. 5.** β-Lap suppresses the angiogenesis and growth of human lung cancer xenografts in vivo. Five-week-old male BALB/cA nude mice were randomly divided into separate groups (n = 6 to 7 each group). A549 cells were subcutaneously injected into the left flank of mice. After tumors grew to approximately 100 mm³, tumor-bearing mice were treated with or without β-lap–HP–β-CD (25 mg/kg per day) or HP–β-CD via tail-vein injection every other day. (A) β-Lap inhibited the tumor volume of A549 xenografts. Dots indicate the mean; bars indicate the S.E. (B) β-Lap led to reduced tumor weight and angiogenesis. (i) β-Lap reduced the tumor weight. Columns indicate the mean; bars indicate the S.E. (P < 0.01 versus the vehicle group). (ii) β-Lap inhibited neovascularization in tumor xenografts. (C) Hematoxylin and eosin (H&E) staining and immunohistochemical staining of CD31, AKT, and CDK4 on tumor sections. Representative images from three random solid tumors in each group are shown. (D) Western blotting results of RIP, AKT, and CDK4 expression. Three random tumors were selected in each group and the corresponding protein was applied to Western blot analysis. The representative blots (left) and the relative band density (right) are shown. Original magnification, ×400 in (C).

![Image of β-Lap leads to structure-specific cleavage of Hsp90](image2)

**Fig. 6.** β-Lap leads to structure-specific cleavage of Hsp90. A549 cells were treated with β-lap (6 μM for 8 hours), streptonigrin (STN; 0.4 μM for 2 hours), menadione (MD; 10 μM for 4 hours), mitomycin (MMC; 10 μM for 2 hours), and 17-AAG (1 μM for 24 hours). Free radical–generating agent H₂O₂ (2 mM for 2 hours) served as the control. Cell extracts were immunoblotted with Hsp90 antibody. Images (left) and the relative band density (right) are shown. Data were from three independent experiments.
studies, our results definitely support the statement that Hsp90 represents a new target for oxidant-based anticancer therapies (Clark et al., 2009; Beck et al., 2011a, 2012).

In this study, we further deciphered the preclinical efficacy of β-lap. First, our results demonstrate that β-lap’s anticancer function occurs in an NQO1-dependent manner, which is consistent with previous studies (Bey et al., 2007; Dong et al., 2010). Our study and others revealed that β-lap can effectively kill NQO1-overexpressing cancer cells while causing minimal effects on neighboring cells that have low enzyme levels or lack the enzyme (Li et al., 2011). Second, β-lap–induced inhibition of Hsp90 chaperone activity and the destabilization of its client proteins are dependent on oxidative stress. Cancer cells are often deficient in antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase (Sun et al., 1993), and they exhibit higher basal levels of ROS than their normal counterparts. Therefore, inducing a selective oxidative stress should be a promising and specific approach for cancer treatment. In our study, we found that the redox-modulator NAC evidently reduced the β-lap–triggered Hsp90 cleavage, indicating that oxidative stress induced by β-lap played a pivotal role in this process. The greater sensitivity toward the β-lap–induced oxidative stress was certainly one element that explained the preferential cleavage of Hsp90 in these cells. In addition, Hsp90 itself is more susceptible to oxidative degradation in cancer cells (Workman, 2004b; Trepel et al., 2010). These unique properties of β-lap collectively participated in its exciting antitumor efficacy in our preclinical mouse model in vivo. A recent report documented that oxidative stress generated by ascorbate-driven menadione redox cycling led to Hsp90 cleavage, and the cleavage occurred in a conserved motif of the N-terminal nucleotide binding site, between Ile-126 and Gly-127 in Hsp90α, and between Ile-131 and Gly-132 in Hsp90β, as determined by mass spectrometry analysis (Beck et al., 2012). Because of the similarity of the ROS release pattern between ascorbate/menadione redox cycling and β-lap’s futile cycling, we assume that Hsp90 cleavage induced by β-lap may also occur in the N-terminal nucleotide binding site.

In addition to the molecular mechanism of β-lap action identified in this study, we demonstrated that β-lap suppresses tumor angiogenesis. As depicted in Fig. 4, β-lap inhibited multiple key cellular functions required for angiogenesis. β-lap significantly induced Hsp90 cleavage and reduced the expression of critical Hsp90 client oncoproteins such as VEGFR2, Her-2, AKT, and CDK4 in HVUVCs in an NQO1- and oxidative stress–dependent manner. Because angiogenesis is the rate-limiting process for tumor growth and metastasis, the negative effect of β-lap on tumor angiogenesis definitely potentiates its anticancer efficacy. Based on the results obtained from our human lung cancer xenograft mouse model, we believe that β-lap targets multiple pivotal points in both tumor cells and endothelial cells, providing a combinatorial blockade of the hallmark phenotypic features of malignancy. Recently, promising activity was reported with 17-AAG in combination with trastuzumab in HER2-positive breast cancer refractory to trastuzumab therapy (Scaltriti et al., 2011), indicating that Hsp90 inhibitors may overcome drug resistance. By considering the low toxicity of β-lap in clinical trials, and on the basis of preclinical results that we obtained in our xenograft mice, we postulate that the combination of β-lap with a molecule-targeted agent, such as the epidermal growth factor receptor inhibitor gefitinib, the HER2 inhibitor trastuzumab, or the VEGF inhibitor bevacizumab, should have a greater therapeutic potential in clinical applications.

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**Authorship Contributions**

**Participated in research design:** Wu, Pang.

**Conducted experiments:** Wu, Wang, Chang, Lu.

**Contributed new reagents or analytic tools:** Lu.

**Performed data analysis:** Wu, Wang, Pang.

**Wrote or contributed to the writing of the manuscript:** Wu, Liu, Pang.

**References**


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