Development of β-Lapachone Prodrugs for Therapy Against Human Cancer Cells with Elevated NAD(P)H:Quinone Oxidoreductase 1 Levels

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Abstract β-Lapachone, an *o*-naphthoquinone, induces a novel caspase- and p53-independent apoptotic pathway dependent on NAD(P)H:quinone oxidoreductase 1 (NQO1). NQO1 reduces β-lapachone to an unstable hydroquinone that rapidly undergoes a two-step oxidation back to the parent compound, perpetuating a futile redox cycle. A deficiency or inhibition of NQO1 rendered cells resistant to β-lapachone. Thus, β-lapachone has great potential for the treatment of specific cancers with elevated NQO1 levels (e.g., breast, non–small cell lung, pancreatic, colon, and prostate cancers). We report the development of mono(arylimino) derivatives of β-lapachone as potential prodrugs. These derivatives are relatively nontoxic and not substrates for NQO1 when initially diluted in water. In solution, however, they undergo hydrolytic conversion to β-lapachone at rates dependent on the electron-withdrawing strength of their substituent groups and pH of the diluent. NQO1 enzyme assays, UV-visible spectrophotometry, high-performance liquid chromatography-electrospray ionization-mass spectrometry, and nuclear magnetic resonance analyses confirmed and monitored conversion of each derivative to β-lapachone. Once converted, β-lapachone derivatives caused NQO1-dependent, μ-calpain-mediated cell death in human cancer cells identical to that caused by β-lapachone. Interestingly, coadministration of *N*-acetyl-L-cysteine prevented derivative-induced cytotoxicity but did not affect β-lapachone lethality. Nuclear magnetic resonance analyses indicated that prevention of β-lapachone derivative cytotoxicity was the result of direct modification of these derivatives by *N*-acetyl-L-cysteine, preventing their conversion to β-lapachone. The use of β-lapachone mono(arylimino) prodrug derivatives, or more specifically a derivative converted in a tumor-specific manner (i.e., in the acidic local environment of the tumor tissue), should reduce normal tissue toxicity while eliciting tumor-selective cell killing by NQO1 bioactivation.

β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-*b*]-pyran-5,6-dione) has demonstrated significant antitumor activity against sarcoma 180 cells *in vitro* (1). Further studies show that β-lapachone elicits significant antitumor activity against Yoshida sarcoma, Walker 256 carcinoma cells (2), and Rauscher leukemia (3) in mice. Data showing activity against a variety of tumors have revitalized interest in this drug (1, 3–5).

Previous studies from our laboratory revealed that β-lapachone was "bioactivated" by the two-electron oxidoreductase, NAD(P)H:quinone oxidoreductase 1 (NQO1; EC 1.6.99.2; ref. 6). Structural similarities between β-lapachone and other members of the naphthoquinone family, such as menadione (vitamin K3, 2-methyl-1,4-naphthoquinone), suggested that NQO1 may be involved in the activation or detoxification of this drug (7–11). Furthermore, the ionizing radiation–inducible properties of NQO1 (i.e., xip3) were consistent with this compound's ability to radiosensitize various human cancer cells (12). We subsequently discovered that NQO1 was the primary intracellular determinant of β-lapachone cytotoxicity (6, 13) and noted that all NQO1$^+$ cell lines examined were more sensitive to β-lapachone. Furthermore, the cytotoxicity of β-lapachone was prevented by coadministration of dicoumarol (a NQO1 inhibitor), and cells lacking NQO1 were inherently more resistant than NQO1-expressing cells. In contrast, NQO1-expressing cancer cells were resistant to menadione, and coadministration of dicoumarol potentiated menadione lethality.

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We showed previously that β-lapachone killed cells through a unique apoptotic pathway. Cell death caused by many chemotherapeutic agents typically involves activation of cysteine proteases (e.g., caspases; refs. 14, 15). Apoptosis induced by β-lapachone in NQO1$^+$ human breast or prostate cancer cell lines was unique, causing a pattern of poly(ADP-ribose) polymerase (PARP) and p53 proteolysis distinct from that caused by other agents (e.g., staurosporine) that caused cell death through activation of caspases (6, 13, 16–18). We noted concomitant atypical PARP (yielding an ~60-kDa fragment) and p53 (yielding an ~40-kDa fragment) cleavage in β-lapachone-treated NQO1$^+$ cells caused by activation of the neutral Ca^{2+}-dependent cysteine protease, μ-calpain (18–20). Certain aspects of β-lapachone cytotoxicity were unique (e.g., atypical PARP and p53 proteolysis), whereas other aspects conformed to classic apoptosis (e.g., lamin B proteolysis, DNA condensation, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive cells, sub-G_0-G_1 cells, and trypan blue exclusion; ref. 13).

Although β-lapachone is ideally suited for use as an antitumor agent because it targets an enzyme elevated in various tumors (21–24), concerns over normal tissue toxicity remain. These concerns could be avoided by the development of a β-lapachone prodrug that would favor conversion to β-lapachone in the microenvironment of the tumor and less so in normal tissues. β-Lapachone holds great promise as an antitumor agent because it (*a*) is "bioactivated" by NQO1 through a futile redox cycle, and NQO1 levels are elevated in numerous cancers (21–24); (*b*) kills independent of cell cycle, caspase, pRb, or p53 status; and (*c*) synergistically kills cells in combination with chemotherapeutic agents as well as ionizing radiation (25). Ideally, β-lapachone prodrugs would become active in a tumor-selective or spontaneous manner with identical or more potent antitumor activity than β-lapachone. The active form of such prodrugs would kill by the same NQO1-dependent manner but avoid normal tissue toxicity depending on their mechanism and rate of activation.

We report that biologically inactive mono(arylimino) derivatives of β-lapachone seem to act as Schiff's bases and are converted to β-lapachone through a spontaneous hydrolytic reaction. The rates of hydrolysis of β-lapachone derivatives varied as a function of the strength of the electron-withdrawing substituent groups in the *para* position of the mono(arylimino) leaving group as well as the pH of the solution (Fig. 1, R group; Supplementary Fig. 1). Thus, mono(arylimino) derivatives may represent usable nontoxic, inactive, and pH-sensitive (26–30) precursors to β-lapachone.

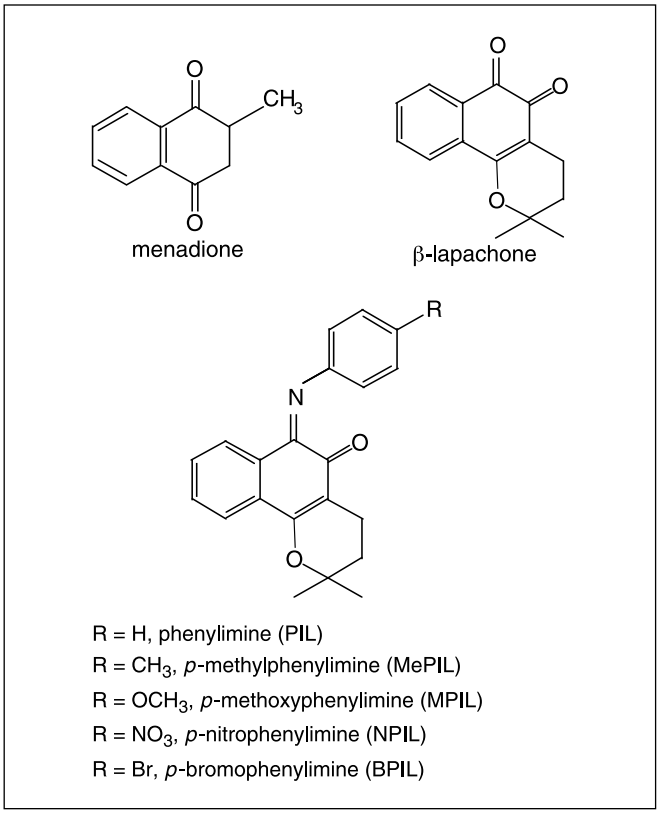

Fig. 1. Structures of β-lapachone derivatives. Structures for menadione, β-lapachone, PIL, MePIL, MPIL, NPIL, and BPIL.

Materials and Methods

Reagents. β-Lapachone was synthesized by us, confirmed by nuclear magnetic resonance (NMR), dissolved in DMSO at 40 mmol/L, and concentrations verified by spectrophotometry (13, 16). β-Lapachone stock solutions were stored at −80°C until used. β-Lapachone mono(arylimino) derivatives [phenylimine lapachone (PIL), 2,2-dimethyl-(*Z*)-6-phenylimino-3,4,5,6-tetrahydro-2H-naphtho[1,2-*b*]oxin-5-one; *p*-methylphenylimine lapachone (MePIL), 2,2-dimethyl-(*Z*)-6-(4-methyl-phenylimino)-3,4,5,6-tetrahydro-2H-naphtho[1,2-*b*]oxin-5-one; *p*-methoxyphenylimine lapachone (MPIL), 2,2-dimethyl-(*Z*)-6-(4-methoxyphenylimino)-3,4,5,6-tetrahydro-2H-naphtho[1,2-*b*]oxin-5-one; and *p*-nitrophenylimine lapachone (NPIL), 2,2-dimethyl-(*Z*)-6-(4-nitrophenylimino)-3,4,5,6-tetrahydro-2H-naphtho[1,2-*b*]oxin-5-one] were synthesized as described (31). *p*-Bromophenylimine lapachone (BPIL), 2,2-dimethyl-(*Z*)-6-(4-bromophenylimino)-3,4,5,6-tetrahydro-2H-naphtho[1,2-*b*]oxin-5-one, was prepared from 4-bromoaniline and β-lapachone as described previously (31), with 90% yield as a brown solid that was recrystallized from cyclohexane. ^{1}H NMR ($CDCl_3$, 200 MHz): 8.19 (dd, *J* = 7.1 and 1.7, 1H, H-7), 7.83 (dd, *J* = 7.1 and 1.7, 1H, H-10), 7.50 (m, 2H, H-8, 9), 7.43 (d, *J* = 8.8, 2H, H-2′), 6.62 (d, *J* = 8.8, 2H, H-3′), 2.41 (t, *J* = 6.7, 2 H, H-4), 1.79 (t, *J* = 6.7, 2 H, H-3), 1.44 (s, 6 H, 2-CH3); ^{13}C NMR ($CDCl_3$, 50 MHz): 177.5 (C-5), 161.6 (C-10b), 152.1 (C-1′), 150.9 (C-6), 132.4 (C-10a), 131.7 (C-3′), 131.3 (C-8), 130.2 (C-9), 130.0 (C-6a), 127.0 (C-7), 123.3 (C-10), 117.6 (C-2′), 115.5 (C-4′), 111.9 (C-4a), 78.5 (C-2), 31.7 (C-3), 26.7 (2-CH_3), 16.0 (C-4). Seventy EV electron impact ionization mass spectroscopy, *m/z* (%): 397 (M^+ + 2, 43), 395 (M +, 43), 316 (48), 260 (93), 232 (100), 203 (35), 76 (32), 41 (37). Aryliminoquinones were dissolved in DMSO at 50 mmol/L. Menadione was obtained from Sigma Chemical Co. (St. Louis, MO), dissolved in DMSO at 100 mmol/L, and stored at −80°C. Drugs [β-lapachone, menadione, and β-lapachone mono(-arylimino) derivatives] were added to medium at a 1:1,000 dilution immediately before administration to cells; control cells received ≤0.1% DMSO for vehicle alone treatment. Dicoumarol (Sigma Chemical) was used as described (6). *N*-acetyl-L-cysteine (NAC; Sigma Chemical) was dissolved in water, pH adjusted to 7.4 using NaOH, and stored at 4°C. Acetonitrile (high-performance liquid chromatography grade), methanol (high-performance liquid chromatography grade), and DMSO (ACS Certified Spectranalyzed grade) were purchased from Fisher Scientific (Pittsburgh, PA). Ammonium formate was purchased from Sigma Chemical. Medipure-grade liquid nitrogen was obtained in high pressure, gas-withdrawal containers from Praxair, Inc. (Cleveland, OH) for use as the sheath and auxiliary gas in the electrospray ion source of the mass spectrometer.

Argon (5.0 ultrahigh purity grade) was obtained from Praxair for use as the collision gas during tandem mass spectrometric experiments.

Cell culture. NQO1-deficient (NQO1$^-$) MDA-MB-468 and MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA) and stably transfected with the cytomegalovirus-driven human NQO1 cDNA in the pcDNA3 mammalian expression vector as described (6). NQO1$^+$ 468-NQ3 and 231-NQ6 as well as NQO1$^-$ vector-alone 468-Vec4 and 231-NQ2 clones were isolated; 231-NQ2 did not express NQO1 protein or enzyme levels. MCF-7:WS8 (MCF-7) cells were obtained and grown as described (6). A549 cells were obtained

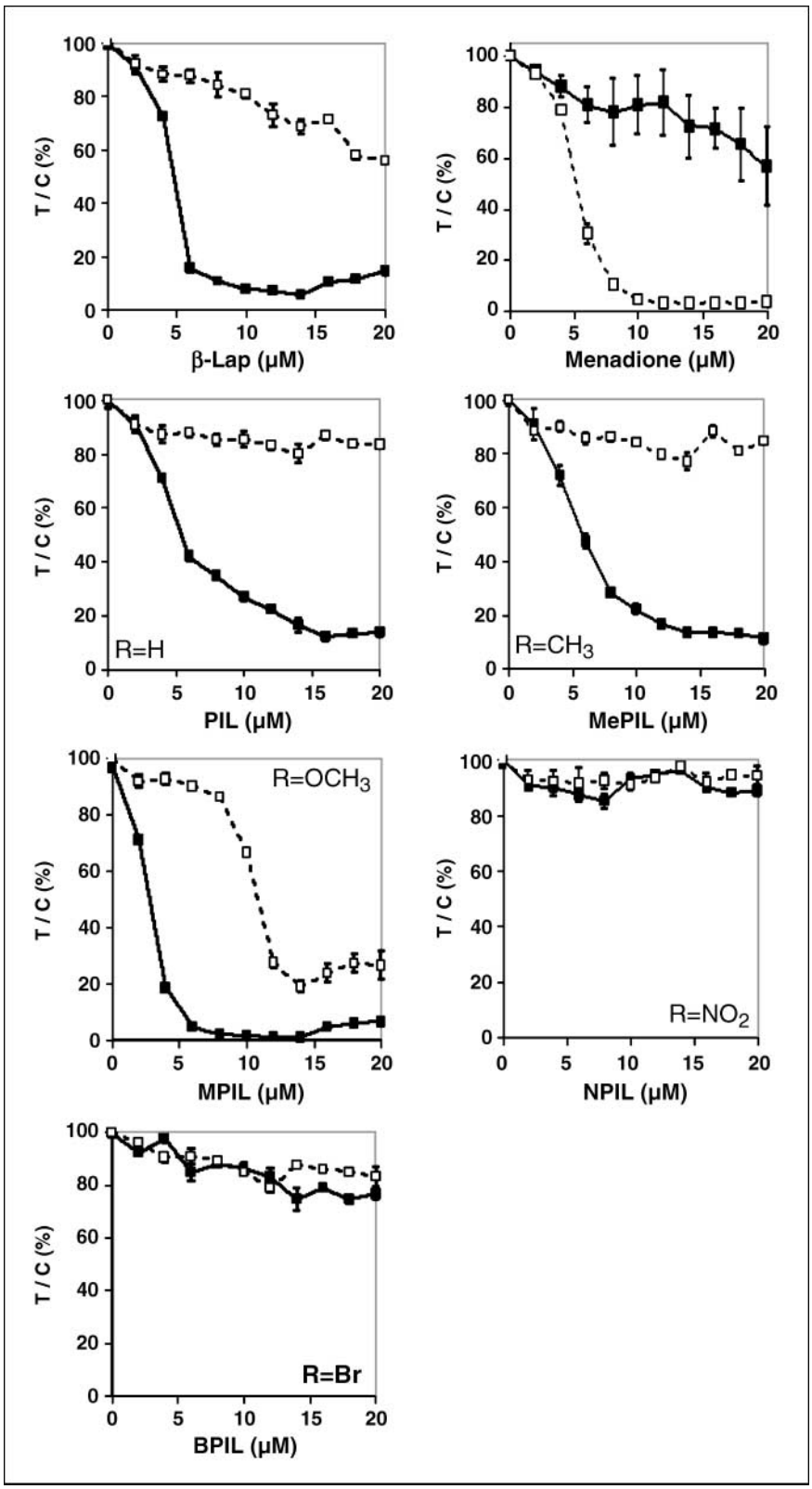

Fig. 2. β-Lapachone (*β-Lap*) mono(arylimino) derivatives resemble β-lapachone in mediating NQO1-dependent cytotoxicity. NQO1$^+$ 231-NQ6 cells were seeded into 48-well plates (1×10^4 cells per well) and allowed to attach overnight. Medium containing various drug concentrations (β-lapachone, menadione, PIL, MePIL, MPIL, NPIL, or BPIL as indicated) either alone (■ and *solid lines*) or coadministered with 40 μmol/L dicoumarol (□ and *dashed lines*) was added for 4 hours. Medium was then removed, fresh drug-free medium was added, and cells were monitored for changes in relative survival. Relative DNA content per well was determined by Hoechst dye fluorescence, and relative growth (T/C) was plotted as described in Materials and Methods. Points, mean of three independent wells from three independent experiments; *bars*, SE.

from Dr. Steven Dubinett (University of California-Los Angeles Medical Center, Los Angeles, CA) and grown as described below. PC-3 cells were obtained and grown as described (32). All cells were grown in RPMI 1640 supplemented with 200 μg/mL geneticin (except for parental MCF-7 and A549 cells), 5% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin (13, 16) in a 37°C humidified incubator with a 5% CO_2, 95% air atmosphere (13, 16). All cells were routinely monitored for, and were free of, *Mycoplasma* contamination (6).

Cell growth assays. Cell growth was assessed using DNA content (6, 13). Briefly, PC-3 (seeded at 5×10^3 per well), A549, MCF-7, 231-NQ6, 231-NQ2 (seeded at 1×10^4 per well), 468-Vec4, or 468-NQ3 (seeded at 4×10^4 per well) cells were plated in 48-well plates and allowed to attach overnight. Cells were then treated with 4-hour pulses of drug [0-20 μmol/L β-lapachone, menadione, or β-lapachone mono(arylimino) derivatives; Fig. 2] alone or in conjunction with 40 μmol/L dicoumarol or 5.0 mmol/L NAC; 40 μmol/L dicoumarol prevented NQO1 activity in all cells examined, and NAC was used to scavenge reactive oxygen and nitrogen species (33). Medium was removed, fresh medium was added, and cells were grown for at least 7 days. DNA content was determined by fluorescence using a Perkin-Elmer HTS 7000 Bio-Assay Reader microtiter plate reader (Boston, MA) (6), and data were expressed as relative growth [treated versus control (T/C)] from three wells per treatment. Each experiment was repeated at least thrice, and data were expressed as mean ± SE. Prior analyses with β-lapachone and other β-lapachone analogues showed that cytotoxicity monitored by growth assays correlated directly with changes monitored by colony forming ability assays (6).

UV-visible spectrophotometric analyses. UV-visible spectrophotometric analyses of β-lapachone mono(arylimino) derivative hydrolysis in water were done in the presence or absence of 5 mmol/L NAC. Analyses of MPIL hydrolysis at varied pH were done in 50 mmol/L Tris-HCl buffer at pH 6, 7, or 8 and the ratio of peak absorbance at 258 and 289 nm was plotted over time as a measure of MPIL hydrolysis to β-lapachone. Analyses were made using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA) scanning 200 to 500 nm wavelengths. Stock solutions (50 mmol/L) of each β-lapachone mono(arylimino) derivative dissolved in DMSO were diluted to 1 mL with water and subjected to UV-visible wavelength scans every 30 minutes for 4 hours. Scans were compared with β-lapachone.

NAD(P)H:quinone oxidoreductase 1 enzyme assays. S9 supernatants were prepared as described (6), aliquoted, and stored at −80°C before analysis. NQO1 enzymatic activities were measured using cytochrome *c* (practical grade; Sigma Chemical) in Tris-HCl buffer (50 mmol/L, pH 7.5; ref. 6). NADH (200 μmol/L) was the immediate electron donor, and β-lapachone, menadione, or β-lapachone mono(arylimino) derivatives (10 μmol/L) were intermediate electron acceptors as monitored using a Beckman DU 640 spectrophotometer. Activities attributed to NQO1 were levels inhibited by 10 μmol/L dicoumarol (34) and calculated as nmol cytochrome *c* reduced/min/μg protein based on initial rate of change in absorbance at 550 nm and an extinction coefficient for cytochrome *c* of 21.1 mmol/L/cm. Results are average enzyme activities for three separate cell extracts ± SD.

NADH recycling assays. NADH recycling assays were done using NQO1-containing S9 extracts from MCF-7 cells (6). NADH oxidation was monitored by changes in absorbance at 340 nm measured for 5 minutes with a Beckman DU 640 spectrophotometer. Protein concentrations were determined by Bradford assays (6). Enzyme activities were expressed as T/C where mol NADH reduced in 5 min/mol substrate [β-lapachone, menadione, or mono(arylimino) derivative] added per μg protein are compared with mol NADH reduced in 5 min/mol menadione added per μg protein.

Western blot analyses. Western blot analyses of DMSO control or drug-treated MCF-7 cells were done as described (13, 16, 18). Loading equivalence and transfer efficiency were monitored by Western blot analyses of proteins not altered in cells following β-lapachone exposure as described (13). Dilutions of 1:2,000 for α-PARP SC-8007 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 1:2,000 for α-p53 D01 antibody (Santa Cruz Biotechnology) were used to monitor apoptotic proteolyses of these proteins.

^{1}H nuclear magnetic resonance spectroscopy. For pure compound spectra, MPIL or NAC (1.0 mg) was dissolved in deuterated DMSO (DMSO-d^6) before acquiring NMR spectra. For 1:1 molar ratio MPIL:NAC spectra, MPIL (2.9 mol) was dissolved in DMSO-d^6, diluted into water containing 2.9 mol NAC, and incubated at room temperature for ≥4 hours. For MPIL hydrolysis analysis, MPIL (1 mg) was dissolved in DMSO-d^6, diluted into water, and allowed to incubate at room temperature for ≥4 hours. The water was evaporated to a volume of 7 mL. All samples were filtered (0.2 μm nylon syringe filter), freeze-dried in a Labconco Freezone 4.5 lyophilizer (Kansas City, MO), and redissolved in 1 mL DMSO-d^6 before acquiring NMR spectra. All ^{1}H NMR spectra were recorded on a 200 MHz Varian Gemini Fourier transform NMR spectrometer (Palo Alto, CA).

High-performance liquid chromatography-electrospray ionization-mass spectrometry measurement of β-lapachone derivative hydrolysis. β-Lapachone mono(arylimino) derivative stock solutions in DMSO ($\sim 3 \times 10^{-3}$ mol/L) were diluted 1:100 (v/v) in methanol. Methanolic working solutions were diluted immediately 1:100 (v/v) in water contained by autosampler vials. Aliquots (5 μL) were injected into the instrument at precisely measured intervals of ~1 hour for 30 hours. Chromatographic separations were done using reverse-phase Polaris C18-A (3 μm particle diameter) medium contained in a 0.46 cm i.d. × 5.0 cm liquid column (Ansys Metachem, Lake Forest, CA). The chromatographic mobile phase consisted of aqueous ammonium formate (2.5×10^{-2} mol/L, pH not adjusted)/acetonitrile (30:70, v/v). Column temperature and mobile phase flow rate were 40°C and 0.5 mL/min, respectively. The mass spectrometer was operated with heated capillary and analyzer manifold temperatures of 350°C and 70°C, respectively. Ion source sheath gas pressure was 80 p.s.i.g., and the auxiliary gas flow rate was set to 40 dimensionless units. The instrument was operated in positive ion mode, the ion source electrospray potential was 3.5 kV, and the collision cell argon pressure was 2.0×10^{-5} mTorr. β-Lapachone and β-lapachone mono(arylimino) derivatives ionized readily as positively charged proton adducts and underwent a characteristic collision-induced loss of *m/z* 56 in the mass spectrometer. Transitions were monitored [precursor ion (*m/z*), product ion (*m/z*), and collision cell offset potential (V)]: β-lapachone (243, 187, −24), PIL (318, 262, −25), MePIL (332, 276, −25), MPIL (348, 292, −25), NPIL (363, 307, −25), and BPIL (396, 340, −25). Acquisition dwell time and product ion scan width were 0.1 second and *m/z* 1.0, respectively, for all monitored transitions. Chromatographic peaks were observed for β-lapachone, PIL, MePIL, MPIL, NPIL, and BPIL at 2.0, 5.1, 6.3, 4.6, 5.1, and 7.8 minutes, respectively. β-Lapachone mono(arylimino) derivative concentration versus time data sets was modeled as an irreversible first-order process according to the equation:

$$C(t)_{\text{prodrug}} = C(0)_{\text{prodrug}} \exp(-k_1 * t),$$

where k_1 is the first-order rate constant and $C(t)_{\text{prodrug}}$ and $C(0)_{\text{prodrug}}$ are the concentrations of the prodrug at time *t* and 0, respectively. The production of β-lapachone during β-lapachone mono(arylimino) derivative hydrolysis was modeled as an irreversible first-order process according to the equation:

$$C(t)_{\beta-\text{lapachone}} = C(0)_{\text{prodrug}}(1 - \exp(-k_2 * t)),$$

where k_2 is the first-order formation constant of β-lapachone and $C(t)_{\beta\text{-lapachone}}$ is the concentration of β-lapachone at time *t*, respectively.

Results

Phenylimine lapachone, p-methylphenylimine lapachone, and methoxyphenylimine lapachone β-lapachone mono(arylimino) derivatives mimic β-lapachone cytotoxicity. Various mono(arylimino) substituted β-lapachone derivatives were synthesized

Table 1. Comparison of lethality of β-lapachone analogues in NQO1$^+$ and NQO1$^-$ MDA-MB-231 breast cancer cells by LD_{50} values (μmol/L)

	Compound alone		Compound + dicoumarol		Compound + NAC	
Compound	NQO1^{+*}	NQO1$^{-\dagger}$	NQO1$^+$	NQO1$^-$	NQO1$^+$	NQO1$^-$
β-Lapachone	6.6 ± 1.4[‡]	14.2 ± 2.1	26 ± 0.5	30 ± 0.5	5.5 ± 0.4	15.3 ± 2.1
PIL	5.9 ± 1.3	16.7 ± 1.8	21 ± 0.5	29 ± 0.5	25 ± 0.5	29 ± 0.5
MePIL	5.7 ± 1.0	15.3 ± 2.3	30 ± 1.0	40 ± 0.5	15 ± 0.5	40 ± 0.5
MPIL	3.7 ± 0.5	9.7 ± 1.3	17.8 ± 1.9	18.0 ± 1.6	8.5 ± 0.4	15.3 ± 1.6
NPIL	>50[§]	>50	>50	>50	>50	>50
BPIL	>50	>50	>50	>50	>50	>50

NOTE: Log-phase cells were treated with a 4-hour pulse of compound (with or without 40 μmol/L dicoumarol or 5 mmol/L NAC). Compound medium was replaced with fresh medium and the cells were allowed to grow for 7 days. Relative DNA per well was determined by Hoechst fluorescence, and relative growth (T/C DNA) was plotted as described in Materials and Methods.

*NQO1$^+$ MDA-MB-231 cells generated as described in Materials and Methods.

†NQO1$^-$ MDA-MB-231 cells generated as described in Materials and Methods.

‡Values were determined from relative growth assays done at least thrice in triplicate.

§Values of >50 μmol/L LD_{50} indicate <50% lethality at 50 μmol/L drug, the upper limit of toxic doses used in assaying that drug.

(31) with leaving groups of different electron-withdrawing strengths (Fig. 1). Because NQO1 reduces quinones (in either *para* or *ortho* position; ref. 31), mono(arylimino) derivatives of β-lapachone were not expected to be substrates for NQO1, nor were they expected to kill NQO1$^+$ human cancer cells as noted with β-lapachone (6). Relative growth assays using NQO1$^+$ (e.g., 468-NQ3, 231-NQ6, MCF-7, A549, and PC-3 cells) compared with NQO1$^-$ (e.g., 468-Vec4, and 231-NQ2) cells were done for each β-lapachone derivative. Log-phase cells were exposed for 4 hours to various concentrations of β-lapachone alone, menadione alone, each β-lapachone mono(arylimino) derivative alone, or each compound in combination with 40 μmol/L dicoumarol (Fig. 2). β-Lapachone mono(arylimino) derivatives PIL, MePIL, and MPIL were as selectively toxic to NQO1$^+$ breast cancer cells as β-lapachone, with comparable LD_{50} values of 5.9 ± 1.3, 5.7 ± 1.0, 3.7 ± 0.5, and 6.6 ± 1.4 μmol/L, respectively (Fig. 2; Table 1). In contrast, NPIL and BPIL were significantly less toxic, with LD_{50} values of >50 μmol/L (Fig. 2; Table 1). Dicoumarol prevented PIL, MePIL, and MPIL cytotoxicities in a manner similar to that observed with β-lapachone in NQO1$^+$ breast cancer cells, increasing LD_{50} values to 21 ± 0.5, 30 ± 1.0, 17.8 ± 1.9, and 26 ± 0.5 μmol/L, respectively (Table 1). MPIL was not as toxic as β-lapachone in NQO1$^+$ lung cancer (A549) cells, with LD_{50} values of 8.1 ± 0.2 and 5.3 ± 0.6 μmol/L, respectively (Supplementary Table 1). MPIL was also not as toxic as β-lapachone in NQO1$^+$ prostate cancer (PC-3) cells, with LD_{50} values of 6.5 ± 0.3 and 3.5 ± 0.5 μmol/L, respectively (Supplementary Table 1). Dicoumarol prevented MPIL cytotoxicities in both A549 and PC-3 cells, increasing LD_{50} values to >50 μmol/L (Supplementary Table 1). NQO1$^-$ 468 and 231 cells were significantly more resistant to all drugs examined (Table 1). Overall, these data suggested that β-lapachone mono(arylimino) derivatives may be converted to β-lapachone or β-lapachone-like compounds to kill cancer cells in an NQO1-dependent manner.

Evidence for conversion of β-lapachone mono(arylimino) derivatives to β-lapachone. Direct data supporting the hypothesis that β-lapachone mono(arylimino) derivatives were converted to β-lapachone were then sought. Stock solutions of β-lapachone mono(arylimino) derivatives in DMSO were diluted in water (15:10,000) and subjected to UV-visible spectrophotometric wavelength scans of 200 to 500 nm every 30 minutes for 4 hours, representing the time of drug exposure used in cytotoxicity experiments (Fig. 2). The UV-visible spectra of β-lapachone mono(arylimino) derivatives PIL, MePIL, and MPIL significantly changed, ultimately resembling the spectra of β-lapachone (Supplementary Fig. 2A-C). In contrast, the β-lapachone spectra remained unaltered over the 4-hour period. In contrast, scans of NPIL and BPIL [less toxic derivatives (Fig. 2)] showed a time-dependent change in their spectra, yet the resulting spectra did not resemble the spectra of β-lapachone (Supplementary Fig. 2D and E), suggesting that these compounds did not convert to β-lapachone.

To determine the effect of pH on the hydrolysis of β-lapachone mono(arylimino) derivatives, a stock solution of MPIL in DMSO was diluted in 50 mmol/L Tris-HCl buffers of pH 6, 7, and 8 (5:1,000), and UV-visible spectra measured every 30 minutes for 4 hours. The spectrum of β-lapachone is characterized by a major peak at 258 nm and a minor peak at 289 nm, giving a signature ratio of 2.7. The spectrum of unhydrolyzed MPIL also has a major peak at 258 and a minor peak at 289 nm; however, the ratio of these two peaks is 1.16. As MPIL hydrolyzes to β-lapachone, the ratio increases from 1.16 to 2.7. For each different pH, the ratio of peak absorbance for MPIL at 258 and 289 nm (258/289 nm) was plotted over time as a measure of hydrolysis of MPIL to β-lapachone (Supplementary Fig. 1). MPIL was converted to β-lapachone more rapidly at pH 6 (filled circles) than at pH 7 (filled squares) or pH 8 (filled triangles), demonstrating that MPIL converted to β-lapachone more efficiently in acidic conditions. The ratio at pH 6 does not reach 2.7 within 4 hours, indicating that MPIL does not convert to β-lapachone completely within 4 hours.

Using high-performance liquid chromatography-electrospray ionization-mass spectrometry, the conversion in water of PIL, MePIL, MPIL, and NPIL to β-lapachone over time was monitored

(Supplementary Fig. 3; Supplementary Table 2). The rates of conversion of these β-lapachone derivatives from highest to lowest were MPIL > MePIL > PIL > NPIL, with rate constants of derivative loss ranging from −0.693 to −0.001 h^{-1} and rate constants of β-lapachone formation ranging from 0.221 to 0.034 h^{-1}. NMR spectra acquired before (Supplementary Fig. 4A) and after incubating MPIL with water for ≥4 hours (Supplementary Fig. 4C) confirmed disappearance of the mono(arylimino) derivative and a concomitant increase in β-lapachone formation. The appearance of the doublet at 7.9 ppm, as well as the loss of intensity of the 8.2 ppm doublet, suggests that MPIL was hydrolyzed to β-lapachone (Supplementary Fig. 4C). The rates of loss of parent compounds (Supplementary Table 2) correlated well with the relative cytotoxicities of the compounds (Fig. 2; Table 1; Supplementary Table 1). For example, the low rate of conversion of NPIL to β-lapachone was consistent with the lowered cytotoxicity of this set of compounds (e.g., NPIL and BPIL).

β-Lapachone mono(arylimino) derivatives become NAD(P)H: quinone oxidoreductase 1 substrates. Data from Fig. 2, Table 1, Supplementary Fig. 2, and Supplementary Table 1 suggest that β-lapachone mono(arylimino) derivatives can be converted to β-lapachone. Therefore, we did NQO1 enzyme activity assays after immediate (t = 0) or 1 or 4 hours of diluting each β-lapachone derivative in aqueous buffer. NQO1 activities were expected to reflect conversion of biologically inactive β-lapachone mono(arylimino) derivatives to β-lapachone. β-Lapachone and menadione were included as controls. S9 supernatants from MCF-7 cells were used as a source of NQO1 for enzymatic reactions as described in Materials and Methods. As expected at time 0, all β-lapachone mono(arylimino) derivatives examined were poor NQO1 substrates, reflected by low NQO1 activities (1.17-6.49 μmol cytochrome c/min/μg) compared with menadione (21.23 μmol cytochrome c/min/μg) or β-lapachone (13.34 μmol cytochrome c/min/μg; t = 0; Fig. 3A). However, after a 1-hour incubation in reaction buffer, PIL, MePIL, and MPIL became NQO1 substrates, resulting in NQO1 enzyme activities ranging from 9.48 to 13.49 μmol cytochrome c/min/μg (t = 1 hour; Fig. 3A), similar to β-lapachone. In contrast, the relatively nontoxic derivatives, BPIL and NPIL, remained poor substrates for NQO1 (1.34-2.25 μmol cytochrome c/min/μg). After an incubation time of 4 hours, PIL, MePIL, and MPIL were better substrates for NQO1, eliciting approximately three to five times the NQO1 enzyme activity initially observed, rising to 14.7 to 18.6 μmol cytochrome c/min/μg (t = 4 hours; Fig. 3A). NPIL elicited approximately twice the NQO1 enzyme activity (2.9 μmol cytochrome c/min/μg), and BPIL elicited approximately thrice the NQO1 enzyme activity initially observed, rising to 4.6 μmol cytochrome c/min/μg. Thus, biologically inactive β-lapachone mono(arylimino) derivatives were converted into NQO1 substrates at times that correlated with cytotoxic exposure times for each derivative in $NQO1^+$ cells (Fig. 2) and hydrolytic rates determined by high-performance liquid chromatography-electrospray ionization-mass spectrometry (Supplementary Fig. 3; Supplementary Table 2).

When NQO1 metabolizes β-lapachone in the absence of an electron acceptor (e.g., cytochrome c), a futile cycle of NADH oxidation occurs (6). Up to 10 mol NADH may be oxidized per mol β-lapachone in 5 minutes (Fig. 3B). In contrast, when menadione was used in the same reaction, ~1 mol NADH was oxidized per mol menadione in 5 minutes (Fig. 3B; ref. 35). When β-lapachone mono(arylimino) derivatives were examined immediately after dilution with water, these compounds were not efficient NQO1 substrates and therefore elicited minimal futile redox cycling in this enzymatic assay. Consistent with the finding that PIL, MePIL, and MPIL were converted to β-lapachone (a known NQO1 substrate; ref. 6), at 4 hours, these same compounds elicited significant NQO1 futile cycling; for example, 7.25- to 12.9-fold T/C were noted, equal to the futile cycling noted for β-lapachone (9.98-fold T/C). Consistent with the marginal cytotoxicity of NPIL and BPIL (Fig. 2; Table 1), these compounds elicited little or no NQO1-dependent recycling activities (2.37- to 2.73-fold T/C; Fig. 3B).

β-Lapachone mono(arylimino) derivatives induce atypical poly(ADP-ribose) polymerase and p53 cleavage in NAD(P)H: quinone oxidoreductase 1-positive MCF-7 cells. β-Lapachone-induced apoptosis was characterized by atypical proteolyses of PARP (resulting in an ~60-kDa fragment; refs. 6, 16, 18, 32) and p53 (giving an ~40-kDa fragment; ref. 18), indicative of μ-calpain-mediated cell death (Fig. 4A and C; ref. 18). $NQO1^+$ MCF-7 cells were treated for 4 hours with either 5 μmol/L PIL, MePIL, or MPIL as well as 20 μmol/L NPIL or BPIL. Because NPIL and BPIL were relatively nontoxic at concentrations below 15 μmol/L (Fig. 2), a higher concentration of these compounds was used. PIL, MePIL, and MPIL induced atypical PARP (Fig. 4B; Supplementary Fig. 5A and B) and p53 (Fig. 4C and D) proteolyses. Proteolyses were abrogated by 40 μmol/L dicoumarol cotreatment as shown for β-lapachone (6) and were not observed in $NQO1^-$ 231 or 468 cells (data not shown). Unlike β-lapachone, PARP and p53 proteolyses induced by PIL, MePIL, or MPIL were blocked by cotreatment with 5 mmol/L NAC (Fig. 4; Supplementary Fig. 5), consistent with the protection afforded by NAC against PIL, MePIL, and MPIL cytotoxicities (Table 1). NPIL and BPIL did not induce atypical proteolyses of PARP or p53 (Fig. 4; Supplementary Fig. 5), consistent with their inability to induce cell death after a 4-hour exposure (Fig. 2; Table 1).

N-acetyl-L-cysteine prevents β-lapachone mono(arylimino) derivative conversion to β-lapachone and subsequent cytotoxicity. Coadministration of NAC did not affect the cytotoxicity of β-lapachone, suggesting that reactive oxygen species produced by this drug (36–38) may not play a role in its lethality or that the reactive oxygen species formed by treatment of $NQO1^+$ cells with β-lapachone are compartmentalized (Table 1; Supplementary Table 1). Preliminary data suggest that NAC pretreatment and cotreatment of cells did not block reactive oxygen species formation induced by β-lapachone exposure.[10] In contrast, cotreatments of $NQO1^+$ breast cancer cells with 5 mmol/L NAC in combination with PIL, MePIL, or MPIL dramatically protected these cells (~3-fold or greater) from lethality; LD_{50} values increased to 25 ± 0.5, 15 ± 0.5, and 8.5 ± 0.4 μmol/L, respectively (Table 1). In $NQO1^+$ A549 and PC-3 cells, cotreatments with NAC also increased LD_{50} values to 16.1 ± 0.2 and 11.3 ± 0.4 μmol/L, respectively (Supplementary Table 1). To determine if NAC was directly interacting with

[10] K.E. Reinicke et al. Investigating the role of reactive oxygen species in β-lapachone-mediated cell death, unpublished data.

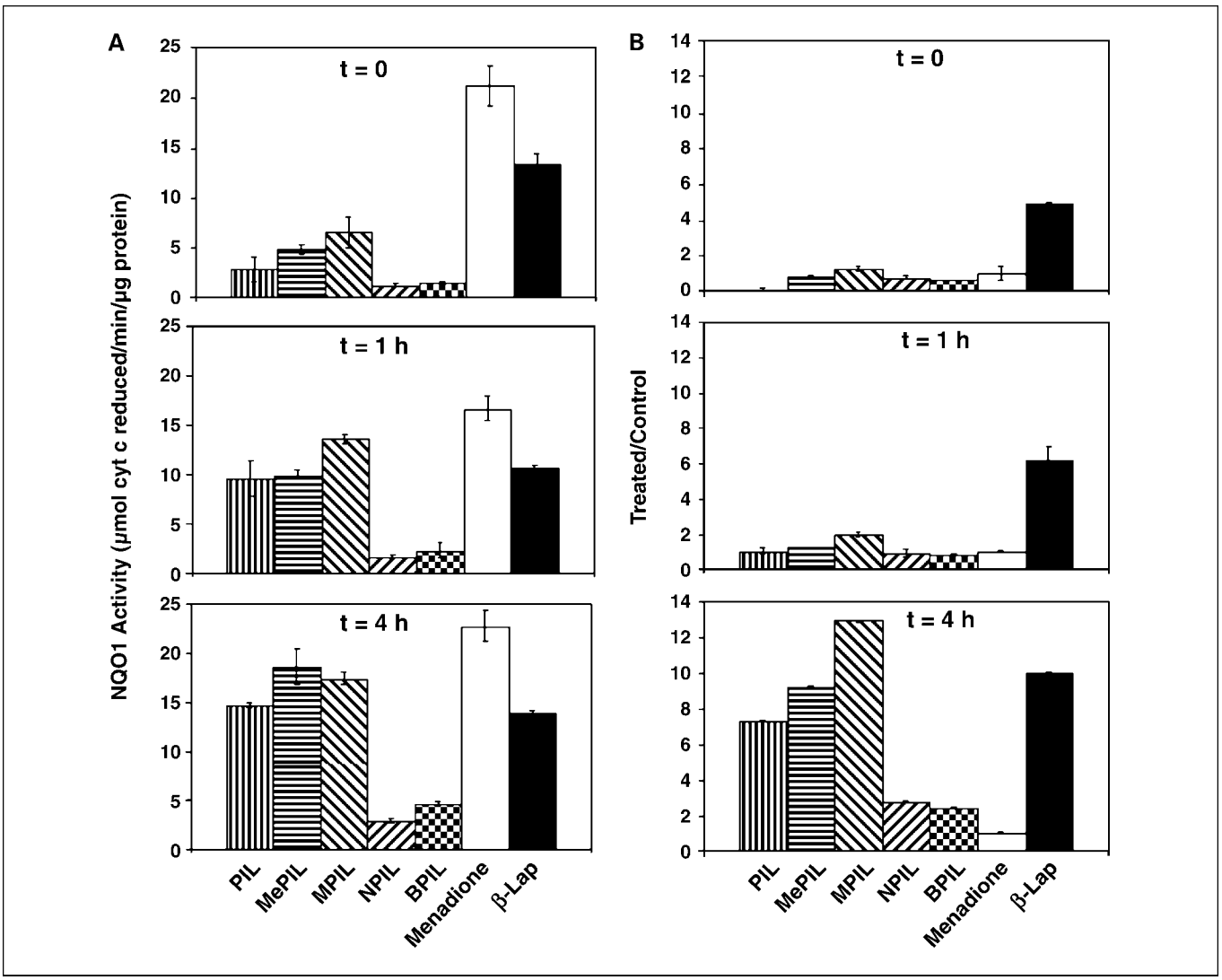

Fig. 3. β-Lapachone mono(arylimino) derivatives become NQO1 substrates and enter into futile cycling reactions of NADH oxidation similar to β-lapachone. *A*, NQO1 enzyme activity was assayed as described in Materials and Methods (1) after allowing the β-lapachone mono(arylimino) derivatives to solubilize in water for 0, 1, or 4 hours (*t* = 0, 1, or 4 hours). Enzyme activities were calculated as μmol cytochrome *c* reduced/min/μg protein. Columns, average NQO1 enzyme activities for three separate cell extractions; *bars*, SD. B, futile cycling activity was assayed as described in Materials and Methods (6) after dissolving β-lapachone mono(arylimino) derivatives in water for 0, 1, or 4 hours (*t* = 0, 1, or 4 hours). NADH oxidation was monitored by changes in UV-visible absorbance at 340 nm. Results are expressed as T/C, where average mol NADH oxidized/mol substrate (compound)/5 min/μg protein ± SD are compared with mol NADH oxidized/mol menadione/5 min/μg protein ± SD. Averages were determined from experiments done thrice.

β-lapachone mono(arylimino) derivatives to prevent their conversion to β-lapachone, we collected UV-visible scans of PIL, MePIL, MPIL, and BPIL, with and without 5 mmol/L NAC, every 30 minutes for 4 hours (Supplementary Fig. 6). We observed that conversion of PIL, MePIL, MPIL, and BPIL to β-lapachone was blocked by NAC (Supplementary Fig. 6B-E), whereas scans of β-lapachone with or without 5 mmol/L NAC remained unchanged (Supplementary Fig. 6A). NMR analyses confirmed the direct interaction and derivitization of MPIL with NAC (Supplementary Fig. 7). A 1:1 molar ratio mixture of MPIL and NAC incubated at room temperature in aqueous solution showed the appearance of doublets in the 6.5 to 7.5 ppm range, suggesting chemical modification of MPIL near the imine moiety of the molecule (Supplementary Fig. 7C). The appearance of a multiplet at 4.35 ppm is indicative of participation of NAC in the chemical modification (Supplementary Fig. 7C).

Discussion

Our data support the hypothesis that mono(arylimino) derivatives of β-lapachone could be potentially useful prodrugs for therapy against human cancers with elevated NQO1 levels. Five derivatives with unique chemical structures (Fig. 1; ref. 31) were directly compared with β-lapachone and menadione for apoptotic responses and lethality. Three derivatives (PIL, MePIL, and MPIL) exhibited cytotoxicities equivalent to, or slightly greater than, β-lapachone in NQO1$^+$ cells (Fig. 2; Table 1; Supplementary Table 1). Our results indicated that these biologically inactive β-lapachone derivatives hydrolyzed to β-lapachone (Fig. 5; ref. 39) in a manner consistent with Schiff's base hydrolysis. Relative cell growth assays supported the theory that these derivatives were converted to β-lapachone within 4 hours after diluting the compounds in aqueous solutions (Fig. 2;

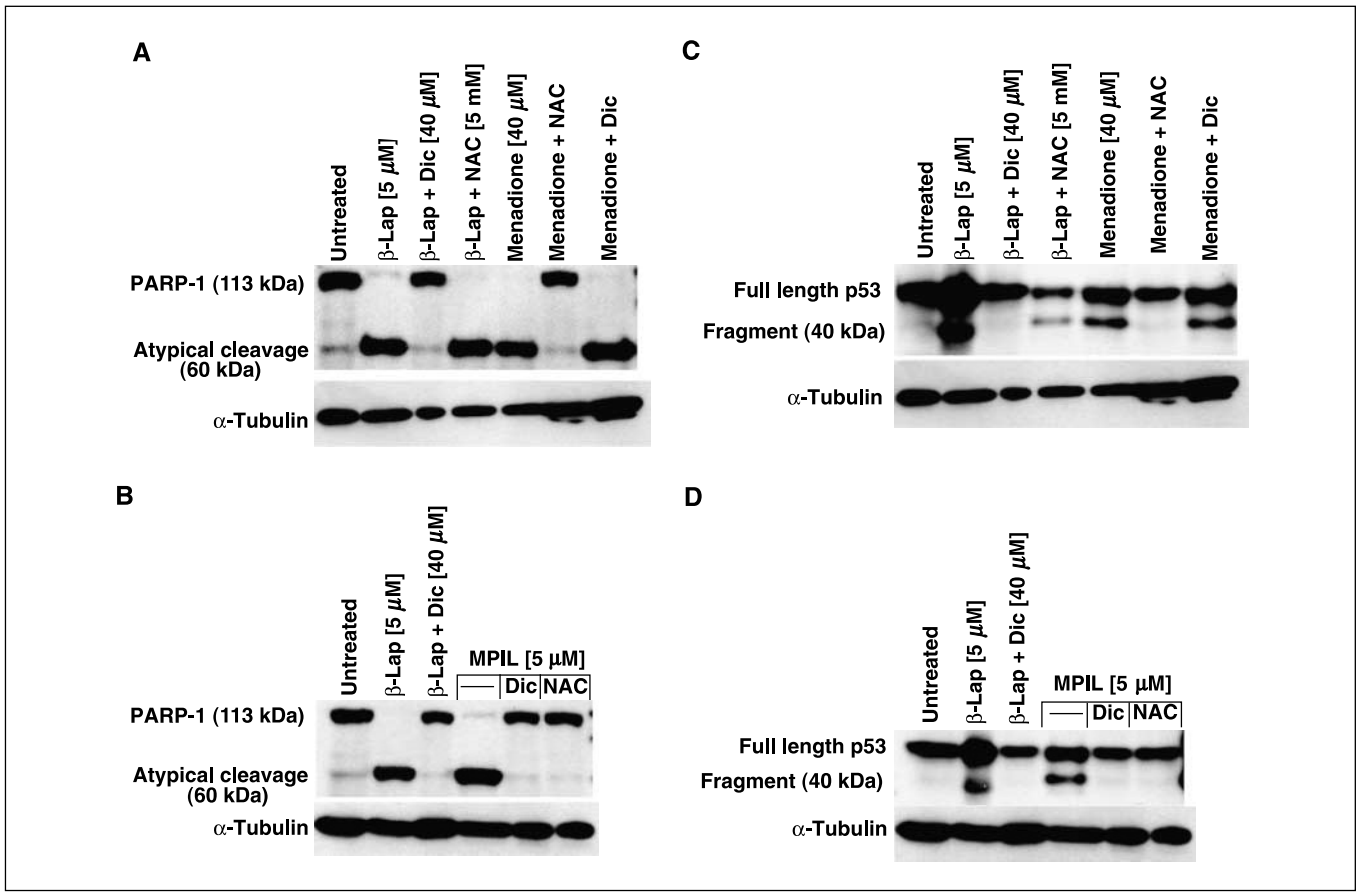

Fig. 4. β-Lapachone mono(arylimino) derivatives cause NQO1-dependent, atypical PARP and p53 apoptotic cleavage in MCF-7 cells similar to β-lapachone. MCF-7 cells were treated with 4-hour pulses of (*A*) 5 μmol/L β-lapachone or 40 μmol/L menadione or (*B*) 5 μmol/L MPIL ± 40 μmol/L dicoumarol (*Dic*) or 5 mmol/L NAC. A higher dose of menadione was necessary to induce apoptosis due to high levels of NQO1 in MCF-7 cells (6). Whole cell extracts were prepared at 24 hours and analyzed using standard Western analyses as described in Materials and Methods. Blots were probed with α-PARP antibody. Representative of experiments done at least thrice. MCF-7 cells were treated with 4-hour pulses of 5 μmol/L β-lapachone, 40 μmol/L menadione, or DMSO alone (*C*) or 5 μmol/L MPIL ± 40 μmol/L dicoumarol (*Dic*) or 5 mmol/L NAC (*D*). A higher dose of menadione was needed to induce apoptosis due to the elevated levels of NQO1 in MCF-7 cells (6). Whole cell extracts were prepared 24 hours post-treatment and analyzed using standard Western blot analyses as described in Materials and Methods. Blots were probed with α-p53 antibody. Representative of experiments done at least thrice.

Table 1; Supplementary Table 1). β-Lapachone prodrug-induced cell death was significantly less in NQO1$^-$ cells and was inhibited by dicoumarol in NQO1$^+$ breast, prostate, or non–small cell lung cancer cells, similar to β-lapachone (6, 18, 32). These results were consistent with hollow fiber assays (31), indicating that mono(arylimino) derivatives of β-lapachone may have an advantage over β-lapachone.

Direct evidence that specific β-lapachone mono(arylimino) derivatives spontaneously convert to β-lapachone in aqueous solution was provided by UV-visible spectrophotometric analyses (Supplementary Fig. 2), NMR analyses (Supplementary Fig. 4), high-performance liquid chromatography-electrospray ionization-mass spectrometry analyses (Supplementary Fig. 3; Supplementary Table 2), and preferential lethality in NQO1$^+$ cells (Table 1; Supplementary Table 1). The data presented in Fig. 3A support our hypothesis that these β-lapachone mono(arylimino) derivatives need to hydrolyze to β-lapachone for reduction by NQO1. The rate of hydrolysis was dependent on the electron-withdrawing strength of the unique substituent phenylimine group of the compounds (Figs. 1 and 3; Supplementary Figs. 2 and 3; Supplementary Table 2; ref. 39). The ability of these derivatives to induce NQO1-dependent atypical PARP and p53 cleavage (Fig. 4; Supplementary Fig. 5) confirmed their conversion to the biologically active β-lapachone parent compound (6, 16, 18, 32).[11] Conversion of β-lapachone mono(arylimino) derivatives to β-lapachone was noted by Misico and Forzani (39), where the hydrolytic decay time constants of β-lapachone mono-(arylimino) derivatives in aqueous solution were measured by cyclic voltammetry. In these studies, increased conversion was noted at lower pH. However, the biological consequences of the proposed conversion were not evaluated. Here, we show that β-lapachone mono(arylimino) derivatives are converted to active β-lapachone. Because conversion is enhanced at lower pH (Supplementary Fig. 1; refs. 26–30, 39), use of these β-lapachone derivatives for increased tumor-selective conversion in the acidic microenvironment of tumors (40–42) could be achieved.

Interestingly, the biologically active β-lapachone mono(arylimino) derivatives were fundamentally different from β-lapachone, because their cytotoxicity was blocked by NAC (5 mmol/L) cotreatments (Table 1; Supplementary Table 1).

[11] R.I. Misico, G. Arzac, G. Burton, J.J. Pink, M. Beman, E. Hohler, D. Boothman. 23rd IUPAC International Symposium on the Chemistry of Natural Products; Florence, Italy; July 28 to August 2, 2002.

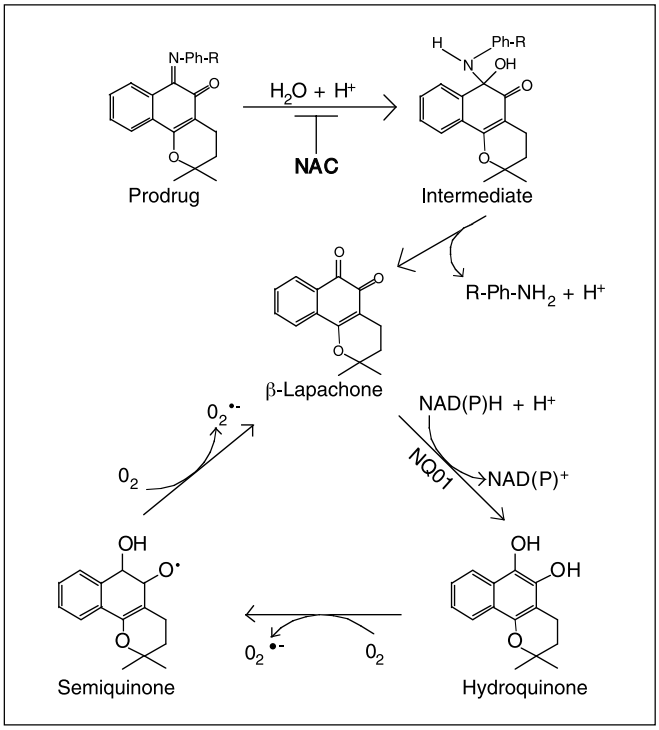

Fig. 5. Proposed mechanism of β-lapachone mono(arylimino) derivative conversion to β-lapachone followed by β-lapachone redox cycling. Our data strongly suggest that β-lapachone mono(arylimino) derivatives undergo a spontaneous Schiff's base hydrolytic reaction, converting the original nontoxic β-lapachone derivatives into the NQO1-dependent cytotoxic β-lapachone parent compound. The rate of this reaction seems to be dependent on the electron-withdrawing effect of the R group on the mono(arylimino) phenyl ring (Fig. 1). β-Lapachone undergoes a futile redox cycle with NQO1 wherein the hydroquinone form is unstable and through two one-electron oxidation steps converts back to the parent β-lapachone molecule. This cycling "bioactivates" β-lapachone and results in Ca^{2+}-dependent, μ-calpain-mediated apoptotic cell death responses in which p53 and PARP are selectively cleaved (Fig. 4; Supplementary Fig. 5).

Consistent with the ability of NAC to inhibit the cytotoxicities of these β-lapachone derivatives, addition of NAC prevented spontaneous conversion to β-lapachone in the UV-visible spectrophotometric wavelength scans observed with these compounds in aqueous solutions (Supplementary Fig. 6). NMR data suggest that NAC directly interacts with these agents to create NAC-derivative conjugates (Supplementary Fig. 7), thereby preventing conversion to β-lapachone. Consequently, NAC exposure rendered the derivatives unable to interact with NQO1 and cause cell death. A similar phenomenon of lapachones undergoing sulfur-mediated Michael reactions was noted previously using L-cysteine and β-mercaptoethanol (43).

The rationale underlying development of this class of mono(arylimino) prodrugs for the delivery of β-lapachone for chemotherapy and/or radiotherapy is based on delayed, pH-dependent activation (26–30). After systemic administration of a potent β-lapachone mono(arylimino) prodrug, the chemotherapeutic agent must traverse the body through zones of neutral pH via the bloodstream and lymphatic circulation, eventually reaching its target, acidic solid tumor tissue (40–42). We believe that mono(arylimino) derivatives of β-lapachone would cause minimal damage to normal tissues with neutral pH microenvironments during circulation. Ideally, these β-lapachone mono(arylimino) prodrugs will remain in inactive forms during the initial systemic distribution and will not become active until they successfully reach the tumor, which commonly has an acidic microenvironment (40–42). The resultant β-lapachone produced is then "bioactivated" by NQO1 (6), an enzyme found in human tissues at low levels (10, 44, 45) but expressed at high levels in many human cancers (21–24). Therefore, a mono(arylimino) prodrug of β-lapachone would remain inactive in circulation through neutral pH zones until it reached its target tissue (acidic tumors with elevated NQO1 levels). There, it would spontaneously become a high affinity substrate for NQO1 (6). Retention in tumor over normal tissue could be enhanced in NQO1-overexpressing cells due to futile cycling of the converted, and now active, β-lapachone compound.

The pharmacokinetics of a prodrug are expected to be vastly different from those of the parent drug. This was clearly noted in the development of CPT-11, a camptothecin derivative that undergoes de-esterification *in vivo* to yield SN-38 in the blood of mammals (46). We are in the process of analyzing the pharmacokinetics of β-lapachone compared with specific β-lapachone mono(arylimino) derivatives. The ideal β-lapachone prodrug may be one that requires a longer time to become active (e.g., NPIL or BPIL) and is possibly accelerated in an $NQO1^+$ acidic tumor environment compared with a compound that is more rapidly activated (e.g., PIL, MePIL, or MPIL; ref. 39).

Collectively, the data presented in this article strongly suggest that some form of β-lapachone mono(arylimino) derivative can be developed for use as a prodrug. The observation that the electron-withdrawing group on the arylimino substitution group as well as the pH of the surrounding aqueous environment can directly influence conversion of the prodrugs to β-lapachone should be invaluable in developing these active chemotherapeutic agents. Analyses of this class of β-lapachone prodrugs could lead to potent, tumor-selective, antitumor agents. We are currently working on developing a β-lapachone mono(arylimino) compound with a leaving group that is specifically cleaved through a reaction catalyzed by a tumor-selective enzyme. Additionally, we are developing a derivative of β-lapachone with a cytotoxic leaving group that could further enhance the antitumor efficacy of these prodrugs, particularly in an acidic tumor microenvironment.

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Development of β-Lapachone Prodrugs for Therapy Against Human Cancer Cells with Elevated NAD(P)H:Quinone Oxidoreductase 1 Levels

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