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Preclinical paper

Effects of 1,2-naphthoquinones on human tumor cell growth and lack of cross-resistance with other anticancer agents

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The sensitivity of human tumor and rat prostate tumor cells to a series of naphthoquinones, including tricyclic compounds of the β-lapachone and dunnione families as well as 4-alkoxy-1,2-naphthoquinones, was evaluated. To better understand the mechanism of cytotoxicity of 1,2-naphthoquinones, the roles of various resistance mechanisms including P-glycoprotein, multidrug resistant associated protein, glutathione (GSH) and related enzymes, altered topoisomerase activity, and overexpression of genes that control apoptosis (*bcl*-2 and *bcl*-x_L) were studied. MCF7 cells were most sensitive to the naphthoquinones with IC_{50} values ranging from 1.1 to 10.8 μM, as compared to 2.5 to >32 μM for HT29 human colon, A549 human lung, CEM leukemia and AT3.1 rat prostate cancer cells. MCF7 ADR cells, selected for resistance to adriamycin (ADR), displayed cross-resistance to the tricyclic 1,2-naphthoquinones. Drug efflux via a P-glycoprotein mechanism was ruled out as a mechanism of resistance to 1,2-naphthoquinones, since KB-V1 cells expressing high levels of P-glycoprotein and the KB-3.1 parent line were equally sensitive to these compounds. Any resistance of the tricyclic naphthoquinones noted in ADR-resistant cells appeared to relate to the GSH redox cycle and could be circumvented by exposure to buthionine sulfoximine or by changing the structure from a tricyclic derivative to a 4-alkoxy-1,2-naphthoquinone. The 1,2-naphthoquinones were found to be cytotoxic against CEM/VM-1 and CEM/M70-B1 cells that were selected for resistance to teniposide or merbarone, respectively. In addition, cells overexpressing *bcl*-2 or *bcl*-x_L proteins were as sensitive to 1,2-naphthoquinones as were control cells. Because of their effectiveness in drug-resistant cells, these agents appear to hold promise as effective chemotherapeutic agents. [© 1998 Lippincott-Raven Publishers.]

Key words: Anticancer drug, cytotoxicity, glutathione, resistance, naphthoquinones.

Introduction

Many efficient antineoplastic drugs are either quinones (anthracycline derivatives, mitoxantrone, actinomycin), quinonoid derivatives (quinolones, genistein, bactracyclin) or drugs that can easily be converted to quinones by *in vivo* oxidation (etoposide).[1] The literature on quinone-DNA interactions is replete with references to quinones having the potential to undergo redox cycling, with the formation of highly reactive oxygen species that are thought to relate to their cytotoxic antitumor activity.[2] It has also been shown that many quinones are efficient modifiers of topoisomerase II (Topo II) activity.[3] The antitumor activities of the quinones were revealed more than two decades ago when the National Cancer Institute published a report in which 1500 synthetic and natural quinones were screened for their anticancer activities.[4] One of these quinones was β-lapachone **1** (Figure 1), a natural product that inhibited tumor growth in rats implanted

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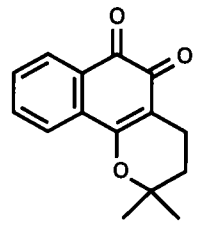
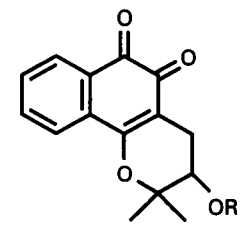
with W-256 carcinoma. β-Lapachone **1** also showed antiparasitic activity,[5-7] and was later found to inhibit reverse transcriptase[8] and prolong the survival of mice infected with Rausher leukemia virus.[9] β-Lapachone also acts as a DNA repair inhibitor[10] and as a cytotoxic drug against human cancer cell lines.[11-13]

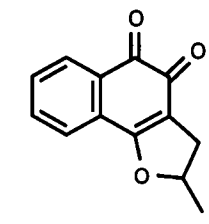
Recently, it has been noted that human prostate cancer cells were most sensitive to β-lapachone and that they were induced to undergo apoptosis independent of p53 and *bcl*-2.[12,13] The molecular mechanism by which β-lapachone exerts its apoptotic effect remains to be determined; however, Topo is a possible candidate. Li *et al.* reported that β-lapachone inhibits Topo I with a mode of action different from camptothecin.[11] More recently, Frydman *et al.* demonstrated that several 1,2-naphthoquinone derivatives, including β-lapachone, induce DNA Topo II-mediated DNA cleavage in an ATP-independent manner and that this effect may be crucial for the cytotoxicity of the 1,2-naphthoquinones.[14]



1
(SL11001)



2a R=H
(SL11002)
2b R=$COCH_2CO_2H$
(SL11003)



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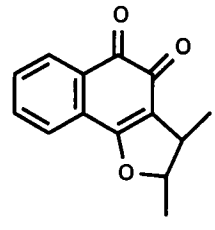

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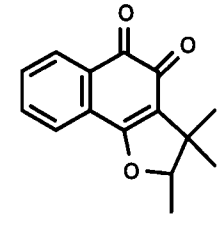
(SL11009)



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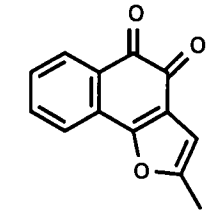
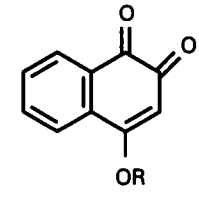


6
(SL11011)



7a R=CH_2CH_3
(SL11039)
7b R=$(CH_2)_4$ CH_3
(SL11013)
7c R=$(CH_2)_6$ CH_3
(SL11021)

8a R=$CH_2(CH_3)_2$
(SL11016)
8b R=CH_2CH $(CH_3)_2$
(SL11014)
8c R=$(CH_2)_2$ $CH(CH_3)_2$
(SL11015)

9a R=$CH_2CHCHCH_3$
(SL11017)
9b R=CH_2 $CHCHCH_2CH_3$
(SL11020)

10 R=$CH_2C_6H_5$
(SL11018)
11 R=CH_2 C_6CH_{11}
(SL11019)

Figure 1. Structures of 1,2-naphthoquinones. Numbers in parentheses refer to S'LIL pharmaceutical drug designations.

This report extends previous studies by including an evaluation of the cytotoxic activity of a series of β-lapachone analogs, dunnione analogs and novel synthetic 1,2-naphthoquinones against both drug-sensitive and drug-resistant cell lines. The structural similarity of naphthoquinones to anticancer drugs such as adriamycin (ADR)[4] prompted us to evaluate cross-resistance in MCF7 ADR breast cancer cells that were selected for resistance to ADR,[15] as well as in other human tumor cell lines that overexpress distinct proteins involved in multidrug resistance (MDR). These cell lines include: HL60 ADR cells that were selected for resistance to adriamycin and that overexpress multidrug resistant associated protein (MRP) but not P-glycoprotein;[16] KB-VI cells that are resistant to vinblastine due to the expression of high levels of P-glycoprotein; MCF7 GPx cells that exhibit elevated selenium-dependent glutathione (GSH) peroxidase (GPx) expression due to transfection with a GPx expression construct; murine FL5.12 cells overexpressing *bcl*-2[17] or *bcl*-x_L,[18] proteins that protect against programmed cell death; and CEM/VM-1[19] and CEM/M70-B1,[20] cells that were selected for resistance to teniposide or merbarone, respectively. In CEM/VM-1 cells decreased Topo II α activity is associated with mutations in the Topo II gene, while in CEM/M70-B1 cells decreased Topo II activity is associated with decreased expression of the Topo II gene. We found that β-lapachone and its tricyclic analogs, but not 4-alkoxy-1,2-naphthoquinones, were affected only by resistance that involved the GSH redox cycle. Resistance could be overcome by exposure to L-buthionine sulfoximine (BSO).

Materials and methods

Chemicals

The 1,2-naphthoquinones used in this study are shown in Figure 1. β Lapachone **1**, 3-hydroxy-β-lapachone **2a** and 3-malonyloxy-β-lapachone **2b** were obtained from synthetic lapachol. The latter was in turn obtained by a preparative alkylation of 2-hydroxy-1,4-naphthoquinone with dimethylallyl bromide.[21] Two alkylated products were obtained, the C3-alkylated product (lapachol) and the *O*-(γ,γ′-dimethyl) allyl ether of 2-hydroxy-1,4-naphthoquinone. Lapachol was either cyclized to β-lapachone **1** with acid[22] or oxidized with *m*-chloroperbenzoic acid to its epoxide.[21] The latter was cyclized with acid to give **2a**. Acylation of **2a** with *t*-butyl malonate followed by cleavage of the *t*-butyl residue with trifluoroacetic acid yielded the 3-malonyloxy derivative **2b**. By heating *O*-(γ,γ′-dimethyl) allyl ether at 120°C it underwent a Cope transposition to give the C3 (α,α′-dimethyl) allyl substituted 2-hydroxy-1,4-naphthoquinone.[23] The latter was cyclized with acid to give **5**. Quinones **3** and **4** were obtained by a similar reaction sequence starting from the *O*-crotyl and *O*-allyl ethers of 2-hydroxy-1,4-naphthoquinone. Compound **6** was prepared by condensation of the latter quinone with propionaldehyde, followed by an oxidative cyclization to form the furan ring 4-alkoxy-1,2-naphthoquinones. **7a–11** were obtained by alkylation of the silver salt of 2-hydroxy-1,4-naphthoquinone following established procedures.[24] The 4-alkoxy residues were either of the *n*-alkyl type (**7a–c**), of the branched alkyl type (**8a–c**), of the alkene type (**9a–b**), as well as a benzyl residue (**10**) and an alicyclic residue (**11**). MTT, *N*,*N*-dimethylformamide, lauryl sulfate, BSO, *N*-acetylcystine, GSH, NADPH, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and GSH reductase were purchased from Sigma (St Louis, MO). BioRad protein determination solution was obtained from BioRad (Richmond, CA). Other biochemicals were purchased from Mediatech (Herndon, VA). The photoaffinity analog of verapamil, [^{125}I]NAS-VP (1100 Ci/mmol), was synthesized as described previously.[25]

Cell culture

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (Rockville, MD), and was grown in Ham's F-12K medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The human colon carcinoma cell line HT29 was a gift from Dr LC Erickson (Loyola Medical Center, Maywood, IL), and was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The metastatic rat prostate cell line AT3.1 was a gift from Dr CW Rinker-Shaeffer (Department of Surgery, University of Chicago) and was grown in RPMI medium supplemented with 8% fetal bovine serum. The human leukemia cell lines were CEM, CEM/VM-1[19] and CEM/M70-B1,[20] and were grown in RPMI medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. MCF7 ADR originated in Dr K Cowan's laboratory (NCI, Bethesda, MD). All MCF7 cells were grown in Richter's improved modified Eagle's medium supplemented with 10% fetal bovine serum and 2.2 g/l sodium bicarbonate. MCF7 GPx media contained 30 nM selenium. The human leukemia cells, HL60 and HL60 ADR were originally provided by Dr Melvin Center (Kansas State University, Manhattan, KS). HL60 and HL60 ADR were grown

in RPMI medium supplemented with 10% fetal bovine serum. The human epidermoid carcinoma cell line KB3.1 and its multidrug resistant variant KB-V1 were originally provided by Dr IB Roninson (Department of Genetics, University of Illinois). KB3.1 and KB-V1 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The murine lymphoma cell lines FL5.12 *neo*, *bcl*-x_L and *bcl*$_2$ were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10% IL-3, 1.7 mM L-glutamine, 1 μg/ml geneticin, 0.5 μM β-mercaptoethanol and 17 mM HEPES buffer. IL-3 was harvested from cultures of the WEHI 3B murine myelo-monocytic leukemia cell line. All cells were grown in 100 units/ml penicillin and 100 μg/ml streptomycin, except for HT29 which utilized 50 μg/ml gentamycin and the CEM lines which were grown in antibiotic-free medium. All cell cultures were maintained at 37°C in 5% CO_2/95% humidified air.

GPx expression constructs

An expression construct composed of the bovine GPx-1 cDNA, including the 3′ untranslated region required for the recognition of the in-frame UGA codon as selenocysteine, was constructed. The EFα-1A untranslated leader was placed 5′ to the cDNA to provide maximal translational efficiency (provided by M Sandbaken and R Gesteland, University of Utah). These sequences were inserted into the pLNCX retroviral vector that sustains high levels of transcription from a CMV promoter, provides a polyadenylation signal and contains an antibiotic resistance marker capable of conferring resistance to geneticin G418.[26] MCF7 cells were chosen as recipients due to their low levels of total GPx activity. The GPx expression construct was introduced into MCF7 cells by calcium phosphate-mediated transfection. Individual G418-resistant colonies were isolated with cloning cylinders and expanded.

Murine cells expressing *bcl*-2, *bcl*-x_L and *neo*

Murine FL5.12 cells were electroporated with a pSFFV-Neo plasmid containing a human *bcl*-x_L cDNA cloned into the *Eco*RI site as described.[18] As a negative control, transfections were also performed with the pSFFV-Neo plasmid without an insert. FL5.12 cells were transfected with *bcl*-2 using electroporation and selection for neomicin resistance with G418 as previously described.[18]

Cytotoxicity assay

Cytotoxicity was evaluated using either a colony forming efficiency assay or modifications of a described MTT assay.[27] For colony forming efficiency, cells plated at a density of 5×10^5 cells per 25 cm^2 were exposed to increasing concentrations of drug for 4 h prior to replating at densities between 50 and 800 cells in 60 mm dishes. Colonies were stained 15 days later with Trypan blue and counted. For the MTT assay, cells growing as a monolayer were plated at a density of 500 cells/well in 96-well plates and allowed to grow for 24 h. Following a 4 h incubation with drug, the drug-containing media was replaced with fresh media. Six days after drug treatment, 25 μl of a 5 mg/ml solution of MTT was added to each well followed 4 h later by the addition of 100 μl lysing buffer (20% sodium dodecyl sulfate, 50% *N*,*N*-dimethylformamide and 0.8% acetic acid, pH 4.7) for an additional 22 h. A microplate reader set at 570 nm was used to determine the absorbance. Results were expressed as the fraction of the optical density determined for cells treated with drug compared to those treated with vehicle. Cells grown in suspension were plated at a density of 3.1×10^4 cells/ml and incubated for 4 h with drug at various concentrations, as described above. The cells in suspension were centrifuged at 250 *g* for 10 min and the media was replaced with fresh media. After 3–6 days, 25 μl of a 5 mg/ml solution of MTT was added to each well followed by the procedure noted above. For glutathione depletion experiments, cells were pre-treated with BSO (100 μM) for 16 h and then for an additional 4 h during exposure to the 1,2-naphthoquinones. Following drug treatment, fresh medium was added to cells and they were grown for 6 days. MTT followed the procedure described above.

FL5.12 cells were initially cultured at 5×10^5 cells/ml in 24-well plates and treated with increasing concentrations of 1,2-naphthoquinone. Viability was determined at each time point by centrifuging cells for 10 min at 1400 r.p.m. at 4°C, resuspending the cell pellet in 500 μl of fluorescence-activated cell sorting (FACS) buffer (0.1% sodium azide, 1% bovine serum albumin, in 1 × phosphate-buffered saline, pH 7.4) with 2 μl of 0.5 mg/ml propidium iodide and analyzing the cells for uptake of propidium iodide on a fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA).

Enzyme assays

Total intracellular GSH levels were measured using the enzyme recycling assay described by Tietze[28] and GST activity was assayed as described by Habig,[29] using

1 mM GSH and 1 mM CDNB. Protein determinations were carried out using the Bradford method.[30] GPx activity was measured by an assay which spectrophotometrically measures the oxidation of NADPH in a coupled system containing reduced GSH, GSH reductase, cellular extracts and hydrogen peroxide as the substrate.[31]

Inhibition of verapamil binding

Photoaffinity labeling was carried out using exponentially growing cells (5×10^5 cells per assay). KB-V1 cells were suspended in Ca^{2+}/Mg^{2+}-free Dulbecco phosphate-buffered saline containing 4% (v/v) dimethyl sulfoxide and 1 mM [^{125}I]NAS-VP (specific activity 1100 Ci/mmol), the photoaffinity analog of verapamil, in a final volume of 0.050 ml. The cells were then preincubated for 30 min at room temperature in the absence or presence of verapamil and 1, 10 or 100 μM compound **1**, **5** or **7b**. Photoaffinity labeling was carried out as previously described.[25] The photolabeled samples were then processed for SDS-PAGE as described previously.[25]

Results

Cytotoxic effects of 1,2-naphthoquinones on human and rat tumor cells

A systematic study of the antitumor effects of naphthoquinones was undertaken in an effort to relate structure to cytotoxic activity. The lead compound was the natural product β-lapachone **1**, a tricyclic 1,2-naphthoquinone that was assayed along with its derivative 3-hydroxy β-lapachone **2a** and its malonyl derivative **2b**, a water soluble quinone. Dunnione **5**, also a tricyclic natural quinone, was assayed together with its analogs **3** and **4**, and the fully aromatic tricyclic 1,2-naphthoquinone **6**, where the dihydrofuran ring is oxidized to a furan ring. Finally, in order to preserve the 4-*O*-alkyl substitution present in structures **1-6** while eliminating the third heterocyclic ring, 1,2-naphthoquinones **7a-11** were prepared and screened for antitumor activity. The structures of all 1,2-naphthoquinones are illustrated in Figure 1. Each 1,2-naphthoquinone was evaluated for cytotoxicity against several human tumor and rat cell lines, measured by colony forming efficiency and/or MTT cell growth inhibition. The doses required to inhibit 50% cell growth of HT29 human colon tumor cells, A549 human lung tumor cells, AT3.1 rat prostate tumor cells and KB3.1 human epidermoid carcinoma cells are shown in Table 1. With the exception of naphthoquinones **2a** and **3**, all compounds inhibited 50% cell growth with an IC_{50} in the range of 2-11 μM. Alterations at the 3-position of β-lapachone resulted in much less active derivatives, as illustrated by a 2- and 8-fold decrease in potency for naphthoquinones **2a** and **3**, respectively, compared to β-lapachone **1**, in HT29 cells. Similar IC_{50} values were obtained using either MTT or colony forming assay. It was noted in colony forming assays that β-lapachone and derivatives produced a steep dose response with a 2-3 log cell kill against MCF7 and HT29 cells at 5 μM and higher concentrations with little to no cell kill at lower concentrations (data not shown). The plateau may be indicative of a resistance mechanism that is overcome at higher doses of drug.

1,2-Naphthoquinone cross-resistance with ADR: role of P-glycoprotein, MRP and the GSH redox cycle

Because of the well-known MDR associated with exposure to the anthracycline quinones and the quinoid-forming etoposides,[32] we evaluated cross-resistance between the 1,2-naphthoquinones and ADR using MCF7 and MCF7 ADR cells. The effect of ADR on MCF7 ADR cells was used as a positive

Table 1. Effect of 1,2-naphthoquinones on tumor cell growth[a]

Drug	IC_{50} (μM)			
	HT29	A549	AT3.1	KB3.1
1	3.0±0.1	6.1±0.1	3.5±0.2	3.5±0.2
2a	6.9±0.8	23±2.0	ND[c]	6.3±0.7
2b	24.4±0.6	>32	ND	ND
3	3.3±0.2	7.1±0.2	6.6±0.1	4.2±0.3
4	3.1±0.1	7.3±0.1	3.6±0.1	3.4±0.1
5	2.8±0.1	5.5±0.6	2.6±0.4	2.5±0.4
6	2.9±0.2	11.1±2.5	6.3±0.6	3.6±0.5
7b	2.7±0.2	4.6±0.7	3.3±0.4	3.5±0.1

[a]Exponentially growing cells were exposed to increasing concentrations of 1,2-naphthoquinones for 4 h. Six days following treatment, a MTT assay was performed to determine cell growth. IC_{50} values were determined by extrapolation from a graph of MTT growth inhibition (drug treated/control) versus drug concentrations. Each point represents the mean (±SD) of triplicate determinations of a single experiment.
[b]IC_{50} represents the concentration required to inhibit 50% tumor cell growth.
[c]Not done.

control. Cross-resistance was observed between ADR and the tricyclic 1,2-naphthoquinones **1-5** (Table 2) with MCF7 ADR cells being 3.5- to 6.2-fold less sensitive to these quinones. Although cross-resistance was observed with 4-alkoxy-1,2-naphthoquinones **7a**, **7b** and **8a**, analogs **7c**, **8b**, **9a** and **9b** are equally potent in both cell lines, and compounds **8c**, **10** and **11** were more potent in MCF7 ADR cells than in MCF7 cells. The potency of 4-alkoxy-1,2-naphthoquinones in MCF7 cells remained relatively constant as the length of the substituent at the 4-position increased; however, there was a trend towards increased potency in MCF7 ADR cells as the length or hydrophobicity of the 4-alkoxy chain increased. The ratios of MCF7 ADR/MCF7 IC_{50}s for $R = CH_2CH_3$ (**7a**), $R = (CH_2)_4CH_3$ (**7b**) and $R = (CH_2)_6CH_3$ (**7c**) decreased from 2.3 to 1.6 to 0.5, respectively. Similarly, the ratios for $R = CH(CH_3)_2$ (**8a**), $R = CH_2CH(CH_3)_2$ (**8b**) and $R = (CH_2)_2CH(CH_3)_2$ (**8c**) decreased from 1.9 to 0.9 to 0.5, respectively.

Resistance of MCF7 ADR cells to ADR may be due to several mechanisms including decreased drug accumulation secondary to increased activity of the ATP-driven P-glycoprotein efflux pump and/or altered GSH redox cycling.[33] The possible involvement of either mechanism, as well as the involvement of MRP, in resistance to 1,2-naphthoquinones was therefore evaluated.

Table 2. Cytotoxic activity of 1,2-naphthoquinones against MCF7 and MCF7 ADR cells

Drug	IC_{50} (μM)[a]		Ratio[b]	*p* value[c]
	MCF7	MCF7 ADR		
1	1.8±0.8	9.0±0.2	5.0	<0.001
3	2.5±1.0	9.8±2.8	3.9	<0.001
4	2.1±1.0	7.4±1.6	3.5	<0.001
5	1.1±0.5	6.8±0.0	6.2	<0.001
7a	6.0±0.3	13.7±0.3	2.3	<0.001
7b	1.5±0.4	2.4±1.2	1.6	<0.05
7c	3.3±1.5	1.7±0.2	0.5	0.14
8a	3.0±0.4	5.8±0.6	1.9	<0.001
8b	2.9±0.3	2.7±0.4	0.9	0.31
8c	4.0±2.1	1.9±0.5	0.5	<0.05
9a	4.7±1.5	4.5±1.3	1.0	0.87
9b	3.5±0.4	4.0±0.2	1.1	0.13
10	4.6±1.8	2.8±0.5	0.6	<0.05
11	3.7±2.2	1.3±0.3	0.4	<0.05
ADR	0.03±0.02	0.83±0.4	28	<0.001

[a]MCF7 breast and MCF7 ADR cells resistant to adriamycin were exposed to increasing concentrations of 1,2-naphthoquinones for 4 h. MTT analysis was performed to determine cell density 6 days following treatment. IC_{50} refers to the concentration required to inhibit 50% cell growth relative to control (zero drug addition). Each point represents the mean of results from two to four separate experiments each run in triplicate (except compound **7a** which was done once in triplicate).

[b]Ratio of concentration required to inhibit 50% cell growth in MCF7 ADR compared to that required in MCF7 cells.

[c]*p* value as determined by Student's two-sided *t*-test comparing IC_{50} values for MCF7 ADR cells and MCF7 cells.

The sensitivity of KB-V1 cells, selected for resistance to vinblastine (a known P-glycoprotein substrate), to 1,2-naphthoquinones was compared to that of KB3.1 parent cells. KB-V1 cells express high levels of P-glycoprotein. Naphthoquinones **1-6** and **7b** induced the same inhibition of cell growth in resistant KB-V1 and in sensitive KB-3.1 cells, suggesting that these agents are not substrates for P-glycoprotein. Figure 2 illustrates the cytotoxic effect of increasing concentrations of compounds **3** and **6** against KB-3.1 and KB-V1 cells. Importantly, the binding of [^{125}I]NAS-VP, a photoaffinity analog of verapamil, to P-glycoprotein was not inhibited in KB-V1 cells with 1, 10 or 100 μM **1**, **5** or **7b** (data not shown), thus lending further support to the finding that 1,2-naphthoquinones are not exported from these cells via P-glycoprotein. This is consistent with a previous report demonstrating β-lapachone is unaffected by MDR1-mediated resistance.[14]

To determine whether glutathione-related mechanisms play a role in the resistance of MCF7 ADR cells to 1,2-naphthoquinones, the effect of BSO, a γ-glutamyl cysteine synthetase inhibitor, on the sensitivities of cells to 1,2-naphthoquinones was evaluated. Depletion of GSH levels in MCF7 ADR cells to 0.7% of basal activity occurred in the presence of 100 μM BSO for 16 h (Table 3). MCF7 ADR cells exposed to BSO were more sensitive to increasing concentrations of 1,2-naphthoquinones than control MCF7 ADR cells (Figure 3 and data not shown). The concentration required to inhibit the growth of 50% of MCF7 ADR cells following exposure to the tricyclic 1,2-naphthoquinones **1** and **3-5** decreased between 6- and 11-fold in the presence of BSO, indicating that GSH plays a role in resistance to β-lapachone and dunnione derivatives. The increase in sensitivity to compounds **7b**, **8c** and **11** and ADR following exposure to BSO was approximately 3-fold. Our data indicating a partial reversal of resistance of MCF7 ADR to ADR upon pretreatment to BSO is consistent with the literature reports.[33,34] The sensitivity of MCF7 cells to all compounds tested was equivalent in the presence and absence of BSO.

Because MRP confers resistance to glutathione-conjugated drugs and BSO reverses resistance through GSH depletion,[16] it was of considerable interest to determine whether this protein confered resistance to 1,2-naphthoquinones. The cytotoxic effects of 1,2-naphthoquinones on HL60 and HL60 ADR cells that have elevated MRP were evaluated. Figure 4 illustrates a lack of cross-resistance of HL60 ADR cells to compounds

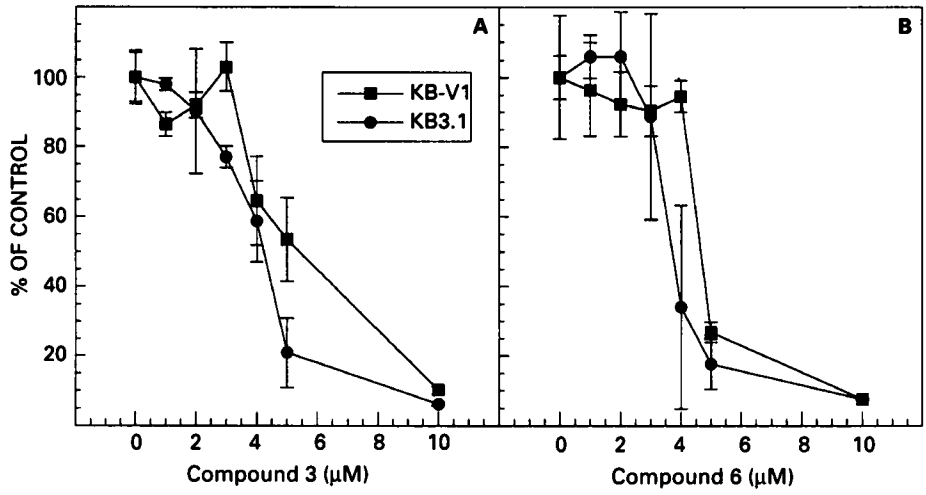


Figure 2. Effect of 1,2-naphthoquinones on KB3.1 and KB-V1 cells. (A) KB3.1 (●) and KB-V1 (■) cells were exposed to fresh medium containing increasing concentrations of compound **3** (A) or compound **6** (B) for 4 h. After 6 days, MTT analysis was performed to determine cell density. Inhibition of cell growth relative to control (zero drug addition) was calculated for each concentration. Each point represents the mean (±SD) of three determinations.

Table 3. GSH and GSH-related enzyme activity

Cell line	GSH (nmol/10^6 cells)		GST (nmol/min /mg protein)	GPx[a] (nmol/min /mg protein)
	−BSO	+BSO[b]		
MCF7	4.5	1.9	8±7	1.7±0.6
MCF7 ADR	13.7	0.1	123±8	7.9±0.9
MCF7 GPx	ND[c]	ND	ND	110±21
HL60	153±2	10±2	126±3	ND
HL60 ADR	182±24	0.5	144±3	ND

[a]nmol NADPH oxidized/min/mg protein using H_2O_2 as a substrate
[b]GSH activity following exposure to 100 μM for 16 h.
[c]ND, not done

7b and **8c**, indicating that the 4-alkoxy-1,2-naphthoquinones are not substrates for this MRP. Both HL60 and HL60 ADR cell lines express high levels of GSH in the absence of BSO and low levels in the presence of BSO (Table 3). The sensitivity of both HL60 and HL60 ADR cells to compounds **7b** and **8c** following exposure to BSO was enhanced, further suggesting a role for GSH in resistance to these compounds (Figure 4).

Role of GPx in resistance to tricyclic 1,2-naphthoquinones

MCF7 ADR cells are known to have a significantly elevated level of selenium-containing GPx.[33] It is known that GPx contributes to cellular protection against oxidative damage.[33] For this reason, we evaluated the possible independent contribution of GPx to resistance to 1,2-naphthoquinones. This was accomplished by evaluating the sensitivity of cells transfected with an active GPx expression construct, versus the sensitivity of the parent line, to 1,2-naphthoquinones. MCF7 cells transfected with the GPx cDNA expression construct exhibited a 65-fold increase in GPx activity, in the presence of selenium (Table 3). The transfected cell line was no more resistant to the tricyclic 1,2-naphthoquinones or to the 4-alkoxy-1,2-naphthoquinone than was the parent line, indicating that selenium dependent GPx alone did not confer resistance to 1,2-naphthoquinones (data not shown).

Effect of 1,2-naphthoquinones in cell lines selected for resistance to inhibitors of DNA Topo II

To ascertain whether cells selected for resistance to the Topo II inhibitor teniposide (VM-26), a Topo II ATP-dependent drug, were cross-resistant to naphthoquinones, a cell line with altered Topo II activity (CEM/VM-1)[19] was compared for sensitivity to compounds **1**, **3**, **5** and **7b** with the CEM parent line that originated from a patient with acute lymphoblastic leukemia.[35] CEM/VM-1 cells were slightly more resistant to β-lapachone **1** and dunnione **5**, at lower concentrations, when compared to CEM cells; however, this difference was not observed for quinones **3** or **7b** (Figure 5). CEM/M70-B1 cells were selected for resistance to the catalytic Topo II inhibitor, merbar-

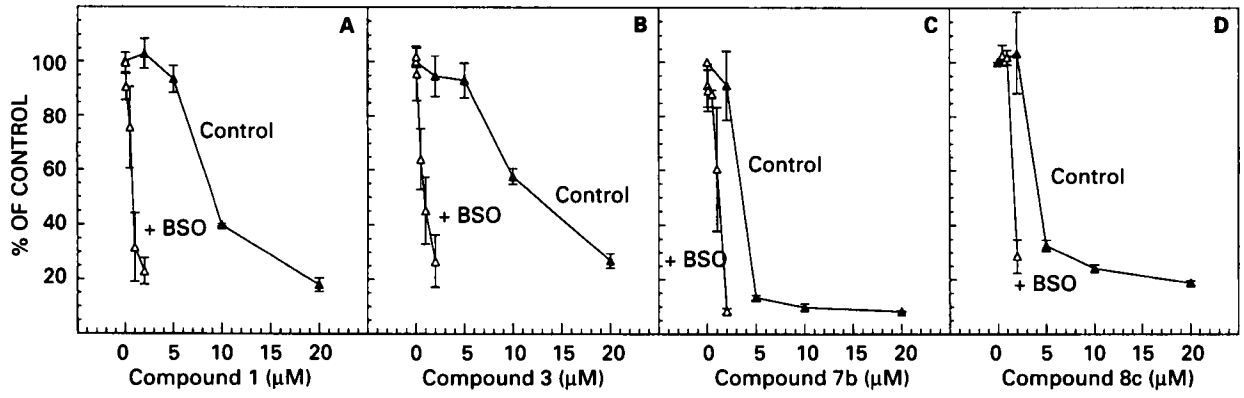


Figure 3. Effect of 1,2-naphthoquinones on MCF7 ADR cells in the presence and absence of BSO. (A) MCF7 ADR cells were exposed to fresh medium (▲) or medium containing 100 μM BSO (△) 16 h prior to and during exposure to increasing concentrations of compound **1** (A), compound **3** (B), compound **7b** (C) or compound **8c** (D) for 4 h. After 6 days, MTT analysis was performed to determine cell density. Inhibition of cell growth relative to control (zero drug addition) was calculated for each concentration. Each point represents the mean (±SD) of three determinations.

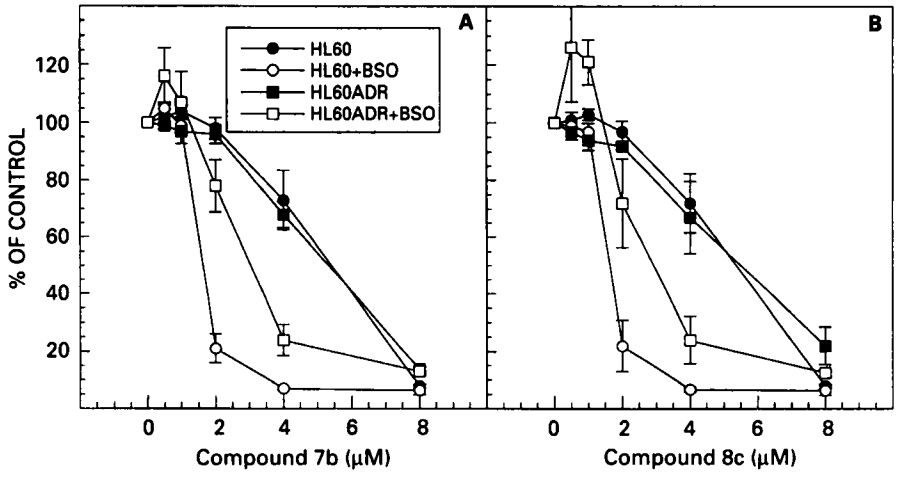


Figure 4. Effect of 1,2-naphthoquinones on HL60 and HL60 ADR cells in the presence and absence of BSO. (A) HL60 (circles) or HL60 ADR (squares) cells were exposed to fresh medium (closed symbols) or medium containing 100 μM BSO (open symbols) 16 h prior to and during exposure to increasing concentrations of compound **7b** (A) or compound **8c** (B) for 4 h. After 6 days, MTT analysis was performed to determine cell density. Inhibition of cell growth relative to control (zero drug addition) was calculated for each concentration. Each point represents the mean (±SD) of three determinations.

one.[20] There was no difference in the sensitivities of CEM/M70-B1 and CEM cells to the cytotoxic effects of any agent tested (Figure 5 and data not shown). The lack of resistance of these cell lines to 1,2-naphthoquinones lends support to the finding that these compounds inhibit Topo II by an ATP-independent mechanism.[14]

Role of *bcl*-2 and *bcl*-x_L in resistance to 1,2-naphthoquinones

Previous studies indicated that β-lapachone induces cell death with characteristics of apoptosis in human prostate cells. Expression of *bcl*-2 or *bcl*-x_L has been implicated in resistance to chemotherapeutic drugs.[17,18] To determine whether overexpression of *bcl*-2 or *bcl*-x_L prevented cytotoxicity induced by 1,2-naphthoquinones, we investigated the toxicity of **1**, **5** and **7b** against FL5.12 cells transfected with the *neo* gene (control), *bcl*-2 cDNA or *bcl*-x_L cDNA (Figure 6). Neither *bcl*-2 nor *bcl*-x_L overexpression resulted in resistance to β-lapachone (**1**), a tricyclic analog (**5**) or a 4-alkoxy-1,2-naphthoquinone analog (**7b**) at 48 h post-treatment. Similar results were observed at 24 and 72 h post-treatment (data not shown). In contrast,

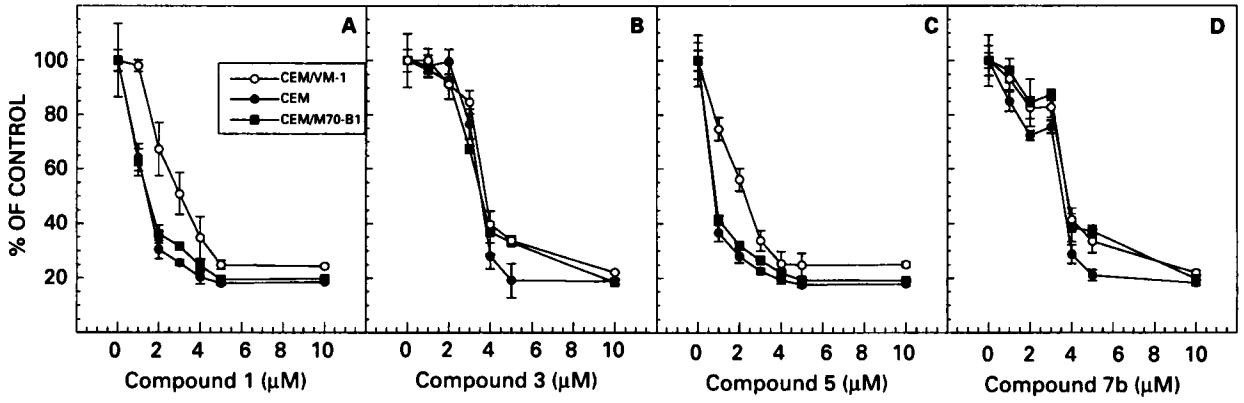


Figure 5. Effect of 1,2-naphthoquinones on the human leukemia cell lines: CEM, CEM/VM-1 and CEM/M70-B1. CEM (●), CEM/VM-1 (○) and CEM/M70-B1 (■) cells were exposed to fresh medium containing increasing concentrations of **1** (A), **5** (B), **3** (C) or **7b** (D) for 4 h. After 6 days, MTT analysis was performed to determine cell density. Inhibition of cell growth relative to control (zero drug addition) was calculated for each concentration. Each point represents the mean (±SD) of three determinations.

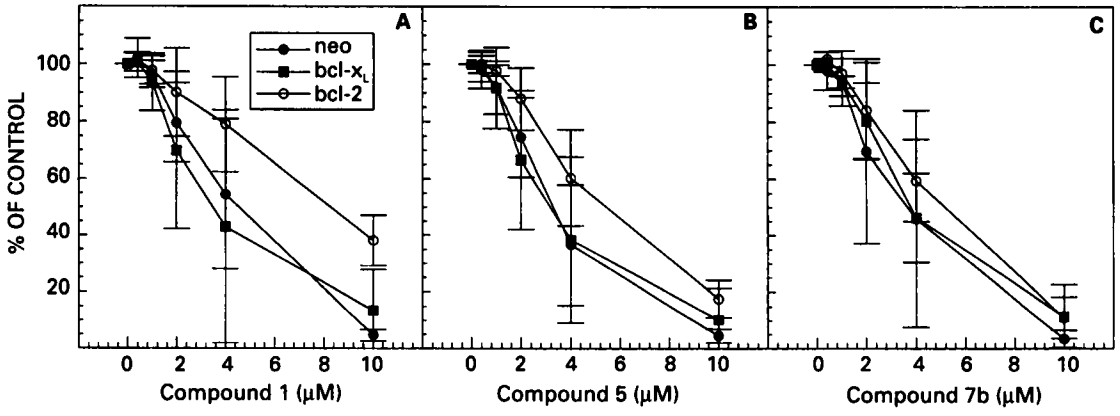


Figure 6. Effect of 1,2-naphthoquinones on the murine lymphoma line FL5.12 transfected with *neo*, *bcl*-2 or *bcl*-x$_L$. FL5.12 cells transfected with *neo* (●), *bcl*-x$_L$ (■) and *bcl*-2 (○) cells were exposed to fresh medium containing increasing concentrations of **1** (A), **5** (B) or **7b** (C) for 4 h. After 48 h, cells were stained with propidium iodide and analyzed by flow cytometry. Each point represents the mean (±SD) of results from three separate experiments with each experiment run in triplicate.

cells overexpressing *bcl*-2 and *bcl*-x$_L$ were less sensitive to cisplatin and vincristine than were control cells (data not shown).

Discussion

A major impediment to the effective chemotherapy of neoplastic diseases is the development of resistance by malignant cells. Compounding this problem is the observation that certain tumors demonstrate multidrug resistance, in which cross-resistance to a variety of agents that differ in both chemical structure and putative mechanisms of action is evident.[36,37] Recent investigations have revealed several markedly different biochemical and molecular characteristics of drug-resistant tumors, when compared to their drug-sensitive counterparts. Mechanisms of resistance include: (i) elevation of reduced glutathione or of glutathione-S-transferase activity, an enzyme that catalyzes the conjugation of glutathione to a variety of electrophilic compounds,[39] (ii) decreased intracellular accumulation of drug in association with the increased production of a membrane P-glycoprotein, P170,[37] or the overexpression of a MRP,[16] (iii) an altered topoisomerase enzyme with decreased catalytic activity[38] and (iv) overexpression of proteins that control apoptosis, *bcl*-2[17] or *bcl*-x$_L$.[18] We found that

while MCF7 ADR cells that are resistant to ADR are approximately 5-fold more resistant to tricyclic 1,2-naphthoquinones **1-5** than are parent MCF7 cells, 4-alkoxy-1,2-naphthoquinones **7a-11** activity exhibited a trend from only 2-fold resistance to actual collatoral sensitivity against MCF7 ADR cells, as the length of the 4-alkoxy group increased. It is possible that hydrophobicity plays a role in the uptake and/or retention of 1,2-naphthoquinones in MCF7 ADR cells.

Our data also shows that compounds **1-5** do not share cross-resistance with the Topo II inhibitors vinblastine and merbarone, that it is unlikely that MRP is involved in resistance to 1,2-naphthoquinones, since both HL60 and HL60 ADR cells are equally sensitive to the cytotoxic effects of the 4-alkoxy-1,2-naphthoquinones. Furthermore, while the 1,2-naphthoquinones, including β-lapachone, are not substrates for the P-glycoprotein pump, these compounds may react with GSH in a reaction that can either take place spontaneously or that can be catalyzed by glutathione-S-transferase. It is known that glutathione can react with quinones by either 1,2 or 1,4 Michael addition reactions.[39] The degree of GSH-related resistance of the 4-alkoxy-1,2-naphthoquinones is less than the degree of resistance to the tricyclic naphthoquinones. This fact may be a function of the slower reaction rates of the 4-alkoxy-1,2-naphthoquinones with soluble thiols, in contrast to the faster reaction rate of β-lapachone with the same thiols.[40]

To examine the possible role of oxidant radicals in tumor killing by 1,2-naphthoquinones, we evaluated the cytotoxicity of 1,2-naphthoquinones in cells expressing high levels of GPx. β-Lapachone has been shown to stimulate superoxide anion and H_2O_2 production in mitochondrial and microsomal suspensions as well as in intact cells.[41,42] It has been suggested that production of H_2O_2 is a mechanism through which β-lapachone kills mammalian cells.[42] Hydrogen peroxide can directly react with cellular components to produce a toxic effect. Detoxification of H_2O_2 by GPx may be a pathway through which cells become resistant to 1,2-naphthoquinones. GPx uses GSH to reduce H_2O_2 and various organic peroxides to water and the corresponding organic alcohols.[38,44] GPx levels in MCF7 GPx cells were increased by 65-fold, yet the transfected cell line and the parent line were equally sensitive to 1,2-naphthoquinones **1**, **3**, **5** and **7b**. Obviously, selenium-dependent GPx alone does not confer resistance to 1,2-naphthoquinones. Although Doroshow[44] reported that overexpression of the cDNA for GPx increased the tolerance of these cells to the oxidative stress induced by ADR, Liebman *et al.*[45] reported that overexpression of GPx in cells did not significantly change their resistance to ADR. In support of the data by Liebman *et al.*,[45] our transfectant MCF7 GPx cells did not show increased resistance to ADR (data not shown). While GPx may contribute to 1,2-naphthoquinone or ADR resistance under some circumstances, it is insufficient to confer this phenotype without other cellular changes. It is possible that GPx in combination with catalase or superoxide dismutase may play a role in the protection of 1,2-naphthoquinones.

Previous studies have demonstrated that β-lapachone induces apoptosis in prostate cancer cell lines and that this process was not protected by ectopically overexpressed *bcl*-2. We have extended this observation to include a lack of protection of cells overexpressing *bcl*-x_L to the cytotoxic effects of 1,2-naphthoquinones. These results suggest the existence of a cell death program that is independent of both *bcl*-2 and *bcl*-x_L that can be activated by β-lapachone. Further studies are needed to define the molecular target for β-lapachone-induced cell death and the contribution of the poisoning of Topo II.

Conclusion

β-Lapachone and related naphthoquinones are found to be cytotoxic against a panel of drug-sensitive and drug-resistant tumor cell lines, including MDR1 and MRP-overexpressing cell lines, the atypical MDR CEM/V1 and CEM/M70-B1 cell lines, cells overexpressing GPx, and cells overexpressing proteins known to protect from apoptotic cell death (*bcl*-2 or *bcl*-x_L). Resistance noted in MCF7ADR cells was due to the GSH redox cycle and could be circumvented with BSO. The fact that 1,2-naphthoquinones are not affected by those resistance mechanisms associated with other Topo II inhibitors opens new possibilities in the search for antitumor agents.

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