

# Identification of Potential Prostate Cancer Preventive Agents through Induction of Quinone Reductase *in Vitro*<sup>1</sup>

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## Abstract

Human prostate cancer is characterized by an early and near-universal loss of expression of the phase 2 enzyme glutathione *S*-transferase- $\pi$  (GSTP1). We hypothesize that a mechanism-based prostate cancer preventive strategy could involve induction of phase 2 enzymes within the prostate to compensate for the loss of GSTP1 expression. NAD[P]H:(quinone-acceptor) oxidoreductase (quinone reductase or QR) enzymatic activity, a surrogate of phase 2 enzyme response, was measured after treating the human prostate cancer cell line LNCaP with known phase 2 enzyme-inducing agents from 10 distinct chemical classes. QR enzymatic activity was assayed in microtiter plates using the menadione-coupled reduction of tetrazolium dye. Degree of induction was expressed as fold-increase over control and corrected for toxicity. Compounds were also tested in LNCaP-5-aza-C, an LNCaP subline selected in 5-aza-cytidine that expresses GSTP1, and in the human liver cell line HepG2. LNCaP showed robust induction of QR enzymatic activity after treatment with a subset of the phase 2 enzyme-inducing agents. All Michael acceptors were effective at inducing QR activity in LNCaP. Some phenolic antioxidants, heavy metal salts, and quinones also significantly increased QR activity, although inducer potency varied widely within these classes of compounds. Some of the isothiocyanates, mercaptans, bifunctional inducers, and trivalent arsenicals also produced modest QR induction, but peroxides and dithiolethiones were inactive. LNCaP-5-aza-C and LNCaP responded similarly to all compounds, but the pattern of response for HepG2 differed significantly. The differences in QR responsiveness between the prostate cell lines and HepG2 suggest that prostate tissues may have a unique pattern

of response to phase 2-inducing agents distinct from other tissue types. Our data suggest that measurement of QR induction in prostate cancer cell lines may help identify potential cancer chemopreventive agents effective in the prostate.

## Introduction

Prostate cancer is the most commonly diagnosed noncutaneous malignancy and second leading cause of cancer death in American men (1). One striking feature of this disease is the tremendous disparity in incidence and mortality rates worldwide. In contrast to Western industrialized nations, prostate cancer is rarely diagnosed and contributes little to cancer mortality in Asia (2, 3). Migration studies suggest that lifestyle and/or the environment are important determinants of prostate cancer pathogenesis. Men who emigrate from Asia to the United States acquire higher rates of prostate cancer, and subsequent generations of American-born Asian men retain this elevated risk (4–7). Although the environmental factors responsible for this change in risk are unknown, this observation suggests that lifestyle changes may prevent the development of prostate cancer or slow the progression of the disease. The development of preventive intervention strategies has become particularly pressing because large cohorts of men are identified who are at increased risk for prostate cancer, including African-Americans, those with a family history of prostate cancer, and men carrying genetic markers associated with prostate cancer risk (8–12).

The ideal prostate cancer preventive strategy has not been defined. Antiproliferative agents, compounds that induce differentiation, and drugs that alter the androgen milieu of the prostate have all been proposed as potential preventive approaches and are currently being evaluated in clinical trials (13). Another possible strategy, yet untested in prostate cancer, involves induction of enzymes of carcinogen defense (phase 2 enzymes), thereby buttressing the innate defenses of the prostate cell to slow accumulation of genetic alterations responsible for the development and progression of the disease. We have collected provocative evidence that such a strategy may be particularly relevant to prostatic carcinogenesis. Virtually all human prostate cancer cancers, regardless of grade or stage, lack expression of the phase 2 enzyme GSTP<sup>3</sup> (14–16). This loss of expression is associated with extensive methylation of deoxycytidine residues in the 5'-regulatory regions of the *GSTP1* gene. Intriguingly, this alteration appears to be an early event in prostatic carcinogenesis in that it can be found in prostatic intraepithelial neoplasia, a purported prostate cancer

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<sup>3</sup> The abbreviations used are: GSTP, glutathione *S*-transferase  $\pi$ ; QR, quinone reductase;  $I_{max}$ , maximum fold-induction for each of the compounds compared with DMSO-treated controls;  $EC_{100}$ , concentration of each compound at maximum induction;  $IC_{50}$ , concentration toxic to 50% of cells.

precursor lesion (17). Mice genetically lacking *GSTP1* have increased susceptibility to DMBA/TPA-induced skin cancer, suggesting that loss of this enzyme in itself can contribute to carcinogenesis (18).

A large body of evidence suggests that induction of phase 2 enzymes, and in particular the glutathione transferases, will prevent carcinogen-induced tumors in a number of species (19, 20). NAD[P]H:(quinone-acceptor) oxidoreductase or QR, a cytosolic FAD-dependent flavoprotein, is induced coordinately with the glutathione transferases and has served as a surrogate marker of phase 2 enzyme responsiveness *in vivo* and *in vitro* (21–25). QR protects cells against quinones and highly reactive semiquinones by catalyzing an obligate two-electron reduction of quinones to hydroquinones (26). In the prostate, QR has been shown to protect against formation of mutagenic 4-catechol-estrogen DNA adducts in Noble rats (27). *In vitro* methods have been devised to rapidly screen agents for QR induction and have been used to identify synthetic and diet-derived candidate chemopreventive agents (21–23). Several of these compounds have later been shown to prevent carcinogenesis in animal models (28, 29).

Previous *in vitro* screens of phase 2 enzyme-inducing compounds have usually been carried out using the Hepa1c1c7 murine hepatoma cell line. Although this cell line has documented utility in the identification of novel agents, it is unknown whether the responses observed in this cell line can be extrapolated to other tissue or cell types, to responses *in vivo*, or to other species. Because human prostate cancer selectively lacks *GSTP1* expression, we hypothesize that compounds able to induce phase 2 enzyme activity within prostate epithelial cells may hold promise as prostate cancer preventive agents. To evaluate the possibility of phase 2 enzyme induction in human prostatic cells, we screened a diverse set of 55 compounds for their ability to induce QR enzymatic activity in the human prostate cancer cell line LNCaP. Compounds evaluated include monofunctional inducers (Michael reaction acceptors, diphenols, quinones, isothiocyanates, peroxides, azo dyes, and heavy metals), bifunctional inducers, as well as other putative cancer preventive agents. Induction of QR activity was also assayed in an LNCaP subline (LNCaP-5-aza-C) that expresses *GSTP1* and in the human hepatoblastoma cell line HepG2. Measurement of toxicity of agents for each of the cell lines was carried out in parallel plates treated identically.

## Materials and Methods

**Reagents.** Vinylene trithiocarbonate, 1,2-dithiol[4,3-*c*]-1,2-dithiole-3,6-dithione, dimethyl fumarate, dimethyl maleate, 1-nitro-1-cyclohexene, phenyl isothiocyanate, phenethyl isothiocyanate, benzyl isothiocyanate, chalcone, perillyl alcohol, and selenium were obtained from Aldrich Chemical Co. (Milwaukee, WI). Linomide and a related compound, 2,4-quinolinediol, were a gift of Dr. John T. Isaacs (Johns Hopkins Oncology Center). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO).

**Cell Culture.** The human prostate cancer cell line LNCaP and human hepatoblastoma cell line HepG2 were obtained from American Type Culture Collection. The LNCaPazaC cell line was derived from selection of the LNCaP cell line in 5  $\mu$ M 5-aza-cytidine, a noncompetitive inhibitor of DNA methyltransferase, and stably expresses the *GSTP* enzyme (16). LNCaP and LNCaP-5-aza-C cell lines were cultured in 96-well plates at a density of 10,000 cells/well in 200  $\mu$ l of RPMI 1640 and grown in a humidified incubator in 5% CO<sub>2</sub> at 37°C. HepG2 cells were plated at a density of 4000 cells/well and

grown similarly. The following day, the medium was aspirated and replaced with RPMI 1640 supplemented with 10% charcoal-stripped FCS, 100 units/ml penicillin G, 100  $\mu$ g/ml streptomycin, and 0.1% DMSO. Test compounds were dissolved in DMSO and diluted in the medium such that the concentration of DMSO did not exceed 0.1%. Two-fold serial dilutions of each compound were made in the microtiter plates so that an entire row (eight wells) represented a single concentration of the compound. One row treated with DMSO alone served as a control, and another row containing only medium was used as a blank in absorbance determinations. After 48 h of exposure to each compound, plates were assayed for quinone reductase activity.

**Quinone Reductase Assays.** Quinone reductase activity was assessed by the menadione-coupled reduction of tetrazolium dye as modified from Prochaska and Santamaria (30). Medium was gently aspirated, and the cells were lysed by incubation at 37°C with 50  $\mu$ l of 0.08% digitonin and 2 mM EDTA (pH 7.8) with gentle agitation for 30 min. During this incubation, a stock solution was prepared by combining 16.7 mg of BSA, 7.5 mg of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide, 0.6 mg of NADP, 1.25 ml of 0.5 M Tris HCl (pH 7.4), 166.7  $\mu$ l of 1.5% Tween 20, 166.7  $\mu$ l of 150 mM glucose 6-phosphate, 16.7  $\mu$ l of 7.5 mM FAD, 50 units of yeast glucose 6-phosphate dehydrogenase, and distilled water to a final volume of 25 ml for each plate to be assayed. Immediately before use, 25  $\mu$ l of 50 mM menadione dissolved in acetonitrile were added to this stock solution. Two hundred  $\mu$ l of the complete stock solution was added simultaneously to the cell lysate in all 96 wells of the plate. Plates were immediately placed in a Tecan 96-well plate automated optical scanner, and readings at 610 nm were taken every 30 s. In virtually all instances, a change in absorbance attributable to the formation of blue-brown reduced tetrazolium dye was linear for well over 5 min; therefore, a single reading at 5 min was used for all compounds as described by Prochaska *et al.* (21).

**Toxicity Assessment.** Toxicity of the compounds was assessed in parallel plates treated identically to those used in assays for quinone reductase activity (30). After 48 h of exposure to each compound, cells were fixed with methanol and stained with 0.5% crystal violet for 5 min. Plates were then washed with distilled water and allowed to air dry overnight. Bound dye was dissolved with 200  $\mu$ l/well of 1% SDS, and the plates were scanned at 610 nm.

**Inducer Potency.** QR activity, in arbitrary units, was calculated automatically from the mean activity for all eight wells at each concentration for each compound using software developed in our laboratory. Activity was corrected for toxicity at each concentration as described (30). Inducer potency (fold-induction of QR activity) was expressed as the ratio of corrected QR activity for treated cells to corrected QR activity for the vehicle controls.

## Results

**QR Induction in LNCaP.** Phase 2 enzyme-inducing agents comprise a chemically diverse set of compounds and have been demonstrated to prevent carcinogen-induced tumors in a variety of model systems. To characterize the phase 2 enzyme responsiveness of the human prostate cancer cell line LNCaP, we measured QR activity after treatment with 34 different phase 2-inducing agents from 10 distinct chemical classes. Compounds were selected because of their ability to induce phase 2 enzyme activity in Hepa1c1c7 murine hepatoma cells or another model system.

Table 1 Quinone reductase inducer potency and toxicity of compounds of diverse chemical classes in LNCaP cells

Compound	Dose range ( $\mu\text{M}$ )	$I_{\text{Max}}$ Mean $\pm$ SD	$P^a$	$\text{EC}_{100}$ ( $\mu\text{M}$ )	$\text{IC}_{50}$ ( $\mu\text{M}$ )
Bifunctional inducers (PAHs)					
Benzo(a)pyrene	0.040–200	NI <sup>b</sup>		NI	>200
20-Methylcholanthrene	0.78–200	NI		NI	>200
$\beta$ -naphthoflavone	0.008–500	NI		NI	500 $\pm$ 150
Sudan 1	0.020–500	1.59 $\pm$ 0.169	.00008	25	260 $\pm$ 62
Sudan 2	0.20–50	NI		NI	>50
Sudan 3	0.20–500	1.28 $\pm$ 1.89	.0008	50	>500
1-[2-Thiazolylazo]-2-naphthol	0.20–100	NI		NI	13 $\pm$ 2
1-[2-Pyridoylazo]-2-naphthol	0.20–50	NI		NI	7.5 $\pm$ 2
Isothiocyanates					
Benzyl isothiocyanate	0.20–50	1.10 $\pm$ 0.096	.001	1.56	3.1 $\pm$ 0.5
Phenyl isothiocyanate	2.0–500	NI		NI	170 $\pm$ 24
Phenethyl isothiocyanate	0.20–50	1.19 $\pm$ 0.143	.005	3.125	6 $\pm$ 0.9
Phenolic antioxidants					
Butylated hydroxyanisole	0.78–200	1.17 $\pm$ 0.072	.009	25	120 $\pm$ 60
Butylated hydroxytoluene	2.0–500	NI		NI	300 $\pm$ 100
Catechol	0.78–200	2.14 $\pm$ 0.279	.000001	12.5	75 $\pm$ 6
Resorcinol	0.20–5000	NI		NI	5000 $\pm$ 900
Heavy metal salts					
CdCl <sub>2</sub>	0.20–50	1.52 $\pm$ 0.213	.002	3.25	10 $\pm$ 2.5
HgCl <sub>2</sub>	0.20–50	5.54 $\pm$ 0.235		50	50 $\pm$ 12
ZnCl <sub>2</sub>	2.0–500	NI	.00000001	NI	150 $\pm$ 16
Peroxides					
Cumene hydroperoxide	0.39–100	NI		NI	25 $\pm$ 5
Hydrogen peroxide	2.0–500	NI		NI	70 $\pm$ 17
<i>tert</i> -Butyl hydroperoxide	0.39–100	NI		NI	13.5 $\pm$ 5
Mercaptans					
1,2-Ethanedithiol	2.0–500	1.27 $\pm$ 0.109	.0006	31.25	70 $\pm$ 14
Michael acceptors					
Coumarin	0.20–5000	1.22 $\pm$ 0.152	.003	50	2500 $\pm$ 1100
Dimethyl maleate	2.0–500	2.46 $\pm$ 0.312	.000005	62.5	100 $\pm$ 16
Dimethyl fumarate	0.78–200	2.05 $\pm$ 0.151	.000000007	80	100 $\pm$ 23
$\alpha$ -Methylene- $\gamma$ -butyrolactone	0.20–50	2.20 $\pm$ 0.203	.0000003	25	30 $\pm$ 1.4
1-Nitro-1-cyclohexene	0.20–50	1.47 $\pm$ 0.196	.002	3.125	7 $\pm$ 1.1
Quinones					
Hydroquinone	0.20–50	3.54 $\pm$ 0.431	.0000009	12.5	>50
Ethoxyquin	0.20–500	NI		NI	70 $\pm$ 7
Dithiolethiones					
[1,2]Dithiolo-dithiole-dithione	2.0–500	NI		NI	125 $\pm$ 11
Vinylene trithiocarbonate	2.0–500	NI		NI	>500
Trivalent arsenicals					
Phenylarsine oxide	0.008–50	1.54 $\pm$ 0.256	.0008	0.125	0.13 $\pm$ 0.03
Sodium <i>m</i> -arsenite	0.040–10	NI		NI	3.5 $\pm$ 0.5

<sup>a</sup> QR activity of treated cells compared with vehicle-treated controls by a two-tailed Student's *t* test.

<sup>b</sup> NI, not induced.

LNCaP readily responded to several phase 2 enzyme-inducing agents. Table 1 shows  $I_{\text{Max}}$ ,  $\text{EC}_{100}$ , and  $\text{IC}_{50}$ . A typical induction profile is shown in Fig. 1. All Michael acceptors, particularly dimethyl maleate, dimethyl fumarate, and methylene butyrolactone, reliably produced significant induction of QR activity in LNCaP. No other chemical class universally produced robust QR induction in LNCaP, and responses to individual members of each class varied widely. For instance, catechol was the only phenolic antioxidant to robustly increase QR activity; hydroquinone and HgCl<sub>2</sub> were also the only compounds in their classes to induce QR. Two of the bifunctional inducers (planar aromatic hydrocarbons, known to induce both phase 1 and phase 2 enzymes) produced modest induction in LNCaP as did the isothiocyanates, peroxides, mercaptans and trivalent arsenicals. Somewhat surprisingly, the dithiolethiones failed to induce quinone reductase altogether.

**QR Induction in LNCaP by Other Cancer Chemopreventive Agents.** We evaluated whether 21 compounds implicated as potential chemopreventive agents could influence phase 2

enzymatic activity in LNCaP (Table 2). Epigallocatechin was the only tea catechin to produce slight QR induction in LNCaP at near-toxic doses. Two selenium compounds, sodium selenite and selenium dioxide, produced modest elevation of QR activity at concentrations approaching their  $\text{IC}_{50}$  for LNCaP. Of the remaining diverse set of compounds, quercetin (1.66-fold) and chalcone (1.44-fold) produced modest increases in QR activity, whereas curcumin and *para*-coumaric acid produced more significant levels of induction at micromolar doses. Curcumin-treated cells showed QR induction over baseline starting at 6.25  $\mu\text{M}$  that peaked at 2.01-fold at 25  $\mu\text{M}$ . Curcumin was toxic at slightly higher doses ( $\text{IC}_{50}$ , 50  $\mu\text{M}$ ). *para*-Coumaric acid produced QR induction in LNCaP that began at 62.5  $\mu\text{M}$  and increased linearly with dose. *para*-Coumaric acid was not toxic to LNCaP, even at high concentrations ( $\text{IC}_{50}$ , >1000  $\mu\text{M}$ ).

**Induction Patterns in LNCaP Cell Lines Differ from HepG2.** Phase 2 enzyme response has been reported to vary significantly between different species and between different tissue types in single animals (28, 31–35). Because LNCaP

Fig. 1. QR induction over a range of concentrations for LNCaP, LNCaP-5-aza-C, and HepG2 after treatment with HgCl<sub>2</sub>. Similar induction profiles were generated for the 55 compounds screened in the three cell lines. Toxicity profiles were plotted similarly. Bars, SD.

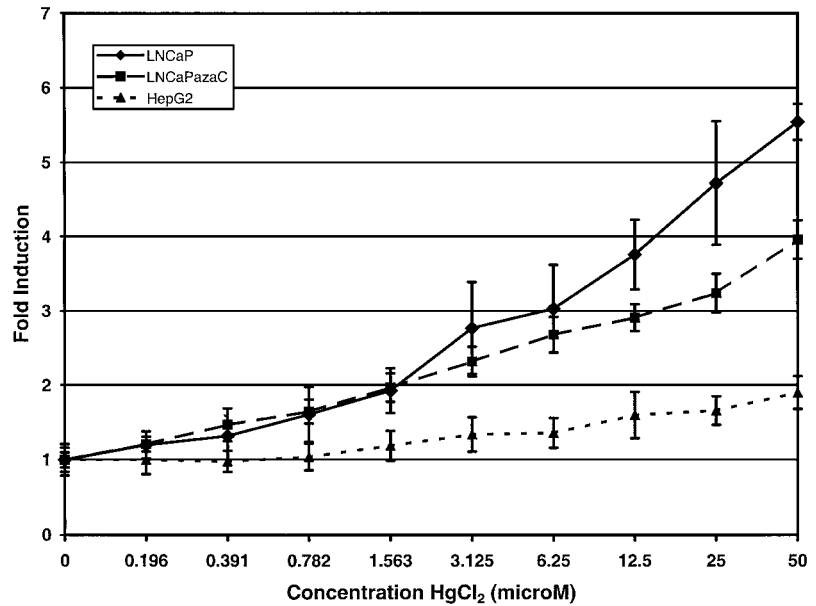


Table 2 Inducer potency and toxicity of putative preventive agents in LNCaP cells

Compound	Dose range (μM)	I <sub>Max</sub> Mean ± SD	P	EC <sub>100</sub> (μM)	IC <sub>50</sub> (μM)
Catechins					
Catechin	2.0–500	NI		NI	>500
Epicatechin	2.0–500	NI		NI	>500
Epicatechin gallate	2.0–500	NI		NI	230 ± 74
Epigallocatechin	2.0–500	1.26 ± 0.287	.0005	62.5	80 ± 16
Epigallocatechin gallate	2.0–500	NI		NI	95 ± 40
Selenium compounds					
Selenium dioxide	0.20–50	1.59 ± 0.132	.0002	6.25	7.5 ± 0.8
Selenium	0.20–50	NI		NI	40 ± 13
Selenocystamine	0.20–50	NI		NI	11 ± 3
Selenocysteine	0.20–50	NI		NI	14 ± 4
Selenomethionine	0.20–50	NI		NI	>50
Selenium sulfate	0.20–50	NI		NI	>50
Sodium selenate	0.20–50	NI		NI	35 ± 13
Sodium selenite	0.20–50	1.48 ± 0.339	.005	3.125	5.5 ± 0.8
Others					
Perillyl alcohol	2.0–500	NI		NI	>500
Quercetin	2.0–500	1.66 ± 0.171	.0005	15.63	62.5 ± 13
Chalcone	2.0–500	1.44 ± 0.118	.000002	31.25	62 ± 12
Lycopene	1.0–250	NI		NI	>250
Curcumin	0.20–50	2.01 ± 0.239	.00002	25	50 ± 9
Limonene	2.0–500	NI		NI	400 ± 180
Linomide	0.20–50	NI		NI	3.9 ± 1.8
2,4-Quinolinediol	2.0–500	NI		NI	>500
para-Coumaric acid	3.91–1000	2.28 ± 0.171	.00000008	1000	>1000

<sup>a</sup> Abbreviations given in Table 1.

displayed a spectrum of induction to the 34 compounds that differed from that reported for Hep1c1c7, we wondered whether those differences were attributable to their species of origin, to their tissue of origin, or to both. In addition, because normal prostatic epithelial cells express GSTP1, we were curious whether reexpression of GSTP1 in LNCaP would affect QR induction (14). Therefore, we evaluated the degree and pattern of QR response to all 55 compounds we had tested in LNCaP in the human hepatoblastoma cell line HepG2 and in an LNCaP cell line selected in 5-aza-cytidine (LNCaP-5-aza-C)

which, unlike the parent cell line, expresses the phase 2 enzyme GSTP (GSTP1; Ref. 16). In both HepG2 and LNCaP-5-aza-C, all compounds were tested over the range of concentrations listed for LNCaP in Table 1. Toxicity measurements were carried out in parallel plates handled identically.

Depicted in Fig. 2 are the 35 compounds that produced a QR response in at least one of the three cell lines. The remaining 20 compounds failed to generate significant QR induction in any of the cell lines and are not shown. Although there were some minor quantitative differences in response between



	LNCaP	LNCaPazaC	HepG2
20-Methylcholanthrene	NI	NI	1.32±0.20
Butylated hydroxyanisole	1.17±0.072	1.55±0.13	1.87±0.21
Butylated hydroxytoluene	NI	NI	1.22±0.09
Benzyl isothiocyanate	1.10±0.096	NI	1.37±0.10
Beta-naphthoflavone	NI	NI	<b>4.98±1.98</b>
Benzo[a]pyrene	NI	NI	1.62±0.13
Catechol	<b>2.14±0.28</b>	<b>2.47±0.38</b>	1.30±0.13
CdCl <sub>2</sub>	1.52±0.21	1.40±0.18	1.21±0.10
Chalcone	1.44±0.12	1.60±0.43	1.41±0.19
Coumarin	1.22±0.15	1.31±0.10	1.42±0.13
Curcumin	<b>2.01±0.24</b>	<b>2.48±0.12</b>	1.74±0.24
Dimethyl fumarate	<b>2.05±0.15</b>	<b>3.76±0.63</b>	1.78±0.18
Dimethyl maleate	<b>2.46±0.31</b>	<b>3.24±0.39</b>	<b>2.25±0.07</b>
Epicatechin	NI	1.36±0.16	NI
1,2-Ethanedithiole	1.27±0.11	1.18±0.13	NI
Epigallocatechin	1.26±0.29	NI	NI
Ethoxyquin	NI	1.53±0.15	1.38±0.09
HgCl <sub>2</sub>	<b>5.54±0.24</b>	<b>3.69±0.24</b>	1.9±0.22
Hydroquinone	<b>3.54±0.43</b>	<b>5.66±0.93</b>	<b>2.12±0.17</b>
Methylene butyrolactone	<b>2.20±0.20</b>	<b>3.61±0.51</b>	1.75±0.14
Nitrocyclohexene	1.47±0.20	1.80±0.19	1.26±0.12
Pyridoylazo-2-naphthol	NI	NI	1.70±0.37
Phenylarsine oxide	1.54±0.26	<b>2.00±0.28</b>	1.23±0.11
Para-coumaric acid	<b>2.26±0.17</b>	<b>2.02±0.15</b>	1.14±0.11
Phenethyl isothiocyanate	1.19±0.14	NI	1.32±0.23
Quercetin	1.66±0.17	1.59±0.22	1.69±0.13
Sodium m-arsenite	NI	NI	1.43±0.10
Selenium dioxide	1.89±0.13	1.54±0.19	NI
Sodium selenite	1.48±0.34	NI	NI
Sudan 1	1.59±0.17	1.90±0.11	<b>2.63±0.19</b>
Sudan 2	NI	NI	<b>2.55±0.28</b>
Sudan 3	1.28±0.19	1.29±0.11	1.87±0.12
Thiazolylazo-2-naphthol	NI	NI	1.47±0.15
ZnCl <sub>2</sub>	NI	<b>1.69±0.31</b>	1.46±0.12

Fig. 2.  $I_{\text{Max}}$ s for LNCaP, LNCaP-5-aza-C, and HepG2 for 35 compounds effective in at least one of the cell lines. All values listed show significant induction of QR in treated cells compared with vehicle-treated control at  $P < 0.05$  by a two-tail Student's  $t$  test. An additional 20 compounds that had no effect in any of the three cell lines are not shown. Differences in the patterns of response are highlighted with darker grays representing greater QR induction. The pattern of response is similar between the prostate cell lines and contrasts sharply with HepG2.

LNCaP and LNCaP-5-aza-C (e.g., HgCl<sub>2</sub>, methylene butyrolactone), in most cases QR responsiveness in the GSTP1-expressing LNCaPazaC cell line was virtually identical to the parental cell line. QR induction in HepG2 differed significantly from the prostate cell lines for most of the compounds tested. HepG2 responded robustly to bifunctional inducing agents including  $\beta$ -naphthoflavone, benzo(a)pyrene, and the azo dyes, whereas the prostate cell lines usually responded meagerly to these agents or failed to respond altogether. Response to most monofunctional inducers, although present, was often blunted somewhat in HepG2 compared with the prostate cell lines. Toxicity profiles and IC<sub>50</sub> levels differed little between the prostate and liver cell lines (not shown). Thus, there appear to be significant qualitative and quantitative differences in the pattern of QR response between cells derived from different tissues.

## Discussion

The human prostate cancer cell line LNCaP appears to be an excellent model for identifying potential prostate cancer preventive agents that act through induction of phase 2 enzymes. LNCaP expresses QR, possesses QR enzymatic activity, and has the capacity to respond to phase 2 enzyme-inducing agents. Because sulforaphane induces several phase 2 enzymes and glutathione synthetic pathways in LNCaP, QR appears to be a valid surrogate of phase 2 enzyme activity in this cell line (36).

Reexpression of GSTP1 by selection with 5-azacytidine did little to alter the pattern or degree of QR responsiveness to chemically diverse compounds. Phase 2 enzyme response in LNCaP and LNCaP-5-aza-C did differ significantly from that of HepG2 and that reported for the murine hepatoma cell line Hepa1c1c7. In part, these differences may be attributable to their tissue of origin, or, for Hepa1c1c7, their species of origin, particularly because rodent cells are more labile in their phase 2 enzyme response than human cells (28, 31–35). Furthermore, the carcinogen *N*-OH-2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine can be activated directly in prostate cancer cell lines, and reexpression of GSTP1 will prevent this activation (37). Therefore, preventive agents that act through induction of phase 2 enzymes may be particularly relevant to human prostate cancer prevention and should be tested for efficacy in human prostatic cell lines. It should be noted, however, that toxic compounds, such as HgCl<sub>2</sub> and some azo dyes, can also induce QR activity. Additional work will be necessary to test whether agents effective in prostate cells *in vitro* are safe and will also produce phase 2 enzyme induction *in vivo*.

The LNCaP cell lines showed a distinct pattern of QR response to monofunctional inducers of several chemical classes. LNCaP and LNCaP-5-aza-C showed robust QR induction when treated with classic Michael reaction acceptors including dimethyl fumarate, dimethyl maleate, and methylene butyrolactone, suggesting this class of compounds may hold promise as prostate cancer preventive agents. Within other chemical classes, QR induction in the LNCaP cell lines was more varied. Both cell lines displayed significant QR induction when treated with catechol, HgCl<sub>2</sub>, and hydroquinone but little or no induction when treated with other members of these chemical classes. Similarly, sulforaphane will produce vigorous QR induction in LNCaP; yet we observed very little response to other isothiocyanates in this study (36). We were surprised that dithiolethiones failed to induce QR in LNCaP, particularly because they are effective in other *in vivo* and *in vitro* model systems and ongoing clinical trials in liver cancer with these agents (38, 39). Our findings raise questions whether dithiolethiones would be effective as prostate cancer chemopreventive agents.

The factors underlying the unique patterns of response in LNCaP and the other cell lines are unknown. Phase 2 enzyme response is regulated transcriptionally by Nrf2 binding at antioxidant response enhancer elements (40). Treatment with phase 2 enzyme-inducing agents activates mitogen-activated protein kinase, protein kinase C, and phosphatidylinositol 3-kinase pathways that lead to release of Nrf2 from Keap1 in the cytoplasm, translocation of Nrf2 to the nucleus, and binding together with Maf to antioxidant response enhancers (41). The induction capacity of any compound will be influenced by its ability to stimulate thiol-dependent sensors in the cytoplasm, a process that depends on the biochemical milieu of the cell. Spencer *et al.* (42) and Talalay *et al.* (43) have proposed that the capacity of a compound to induce phase 2 enzyme expression is directly related to its ability to act as a Michael acceptor. For heavy metal salts, inductive capacity parallels their affinity for sulfhydryl groups (44). Therefore, compounds active in LNCaP may be more prone to exist as Michael acceptors or other chemical species with high affinity for thiol groups (45). Zhang and colleagues (46–48) have reported that phase 2 enzymatic induction by isothiocyanates parallels the accumulation of glutathione conjugates intracellularly, and that this accumulation can be affected by GSH concentration and glutathione transferase activity. We did not observe a direct relationship between GSTP1 expression and inducer potency between the

LNCaP and LNCaP-5-aza-C cell lines, suggesting that factors beyond glutathione transferase activity may account for phase 2 enzyme responsiveness in these prostatic cell lines.

The ability of a compound to act as a phase 2 enzyme-inducing agent may also depend on the unique profile of gene expression in each cell line. Large-scale gene expression profiling has demonstrated that cell lines possess unique gene expression patterns that retain many features of their tissue of origin (49). These findings suggest that the response to chemopreventive agents observed *in vitro* may parallel their effects *in vivo*. The expression data also highlight that the response to any compound will depend upon genes expressed in the cell line in which it is tested. Indeed, the differences in QR response we observed in LNCaP and HepG2 could be attributable to the differences in the genes they express. For instance, LNCaP cells showed little QR induction after treatment with bifunctional inducing agents, whereas these agents were the most potent QR inducers in HepG2. Bifunctional inducers require conversion by phase 1 enzymes into oxidized metabolites that then induce phase 2 enzymatic activity (43). We have observed previously that LNCaP cells are unable to activate the heterocyclic amine PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) by *N*-hydroxylation into carcinogenic *N*-OH-PhIP, suggesting low or absent phase 1 enzymatic activity (37). Thus, one possible explanation for the meager QR induction in LNCaP cells in response to bifunctional inducers is that they do not express the enzymes necessary to metabolize the compounds into QR-inducing agents. In addition to this difference between LNCaP and HepG2, we suspect that the unique patterns of response to diverse phase 2 enzyme-inducing agents in different cell lines may be attributable to other poorly characterized differences in gene expression, such as differences in the pattern of expression of thiol-dependent sensing proteins and cell line-specific expression of metabolic enzymes and signaling pathways. Gene expression profiling and proteomics will help define the molecular underpinnings of the phase 2 enzyme response in different tissues (50).

Our limited survey of candidate chemopreventive agents for QR induction in LNCaP demonstrates the potential for this model system in identifying novel agents for use in prostate cancer. Curcumin reliably produced robust induction of QR at micromolar doses in the prostate cells. The potency of curcumin in LNCaP undoubtedly relates to its ability to act as a classic Michael acceptor (51). Curcumin is also intriguing as a prostate cancer preventive agent because it possesses anti-inflammatory effects and inflammation, and free radical generation has been implicated in prostatic carcinogenesis (52–54). Curcumin can also inhibit cyclooxygenase-2 and inducible nitric oxide synthase, and clinical trials are under way to evaluate cyclooxygenase-2 inhibitors as prostate cancer preventive agents (55). Although there is some debate about curcumin, its ability to quench free radicals as well as induce phase 2 enzymes make it attractive as a prostate cancer preventive agent (56, 57).

The flavonoids quercetin and chalcone both produced modest induction in QR at micromolar doses. Both are distributed widely in plants and have been shown to act as phase 2 enzyme-inducing agents in other systems (58, 59). The ability of these compounds to induce QR in prostatic cells may help explain the observed inverse correlation between vegetable consumption and prostate cancer risk (60–63). Other epidemiological studies have noted an inverse correlation between serum selenium levels and prostate cancer risk, and we observed modest induction of QR by selenium dioxide and sodium selenite (64, 65). Our results suggest that one of the ways

that selenium may act to prevent prostate cancer is by inducing phase 2 enzyme activity.

*para*-Coumaric acid readily induced QR activity with little toxicity at high doses. Tomatoes possess relatively high levels of *para*-coumaric acid, and tomato consumption has been associated with a decreased risk of prostate cancer (66, 67). Previous work has ascribed this preventive effect to lycopene, the most potent quencher of singlet oxygen of all carotenoids but, in our hands, lacking QR inducing activity (68). Our findings raise the intriguing possibility that lycopene and *para*-coumaric acid in tomatoes may act in concert to protect against prostate cancer by quenching free radicals and inducing carcinogen defenses in prostate cells.

In summary, the human prostate cancer cell line LNCaP could serve as a model for future screens to identify phase 2 enzyme-inducing chemopreventive agents with activity in human prostate tissues. Although Michael acceptors appear most promising as prostate phase 2 enzyme-inducing agents, several other classes of compounds also show robust activation not easily predicted by their chemical class. Future efforts will focus on identifying additional phase 2 enzyme-inducing agents effective in the prostate *in vitro* and *in vivo* and in defining the factors responsible for the unique pattern of response to phase 2 enzyme-inducing agents in prostate cells.

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