Screening strategies for the detection of anticarcinogenic enzyme inducers

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The induction of electrophile-processing Phase II enzymes (i.e., glutathione S-transferases, UDP-glucuronosyltransferases, and quinone reductase) is a major mechanism whereby a large group of heterogeneous compounds prevent the toxic, mutagenic, and neoplastic effects of carcinogens. This paper reviews the direct assay of quinone reductase in cultured cells as a method to rapidly detect potentially anticarcinogenic substances. Cells (usually Hepa 1c1c7 murine hepatoma cells) growing in 96-well microtiter plates are exposed to test compounds for 24 to 72 hrs and are then lysed and assayed for quinone reductase activity by measuring the formation of formazan dye from the menadione-dependent reduction of MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide). Reaction velocities are linearly dependent on quinone reductase over a wide range of enzyme concentrations, and the specificity of MTT reduction can be ascertained with dicoumarol, a potent inhibitor of quinone reductase. This in vitro technique reliably detects compounds that effectively induce Phase II enzymes in vivo, and the screening assay has identified hitherto unrecognized and novel inducers from synthetic and natural sources. As inducers of Phase II enzymes are receiving serious consideration as potential human anticarcinogens, the assay described offers the opportunity to rapidly identify, isolate, and characterize inducers of medicinal interest. (J. Nutr. Biochem. 5:360–368, 1994.)

Keywords: chemoprevention; vegetables; Phase II enzymes; enzyme induction; quinone reductase

Introduction

Cancer is potentially a preventable disease. Up to 85% of all human malignancies have been estimated to be caused by extra-genetic factors, which include smoking, diet, ethanol, drugs, pesticides, and infectious agents, as well as sexual and behavioral practices.[1,2] Although causative factors for the genesis of human malignancies have been recognized for more than 200 years, the identification of compounds that prevent cancer is much more recent.[3–5] Compelling evidence has accumulated in the last half century to suggest that a wide variety of compounds are effective inhibitors of experimental carcinogenesis. Accordingly, there is increasing enthusiasm for identifying compounds that could serve as human anticarcinogens.[6–9]

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Initial studies screening for anticarcinogenic activity required the use of long-term carcinogenesis assays. Although candidate chemopreventive agents must be proven to prevent experimentally induced tumors, the identification of the mechanisms that can disrupt the neoplastic process offers the opportunity to develop rapid screening assays. The recognition that many anticarcinogens shared the ability to induce Phase II enzymes[10–17] was a critical advance for two reasons. First, it provided a unifying mechanism for protection because Phase II enzymes divert ultimate carcinogens from reacting with critical cellular macromolecules. Second, if the induction of Phase II enzymes is a causal mechanism for protection, then novel chemoprotective agents should be identifiable by their ability to elevate tissue levels of Phase II enzymes.[10,17] One notable success has been the identification of oltipraz, which is currently undergoing clinical evaluation as a human anticarcinogen.[7,9] Oltipraz was correctly predicted by Bueding to possess anticarcinogenic activity based solely on its ability to induce Phase II enzymes in rodents.[18–21]

Screening compounds for their ability to induce Phase II enzymes in vivo is considerably more rapid than screening compounds for anticarcinogenic activity. Nevertheless, these studies require large numbers of animals, and the assay of multiple tissues is time-consuming and tedious. Talalay and colleagues developed a more rapid screening system by demonstrating that quinone reductase is induced in the Hepa 1c1c7 murine hepatoma cell line by many compounds that induce Phase II enzymes in vivo.[22,23] This cell line and its mutant subclones have been instrumental in determining the molecular mechanisms responsible for Phase II enzyme induction.[22–28] However, the procedures entailed in conventional assays (e.g., harvesting, homogenizing, centrifuging, and assay of quinone reductase and protein content) restricted the usefulness of cultured cells to screen for inductive activity.

Prochaska and Santamaria[29] described a rapid and direct assay of quinone reductase in Hepa 1c1c7 cells grown and induced in microtiter wells. This assay has proven to be an ideal method for screening compounds for inductive activity.[28,30,31] Moreover, as many vegetables and fruits are able to induce Phase II enzymes in vivo,[3,16,20,32] the assay offers the opportunity to purify and characterize the inductive activity from complex plant extracts. Subsequent studies have shown this proposal[29] to be correct: Inductive activity from vegetable extracts can be measured and quantified,[33] and the method has been used to purify sulforaphane, the major inductive activity of SAGA broccoli.[34]

The purpose of this paper is to describe the experimental details for the direct measurement of quinone reductase from cells treated with test compounds and to discuss the advantages and limitations of the method.

Methods and materials

Materials

The reagents required for the assay of quinone reductase can be purchased from the sources cited.[29] In addition, we purchase activated charcoal from Sigma (St. Louis, MO USA). The Hepa 1c1c7 cell line and its mutant subclones were obtained from O. Hankinson, University of California, Los Angeles, and J.P. Whitlock, Jr., Stanford University, Palo Alto, California USA.

Methods

Hepa 1c1c7 culture conditions. Hepa 1c1c7 cells and its mutant subclones are grown at 37°C in humidified incubators containing 5% CO_2. The medium used is α-minimal essential medium (without ribonucleosides and deoxyribonucleosides) supplemented with 10% fetal calf serum. Although our cultures are grown without antibiotics, the media can be supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. We have found that heat- and charcoal-treated fetal calf serum[31,33] renders Hepa cells more sensitive to inducers as well as decreases the basal quinone reductase activity. The net effect of this treatment is that the signal for inductive activity is increased. Technical note: Some cell lines do not grow well in medium supplemented with heat- and charcoal-treated fetal calf serum. Therefore, if another cell line is used, it is important to compare their growth in medium containing heat- and charcoal-treated fetal calf serum with untreated serum.

Growth of cells in microtiter plates. *tert*-Butylhydroquinone (t-BHQ) and β-naphthoflavone (βNF) were chosen as representatives of monofunctional and bifunctional inducers,[25]† respectively, to illustrate the typical results achieved with the screening assay. The compounds were dissolved in dimethyl sulfoxide (DMSO) and were diluted 1:1000 into medium shortly before use. In the example under discussion, Hepa 1c1c7 and c1 cells were plated at densities of 5,000 cells per 0.32 cm^2 microtiter well. BP^{r}c1 cells, which grow more slowly, were plated at a density of 10,000 cells per well. The final volume was 150 μL medium/well. The first column of wells (column "1"; $N = 8$ wells) on the edge of the microtiter plates were left empty to serve as optical blanks for the spectrophotometric assays described below. After 24 hrs of growth, the medium was decanted and all wells except the column of wells next to the cell-free column (column "2") were refed 150 μL/well medium containing 0.1% final concentration DMSO. The empty column of wells (column "2") was then refed 150 μL/well of medium containing either 5 μM βNF or 240 μM t-BHQ dissolved in 0.1% final concentration DMSO. To the adjacent column of wells (column "3"), 150 μL/well of the same medium was added, thoroughly mixed by repeated refilling and gently dispensing the contents of the multichannel pipette, and 150 μL/well was removed and added to the next column of wells. These steps were repeated to generate a series of twofold dilutions down the plate to the tenth column of wells. The last two columns ("11" and "12") served as control wells. Duplicate sets of plates were prepared.

Assay of quinone reductase activity. The method of Prochaska and Santamaria[29] was used to assay for quinone reductase activity, but is described here briefly. After the plates were exposed to inducers for 48 hrs, the media from one set of plates were decanted and the cells were lysed by agitating the plates on an orbital shaker in the presence of 50 μL/well of 0.8% digitonin and 2 mM EDTA, pH 7.8 for 10 min. Before the plates were assayed for quinone reductase activity, a cocktail containing 25 mM Tris•Cl (pH 7.4), 0.67 mg/mL bovine serum albumin, 0.01% Tween-20, 5 μM FAD, 30 μM $NADP^+$, 1 mM glucose 6-phosphate, 2 U/mL Bakers yeast glucose-6-phosphate dehydrogenase, and 0.3 mg/mL MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] was prepared for all plates to be assayed (25 mL/plate). Shortly before each plate was scanned, 25 μL of 50 mM menadione dissolved in acetonitrile was mixed with 25 mL of assay cocktail. After 200 μL/well of the resulting solution was added to the plates, the plates were placed in an uv_{max} microtiter plate scanner (Molecular Devices, Menlo Park, CA USA), and the rate of formation of the formazan dye was quantitated at 610 nm for two min. The dicoumarol-inhibitable rate of MTT reduction was measured by rescanning the plates after the addition of 50 μL/well of 0.3 mM dicoumarol dissolved in 0.5% DMSO and 5 mM potassium phosphate (pH 7.4).

Assay of cytotoxicity. The remaining plates were stained for cell densities with crystal violet.[29] Briefly, the plates were shaken free of media and placed in a vat containing 0.2% crystal violet and 2% ethanol for 10 min. The plates were then washed with tap water for 2 min, and the retained dye was solubilized by warming the plates at 37 to 45°C for 60 to 120 min with 200 μL/well of 0.5% sodium dodecyl sulfate and 50% ethanol. The plates were then scanned at 610 nm.

Calculation of specific activities. Software packages that allow transfer of data to diskettes for processing are available for commercially available microtiter scanners. In our case, the velocities of the quinone reductase activity and the crystal violet absorbancies are stored as *.prn files. The files are then directly imported to prepared Lotus 1-2-3 spreadsheets (Lotus Development Corp., Cambridge, MA USA) that allow the data to be calculated automatically *(Figure 1)*. Depending on the application, spreadsheets are written to determine the parameter desired (mean, standard error of the mean for each condition, ratio of inducer-treated to control cells, etc.). We express quinone reductase activities and cell densities in terms of inducer-treated to control cells, and the increase in specific activity is determined by calculating the ratio of these values.

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†Anticarcinogenic enzyme inducers can be segregated into two families.[3,25] Bifunctional inducers can be characterized as flat planar polycyclic aromatics (i.e., flavanoids, azo dyes, polycyclic aromatic hydrocarbons) and are potent ligands for the *Ah* receptor. These compounds dramatically induce *Ah* receptor-dependent Phase I enzymes as well as induce Phase II enzymatic activities. Monofunctional inducers are structurally diverse (i.e., phenols, isothiocyanates, dithiolethiones, dithiocarbamates) and induce Phase II enzymes with little or no effect on *Ah*-receptor-dependent Phase I enzyme induction.

t-BHQ DOSE RESPONSE IN HEPA 1c1c7 CELLS

		CONCENTRATION [μM]									
	BLANK	240.0	120.0	60.0	30.0	15.0	7.5	3.8	1.9	0.9	0.0	0.0
QR ACTIVITY	1.7	8.9	169.5	221.8	184.0	133.2	99.4	73.9	59.9	52.2	40.0	40.5
	2.5	8.3	188.1	215.2	179.2	133.5	101.2	78.1	60.6	54.7	41.0	39.7
RAW KINETIC	2.2	6.6	174.4	213.3	170.1	134.1	98.4	80.6	59.7	52.5	41.2	39.8
DATA	2.4	6.0	169.5	220.3	172.8	129.8	100.0	73.2	61.2	54.4	41.2	42.1
(mOD/min)	1.3	5.4	178.7	222.3	176.2	134.4	102.7	75.0	59.7	53.3	42.5	41.1
	1.6	5.9	178.5	211.0	166.9	128.4	95.4	78.7	63.3	54.5	39.1	40.6
	2.2	5.2	168.7	205.8	174.7	126.1	95.8	73.2	61.4	52.7	41.7	40.6
	1.1	11.4	158.9	208.4	171.9	121.8	95.0	70.5	59.6	49.9	43.5	39.4
QR ACTIVITY CALCULATIONS												
AVERAGE-BLANK		*5.3*	*171.4*	*212.9*	*172.6*	*128.3*	*96.6*	*73.5*	*58.8*	*51.1*	*39.4*	*38.6*
STANDARD ERROR OF THE MEAN		*0.8*	*3.1*	*2.2*	*1.9*	*1.6*	*1.0*	*1.2*	*0.4*	*0.6*	*0.5*	*0.3*
RATIO OF TREATED/CONTROL		*0.14*	*4.40*	*5.46*	*4.43*	*3.29*	*2.48*	*1.89*	*1.51*	*1.31*	*1.01*	*0.99*
CRYSTAL	0.161	0.138	0.745	1.269	1.421	1.491	1.347	1.359	1.448	1.364	1.354	1.276
VIOLET	0.144	0.147	0.763	1.293	1.420	1.477	1.431	1.384	1.434	1.303	1.409	1.356
STAINING	0.142	0.146	0.698	1.213	1.370	1.422	1.498	1.441	1.468	1.370	1.322	1.392
	0.137	0.147	0.760	1.218	1.406	1.454	1.479	1.467	1.407	1.391	1.358	1.370
RAW DATA	0.138	0.151	0.683	1.201	1.396	1.420	1.436	1.404	1.462	1.405	1.351	1.344
(ABSORBANCE)	0.137	0.155	0.763	1.183	1.387	1.463	1.364	1.332	1.388	1.353	1.403	1.430
	0.062	0.159	0.739	1.187	1.415	1.430	1.377	1.341	1.388	1.329	1.395	1.366
	0.093	0.163	0.715	1.190	1.442	1.397	1.389	1.350	1.388	1.304	1.387	1.302
CRYSTAL VIOLET CALCULATIONS												
AVERAGE-BLANK		*0.024*	*0.607*	*1.093*	*1.280*	*1.318*	*1.288*	*1.258*	*1.296*	*1.226*	*1.246*	*1.228*
STANDARD ERROR OF THE MEAN		*0.003*	*0.011*	*0.014*	*0.008*	*0.011*	*0.019*	*0.017*	*0.012*	*0.013*	*0.011*	*0.017*
RATIO OF TREATED/CONTROL		*0.019*	*0.490*	*0.883*	*1.035*	*1.065*	*1.042*	*1.017*	*1.048*	*0.991*	*1.007*	*0.993*
QR SPECIFIC ACTIVITY												
RATIO OF TREATED/CONTROL		**7.05**	**8.97**	**6.18**	**4.28**	**3.09**	**2.38**	**1.85**	**1.44**	**1.32**	**1.00**	**1.00**

Figure 1 Lotus 1-2-3 spreadsheet printout (using the Wysiwyg publishing feature of Lotus) of raw data and calculated results for the potency of induction of quinone reductase and toxicity of *tert*-butylhydroquinone (t-BHQ) on Hepa 1c1c7 cells. Hepa 1c1c7 cells were grown, induced, and assayed as described in Methods and materials. The rates of MTT reduction for the quinone reductase assay and the absorbancies of retained crystal violet for the cytotoxicity assay are displayed as 8 × 12 matrices. Addition of dicoumarol reduced the rates of MTT reduction by more than 95% (not shown). The boxed values in italics are the average, standard error of the mean, and ratios for inducer-treated to control cells calculated by the spreadsheet. The values at the bottom of the spreadsheet are the ratios of the specific activities for inducer-treated to control cells. Note that >60 μM t-BHQ is cytotoxic (the cell density is reduced by 51% at 120 μM t-BHQ and by 98% at 240 μM t-BHQ). Moreover, the ratio of induction at 240 μM t-BHQ (7.05) is not meaningful because the crystal violet absorbancies are only slightly above the spectrophotometric blanks. Also note that the blank absorbancies in the bottom two wells (0.062, 0.093) are lower than the others (range = 0.137 to 0.161). This artifact, although small and not seen elsewhere on the plate, is produced by uneven rinsing. It is critically important to rinse plates free of excess crystal violet evenly and gently under running tap water.

Results

The assay developed for quinone reductase from lysed cells has been shown to be specific and linearly dependent on added enzyme over a wide range of quinone reductase concentrations.[29] The wide dynamic range of the assay is accomplished by maintaining both substrate (menadione and NADPH) concentrations constant. Thus, large differences in the quinone reductase activities between inducer-treated and control cells can be accurately measured *Figure 1.*

The data generated from quinone reductase activities and crystal violet stains are imported into prepared spreadsheets that automatically calculates the parameters desired. The spreadsheet shown in *Figure 1* analyzes the data for quinone reductase activity per well, cell density per well, and the ratio of these parameters (specific activity). The spreadsheet also permits automated graphical analysis *(Figure 2).*

It is important to note that the variation of quinone reductase activities and crystal violet stains between wells for a given inducer concentration are small *(Figure 1).* This implies that it is not necessary to assay a single compound on a microtiter plate. With the appropriate technical expertise, it is possible to use individual rows on a microtiter plate to assay different chromatographic fractions or test compounds for rapidly surveying a large number of samples. This strategy was used in the purification of sulforaphane from broccoli.[34] Because each chromatographic fraction can be assessed on an individual row or column of wells, it

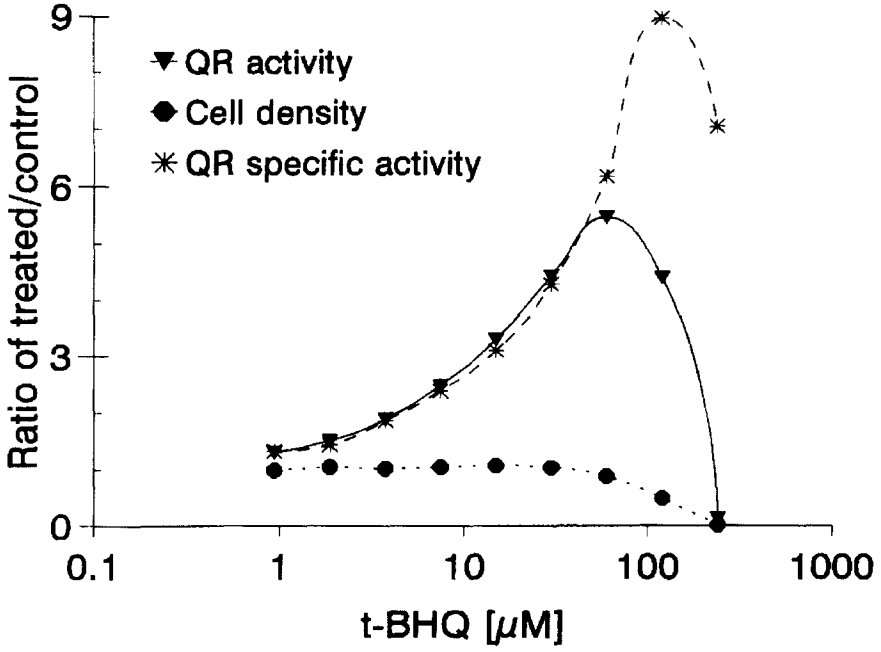

Figure 2 Effect of *tert*-butylhydroquinone (t-BHQ) on quinone reductase (QR) activities, cell densities, and quinone reductase specific activities expressed as ratios of inducer-treated to control cells. The data were imported directly from the spreadsheet shown in *Figure 1* to the Harvard Graphics program (SPC, Santa Clara, CA USA).

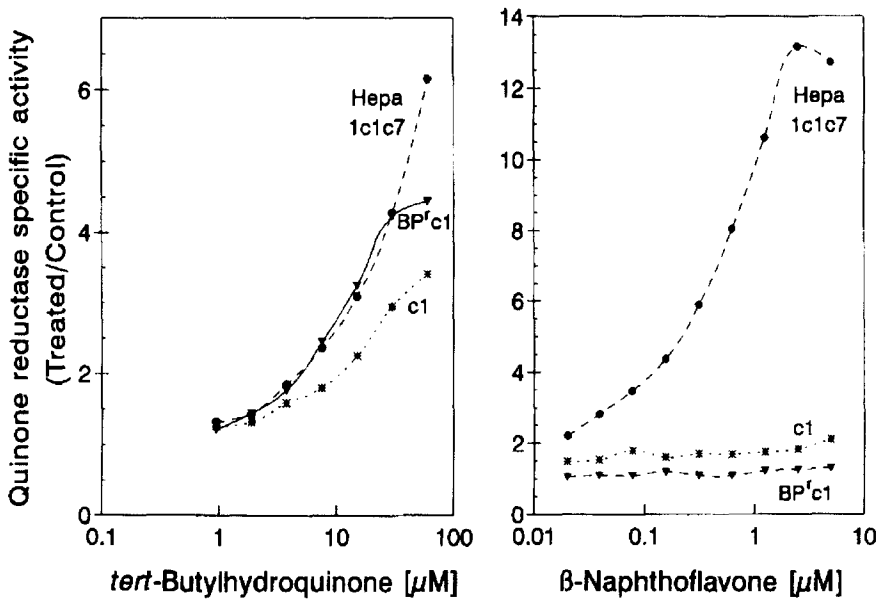

Figure 3 Effect of a monofunctional inducer (*tert*-butylhydroquinone) and bifunctional inducer (β-naphthoflavone) on quinone reductase specific activities in wild-type Hepa 1c1c7 cells and its mutant subclones defective in either a functional *Ah* receptor (BP^{r}c1)[35] or cytochrome P_1-450 gene product (c1).[36] Note that the potency of induction of quinone reductase by *tert*-butylhydroquinone in all three cell lines are similar, whereas the potency of induction of quinone reductase by β-naphthoflavone is at least 1,000-fold higher in the wild-type cells.

is possible to derive information on the potency of induction and toxicity of up to 12 fractions and/or test compounds from a single set of microtiter plates.

Figure 3 shows the dose-response for quinone reductase induction by t-BHQ and βNF in Hepa 1c1c7 cells and its mutant subclones defective in either a functional *Ah* receptor (BP^{r}cl)[35] or cytochrome P_1-450 gene product (cl).[36] The

potency of induction of quinone reductase by t-BHQ is comparable in all three cell lines. βNF, however, is inactive in BP^{r}c1 cells and is only weakly active in c1 cells. Thus, the use of mutants of Hepa 1c1c7 cells permits the rapid categorization of monofunctional and bifunctional inducers.[25] Because the Phase I enzymes induced by bifunctional inducers can activate carcinogens to their ultimate reactive forms, it is likely that monofunctional inducers are better candidates as human anticarcinogens than bifunctional inducers.

Discussion

The screening assay for quinone reductase provides a rapid and reliable method for identifying inductive activity. Although a representative experiment for measuring this activity is presented here, the successful application of the method for measuring the potency of a large series of synthetic inducers[28,30,31] as well as for the characterization of inductive activity from vegetables[33,34] have been published.

There are potential caveats of the screening assay. It is impossible to estimate the effects that pharmacokinetics will have on the potency of a putative inducer in vivo. Moreover, although Hepa 1c1c7 cells can identify inducers that are active in vivo, there are examples where it can fail to identify others. First, BHA (2[3]-*tert*-butyl-4-hydroxyanisole) is a much weaker inducer of Phase II enzymes in vitro than it is in the liver in vivo.[22,23,37] Because it is likely that BHA must be *O*-demethylated to form t-BHQ before it is able to induce Phase II enzymes,[37] the observed ineffectiveness of BHA in Hepa 1c1c7 cells may be due to the loss of enzyme(s) necessary for its conversion to an active metabolite. Second, the screening assay would not have detected indole-3-carbinol as an inducer of Phase II enzymes in vivo. Although this compound is present in cruciferous vegetables, it possesses little inductive activity in vitro because the compound requires the acidic environment of the stomach to form dimers/trimers that are potent ligands for the *Ah* receptor.[38,39]

Although there are potential deficiencies of the assay, there are very significant advantages. First, the assay has been designed so that the substrate concentrations remain constant.[29] The net effect is that quinone reductase levels can be reliably measured over a large range of enzyme concentrations. Second, the assay can be performed rapidly and provides information on the potency and toxicity of test compounds in 3 to 4 days. The same information would take months in rodents or weeks in conventional cell culture systems to obtain. Because data are imported directly and computed in pre-formatted spreadsheets, the processing of information is rapid and less subject to human error. Third, the "inductive activity" of test compounds,[30] plant extracts,[33] or chromatographic fractions[34] can be precisely quantified and compared with other samples. Fourth, the assay is cost-effective because the quantity of reagents (cells, media, etc.) needed to screen for inductive activity is small. Fifth, with mutant subclones of Hepa 1c1c7 cells, it is possible to define whether a test compound is a monofunctional or bifunctional inducer.[25] Sixth, only small quantities of test compounds are required for the assay. Thus, as demonstrated by Zhang et al.,[34] it is possible to purify inductive activity from complex plant extracts without requiring large quantities of starting material or specialized equipment.

Although the Hepa 1c1c7 cell line has become the standard cell line in which to screen for inductive activity, the assay can be applied to other systems *(Table 1)*. In the case of suspension cultures it is necessary to use a cytotoxicity assay other than crystal violet staining. We have used MTT staining of intact cells as described by Alley et al.[45] for these purposes. In the absence of menadione, MTT is reduced by a number of dehydrogenases and is an accurate surrogate marker for viable cells. In addition to immortalized cells, Gordon et al.[46] demonstrated that human peripheral blood mononuclear cells possess a measurable and inducible quinone reductase. It is noteworthy that the H9 cutaneous T-cell lymphoma cell line behaves similarly to peripheral blood mononuclear cells by their inability to elevate quinone reductase in response to bifunctional inducers.[43] This suggests that immortalized cells can serve as useful models in which to study chemopreventive enzyme inducers.

In conclusion, the efficient identification of chemopreventive agents requires

Table 1 Cell lines with inducible quinone reductase activities

Species	Cell line	Cell of origin	Reference
Mouse	Hepa 1c1c7 and mutants	liver	21–25
	3T3	fibroblasts	22
	C3H/10T1/2	embryo fibroblasts	40
	F9	teratocarcinoma	†
Rat	Walker Rat Carcinoma	carcinoma	†
	BRL 3A	liver	†
	Clone 9	liver	†
Human	Hep G2	liver	41, 42
	H9	T-cell	43
	U937	Promonocyte	†
Hybrid	N18-RE-105	mouse neuroblastoma x rat embryo retina	44

†H.J. Prochaska, unpublished observations.

an understanding of the mechanisms that can disrupt the neoplastic process. By the early 1980s, it became clear that many seemingly dissimilar compounds that prevent cancer are also inducers of Phase II enzymes. By exploiting the paradigm that inducers of Phase II enzymes are potential anticarcinogens, candidate anticarcinogens of medicinal interest have been identified (i.e., oltipraz, dimethyl fumarate, sulforaphane).[18,19,30,31,34] With the ability to take advantage of an in vitro assay that can screen the potency and toxicity of hundreds of specimens in a short time, it should be possible to isolate and characterize many other inducers from synthetic and natural sources. Moreover, the screening assay can help us to gain a better understanding of the relationship between dietary sources of inductive activity and the risk of cancer.

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