High-Throughput Library Screening Identifies Two Novel NQO1 Inducers in Human Lung Cells

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Many phytochemicals possess antioxidant and cancer-preventive properties, some putatively through antioxidant response element-mediated phase II metabolism, entailing mutagen/oxidant quenching. In our recent studies, however, most candidate phytochemical agents were not potent in inducing phase II genes in normal human lung cells. In this study, we applied a messenger RNA (mRNA)-specific gene expression-based high throughput *in vitro* screening approach to discover new, potent plant-derived phase II inducing chemopreventive agents. Primary normal human bronchial epithelial (NHBE) cells and immortalized human bronchial epithelial cells (HBEs) were exposed to 800 individual compounds in the Micro-Source Natural Products Library. At a level achievable in humans by diet (1.0 μ M), 2,3-dihydroxy-4-methoxy-4'-ethoxybenzophenone (DMEBP), triacetyresveratrol (TRES), ivermectin, sanguinarine sulfate, and daunorubicin induced reduced nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase 1 (NQO1) mRNA and protein expression in NHBE cells. DMEBP and TRES were the most attractive agents as coupling potency and low toxicity for induction of NQO1 (mRNA level, ≥ 3 - to 10.8-fold that of control; protein level, $\geq$ two- to fourfold that of control). Induction of *glutathione S-transferase pi* mRNA expression was modest, and none was apparent for glutathione S-transferase pi protein expression. Measurements of reactive oxygen species and glutathione/oxidized glutathione ratio showed an antioxidant effect for DMEBP, but no definite effect was found for TRES in NHBE cells. Exposure of NHBE cells to H₂O₂ induced nuclear translocation of nuclear factor erythroid 2-related factor 2, but this translocation was not significantly inhibited by TRES and DMEBP. These studies show that potency and low toxicity may align for two potential NQO1-inducing agents, DMEBP and TRES.

Keywords: *GSTP1*; *NQO1*; phytochemicals; high-throughput screening; gene expression

There is accumulating evidence to support an inverse relationship between regular consumption of fruit and vegetables and risk of specific cancers, including lung cancer (1, 2). To identify more potent foodstuffs or agents, it is important to understand the mechanism by which components of fruit and vegetables prevent cancer. This would allow more efficient evaluation in animals, in advance of being tested in human intervention trials, and certainly before they can be recommended for inclusion in

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CLINICAL RELEVANCE

The study addresses the relative lack of natural and dietary inducers of the antimutagen/antioxidant phase II metabolism pathway with activity in normal target bronchial cells. Using a messenger RNA-specific gene expression-based high-throughput *in vitro* screening approach to screen 800 compounds in a natural products library, this study has identified 2,3-dihydroxy-4-methoxy-4'-ethoxybenzophenone (derivative, myrtle extract, *Myrtus*, *Myrtaceae*) and triacetyresveratrol (source, arctic sponge) as low-toxicity, potent phase II inducers, implying that they are potential new agents for further *in vitro* and preclinical *in vivo* testing for prevention of oxidant-related disease.

dietary supplements. Numerous phytochemicals derived from edible plants have been reported to block or protect against chemical carcinogenesis, mainly by their ability to induce phase II detoxification enzymes, including glutathione S-transferase and reduced nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase 1 (NQO1). Nuclear factor erythroid 2-related factor 2 (*NRF2*) is essential for antioxidant response element (ARE)-mediated induction of genes, including phase II detoxifying enzymes and antioxidant enzymes (3). *NRF2* plays a protective role against oxidative stress, which induces nuclear translocation of *NRF2* (4). Because detoxification enzymes are not necessarily expressed or functioning at maximal capacity, their induction should be an effective strategy for cancer chemoprevention.

Several studies have shown that elevation of glutathione S-transferase pi and NQO1 enzymes correlates with protections against chemical-induced carcinogenesis in animal models (5, 6). Knockout of either *GSTP1* or *NQO1* in mice led to a significant increase in both carcinogen-induced and spontaneous tumorigenesis (7–10). Epidemiological studies in humans have suggested that genetic variants in the *GST* and *NQO1* genes are risk factors for lung cancer, but a large number of studies have reported apparently conflicting results (11–14). We hypothesized that these two enzyme systems could be effectively used to screen potential chemopreventive agents for lung cancer by testing their ability to induce these enzymes in human lung cells. However, in studies to date, most of the candidate agents were inactive in normal human lung cells (15). *In vitro* and *in vivo* screening studies for discovery are clearly needed for identifying new, more potent chemopreventive agents for lung cancer.

In vitro screening assays using induction of phase II enzymes have been used for cell-based bioassay-guided fractionation of natural products for discovery of a potential chemoprevention agent (16, 17). However, assays based on fractionation require multiple iterations to isolate active constituents, and must be accompanied by conventional structure elucidation analysis, such

as nuclear magnetic resonance, spectrophotometry, and mass spectrometry. These procedures are low throughput and labor intensive. For accelerating discovery of new, more potent phase II-inducing chemopreventive agents for lung cancer, a gene expression-based, high-throughput screening of an 800-compound plant-derived library in normal human lung cells at diet-achievable doses was recently developed in our laboratory.

MATERIALS AND METHODS

Cells and Reagents

Normal human bronchial epithelial (NHBE) cells (BioWhittaker, Inc., Walkersville, MD) were maintained in bronchial epithelial growth medium (BioWhittaker, Inc.), and human bronchial epithelial cells (HBECs; genetically engineered for *hTERT* and *CDK4* overexpression, immortalized; courtesy of Dr. J. D. Minna at the University of Texas Southwestern Medical Center [18]) were cultured with keratinocyte serum-free medium (Life Technologies, Gaithersburg, MD), as previously described (15).

The MicroSource Natural Products Library (Discovery Systems, Inc., Gaylordsville, CT) is a 800-compound collection of pure natural products and their derivatives (<http://www.msdiscovery.com/natprod.html>). Compounds in the collections are provided at 10-mM concentrations in DMSO solution in 96-well-plate format. All are fully characterized according to literature reports, and meet the criteria of a minimum of 95% purity.

Primary Screen

Cells were plated in 96-well plates and allowed to grow for 24 hours, then exposed to 1.0 μ M compounds (final concentration) from the MicroSource Natural Products Library or a corresponding amount of DMSO. After 48 hours, total RNA was prepared by the automated SV 96 Total RNA Isolation System (Promega, Madison, WI) on a NextGen expression workstation (NextGen Sciences, Ann Arbor, MI) according to the manufacturer's instructions. Quantitative RT-PCR for *GSTP1* and *NQO1* was performed with Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) in a 384-well optical plate in an ABI 7900HT instrument (Applied Biosystems) using a previously published RNA-specific strategy (19–21). An outline of the primary screen is depicted in Figure E1 in the online supplement.

Cell Viability Assay

Evaluation of cytotoxicity for selected compounds was determined by measuring cell viability using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions (see the online supplement for details).

Validation of Selected Compounds

To reduce the number of false positives or negatives in the first screening, we reassayed all positive results with expression above 2-fold changes and 15% of negative results with expression below 2-fold changes using RNA extracted from the cells cultured in six-well plates. Furthermore, we set up a factorial experiment to test the effect of the selected compounds on *GSTP1* and *NQO1* messenger RNA (mRNA) and protein expression in two cell lines (NHBE and HBEC) at three concentration levels (0.5, 1.0, and 2.0 μ M) and three time points (24 h, 48 h, and 6 d). *GSTP1* and *NQO1* protein assays were performed by Western blots using WesternBreeze Chemiluminescent Western blot immunodetection kit (Invitrogen, Carlsbad, CA), as described previously (15).

Reactive Oxygen Species Determination and Immunofluorescence Staining of NRF2

Reactive oxygen species (ROS) were measured by determining intracellular glutathione (GSH) and oxidized glutathione (GSSG) levels and colorimetric measurement of 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) (22). Nuclear translocation of *NRF2* in NHBE cells was assessed by immunofluorescent staining (23) using anti-NRF2 rabbit polyclonal antibody (Santa Cruz Biotechnology,

Santa Cruz, CA) and goat anti-rabbit IgG-Alexa Fluor 488-conjugated antibody (Invitrogen). The detailed methods are described in online supplement.

Statistical Analysis

Data are means ($\pm$ SEM), and were evaluated by ANOVA with *post hoc* Dunnett's test, as described elsewhere (15). Z factor values were calculated using the method described by Zhang and colleagues (24). All differences were considered significant at a *P* value less than 0.05.

RESULTS

High-Throughput Screening for Compounds Inducing *GSTP1* and/or *NQO1* mRNA Expression

Of the 800 compounds tested, three increased *GSTP1* expression more than twofold in NHBE cells compared with the control DMSO treatment, whereas none increased *GSTP1* expression more than twofold in HBECs (Figure 1). For *NQO1* expression, three compounds in NHBE cells and two compounds in HBECs showed more than twofold increase compared with the DMSO control (Figure 1). The evaluation of the quality of the high-throughput screening assay showed acceptable signal-to-noise ratios (range, 13.2–17.6) and reasonable Z factors ($0.5 < Z$ factor < 1), indicating a very robust RT-PCR assay (Table 1). We selected eight compounds for further consideration, because they increased expression more than twofold for at least three of four experimental conditions: *GSTP1* mRNA expression in NHBE cells; *NQO1* mRNA expression in NHBE cells; *GSTP1* mRNA expression in HBECs; and *NQO1* mRNA expression in HBECs (Table 2). The eight compounds selected were 2,3-dihydroxy-4-methoxy-4'-ethoxybenzophenone (DMEBP), stictic acid (SA), triacetylresveratrol (TRES), 2',4'-dihydroxy-4-methoxychalcone (DMC), 2',3-dihydroxy-4,4', 6'-trimethoxychalcone (DTMC), ivermectin (IVE), sanguinarine sulfate (SS), and daunorubicin (DAU). We illustrated the molecular structures of these selected inducers (Figure E2).

Figure 1. Relative gene expression of *glutathione S-transferase pi* and reduced nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase 1 (*NQO1*) (fold of DMSO-treated control) in normal human bronchial epithelial (NHBE) cells and human bronchial epithelial cells (HBECs) treated with 800 plant-derived compounds. H, HBECs; N, NHBE cells.

TABLE 1. HIGH-THROUGHPUT SCREENING ASSAY PARAMETERS DERIVED FROM DIFFERENT RT-PCR RUNS FOR MEASURING *GSTP1* AND *NQO1* MESSENGER RNA EXPRESSION IN NORMAL HUMAN BRONCHIAL EPITHELIAL AND HUMAN BRONCHIAL EPITHELIAL CELLS

	NHBE Cells		HBECs	
	<i>GSTP1</i>	<i>NQO1</i>	<i>GSTP1</i>	<i>NQO1</i>
Activated hits*				
Mean $\pm$ SD	2.46 $\pm$ 0.11	2.55 $\pm$ 0.13	2.37 $\pm$ 0.14	2.49 $\pm$ 0.12
Signal-to-noise ratio	16.2	15.5	17.1	13.5
Z factor	0.59	0.55	0.52	0.54
Inhibited hits				
Mean $\pm$ SD	-2.40 $\pm$ 0.09	-2.41 $\pm$ 0.11	-2.45 $\pm$ 0.12	-2.45 $\pm$ 0.08
Signal-to-noise ratio	15.6	14.1	17.6	13.2
Z factor	0.61	0.55	0.59	0.61

Definition of abbreviations: *GSTP1*, glutathione S-transferase pi; HBECs, human bronchial epithelial cells; NHBE, normal human bronchial epithelial; *NQO1*, reduced nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase 1.

* Hits were identified as the active compounds that either activate or inhibit *GSTP1* and *NQO1* expression above twofold, and the parameters were calculated using DMSO-treated controls as the control group.

Cytotoxicity Assay of the Selected Inducers

Incubation of NHBE cells with the eight selected compounds caused a dose-dependent inhibition of cell proliferation ($P < 0.05$), with the relative antiproliferation activities being in the order: IVE $>$ SS $>$ DAU $>$ SA $>$ DMEBP $>$ TRES $>$ DMC $>$ DTMC, based on their cytotoxicity concentration that kills 50% of the cells (Figures 2A and 2C). The IC_{50} for IVE, SS, DAU, SA, TRES, DMEBP, DMC, and DTMC in NHBE cells were 1.3 ± 0.4 , 4.5 ± 1.0 , 8.2 ± 1.3 , 26 ± 1.7 , 31 ± 4.1 , 36 ± 3.8 , 76 ± 9.2 , and 78 ± 8.3 μ mol/L, respectively. Similar results were found in HBECs, although there were fewer significant differences among the compounds (Figures 2B and 2D).

Validation of Hit Compounds by RT-PCR and Western Blotting

Consistent with the effect of mRNA expression, the induction of *NQO1* protein expression was found for all eight selected agents in NHBE cells at the same concentration that induced *NQO1* mRNA expression (Figures 3A and 3B). Treatment with DMEBP, TRES, IVE, SS, and DAU significantly induced *NQO1* protein expression to 2- to 4-fold of the control value in NHBE cells. However, *GSTP1* protein expression was not affected by any of the selected agents in NHBE cells. In addition, *GSTP1* and *NQO1*

protein levels were not affected by any of the agents in HBECs (data not shown).

As DMEBP and TRES seemed to combine significant potency and low toxicity for induction of *NQO1*, we further tested the effect of DMEBP and TRES on *GSTP1* and *NQO1* mRNA and protein expression in NHBE and HBECs at three concentration levels (0.5, 1.0 and 2.0 μ mol/L) and three time points (24 h, 48 h, and 6 d). Because TRES is an analog of well studied resveratrol (trans-3,4',5-trihydroxystilbene [RES]), we also included RES in this experiment. *NQO1* mRNA expression in NHBE cells increased to 3- to 10.8-fold of the control value after application of DMEBP, with a maximum increase of 9.8-fold after 48-hour application of 2.0 μ M DMEBP. After application of TRES, *NQO1* mRNA expression in NHBE cells increased up to 4.6-fold of the control value after 48-hour application of 2.0 μ M TRES (Figure 4A). Compared with TRES, RES showed a relatively low potency in induction of *NQO1* mRNA expression (Figure 4A). *NQO1* protein expression in NHBE cells increased to 2- to 3.8-fold of the control value by TRES and DMEBP, with a maximum increase of 2.8-fold after 6-day application of 2.0 μ M DMEBP (Figure 4B). In HBECs, we observed similar effects of these agents on *NQO1* mRNA expression; however, *GSTP1* and *NQO1* protein levels were not affected in any condition tested (data not shown).

TABLE 2. *GSTP1* AND *NQO1* MESSENGER RNA EXPRESSION IN NORMAL HUMAN BRONCHIAL EPITHELIAL AND HUMAN BRONCHIAL EPITHELIAL CELLS TREATED WITH THE SELECTED COMPOUNDS

Agent	Relative mRNA Expression*				Source	Bioactivity
	NHBE Cells		HBECs			
	GSTP1	NQO1	GSTP1	NQO1		
DMEBP	2.0 ± 0.2	2.3 ± 0.2	2.5 ± 0.3	4.2 ± 0.3	Derivative Myrtus communis	undetermined
SA	2.9 ± 0.3	2.2 ± 0.2	1.8 ± 0.2	2.1 ± 0.1	lichen, usnea articulata	antioxidant
TRES	3.8 ± 0.3	2.6 ± 0.2	2.2 ± 0.2	1.7 ± 0.2	kirkpatrickia variolosa (sponge)	antioxidant
DMC	2.9 ± 0.3	2.9 ± 0.2	2.2 ± 0.1	1.1 ± 0.1	Bauhinia manca	undetermined
DTMC	3.1 ± 0.2	2.6 ± 0.1	2.6 ± 0.2	1.5 ± 0.1	merrillia caloxylon	undetermined
IVE	1.3 ± 0.2	4.8 ± 0.3	2.9 ± 0.2	3.0 ± 0.3	streptomyces avermitilis	antiparasitic
SS	2.1 ± 0.2	6.1 ± 0.5	2.5 ± 0.1	2.9 ± 0.2	sanguinaria canadensis	antineoplastic; antiplaque
DAU	4.2 ± 0.2	5.6 ± 0.4	2.0 ± 0.3	4.8 ± 0.5	streptomyces peucetius	antineoplastic

Definition of abbreviations: DAU, daunorubicin; DMC, 2',4'-dihydroxy-4-methoxychalcone; DMEBP, 2,3-dihydroxy-4-methoxy-4'-ethoxybenzophenone; DTMC, 2',3-dihydroxy-4,4',6'-trimethoxychalcone; *GSTP1*, glutathione S-transferase pi; HBECs, human bronchial epithelial cells; IVE, ivermectin; mRNA, messenger RNA; NHBE, normal human bronchial epithelial; *NQO1*, reduced nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase 1; SA, stictic acid; SS, sanguinarine sulfate; TRES, triacetylresveratrol.

* Fold of DMSO-treated control. Data are means $\pm$ SEM ($n = 3$).

Figure 2. Survival of NHBE (A and C) and HBECs (B and D) cells treated with the selected agents. (A and B) Representative cell survival curve of dose-response growth inhibition after 48-hour treatments with the selected inducers. (C and D) The cytotoxicity concentration that kills 50% of the cells (CC₅₀) of three independent assays for the selected compounds. Data are means ($\pm$ SEM) ($n = 3$); * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with ivermectin (IVE)-treated cells. DAU, daunorubicin; DMC, 2',4'-dihydroxy-4-methoxychalcone; DMEBP, 2,3-dihydroxy-4-methoxy-4'-ethoxybenzophenone; DTMC, 2',3-dihydroxy-4,4',6'-trimethoxychalcone; SA, stictic acid; SS, sanguinarine sulfate; TRES, triacetylresveratrol.

ROS Modulated by DMEBP, TRES, and RES

Accumulation of intracellular ROS was determined measuring the levels of GSH and GSSG, and by colorimetric measurement of the redox-sensitive fluorophore, CM-H₂DCFDA. Stimulating the cells with DMEBP increased the GSH:GSSG ratio after 24-hour application, whereas treatment with TRES and RES had no effect (Figure 5A). Quantification of CM-H₂DCFDA showed that all three studied agents decreased the ROS content after 48-hour application in NHBE cells (Figure 5B).

Intracellular Trafficking of NRF2 Mediated by DMEBP, TRES, and RES

Exposure of NHBE cells to 250 μ M H₂O₂ for 30 minutes resulted in nuclear translocation of *NRF2* at 24 hours (Figure E3A, lower panel). However, addition of TRES, DMEBP, or RES for 24 hours did not significantly reduce nuclear translocation of *NRF2* (Figures E3B–E3D).

DISCUSSION

Endogenous mRNA expression has been successfully used as a surrogate of cellular states in high-throughput screening (25) for identification of small molecules that can regulate gene expression (26–28). Here, we have demonstrated the utility of such a system to screen 800 compounds in the MicroSource Natural Products Library, and identify DMEBP, TRES, IVE, SS, and DAU as plant-derived inducers for NQO1 mRNA and protein expression. Furthermore, we demonstrated DMEBP and TRES as the most potent and low-toxicity inducers (≥ 3 - to 10.8-fold of control at mRNA level; ≥ 2 - to 4-fold of control at the protein level), implying that they are potential new agents

for further *in vitro* and preclinical *in vivo* testing for prevention of oxidant-related disease.

TRES was isolated from the sponge, *Kirkpatrickia variolosa*. It is chemically related to the well studied RES, which is a natural compound found in large quantities, most notably in grapes and red wine. RES has been suggested to have a cancer-chemopreventive effect (29) and chemotherapeutic potential (30, 31). RES has been shown to undergo metabolic phase II

Figure 3. GSTP1 and NQO1 protein expression in NHBE cells treated with the selected agents. (A) GSTP1 and NQO1 protein expression (the top gel was pieced together from two gels). (B) Densitometry analysis of relative GSTP1 and NQO1 protein expression in NHBE cells. The relative density ratio of GSTP1 or NQO1 protein band to GAPDH with DMSO-treated cells was arbitrarily set as 1.0. Values are expressed as fold of the DMSO-treated control and are means ($\pm$ SEM) ($n = 3$); ** $P < 0.01$ and *** $P < 0.001$ compared with DMSO-treated control.

Figure 4. *NQO1* messenger RNA (mRNA) (A) and protein (B) expression in NHBE cells treated with TRES, trans-3,4',5-trihydroxystilbene (RES), and DMEBP. The relative density ratio of *NQO1* protein band to GAPDH with DMSO-treated cells was arbitrarily set as 1.0. Values are expressed as fold of the DMSO-treated control and are means ($\pm$ SEM) ($n = 3$); * $P < 0.05$ and ** $P < 0.01$ compared with DMSO-treated control.

reactions involving conjugation with sulfate and glucuronic acid, which may influence the biological effect of the compound. Using a RES-specific bioaffinity approach, Wu and colleagues (32) have identified *GSTP1*, quinone reductase 2, *NRF2*, estrogen receptor β , p53, and p21 as RES-targeting proteins, suggesting that RES affects cellular function at multiple levels, ranging from events at the plasma membrane, to interaction with detoxification enzymes, such as *GSTP1* and quinone reductase 2, and transcription by targeting factors, such as estrogen receptor β and *NRF2*, as well as cell cycle regulation through p53 and p21 (33). In this study, we found that *NQO1* mRNA expression in NHBE cells was induced up to 4.6-fold by TRES, as well as less-potent induction by RES. Several studies have shown that acetylated analogs of RES (TRES) exhibit the same or higher inhibitory effects on various tumor cell lines than RES itself, while being absorbed faster and resisting cellular breakdown (34–36). Because of the centrality of GSH status of a cell for redox homeostasis, which in turn impacts various biological events, we measured total GSH and GSSG:GSH ratios (37). TRES and RES had no effect on the change of total GSH level and GSH:GSSG ratio, although they showed a slight decrease of ROS content. The antioxidant activity of RES and its analogs might be dependent on the position of key hydroxyl groups (38, 39).

An interesting outcome of the present study was the identification of DMEBP as a new phase II inducer. DMEBP is a synthetic derivative, nonnatural, of several benzophenones similar to those found in myrtle plant (*Myrtus*, *Myrtaceae*). Our study showed that DMEBP has a significant induction effect on *NQO1* mRNA and protein expression at the lower concentrations of 0.5 and 1.0 μ M, albeit with some cytotoxicity at higher

Figure 5. Intracellular reactive oxygen species (ROS) content in NHBE cells treated with TRES, RES, and DMEBP. (A) GSH:GSSG ratio. (B) Colorimetric measuring of 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Values are expressed as GSH:GSSG ratio (A) or ROS content (%) of control (B), and are means ($\pm$ SEM) ($n = 3$); * $P < 0.05$ and ** $P < 0.01$ compared with DMSO-treated control.

concentration. Furthermore, we found that DMEBP increased the GSH level and GSH:GSSG ratio after 24-hour application and decreased ROS content after 48 hours at the human achievable concentration. More *in vitro* and *in vivo* studies on this new inducer are warranted.

NRF2 is a critical transcription factor mediating amplification of the mammalian defense system against environmental stresses. It has been clearly demonstrated that *NRF2* translocates into the nucleus and binds to the ARE after activation by redox/oxidative stresses, leading to transcriptional activation of downstream genes, such as *GST*, *g-GCS*, and *NQO1* (4, 40, 41). To identify the potential mechanism by which our newly discovered agents, TRES and DMEBP, activate the phase II metabolic enzyme, *NQO1*, we investigated whether TRES and DMEBP can inhibit H_2O_2 -mediated nuclear translocation of *NRF2*. Our data show nuclear translocation of *NRF2* in NHBE cells stimulated with an oxidant (H_2O_2), and prevention of that translocation by 1–2 μ M TRES and DMEBP, which we've shown do augment *NQO1* expression. These data suggest that *NRF2*-related signaling pathway might not be directly involved in the activation of *NQO1* induced by TRES and DMEBP, but rather gene induction by these agents occurs by other means (e.g. direct redox rebalancing), in turn, perhaps, reducing *NRF2* signaling as a secondary phenomenon.

Among the other agents screened as active (albeit more cytotoxic), sanguinarine, which is a bioactive alkaloid from the toxic plant *Chelidonium majus* (greater celandine) has been shown to have antiviral, antimicrobial, and even antitumor activity (42). IVE, a macrocyclic lactone derived from the bacterium *Streptomyces avermitilis*, is a broad-spectrum antiparasitic agent, and is mainly used in humans in the treatment of onchocerciasis, lymphatic filariasis. Daunorubicin, or daunomycin (daunomycin cerubidine), initially isolated from *Streptomyces peucetius*, is an anthracycline drug used for chemotherapy of some types of cancer, such as leukemia. Here, we showed that SS, IVE, and DAU are able to induce *GSTP1* and *NQO1* expression. Nagai and colleagues (43) showed a three- to fourfold increase in *GSTP1*

mRNA, and a two- to threefold increase in GSTP1 protein levels, 3 days after 100 μM H_2O_2 exposures of K562 cells. In the current study, we could not conclude whether the induction of *GSTP1* and *NQO1* by IVE, SS, and DAU is due to this redox-related toxicity effect of these compounds. However, these three compounds showed significant cytotoxicity (especially at high dose). On the other hand, DMEBP and TRES, as new phase II inducers, show potential cancer-chemoprevention activity over a wide range of doses without showing apparent toxicity.

There are two limitations of our study. First, our initial screen of the compound library was performed at only one concentration (1.0 μM), which entails the risk of missing bioactive molecules that are insufficiently efficacious at the chosen concentration. However, our previous study showed that *NQO1* mRNA expression could be significantly induced by sulforaphane at the lower concentrations of 0.5 and 1.0 μM (15). It has been shown that 1.0 μM is a diet-achievable serum concentration for Epigallocatechin gallate and sulforaphane (44–47). The dose of dietary chemopreventive agent should not be the maximum tolerated dose, but rather, the minimum dose that can induce the optimal biomarker response without any adverse effects. In an effort to identify candidate chemopreventive agents for experimentally augmenting GSTP1 and NQO1 expression at the lower concentration, and handle the throughput, we chose 1.0 μM as the concentration for initial screening of the MicroSource Natural Products Library, and followed it up with more detailed dose-finding studies on DMEBP and TRES. Second, we used normal and immortalized lung cells, but not malignant cell lines for screening. We made this choice explicitly, as it is more suitable to our interest in carcinogenesis than are the cell growth and behavior of already phenotypically malignant cells. Malignant cells are likely to have different genomic, regulatory, and proteomic patterns compared with early neoplastic, or certainly normal, cells. Therefore, we used the primary NHBE and normal but target-gene immortalized HBECs for screening these new chemopreventive agents, as these would seem to be the most appropriate target cells for such efforts.

Overall, these data imply that: (1) many plant compounds show some antioxidant gene induction activity without measurably changing redox status itself; (2) two index ARE-containing genes show divergent responsiveness in lung cells, so coregulatory features are critical; (3) potency and safety of plant compounds can be aligned in some cases, such as for TRES and DMEBP; (4) a broader array of antioxidant/antimutagen gene sampling is warranted to more comprehensively test agent efficacy, over and above these two index genes; and (5) testing of naturally occurring crude mixtures (e.g., grape skin), containing multiple congeners of any one compound, would likely yield a different, perhaps more potent, set of agents and mixtures for preclinical *in vivo* testing to further cancer prevention.

Author disclosures are available with the text of this article at www.atsjournals.org.

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