Generation of a Stable Antioxidant Response Element–Driven Reporter Gene Cell Line and Its Use to Show Redox-Dependent Activation of Nrf2 by Cancer Chemotherapeutic Agents

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Abstract

The NF-E2 p45-related factor 2 (Nrf2) regulates cytoprotective genes that contain an antioxidant response element (ARE) in their promoters. To investigate whether anticancer drugs can induce ARE-driven gene expression, we have developed a stable human mammary MCF7-derived reporter cell line called AREc32, which contains a luciferase gene construct controlled by eight copies of the *cis*-element. In these cells, luciferase activity was increased up to 50-fold following treatment with 50 μmol/L *tert*-butylhydroquinone (*t*-BHQ). Basal and inducible luciferase activities in AREc32 cells were increased by forced overexpression of Nrf2 and reduced by knockdown of endogenous Nrf2 expression with RNA interference. Depletion of cellular reduced glutathione (GSH) by treatment of AREc32 cells with L-buthionine-*S,R*-sulfoximine (BSO) did not influence basal levels of luciferase activity, but pretreatment with BSO augmented induction of luciferase activity by *t*-BHQ. Induction of reporter activity by *t*-BHQ in AREc32 cells was suppressed markedly by the antioxidants *N*-acetylcysteine and GSH but only modestly by vitamins C or E, suggesting that ARE-luciferase expression is induced primarily by thiol-active electrophiles rather than free radicals. The anticancer drugs cisplatin, etoposide, mitoxantrone, chlorambucil, melphalan, and carmustine [1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU)] weakly induced luciferase activity in AREc32 cells. Moreover, treatment of AREc32 cells with BSO immediately before exposure to anticancer drugs enhanced induction of ARE-driven luciferase activity by cisplatin, BCNU, chlorambucil, and melphalan and also induced endogenous *AKR1C* (*AKR1C* refers to *AKR1C1* and *AKR1C2*), a target gene of Nrf2. Our findings show that Nrf2 can be activated by certain anticancer agents, and this will influence the effectiveness of chemotherapy. (Cancer Res 2006; 66(22): 10983-94)

Introduction

Drug-metabolizing enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1), aldo-keto reductase (AKR), and glutathione *S*-transferases (GST), along with reduced glutathione (GSH) and its biosynthetic enzymes glutamate cysteine ligase (GCL, comprising GCLC and GCLM subunits) and GSH synthase, protect cells against carcinogenic electrophiles and reactive oxygen species (ROS; refs. 1, 2). This defense can be induced in response to redox stressors, thereby allowing cells to adapt to the presence of pro-oxidants and electrophiles. It is also overexpressed in certain tumors. Induction of these genes is controlled primarily by NF-E2 p45-related factor 2 (Nrf2; refs. 3, 4), a transcription factor belonging to the family of cap "n" collar (CNC) basic-region leucine zipper (bZIP) proteins (5). Nrf2 mediates induction of detoxication and antioxidant genes that contain an antioxidant response element [ARE, 5′-(A/G)TGACNNNGC(A/G)-3′] in their promoters (2, 5); the ARE has also been referred to as the electrophile response element (EpRE). A key role for Nrf2 in controlling the ability of cells to withstand harmful environmental agents has been revealed by studies in which Nrf2 knockout mice have been shown to exhibit sensitivity to hyperoxia-induced lung injury and increased susceptibility to toxic xenobiotics, including carcinogens (6, 7).

The activity of Nrf2 is repressed by the inhibitory factor Kelch-like ECH associated protein 1 (Keap1) that facilitates degradation of Nrf2 through acting as a Cul3 substrate adaptor (8, 9). Electrophilic agents modify Keap1 and prevent it from targeting Nrf2 for degradation (10). Such inactivation of Keap1 allows Nrf2 to accumulate in the nucleus where it forms a heterodimer with other bZIP proteins and transactivates target genes, including *NQO1*, *AKR*, *GST*, *GCLC*, and *GCLM* (3, 11). Genetic knockout or knockdown of Keap1 also increases expression of the ARE-gene battery (8, 11).

A number of the genes that are regulated by Nrf2 have been linked to drug resistance. For example, the antioxidant GSH, the synthesis of which is primarily dictated by the activity of GCLC and GCLM, has been implicated in resistance of tumor cells to several chemotherapeutic agents, including cisplatin and melphalan (12, 13). Similarly, overexpression of GST isoenzymes, which catalyze the conjugation of GSH with electrophilic compounds (14), has been reported in a large number of tumor types (1) and implicated in resistance towards chemotherapeutic agents (12, 13, 15).

Because the levels of drug-metabolizing enzymes help determine the sensitivity of tumor cells to anticancer agents, it is important to understand how their genes are regulated. Based on the evidence that Nrf2 regulates the expression of ARE-driven antioxidant and detoxication genes in response to Michael reaction acceptors, redox-cycling hydroquinones, quinones, and other electrophiles (16), we postulated that its activity might be modulated by anticancer drugs. For example, we expected that the activity of Nrf2 could be increased by alkylating agents and also possibly by redox-cycling compounds. Based on the facts that chlorambucil, melphalan, carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)], cyclophosphamide metabolites, and thiotepa

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-2298

are electrophilic agents that can be conjugated with GSH (15), and that many agonists of Nrf2 are thiol-active electrophiles (17), it might be anticipated that chemotherapeutic alkylating agents would activate the ARE-gene battery. In the present article, we have tested this hypothesis. We have also examined whether doxorubicin and etoposide can induce ARE-driven gene expression because they are capable of redox-cycling, thereby producing ROS that might activate Nrf2. Furthermore, the anticancer agents cisplatin, methotrexate, mitoxantrone, and paclitaxel, which are neither alkylating agents nor redox-cycling compounds, were also included in the study.

We now describe the generation of a sensitive, stable ARE reporter cell line AREc32, which contains a luciferase gene under the transcriptional control of multiple tandemly arrayed copies of the minimal functional *cis*-element found in the promoters of rat *GSTA2* (18) and mouse *gsta1* (19). To evaluate the usefulness of the reporter lines, we challenged it with the redox-cycling agent *tert*-butylhydroquinone (*t*-BHQ), produced as a cytochrome P450 metabolite of the phenolic antioxidant butylated hydroxyanisole (BHA; ref. 20), and the isothiocyanate sulforaphane (SUL), as both of these compounds induce ARE-driven gene expression (16). *t*-BHQ was used in the original study to define monofunctional inducers (21) and also to define the ARE (18). Induction of the luciferase reporter gene in the stable AREc32 reporter cells by *t*-BHQ and SUL was found to be sensitive to changes in cellular redox status and is regulated specifically by Nrf2. Importantly, we also report that anticancer drugs are capable of inducing reporter gene expression in AREc32 cells, and that depletion of cellular GSH with L-buthionine-*S,R*-sulfoximine (BSO) enhances induction of ARE-driven luciferase by certain anticancer agents. Our study provides evidence that Nrf2 is involved in the responsiveness of mammary tumor cells to chemotherapeutic agents in a redox-dependent fashion.

Materials and Methods

Chemicals and cell culture. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. Ltd. (Dorset, United Kingdom). D,L-sulforaphane was obtained from LKT Laboratories, Inc. (St. Paul, MN). HepG2 (human hepatoblastoma), MCF7 (human breast carcinoma), Hepa1c1c7 (mouse hepatoma), and Chinese hamster ovarian carcinoma (CHO) cell lines were obtained from the cell services of Cancer Research UK (London, United Kingdom). The growth medium for MCF7 and HepG2 cells was DMEM with glutamax supplemented with 10% fetal bovine serum and antibiotics. For Hepa1c1c7 cells, the growth medium was supplemented with 1% nonessential amino acids and 2.5 μg/mL bovine insulin. For CHO cells, the growth medium was supplemented with 16 μmol/L thymidine and 100 μmol/L hypoxanthine. All cells were cultured at 37°C, in 95% air and 5% CO_2, and passaged every 3 to 4 days. All media supplements for cell culture were purchased from Life Technologies, Inc. Ltd. (Paisley, United Kingdom).

Reporter plasmids and expression constructs. The ARE-luciferase reporter plasmids were generated using the pGL3-promoter vector (Promega UK, Southampton, United Kingdom) containing an SV40 promoter upstream of the firefly luciferase gene. The sequences of the inserts used in the different plasmids are summarized in Table 1. They differ in the number of copies of ARE sequences that have been inserted, in head-to-tail orientation, through *Nhe*I and *Xho*I restriction sites upstream of the promoter-*luc*$^+$ transcriptional unit. Five plasmids were made containing either one, two, four, six, or eight copies of the ARE (5′-GTGACAAAGCA-3′, with the minimal functional sequence underlined) present in both rat *GSTA2* and mouse *gsta1*; these were called pGL-*n*xARE (where *n* is the number of concatenated elements). A linker with the sequence of 5′-CCC-3′ and 5′-GGG-3′ on the opposite strand was placed between individual *cis*-elements. In addition, a plasmid named pGL-*GSTA2*.41bp-ARE was generated that represents the 41-bp 5′-GAGCTTGGAAATGGCATTGCTAATGGTGACAAAGCAACTTT-3′ between nucleotides −682 and −722 in the rat *GSTA2* gene promoter (with the minimal functional enhancer shown underlined), driving the luciferase reporter gene. In mouse *gsta1*, this sequence is 5′-TAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTG-3′ (14, 19) and was originally called the EpRE. The oligonucleotides were synthesized by MWG-BIOTECH AG (Eberserg, Germany). After the plasmids were generated, the DNA sequence of the inserts was verified.

Table 1. Sequence of inserts in the pGL3 promoter vector

Plasmid	Sequence of insert (5′→3′)
PGL-1xARE	5′-CCCGTGACAAAGCACCC-3′
PGL-2xARE	5′-GTGACAAAGCACCCGTGACAAAGCA-3′
PGL-4xARE	5′-GTGACAAAGCACCCGTGACAAAGCA CCCGTGACAAAGCACCCGTGACAAAGCA-3′
PGL-6xARE	5′-GTGACAAAGCACCCGTGACAAAGCACCC GTGACAAAGCACCCGTGACAAAGCACCC GTGACAAAGCACCCGTGACAAAGCA-3′
PGL-8xARE	5′-GTGACAAAGCACCCGTGACAAAGCACCC GTGACAAAGCACCCGTGACAAAGCACCC GTGACAAAGCACCCGTGACAAAGCACCC GTGACAAAGCACCCGTGACAAAGCA-3′
PGL-*GSTA2* .41bp-ARE	5′-GAGCTTGGAAATGGCATTGCTAATG GTGACAAAGCAACTTT-3′

NOTE: The letters underlined represent those nucleotides that form the minimal functional ARE.

The pHyg-EF-hNrf2 expression vector encoding a green fluorescent protein (GFP)–tagged human Nrf2, was a gift from Prof. Masayuki Yamamoto (Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Japan). The plasmid pEGFP-N1, a GFP expression vector employed as a negative control, was obtained from BD Clontech UK (Hampshire, United Kingdom).

Transient transfection and analysis of luciferase reporter gene activity. The Dual-luciferase Reporter Assay System (Promega) was used to examine reporter gene activity in transiently transfected cells. Briefly, cells were seeded at a density of 2×10^5 per well in 24-well plates and grown in the appropriate medium. After incubation overnight, the cells were transiently transfected with various ARE-luciferase reporter plasmids. The plasmid pRL-TK, encoding *Renilla* luciferase, was used to control for transfection efficiency. Transfections were done using LipofectAMINE 2000 reagent (Life Technologies, Inc. Ltd., Coventry, United Kingdom) according to the manufacturer's instructions. Following transfection, the culture medium was replaced 24 hours later with fresh growth medium containing 50 μmol/L *t*-BHQ (in a solution giving a final concentration of 0.1% v/v DMSO), which was prepared immediately before each experiment. For control experiments, vehicle alone (0.1% v/v DMSO) was added to the growth medium. Cells were left for 24 hours to respond to xenobiotics before being harvested, and the firefly and *Renilla* luciferase activities in their lysates were measured using a luminometer (Turner Designs Model TD-20/20, Promega) following addition of Luciferase Assay Reagent II (Promega). After quenching the reaction, the *Renilla* luciferase reaction was initiated by adding Stop and Glo Reagent (Promega). The relative luciferase activity was calculated by normalizing firefly luciferase activity to that of *Renilla* luciferase.

Generation of stable ARE-driven reporter systems. The pGL-8xARE, along with the pcDNA3.1 plasmid containing the neomycin selectable marker, was stably transfected into MCF7 cells using the calcium phosphate

method. Transfected cells were selected using 0.8 mg/mL G418 in the media for 3 to 4 weeks. The G418-resistant clones were isolated and screened by measuring their basal and inducible (obtained by treatment with 50 μmol/L *t*-BHQ) luciferase activities as described above. Positive clones, which showed low background and high inducible luciferase activity, were passaged and maintained in growth medium containing 0.8 mg/mL G418.

Treatment of stable ARE-luciferase reporter cells with anticancer drugs. BCNU and melphalan were dissolved in acidified ethanol as 1,000× concentrated solutions. Doxorubicin, epirubicin, cyclophosphamide, methotrexate, and paclitaxel were dissolved in PBS. The other chemotherapeutic agents were prepared as 1,000× concentrated stock solutions in DMSO and were stored at −20°C until use. For treatment with these drugs, cells were seeded at a density of 1.2×10^4 per well in 96-well microtiter plates in growth medium. After overnight recovery, the culture medium was replaced with fresh DMEM supplemented with antibiotics along with the particular drug of interest. An equal volume of vehicle was added to the control wells. After treatment for 24 hours, firefly luciferase activity was determined as described above.

Forced expression of hNrf2 in stable ARE-luciferase reporter cells. For transfection, AREc32 cells were seeded at 1.5×10^4 per well in 100 μL growth medium in 96-well plates. After overnight recovery, the cells were transfected with between 25 and 100 ng/well of the vectors pHyg-EF-hNrf2 or pEGFP-N1 using the LipofectAMINE 2000 reagent. Following a 4-hour recovery period after transfection, the culture medium was replaced with fresh DMEM containing glutamax and 10 μmol/L *t*-BHQ (or DMSO alone) supplemented with antibiotics. An equal volume of DMSO was added to the control wells. Finally, firefly luciferase activity was measured after treatment with *t*-BHQ for 24 hours.

Knockdown of Nrf2. pRS hNrf2, a pSUPER RNA interference (RNAi) vector targeting human Nrf2, was recovered from the glycerol stocks of the SUPER RNAi library (Netherlands Cancer Institute, Amsterdam, The Netherlands). The sequence of the oligo insert in the pRS-hNrf2 used in this study was 5′-GCATTGGAGTGTCAGTATG-3′, corresponding to nucleotides 1921 to 1939 of hNrf2 cDNA (where the nucleotides are numbered with the A in the ATG initiation codon designated as +1). A pSUPER RNAi vector targeting GFP, pRS-GFP, was also obtained from the SUPER RNAi library and used as a negative control.

For transfection with pSUPER RNAi, AREc32 cells were seeded at 1.5×10^4 per well in 100 μL growth medium in 96-well plates. After overnight recovery, between 25 and 100 ng/well of the pRS-hNrf2 or pRS-GFP pSUPER vectors were transfected into the cells using the LipofectAMINE 2000 reagent. Following recovery from transfection (24 hours), the culture medium was replaced with fresh DMEM containing glutamax and 10 μmol/L *t*-BHQ (or DMSO alone) supplemented with antibiotics. After 24 hours of treatment, firefly luciferase activity was measured. The efficacy of the RNAi to knockdown Nrf2 was confirmed by measurement of mRNA using Taqman analysis as described below.

Real-time quantitative PCR (reverse transcription-PCR). Total RNA was isolated with TRIzol and further purified with the RNeasy Mini kit (Qiagen Ltd., Germany) in accordance with the manufacturer's instructions. The A_{260}/A_{280} ratio of total RNA used was typically ≥1.9, and its quality was assessed using the Agilent 2100 Bioanalyzer. Reverse transcription-PCR (RT-PCR) was done as described previously (22). The primers were synthesized by MWG-BIOTECH. The probes, which were labeled with a 5′ fluorescent reporter dye (6-carboxyfluorescein) and a 3′ quenching dye (6-carboxytetramethylrhodamine), were synthesized by Qiagen. Each assay was done in triplicate. The specificity of PCR amplifications from the various sets of oligonucleotide primers was examined routinely by agarose-gel electrophoresis. The results were analyzed by using AB7700 system software. The level of 18S rRNA was used as an internal standard. For measurement of cDNA corresponding to human Nrf2 mRNA, the forward primer was 5′-ACTCCCTGCAGCAAACAAGAG-3′; the reverse primer was 5′-TTTTTCTTAACATCTGGCTTCTTACTTTT-3′; and the probe was 5′-TGGCAATGTTTTCCTTGTTCC-3′. For measurement of cDNA corresponding to human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, the forward primer was 5′-GAAGGTGAAGGTCGGAGTC-3′; the reverse primer was 5′-GAAGATGGTGATGGGATTTC-3′; and the probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. The sequences for the primers and probes for measuring cDNA corresponding to human NQO1, AKR1C, GCLC, and GCLM mRNAs have been described previously (11).

Western blot analysis. For the whole-cell extracts, cells were lysed in an extraction buffer containing 0.1 mol/L HEPES (pH 7.4), 0.5 mol/L KCl, 5 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, and 20% glycerol supplemented with protease inhibitor mixture (Roche Diagnostics, Indianapolis, IN). Nuclear extracts were prepared as described previously (23). Protein samples were separated in SDS-PAGE gels using a standard protocol. Immunoblottings were carried out using antiserum raised against AKR1C1 or Nrf2 as described previously (24, 25). To confirm equal loading, immunoblotting with antibody against actin (Sigma, St. Louis, MO) was also done.

Statistical analysis. Statistical comparisons were done by unpaired Student's *t* tests. $P < 0.05$ was considered statistically significant.

Results

Generation of a stable cell line expressing a functional ARE-driven reporter transgene. In this study, a series of ARE-luciferase reporter plasmids containing either one, two, four, six, or eight copies of the *cis*-element common to the rat *GSTA2* and mouse *gsta1* gene promoters were made; the sequences introduced into the pGL3 promoter vector are listed in Table 1. MCF7 cells were used to test these reporter constructs by transient transfection because Taqman analysis showed they contained substantial levels of mRNA for Nrf2 and Keap1 (data not shown). When MCF7 cells were treated with the monofunctional inducer *t*-BHQ, a marked increase in the amount of Nrf2 protein was detected in whole-cell lysates and nuclei obtained by subcellular fractionation (Fig. 1*A*). Previous work has shown that Nrf2 accumulates in HEK293 cells and HepG2 cells treated with *t*-BHQ (10, 26), findings that are consistent with the discovery that activation of ARE-driven gene expression by monofunctional inducing agents involves stabilization of Nrf2 protein through inhibition of Keap1 (9, 25). Our Western blotting data for Nrf2 protein suggest that the Keap1-Cul3 degradation pathway is functional in MCF7 cells and can be inhibited by *t*-BHQ. The increase in Nrf2 protein we observed following *t*-BHQ treatment of MCF7 cells is comparable with that observed in HEK293 cells (10) but seems to be greater than that reported in HepG2 cells (26).

Upon transfection of the various reporter constructs into MCF7 cells, it was found that increasing the number of copies of the ARE in the promoter of pGL3 had no significant effect on the basal level of luciferase activity observed under normal homeostatic conditions. However, there was a good correlation between the number of ARE copies in the pGL3 promoter vector and the level of induction of luciferase activity by *t*-BHQ in the MCF7 cells (Fig. 1*B*). A similar result was also observed in HepG2 cells transfected with the same reporter constructs (data not shown). These results confirm the findings of Nguyen et al. (27) in which they showed that transfection of reporter plasmids containing multiple copies of the rat *GSTA2*-ARE increased the sensitivity of chloramphenicol acetyltransferase reporter gene activity to induction by *t*-BHQ treatment.

To choose an appropriate cell system for the generation of a stable reporter cell line, pGL-*GSTA2*.41bp-ARE was transfected into HepG2, MCF7, CHO, and Hepa1c1c7 cells. Following transient transfection of this construct into MCF7 cells, luciferase activity was induced up to 50-fold after treatment for 18 hours with 50 μmol/L *t*-BHQ (Table 2). By contrast, the reporter gene was only

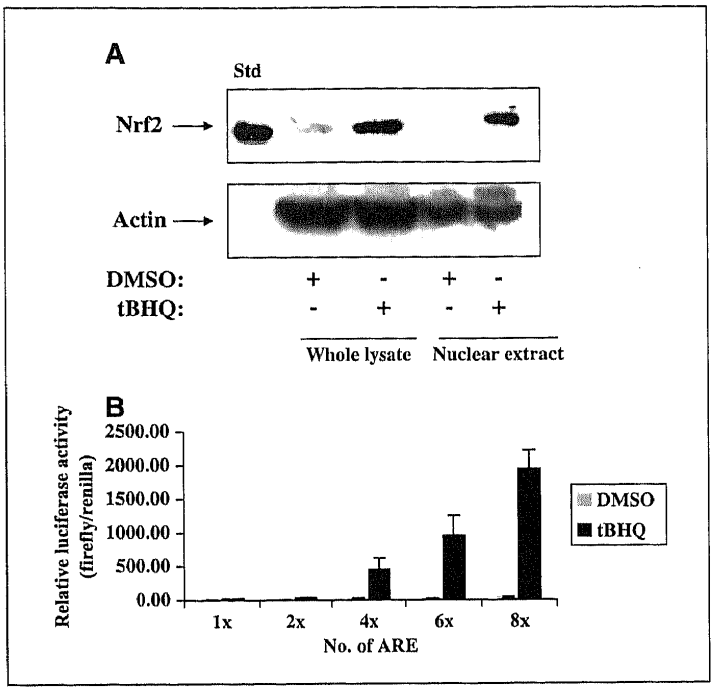

Figure 1. Correlation between ARE copy number and induction of reporter gene activity. *A*, MCF7 cells were cultured in DMEM supplemented with antibiotics containing DMSO or 10 μmol/L *t*-BHQ (*tBHQ*) for 24 hours. Portions (60 μg) whole-cell extracts and 20 μg nuclear extracts (*Nuclear*) were subjected to 7% SDS-PAGE, and the level of Nrf2 protein was measured by Western immunoblotting. *Std*, 1 ng recombinant his-mNrf2. Results from at least three separate experiments. *B*, MCF7 cells were seeded at 2×10^5 per well in 24-well plates, transfected with the various pGL3-nxARE constructs, and treated with 50 μmol/L *t*-BHQ. Luciferase reporter activity was determined 18 hours later as detailed in Materials and Methods. Results of three separate experiments. Each treatment in each experiment has at least three replicates. *Columns*, mean; *bars*, SD.

induced between 2- and 4-fold following similar transfection experiments in HepG2, CHO, or Hepa1c1c7 cells. These results, therefore, show that MCF7 cells provide a particularly sensitive means of measuring ARE-driven transcription.

We decided to employ pGL-8xARE as the plasmid to generate a stable reporter cell line because this construct gave a reasonably high level of inducible luciferase production following treatment with *t*-BHQ. To this end, pGL-8xARE and pcDNA3.1, which contained a neomycin selectable marker, were stably cotransfected into MCF7 cells and selected in the presence of G418. In total, 153 G418-resistant clones were isolated. After the first passage, 32 clones were kept for further monitoring according to their basal and inducible luciferase activities. Among them, one clone, called AREc32, showed low basal and high inducible luciferase activity; an ~10- to 13-fold increase in reporter gene activity was observed following treatment with 10 μmol/L *t*-BHQ. This clone also showed a stable phenotype after >20 passages. The rest of the clones were discarded because they showed either a lower level of induction (2- to 6-fold) by 10 μmol/L *t*-BHQ, or an unstable phenotype upon further passage. Therefore, AREc32 cells were retained for further study.

Induction of ARE-driven luciferase activity in AREc32 cells is mediated by Nrf2. To confirm that the luciferase activity in AREc32 cells was responsive to Nrf2, this CNC bZIP protein was overexpressed in AREc32 cells by transient transfection with the expression construct pHyg-EF-hNrf2. As shown in Fig. 2*A*, the control cells where no DNA was included in the transfection mix gave 13-fold induction of luciferase activity when treated with 10 μmol/L *t*-BHQ. When 25 ng of pHyg-EF-hNrf2 plasmid DNA was used per well, the basal luciferase activity did not change significantly, and the inducible luciferase activity increased to 18-fold. Following transfection with 50 ng of pHyg-EF-hNrf2 per well, the basal level of luciferase activity increased to 2.6-fold, and the inducible level increased to 22-fold. Finally, following transfection with 100 ng of pHyg-EF-hNrf2, the basal reporter gene activity increased to 4-fold and the inducible level to 28-fold. In different wells, the same amount of pEGFP-N1, an expression vector for enhanced GFP (EGFP), was transfected into AREc32 cells as a negative control. Neither the basal nor the inducible luciferase activities were altered by overexpression of EGFP.

To determine whether Nrf2 mediates induction of luciferase activity by *t*-BHQ in AREc32 cells, an RNAi vector was used to knockdown its expression. Figure 2*B* shows that 24 hours after transfection of AREc32 cells with pRS-Nrf2, the endogenous level of mRNA for Nrf2 was reduced to ~40% of the control level, but its abundance was not affected by transfection with the control pRS-GFP vector. Transfection of these cells with either pRS-hNrf2 or pRS-GFP did not affect the level of GAPDH mRNA (Fig. 2*B*). This finding indicates that transfection of AREc32 cells with pRS-hNrf2 specifically suppressed expression of the CNC bZIP factor. AREc32 cells were treated with variable amounts of RNAi against Nrf2 and the effect on both basal and inducible luciferase reporter gene activity examined. Transfection of these cells with 25 to 100 ng pRS-hNrf2 per well reduced the basal level of ARE-driven luciferase activity to 60% of the control level (Fig. 2*C*).

Table 2. Identification of MCF7 cells for optimal use of ARE reporter system

Cell line	Relative luciferase activity (DMSO treated)	Relative luciferase activity (*t*-BHQ treated)	Ratio (*t*-BHQ/DMSO)
HepG2	1.0 ± 0.3	2.8 ± 0.9	2.8 ± 0.9
MCF7	43.8 ± 3.5	2276.1 ± 521.1	52.0 ± 11.9
CHO	426.1 ± 64.7	1171.6 ± 8.8	2.7 ± 0.1
Hepa1c1c7	39.2 ± 1.4	140.7 ± 19.6	3.6 ± 0.5

NOTE: MCF7, HepG2, CHO, and Hepa1c1c7 cells were seeded at 2×10^5 per well in 24-well plates, before being transfected with pGL-*GSTA2*.41bp-ARE construct. The plasmid pRL-TK was used as internal control in each transfection. The cells were treated with 50 μmol/L *t*-BHQ, and luciferase reporter activity was determined as detailed in Materials and Methods. For control experiments, the same volume of DMSO was added to the medium. The value of relative luciferase activity of HepG2 cells treated with DMSO was set at 1. Data are expressed as the mean ± SD. This represents the results of three separate experiments. Each treatment in each experiment has at least three replicates.

When the effect of pRS-hNrf2 on induction of reporter gene activity by 10 μmol/L *t*-BHQ was tested, a dose-dependent inhibition was observed. The inducibility of luciferase activity was not affected significantly following transfection with 25 ng of pRS-hNrf2 per well, when compared with the mock-transfected control cells (9-fold induction). When 50 ng of pRS-hNrf2 DNA was used to transfect cells in each well, induction of luciferase activity by 10 μmol/L *t*-BHQ was reduced to 8-fold. When 100 ng of pRS-hNrf2 DNA was used per well, only 5-fold induction by 10 μmol/L *t*-BHQ was detected. In different wells, the basal and inducible luciferase activities were not affected when AREc32 cells were transfected with the same amount of pRS-GFP DNA, which targeted GFP mRNA (Fig. 2*C*). These data indicate both basal and inducible ARE-driven gene expression is mediated by Nrf2 in the AREc32 cells.

Time- and dose-dependent induction of luciferase in AREc32 cells. Luciferase activity in AREc32 cells could be induced in a time- and dose-dependent manner; after treatment for 24 hours, luciferase activity was increased 2-fold by 1 μmol/L *t*-BHQ and 5-fold by 5 μmol/L *t*-BHQ (see Fig. 3*A*; Table 3). A maximum luciferase activity (around 10-fold increase) was seen following treatment with 10 μmol/L *t*-BHQ. Induction of luciferase activity by *t*-BHQ was also time dependent; it increased 4-fold after 8 hours of treatment with 10 μmol/L *t*-BHQ and reached 10-fold after 18 hours of treatment with the same dose of *t*-BHQ (Fig. 3*B*). A similar magnitude of induction of luciferase activity in AREc32 cells was observed after 24 hours of exposure to 10 μmol/L SUL (see later), a potent NQO1 and AKR enzyme inducer in human HaCaT, LS174, and Caco2 cell lines (11, 28).

Expressions of endogenous AKR1C and luciferase reporter activity in AREc32 cells are both increased by monofunctional inducing agents. To test whether luciferase activity in AREc32 cells reflected expression of endogenous ARE-driven genes, its induction by *t*-BHQ was compared with that of *NQO1, GCLC, GCLM,* and *AKR1C*. After 24 hours of treatment with 10 μmol/L *t*-BHQ, the levels of mRNAs for NQO1, GCLC, GCLM, and AKR1C were increased 3.0-, 1.7-, 1.7-, and 15-fold, respectively (Fig. 4). By contrast, luciferase activity was induced 10-fold.

Luciferase activity in AREc32 cells was increased significantly by monofunctional and bifunctional inducing agents (for a review of such compounds, see ref. 2) and other Nrf2 activators (Table 3; Supplementary Fig. S1), including acrolein, SUL, BHA, ethoxyquin, pyrrolidine dithiocarbamate (PDTC), indole-3-carbinol, H_2O_2, and β-naphthoflavone (data not shown). In addition, we found that 7-ethoxycoumarin and methyl methanesulfonate (MMS) were effective inducers of luciferase activity in AREc32 cells (Table 3; Supplementary Fig. S1). In our stable reporter cell line, butylated hydroxytoluene (BHT) had no effect on the luciferase activity.

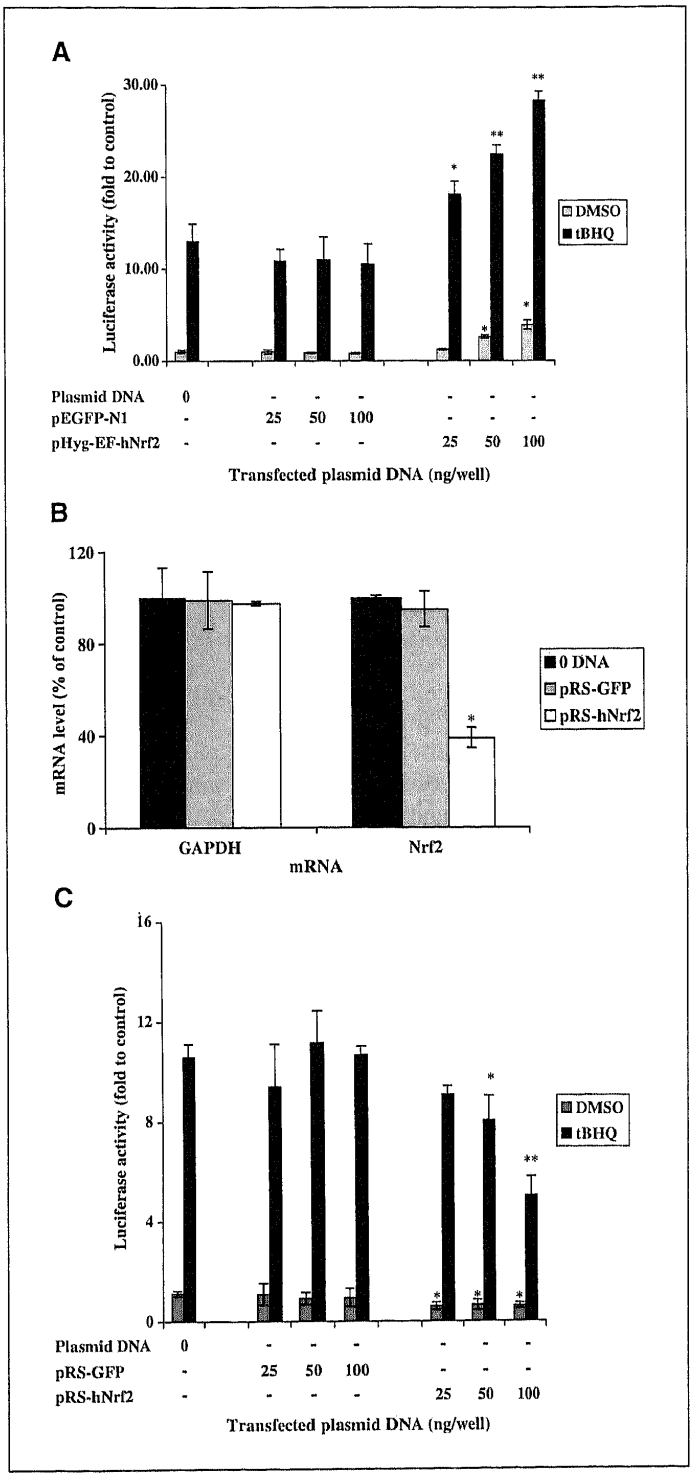

Figure 2. Luciferase reporter activity in AREc32 cells was mediated by Nrf2. *A*, overexpression of Nrf2 in AREc32 cells increased both the basal and the inducible luciferase reporter activity. AREc32 cells were seeded in a 96-well plate at 1.5×10^4 per well and transfected with either 25, 50, or 100 ng/well of pEGF-hNrf2. The same amount of pEGFP-N1 was transfected as a negative control. After transfection (24 hours), the cells were treated for further 24 hours with either DMSO alone or 10 μmol/L *t*-BHQ (in DMSO). The luciferase activity was assayed as described in Materials and Methods. *Control,* DNA was absent, and the transfection reagent was only added to the cells and treated with DMSO for 24 hours. *B*, knockdown of Nrf2 by RNAi vector in AREc32 cells. The AREc32 cells were seeded in 100-mm dishes at 8×10^6 per dish in growth medium. Twenty-four hours later, the cells were transfected with either 24 μg pRS-hNrf2 or 24 μg pRS-GFP per plate. After a further 24 hours had elapsed, total RNA was extracted from the cells, and levels of Nrf2 and GAPDH mRNAs were measured by Taqman. The level of 18S rRNA was used as an internal standard. The mRNA level from cells that had been mock transfected (control) was set at 100%. Suppression of Nrf2 expression in AREc32 cells reduces the basal and inducible luciferase reporter activity. *C*, in a parallel experiment to (*B*), AREc32 cells were seeded in a 96-well plate at 1.5×10^4 per well and transfected with 25, 50, and 100 ng/well pRS-hNrf2. The same amount of pRS-GFP was transfected as negative control. Twenty-four hours after the transfection, the cells were treated with DMSO or 10 μmol/L *t*-BHQ. Luciferase activity was assayed as described in the text. *Control,* DNA was absent, and the transfection reagent was only added to the cells and treated with DMSO for 24 hours. *Columns,* mean from triplicate samples; *bars,* SD. Each treatment in each experiment has at least three replicates. The significant of the differences between luciferase activity from cultures transfected with pRS-hNrf2 or pEGF-Nrf2, and the control was assessed by unpaired Student's *t* test. *, $P < 0.05$; **, $P < 0.005$.

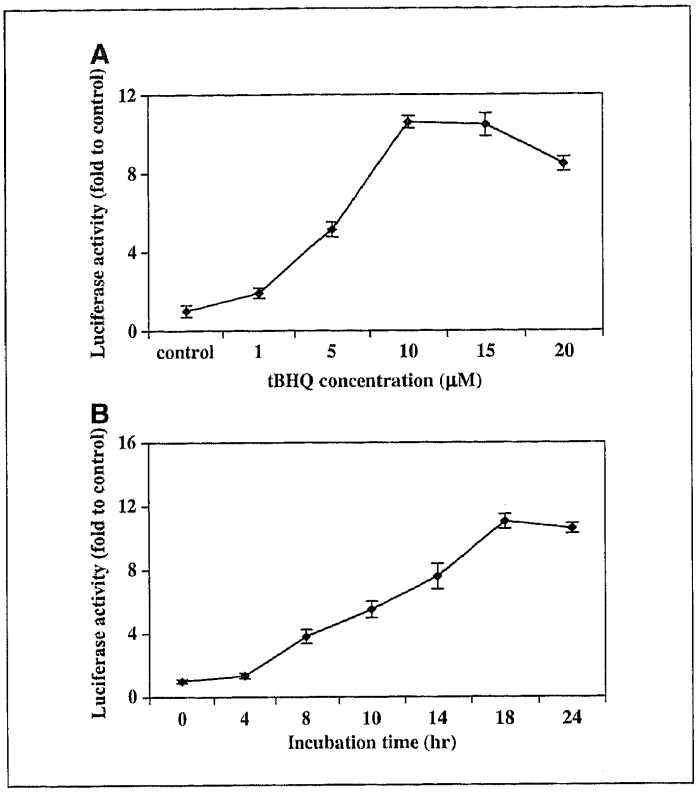

Figure 3. *t*-BHQ induces ARE reporter activity in MCF7 cells in a time- and dose-dependent manner. *A*, cells were seeded in a 96-well plate at 1.2×10^4 per well in the growth medium. After 24 hours of recovery, the culture medium was replaced with fresh DMEM supplemented with antibiotics containing 1 to 20 μmol/L *t*-BHQ. The cells were then incubated for between 4 and 24 hours and assayed for luciferase activity as detailed in Materials and Methods. The value of luciferase activity of cells treated with DMSO (0.1% v/v; control) was set at 1. *B*, time course of the induction of luciferase induction in response to 10 μmol/L *t*-BHQ in AREc32 cells. Dose response of luciferase induction to *t*-BHQ in AREc32 cells. Results of three separate experiments. Each treatment in each experiment has at least three replicates. *Points*, mean-fold increase relative to control value; *bars*, SD.

As endogenous *AKR1C* in AREc32 cells proved to be highly responsive to *t*-BHQ treatment, we tested whether it was also induced by the compounds that increased luciferase activity. As shown in Table 4, AKR1C mRNA was increased substantially by these compounds, with acrolein, ethoxyquin, and PDTC proving to be particularly effective.

Redox status influences reporter luciferase activity in AREc32 cells. It is unclear whether antioxidants can inhibit inducible ARE-driven gene expression because contradictory data have been published. For example, Pinkus et al. (29) reported that induction of transcription through the mouse *gsta1*-ARE in HepG2 cells by 100 μmol/L BHA was inhibited by inclusion of 30 mmol/L GSH in the medium, whereas Lee et al. (30) reported that induction of gene expression through the human *NQO1*-ARE in IMR-32 cells by 10 μmol/L *t*-BHQ was not inhibited by 2 hours of pretreatment with either 5 mmol/L GSH or 2.5 mmol/L *N*-acetylcysteine (NAC). In our study, either NAC or GSH was added simultaneously to AREc32 cells along with either *t*-BHQ or SUL, and their effects on reporter gene expression were examined. As shown in Fig. 5, neither NAC nor GSH influenced basal ARE activity in the stable reporter cells. The induction of luciferase activity by *t*-BHQ was, however, reduced to only 20% in the presence of 5 mmol/L NAC and to just 30% by 5 mmol/L GSH (Fig. 5*A*). A similar degree of inhibition of induction was also observed in AREc32 cells treated with SUL (Fig. 5*B*), where the presence of 5 mmol/L NAC or 5 mmol/L GSH diminished its ability to increase luciferase activity to only 30% of the level seen in the absence of antioxidant. By contrast, the free radical scavengers vitamin E and vitamin C did not markedly inhibit induction of luciferase activity by *t*-BHQ or SUL. Close examination of Fig. 5*A* and *B* suggests that vitamin E can modestly reduce induction of luciferase activity by *t*-BHQ, but it had no effect on induction by SUL.

To examine whether the intracellular level of GSH itself might negatively control induction of ARE-driven luciferase activity, the cellular GSH pool was depleted using BSO before treatment with *t*-BHQ or SUL. As shown in Fig. 5*C*, treatment with BSO alone did not affect the luciferase activity. However, when the cells were pretreated for 24 hours with 50 μmol/L BSO before challenge with *t*-BHQ alone, the induction of luciferase activity by 10 μmol/L *t*-BHQ increased from 10- to 25-fold. Furthermore, if the cells were not only pretreated for 24 hours with BSO, but this GSH synthesis inhibitor was also included in the media during challenge with *t*-BHQ, luciferase activity increased further to 40-fold. In the case of induction by 10 μmol/L SUL, BSO pretreatment resulted in an increase in luciferase activity from 13- to 21-fold, and when BSO treatment was continued in the presence of SUL, luciferase activity increased further to 34-fold.

Anticancer drugs can activate ARE-reporter gene expression. To find out whether cancer chemotherapeutic agents modulate the Nrf2-ARE system, a number of anticancer drugs were screened using AREc32 cells. Based on the IC_{50} results (data not shown), AREc32 cells were treated for 24 hours with multiple sublethal doses of the therapeutic agents. According to their effect on luciferase activity, these drugs were divided in Table 5 into three groups: those with no significant effect on luciferase activity, modest activators, and strong activators. Thus, doxorubicin,

Table 3. Inducers of luciferase activity in AREc32 cells

Compound	CD (μmol/L)
t-BHQ	1
SUL	2
Acrolein	2
Ethoxyquin	5
BHA	20
I3C	20
PDTC	20
MMS	100
7-Ethoxycoumarin	100
H_2O_2	300

NOTE: Cells were seeded in a 96-well plate at 1.2×10^4 per well in the growth medium. After 24 hours of recovery, the culture medium was replaced with fresh DMEM supplemented with antibiotics containing various concentrations of the compounds listed. The cells were incubated for a further 24 hours before luciferase activity was measured as detailed in Materials and Methods. The value of luciferase activity of cells treated with DMSO (0.1% v/v) was set at 1. The results presented represent results from three separate experiments. Each treatment in each experiment has at least three replicates.

Abbreviations: CD, concentration of inducing agent that doubled luciferase reporter activity; I3C, indole-3-carbinol.

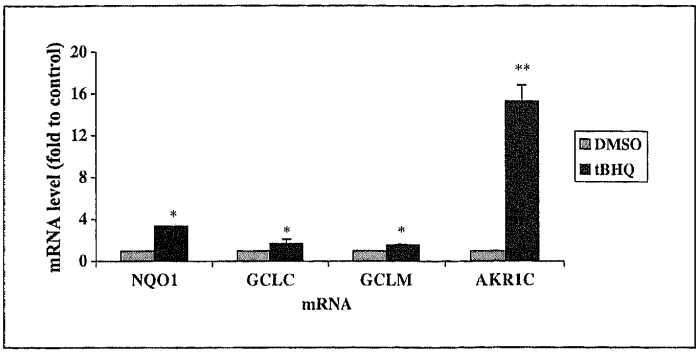

Figure 4. Induction of endogenous *NQO1, GCLC, GCLM,* and *AKR1C* by *t*-BHQ in AREc32 cells. Total RNA was extracted from AREc32 cells, which had been treated with 10 μmol/L *t*-BHQ for 24 hours. The mRNA levels of NQO1, GCLC, GCLM, and AKR1C were measured by Taqman analysis as described in ref. 11. The level of 18S rRNA was used as an internal standard. Control, cells were treated with DMSO only. *Columns,* mean from triplicate samples; *bars,* SD. Results of three separate experiments. The significant of the differences between mRNA levels from cultures treated with *t*-BHQ and the control was assessed by unpaired Student's *t* test. *, $P < 0.05$.

epirubicin, paclitaxel (Taxol), methotrexate, and thiotepa treatment had no effect on the level of luciferase activity in AREc32 cells. By contrast, cisplatin, melphalan, and etoposide modestly increased luciferase activity. Treatment of these cells with the alkylating agents chlorambucil, BCNU, and the cytotoxic antibiotic mitoxantrone produced a stronger induction of luciferase activity of between 2- and 4-fold.

Using AREc32 cells, we found that cyclophosphamide treatment had no effect on ARE-luciferase activity. By contrast, acrolein, a major metabolite of cyclophosphamide was found to be a potent ARE activator; 10 μmol/L acrolein gave a 27-fold increase in luciferase activity.

Activation of ARE-driven gene expression by anticancer drugs is redox dependent. To examine whether cellular GSH level has any effect on the ability of anticancer drugs to activate luciferase activity, we pretreated AREc32 cells with 50 μmol/L BSO for 24 hours before challenging them with chemotherapeutic agents. As can be seen in Fig. 6*A*, pretreatment with BSO caused the induction of luciferase activity by cisplatin and melphalan to be increased to 3- and 5-fold, respectively. More remarkably, BSO caused the induction of luciferase activity by chlorambucil and BCNU to be increased to >10-fold. Such inductions were nearly completely repressed by the addition of 5 mmol/L NAC. For treatments with etoposide and mitoxantrone, we found that BSO pretreatment did not change luciferase activity significantly (data not shown).

To find out whether anticancer drugs similarly activate the expression of an endogenous Nrf2-regulated gene, we examined expression of *AKR1C* in AREc32 cells. Without pretreatment with BSO, the mRNA level of AKR1C was only slightly increased by the treatment of the reporter cell line with melphalan, cisplatin, or chlorambucil. However, when the cells were pretreated with 50 μmol/L BSO for 24 hours, melphalan and cisplatin increased the expression of AKR1C mRNA by 3- and 4-fold, respectively, and chlorambucil increased this mRNA 31-fold (Fig. 6*B*). Treatment with BCNU induced the expression of AKR1C mRNA 3-fold, and with BSO pretreatment, BCNU induced AKR1C mRNA 42-fold (Fig. 6*B*). Immunoblotting revealed that AKR1C protein was also increased by these anticancer drugs (Fig. 6*C*). BSO pretreatment did not further enhance the expression of AKR1C protein by *t*-BHQ treatment. This is, however, possibly because the induction of AKR1C by 10 μmol/L *t*-BHQ alone has already reached the maximum level.

Discussion

We have generated a stable ARE-reporter human mammary cell line called AREc32, derived from MCF7 cells, in which only the minimal enhancer sequence (5′-GTGACAAAGCA-3′) is present to direct expression of the luciferase transgene. The ARE employed for this purpose was designed around that found in the promoters of both rat *GSTA2* and mouse *gsta1*. In the latter gene, both its basal and inducible expressions have been shown to be regulated by Nrf2 *in vivo* (31). It should also be noted that our constructs did not contain the upstream 5′-T($^{A}/_{C}$)A-3′ trinucleotide sequence that is found in the extended ARE (32, 33); in the rat *GSTA2* and mouse *gsta1* genes, this is 5′-TAATGGTGACAAAGCA-3′, with the relevant upstream trinucleotides underlined.

We have shown that in our AREc32 cells, expression of luciferase activity is mediated by Nrf2 and is sensitive to redox status. This cell line gave a ~10-fold increase in reporter gene activity by treatment with relatively modest doses of typical monofunctional inducers and therefore provides a good model system that can be used to screen chemical libraries to identify agonists of Nrf2.

Previously, Zhu and Fahl (34) generated a stable ARE-GFP reporter HepG2 cell line. The reporter construct they employed contained four concatenated copies of the 41-bp ARE-containing

Table 4. Inducers of AKR1C in AREc32 cells

Compound	Fold increase* in AKR1C mRNA	Fold change† in GAPDH mRNA
100 μmol/L Ethoxycoumarin	1.3 ± 0.01‡	1.0 ± 0.10
500 μmol/L H_2O_2	3.2 ± 0.2	0.7 ± 0.10
100 μmol/L BHA	6.5 ± 1.5	1.3 ± 0.08
100 μmol/L MMS	8.6 ± 0.8	0.7 ± 0.08
100 μmol/L I3C	14.3 ± 1.3	1.0 ± 0.10
5 μmol/L acrolein	96.1 ± 8.0	0.9 ± 0.11
20 μmol/L ethoxyquin	175.2 ± 17	1.1 ± 0.06
100 μmol/L PDTC	183.9 ± 10.8	0.7 ± 0.08

NOTE: Cells were seeded in 100-mm dishes at 2×10^6 per dish in the growth medium. After 24 hours of recovery, the culture medium was replaced with fresh DMEM supplemented with antibiotics containing 100 μmol/L ethoxycoumarin, 500 μmol/L H_2O_2, 100 μmol/L BHA, 100 μmol/L MMS, 100 μmol/L I3C, 5 μmol/L acrolein, 20 μmol/L ethoxyquin, or 100 μmol/L PDTC. The cells were then incubated for 24 hours. Total RNA was extracted, and the Taqman RT-PCR analysis for AKR1C mRNA was carried out as detailed in Materials and Methods. The mRNA level of GAPDH relative to 18S RNA was not affected by the majority of treatments and was used as the internal standard for the AKR1C results. The mRNA level of AKR1C from cells treated with DMSO (0.1% v/v; control) was set at 1. The results presented represent results from two separate experiments. Each treatment in each experiment has at least three replicates.

Abbreviation: I3C, indole-3-carbinol.

*Data expressed as mean-fold increase relative to control value ±SD.

† mRNA level of GAPDH was normalized by 18S RNA level and data expressed as mean-fold relative to control.

‡ $P < 0.05$.

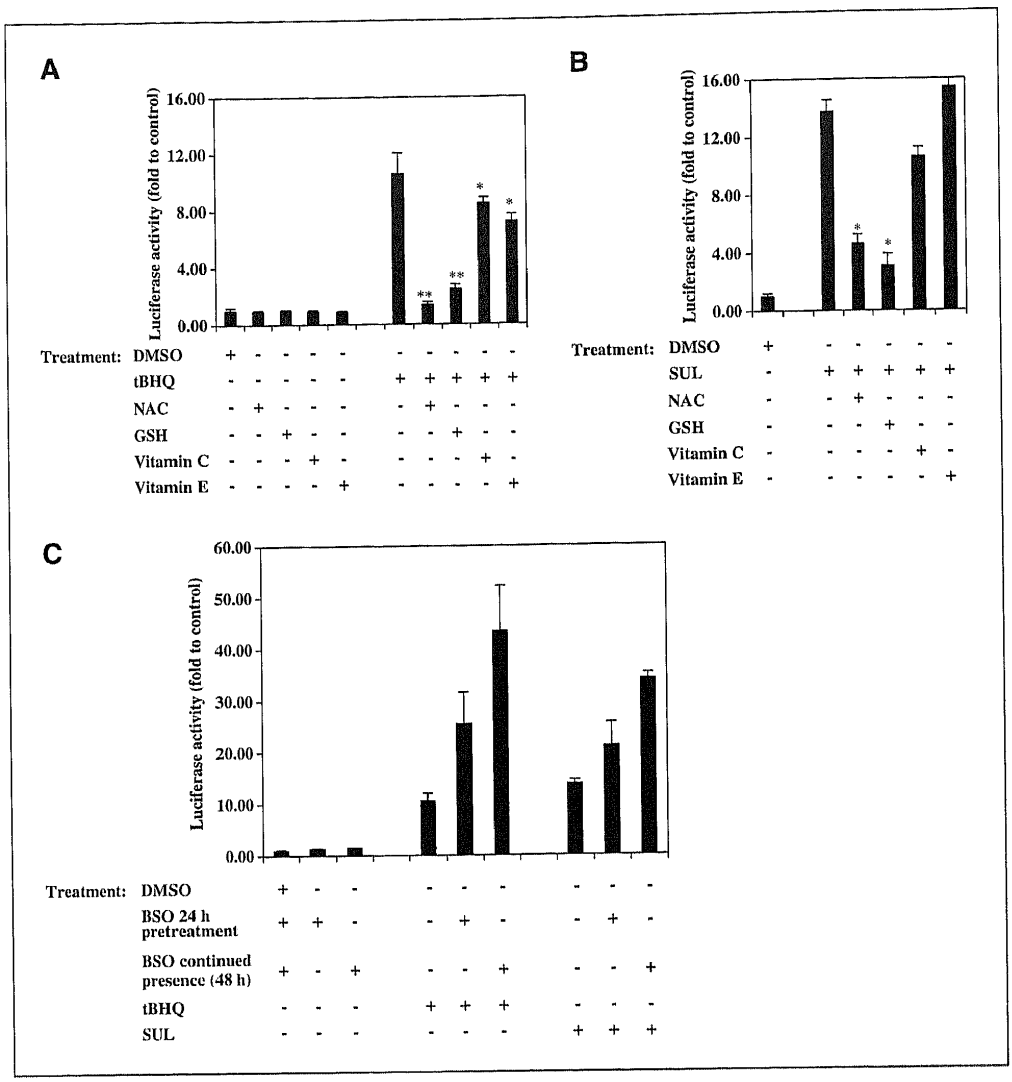

Figure 5. Induction of reporter gene activity in AREc32 cells is sensitive to cellular redox status. *A*, NAC and GSH, but not vitamin C or vitamin E, repressed the induction of luciferase activity by *t*-BHQ in AREc32 cells. The AREc32 cells were seeded in a 96-well plate at 1.2×10^4 per well. After incubation for 24 hours, the culture medium was replaced with fresh DMEM supplemented with antibiotics containing either DMSO (control), or 10 μmol/L *t*-BHQ, or 10 μmol/L *t*-BHQ + 5 mmol/L NAC, or 10 μmol/L *t*-BHQ + 5 mmol/L GSH, or 10 μmol/L *t*-BHQ + 100 μmol/L vitamin C, or 10 μmol/L *t*-BHQ + 100 μmol/L vitamin E. After 24 hours of treatment, luciferase activity in the cells was assayed as described in Materials and Methods. *B*, NAC and GSH, but not vitamin C or vitamin E, repressed the induction of luciferase activity by SUL in AREc32 cells. AREc32 cells were seeded in a 96-well plate at 1.2×10^4 per well. After 24 hours of incubation, the culture medium was replaced with fresh DMEM supplemented with antibiotics containing either DMSO (control), or 10 μmol/L SUL, or 10 μmol/L SUL + 5 mmol/L NAC, or 10 μmol/L SUL + 5 mmol/L GSH, or 10 μmol/L SUL + 100 μmol/L vitamin C, or 10 μmol/L SUL + 100 μmol/L vitamin E. After 24 hours of treatment, luciferase activity was measured. *C*, BSO enhanced the induction of luciferase activity in AREc32 cells by *t*-BHQ or SUL. The cells were seeded in a 96-well plate at $0.4 \cdot 10^4$ per well. After 24 hours of recovery, the culture medium was replaced with growth medium containing 50 μmol/L BSO. Twenty-four hours later, the culture medium was replaced with fresh DMEM supplemented with antibiotics containing DMSO (control), 10 μmol/L *t*-BHQ, or 10 μmol/L SUL with or without 50 μmol/L BSO. After the cells were incubated for 24 hours, luciferase activity was determined. The value of control cells treated with DMSO was set at 1. Results of three separate experiments. Each treatment in each experiment has at least three replicates. *, $P < 0.05$; **, $P < 0.005$.

promoter sequence from mouse *gsta1* ligated to the thymidine kinase promoter driving GFP and gave just 3-fold induction following treatment with 90 μmol/L *t*-BHQ. Based on induction of reporter genes by *t*-BHQ, it is apparent that our AREc32 cells gave very substantially greater levels of induction than was found in HepG2 cells. A murine Hepa1c1c7 cell line stably transfected with the mouse 41-bp mouse *gsta1*-ARE driving the luciferase gene in pGL3-basic has been described recently (35), and it seems to give comparable levels of induction as we find in our AREc32 cells.

Mechanism of ARE-driven gene induction by *t*-BHQ. We have employed *t*-BHQ as our principal monofunctional inducer to challenge AREc32 cells because it was used in the original experiments to define both this type of agent (21) and the ARE consensus sequence (18). In this case, *t*-BHQ probably induces ARE-driven genes because it undergoes a two-electron oxidation to form an electrophilic quinone. Correlation of the relative abilities of *t*-BHQ and its analogue 2,5-di-*tert*-butylhydroquinone to induce rat *GSTP1* with their relative redox-cycling activities, their abilities to produce superoxide, or their electrophilic properties has established that it is the last characteristic that is most important for activation of ARE-driven gene expression (35). In particular, Nakamura et al. (36) found that *t*-BHQ can form a quinone and is a good inducer of *GSTP1*, whereas 2,5-di-*tert*-butylhydroquinone cannot form a quinone and is not a good inducer of *GSTP1*. As these workers showed that both compounds could be oxidized to their respective benzoquinones and both could generate superoxide by redox-cycling, but only *t*-BHQ and not 2,5-di-*tert*-butylhydroquinone could form a quinone that would react with GSH, they considered that the ability to form a thiol-reactive

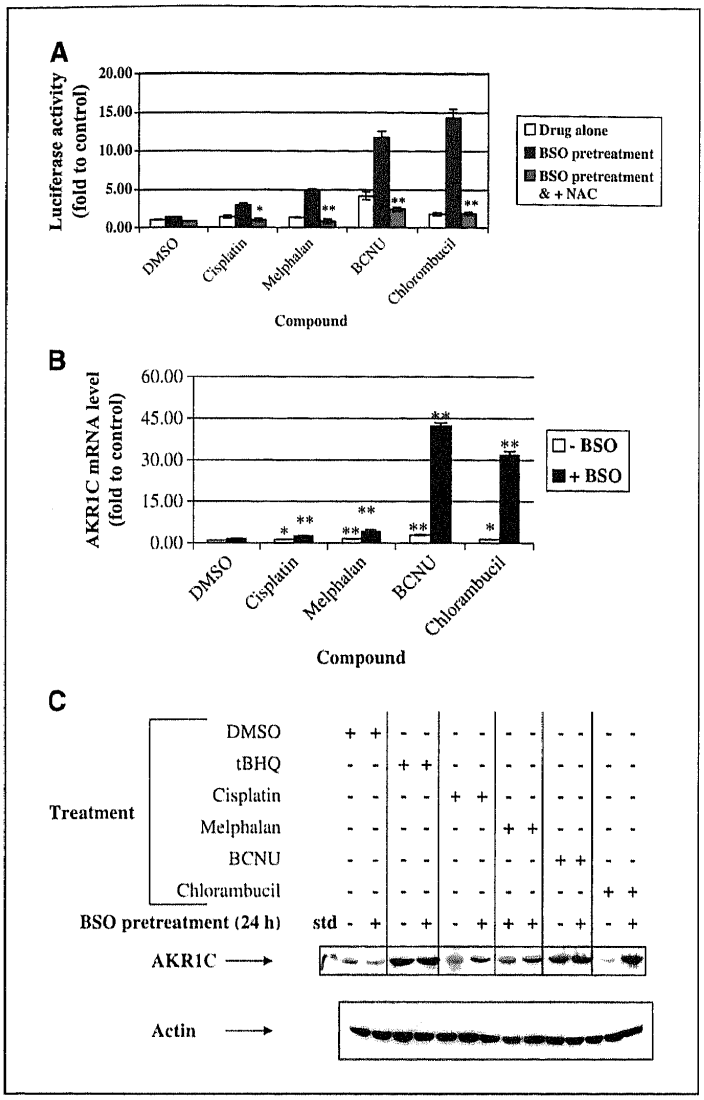

Figure 6. Induction of ARE-driven gene expression by anticancer drugs is redox dependent. *A*, BSO enhanced the induction of luciferase reporter gene activity in AREc32 cells by anticancer drugs. AREc32 cells were seeded in a 96-well plate at 0.4×10^4 per well, and after 24 hours of recovery, the culture medium was replaced with growth medium containing 50 μmol/L BSO. Equal volume of PBS was added to the cells that were not subjected to BSO pretreatment. Twenty-four hours later, the culture medium was replaced with fresh DMEM supplemented with antibiotics containing DMSO (control), or 10 μmol/L cisplatin, or 20 μmol/L melphalan, or 100 μmol/L BCNU, or 100 μmol/L chlorambucil with or without 5 mmol/L NAC and incubated for 24 hours. The cells were assayed for luciferase activity as detailed in Materials and Methods. The value of control cells treated with DMSO was set at 1. *Columns*, mean from triplicate samples; *bars*, SE. The significance of the differences between luciferase activity from cultures exposed to the anticancer agents with NAC and cultures treated with the anticancer agents alone was assessed by unpaired Student's *t* test. Results of three separate experiments. *, $P < 0.05$; **, $P < 0.005$. *B*, endogenous AKR1C mRNA in AREc32 cells was induced by anticancer drugs in a redox-dependent manner. AREc32 cells were seeded in 100-mm dishes at 2×10^6 per dish in the growth medium. After 24 hours of recovery, the culture medium was replaced with growth medium containing 50 μmol/L BSO. Twenty-four hours later, the culture medium was replaced with fresh DMEM supplemented with antibiotics containing either DMSO, 10 μmol/L *t*-BHQ, 20 μmol/L melphalan, 10 μmol/L cisplatin, 100 μmol/L BCNU, or 100 μmol/L chlorambucil and incubated for a further 24 hours. After 24 hours of treatment, the cells were harvested. The expression of AKR1C mRNA was measured by Taqman analysis (11). The mRNA level of AKR1C of cells treated with DMSO (control) was set at 1. The significance of the differences between AKR1C mRNA level from cultures exposed to the anticancer agents and those to DMSO was assessed by unpaired Student's *t* test. Two separate experiments. Each treatment in each experiment has three replicates. *, $P < 0.05$; **, $P < 0.005$. *C*, in a parallel experiment to (*B*), 30 μg whole-cell extracts were separated on 15% SDS-PAGE. The expression of AKR1C was measured by Western immunoblotting with antibody specific to AKR1C. Results from three separate experiments.

electrophile is the essential characteristic required for induction of ARE genes (36).

Response of AREc32 cells to monofunctional inducers. Initial characterization of our stable reporter cell line showed that ARE-driven luciferase activity could be induced up to 70-fold by treatment with 50 μmol/L *t*-BHQ (with serum). However, in most experiments, we used just 10 μmol/L *t*-BHQ (without serum), and this elicited an ~10-fold increase in reporter activity. Treatment of AREc32 cells with 10 μmol/L SUL, another widely used monofunctional inducer, resulted in a similar increase in luciferase activity. We also established that the endogenous ARE gene battery is inducible in the stable cell line. Taqman RT-PCR and Western blotting showed that endogenous members of the ARE gene battery, such as *AKR1C, NQO1, GCLC,* and *GCLM,* could be induced by *t*-BHQ in our stable reporter cell line. The increase in expression of these genes was similar to the increases seen in Caco2 and LS174 cells treated with *t*-BHQ or SUL (11, 28).

Response of AREc32 cells to metabolizable phenolic antioxidants. Following confirmation that monofunctional inducers increase the expression of endogenous genes in AREc32 cells, we tested whether BHA and ethoxyquin were capable of inducing luciferase reporter activity in these cells because they are classic chemopreventive-blocking agents. In rodents, these antioxidants are each strong inducing agents. This property is not, however, thought to be due to their antioxidant properties. Rather, it is thought that it is necessary for them to be metabolized into compounds that contain quinone or α,β-unsaturated carbonyl groups in order for them to induce ARE-driven genes (20). For example, BHA is *O*-demethylated by cytochrome P450 to yield *t*-BHQ that, as described above, is susceptible to a two-electron oxidation to form *tert*-butylbenzoquinone (20). This latter metabolite is thiol-active and is postulated to be the ultimate inducing agent (36). In AREc32 cells, *t*-BHQ was 20-fold more potent than BHA at inducing luciferase activity (Table 3), an observation that is consistent with the hypothesis that BHA requires to be metabolized via *t*-BHQ to *tert*-butylbenzoquinone to induce gene expression. We also found that ethoxyquin is a relatively potent inducer of reporter gene activity in AREc32 cells, which suggests it is efficiently converted by *O*-de-ethylation and oxidation reactions to quinoline- and quinolone-containing metabolites, which are considered to be the ultimate inducers (20). In further experiments, we found that BHT did not induce gene activity in our stable reporter cell line despite it being a potent inducing agent in rats (14). Unfortunately, we do not know which cytochrome P450s are expressed in our stable reporter cell line, although in terms of explaining the different potency of induction affected by the antioxidants, it would be interesting to know if the AREc32 cells catalyze *O*-de-alkylation of BHA and ethoxyquin but not of BHT.

Besides BHA, ethoxyquin, and BHT, we have examined the effect of PDTC on luciferase activity in AREc32 cells. In this case, the dithiocarbamate was shown to be a robust inducer of ARE-driven reporter gene expression and endogenous AKR1C. This is not surprising as PDTC has previously been found to induce *GCLM* in HepG2 cells (37), and there is good evidence that following the conversion of PDTC to thioram disulfides, it is able to oxidize GSH and protein sulfhydryls (37). Taken together with previous studies (17, 38, 39), our findings support the hypothesis that synthetic antioxidants are not themselves inducers, but those that are metabolized to thiol-active quinones, quinolones, α,β-unsaturated carbonyls, or thioram disulfides can activate transcription of the ARE gene battery.

Modulation of reporter gene activity in AREc32 cells by redox status. The contribution that ROS may make to Nrf2-mediated gene transcription is difficult to determine because they can generate α,β-unsaturated carbonyls, such as acrolein and 4-hydroxynonenal, through modification of intracellular macromolecules that are potent inducing agents (14). In the present study, we found H_2O_2, which is included among ROS, to be a relatively poor inducer of luciferase reporter activity and *AKR1C* (Tables 3 and 4), but it is not known if this modest level of induction is a direct or indirect effect of the oxidant. To address the question of whether ROS contribute to homeostatic ARE-driven transcription, we treated AREc32 cells with the thiol antioxidants NAC and GSH and with the free radical scavengers vitamin C and vitamin E. None of these compounds influenced basal luciferase reporter activity in AREc32 cells. We also treated the reporter cells with BSO to deplete GSH and thereby increase the intracellular level of ROS, and this was also found to have no effect on basal luciferase activity (Fig. 5*C*). Whereas these experiments do not exclude ROS as agonists of ARE-driven gene expression, our data suggest that these agents are not potent inducers.

Although NAC and GSH did not have any effect on basal luciferase activity in AREc32 cells, they substantially attenuated induction of ARE-driven luciferase by either *t*-BHQ or SUL. These findings are consistent with the hypothesis that the intracellular GSH pool acts as a buffer to negatively regulate the ARE gene battery by preventing thiol-active inducing agents from interacting with critical cysteine residues in proteins including Keap1 (38, 40). By comparison, vitamin C and vitamin E only modestly inhibited induction of luciferase by *t*-BHQ. This small decrease in induction by *t*-BHQ is presumably because vitamins C and E inhibit its two-electron oxidation to *tert*-butylbenzoquinone, the ultimate inducing agent. Neither vitamin C nor vitamin E inhibited induction of luciferase by SUL presumably because the isothiocyanate itself is the inducer and does not require further oxidation to activate Nrf2.

Our finding that NAC can substantially repress induction of ARE-driven gene expression by *t*-BHQ is in marked contrast to the results of Lee et al. (30) who reported that NAC did not influence induction of *NQO1* in IMR-32 cells by *t*-BHQ. A probable explanation for this discrepancy is that the human *NQO1*-ARE contains an embedded AP1 site (5′-GTGACTCAGCA-3′), whereas the ARE in rat *GSTA2* and mouse *gsta1* (5′-GTGACAAAGCA-3′) does not. It is distinctly possible that transcriptional activation of the human *NQO1* gene via its ARE involves Jun family members as well as Nrf2 (2). The ARE in the promoter of the human *ferritin H* gene contains an embedded AP1 site and can recruit JunD (41). Significantly, c-Jun and c-Fos can be induced by *t*-BHQ (42), suggesting that temporal differences may occur in the complexes recruited to the human *NQO1*-ARE. Our interpretation of these apparently contradictory findings is that antioxidants can inhibit thiol-active inducers from activating Nrf2, but they do not have a similar effect on c-Jun and c-Fos. This hypothesis requires to be tested.

Induction of reporter gene activity in AREc32 cells by mutagens and anticancer drugs. Monofunctional inducers that activate ARE-driven transcription are all soft electrophiles as they react with soft nucleophiles, such as SH groups, rather than reacting with N or O atoms in macromolecules (43). We found that the strong mutagen MMS, that can be classed as a hard electrophile because it is capable of *N*-methylating basic amino acids in protein as well as both *N*- and *O*-methylating nucleic acid bases in DNA (44), was able to induce ARE-luciferase activity in AREc32 cells. Interestingly, MMS can also methylate thiol compounds and deplete cellular GSH (45). Based on our data on MMS, it seems that both hard and soft electrophiles can activate ARE-driven transcription.

During the present study, AREc32 cells were used to examine whether chemotherapeutic drugs that produce electrophilic metabolites, such as alkylating agents, can induce ARE-driven gene expression. We found that melphalan, chlorambucil, and

Table 5. Effect of the treating AREc32 cells with anticancer drugs and their metabolites

Type of modulation	Drugs and metabolites	Class of drug	Fold increase*	Concentration of xenobiotic
Inactive	Doxorubicin	Cytotoxic antibiotic	1.0 ± 0.04	1.0 μg/mL
	Epirubicin	Cytotoxic antibiotic	1.1 ± 0.03	1.0 μg/mL
	Cyclophosphamide	Alkylating agent	1.0 ± 0.05	100 μmol/L
	Methotrexate	Antimetabolite	1.1 ± 0.06	10 μmol/L
	Paclitaxel	Taxane	1.1 ± 0.05	5 nmol/L
	Thiotepa	Alkylating agent	1.1 ± 0.1	20 μmol/L
Weak inducers	Cisplatin†	Platium compound	1.3 ± 0.06	10 μmol/L
	Melphalan†	Alkylating agent	1.3 ± 0.06	20 μmol/L
	Etoposide†	Topo 2 inhibitor	1.3 ± 0.07	10 μmol/L
	Chlorambucil†	Alkylating agent	1.8 ± 0.19	100 μmol/L
	Mitoxantrone†	Cytotoxic antibiotic	2.1 ± 0.08	1 μmol/L
	BCNU†	Alkylating agent	4.1 ± 0.15	100 μmol/L
Strong inducer	Acrolein		27 ± 2.5	10 μmol/L

NOTE: Treatment was for 24 hours as detailed in Materials and Methods. For control cells, the same volume of 0.1% (v/v) of vehicle was added to the medium. The significance of differences between the luciferase activity from cultures exposed to the anticancer agents and cultures treated with the DMSO was assessed by unpaired Student's *t* test. This represents the results of three separate experiments.

*Data are expressed as the mean-fold increase relative to control value ±S.D.

†$P < 0.05$.

BCNU were effective inducers of luciferase reporter gene activity. Induction of ARE-driven gene expression by these alkylating agents was redox sensitive, insofar as it was augmented by BSO pretreatment and suppressed by NAC. It therefore seems most probable that melphalan, chlorambucil, and BCNU induce the ARE gene battery through modifying cysteine residues in Keap1. Further studies are required to test this prediction. We also found that thiotepa and cyclophosphamide, which are alkylating agents, did not induce luciferase reporter gene activity. In both cases, the agents are prodrugs and require to be metabolized to be effective. It therefore seems probable that the cytochrome P450s required for their activation are not expressed in our stable reporter cell line.

In addition, to induction of ARE-driven gene expression by certain alkylating agents, we found that mitoxantrone (a cytotoxic antibiotic), etoposide (a topoisomerase 2 inhibitor), and cisplatin (a platin compound that cross-links DNA strands) were all able to induce luciferase reporter gene activity. The metabolic processes by which these agents act as inducers of ARE genes, and how they impinge on Keap1, is not known. It should, however, be noted that induction by cisplatin was increased by pretreatment of AREc32 cells with BSO, an observation that is consistent with the notion that thiol chemistry underpins the process.

As mentioned above, the role of ROS in inducing ARE genes is poorly understood. In the course of this study, we found that the cytotoxic antibiotic doxorubicin did not induce luciferase activity in AREc32 cells. The fact that doxorubicin is thought to act as a redox-cycling agent and can therefore produce free radicals suggests that ROS may not be particularly potent at inducing ARE-driven genes.

Contribution of Nrf2 to drug resistance. The older literature focused on the contributions of the P-glycoprotein (MDR1) and GST to acquired resistance of tumor cells to chemotherapeutic drugs (1). There is, however, increasing evidence that the redox status of cells is an important factor in determining whether tumor cells can withstand chemotherapy (12). The fact that Nrf2 controls the expression of GCLC and GCLM, which together catalyze the rate-limiting step in GSH biosynthesis, may strongly influence cell survival through inhibition of apoptosis. In particular, ROS have been found to inhibit the growth of malignant cells and/or to induce apoptosis (46, 47). Increases in GSH levels would be expected to reduce ROS levels and antagonize apoptotic signals. Studies in a variety of tumor cell types have suggested that cancer chemotherapy drugs induce apoptosis in part by generating endogenous oxidants (48). Previous work has shown that an association exists between a coordinated increase in the expression of antioxidant proteins and resistance to anticancer drugs. For example, high levels of GCLC and MRP have reported to be associated with increased resistance towards doxorubicin in mesothelioma cells (49), and an increase in GCLC has been associated with resistance to cisplatin in ovarian cells (50). Recently, it has been reported that acquired resistance of KCL22 human chronic myelogenous leukemia cells to imatinib was linked to increases in both GSH levels and the amounts of nuclear Nrf2 (51). Treatment with vitamin C diminished the amount of nuclear Nrf2 and restored sensitivity to imatinib (51). The molecular mechanism responsible for this finding is unknown, but it infers that Nrf2 and GSH can play a pivotal role in drug resistance.

Concluding comments. We have developed a stable human mammary reporter cell line to allow inducers of ARE-driven genes to be identified. These cells have been used to show that alkylating agents and some other anticancer drugs can induce the ARE gene battery. Furthermore, in some instances, this induction can be dramatically enhanced by depletion of intracellular GSH. An important inference from these observations is that suboptimal treatment of cancer by chemotherapeutic drugs may induce adaptive cytoprotective genes in tumors. It is also clear that the redox status of cells in the tumor will influence their ability to activate such defenses. Thus, suboptimal treatment may cause depletion of GSH that could in turn allow increased up-regulation of cytoprotective genes. Other types of therapeutic agent, such as the analgesic acetaminophen, can deplete GSH, and these may similarly allow augmented induction of cytoprotective genes in tumor cells by anticancer drugs.

Acknowledgments

Received 6/22/2006; revised 8/17/2006; accepted 9/12/2006.

Grant support: Imperial Cancer Research Fund Incubator Grant (C.R. Wolf and J.D. Hayes).

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We thank Prof. Masayuki Yamamoto and Dr. Ken Itoh (Institute of Basic Medical Sciences, University of Tsukuba, Japan) for providing the pHyg-EF-hNrf2 construct and Lesley McLaughlin for helpful comments about the characteristics of the chemotherapeutic drugs studied.

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