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GRANT NUMBER DAMD17-98-1-8286

TITLE: Glutathione Transferases and the Multidrug Resistance -
Associated Protein in Prevention of Potentially Carcinogenic
Oxidant Stress in Breast Cancer

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REPORT DATE: June 1999

TYPE OF REPORT: Annual Summary

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. AGENCY USE ONLY <i>(Leave blank)</i>	2. REPORT DATE June 1999	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jun 98 - 31 May 99)		
4. TITLE AND SUBTITLE Glutathione Transferases and the Multidrug Resistance - Associated Protein in Prevention of Potentially Carcinogenic Oxidant Stress in Breast Cancer		5. FUNDING NUMBERS DAMD17-98-1-8286		
6. AUTHOR(S) Robin L. Haynes				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Bowman Gray School of Medicine Winston-Salem, North Carolina 27157		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		12b. DISTRIBUTION CODE		
13. ABSTRACT <i>(Maximum 200 words)</i> <p style="margin-left: 40px;">The purpose of this project is to determine : a) mechanisms of toxicity or DNA damage by 4-hydroxy-nonenal (HNE), an oxidative stress-induced lipid aldehyde, and b) the protective functions of several key cellular enzymes against HNE and other potentially carcinogenic redox-active electrophiles. The focus is on the application of transgenic cell lines that express specific protective genes. The findings to date are : 1) the most efficient GST isozyme tested does not confer resistance to HNE; 2) GST and MRP do not cooperate to provide resistance to HNE or to tBuOOH; 3) the toxicity of HNE is mediated primarily via apoptosis; 4) the aldehyde function is required for HNE toxicity; 5) toxicity and apoptosis, and covalent modification of proteins by HNE can be completely blocked by transfected human aldehyde dehydrogenase-3; 6) the C2=C3 double bond also makes an important contribution to HNE reactivity, but is less critical than the -CHO; 7) increasing chain length enhances toxicity of HNE (least impact); 8) expression of the antioxidant gene Bcl-2 also protects against apoptosis by HNE.</p>				
14. SUBJECT TERMS Breast Cancer oxidative stress; lipid peroxidation; 4-hydroxynonenal; apoptosis; aldehyde dehydrogenase; glutathione; oxidative damage resistance; chemoprevention; aldehyde dehydrogenase; glutathione S-transferase; multidrug resistance-associated protein.		15. NUMBER OF PAGES 53		
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

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Introduction

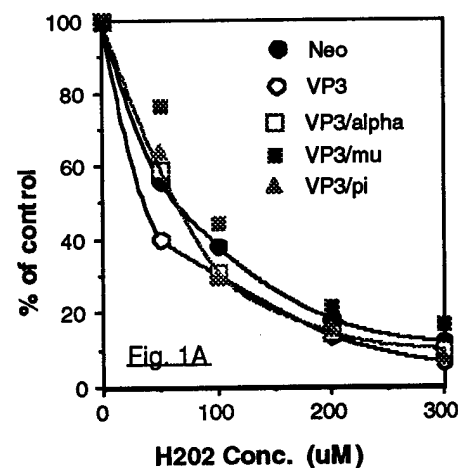
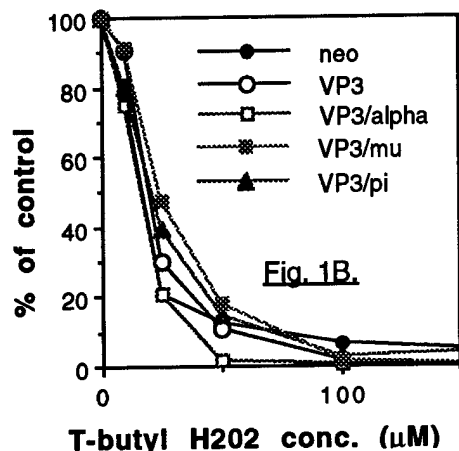
The increasing incidence of breast cancer indicates a clear need for research aimed at decreasing the susceptibility to factors which initiate the promotion and progression of breast neoplasia. Oxidative stress, a subject of recent study, is generated thru the accumulation of excess reactive oxidative species (ROS) which are natural byproducts of aerobic metabolism. These ROS include hydrogen peroxide, oxygen derived free radicals, and aldehyde products of lipid peroxidation. Cellular macromolecules including DNA, proteins, and lipids are potential targets for oxidative stress damage. In breast cancer, oxidative stress damage has been linked to susceptibility primarily through the study of antioxidants such as isoflavins and vitamin E that have been shown to be successful anticarcinogenic agents in breast tissue. The high susceptibility of breast tissue to lipid peroxidation due to the high fat content of breast tissue suggests that prevention of oxidative stress is important in preventing the development of breast neoplasia. Hence, the subjects of this project are : a) mechanisms of oxidative damage, and b) pathways whereby cells defend against the potentially carcinogenic effects of reactive oxygen species (ROS). The purpose of this project is to determine : a) mechanisms of toxicity or DNA damage by 4-hydroxy-nonenal (HNE), an oxidative stress-induced lipid aldehyde, and b) the protective functions of several key cellular enzymes against HNE and other potentially carcinogenic redox-active electrophiles. The approach is via the application of transgenic cell lines that express specific protective genes. Based on our recent findings, the scope of the project has been modified to emphasize one specific ROS (4-hydroxynonenal), and the aldehyde dehydrogenase (ALDH) detoxification pathway, although studies on MRP and GST are still being conducted. The progression of this project has been quite successful, despite initial difficulties, as described below.

Summary of Year 1

Initial efforts were directed toward obtaining further preliminary data to determine the approaches most likely to provide useful insights among those originally proposed. Toward this end, several cell lines were tested for sensitivity to specific ROS or prooxidant compounds. The experiments with the MCF-7 lines expressing MRP1 only (VP3), or MRP1 together with GST isozymes will be described first. All data are representative examples of two or more experiments.

1. MRP1 and GST

Cells expressing MRP1 alone (MCF-7/VP3), or MRP1 + GST alpha (VP3/mu), or + GST mu (VP3/pi) (provided by Dr. C.S. Morrow, a collaborator and a member of the dissertation committee) were tested for sensitivity to several ROS or prooxidant agents in comparison with the parent cell line (MCF-7/neo).

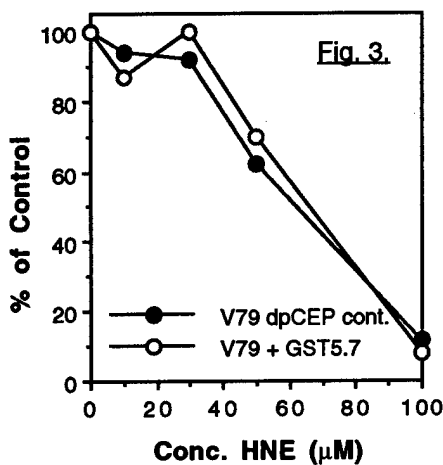


There was no real difference in sensitivity to H₂O₂ (Figure 1A) or tert-butyl hydroperoxide (Figure 1B). A slight sensitization to H₂O₂ was often seen in the MRP1 +

GST alpha line. Similarly negative results were obtained with the free radical generating agents paraquat or tert-butyl hydroquinone (*not shown*).

Since the most important lipid aldehyde generated by lipid peroxidation (HNE) is metabolized significantly to a GSH conjugate in mammalian tissues, I next examined the toxicity of HNE in these MCF-7 cells expressing MRP1 + GSTs. Again, there was no protection against HNE cytotoxicity (Figure 2). While the data in Figure 2 suggested sensitization in the MRP1 expressing lines, repeat experiments showed no differences.

To further explore the effects of HNE conjugation, I transfected the mutagenicity tester cell line V79 with



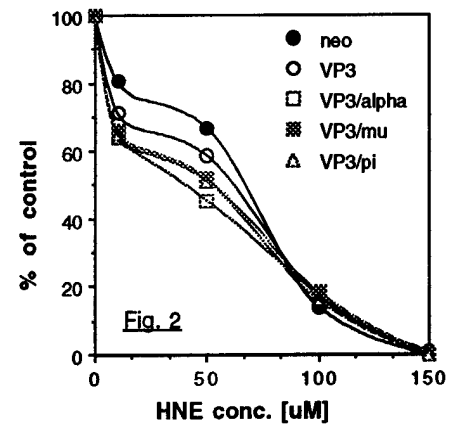
mGSTA4-4 (AKA mGST5.7), one of the most efficient GST isozymes for the conjugation of HNE with GSH. The cells expressed high levels of mGSTA4-4, as shown by the rapid complete conjugation of 0.1 mM HNE in a 1 ml solution by 10 µL of added cell lysate, as compared to very little conjugation by control lysate even after 10 minutes (data not shown). The activity for CDNB (a poor substrate for this isozyme) was a robust 500 – 700 mU/mg higher than in control (empty vector) transfected cells (100 mU/mg). Still, the cells were not protected against HNE cytotoxicity, even at this high level of conjugation activity (Fig. 3). The HNE was likely conjugated, as shown by a faster rate of depletion of GSH in the mGSTA4-4 expressing cells.

Further studies with this cell line indicated no protection against several other agents known to produce ROS, including tert-butyl hydroperoxide, the redox-cycling anticancer drug doxorubicin, and the diuretic drug ethacrynic acid (data not shown).

The above studies were undertaken based on our previous observations of strong protection by GSTs alone against DNA damage (Carcinog. 15:1155, 1994) and synergy observed in collaboration with Dr. C.S. Morrow (Carcinog. 19: 109-115, 1997) between MRP1 and GST for protection against 4-nitroquinoline-1-oxide, a carcinogen that exerts toxicity by direct alkylation as well as via oxidative stress. The negative results described above have led us to conclude that conjugation with GSH, and subsequent efflux by MRP1 do not appear to protect cells against the toxicity of HNE either added to medium or generated via exposure to other ROS. The reason may relate to the fact that GSH conjugation occurs at the C3 position, which leaves the aldehyde function free to tautomerized between the thiohemiacetal and open chain aldehyde forms. Thus, conjugation with GSH could cause accumulation of these intermediates which retain toxicity, a scenario analogous to the results with NQO, except that MRP1 does not enable synergistic GST protection against HNE, even though the conjugate has been reported to be a substrate for MRP1 efflux. These results, together with difficulties in expression of MRP1 by transfection have led me to substitute a new component to the project, as described below. We do plan, however, to test the ability of a second human MRP homolog, MRP2, to enable protection against HNE by GST-catalyzed conjugation with GSH.

2. Mechanisms of HNE Toxicity and Cellular Resistance to HNE

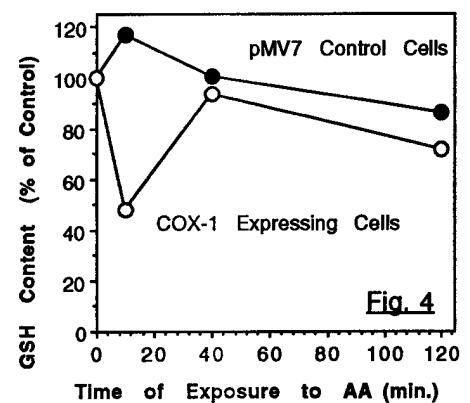
In order to better understand how HNE may exert toxicity, we first examined the contributions of each of the structural components of HNE to overall toxicity using analogous



compounds with each of the components altered to eliminate its respective contribution. These studies were done with the RAW 264.7 macrophage cell line because the MCF-7 cell line does not exhibit DNA fragmentation, the most readily accessible marker of apoptosis; and because it has a normal p53 tumor suppressor gene that is responsive to DNA damage. The attached manuscript describes data that demonstrate that: 1) HNE induces toxicity via induction of apoptosis (Fig. 2); 2) the aldehyde function is required for HNE toxicity (Figs. 1, 3); 2) toxicity and apoptosis, and covalent modification of proteins by HNE can be completely blocked by transfected human aldehyde dehydrogenase-3 (Figs. 5, 6A/B); 3) the C2=C3 double bond also makes an important contribution to HNE reactivity, but is less critical than the -CHO (Figs. 1, 3); and 4) increasing chain length enhances toxicity of HNE (least impact) (Figs. 1, 4). Protection by ALDH3 against cytotoxicity of HNE has also been demonstrated in V79 cells, and corollary studies are planned in MCF-7 cells expressing a transfected ALDH-3 (JBC 269 : 23197, 1994).

3. Cyclooxygenase as a Model for Intracellular Lipid Peroxidation

The CHO cell lines expressing human cyclooxygenases (COX-1 or COX-2) were originally envisioned as oxidative stress models for intracellular generation of HNE, MDA, and other products of endogenous lipid peroxidation. This was suggested by the observations that: 1) the COX-expressing cells are hypersensitive to the GSH depleting agent BSO, and 2) when the COX-1 expressing cells are pretreated with BSO, they are hypersensitive to apoptosis induction by arachidonic acid (AA). I have determined that this is not likely due to oxidative stress *per se*, since GSH is only depleted 40-50% and only for a short time after addition of toxic amounts of AA (Fig. 4). Furthermore, expression of COX-1 renders cells more sensitive to alkylating agents even in the absence of added AA or changes in GSH status, suggesting that signal transduction may be the operant mechanism. This aspect is being pursued by others in a project focused on AA as a drug response modifier.



4. Additional Ongoing Studies on Cellular Effects of HNE and Resistance to HNE Toxicity

A. Induction of apoptosis by HNE may proceed via various signalling pathways, triggered by DNA damage (p53-mediated) or several other pathways that operate via mitochondrial effects or other pathways leading to activation of caspase-3. I have examined p53 levels in RAW 264.7 cells after exposure to 40 μ M HNE for one hour. A western blot is shown above in Figure 5, which indicates that exposure to HNE induces p53 protein accumulation to a similar extent as cis-platinum (CDDP), a known DNA damaging p53 inducer.

Hrs. 0 0.5 1 2 3 6 9 CDDP



Fig. 5. Time course of induction of p53 protein after exposure to HNE.

B. The Bcl-2 gene was originally characterized as an oncogene, but more recently has been shown to function as an anti-apoptotic and antioxidant protein, likely mediated via effects on the integrity of mitochondria. As shown in Figure 6 on the following page, expression of transfected Bcl2 in RAW 264.7 cells (provided by Dr. B. Brüne, Erlangen, Germany) resulted in complete protection against HNE-induced apoptosis. This cell line will help us to trace the events that are triggered by HNE exposure, and the role of p53 induction in relation to potential DNA damage or other signal transduction mechanisms. I am currently conducting experiments to determine how p53 levels are affected by HNE in this line. I will also determine the overall level of oxidant stress in these cells by DCF fluorescence.

Protection Against Apoptosis by BCL-2 Overexpression

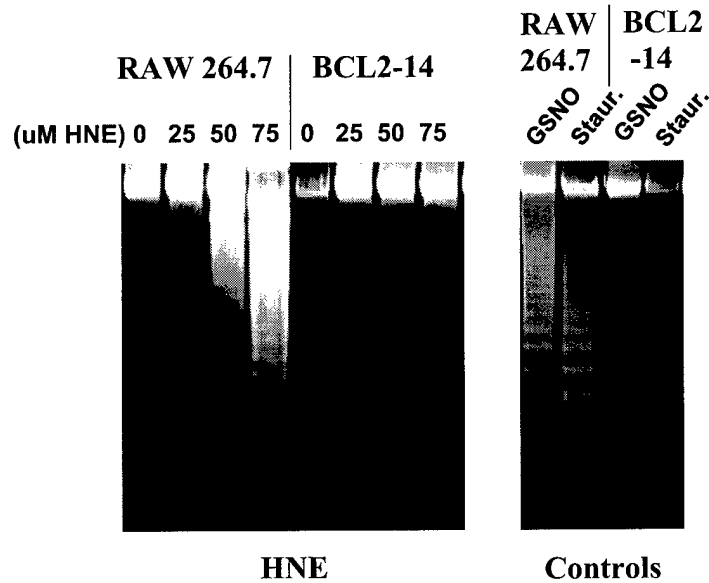


Figure 5. Prevention of HNE-Induced Apoptosis by Bcl-2 Expression. The Bcl-2 expressing cells were protected against apoptosis as indicated by DNA fragmentation in cells exposed to HNE, or in the positive control inducers nitroglutathione (an NO releasing agent) or staurosporin.

Key Research Accomplishments

The key findings to date are :

- 1) The most efficient GST isozyme tested does not confer resistance to HNE.
- 2) GST and MRP do not cooperate to provide resistance to HNE or to tBuOOH.
- 3) The aldehyde function is required for HNE toxicity, which can be blocked by transfected human aldehyde dehydrogenase-3.
- 4) The C2=C3 double bond also makes an important contribution to HNE reactivity, but is less critical than the -CHO.
- 5) Increasing chain length enhances toxicity of lipid aldehydes (least impact);
- 6) Several GSTs confer resistance to DNA damage but not cytotoxicity of 4-NQO.
- 7) MRP and hGSTP1 do cooperate to confer high-level resistance to 4-NQO toxicity.
- 8) Expression of the antioxidant gene Bcl-2 also protects against apoptosis by HNE.

Reportable Outcomes

Abstracts : (copies attached)

1. Townsend, A.J., Leone-Kabler, S., Doss, A.J., and Haynes, R.L. Effect of cyclooxygenase (COX) expression on cellular sensitivity to GSH depletion, apoptosis, and cytotoxic drug sensitivity in transfected Chinese hamster ovary (CHO/AS52) cells. *Proc. Amer. Assoc. Cancer Res.* **40**: # 4453, 1999.
2. Haynes, R. L. , Willingham, M., Szweda, L., and Townsend, A. J. Structure-Activity and Mechanistic Studies on Induction of Apoptosis by 4-Hydroxy-2-nonenal in RAW 264.7 Cells. *Proc. Keystone Symp. On Apoptosis, Keystone, CO , April 1999.*

Manuscripts : (copies attached)

1. Townsend, A. J., Fields, Wanda R., Haynes, R. L., Karper , A. J., Li, Ying, Doehmer, J., and Morrow, C. S. Chemoprotective functions of glutathione s-transferases in cell lines induced to express specific isozymes by stable transfection. *Chem.-Biol. Interact.* **111**: 389-407, 1998.
2. Haynes, R.L., Szweda, L., and Townsend, A.J. Structure-Activity Relationships for Growth Inhibition and Induction of Apoptosis by 4-Hydroxy 2-nonenal in RAW 264.7 Murine Macrophage Cells. *Manuscript in preparation (copy attached).*

Cell Lines, Expression Vectors, Etc.

1. Cell lines : a) Several V79 cell lines stably transfected with mGSTM1 expression vector.
b) One RAW 264.7 cell line stably transfected with hALDH-3 expression vector.
2. Vectors : The two mammalian plasmid expression vectors used to create these cell lines.

**Structure-Activity Relationships for Growth Inhibition and
Induction of Apoptosis by 4-Hydroxy 2-nonenal in Raw 264.7
Cells.**

Robin Haynes, Luke Szweda, and Alan Townsend

Abstract

4-Hydroxy-2-nonenal (HNE) is a highly reactive lipid aldehyde byproduct of the peroxidation of cellular membranes. The deleterious effects of HNE exposure are numerous ranging from protein alkylation to thiol depletion and most recently apoptosis induction. A unique feature of HNE is the presence of three functional groups which react in concert to yield the full toxicity of the compound. These functional groups include a C1 aldehyde, a C2=C3 double bond, and a C4- hydroxyl group. The contribution of each of these to the total reactivity of HNE was examined using a global toxicity endpoint growth inhibition/ cell survival. In addition, we determined that the specific mode of cell death was primarily via apoptosis, and examined the importance of the three functional groups to the apoptotic potential of HNE. These experiments were done with a mouse alveolar macrophage RAW 264.7 cell line, using analogous compounds which lacked one or more of the structural moieties. We also examined the importance of the lipophilicity of HNE with analogous aldehydes each with a different lipid chain length. Both the growth inhibition and the apoptotic potential assays yielded parallel results: the rank order of increasing contribution to toxicity was hydroxyl < C2=C3 double bond < C1 aldehyde. Chain length was also found to play an important role in a series of α,β -unsaturated alkenyl aldehydes, with increasing chain length yielding increasing toxicity.

no indent

To further our studies and verify the importance of the aldehyde moiety, a RAW 264.7 cell line overexpressing the human aldehyde metabolizing enzyme aldehyde dehydrogenase-3 (hALDH3) was produced. This cell line showed protection against HNE-induced apoptosis as well as a dramatic reduction in HNE-protein adduct formation. This result confirms the importance of the aldehyde moiety in the mechanism of HNE-induced apoptosis.

The condition of oxidative stress involves a variety of cellular changes and reactions that lead to the accumulation of oxidatively damaged macromolecules and ultimately to the demise of the cell. The polyunsaturated fatty acids comprising biological membranes are susceptible to oxidative damage. When fatty acids such as arachidonic acid interact with free radical species in the presence of molecular oxygen, a self propagating lipid peroxidation reaction occurs and results in the formation of byproducts such as hydroperoxides and reactive aldehydes. Of particular interest to our lab is the α,β -unsaturated aldehyde 4-hydroxyl -2 nonenal (HNE). It is the most reactive and cytotoxic of the aldehyde byproducts of lipid peroxidation and has been shown to induce a variety of cellular damage including protein adduct formation, protein thiol modification, glutathione depletion, DNA and RNA synthesis inhibition, inhibition of mitochondrial respiration, and calcium homeostasis disturbances. HNE-induced protein damage has been associated with several injury and disease conditions such as ischemia-reperfusion injury, atherosclerosis, alcoholic liver disease, alzheimer's disease, and general cellular aging.

Recently HNE has been shown to induce apoptosis in certain cell lines suggesting a possible connection between oxidant stress generated lipid peroxidation byproducts and oxidant stress induced apoptosis. This observation presents questions regarding the role that HNE plays in oxidant induced apoptosis, the specific mechanism of HNE initiation of apoptosis, and contributions of HNE structural features to the potency for initiation of apoptosis. It is the correlation of HNE structural components to the toxicity of HNE and more specifically to the onset of apoptosis that is the primary focus of this paper.

Unique structural features of HNE include the presence of two structural domains, a lipophilic tail and a polar head comprised of multiple reactive functional groups. The polar head contains an aldehyde at the C-1 position, a C2=C3 double bond, and a hydroxyl group at the C-4 position. These groups may participate independently or cooperatively to interact with cellular molecules. One way to evaluate the importance of each moiety in the toxicity of HNE is to compare the effects of HNE to analogous compounds which either

vary in fatty acid chain length or lack a specific functional group. For example, compounds such as trans-2 hexenal and trans-2 octenal vary in fatty acid chain length while compounds such as nonanal and nonenoic acid lack the C2=C3 double bond and the C-1 aldehyde respectively. By removing the effect of a certain functional group of HNE it is possible to determine the contribution of that group to the toxicity of HNE.

In addition to using compounds analogous to HNE it is also possible to enzymatically modify specific functional groups and determine their contribution to toxicity by observing changes in overall HNE toxicity. The biological mechanism of HNE detoxification primarily involves four phase II detoxification enzymes: aldehyde dehydrogenase (ALDH), glutathione S-transferase (GST), alcohol dehydrogenase (ADH), and aldose reductase. Here we present data using human class 3 ALDH as a primary means of aldehyde removal. The result confirms that the aldehyde moiety is the most potent component contributing to HNE toxicity.

Materials and Methods

Cell Culture Conditions and Reagents -- Mouse alveolar macrophage Raw 264.7 cells were grown at 37°C in a 5% CO₂ atmosphere in DMEM medium (GIBCO) supplemented with 10% fetal bovine serum. 4-Hydroxynonenal was kindly provided by the lab of Dr. Herman Esterbauer (University of Graz, Austria). Analogous aldehydes trans-2 hexenal, trans-2 octenal, trans-2 nonenal, and nonenal were purchased from Aldrich (Milwaukee, WI) while nonenoic acid was purchased from TCI (Portland, OR).

Growth Inhibition/ Cell Survival -- 1.2×10^6 cells were treated in suspension in 5ml PBS plus chemical agent for 30 minutes at 37°C. Cells were pelleted by centrifugation (1000 rpm for 5 minutes) and resuspended in DMEM complete media. 6×10^5 cells were plated in 6-well dishes and allowed to grow for 2 days at which time cells were trypsinized and counted.

DNA Fragmentation Assay -- Cells were plated at 2×10^6 cells per 60mm petri dish. 16- 20 hours later cells were rinsed in serum-free media and exposed for 1 hour in serum-free, chemical-containing media. After 1 hour, media was removed and replaced with DMEM complete media. Cells were allowed to incubate for an additional 9 hours at which time they were harvested in phosphate buffered saline (PBS) (pH 7.4), and centrifuged at 4°C, 1000 RPM for 5 minutes. Cells were then lysed in 20mM EDTA, 100mM Tris (pH 8.0), 0.8% sodium lauryl sarcosine and subjected to RNase treatment (.5mg/ml) for 1 hour at 37°C followed by proteinase K treatment (5mg/ml) for 6-12 hours at 55°C. Nonfragmented chromosomal DNA was removed by filtering the lysate through 45µm syringe filters pretreated with .2 mg/ml bovine serum albumin (BSA). Fragmented DNA was then precipitated with 1/10 volume 3M sodium acetate (pH 5.2) and 2.5 volumes 100% ethanol. DNA was run on a 1.8% agarose gel.

Transfection of hALDH3 -- The cDNA for human class 3 ALDH was previously amplified by polymerase chain reaction from human stomach cDNA and subcloned into the XhoI site of the ΔpCEP4Δ mammalian expression vector, a derivative of

the pCEP4 vector (Invitrogen) that was modified to prevent episomal replication and favor host integration (XXX). Both the Δ pCEP4 Δ /hALDH3 vector and Δ pCEP4 Δ (empty vector) were introduced into RAW 264.7 cells using the cationic liposome reagent *Escort* (Sigma). Briefly, cells were plated in 100mm petri dishes and grown to 70-80% confluency. 30-50 μ L of *Escort* was incubated with 15-25 μ g of DNA in 800 μ L of transfection media Opti-MEM (GIBCO) for 15 minutes. Opti-MEM was added to *Escort*/DNA mixture to total 8mL. Cells were then allowed to incubate in transfection media for 6 hours. 24 hours later cells were subcultured and selection media (DMEM(10% FBS) with .7 mg/ml hygromycin) was added. Once hygromycin resistant colonies grew, they were cloned and expanded for ALDH screening.

Analysis of ALDH Expression --Enzyme activity assays were done according to XXXX using crude cytosol with benzaldehyde(1mM) as a substrate and NADP+ (1mM) as a cofactor.

For protein detection, 50 μ g total protein was electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose. The nitrocellulose was probed with a rabbit anti-rat class 3 ALDH antisera (kindly provided by Dr. Ronald Lindahl, University of South Dakota) that was cross-reactive with human ALDH-3 (1:3000 dilution). After probing with secondary antibody, goat anti-rabbit (HRP conjugated (BioRad)), protein was detected with chemiluminescence.

HNE Protein Adduct Detection --Cells were plated at 2.5×10^6 cells / 60mm dish. 16-20 hours later cells were dosed for 1 hour in serum -free media. 10% FBS was added at 1 hour and cells were allowed to incubate for an additional hour. Cells were harvested in PBS, centrifuged, and the pellets lysed in 50mM Tris, 5mM EDTA, 1mM PMSF. Lysates were centrifuged at 14,000xg for 10 minutes at 4°C and protein (50 μ g/lane) was run on a 10% SDS-PAGE gel and transferred by semi-dry electrophoresis to nitrocellulose. Adducts were detected using an anti HNE-protein adduct antibody (XXXXXX) at a dilution of 1:2500. After probing with goat anti rabbit, HRP conjugated

secondary antibody (BioRAD, Hercules, CA.) (1:3000) the protein was detected using *Renaissance* chemiluminescence reagent (NEN Life Science Products, Boston, MA).

Results

Comparison of the effects of HNE and analogous compounds on growth inhibition and cell survival. To compare the contributions that each of the functional groups have on the overall toxicity of HNE, growth inhibition and cell survival assays were done using different congeners, each analogous to HNE but lacking a functional group. Growth inhibition data for HNE, trans-2 nonenal (lacks the OH), nonenal (lacks the C2=C3 double bond), and nonenoic acid (lacks the aldehyde) yields IC_{50} 's of 9 +/- 1.1 μ M, 24 +/- 4.3 μ M, 308 +/- 34.9 μ M, 1770 +/- 342 μ M respectively (Figure 1A). The closeness of the IC_{50} 's of HNE and trans-2 nonenal suggest that the hydroxyl group contributes a relatively minor fraction of the toxicity of HNE. For this reason, and reasons of availability, all other lipid compounds used in the study (nonanal, nonenoic acid, trans-2 octenal, trans-2 hexenal) lack the hydroxyl group, presumably with little effect on the results.

To examine the effect of the lipophilicity on growth inhibition, cells were treated with analogous aldehydes of different chain lengths (Figure 1B). These experiments show increased toxicity with increased chain length as shown by IC_{50} values of 99 +/- 20 μ M, 30.2 +/- 5 μ M, 24.3 +/- 4.35 μ M for trans-2 hexenal, trans-2 octenal, and trans-2 nonenal respectively.

HNE-induced apoptosis in RAW 264.7 cells. In order to determine the dose-responsiveness of apoptosis induction we exposed the cells to increasing concentrations of HNE and used internucleosomal DNA fragmentation as an index of apoptosis. This HNE dose response (Figure 2) shows DNA laddering at HNE concentrations in media as low as 30 μ M. As the HNE concentration increases, the amount of laddering increases indicating an increase in the population of cells undergoing apoptosis. This response is relatively rapid, within 10 hours after a 1 hour exposure to HNE.

A second apoptotic endpoint, caspase 3 activation, was also examined to show dose responsiveness to HNE exposure. Using caspase substrates which become fluorescent

upon cleavage we showed caspase activities of .31 nmole/min/mg, .64 nmole/min/mg, and .79 nmole/min/mg with exposure to 0 μ M, 40 μ M, and 70 μ M HNE respectively (data not shown). This experiment not only confirms the dose dependent nature of the response but also yields a second positive apoptotic endpoint indicating that cells are dying by apoptotic death rather than necrotic death. In addition, using time lapse video microscopy we have shown that at 50 μ M HNE greater than 90% of the cells have formed apoptotic blebs, blistered, and lysed by 18 hours (R.Haynes and M.Willingham, unpublished observations). Again this confirms apoptosis as the primary mode of cell death.

Importance of reactive functional groups on the induction of apoptosis.

While the growth inhibition studies give a global view of the relative toxicity of each of the compounds and the importance of each of the functional groups, we wanted to examine whether this relationship holds true for the more specific form of cell death, apoptosis. As seen in figure 2, HNE induces detectable DNA laddering indicative of apoptosis at concentrations as low as 30 μ M. To determine the extent to which the various functional groups influence this apoptotic induction, a dose response using the compounds in Figure 1A was done with DNA laddering as an index of apoptosis (Figure 3). As the HNE exposure increased from 25 μ M to 75 μ M there is a clear increase in apoptosis induction. Trans-2 nonenal yields a dose response similar to HNE, confirming the similarity of trans-2 nonenal to HNE, with only a slight loss of toxicity in the absence of the hydroxyl. Both nonenoic acid and nonanal show no apoptotic laddering within the concentration range tested (25 - 75 μ M). However, as shown in Fig. 1A, these concentrations may not be toxic enough to induce significant amounts of apoptosis. Indeed, cells treated with nonanal or nonenoic acid concentrations in the IC₅₀ to IC₉₀ range have shown significant amounts of laddering (data not shown), verifying an apoptotic form of cell death with these compounds as well.

Effect of fatty acid chain length on apoptosis induction. To examine the role of chain length in HNE-induced apoptosis, a dose response similar to Figure 3 was done

using analogous aldehydes with varying chain lengths and apoptotic laddering as an endpoint (Figure 4). Trans-2 hexenal (6 carbons), in the concentration range tested, yields very little apoptosis induction. Increasing the length of the chain by 2 carbons (trans-2 octenal) results in a significant increase in laddering. The addition of a ninth carbon (trans-2 nonenal) further enhances the apoptotic induction. This data parallels the growth inhibition data seen in figure 2B; apoptotic potential increases with increasing chain length, in the order trans-2 hexenal < trans-2 octenal < trans-2 nonenal.

From the growth inhibition it is evident that the aldehyde plays a key role in the toxicity of HNE. Cells have evolved ways to enzymatically detoxify this aldehyde and to metabolize it to nontoxic byproducts. To determine whether the enzymatic removal of this aldehyde would protect the cell from the toxicity of HNE and render it resistant to apoptosis induction, the human enzyme class 3 aldehyde dehydrogenase (ALDH) was overexpressed by stable transfection in RAW 264.7 cells. Figure 5 shows the overexpressed protein in the clone hALDH3-109 compared to the empty vector control Δ pCEP4 Δ -16. Activity assays yielded an ALDH activity of 100 +/- 4 U/mg in clone 109. Control activities were nondetectable.

ALDH protection against HNE induced apoptosis and protein adduct formation. When controls cell and ALDH transfected cells were exposed to HNE, ALDH overexpression rendered protection against apoptosis induction throughout the concentration range tested (Figure 6A). To further characterize the protection given by ALDH, a more specific endpoint, HNE - protein adduct formation was examined. Aldehydes are known to be extremely reactive to proteins, in particular the thiol groups of cysteine residues. It is possible to quantitate this HNE- protein interaction with an antibody specific to HNE-modified amino acid residues (XXXX). Figure 6B shows the dose dependent nature of HNE- protein adduct formation in the empty vector Δ pCEP4 Δ . Cells

overexpressing hALDH3 show essentially complete protection as seen by extremely low levels of adduct formation throughout the concentration range tested.

Discussion

The mechanisms involved in the biological interactions of lipid peroxidation products have been studied in detail and reactions with macromolecules such as protein and DNA are well documented. α,β -unsaturated aldehydes such as HNE are known to chemically interact with macromolecules, in particular proteins, in a variety of ways depending on the amino acid residue and its surrounding environmental conditions (pH, proximal amino acids). The nature of the protein-HNE interaction is complex due to the multiple reactive groups comprising the polar head of HNE. Structure-activity correlates with compounds analogous to HNE allow assessment of the contribution of individual functional groups to the overall toxicity of HNE, and in this case the more specific endpoint, apoptosis induction.

We have shown from the growth inhibition data that the aldehyde is the most essential functional group that determines the toxicity of HNE. This is seen in the dramatic loss of toxicity when cells are exposed to nonenoic acid which lacks the aldehyde yet retains the double bond (IC_{50} of 1.77mM compared to IC_{50} of 9 μ M for HNE). It is well documented that a common HNE induced cellular change in some cells is depletion of both protein thiols and low molecular weight thiols such as glutathione(GSH). In the HNE-thiol reaction, there is an initial depolarization of an electron from the aldehyde group which facilitates the addition of the thiol group across the C2=C3 double bond. Removal of C1-aldehyde prevents this interaction and therefore greatly reduces the reactivity toward thiols. In other cell lines HNE has been shown to rapidly deplete cellular glutathione (GSH). In the RAW 264.7 cell line used here we have shown that HNE exposure does not significantly reduce GSH levels (data not shown), perhaps due to the low activity levels of GSH conjugating enzyme, GST.

A second compound, nonanal, shows intermediate toxicity, falling between HNE and nonenoic acid (IC_{50} of 308 μ M). While nonanal retains the aldehyde it lacks the C2=C3

double bond. The loss of this double bond, similar to the loss of the aldehyde, prevents Michael addition of the thiol at the C3 position. However, saturated aldehydes have been shown to interact with proteins, primarily through a Schiff base reaction with amino groups of lysine residues. Unlike the thiol addition, this interaction is thought to be weak and readily reversible, possibly explaining the intermediate toxicity.

It is clear from the trans-2 nonenal data that both the aldehyde and C2=C3 double bond are essential for HNE's full toxic effect. As stated in the Results section, the third reactive group, the hydroxyl, does not strongly contribute to HNE's toxicity. The IC_{50} of trans-2 nonenal is 24 μ M compared to IC_{50} of 9 μ M for HNE. In the mechanism of the HNE - thiol reaction, believed to be the predominant reaction, the hydroxyl group contributes only to a final cyclization of the product yielding a cyclic hemiacetal. For trans-2 nonenal, the noncyclized product of the addition of the thiol to the C3 position is the final product. The thiol adduct of trans-2 nonenal may be less stable and more readily reversible than the cyclized product therefore explaining the slight loss of toxicity when comparing trans-2 nonenal to HNE.

The growth inhibition data demonstrates not only the importance of the functional groups, it also shows the importance of the length of the fatty acid chain. It is clear from the decreasing IC_{50} values that increasing the fatty acid chain from 6 (trans-2 hexenal), to 8 (trans-2 octenal), to 9 (trans-2 nonenal) increases the toxicity of the compound. When lipid compounds are used in a biological system, the question of solubility often becomes an issue. Hydrophobicity constants are known for each of the lipid aldehydes used in this study; trans-2 hexenal-.85, trans-2 octenal-1.89, trans-2 nonenal-2.30, and HNE-1.01. Although the hydrophobic nature of the compounds increase with increasing chain length, it is clear from the increasing toxicity associated with increased chain length that the lipophilicity of the compound plays an important role in the toxicity. HNE appears to be a possible exception to this. The low hydrophobicity constant is due to the presence of the

hydroxyl which makes it more readily soluble after diffusing through the membrane and thus more available for cytosolic interactions.

While the growth inhibition gave important information on the overall toxicity of each of the compounds, we wanted to determine the particular mode of cell death and see if the results of the two studies parallel each other. The ability of each of the compounds to induce programmed cell death or apoptosis was studied. Here it is appropriate to address the issue of biologically relevant concentrations. While normal HNE concentrations in a cell are relatively low it is thought that localized HNE concentrations can increase to as high as 4.5mM within the space of peroxidizing membranes. In particular, mitochondrial membranes may accumulate high HNE concentrations due to the proximity to reactive oxygen species leakage. Oxidant stress induces lipid peroxidation and formation of HNE, which we have shown to be a potent trigger for apoptosis induction in RAW cells. As with the growth inhibition, removing functional groups decreases the potency of the apoptotic induction, with entirely analogous structure-activity relationships.

Since the toxic compounds used in this study were added to the extracellular medium, we wanted to verify that the aldehyde group exerted its toxicity intracellularly, rather than at the cell surface. We generated a system in which the aldehyde metabolizing enzyme, aldehyde dehydrogenase, is overexpressed. This system allows us to enzymatically remove the aldehyde and measure the protection rendered against cytotoxicity and apoptosis, as well as protein modification. It is clear from laddering experiments that intracellular removal of the aldehyde by ALDH oxidation completely protects the cells from apoptotic induction. This confirms that the aldehyde is the essential component of HNE. A second endpoint was examined in the overexpression system, protein adduct formation. Clearly, removal of the aldehyde from the intracellular space protects the cell from protein-HNE interactions that may lead to adduct formation. This observation suggests that the protein modification may play a role and is likely a trigger mechanism for the apoptotic induction. There is current literature that emphasizes the role of certain protein thiols as

critical sensors and accentuates the modification of their thiols as important triggers for apoptotic events. The most well documented example of this is the modification of critical thiols on the mitochondrial permeability transition pore leading to the induction of the permeability transition which is thought to be an integral part of the apoptotic pathway.

Although there is much work that remains to be done to elucidate the mechanism of HNE induced apoptosis and the role that HNE has in oxidant induced apoptosis, these data indicate critical interactions that may be focused on for future studies aimed at this goal.

Figure Legends

Figure 1A. Growth inhibition/ cell survival to determine the importance of individual functional groups. Cells were dosed with analogous compounds as described in Materials and Methods and allowed to grow for 2 days at which time they were trypsinized and counted. Analogous compounds used include HNE (○); trans 2- nonenal (●) (lacks the hydroxyl); nonanal (□) (lacks the C2=C3 double bond and hydroxyl); and nonenoic acid (■) (lacks the C1 aldehyde and hydroxyl). IC₅₀s for each are 9 +/- 1.1μM, 24 +/- 4.3μM, 308 +/- 34.9μM, and 1770 +/- 342μM for HNE, trans 2- nonenal, nonanal, and nonenoic acid respectively. The number of repetitions equals 5.

p values(?)

Figure 1B. Growth inhibition/ cell survival to determine the importance of chain length.

Cell were dosed as described above with analogous aldehydes trans-2 nonenal (●) (9 carbons); trans-2 octenal (○) (8 carbons); trans-2 hexenal (□) (6 carbons). IC₅₀'s for each are 24 +/- 4.3μM, 30 +/- 5μM, and 99 +/- 20μM for trans-2 nonenal, trans-2 octenal, and trans-2 hexenal respectively. The number of repetitions equals 5.

need to fix

p values(?)

Figure 2. HNE induced DNA internucleosomal fragmentation. RAW cells were treated with increasing concentrations of HNE for 1 hour then placed in fresh medium without HNE. 10 hours after exposure, cells were harvested and DNA was isolated as described in Material and Methods.

Figure 3. DNA fragmentation induced by 9-carbon analogous compounds. Cells were exposed for 1 hour in serum-free media containing the indicated concentrations of one of the following compounds: nonenoic acid (lacks the C1 aldehyde and hydroxyl), nonanal (lacks the C2=C3 double bond and hydroxyl), trans-2 nonenal (lacks the hydroxyl), or HNE. Cells were harvested and lysed 10 hours after exposure.

Figure 4. DNA fragmentation induced by lipid aldehydes of increasing chain length. Cells were exposed for 1 hour in serum-free media containing the indicated concentrations of one of the following lipid aldehydes; trans-2 hexenal (6 carbon), trans-2 octenal (8 carbon), or trans-2 nonenal (9 carbon). Cells were harvested and lysed 10 hours after exposure.

Figure 5. Expression of human class 3 aldehyde dehydrogenase. Raw cells were transfected with a $\Delta pCEP4\Delta$ control (empty) vector or with a $\Delta pCEP4\Delta$ vector containing human ALDH3 cDNA. 50 μ g of cytosolic protein was run on a 10% SDS-PAGE gel and transferred to nitrocellulose which was probed with 1:3000 dilution of tALDH antisera. Enzyme activity assays were done as described in Materials and Methods and yielded hALDH3 activity levels nondetectable in control cells and 100mU (+/- 4) in the ALDH 3 clone. A V79 cell line overexpressing hALDH3 was used as a positive control (left).

Figure 6A. ALDH protection against HNE induced DNA fragmentation. $\Delta pCEP4\Delta$ control cells and cells overexpressing hALDH3 were dosed for 1 hour in serum-free media containing increasing concentrations of HNE. Cells were harvested and lysed 10 hours after exposure.

Figure 6B. ALDH protection against HNE induced protein adduct formation. $\Delta pCEP4\Delta$ control cells and cells overexpressing hALDH3 were dosed for 2 hours with increasing concentrations of HNE. Cells were lysed and 50 μ g of cytosolic protein were run on a 10% SDS-PAGE gel and transferred to nitrocellulose. Adducts were detected using a HNE adduct specific antibody (1:2500).

Figure 1A

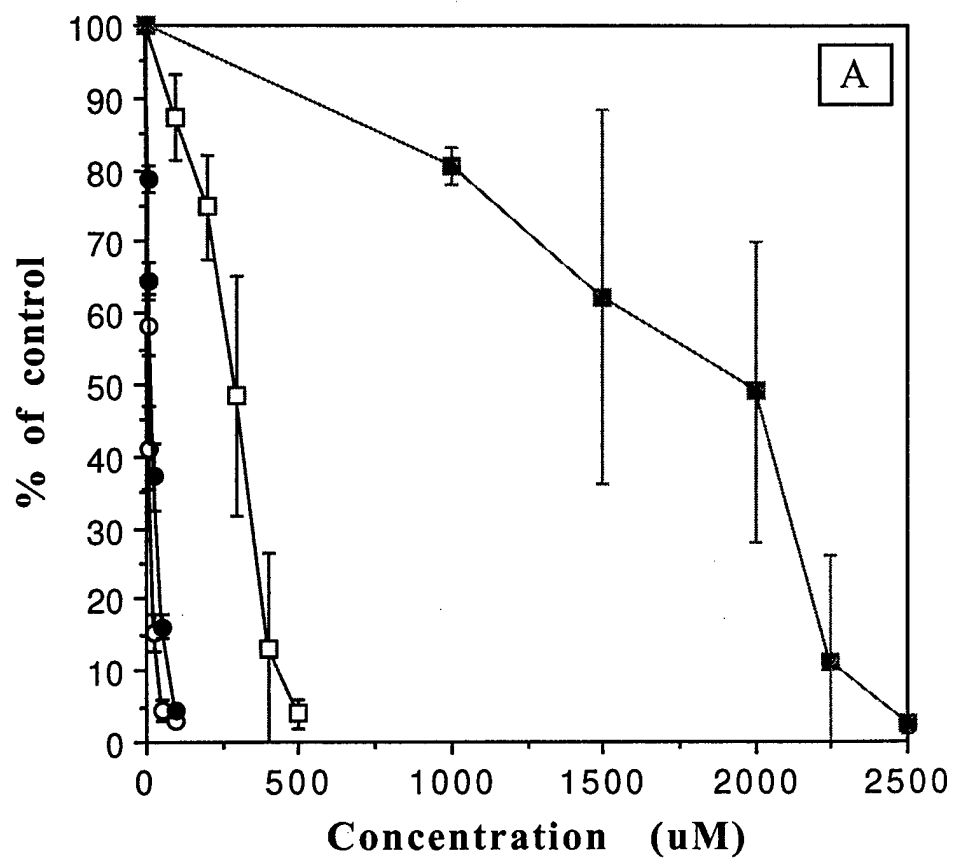


Figure 1B

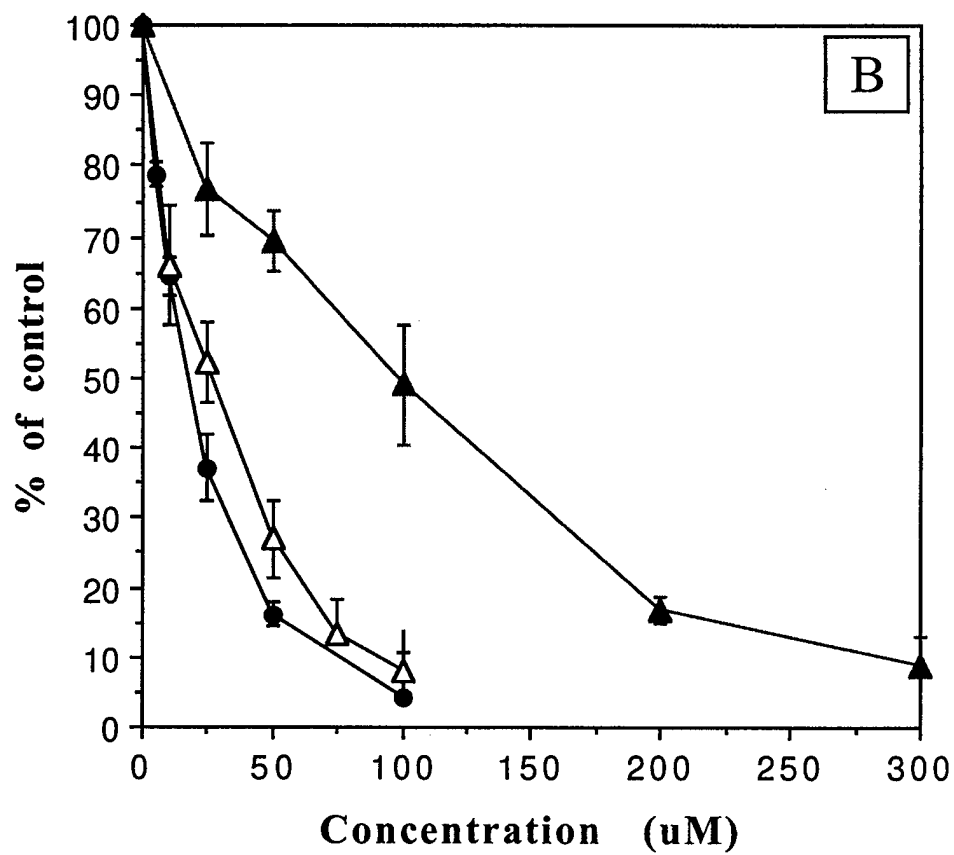


Figure 2

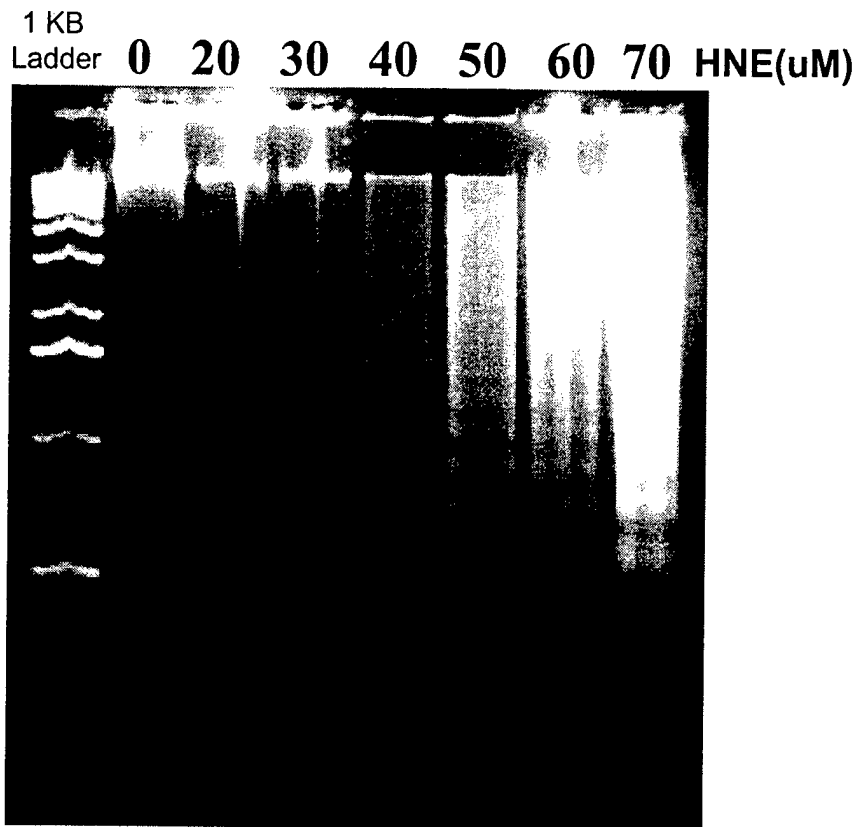


Figure 3

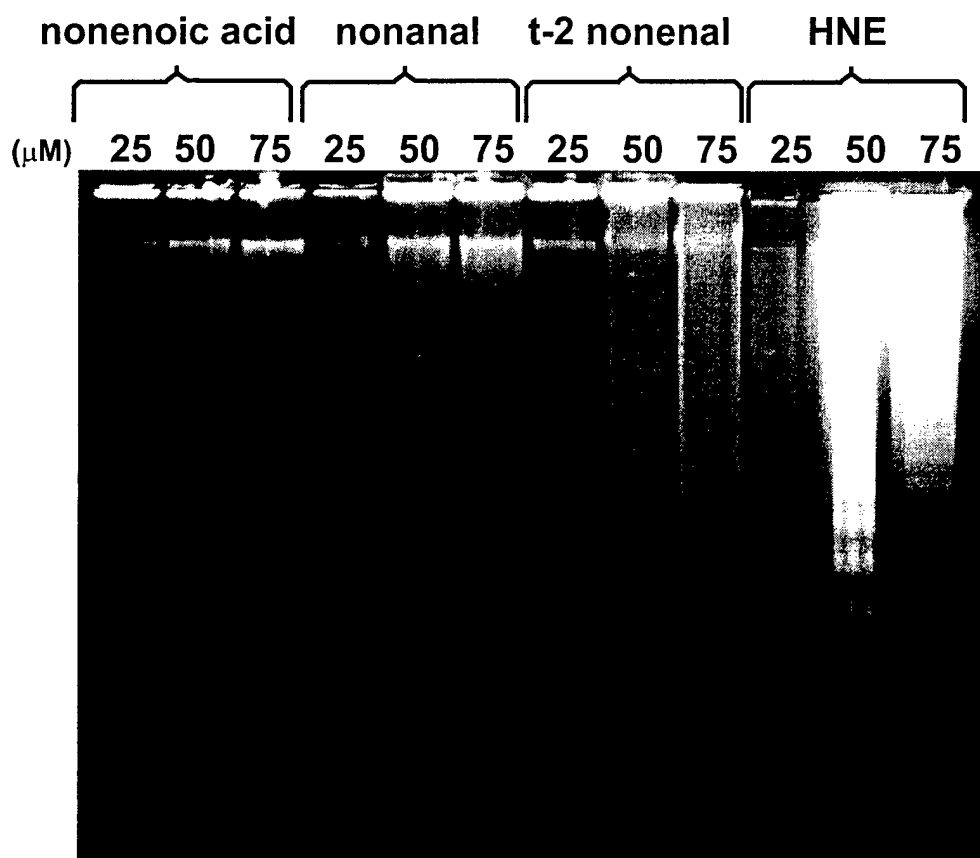


Figure 4

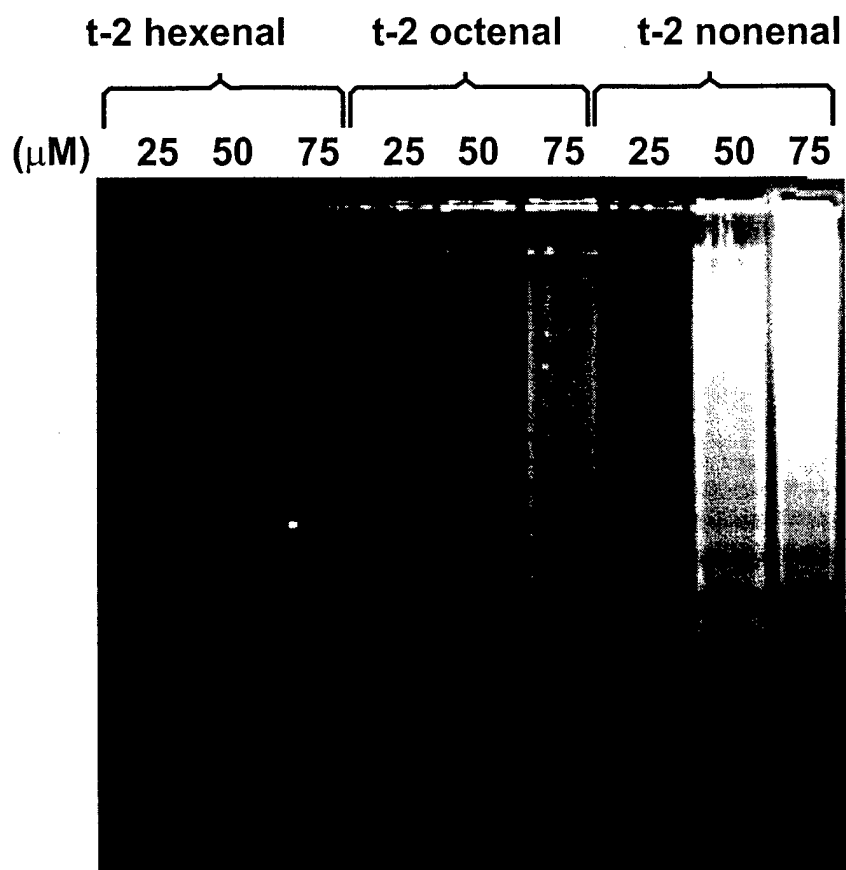


Figure 5

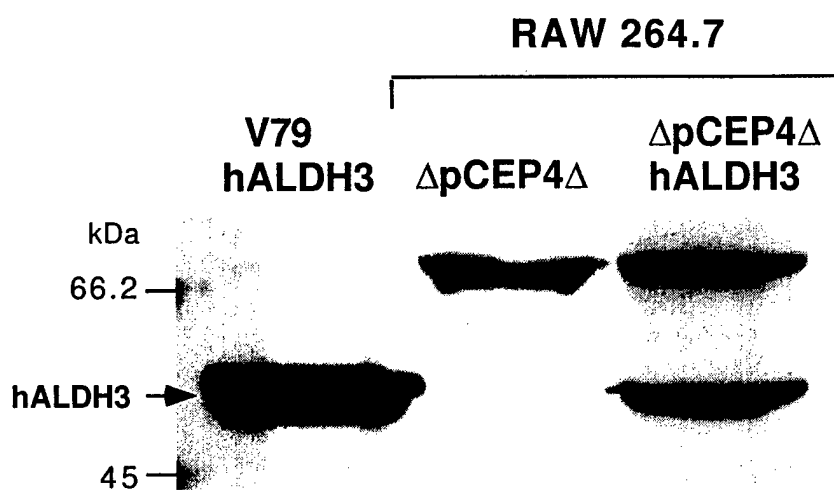


Figure 6A

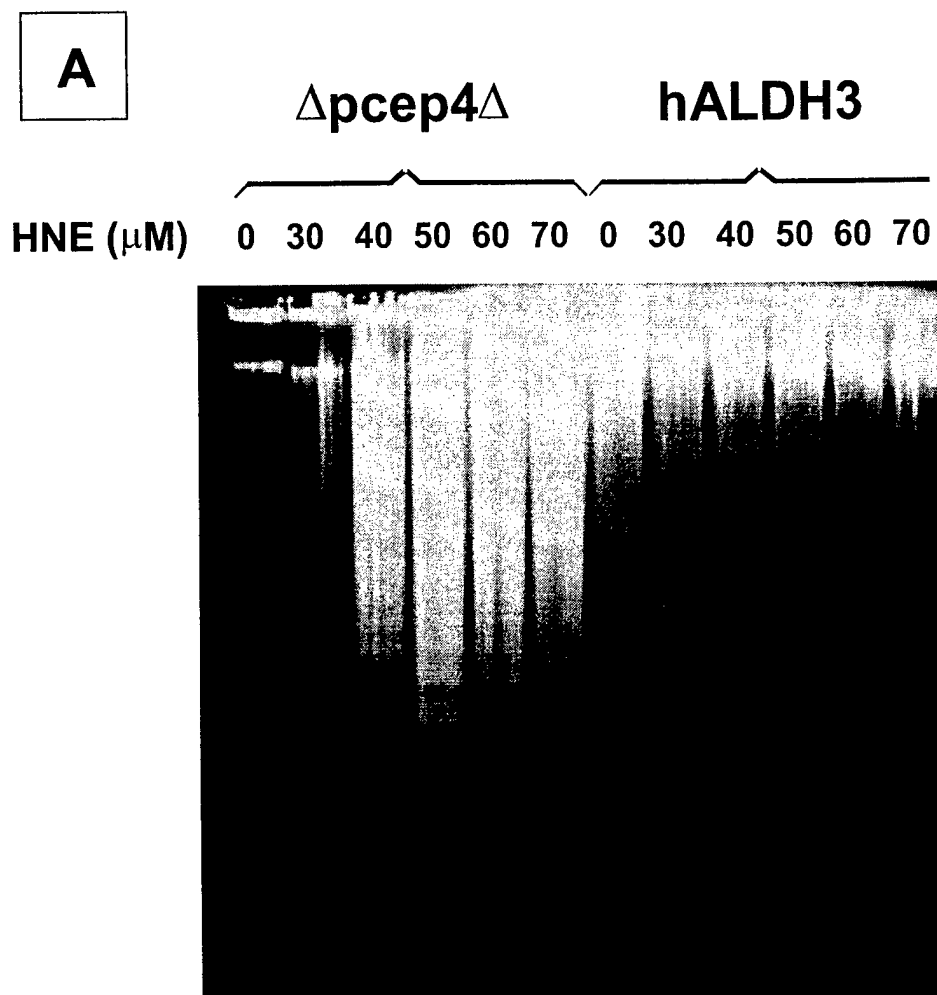
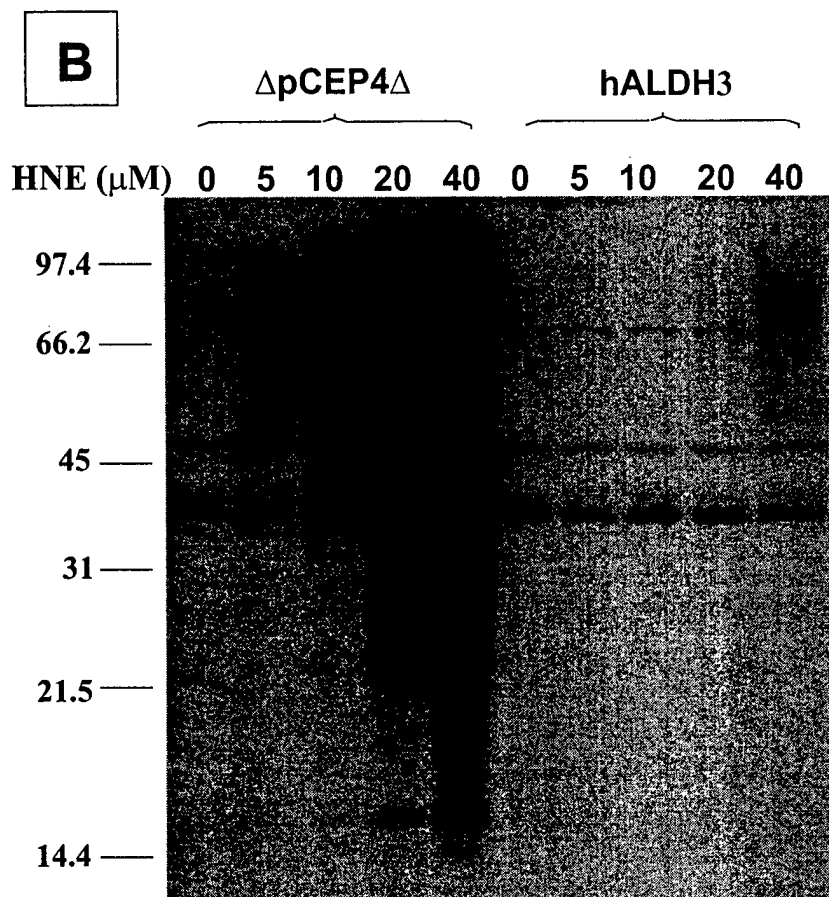


Figure 6B





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Chemico-Biological Interactions 111-112 (1998) 389-407

Chemico-Biological
Interactions

Chemoprotective functions of glutathione S-transferases in cell lines induced to express specific isozymes by stable transfection¹

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Abstract

The authors have shown that expression of mGSTM1-1 or hGSTP1-1 in MCF-7 cells protects against DNA alkylation by 4-nitroquinoline-1-oxide (NQO) in an isozyme-specific manner and is commensurate with relative specific activity. Expression of GSTs also conferred protection against both DNA strand breaks and sister-chromatid exchange induced by NQO. Interestingly, GST expression did not protect against NQO cytotoxicity in

Abbreviations: GST, glutathione S-transferase; hGSTM1-1, human glutathione S-transferase μ -1; hGSTP1-1, human glutathione S-transferase π ; hGSTA2-2, human glutathione S-transferase α -2; mGSTM1-1, murine glutathione S-transferase μ -1; mGSTP1-1, murine glutathione S-transferase π ; mGST-Yc, murine glutathione S-transferase Yc (or mGSTA3-3); GSH, glutathione; MRP, multidrug resistance associated protein; SCE, sister chromatid exchange; BPDE, benzo[a]pyrene-7,8 diol-9,10 epoxide; B[a]P, benzo[a]pyrene; AFB1, aflatoxin B1; AFBO, aflatoxin B1-oxide; CDNB, 1-Cl-2,4-dinitrobenzene; TBS, Tris-buffered saline; FBS, fetal bovine serum; BrdU, bromodeoxyuridine; CYP1A1, cytochrome P450-1A1; CYP2B1, cytochrome P450-2B1.

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¹ Presented by A.J. Townsend at The International Conference on Glutathione and Glutathione-Linked Enzymes in Human Cancer and Other Diseases, Oct. 31-Nov. 3, 1996 at Hilton Head, S.C.

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PII S0009-2797(97)00175-0

[1]. GSTs are thought to play an important role in constitutive cellular defenses against toxic and mutagenic electrophiles and as a contributor to the mechanism of some cancer chemopreventive agents via induction. However, while it is logical to predict such a role in general, the functional protection provided is likely to vary greatly depending on factors such as the dynamics of uptake or metabolic activation in relation to the kinetics and capacity for conjugation. Other related factors that could determine protective capacity of GST expression may include GSH supply, cellular localization of the target macromolecule(s) and ability to dispose of the thioether conjugates, which may cause product inhibition of GST or even toxicity if the charged conjugates accumulate. In addition, conjugation with GSH fails to detoxify some electrophiles and in some cases may increase their toxicity or mutagenicity [2,3].

The above considerations complicate determination of GST chemoprotective functions by comparison of cell lines or tissues that express different or multiple GST isozymes, even when the cell lines are autologously derived, as in the case of drug-selected cell lines. This problem led us to develop a series of stably transfected transgenic model cell lines for comparison with the parental cell line and or with a control line stably transfected with an empty expression vector and selected with the same resistance marker. These model cell lines represent an improvement over toxin-selected cell lines, in the sense that each line differs only in expression of a single GST isozyme. A limitation of this system, however, is loss of the selection pressure for related cellular cofactors that may be necessary for effective protection by GST isozymes. Nonetheless, this approach allows clear assessment of the protective capacity of the conjugation event itself and determination of the effective isozymes and examination of the relationship between cellular specific activity and degree of protection against specific electrophilic toxins that are GST substrates. The authors have utilized stably transfected cell lines that express individual GST isozymes to assess the sufficiency of GST expression for protection of cells against toxicity due to exposure to electrophiles that are known, or suspected to be, substrates for GSH GST-dependent detoxification.

Another limitation related to the use of immortal cell lines is the frequent absence of expression of enzymes, such as cytochrome P-450, that are necessary for activation of some compounds such as polycyclic aromatic hydrocarbons (PAHs). This problem has been circumvented when activation capacity is needed by using clonal cell lines previously transfected with the required cytochrome P-450 isozymes [4,5] as recipient lines for super-transfection with expression vectors for individual GST isozymes. The authors have used the resultant cell lines expressing stably transfected GST isozymes as genetically defined model systems to examine protective functions against specific agents in intact cells. With these cell lines, the authors have been able to examine the ability of specific GST isozymes to protect against carcinogens such as aflatoxin and benzo[a]pyrene that are relatively nontoxic as the parent compounds.

2. Materials and methods

2.1. Materials

[3H]NQO (2.5 Ci/mmol), [3H]BPDE (0.975 Ci/mmol) and unlabeled BPDE were purchased from Chemsyn Science Laboratories (Lenexa, KS). [3H]AFB₁ (18 Ci/mmol) was purchased from Moravak Biochemicals (Brea, CA). Unlabeled NQO, AFB₁, B[a]P and other chemicals were of reagent grade or higher and were purchased from Sigma Chemical Company (St. Louis, MO), Aldrich Chemical Company (Milwaukee, WI), or Fisher Scientific (Pittsburgh, PA).

2.2. Cell lines and expression vectors

Establishment of clonal human MCF-7 cell lines stably transfected with human GSTP1-1, hGSTM1-1, hGSTA2-2 and murine mGSTM1 has been previously described [6-8]. The MCF-7 and T47D transgenic cell lines were all derived using an expression vector (pMTP) which places the inserted cDNA under the control of a human metallothionein IIA promoter. Variable constitutive expression of the inserted sequences was generally, but not always, correlated with the number of vector copies integrated into the genome. Control (empty vector) and GST-expressing MCF-7 and T47-D clones were isolated using the aminoglycoside G-418 to select for the co-transfected pSV2-neo plasmid. Clonal cell lines were subcultured as a monolayer in a 1:1 mixture of RPMI 1640 and Ham's F12 media (Gibco, Long Island, NY) containing 5% fetal bovine serum (Gibco) and 50 µg/ml gentamycin (Gibco), at 37°C in a humidified 95% air + 5% CO₂ atmosphere.

The vectors used for stable transfection of the V79 cell lines (Δ pCEP4) were derived from the pCEP4 vector (Invitrogen) by deletion of the replication origin and the Epstein-Barr nuclear antigen sequences required for episomal replication, as previously described [9]. This vector contains a hygromycin resistance selectable marker gene and positions inserted GST cDNA sequences in the multiple cloning site sequence just downstream of a cytomegalovirus early promoter. The cDNA for human GSTP1 was provided by Dr Jeff Moscow (NCI)[10]; cDNAs for the human GSTM1 and GSTA2 were provided by Dr Chen-Pei Tu (Pennsylvania State University) [11,12]; and the cDNA for the murine GST-Yc was provided by Dr David Eaton (University of Washington) [13]. The murine GSTM1 was cloned by A. Townsend [14] and the murine GSTP1 was cloned by PCR from cDNA prepared from L929 cells (details to be published elsewhere). The V79 cell lines used as recipients for the GST expression vectors were either the parent cell line or transfected derivatives of V79 that expressed either rat cytochrome P450-1A1 (V79MZr1A1; previous designation V79 5D1) [5] or P450-2B1 (V79MZr2B1; previous designation V79/XEM2) [4], in order to allow intracellular activation of benzo[a]pyrene or aflatoxin B1, respectively. Cell lines were routinely passaged as a monolayer in DMEM (Gibco) containing 5% fetal bovine serum (FBS) (Gibco) and 0.7 mg/ml of hygromycin and maintained at 37°C in a humidified 95% air/5% CO₂ atmosphere. Cells were switched to medium without hygromycin 16-18 h prior to each experiment.

2.3. Cytotoxicity assays

Clonogenic survival assays were carried out as previously described [7], in which 200-400 cells were plated in 6-well culture dishes from a single-cell suspension 24 h prior to exposure to electrophilic agents. Concentrations and times of exposure are indicated in the figure legends. The BPDE cytotoxicity assays for the V79 cell lines was done by a sulforhodamine B microplate staining assay in which 250 cells were plated in 96-well plates and exposed to BPDE the following day, then grown for 4 additional days and processed for reading on a microplate reader to determine absorbance, an indirect measure of cellular protein and proliferation [15]. The clonogenic and microplate assays yielded qualitatively similar results.

2.4. Alkylation of cellular macromolecules by radiolabeled carcinogens

Cells were exposed to the labeled carcinogens at the concentrations and time intervals indicated in the figure legends. Carcinogen exposures were in Ham's F12 (MCF-7, T47D lines) or DMEM (V79 lines) medium, since these do not contain GSH. Serum-free medium was used for short exposures (≤ 1 h). Cells were harvested and analyzed for covalent labeling of either total cellular macromolecules (as TCA insoluble radiolabel), or total nucleic acids (by SDS/proteinase K digestion and ethanol precipitation) as previously described [8]. Results were expressed as either CPM/mg protein or CPM/mg total nucleic acids.

2.5. Alkaline elution assay for DNA strand scission

Alkaline elution was done essentially as described by Kohn [16]. Cells were treated with NQO for 20 min, then harvested with trypsin + EDTA and gently layered onto 0.2 μm polycarbonate filters in an apparatus with 8 filter manifold channels. Cells were lysed by overlay and gentle elution with 3 ml of 2% SDS glycine buffer, pH 9.6 + 40 mM EDTA, followed by a 20 min digestion with proteinase K (0.5 mg/ml in 2% SDS glycine buffer) and DNA was eluted in 2% tetrapropylammonium hydroxide solution (pH 12.3) for 15 h through a 75 mm 2 μm polycarbonate nucleopore filter. DNA in eluted fractions and filter fractions was quantitated by a fluorometric assay [17].

2.6. Sister chromatid exchange assay

Cells were plated at 2×10^5 cells per flask to yield 20-25% confluence the following day. The optimal schedule was determined to be addition of 10 μM BrdU 20 h prior to NQO exposure and cells were harvested for sister chromatid exchange (SCE) analysis 20 h after NQO exposure. Cells were treated with 0.02 $\mu\text{g}/\text{ml}$ colcemid for 2 h at 37°C in the culture medium, then harvested with trypsin/EDTA and swollen by addition of hypotonic solution (0.075 M KCl) for 14 min at 37°C, centrifuged and fixed by gradual addition of cold methanol/acetic acid (3:1). Slides were prepared by dropping the fixed cell suspension dropwise at room temperature

onto tilted glass slides. Slides were stained in PBS + Hoechst 33528 dye (0.01 mg/ml) for 12 min, rinsed in PBS and exposed under a wetted cover slip to 366 nm UV light for 13 min at a distance of 13 cm from the source (Spectroline model EN-280L). Slides were washed at 65°C in 2X SSC buffer, then stained in 3% Giemsa for 15 min, rinsed and dried. Slides were analyzed and reported as average SCEs per metaphase spread at high magnification for 5-10 cells per experimental condition.

3. Results

3.1. Transgenic cell lines

In general, the expression levels achieved with the V79 transgenic cell lines were much higher than the GST activity obtained in the MCF-7 transfectant lines (compare specific activity data in Table 1 Table 3). The single T47D clonal transfectant line (T47D π) also expressed very high hGST π activity (Table 1). Expression of GST mRNA, enzyme protein and enzymatic activity were generally proportionate to the degree of incorporation of vector copies into cellular DNA, as indicated by a correlation between measured specific activity and the relative intensities of the respective DNA, RNA, or protein bands probed with radiolabeled cDNAs or by Western blotting with class-specific affinity-purified polyclonal antibodies (data not shown). Only the transfected isozymes were overexpressed, as

Table 1
Specific activities of GST isoenzymes in stably transfected MCF-7 and T47D cell lines

Cell line	Specific activity with CDNB (nmol min per mg)	Relative specific activity for NQO ^a
Parent MCF-7 ^b	9.8 ± 0.4	0.4
MCF-7 Neo-1 ^b	10.7 ± 0.3	0.4
hGST α 2-3 ^b	52 ± 2	0.1
hGST μ -3 ^b	340 ± 26	14
hGST1 ^b	91	31
mGST μ 1-33	75 ± 12	71
T47D neo	15.9 ± 0.2	0.7
T47D π	4411 ± 183	1502

Cells were harvested in log phase and GST activity determined as described in Section 2. Relative specific activity was calculated from specific activities of purified enzymes as described below. Reprinted from Table 1 in [8]; used with permission.

^a Specific activities (nmol min per mg) for 4-NQO conjugation in each transfected cell line, calculated from the specific activity of the purified enzymes for NQO and normalized to the relative activities in each cell line measured with the universal substrate chloro-dinitrobenzene (CDNB). Purified GST isozymes were obtained by GSH affinity chromatography of extracts from prokaryotic expression vectors in *E. coli*, except for hGST π (Sigma, St. Louis, MO).

^b Previously reported in [8]; the low activities in empty vector-transfected control lines was μ class, as determined by Western blotting (not shown).

Table 2
Expression of murine GSTM1-1 or hGSTP1-1 fails to protect V79 cells against cytotoxicity of NQO

	GST specific activity (CDNB) (μ U/mg) ^a	IC ₅₀ for NQO Cytotoxicity (IC ₅₀ , μ M) ^a
Control V79 Δ pCEP	119 \pm 11	7.7 \pm 1.25
V79 mGSTM1-1 (clone B -15)	133 \pm 36	2.5 \pm 0.33
V79 hGSTP1-1 (clone π -21)	357.4 \pm 210	(1.7) ^b

Control (empty vector transfected) and GST-transfected cell lines were exposed to NQO for 20 min, then analyzed for sensitivity to cytotoxicity using the rhodamine red staining microplate assay as described in Section 2. Both of the GST-expressing cell lines were about 3-fold more sensitive to NQO than the control cell line.

^a Results are the mean \pm S.D. of three or more experiments.

^b Result of a single experiment.

shown by Western blot analysis of cytosolic protein (not shown). The hamster V79 fibroblastic lung tumor cell line has moderate activity (\sim 200 mU/mg) of a hamster π class GST. However, this isozyme has been shown to be ineffective for conjugation of BPDE [18], nor is it expected to significantly conjugate AFB₁-8,9-oxide based on studies with π class GST from other species [19,20]. Hamster π class GST may conjugate NQO effectively, but this activity is an order of magnitude lower than the expressed levels of the two GST isozymes transfected into V79 cells for purposes of the NQO studies.

3.2. Effect of GST expression on macromolecular damage and cytotoxicity induced by NQO

Expression of either transfected hGSTP1-1 or mGSTM1-1, isozymes that exhibit high specific activity for conjugation of NQO, reduced alkylation of total cellular macromolecules by [³H]NQO by 70 or 92%, respectively (Fig. 1A). Analysis of alkylation of total nucleic acid revealed quantitatively similar results (not shown). The degree of protection against alkylation was directly and closely correlated with the relative specific activity for NQO conjugation by the respective GST isozymes expressed in these MCF-7 transfectant lines, as shown in the inset panel of Fig. 1A. However, this level of GST expression did not confer significant protection against NQO cytotoxicity in these cells (Fig. 1B).

Induction of SCE is a sensitive index of DNA damage by alkylating carcinogens [21]. The MCF-7 cells expressing either hGSTP1-1 or mGSTM1-1 exhibited a 50% reduction in SCE induction compared with either control or hGSTA2-2 expressing cells (Fig. 2). An intermediate number of SCEs was observed in the hGSTM1-1 transfected MCF-7, consistent with the earlier observed correlation between relative specific activity for NQO conjugation in the respective cell lines (Fig. 1).

Treatment of the MCF-7 cells with NQO also causes DNA strand breaks, as measured by the alkaline elution technique, which measures an increased rate of

elution of DNA containing strand breaks through a microporous filter under alkaline conditions [16]. The control MCF-7 Neo-1 cell line exhibited a steep slope indicative of extensive DNA strand breakage in the initial period of NQO exposure, while DNA from untreated control or GST transfected cells remained on the filter as high molecular weight undamaged DNA (Fig. 3). The GST-transfected cell lines all exhibited an intermediate elution pattern, indicating that they are less susceptible to NQO induction of DNA strand breaks. An even more striking protection against NQO induction of DNA strand breaks was observed when the same experiment was carried out with paired control T47D/Neo and the hGSTP1-1 transfected T47D π cell line, which expresses very high activity of hGSTP1-1 (Table 1). While significant DNA strand breakage was observed in the T47D/Neo control cells even at 0.1 μ M, the lowest NQO concentration tested, no strand scission was induced in the T47D π transfectant line even at 1.6 μ M, a 16-fold higher NQO concentration (Fig. 4).

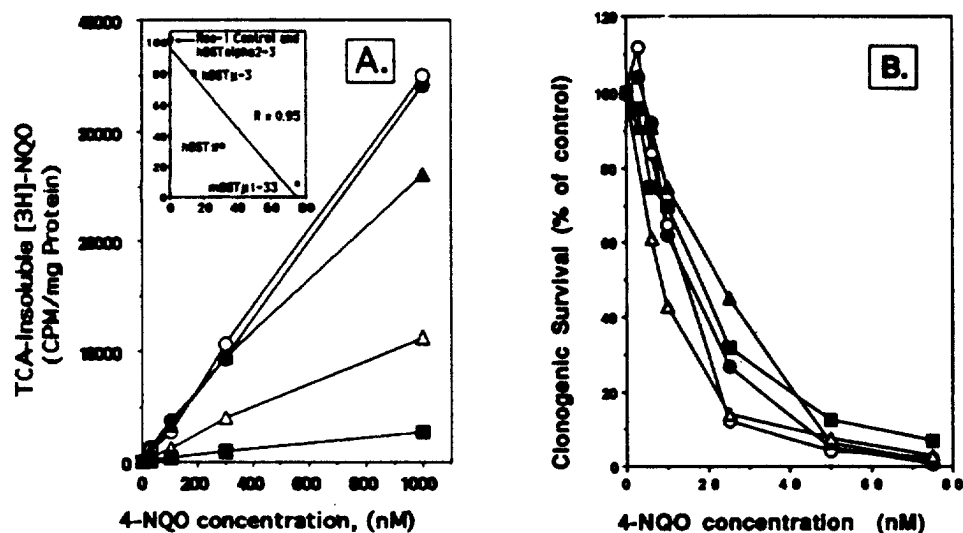


Fig. 1. (A) Dose-response for alkylation by NQO in the control and GST-transfected MCF-7 cell lines. Alkylation was measured as TCA-insoluble covalent labeling of total cellular macromolecules as previously described ([8] no. 2673), following incubation of cells with the indicated concentrations of [3 H]NQO for 20 min. Symbols: neo-1 Control (●); hGST α 2-3 (hGSTA2-2) (○); hGST μ -3 (hGSTM1-1) (▲); hGST π (hGSTP1-1) (△) and mGST μ 1-33 (mGSTM1-1) (■). A representative experiment is shown (see Table 1 for additional data). Inset: the close inverse correlation between the relative specific activity for NQO conjugation (x-axis) and the relative amount of alkylation of total cellular macromolecules (y-axis) is shown. (B) Cytotoxicity of NQO in control and GST-transfected MCF-7 cell lines. Cells were exposed to the indicated concentrations of NQO for 1 h in Ham's F-12 medium (GSH-free), then the medium was changed to the usual growth medium and colonies were allowed to form for 2 weeks. Colonies were fixed and stained with methylene blue and counted. Clonogenic survival is expressed as percent of the untreated control for each cell line. Symbols: neo-1 control (●); hGST α 2-3 (hGSTA2-2) (○); hGST μ -3 (hGSTM1-1) (▲); hGST π (hGSTP1-1) (△); and mGST μ 1-23 (mGSTM1-1) (■). From Fig. 2A and 5 in [8]; used with permission.

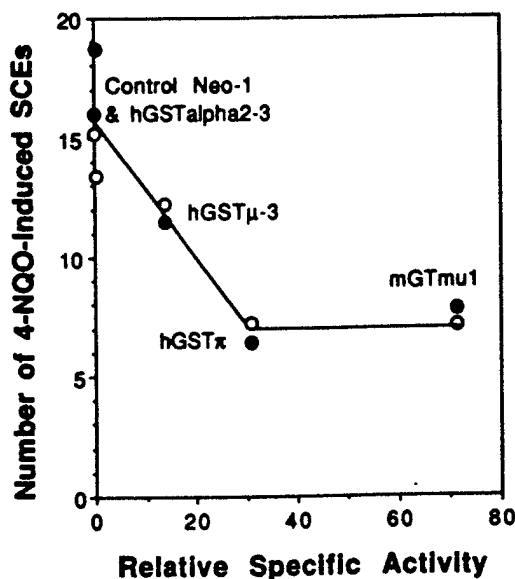


Fig. 2. Dose-response for induction of sister-chromatid exchange by NQO in the control and GST-transfected MCF-7 cell lines. Cells were treated with NQO and processed for SCE analysis as described in Section 2. (●) Indicate a 1 h exposure to 0.3 μ M NQO and (○) indicate continuous exposure to 0.1 μ M NQO.

Interestingly, the T47D π cell line did exhibit a high degree of resistance (24-fold) to NQO cytotoxicity (Fig. 5), in contrast to the results with the MCF-7 derivatives which expressed much lower GST activities. While the reason for this high level of resistance would seem to be due to the much higher (44-fold) expression of hGSTP1-1 as compared with the MCF-7/hGST π transfectant line, other recent results suggest that the explanation may not be that straightforward. The authors have also tested NQO cytotoxicity in V79 cells expressing high levels of hGSTP1-1 or mGSTM1-1 and there was no protection in either GST transfectant line: in fact, the GST-transfected cells were 2-3-fold more sensitive to NQO (Table 2). The most direct conclusion from these experiments is that GST expression and by extension, conjugation of NQO alone, is not sufficient to protect against NQO cytotoxicity, although it does appear to reduce the degree of alkylation and other indices of damage to cellular macromolecules. Potential reasons for these observations are considered in the discussion section.

3.3. Protection by transfected mGST-Yc against nucleic acid alkylation and cytotoxicity induced by aflatoxin B1

Considering the results with NQO, the initial aim of this series of experiments was simply to determine whether expression of the murine GST-Yc, which is known to have one of the highest conjugation efficiencies among GST isozymes that

conjugate activated AFB₁ [22-24], would confer protection against AFB₁ alkylation and cytotoxicity. Since AFB₁-8,9-oxide, the active metabolite of AFB₁, is not available commercially and difficult to prepare and store due chemical instability, the experimental design for this series was based on in situ metabolic activation of AFB₁ in cell lines that were constructed to incorporate expression of a rat cytochrome P-450-2B1 isozyme as the starting point. This was accomplished by supertransfection of our hygromycin-selectable GST-Yc expression vector into the V79MZr2B1 cell line which had been previously stably transformed with a retroviral expression vector containing both the P-450 cDNA and the neo selectable marker gene selected with G418 [4].

Western blot analysis indicated that the mGST-Yc was relatively highly expressed in the transfected cell line while no α class GST was expressed in the V79MZr2B1 parental line (not shown). The activity in the GST-Yc transfectant line, measured by subtraction of the conjugation activity in the control cells, was 266 mU mg with CDNB. However, this is a significantly weaker substrate for mGST-Yc and other α class GST isozymes as compared with π or μ isozymes [25,26]. Expression of mGST-Yc reduced alkylation of total nucleic acids isolated from AFB₁-treated cells by 69%, or more than 3-fold relative to the empty vector-transfected control line (Table 3). Limited analysis of another mGST-Yc transfectant clone yielded quanti-

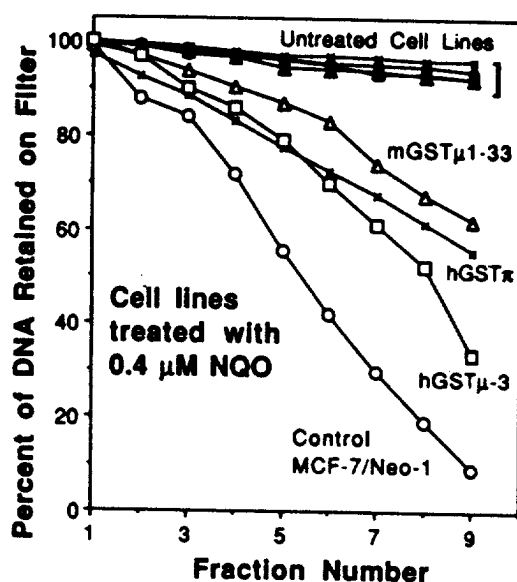


Fig. 3. Alkaline elution assay for induction of single-strand breaks in DNA by NQO in the control and GST-transfected MCF-7 cell lines. Cells were treated with NQO as described in Section 2 and processed for alkaline analysis. Closed symbols indicate control untreated cells and open symbols indicate cells treated with 0.4 μ M NQO.

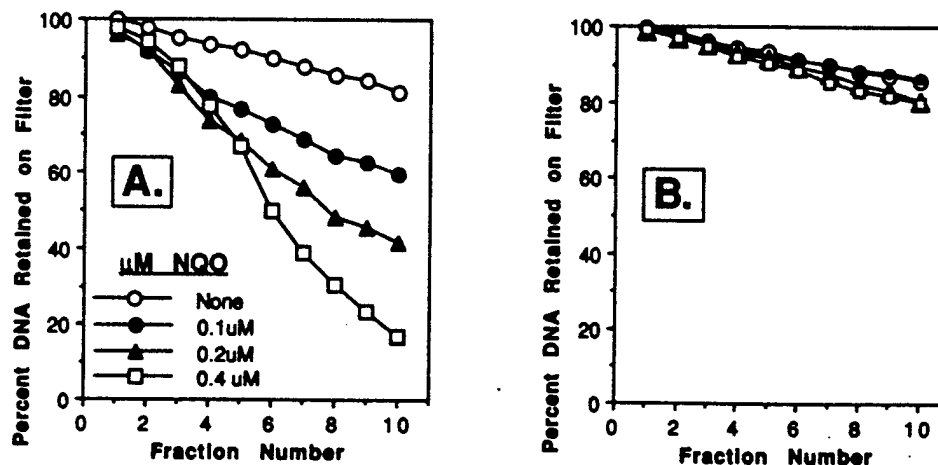


Fig. 4. Alkaline elution assay for induction of single-strand breaks in DNA by NQO in the control and GST-transfected T47D cell lines. Cells were treated with the indicated concentrations of NQO and processed for alkaline elution analysis as described in Section 2. The slope of the elution profile is proportional to the degree of DNA strand scission.

tatively similar results for protection against DNA alkylation (not shown). Expression of mGST-Yc also conferred almost 5-fold resistance to the cytotoxic effects of AFB₁ as compared with either the empty vector-transfected (Table 3) or parental (not shown) cell lines.

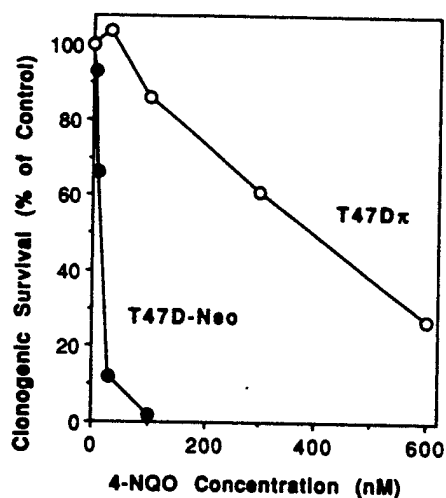


Fig. 5. Clonogenic survival cytotoxicity assay for inhibition of colony formation by NQO in the control and GST-transfected T47D cell lines. The indicated concentrations of NQO were added 24 h after plating and cells were processed for clonogenic survival assay as described in Section 2 and the legend to Fig. 1. Clonogenic survival is expressed as percent of the untreated control for each cell line.

Table 3

Expression of murine GST-Yc protects V79 cells against alkylation of total cellular nucleic acids and cytotoxicity following exposure to aflatoxin B₁

	V79-SDP-1 (control)	V79-SΔYc1-4
Alkylation of total nucleic acids by 300 nM [³ H]AFB ₁ (CPM mg) ^b	1476 ± 324	458 ± 183 ^c (31%) (3.3-fold resistant)
Cytotoxicity (nM AFB ₁) (IC ₅₀ for AFB ₁)	14 ± 4.7	69 ± 9.2 ^d (4.9-fold resistant)

Control (empty vector transfected) and mGST-Yc transfected V79MZr2B1 cells^a were exposed to 300 nM [³H]AFB₁ for 4 h in serum-free medium, then serum was added and the incubation continued for an additional 20 h. Total nucleic acids were isolated and analyzed as described in Section 2. Cytotoxicity was determined by clonogenic survival as described in Section 2.

^a The GST-Yc1 specific activity was 266 mU mg as measured by difference between SΔYc1-4 and control SDP-1 cells. Western blotting showed that α class GST was expressed only in the SΔYc1-4 cell line (not shown).

^b Results (CPM mg) are the mean ± S.D. of triplicate samples from three independent experiments; 1.0 pmol AFB₁ results in 18 000 cpm of counted signal.

^c Significant. *P* = 0.01.

^d Significant. *P* = 0.001.

3.4. Protection by transfected human or murine π class GST against alkylation, but not cytotoxicity induced by benzo[a]pyrene diol-epoxide

The V79MZr1A1 cell line was used as recipient for the π class GST transfections because it expresses a rat cytochrome P-450 1A1 isozyme. Since this is the primary isozyme involved in activation of B[a]P to the ultimate carcinogen BPDE, this approach allows examination of effects of either BPDE or the parent compound B[a]P in the same clonal cell line. Both mGSTP1-1 and hGSTP1-1 were similarly highly expressed in the V79MZr1A1 recipient line, with activities in the range of the highest expressing tissues such as placenta or mouse liver (> 3000 mU mg). This was reflected in strong protein bands on Western blots compared with the controls which expressed much lower activity of hamster GSTP1-1 (not shown). The effect of GST expression in the MCF-7 series was modest, with 10% reduction in the hGST μ -3 line (not significant) and 29% reduction in the hGST π line, an effect with only marginal significance (Table 4). These results reflected the greater activity and selectivity of hGSTP1-1 than hGSTM1-1 for conjugation of BPDE [27].

Since the level of expression in V79MZr1A1 was considerably (5-30-fold) higher than the study was able to obtain in MCF-7, the authors repeated the alkylation studies in two clonal transfectant lines that expressed high levels of murine GSTP1-1 or hGSTP1-1. A second purpose of the V79 experiments was to compare human and murine π class GSTs with respect to their ability to protect against macromolecular damage by BPDE. Expression of either isozyme reduced alkylation of total cellular nucleic acids to 36% (hGSTP1-1) or 19% (mGSTP1-1) of control

levels (i.e. 64–81% reduction). Alkylation of total macromolecules (TCA-insoluble signal) was reduced by 40–50% (not shown), indicating a preferential protection of nucleic acids, as compared with protein by GST expression. In contrast to the protection against alkylation, however, the authors have not found any protection against cytotoxicity by BPDE in the V79 cell lines, with similar lethality in control (XΔP-1, $IC_{50} = 0.15, 0.14$) and GSTP1 transfectant lines (Xmπ-6, $IC_{50} = 0.22, 0.20$; Xhπ-33, $IC_{50} = 0.20, 0.20$). Thus, this represents another alkylating carcinogen besides NQO for which the effect of GST expression on toxic effects appears to be governed by considerations beyond simple enzyme kinetics.

4. Discussion

Chemoprevention of cancer is a relatively new area of research emphasis with the goal of reducing susceptibility to malignancy by dietary or pharmacologic intervention strategies aimed at preventing genetic damage, or slowing further progression in 'initiated' mutant cells [28]. Chemopreventive agents such as the prototypic experimental drug oltipraz can reduce chemical damage to DNA by direct inhibition of carcinogen activation [29], or it may act indirectly via enhancement of expression of natural cellular pathways of defense against electrophilic damage [30,31]. As previously mentioned, this role as a 'blocking agent' to prevent DNA

Table 4
Expression of **Pi class GST** protects V79 cells against alkylation of total cellular nucleic acids following exposure to benzo[a]pyrene diol-epoxide (BPDE)

	Alkylation of total nucleic acids by [³ H]BPDE (CPM mg ⁻¹)	Percent of control
MCF-7 cell lines		
MCF-7 neo-1	8699 ± 1196	100
hGSTμ-3	7793 ± 222 ^b	90
hGSTπ	6173 ± 843 ^c	71
V79 cell lines		
XΔP-1 (control)	3313 ± 573	100
Xmπ-6	631 ± 82 ^d	19
Xhπ-33	1111 ± 117 ^e	36

Control (empty vector transfected) and GST-transfected cell lines were exposed to [³H]BPDE and the total nucleic acids were isolated and analyzed as described in Section 2. Results (CPM/mg) are the mean ± S.D. of triplicate samples from three independent experiments.

^a Results are the mean ± S.D. of three or more experiments; 1 pmol BPDE results in approximately 975 cpm of counted signal.

^b Not significant. $P > 0.02$.

^c Marginally significant. $P = 0.04$.

^d Significant. $P = 0.001$.

^e Significant. $P = 0.003$.

damage or reduce it early in the evolution of carcinogenesis has far-reaching implications for a disease such as cancer that results from a series of stepwise cascading mutational events. It is reasonable to suggest that the probability for further oncogenic progression increases with accumulation of alterations in genes required for critical homeostatic processes such as DNA replication and repair, cell cycle control and signal transduction. This concept is consistent with the exponential rise in cancer incidence in the elderly. Thus, prevention or delay of any significant fraction of early DNA damage events might postpone the development of most sporadic (i.e. non-hereditary) cancer beyond the normal life span in many individuals.

A significant body of literature now supports the notion that dietary or pharmacologic agents that induce phase 2 detoxifying enzymes, without concomitant induction of phase 1 activating systems, have potent chemopreventive activity against the carcinogenic effects of certain chemicals [32-34]. In many cases, the mechanism of protection by these compounds has been closely correlated with induction of expression of GST isozymes and in some cases also with increased formation of detoxified metabolites and decreased formation of DNA adducts in vivo [26,35-37]. Further, the substrate specificity and kinetics of GST isozymes support a role for these detoxifying gene products in resistance to toxic electrophiles and prevention of carcinogenesis [25,26,36,38]. However, multiple GSTs are expressed in many tissues or cell lines and chemoprotective agents often induce other protective enzymes in addition to GST that complicate interpretation of the contributions of the various contributing mechanisms. Thus, although evidence continues to accumulate in support of a key role for GST isozymes in chemoprevention of mutagenesis and carcinogenesis, more detailed and defined studies are needed to assess the protective efficacy and capacity of specific GST isozymes at the cellular level against the detrimental effects of individual carcinogens and other toxic species.

The genetically engineered models created for use in the studies presented herein have inherent advantages over systems where activated metabolites are added, or that generate the active species outside of the target cell via microsome- or whole cell-mediated ('co-culture') methods. These include first and most importantly, the power of the genetic approach. Since the transfection recipient 'host' line is ideally chosen for a low background activity of the gene being studied, each expressing transfectant derivative line can be readily compared with the isogenic control (empty vector-transfected) and/or parental lines to deduce biological activities of the introduced gene product. Thus, an entire gene family can be compared in a single biological context (i.e. cell line), allowing enzyme function to be clearly distinguished from background influences. Other advantages include more relevant cellular pharmacokinetics and compartmentation of metabolism and the ability to study more than one gene product in the same cell to assess co-operativity of complex protective mechanisms.

The first carcinogen examined was NQO and using the MCF-7 series of transfected cell lines, the authors found highly effective protection (92% reduction) against alkylation of cellular macromolecules by [³H]NQO in GST-transfected

MCF-7 lines expressing the murine mGSTM1-1, which has the highest specific activity for NQO conjugation of the GSTs tested. Expression of human hGSTP1-1 reduced alkylation by 70%, while only a modest reduction by hGSTM1-1 was observed. A similar result was obtained for labeling of total nucleic acids or purified DNA (not shown). An important observation was the close linear relationship between the relative specific activity in each cell line (i.e. the specific activity of each separate GST isozyme toward NQO, adjusted for the amount expressed) and the reduction in DNA covalent labeling. In addition, the protection was fully reversible by pretreatment with the GST substrate inhibitor ethacrynic acid (not shown).

Expression of these GST isozymes in MCF-7 cells also correlated well with protection against NQO induction of sister chromatid exchange, a sensitive index of genotoxic stress. The authors also observed partial protection against NQO induction of DNA strand breaks in the MCF-7 control and transfected cell lines and complete abrogation of DNA strand breaks (> 16-fold resistance) in the T47D π line, which expresses much higher (> 40-fold) activity of the hGSTP1-1 isozyme. Interestingly, however, there was no protection by GST expression against the cytotoxic effects of NQO as measured by clonogenic survival assays with the MCF-7 derivatives. This result was surprising, in view of the protection against DNA alkylation and strand breaks, but could be the result of a different target, metabolite, or mechanism involved in cytotoxicity (e.g. the GSH conjugate may be cytotoxic). The authors also observed this lack of protection against NQO cytotoxicity even at much higher levels of mGSTM1-1 expression in the V79.hGSTP1-1 clone π -21 cell line, indicating that a high expression level per se could not ensure sufficient detoxification to prevent NQO lethality. When the T47D π cell line was tested, however, the authors found that this transfectant line, which expresses a similarly high activity of hGSTP1-1, was highly resistant to NQO cytotoxicity. One potential explanation for the discrepancy in results between the T47D π and V79.hGSTP1-1 derivative lines could relate to DNA strand breakage, which was completely prevented in the T47D π line. This is consistent with the much weaker reduction in strand breaks in the MCF-7 lines, which were not resistant to NQO cytotoxicity. Other possibilities could include the rapid doubling time for V79 cells (14 h, compared with about 30 h in MCF-7 and T47D), different mechanisms or targets for NQO toxicity, or variable expression of membrane transport proteins required for removal of GSH conjugates of NQO, such as MRP. Further experiments will be required to resolve these questions.

One potential reason for the lack of protection against NQO cytotoxicity could be if the GSH conjugate was unable to exit the cell. The conjugate might then cause feedback inhibition of further GST conjugation, or it might accumulate and cause cytotoxicity itself. Recent studies have shown that when the multidrug resistance associated protein (MRP) is expressed in MCF-7 VP cells [15], expression of transfected hGSTP1-1 does result in high level (40-fold) resistance to NQO cytotoxicity [39]. This represents a synergistic interaction that appears to enable GST-mediated protection, since the degree of resistance with expression of MRP and hGSTP1-1 together (40-fold) is greater than the expected additive resistance based on the amount of resistance conferred individually in cells expressing either MRP (4-fold) or GSTP1-1 (2-fold) alone.

Another toxin chosen for study was AFB₁, a highly hepatotoxic food contaminant and suspected human liver carcinogen produced by the mold *Aspergillus flavus*. There is a wide range of species and organ sensitivity to AFB₁ toxicity and carcinogenesis and at least a portion of this is related to differential expression and activity of GST isozymes [23,24,40]. For example, one reason that has been proposed for the relative resistance of mice to the toxicity and carcinogenicity of AFB₁ as compared with rats and humans is the constitutive expression in mouse liver of an α class GST that has high specific activity for conjugation of AFB₁-8,9-epoxide [22-24]. An orthologous GST is inducible in rats by anticarcinogenic agents such as ethoxyquin and oltipraz and this apparently accounts for a major share of the potent chemoprotective effects of these agents against AFB₁ hepatocarcinogenesis in rats [23,35,41]. However, humans apparently lack a similar GST isozyme with exceptional activity for conjugation of AFB₁-8,9-oxide [23].

The experiments with the V79MZR2B1 cells were possible because the parent cell line expresses a rat cytochrome P-450 gene introduced by DNA-mediated transfection [4]. These studies indicated that mGST-Yc was capable of reducing nucleic acid alkylation by about 3-fold. In addition, nearly 5-fold resistance to AFB₁ cytotoxicity was observed. Since the human GSTM1-1 is inducible by chemopreventive agents and is the most active human isozyme for conjugation of AFB₁-8,9-oxide, it will be interesting to determine whether it can reduce DNA damage or cytotoxicity and if so what activity will be required for significant effects. Interesting studies recently published have shown that AFB₁ is a substrate for the MRP transmembrane ATP-dependent efflux transporter [42]. Thus, it is possible that the MRP efflux pump may co-operate with the lower activity human GSTM1-1 to yield an additive protective effect by removing both AFB₁ and its GSH conjugate.

The final series of experiments demonstrated that in MCF-7 cells, hGSTP1-1 provided marginally significant protection against DNA alkylation by BPDE, whereas hGSTM1-1 did not significantly reduce alkylation. The V79 derivatives were used to compare human and murine GSTP1-1 at much higher levels of expression and the results indicated a much stronger protective effect (64-81% reduction) against nucleic acid alkylation, with the murine isozyme yielding slightly greater efficacy. Once again, however, there was no protection against cytotoxicity in the V79 series, in spite of the high level of expression. The reasons for the preferential protection against DNA damage end-points as compared with cytotoxicity are intriguing and merit further investigation. The differences in the cytotoxicity of NQO in the V79 and T47D derivative lines expressing similarly high hGSTP1-1 activities will offer an interesting point of departure for initial examination of the issues discussed above.

Acknowledgements

Supported by USPHS grants no. 1-ROI-ES-06006 from the NIEHS and CA-70338 from the NCI, and #DO-242.6-2 from the Deutsche Forschungsgemeinschaft.

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