**Carcinogenesis** vol.28 no.1 pp.207–214, 2007
doi:10.1093/carcin/bgl125
*Advance Access publication August 2, 2006*

# Expression of human glutathione *S*-transferase P1 confers resistance to benzo[*a*]pyrene or benzo[*a*]pyrene-7,8-dihydrodiol mutagenesis, macromolecular alkylation and formation of stable N2-Gua-BPDE adducts in stably transfected V79MZ cells co-expressing hCYP1A1

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**Transgenic cell lines were constructed to study dynamic competition between activation versus detoxification of benzo[*a*]pyrene (B[*a*]P) and its metabolites. Transfected V79MZ cells expressing human cytochrome P4501A1 (hCYP1A1) alone, or expressing hCYP1A1 in combination with human glutathione *S*-transferase P1 (hGSTP1), were used to determine how effectively GST protects against macromolecular damage or mutagenicity of B[*a*]P or its enantiomeric dihydrodiol metabolites (+)-benzo[*a*]pyrene-7,8-dihydrodiol [(+)B[*a*]P-7,8-diol] and (−)-benzo[*a*]pyrene-7,8-dihydrodiol [(−)-B[*a*]P-7,8-diol]. Mutagenicity of B[*a*]P at the *hprt* locus was dose- and time-dependent in cells that expressed hCYP1A1. Mutagenicity was reduced in cells further modified to co-express hGSTP1. Dose-response and time-course studies indicated that mutagenicity was reduced up to 3-fold by hGSTP1 expression, compared with cells expressing hCYP1A1 alone. Mutagenicity induced by the B[*a*]P 7,8-dihydrodiols was also dose-dependent, and was reduced 2- to 5-fold by hGSTP1. Expression of hGSTP1 reduced B[*a*]P adducts in total cellular macromolecules by 3.8-fold, which correlated with the reduction in B[*a*]P mutagenicity and with reduction in the formation of the proximate metabolite B[*a*]P 7,8-dihydrodiols from B[*a*]P. However, measurement of total B[*a*]P metabolites bound to DNA isolated from cells incubated with [$^3$H]-B[*a*]P revealed only 12, 33 and 24% reduction at 12, 24 and 48 h, respectively, by GSTP1 expression. Nevertheless, $^{32}$P-post-labeling analysis demonstrated nearly total prevention of the known B[*a*]P-DNA adduct, N2-guanine-benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE), in cells co-expressing hGSTP1. This adduct, thought to be the most mutagenic of the stable B[*a*]P adducts, accounts for 15% or less of the total DNA adducts observed. These results indicate that the reduction in hCYP1A1-mediated B[*a*]P mutagenesis by hGSTP1 is probably largely due to prevention of the N2-guanine-BPDE adduct. However, the significant fraction (30–40%) of this mutagenesis and the majority of the total DNA binding that are not prevented together suggest formation by hCYP1A1 of a subset of mutagenic metabolites of B[*a*]P that are not effectively detoxified by hGSTP1.**

**Abbreviations:** B[*a*]P, benzo[*a*]pyrene; B[*a*]P-7,8-diol, benzo[*a*]pyrene-7,8-dihydrodiol; BPDE, benzo[*a*]pyrene-7,8-diol-9,10-epoxide; EROD, ethoxyresorufin-*O*-deethylase; hCYP1A1, human cytochrome P4501A1; hGSTP1, human glutathione *S*-transferase P1; PAH, polycyclic aromatic hydrocarbon.

## Introduction

Polycyclic aromatic hydrocarbons (PAH) are common environmental pollutants, formed during incomplete combustion of organic matter, and are classified as putative human carcinogens. The parent PAH compounds themselves are relatively unreactive, but become toxic upon bioactivation by cytochrome P450 (CYP) dependent monooxygenases ('Phase I' biotransformation reactions) to multiple reactive electrophiles (1). These reactive electrophiles often serve as activated substrates for 'Phase II' conjugation reactions that render the conjugated product less reactive and more water soluble (2). A third 'Phase III' component for active export of the conjugated product may be required to complete the detoxification process (3,4). The relative impact of these components of xenobiotic metabolism have been characterized separately for a number of relevant toxic or mutagenic environmental contaminants. However, the dynamics of interactions among the pathways, and the underlying mechanisms of this interplay are not well understood.

An extensively studied PAH is benzo[*a*]pyrene (B[*a*]P), a constituent of coal tar, diesel exhaust and cigarette smoke. Activation of PAH is catalyzed by CYP1A1, CYP1A2, CYP1B1 and CYP3A4 (5). In humans and rodents, CYP1A1 is thought to play a key role in the stereoselective biotransformation of B[*a*]P to highly reactive epoxides. The oxygenation of B[*a*]P catalyzed by CYP1A1 results in a pair of diasteromeric (+/−)-benzo[*a*]pyrene-7,8-dihydrodiols (B[*a*]P-7,8-diols), and from these, two pairs of diastereomeric (+/−)-*syn* and (+/−)-*anti* diol-epoxides are formed (6). The benzo[*a*]pyrene diol-epoxide (BPDE) metabolites can bind covalently to cellular macromolecules, for example, to thiol groups on proteins (7), or to the exocyclic nitrogen groups of guanine and adenine in nucleic acids (8). The (+)-*anti*-benzo[*a*]pyrene-7,8-diol-9,10-epoxide [(+)-*anti*-BPDE] is the most mutagenic and carcinogenic metabolite among the four BPDE stereoisomers (5). These BPDE metabolites may be detoxified via spontaneous or enzymatic hydrolysis to yield triols or tetrahydroxyl (tetrol) derivatives (6). Alternatively, they may be conjugated spontaneously or enzymatically with cellular glutathione (GSH) (9). Both processes result in elimination of the reactive epoxide moiety and loss of reactivity toward nucleophilic sites in cellular DNA.

Glutathione *S*-transferase (GST) enzymes are thought to play a major role in detoxification of reactive electrophiles and hence in prevention of their cytotoxic and genotoxic

 

damage via catalysis of conjugation of the thiol group of GSH to reactive electrophiles. There is much evidence in support of GST expression as a key component of cellular defenses against cytotoxic and genotoxic damage (10). The GST superfamily catalyzes conjugation of a diverse array of different substrates, including electrophiles that are cytotoxic, genotoxic, or both (10,11). Induction of GST by chemopreventive agents is associated with reduced chemical-induced DNA damage and carcinogenesis in rodent models (2). Systematic investigations into the substrate selectivity of GSTs has demonstrated that human pi class GST (hGSTP) shows preferential activity for conjugation of the *anti*-diastereoisomers of BPDE (12). Expression of this isozyme has been demonstrated by our group and others to be protective against cytotoxicity or genotoxicity of carcinogens (13–15). Furthermore, genetic epidemiology studies involving GST polymorphisms have suggested that altered GST expression or human glutathione *S*-transferase P1 (hGSTP1) allelic variation may be associated with an increased risk of certain human cancers (16,17).

Previous studies in intact cells have focused either on P450 activation of B[*a*]P or on detoxification of the ultimate activated metabolites of B[*a*]P by GST expression. Our intent was to model the dynamic competition between the human cytochrome P4501A1 (hCYP1A1) activation pathway and the hGSTP1 detoxification pathway in order to assess GST-mediated protection against mutagenicity of B[*a*]P under more relevant conditions. The V79 Chinese hamster lung fibroblast cells previously engineered to express hCYP4501A1 alone (18) were further modified to express hGSTP1 by stable transfection. The cell lines were then exposed to B[*a*]P or the intermediate metabolite B[*a*]P-7,8-diol, and protection afforded by GST was determined for different biological endpoints, including mutagenicity at the *hprt* locus, whole cell macromolecular adduct formation and DNA adduct formation, including quantification of specific adducts by $^{32}$P-post-labeling analysis. The results of these studies have important implications for the roles of CYP activation versus GST detoxification in the induction and control of genotoxicity.

## Materials and methods

*Materials and chemicals*

Caution: The PAH and PAH metabolites described herein are potential chemical carcinogens and must be handled with care as outlined in the National Cancer Institute Guidelines.

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco BRL (Grand Island, NY). Fetal bovine serum was purchased from Cellgro/Mediatech (Herndon, VA). B[*a*]P and the pure enantiomers (+) and (−)-B[*a*]P-*trans*-7,8-dihydrodiol were purchased from Midwest Research Institute (Lexena, KS). $^{3}$H-B[*a*]P was purchased from American Radiolabeled Chemicals (ARC, St. Louis, MO). Puregene™ Cell Lysis Solution and Puregene™ Protein Precipitation Solution were purchased from Gentra Systems (Minneapolis, MN). All other reagents were of analytical grade and purchased from Fisher Scientific (Raleigh, NC) or Sigma (St. Louis, MO).

*Cell culture and cell lines*

The parental V79MZ Chinese hamster lung fibroblast cell line is described by Glatt *et al*. (19), and the modification of the V79MZ cell line to express human CYP1A1 has been described by Schmalix *et al*. (18). The V79MZ cell line expressing human CYP1A1 was stably transfected with a hGSTP1 expression vector by the method of calcium phosphate-mediated transfection, as described previously (14). The expression vector utilizes the CMV early promoter to drive expression of the downstream hGSTP1 cDNA and has a hygromycin resistance selectable marker gene. All cell lines were grown and maintained in DMEM medium supplemented with 5% FBS at 37°C in 5% $CO_2$. All hCYP1A1-expressing cells were subcultured at 1:20 every 2 days, and selected periodically with 400 μg/ml G418 (Cellgro/Mediatech, Herndon, VA), to select for retention of the *neo* resistance marker gene and also hCYP1A1 expression. Activities of hCYP1A1 and GST were assayed at least once a month to verify stable expression levels.

*Enzyme assays*

The GST assay is a modification of the method described by Habig (20). Cells were plated on 100 mm culture dishes and harvested at 70–80% confluence in cold phosphate-buffered saline (PBS), pelleted by centrifugation at 500 × *g*, sonicated in 50 mM Tris, 1 mM EDTA and centrifuged at 10 500 r.p.m. for 5 min to separate the cytosol from particulates. Briefly, 2–5 μl of the resulting supernatant was assayed at room temperature in a solution of 0.1 M $K_2PO_4$, pH 6.5, and 1 mM glutathione (GSH). The reaction was initiated with 1 mM (final concentration) 1-chloro-2,4-dinitrobenzene (CDNB) in ethanolic solution. Change in absorbance was monitored at 340 nm for 90 s. Activity was calculated using the ΔA/minute and extinction coefficient, and was reported as nmol/min/mg protein. Protein concentrations were determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

The CYP1A1 activity was assayed as ethoxyresorufin-*O*-deethylase (EROD) activity by the method of Burke *et al*. (21). Cell lysates of 150–200 μl were obtained as described for the GST assay and assayed in a volume of 1 ml containing 100 mM Tris/HCl (pH 7.4), 50 mM $MgCl_2$, 10 mM glucose-6-phosphate, 2 U/ml glucose-6-phosphate dehydrogenase, 20 μM dicumarol and 200 μM β-NADPH. Reactions were initiated by the addition of 10 μM (final) ethoxyresorufin and incubated at 37°C for 1 h with gentle shaking, and the reaction was stopped by the addition of 1 ml cold MeOH. Samples were centrifuged at 500 × *g* to remove any particulate matter and then read on a Perkin-Elmer LS-3B spectrofluorometer (Perkin-Elmer, Norwalk, CT) with excitation at 522 nm and emission at 586 nM, and activity calculated by comparison with a standard curve of resorufin, and normalized to protein content, measured by the BCA protein assay.

*hprt mutagenicity assay*

Cells were plated at a density of $5 \times 10^5$ cells/100 mm plate in the absence of G418 selection for 48 h dose-response studies, or $2.5 \times 10^5$ cells/100 mm plate for time-course studies. After allowing cells to adhere to the dishes overnight, cells were treated with 100, 300 or 600 nM B[*a*]P for a 48 h dose-response study, or 300 nM B[*a*]P for a 72 h time-course study. B[*a*]P was delivered to the cell cultures in DMSO, and all plates were adjusted to contain 0.1% DMSO. At the end of the exposure period, media containing PAH was removed, and 10 ml fresh medium was added and left on the cultures overnight. The next day, cells were then subcultured at $5 \times 10^5$ cells/100 mm plate and grown for 6 days, with one subculture at 3 days, to allow for phenotypic development. After this period, cells were subcultured once more and plated at a density of $5 \times 10^5$ cells/100 mm plate for 6-thioguanine (6-TG) selection (10 μg 6-TG/ml media) in order to select for *hprt* mutants. Cells were maintained under 6-TG selection for 10 days, with one change of media + 6-TG after 3 days, after which mutant *hprt* colonies were stained with 5 mM methylene blue in methanol and counted. The mutation frequency is expressed as mutant colonies per million cells.

*HPLC analysis of B[a]P metabolism*

Cells were plated at $5 \times 10^5$/100 mm plate in 10 ml complete medium 24 h before addition of 10 μl of 1 mM B[*a*]P in DMSO to each (1.0 μM final). Medium was harvested at the indicated times and frozen until analysis. B[*a*]P and metabolites were extracted by gravity flow through C18 solid-phase extraction columns (50 mg MFC18 mini-columns, IST/Argonaut, Redwood City, CA) that were preconditioned with 1 ml methanol and then 1 ml medium + 5% FBS. Columns were washed with 1 ml 10% acetonitrile in $dH_20$ and eluted with 2 × 0.5 ml 100% acetonitrile. The eluate was dried on a lyophilizer and re-dissolved in 50 μl acetonitrile, and 20 μl was injected onto a C18 column (Onyx 5 micron ODS, 150 × 4.6 mm; Phenomenex, Torrance, CA) attached to a Shimadzu VP HPLC system. The solvent gradient program was 40% acetonitrile for 2 min, then a linear gradient to 80% from 2 to 5 min, followed by a linear gradient to 100% from 5 to 20 min. The 100% acetonitrile was maintained for 7 min, and returned to 40% in preparation for the next sample. Peaks were identified by comparison of peak spectra with those of pure standards using a diode array detector, and by comparison of retention times with pure standards. Peak areas were quantified using a Hitachi F1000 fluorescence detector with excitation at 348 nm and detection at 395 nm. Retention times for B[*a*]P (22.5 min) and (+/−)-B[*a*]P-*trans*-7,8-dihydrodiol (14.4 min) were determined with standards, which were also used to calculate the absolute amounts in each sample based on relative fluorescence yield in comparison with standard curves determined for B[*a*]P or racemic (+/−)-B[*a*]P-*trans*-7,8-dihydrodiol. Correction factors were

applied to medium samples on the basis of observed recovery losses for each analyte during SPE extraction and drying/re-dissolution.

*Total DNA alkylation*

Cells were plated at a density of $2.5 \times 10^5$ cells/60 mm plate in the absence of G418 selection. Cells were allowed to attach at 37°C for 16–24 h in a humidified $CO_2$ incubator before use. For studies examining the binding of $^3$H-B[*a*]P (50 Ci/mmol) to DNA, the titrated material was dried from toluene before dissolving in DMSO and added to 3 ml pre-warmed DMEM to give 100 nM, and cell cultures were treated at a final concentration of 0.1 μM for 12, 24 and 48 h. After the exposure period, cells were scraped, washed in cold PBS and pelleted at 500 × *g*, and samples were stored at −20°C until ready for processing. Cell pellets were lysed using 300 μl Puregene™ Cell Lysis Solution (Gentra Systems). Lysates were then treated with 20 μg/ml DNase-free RNase (Gentra Systems) for 30 min at 37°C. After cooling on ice for 1 min, lysates were treated with 100 μl Puregene™ Protein Precipitation Solution (Gentra Systems) with vigorous vortexing. Samples were then centrifuged at 14 000 × *g* to pellet protein. Supernatants were recovered and DNA precipitated with 300 μl of 100% isopropanol. DNA was pelleted in a microcentrifuge at 14 000 × *g* for 10 min, then rinsed with 70% ethanol, pelleted again and then resuspended in 400 μl TE buffer. Proteinase K (0.1 mg/ml) and SDS (0.1%) were added and the samples were digested for at least 4 h at 50°C, then extracted sequentially with equal volumes of phenol saturated with 10 mM Tris/HCl, pH 7.5, then phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and then $CHCl_3$. DNA was re-precipitated by addition of 40 μl 3 M sodium acetate, pH 5, and 1.0 ml 100% ethanol. DNA was pelleted, rinsed with 70% ethanol, pelleted again and then resuspended in 800 μl TE buffer and quantified by absorbance at 260 nm. DNA was quantified spectrophotometrically by absorbance at 260 nm, and radioactivity was analyzed by liquid scintillation counting; then adduct levels were calculated from the specific activity and expressed as pmol/mg DNA. This method gave similar results and less variation than purification of DNA by two other protocols, including extended digestion with proteinase K followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1), or isopyknic CsCl gradient ultracentrifugation. The Student's *t*-test was used to determine *P*-values.

*Total cellular macromolecular adducts*

Cell pellets were initially resuspended in 200 μl $ddH_2O$ and then sonicated for 20 s, and 20 μl was taken for protein concentration analysis. To the remaining volume, 500 μl cold methanol + 1% perchloric acid was added. The suspension was incubated on ice for 10 min and then 700 μl hexane was added, and the hexane/MeOH suspension was mixed by inversion for 15 min. Cells were pelleted at 14 000 × *g* and pellets were resuspended in 600 μl 70% MeOH/1% perchloric acid, followed by a 20 s sonication. Hexane (600 μl) was then added and the suspension was once again mixed by inversion for 15 min. The suspension was pelleted at 14 000 × *g* and resuspended in 500 μl 70% MeOH, followed by 20 s sonication. Suspensions were analyzed by scintillation counting and final DPM normalized to milligram protein as determined by BCA assay. The Student's *t*-test was used to determine *P*-values.

*$^{32}$P-post-labeling of DNA*

The samples were analyzed by a nuclease P1-mediated $^{32}$P-post-labeling method with modifications (22,23). Briefly, 10 μg DNA was digested with micrococcal nuclease and spleen phosphodiesterase for 5 h at 37°C, and 2 μg was removed and used for calculation of total nucleotide levels. The remaining 8 μg was treated with nuclease P1 for 1 h at 37°C. The enriched adducts were labeled with 80 μCi [γ-$^{32}$P]-ATP in the presence of. T4 polynucleotide kinase. Labeled adducts were resolved by multidirectional PEI-cellulose TLC with the following solvents: D1 = 1.4 M sodium phosphate, pH 5.8, onto a Whatman #17 wick; D3 = 4 M lithium formate : 8.5 M urea; D4 = isopropanol : 4 M ammonium hydroxide (1 : 1); D5 = 1.7 M sodium phosphate, pH 5.8. Adducts were visualized using a Packard InstantImager. Total nucleotides were analyzed by labeling 2.0 ng of non-enriched digest in parallel with adducts, and normal nucleotides resolved with 0.5 M acetic acid and 2N formic acid solvent following conversion to monophosphates by treatment with nuclease P1. Relative Adduct Labeling was calculated and the adduct levels were expressed as adducts/$10^9$ nt.

## Results

*Transgenic cell lines*

The human GSTP1 was stably expressed after transfection into the hCYP1A1-expressing V79MZh1A1 cell line (18). The GST specific activity of the dual-transfectant line (V79MZh1A1/P1-23) was 2881 ± 508 nmol/min/mg (Table I). Background GST activity was <400 nmol/min/mg in V79MZ and V79MZh1A1 cells, owing to relatively low level expression of hamster GSTP1, an isozyme previously shown to have negligible activity for conjugation of BPDE in V79 cells (24). CYP1A1 activities measured in the EROD assay were comparable across all cell lines expressing the CYP1A1 transgene (Table I). Parental V79 cells have no significant CYP activity, and CYP1A1 is not inducible in this cell line (18).

**Table I.** Specific activities of human CYP1A1 and hGSTP1 expressed in the transgenic cell lines

| Cell line | Genes expressed | CYP1A1 (EROD) specific activity (pmol/min/mg) | GSTP1-specific activity (nmol/min/mg) |
|---|---|---|---|
| V79MZ (control) | No transgenes | 0.23 ± 0.22 | 385 ± 112 |
| V79MZh1A1 | hP4501A1 | 15.8 ± 1.9 | 359 ± 133 |
| h1A1/hGSTP1-23 | hP4501A1 + hGSTP1 | 15.9 ± 1.4 | 2881 ± 508 |

Cytochrome P4501A1 activity was measured by the EROD assay, and GST was measured using CDNB as substrate, as described in Materials and methods. Results are the average of at least three independent determinations.

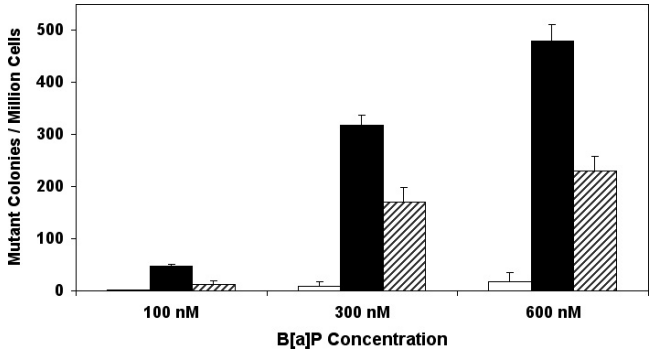


**Fig. 1.** Mutagenicity of B[*a*]P at different concentrations in transgenic cell lines. V79MZ cells genetically modified to express hCYP1A1 (V79MZh1A1), or V79MZh1A1 cells further modified to express both hCYP1A1 and hGSTP1 (V79MZh1A1/P1-23) were exposed to 100, 300 or 600 nM B[*a*]P for 48 h, and the *hprt* mutagenicity assay was performed as described in Materials and methods. Legend: open bars, V79MZ control; filled bars, V79MZh1A1; cross-hatched bars, V79MZh1A1/hGSTP1-23. Results are the mean ± standard deviation (SD) of at least four independent assays.

*Mutagenicity of B[a]P and pure enantiomeric B[a]P-7,8-dihydrodiols*

Mutagenicity was measured by frequency of mutation at the *hprt* locus following exposure of the cells to B[*a*]P. Treatment with 100, 300 and 600 nM B[*a*]P indicated that the frequency of mutation is dependent upon the dose of B[*a*]P administered. The results indicated a highly significant 2-fold protection against mutagenicity at the *hprt* locus by co-expression of hGSTP1 in cells expressing hCYP1A1 at 300 or 600 nM, and 3.6-fold protection at 100 nM (Figure 1, $P \leq 0.0006$). Time-course studies shown in Figure 2 demonstrated that induction of mutagenicity at 300 nM B[*a*]P was time-dependent and confirmed that protection afforded by hGSTP1 expression was highly significant at all time points ($P \leq 0.004$). Mutant formation was readily

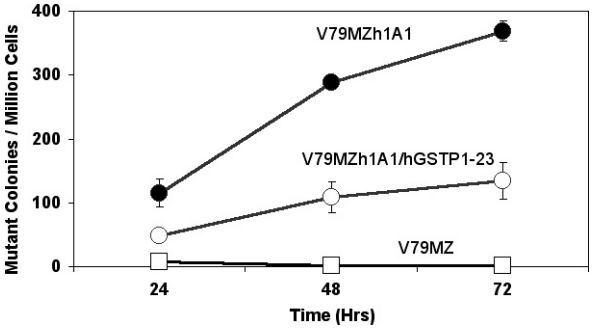


**Fig. 2.** Time course of B[*a*]P mutagenicity in transgenic cell lines. V79MZ cells genetically modified to express hCYP1A1 (V79MZh1A1), or V79MZh1A1 cells further modified to express both hCYP1A1 and hGSTP1 (V79MZh1A1/P1-23) were exposed to 300 nM B[*a*]P for the indicated times, and the *hprt* mutagenicity assay was performed as described in Materials and methods. Symbols: open squares, V79MZ control; filled circles, V79MZh1A1; open circles, V79MZh1A1/hGSTP1-23. Results are the mean ± SD of at least three independent assays.

detectable after 24 h, and began to plateau over the course of 48–72 h, probably due to depletion of the parent B[*a*]P from the cell culture medium, as described below.

Cells were also exposed to the pure enantiomers of the 7,8-dihydrodiol metabolites of B[*a*]P, (+)-B[*a*]P-7,8-diol and (−)-B[*a*]P-7,8-diol, in order to determine protective efficacy of GST expression against mutagenicity of these compounds. The mutagenicity of the B[*a*]P-7,8-diols increased in proportion to concentration over a range from 3 to 100 nM of either the (+)-B[*a*]P-7,8-diol or the (−)-B[*a*]P-7,8-diol (Figure 3A and B). The (−)-B[*a*]P-7,8-diol yielded a greater number of mutants than the (+)-B[*a*]P-7,8-diol at each concentration tested, consistent with earlier reports that (−)-B[*a*]P-7,8-diol is the precursor of (+)-*anti*-BPDE, the most mutagenic and carcinogenic among the four enantiomers (25). The degree of protection by hGSTP1 was 3- to 5-fold for both B[*a*]P-7,8-diol enantiomers in the range of 3 to 30 nM, and at 100 nM (+)-B[*a*]P-7,8-diol, but decreased to 2-fold protection in cells exposed to 100 nM (−)-B[*a*]P-7,8-diol. Differences were significant for comparison of V79MZh1A1 versus V79MZh1A1/hGSTP1-23 in cells exposed to (+)-B[*a*]P-7,8-diol ($P \leq 0.006$) or for (−)-B[*a*]P-7,8-diol ($P \leq 0.003$).

*Analysis of B[a]P metabolites in culture medium*

Cell culture medium was analyzed by reverse-phase HPLC over a 48 h time period after addition of 1.0 μM B[*a*]P. The concentration of B[*a*]P decreased ~30% within the first half hour after addition to medium, in the presence or absence of cells, from 1.0 μM to ~0.7 μM (Table II, legend). Comparison of medium added to the polystyrene culture plates with medium kept in polypropylene tubes indicated that the initial loss of B[*a*]P was due to binding to the culture dish plastic. This loss due to binding continued at a slower rate and stabilized at 70–75% decrease in B[*a*]P in the medium of the unmodified V79MZ cells after 24 h (Table II). Experiments with [$^3$H]-B[*a*]P confirmed rapid initial loss from the medium and indicated that <2% of the labeled B[*a*]P was associated with cells harvested from the plates after a 30 min incubation (data not shown). In contrast, nearly all of the unmodified B[*a*]P in solution was consumed from the medium by 48 h in both of the cell lines expressing hCYP1A1. Interestingly, the depletion of B[*a*]P from the medium was significantly faster and more complete with cells expressing both hCYP1A1 and hGSTP1 than with cells expressing only hCYP1A1, even though the hCYP1A1 expression levels were the same. The intermediate B[*a*]P-7,8-diol metabolite initially formed at a similar rate for the first 12 h in both the V79MZh1A1 and the V79MZh1A1/hGSTP1-23 cell lines, and then decreased to nearly undetectable levels over the next 36 h in the hCYP1A1 + hGSTP1-expressing cells, but persisted at higher levels in the cells

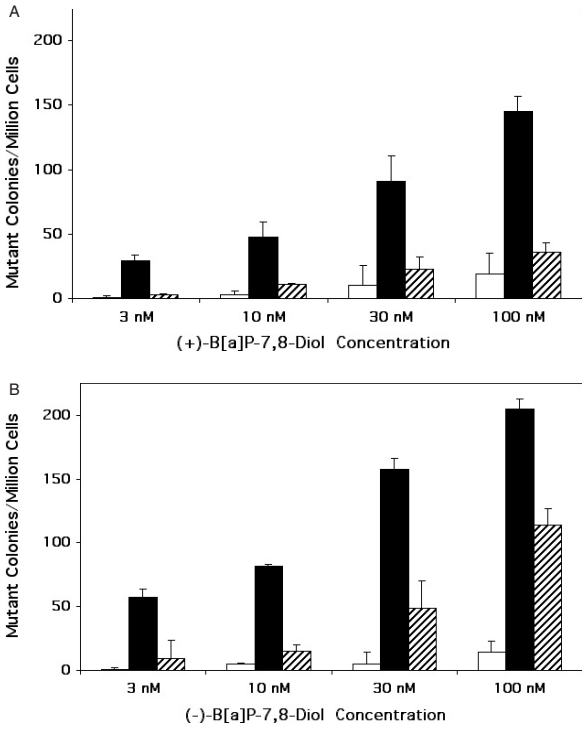


**Fig. 3.** Mutagenicity of B[*a*]P-7,8-dihydrodiol enantiomers at different concentrations in transgenic cell lines. V79MZ cells (open bars) genetically modified to express hCYP1A1 (V79MZh1A1; filled bars), or V79MZh1A1 cells further modified to express both hCYP1A1 and hGSTP1 (V79MZh1A1/P1-23, cross-hatched bars) were exposed to 3, 10, 30 or 100 nM (+)-B[*a*]P-7,8-diol (**A**) or (−)-B[*a*]P-7,8-diol (**B**) for 48 h, and the *hprt* mutagenicity assay was performed as described in Materials and methods. Results are presented as the mean ± SD of at least three independent experiments.

**Table II.** Metabolism of B[*a*]P by transgenic cell lines expressing human CYP1A1 without or with hGSTP1

| Cell line | 12 h | 24 h | 36 h | 48 h |
|---|---|---|---|---|
| B[*a*]P (pMol/plate) | | | | |
| V79MZ | ND | 3089 ± 550 | ND | 2419 ± 606 |
| CYP1A1 | 974 ± 134 | 547 ± 75 | 209 ± 22 | 145 ± 76 |
| 1A1 + GSTP1 | 1107 ± 291 | 189 ± 95 | 46 ± 8 | 23 ± 5 |
| B[a]P-7,8-diol (pMol/plate) | | | | |
| V79MZ | ND | 0.3 | ND | 0.3 |
| CYP1A1 | 83 ± 13 | 96 ± 4 | 83 ± 10 | 57 ± 24 |
| 1A1 + GSTP1 | 70 ± 7 | 37 ± 4 | 14 ± 4 | 1.8 ± 0.8 |

Cells were exposed to 1.0 μM B[a]P, and cell culture medium (10 ml) was harvested at the indicated times and processed for HPLC analysis as described in Materials and methods. Medium from V79MZ control cells contained 6985 ± 917 pMol B[a]P (~ 0.7 μM) at 30 min after addition to the plates. Results, expressed as the total pMol/plate, are the mean ± SEM of three independent experiments. ND, not determined.

**Table III.** $^{32}$P-post-labeling analysis of DNA adducts

| Cell line | Treatment time (hours) | N2-Gua-BPDE adducts/$10^9$ nt |
|---|---|---|
| V79MZh1A1 | 12 | 177 |
| V79MZh1A1/hGSTP1-23 | 12 | 19.2 |
| V79MZ (control) | 24 | 0 |
| V79MZh1A1 | 24 | 111 |
| V79MZh1A1/hGSTP1-23 | 24 | 2.8 |
| V79MZh1A1 | 48 | 67.7 |
| V79MZh1A1/hGSTP1-23 | 48 | 2.4 |

Cells were exposed to 100 nM B[a]P for 12, 24 or 48 h and then DNA was isolated and analyzed as described in Materials and methods. No adducts were detected in B[a]P-treated parental V79MZ cells without hCYP1A1 at 24 h (also see Figure 6), nor in untreated V79MZh1A1 or V79MZh1A1/P1-23 cells (not shown). Results are the average of two experiments with similar results.

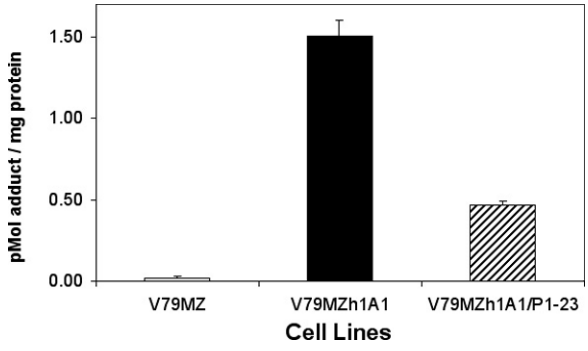


**Fig. 4.** Total cellular macromolecular alkylation in transgenic cell lines exposed to 3H-B[*a*]P. V79MZ cells genetically modified to express hCYP1A1 (V79MZh1A1), or V79MZh1A1 cells further modified to express both hCYP1A1 and hGSTP1 (V79MZh1A1/P1-23) were exposed to 100 nM $^3$H-B[*a*]P for 48 h, and total cellular adducts were measured as described in Materials and methods. Results are presented as the mean ± SD of at least three independent experiments.

with only hCYP1A1 expression even at 48 h (Table II). Significantly, the amount of B[*a*]P-7,8-diols available over the exposure period was 2.6-fold less in the cells with both hCYP1A1 and hGSTP1 expression, a reduction that is similar to the fold protection against mutagenicity. A similar reduction in the B[*a*]P-9,10-diols was observed, but the absolute amounts were ~5-fold lower (data not shown).

### *Total cellular macromolecular and DNA adducts of [$^3$H]-B[a]P*

The accumulation of covalent adducts of B[*a*]P was examined initially by exposing cells to 100 nM [$^3$H]-B[*a*]P over a 48 h period. Expression of hGSTP1 resulted in a 74% (3.8-fold) reduction in total cellular covalent macromolecular adducts in cells expressing hCYP1A1 (Figure 4, $P \leq 0.0004$). This was closely correlated with the reduction in B[*a*]P mutagenicity by hGSTP1, although the adducts in isolated DNA accounted for only 5% of the total bound radioactivity in the V79MZh1A1 cell line.

We next examined the ability of B[*a*]P to form DNA-binding species following exposure of cells to 100 nM [$^3$H]-B[*a*]P for 12, 24 or 48 h (Figure 5). Surprisingly, there was little or no protection by hGSTP1 against the binding of the $^3$H-B[*a*]P to DNA at 12 h ($P > 0.3$), and only a 24% reduction at 48 h ($p < 0.02$). The expression of hGSTP1

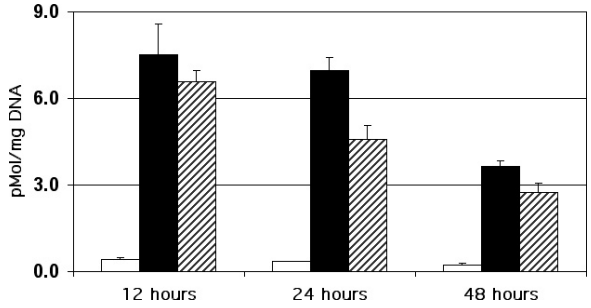


**Fig. 5.** Total DNA adducts in transgenic cell lines exposed to 3H-B[*a*]P. Cells were exposed to 100 nM $^3$H-B[*a*]P for the indicated times, DNA was isolated and analyzed as described in Materials and methods, and results were expressed as pMol/mg DNA. Legend: open bars, V79MZ control; filled bars, V79MZh1A1; cross-hatched bars, V79MZh1A1/hGSTP1-23. Results are the mean ± SD of at least three independent experiments.

reduced DNA binding by 33% at 24 h ($P < 0.005$), but this was still a smaller reduction than expected on the basis of the results of the mutagenicity studies. This result was verified after purification of DNA by two other methods, CsCl isopyknic gradient ultracentrifugation or the standard exhaustive proteinase K digestion and phenol/chlororform/isoamyl alcohol extraction, with comparable results (data not shown).

### *$^{32}$P-post-labeling of DNA adducts*

The results of the DNA-binding studies led us to further investigate the formation of DNA adducts in this model system. The sensitive $^{32}$P-post-labeling technique was employed to identify and quantify specific DNA adducts formed from B[*a*]P in these cells. One major DNA adduct was detected in DNA from V79MZh1A1 cells that was identified by co-migration compared with known standards as N2-Gua-BPDE, the known principal stable DNA adduct formed from BPDE (Figure 6D). This adduct was virtually eliminated (>95% reduction) at 24 or 48 h, while the reduction at 12 h was 89% in V79MZh1A1 cells expressing hGSTP1-1 as compared with cells expressing only hCYP1A1 (Table III and Figure 6F). A minor adduct was also detected, which also disappeared in V79MZh1A1 cells expressing hGSTP1-1. No adducts were detected in the original V79MZ cells without hCYP1A1 (Figure 6B), nor in the untreated cell lines (Figure 6A, C and E). The adduct levels observed using the $^{32}$P-post-labeling method were ~6-fold lower than adducts detected by the [$^3$H]-B[*a*]P covalent binding assay. These results indicate that while the [$^3$H]-B[*a*]P binding assay is measuring other adducts in addition to the major stable N2-Gua-BPDE adduct detected in the post-labeling assay, much of the reduction by hGSTP1 in [$^3$H]-B[*a*]P DNA adducts is accounted for by the strong prevention of the highly mutagenic N2-Gua-BPDE adduct.

## Discussion

A large body of evidence indicates that expression of the Phase II enzyme GST is protective against the genotoxic or cytotoxic effects of various carcinogenic electrophiles (10,11). Experimental evidence from our laboratory demonstrated that GST expression alone was protective against DNA modification (13) but not cytotoxicity of 4-nitroquinoline-1-oxide (NQO). Further studies by Morrow *et al*. (4) also demonstrated that GSH conjugate efflux (phase III), for example by

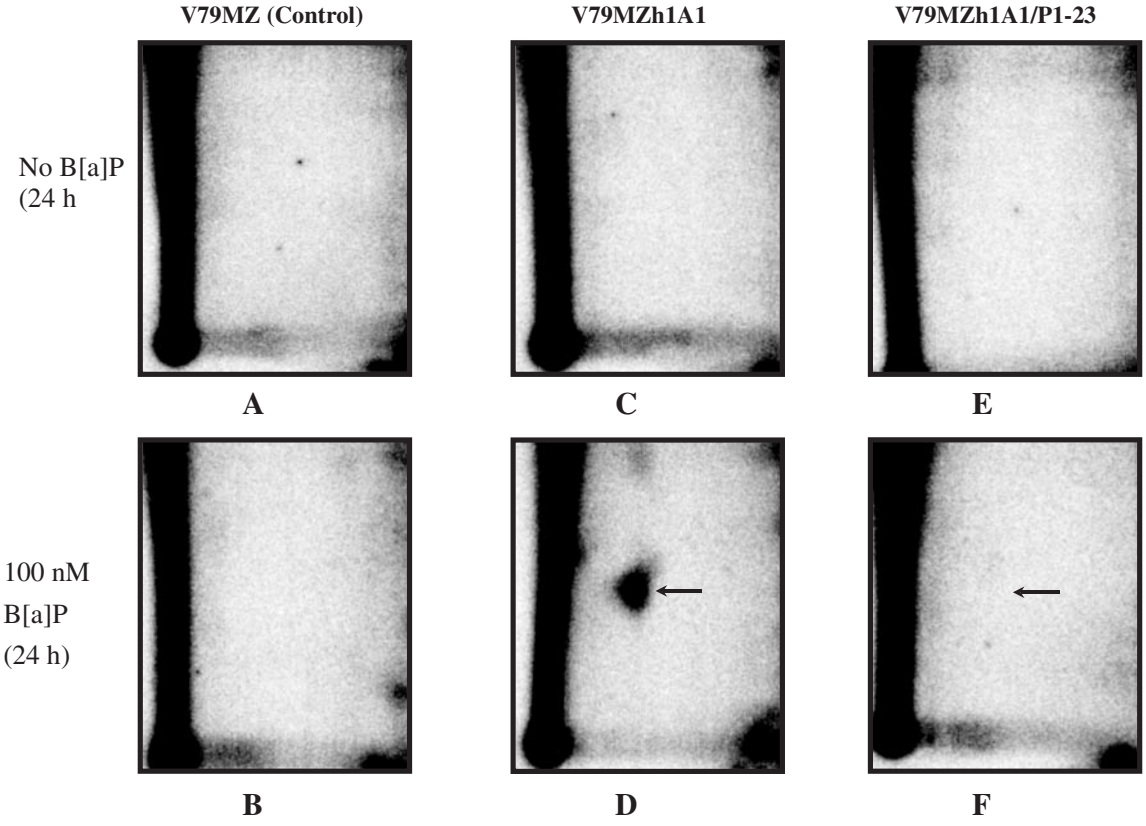


**Fig. 6.** Analysis of B[*a*]P-DNA adducts in transgenic cell lines by 32P-post-labeling. Cells were treated with vehicle (upper figures) or 100 nM B[*a*]P (lower figures) for the indicated times, and DNA was isolated and adducts were identified and measured by $^{32}$P-post-labeling as described in Materials and methods. Cell lines: (**A** and **B**) V79MZ control; (**C** and **D**) V79MZh1A1; (**E** and **F**) V79MZh1A1/hGSTP1-23. Arrow, migration position of the N2-guanine-BPDE adduct.

the multidrug transport protein MRP-1, a glutathione conjugate transport ATPase, was necessary to confer protection against lethality of NQO. We have most recently examined the competitive dynamics of the interaction between Phase I activation versus Phase II detoxification in a transgenic co-expression model system, which allows variables to be tightly controlled. We developed stably transfected cell lines for investigation of protection against the genotoxicity of PAH by co-expression of hGSTP1 in direct competition with Phase I activation by hCYP1A1 in the same cell. These were used to test whether expression of hGSTP1-1 is effective for protection against adduct formation or mutagenicity induced by B[*a*]P, or enantiomers of the stable metabolic intermediate B[*a*]P-7,8-diol, in the context of ongoing activation by hCYP1A1.

Mutagenicity induced by B[*a*]P in the V79MZh1A1 cell line was both time- and dose-dependent, and the protection conferred by expression of hGSTP1 was ~2- to 3-fold at each concentration of B[*a*]P tested and at each time point examined in the time-course study. Protection by hGSTP1 against mutagenicity of B[*a*]P-7,8-diols ranged higher, up to 5-fold protection for both enantiomers. This is consistent with a previous report by Sundberg et al (12) that showed that hGSTP1 exhibited comparable Kcat/Km values for conjugation of (+)-*anti*-BPDE and (+)-*syn*-BPDE, products derived from the (−)-B[*a*]P-7,8-diol and the (+)-B[*a*]P-7,8-diol, respectively. The protection against mutagenicity is also consistent with the lower amounts of both B[*a*]P and B[*a*]P-7,8-diol from 24 to 48 h in the medium of cells expressing hGSTP1 together with hCYP1A1, as compared with the cells with only hCYP1A1 expression. Particularly noteworthy is the fact that the total amount of B[*a*]P-7,8-diols measured in medium over the 48 h exposure period is 2.6-fold lower in the cells with hGSTP1 expression, a reduction that correlates well with the decrease in mutagenicity and total cellular adducts of B[*a*]P in these cells. Since the (+/−) B[*a*]P-7,8-diols are the immediate precursors of the ultimate mutagenic species BPDE, and since their formation from B[*a*]P is rate-limiting for formation of BPDE, the reduction in the amount of this intermediate species is likely to explain much if not all of the protection against mutagenicity. The reason for the faster clearance of B[*a*]P and the decrease in B[*a*]P-diol levels was not immediately clear, since the hCYP1A1 activities were nearly identical in the two cell lines. One possibility is that inactivation of the ultimate reactive species BPDE by conjugation accelerates metabolism of both B[*a*]P and the 7,8-diol by preventing BPDE from inhibiting hCYP1A1. Thus, the increased activation enabled by hGSTP1 expression may actually partially attenuate the protection it confers via conjugation. An alternative potential explanation for the reduction in B[*a*]P-diol may be that the B[*a*]P-7,8-oxide intermediate may itself be conjugated by hGSTP1, preventing formation of B[*a*]P-7,8-diol. This could also prevent competitive inhibition of hCYP1A1 activation of B[*a*]P by B[*a*]P-7,8-diol, enabling faster clearance of B[*a*]P.

The observed protection against mutagenicity, while substantial, was considerably lower than the 16-fold resistance to B[*a*]P cytotoxicity observed in our previous experiments

(S.L. Kabler, C.S. Morrow, J. Doehmer and A.J. Townsend, manuscript in preparation). Therefore, we examined alkylation of total cellular macromolecules to compare the extent of protection by hGSTP1 against either protein or DNA adducts. Measurement of [$^3$H]-B[*a*]P bound to total cellular macromolecules, primarily protein, in cells exposed to [$^3$H]-B[*a*]P demonstrated that adduct formation was dependent on the presence of hCYP1A1, and also showed that protection by hGSTP1 expression against total cellular macromolecular adducts correlated well with the decrease in mutagenicity, exhibiting a 74% reduction relative to cells expressing only hCYP1A1. Surprisingly, however, measurement of the [$^3$H]-B[*a*]P DNA binding, which account for only 5% of the total cellular adducts in the V79MZh1A1 cell line, indicated less than half of the expected reduction by hGSTP1 at 48 h of exposure. This was even more surprising in light of the results of the $^{32}$P-post-labeling analysis of DNA adducts, which showed nearly complete prevention of the N2-Gua-BPDE adduct. Thus, if this were the principal mutagenic stable DNA adduct, as reported in other systems, we would expect ~3-fold (60–70%) reduction by hGSTP1 expression, rather than the >20-fold reduction observed in this key adduct.

There are several possible explanations for the lack of a strong correlation between DNA adducts and mutagenicity. One possibility is that hGSTP1 may most effectively prevent the formation of the most highly mutagenic lesions that may comprise a small fraction of the total stable DNA adducts, via conjugation of a subset of reactive metabolites. The adducts that remain, and are measured in cells expressing hGSTP1 as two-thirds or more of the [$^3$H]-labeled DNA observed in the control cell line, would thus be presumed to be less effectively detoxified by hGSTP1 but also proportionally less mutagenic, accounting for only the remaining one-third or less of the mutations. This explanation is supported by the differential results with the two methods of analysis of B[*a*]P metabolites bound to DNA. A second possibility could be that a subset of adducts formed from metabolites that are substrates of hGSTP1 may be rapidly removed by a promutagenic repair process (26). Thus, if hGSTP1 prevented the formation and promutagenic repair turnover of a key subset of adducts, this might reflect a disproportionate reduction in mutation at a number of damage sites that would not be equally represented in the measurable adduct pool. The remaining poorly repaired and also less mutagenic stable adducts would constitute the bulk of the measured [$^3$H]-labeled DNA adducts. A variation on this theme is that the lack of correlation could be due to poor prevention by hGSTP1 of a portion of the mutagenic damage caused by unstable adducts or abasic sites induced by B[*a*]P metabolites formed via an alternate one-electron oxidation pathway (27), which again would not be proportionately reflected in changes in the stable adduct pool. Finally, the close correlation between mutagenicity and total cellular adducts raises the interesting possibility that damage to cellular protein might also contribute to mutagenicity, for example, by reducing the fidelity of replication through damage to key enzymes, or by inhibiting the action of enzymes involved in DNA replication, processing or repair (28).

The (+)-*anti*-BPDE metabolite is thought to be responsible for the majority of the mutagenicity seen in bacterial and mammalian cell culture models (29), and for tumorigenicity in animals (30). Studies with purified hGSTP1-1 have shown that this metabolite is an excellent substrate (12), and the data presented in Figure 6 clearly demonstrate that formation of the adduct from this species is almost completely prevented in intact cells expressing hGSTP1. The BPDE adducts detected by $^{32}$P-post-labeling accounted for ~15% of the total [$^3$H]-B[*a*]P binding to DNA, which is approximately equal to the reduction in the latter in cells expressing hGSTP1. This also corresponds with the reported fraction of B[*a*]P metabolites formed by hCYP1A1-mediated metabolism via initial epoxidation at the 7,8-position (31). Thus, in this system the major mutagenic B[*a*]P metabolite is effectively detoxified by hGSTP1-1, but other potentially mutagenic species that are not subject to GST-mediated GSH conjugation remain bound to DNA. These results support the value of GST expression in protection against mutagenicity of B[*a*]P and its B[*a*]P-7,8-diol metabolites, but also indicate that a significant component of B[*a*]P mutagenesis resulting from hCYP1A1-mediated activation to reactive electrophiles may escape detoxification by GST-mediated conjugation. Further studies will be necessary to determine the nature of the poorly conjugated metabolites and DNA adducts formed from them, and whether they can be detoxified by other protective pathways or if they avoid metabolic detoxification altogether.

## Acknowledgements

Supported in part by the following grants from NIH: ES10175 and CA77114.

*Conflict of Interest Statement*: None declared.

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*Received July 19, 2005; revised May 16, 2006; accepted June 23, 2006*