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Effects of Chronic Ethacrynic Acid Exposure on Glutathione Conjugation and MRP Expression in Human Colon Tumor Cells

Paul J. Ciaccio,* Hongxie Shen,* Gary D. Kruh,† and Kenneth D. Tew*

**Department of Pharmacology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111 and †Medical Oncology, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111*

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Chronic exposure to ethacrynic acid of a subcloned HT29 human colon cancer cell line produces a 3- to 4-fold increase in the level of resistance to this agent. The resistant cells (HT6-8) have an enhanced capacity to metabolize the parent drug and efflux it from the cell. This is reflected in a 5-fold enhanced decompositioning rate constant for ethacrynic acid in HT6-8 (3.47×10^{-3} min^{-1}) versus wild type cells (1.58×10^{-2} min^{-1}). We observed that the glutathione conjugate of ethacrynic acid is an effective competitive inhibitor for binding to the multidrug resistance-associated protein by [^{35}S]azidophenacyl-glutathione, a photoaffinity analog of glutathione. In addition, the HT6-8 cells overexpressed multidrug resistance-associated transcript 2- to 3-fold. These results suggest that resistance to ethacrynic acid results from a concerted, coordinate increase in defense mechanisms which detoxify the drug and remove its conjugate via plasma membrane efflux.

Inductive stress responses underlie the mechanisms by which cells express resistance to drugs and xenobiotics. In most cases, natural selection will favor a pleiotropic enhancement of the cell's detoxification machinery as a means of responding to an acute or chronic chemical threat. We have a long standing interest in a plant phenolic acid, ethacrynic acid (EA), as an example of a drug which elicits a complex response in tumor cells (1). EA has an α,β-unsaturated carboxyl moiety which gives the compound Michael addition properties and results in binding to cellular nucleophiles. The drug is conjugated to GSH via GST catalysis (2). Both the parent molecule and the EA-SG conjugate are reversible inhibitors (non-competitive and competitive, respectively) of the three major cytosolic GST families π, α and μ (3). EA can also bind covalently with GSTπ, but the binding is reversible since EA is transferable to other cellular sulfhydryls (3). Because of the reactivity of EA-SG conjugates, their removal from the cell milieu would serve a potentially important detoxification function.

We previously characterized HT6-8, an EA-resistant cell line which grows in 72 μM EA (approximately 4-fold resistant compared to wild type HT4-1). These cells were originally found to overexpress GSTπ and GSH (5). More recently we reported that a number of other detoxification gene products, including γ-GCS (the rate-limiting enzyme in *de novo* GSH biosynthesis) and three distinct DDH's are elevated (6,7). These findings underscore the pleiotropic nature of the cell response and the idea that chemicals which upregulate detoxification proteins are often substrates of them. Moreover, it is now apparent that MRP, a member of the ABC transporter family (8), can function as an efflux pump for the GSH conjugates of endogenous and exogenous molecules (9,10,11). Because EA detoxification involves coordination of GST catalysis with GSH as a cofactor, and cellular efflux of the conjugate, we have designed a series of experiments to determine the extent by which EA metabolism and MRP-mediated efflux governs the acquired resistance phenotype associated with this agent.

Abbreviations: [^{35}S]APA, [^{35}S]azidophenacyl-glutathione conjugate; ARE, antioxidant response element; EA, ethacrynic acid; EA-SG, ethacrynic acid glutathione conjugate; DDH, dihydrodiol dehydrogenase(s) (EC1.3.1.20); γ-GCS, γ-glutamylcysteine synthetase; GSH, glutathione; GST, glutathione S-transferase(s) (EC2.5.1.18); MRP, multidrug resistance-associated protein.

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MATERIALS AND METHODS

Cell lines. HT4-1 and HT6-8 are lines cloned from the parental HT29 human colon carcinoma cell line. HT6-8 are maintained in 72 μM EA and express 3- to 4-fold resistance to the drug (5). HL50 AR cells (12) were used for photolabeling experiments.

Metabolism of EA. To monitor the spontaneous or cell-mediated metabolism of EA, an isocratic reversed-phase chromatography method was used. This method was optimized for measuring the disappearance of EA from serum, where drug concentrations in the range of 1 to 150 μM were standard (13,14). For spontaneous conjugation, 200 μM EA was incubated in serum-free medium with 5 mM GSH. Cell pellets (5×10^6 cells/assay) were prepared from cells treated with 200 μM EA for 1 hr. At specific time points cells were washed free of unincorporated EA, trypsinized and pelleted by centrifugation. Organic extraction procedures and HPLC conditions were performed as described previously by LaCreta et al. (13).

Synthesis of drug-GSH conjugates. [^{35}S]APA-SG was prepared as described (15), with modifications. Direct exposure to light was avoided during operations, and steps prior to thin layer chromatography were conducted under nitrogen or with nitrogen-saturated solutions. Two hundred and fifty μCi of [^{35}S]GSH (517.3 Ci/mmol, NEN, Boston, MA) was freed of dithiothreitol by ethyl acetate extraction, and added to a reaction mixture containing potassium phosphate buffer (50 mM, pH 7.4), 4-azidophenacylbromide (10 mM), GSH reductase (120 mU) and NADPH (1 mM). The reaction was allowed to proceed at room temperature for 1 hr and the products were separated by Silica G thin layer chromatography using 1-propanol/water (7:3, v/v) as developer. The radioactive GSH conjugate was located by autoradiography, scraped off the plate and extracted with water. After filtration through a 0.2 μm Gelman filter, the extract was concentrated under nitrogen.

EA-SG was synthesized as described by Ploemen et al. (3) with slight modification. Fifty milligrams of GSH in 5 ml of water was slowly mixed with 100 mg of EA in 10 ml of ethanol/water (1:1) containing 0.5 ml of saturated $NaHCO_3$ solution. The reaction was allowed to proceed for 48 hr at room temperature with continuous stirring. The solvents were then removed by evaporation and the residue was dissolved in 5 ml of saturated $KHCO_3$. The product was then precipitated by adding 1% H_3PO_4, and lyophilized. The powder was redissolved in 1 ml of 10 mM Tris-HCl (pH 7.4) and applied onto a Silica G thin layer chromatography plate that was developed in acetonitrile/water (7:2 (v/v)). The product was extracted from the gel and lyophilized.

Preparation of membranes for photoaffinity labeling. Cells were harvested from HL60 AR cells (12) by centrifugation at 1200 X g for 10 min, and washed twice in ice cold phosphate buffered saline. The cell pellet was diluted 10 fold with hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$), supplemented with protease inhibitors (0.1 mM PMSF, 1 mM Leupeptin, 0.3 mM Aprotinin), and gently stirred on ice for 10 min. The suspension was then homogenized in a chilled Potter-Elvehjem homogenizer using 30 strokes of the pestle. The homogenate was centrifuged at 4000 X g at 4°C for 10 min to remove nuclei and non-lysed cells, and the resulting supernatant was centrifuged at 100,000 X g for 1 hr at 4°C. The pellet was then resuspended in 10 mM Tris-HCl (pH 7.6)/250 mM sucrose containing protease inhibitors, aliquotted and stored at −80°C.

Labeling of membrane proteins with [^{35}S]*APA-SG.* Membrane preparations (100 μg of protein) were incubated in 100 mM AT-125/10 mM Tris-HCl (pH 7.4)/250 mM sucrose for 30 min at 4°C. For competition studies, membrane preparations were incubated for an additional 30 min at 4°C after the addition of various concentrations of competitors. The labeling reaction was conducted in a final volume of 100 μl, and initiated by the addition of [^{35}S]APA-SG (3 μCi). After 30 min of incubation at 4°C in the dark, the samples were quickly photolyzed in a Stratagene UV Stratalinker (energy = 200,000 μJ). Following photolysis, SDS-PAGE sample buffer containing 1% β-mercaptoethanol was added and the labeled proteins analyzed by 7% SDS-PAGE and autoradiography.

Northern blot analysis. Total RNA was extracted from cell pellets by the guanidinium isothiocyanate-phenol-chloroform extraction method. EA and reagents for RNA extraction were purchased from Sigma (St. Louis, MO). Twenty microgram samples of total RNA were denatured, electrophoresed, and transferred onto a Magna nylon membrane (Micron Separations, Inc., Westboro, MA). Membranes were hybridized with an MRP probe consisting of nucleotides 3574 to 4254 of the cDNA (8). Hybridization with a probe for the 36B4 estradiol-independent ribosomal phosphoprotein gene was used as a control for RNA loading (16). DNA probes were labeled with [^{32}P]dCTP by the random primer method using a Boehringer Mannheim kit (Indianapolis, IN). Membranes were hybridized overnight at 45°C, washed under stringent conditions and exposed to x-ray film (Kodak) overnight as described previously (6,7). Signal intensities were measured using an LKB Ultrascan XL densitometer and confirmed by phosphorimaging with a short exposure interval (6 h).

RESULTS

The Michael addition properties of EA produce electrophilic intermediates which are reactive with GSH. Figure 1 shows the *in vitro* disappearance of EA as a function of time in an aqueous buffer environment containing GSH. In a cellular environment the rate of disappearance of EA is influenced by competing electrophiles and by the capacity of the cell to remove the resulting conjugate. Figure 2 compares the intracellular concentrations of EA in wild type (HT4-1) and EA-resistant populations after the cells are incubated in EA-containing media for 1 hr, and the

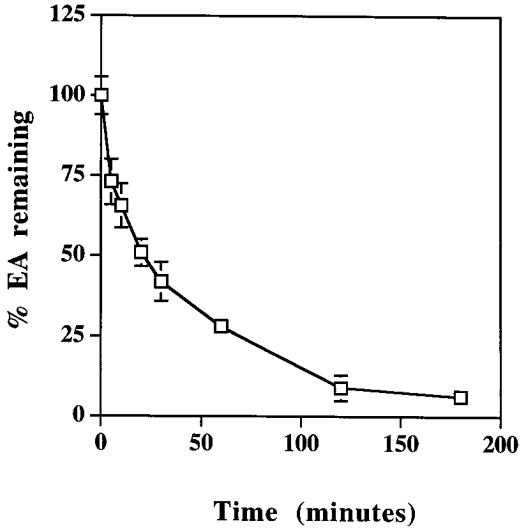

FIG. 1. Spontaneous reaction of EA with GSH. EA (200 μM) was incubated in serum-free medium containing 5 mM GSH. Aliquots were assayed for parent drug (X±S.D.) by HPLC as described in the Methods section (and in references 13 and 14).

medium changed to drug-free medium. The decomposition rate constants (k) for decay from steady state reflect the enhanced capacity of HT6-8 cells to metabolize and efflux EA. These values were calculated as $1.58 \times 10^{-2}\text{min}^{-1}$ for HT4-1 and $3.47 \times 10^{-3}\text{min}^{-1}$ for HT6-8, an approximate 5-fold enhancement in the resistant cell line.

The reactivity of [^{35}S]APA-SG with MRP is demonstrated in Figure 3. Given the limitation of sensitivity in photoaffinity labeling technology, we utilized the overexpressing HL60/ADR cell line as a source for membrane preparations, in lieu of preparations from HT29 cells. High micromolar concentrations of unlabelled APA-SG competed for binding sites on the protein and reduced the

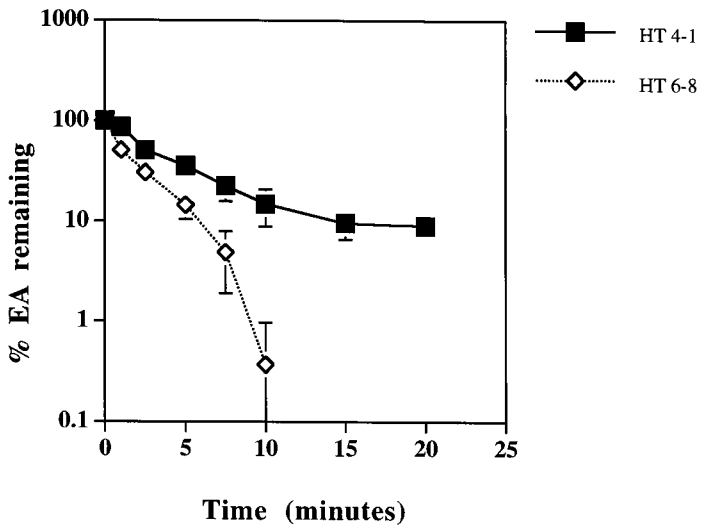

FIG. 2. Cell mediated metabolism of EA by HT29 cells. Cells were treated with 200 μM EA for 1 h. Cells were pelleted and washed free of drug at various time points and analyzed for EA parent (X±S.D.) as described in the Methods section (and in reference 13).

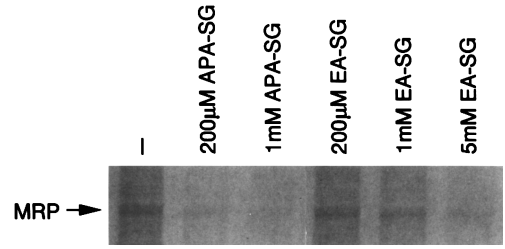

FIG. 3. Competition of [^{35}S]APA-SG photoaffinity labeling of MRP by drug-GSH conjugates. Labeling experiments were performed in the presence of various concentrations of APA-SG, and the GSH conjugate of ethacrynic acid (EA-SG), as described in the Methods section.

photolabeling. Similarly, EA-SG caused a concentration-dependent reduction in photolabeling of MRP protein, albeit at higher concentrations of unlabeled competitor.

Having established EA-SG as an MRP substrate, we examined whether EA influenced the expression of MRP transcript. Figure 4 shows that both acute and chronic EA treatment caused an enhancement in HT39 cell MRP expression. Using 36B4 estradiol independent human acidic ribosomal phosphoprotein as a normalized control transcript, 2.0M- and 2.4-fold increases in MRP transcript were observed for HT4-1 cells exposed to EA for 24 hr and in the resistant HT6-8 cell line, respectively.

DISCUSSION

Detoxification of EA is achieved in a multistep fashion requiring catalytic GSH conjugation and elimination of the conjugate from the cell. The selected EA resistant cell line is able to metabolize and remove EA with rate constants approximately 5 times higher than that of the wild type cells. The more efficient clearance of EA presumably produces the 3- to 4-fold resistance observed in HT6-8 cells. These same cells express low levels of resistance ($\sim$2-fold) to chlorambucil, melphalan, phenylglyoxal, Mitomycin C, prostaglandin D_2 and adriamycin (6). In general, acquired resistance by other cell lines to many of the agents in this list represents a stable phenotype. In contrast, EA resistance is contingent upon the continued presence of the drug and cells will revert to sensitivity upon removal of EA from the culture media (5,6).

Because our photoaffinity studies suggest that GSH conjugates of EA are MRP substrates, the induction of MRP expression by EA is likely to be of functional significance. Both GSTπ and

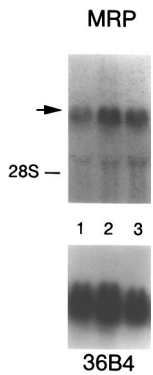

FIG. 4. MRP expression in HT29 colon cancer cells treated with EA. Total RNA was prepared from (1) HT29 subclone HT4-1, (2) HT4-1 cells treated with 50 μM EA for 24 h, and (3) HT6-8 EA-resistant cells which were derived from HT4-1 by continuous growth in 72 μM EA. RNA samples (20 μg) were separated by denaturing gel electrophoresis and transferred to membranes as described in the Methods section. The membranes were hybridized with an MRP probe or a control probe for the 36B4 estradiol-independent human acidic ribosomal phosphoprotein.

γ-GCS are rate limiting enzymes in the catalytic formation of these conjugates (2). Since both are expressed at high basal levels in HT4-1 cells, which we previously reported, the 2- to 3-fold induction of GSTπ and 4- to 5-fold induction of γ-GCS by EA constitutes a significant enhancement of enzyme activity (5,7). Therefore, the 2- to 3-fold elevation in MRP transcript would be consistent in a quantitative sense, and suggests regulation of a series of genes involved in EA detoxification. In the HT6-8 cells, enhanced transcription and prolongation of mRNA half-life by EA were shown previously to contribute to the elevated functional activity and protein expression of a number of candidate genes, including γ-GCS, GSTπ and DDH (7,17). The ARE has been implicated in the inductive effect by Michael acceptors such as EA for phase II detoxification enzymes. Potential ARE elements have been identified in the GSTπ (18) and γ-GCS (19) genes. However, this element was not identified in the recently reported 5′ genomic sequence of MRP (20). It is possible that this motif is present in MRP sequences located further upstream, or that additional unidentified motifs involved in EA induction remain to be elucidated.

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