Cancer Chemother Pharmacol (1997) 40: 215–224

ORIGINAL ARTICLE

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Multienzyme-mediated stable and transient multidrug resistance and collateral sensitivity induced by xenobiotics

Received: 30 August 1996 / Accepted: 16 November 1996

Abstract *Background*: Determinants of cellular sensitivity to anticancer drugs include enzymes that catalyze their biotransformation. Coordinated induction of some of these enzymes is known to be caused by a number of dietary constituents, environmental contaminants, pharmacological agents and other xenobiotics, e.g. 3-methylcholanthrene and catechol. Despite the potential for inducing simultaneous changes in tumor cell sensitivity to a wide range of drugs, scant attention has been paid to the impact that dietary constituents and other xenobiotics might have on the therapeutic outcome of cancer chemotherapy. *Purpose*: The aim of this investigation was to demonstrate the potential of xenobiotic-induced multienzyme-mediated stable and transient multidrug resistance/collateral sensitivity in a model system. *Methods*: Human breast adenocarcinoma MCF-7/0 cells and a stably oxazaphosphorine-resistant subline thereof, MCF-7/OAP, were grown in the presence of 3-methylcholanthrene (3 μM), catechol (30 μM), or vehicle for 5 days. Spectrophotometric and spectrofluorometric assays were used to quantify catalytic activities and thus cellular levels of cytosolic class 3 aldehyde dehydrogenase, glutathione *S*-transferase, DT-diaphorase, UDP-glucuronosyl transferase and cytochrome P450 1A1. A colony-forming assay was used to quantify cellular sensitivities to several anticancer drugs. *Results*: Relative to their untreated counterparts, MCF-7/0 and MCF-7/OAP cells treated with 3-methylcholanthrene or catechol transiently expressed elevated levels of cytosolic class 3 aldehyde dehydrogenase, glutathione *S*-transferase, DT-diaphorase and UDP-glucuronosyl transferase, and were transiently, more resistant to mafosfamide, melphalan, and mitoxantrone, and more sensitive to EO9. Further, MCF-7/0 and MCF-7/OAP cells treated with 3-methylcholanthrene, but not those treated with catechol, transiently expressed elevated levels of cytochrome P450 1A1 and were transiently more sensitive to ellipticine. Relative to MCF-7/0 cells, MCF-7/OAP cells stably overexpressed all but cytochrome P450 1A1 and were stably, more resistant to mafosfamide, melphalan and mitoxantrone, and more sensitive to EO9. Inclusion of relatively specific inhibitors of, or alternative substrates for, the enzymes of interest during drug exposure negated the influence of enzyme overexpression on cellular sensitivities to these agents. Untreated, and 3-methylcholanthrene- or catechol-treated, MCF-7/0 and MCF-7/OAP cells were equisensitive to vincristine and nearly so to doxorubicin. *Conclusions*: Collectively, these experiments illustrate the potential for both stable and transient xenobiotic-induced multienzyme-mediated multidrug resistance/collateral sensitivity that, although also the result of a single event, is mechanistically different from, and pertains to a largely different group of anticancer agents than does, the multidrug resistance caused by cell surface multidrug transporters.

Key words Aldehyde dehydrogenase · DT-diaphorase · Glutathione *S*-transferase · UDP-glucuronosyl transferase · Cytochrome P450 1A1 · Mafosfamide · Cyclophosphamide · Ifosfamide · Oxazaphosphorines · EO9 · Melphalan · Mitoxantrone · Ellipticine · Doxorubicin · Vincristine · Multidrug resistance · Multidrug collateral sensitivity · Breast cancer · Monofunctional inducers · Bifunctional inducers · Phase 1 drug-metabolizing enzymes · Phase 2 drug-metabolizing enzymes

Abbreviations *ALDH-3* cytosolic class-3 aldehyde dehydrogenase · *ARE* antioxidant responsive element · *CHAPS* 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate · *CYP1A1* cytochrome P450 1A1 ·

Supported by USPHS Grant CA 21737 and DOD Grant DAMD 17-94-J-4057

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DT-D DT-diaphorase [NAD(P)H:quinone oxidoreductase, NQO1] · *ELISA* enzyme-linked immunosorbent assay · *GST* glutathione *S*-transferase · *MRP* multidrug resistance-associated protein · *TCDD* tetrachlorodibenzo-*p*-dioxin · *UDP-GT* UDP-glucuronosyl transferase

Introduction

Anticancer drugs, like other pharmacological agents, are often substrates for enzymes that catalyze their conversion to pharmacologically more/less potent metabolites. Amongst these enzymes are ALDH-3, GST, DT-D, UDP-GT and CYP 1A1. ALDH-3 catalyzes the detoxification of the oxazaphosphorines cyclophosphamide, ifosfamide, mafosfamide and 4-hydroperoxycyclophosphamide (reviewed in reference 45), GST catalyzes the detoxification of melphalan, chlorambucil and phosphoramide mustard (reviewed in reference 23), UDP-GT catalyzes the detoxification of mitoxantrone [57], DT-D catalyzes the toxification of mitomycin C and the indoloquinone EO9 (reviewed in references 39 and 58) and CYP 1A1 catalyzes the toxification of ellipticine (reviewed in reference 34). These enzymes are known to be coordinately induced by so-called bifunctional inducers[1] and, except for CYP 1A1, by so-called monofunctional inducers (reviewed in references 3, 6, 7, 26, 45 and 52). Bifunctional inducers include TCDD, indole-3-carbinol and polycyclic aromatic hydrocarbons such as 3-methylcholanthrene and 3,4-benzpyrene. Monofunctional inducers include oltipraz and phenolic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene and catechol.

Although yet to be demonstrated, it follows that when coordinated induction of these enzymes occurs in tumor cells by the action of either bifunctional or monofunctional inducers, cellular sensitivity to relevant antitumor agents should be coordinately altered as well, that is, decreased in those cases where the antitumor drug is converted to a less active metabolite and increased in those cases where the antitumor drug is converted to a more active metabolite. Cellular sensitivity to such anticancer agents would be expected to return to control levels upon removal of the inducer from the environment, i.e. resistance and collateral sensitivity would both be transient.

Coordinated induction of these enzymes could also be caused by a permanent change (mutation) in a signaling mechanism that upregulates gene transcription and is common to the enzymes of interest. In this case, resistance and collateral sensitivity to relevant antitumor agents would be expected to be stable.

Each of these expectations was fully realized in a human breast adenocarcinoma MCF-7 cultured cell model, thereby illustrating the previously unrecognized potential for both stable and transient xenobiotic-induced multidrug resistance (and collateral sensitivity) that is mechanistically different from, and pertains to a largely different group of anticancer agents than, transporter-mediated multidrug resistance.

Materials and methods

Materials

Mafosfamide, melphalan hydrochloride, mitoxantrone hydrochloride and E09 [3-hydroxymethyl-5-aziridinyl-1-methyl-2-(H-indole-4, 7-indione)-propenol] were provided, respectively, by: Dr. J. Pohl, Asta Medica, Frankfurt, Germany; Dr. G. M. Lyon Jr, Burroughs Wellcome & Co., Research Triangle Park, N.C.; Dr. F. E. Durr, American Cyanamid Company, Medical Research Division, Lederle Laboratories, Pearl River, N.Y.; and Dr. H. R. Hendriks, New Drug Development Office, EORTC, Free University Hospital, The Netherlands. Phosphoramide mustard · cyclohexylamine and doxorubicin hydrochloride were supplied, respectively, by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., and Adria Laboratories, Wilmington, Del. Purified human GST α, μ and π, and affinity-purified polyclonal antibodies specific for each of these isoenzymes [54], were provided by Dr. A.J. Townsend, Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, N.C. Ellipticine, vincristine sulfate, antirabbit IgG alkaline phosphatase conjugate, *p*-nitrophenyl phosphate, α-naphthyl β-D-glucuronide, uridine 5′-diphosphoglucuronic acid (UDP-glucuronic acid), α-naphthol, α-naphthaflavone and CHAPS were purchased from the Sigma Chemical Co., St. Louis, Mo. All other chemicals and reagents were obtained from the sources reported in previous publications [46, 48, 51].

Homogenization medium (1.15% w/v KCl and 1 m*M* EDTA in aqueous solution, pH 7.4), drug-exposure medium (10% horse serum in a phosphate-buffered saline-based aqueous solution, pH 7.4), growth medium (10% horse serum in Dulbecco's modified Eagle's medium supplemented with 2 m*M* L-glutamine, 3.7 g/l sodium bicarbonate and 50 mg/l gentamicin), and Lubrol-treated whole homogenates were prepared as described previously [46]. Microsomal fractions were also prepared as described previously [51], except that they were ultimately suspended in a 0.25 *M* aqueous sucrose solution when UDP-GT activity was to be quantified.

Cell culture

Human breast adenocarcinoma MCF-7/0 cells and a stably oxazaphosphorine-resistant subline (MCF-7/OAP), generated by growing the parent MCF-7/0 cells in the presence of gradually increasing concentrations of 4-hydroperoxycyclophosphamide over a period of several months [18], were obtained originally from Dr. B. Teicher, Dana-Farber Cancer Institute, Boston, Mass., and were cultured (monolayer) as described previously [46, 48]. Exponentially growing MCF-7/0 and MCF-7/OAP cells were continuously

[1] Bifunctional inducers are defined here as agents that coordinately induce cytosolic class 3 aldehyde dehydrogenase, glutathione *S*-transferases, DT-diaphorase, UDP-glucuronosyl transferase, epoxide hydrolase, dihydrodiol dehydrogenase, carbonyl reductase and cytochrome P450s 1A1, 1A2 and 1B1; xenobiotic responsive elements (XRE) present in the 5′-upstream regions of the genes that code for these enzymes are essential components of the signaling mechanism by which induction is effected [13, 23, 26, 33, 41, 45, 52]. Monofunctional inducers are defined here as agents that coordinately induce all of the foregoing enzymes except cytochrome P450s 1A1, 1A2 and (putatively) 1B1; in this case, antioxidant responsive elements (ARE) present in the 5′-upstream regions of the genes that code for these enzymes are essential components of the signaling mechanism by which induction is effected [13, 23, 26, 38, 41, 45, 52].

exposed to 3 μ*M* 3-methylcholanthrene or 30 μ*M* catechol for 5 days, as described previously, to obtain MCF-7/0/MC and MCF-7/OAP/MC, and MCF-7/0/CAT and MCF-7/OAP/CAT, cells, respectively [48, 51].

Cultured cells were harvested, resuspended in growth medium, and checked for viability (usually greater than 95% as judged by trypan blue exclusion) as described previously [46, 48]; preparations exhibiting less than 95% viability were discarded. Accepted preparations were again harvested, washed once, and resuspended in drug-exposure medium when cellular sensitivity to the cytotoxic action of various drugs was to be quantified, or in homogenization medium when enzyme activities in microsomal fractions or Lubrol-treated whole homogenates were to be quantified.

Enzyme assays

ALDH-3, pan-GST, DT-D and CYP 1A1 activities, and protein concentrations, were determined as described previously [46, 51].

The spectrofluorometric method described by Mackenzie and Hänninen [30] was used with modifications to quantify UDP-GT activity. The reaction mixture, minus UDP-glucuronic acid, was prepared at 4 °C and contained 50 m*M* potassium phosphate, 4 m*M* $MgCl_2$, 0.05 m*M* α-naphthol (originally dissolved in 0.25% v/v dimethyl sulfoxide in water; the final concentration of dimethyl sulfoxide in the reaction mixture was 0.00625%; this amount of dimethyl sulfoxide did not interfere with the assay), 0.4 m*M* CHAPS and microsomal fractions obtained from 2.5×10^6 cells. It was allowed to stand for 10 min at 4 °C, after which time it was incubated in a waterbath at 37 °C for 5 min. UDP-glucuronic acid was then added to start the reaction (the final UDP-glucuronic acid concentration, reaction volume and pH of the reaction mixture were 2 m*M*, 1 ml and 7.4, respectively). Accumulation of naphthyl glucuronide at 37 °C was continuously quantified and recorded with the aid of an automated spectrofluorometer (Fluorolog-2, SPEX Industries, Edison, N.J.) equipped with a circulating waterbath; excitation and emission wavelengths were 293 and 335 nm, respectively. Reagent blanks lacked UDP-glucuronic acid. Authentic α-naphthyl glucuronide was used to prepare standards.

Enzyme levels are reported in terms of milli-International Units (mIU) of enzyme activity (nmol/min NADPH formed in the case of ALDH-3 activity, nmol/min S-(2-chloro-4-nitrophenyl)glutathione formed in the case of pan-GST activity, nmol/min 2,6-dichlorophenol-indophenol reduced in the case of DT-D activity, nmol/min α-naphthyl glucuronide formed in the case of UDP-GT activity, and nmol/min resorufin formed in the case of CYP 1A1 activity).

Enzyme-linked immunosorbent assay

GST α, μ and π were quantified using an ELISA [24]. Lubrol-treated homogenates obtained from 1–10 × 10^5 cells were diluted with coating buffer (100 m*M* sodium carbonate in aqueous solution, pH 9.6) and placed into the wells of 96-well microtiter plates (100 μl/well). Adhesion of proteins to the surface of the wells was achieved by incubating the plates in a humidified air incubator for 2 h at 37 °C. The plates were then thoroughly washed with a washing buffer (phosphate-buffered saline-based aqueous solution, pH 7.4, containing 0.05% Tween-20) after which 200 μl of blocking solution (phosphate-buffered saline-based aqueous solution, pH 7.4, containing 0.25% bovine serum albumin and 0.05% Tween-20) were added to each of the coated wells and incubation was continued for 2 h to prevent nonspecific binding of the antibody to the wells. The blocking solution was then removed from the wells, 100 μl aliquots of the primary antibody (antibodies specific for human GST α, μ or π) in blocking solution were added to the blocked wells, and incubation was continued for another 2 h. At the end of this time, the wells were washed with washing buffer, 100 μl aliquots of a secondary antibody (antirabbit IgG alkaline phosphatase conjugate) in blocking solution were added to the wells, and incubation was continued for yet another 2 h. Following this incubation, the wells were washed thoroughly with washing buffer, 200 μl aliquots of the substrate solution (0.1 m*M* *p*-nitrophenyl phosphate dissolved in Tris-buffered saline-based aqueous solution, pH 9.0) were added to the wells, and incubation at 37 °C was carried out for 5 to 10 min. Absorbance resulting from the formation of *p*-nitrophenol (405 nm) was then recorded using a microtiter plate reader (UV-Max Kinetic Microplate Reader, Molecular Devices Corporation, Calif.) connected to a Macintosh computer equipped with SOFTmax software. Purified human GST α, μ and π (specific activities 44,500 mIU/mg, 24,100 mIU/mg and 56,700 mIU/mg protein, respectively) were used to generate standard curves.

Drug-exposure and colony-forming assay

Drug-exposure and the colony-forming assay used to determine surviving fractions were as described previously [46]. Briefly, freshly harvested cells were diluted with drug-exposure medium to a concentration of 1 × 10^5 cells/ml and then exposed to drug or vehicle for 30 (mafosfamide, phosphoramide mustard, melphalan, mitoxantrone, EO9, doxorubicin or vincristine) or 180 (ellipticine) min at pH 7.4 and 37 °C after which they were harvested and cultured in drug-free growth medium for 15 (MCF-7/0, MCF-7/0/CAT, MCF-7/OAP, MCF-7/OAP/CAT) or 30 (MCF-7/0/MC and MCF-7/OAP/MC) days. Colonies (≥ 50 cells) were then visualized with methylene blue dye and counted.

Stock solutions of EO9 were prepared by first dissolving it in dimethyl sulfoxide and then diluting this solution with drug-exposure medium. Stock solutions of melphalan and ellipticine were prepared by dissolving them in an acidified ethanol solution (5% 0.1 *N* HCl v/v). Stock solutions of mafosfamide, phosphoramide mustard, mitoxantrone, doxorubicin and vincristine were prepared by dissolving them in water. All drug solutions were prepared just before use. In some experiments, cells were preincubated with benzaldehyde (5 m*M*), dicumarol (25 μ*M*), ethacrynic acid (25 μ*M*), α-naphthol (1 m*M*), α-naphthaflavone (50 μ*M*), or vehicle at 37 °C for 5 (all but α-naphthol) or 30 (α-naphthol) min prior to the addition of mafosfamide, EO9, melphalan, mitoxantrone or ellipticine, respectively, after which incubation was continued for 30 or 180 min as before. Stock solutions of benzaldehyde were prepared by dissolving it in water. Stock solutions of dicumarol were prepared by dissolving it in an alkaline (pH 10.0) aqueous solution and then diluting this solution with drug-exposure medium. Stock solutions of ethacrynic acid were prepared by dissolving it in ethanol and then diluting this solution with drug-exposure medium. Stock solutions of α-naphthaflavone and α-naphthol were prepared by dissolving them in dimethyl sulfoxide and then diluting them with drug-exposure medium.

The concentration of dimethyl sulfoxide or ethanol ultimately present before and during drug exposure did not exceed 0.1%; this concentration did not affect the rate of cell proliferation or induce any of the enzymes of interest. Control experiments established that, at the concentrations used, benzaldehyde, dicumarol, α-naphthol and α-naphthaflavone were without cytotoxic effect against MCF-7/0 and MCF-7/OAP cells. Ethacrynic acid (25 μ*M*) showed a low level of cytotoxicity (11% cell-kill). This was taken into account when calculating the effect of including ethacrynic acid in the drug-exposure medium on the LC_{90} (concentration of drug required to effect 90% cell-kill) values for melphalan.

Data analysis

Computer-assisted unweighted regression analysis was carried out using the STATView (Brainpower, Calabas, Calif.) statistical program to generate all straightline functions.

Table 1 Enzyme activities in methylcholanthrene- and catechol-treated human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells. Human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells were cultured in the presence of vehicle (MCF-7/0, MCF-7/OAP), 3 μ*M* methylcholanthrene (MCF-7/0/MC, MCF-7/OAP/MC) or 30 μ*M* catechol (MCF-7/0/CAT, MCF-7/OAP/CAT) for 5 days, and ALDH-3, Pan-GST, DT-D, UDP-GT and CYP 1A1 activities were quantified as described in Materials and methods. Substrates were 4 m*M* benzaldehyde, 1 m*M* 1-chloro-2,4-dinitrobenzene, 0.04 m*M* 2,6-dichlorophenol-indophenol, 0.05 m*M* α-naphthol and 0.005 m*M* 7-ethoxyresorufin, respectively. Also quantified at this time were GST α, μ and π levels. ELISAs were used for this purpose as described in Materials and methods. Values are the means of duplicate or triplicate determinations made in each of at least two separate experiments

Cells	Enzyme activity (mIU/10^7 cells)							
	ALDH-3[a]	GST				DT-D[a]	UDP-GT	CYP 1A1[a]
		pan[a]	α	μ	π			
MCF-7/0	2	25	9	13	11	82	0.04	0.03
MCF-7/0/MC	310	150	20	113	34	495	0.78	0.48
MCF-7/0/CAT	768	250	29	121	62	6395	0.07	0.03
MCF-7/OAP	254	157	27	81	32	340	0.16	0.03
MCF-7/OAP/MC	3534	192	38	96	36	15300	1.30	0.67
MCF-7/OAP/CAT	1593	227	36	135	51	12400	0.23	0.03

[a]Values are from previous publications [45–48, 51]; they are included here for comparative purposes. Reduced glutathione levels were approximately 185 nmol/10^7 cells for each of the six cell preparations [45]

Results

The ALDH-3, pan-GST, DT-D, UDP-GT and CYP 1A1 levels in untreated, and in 3-methylcholanthrene- and catechol-treated, human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells are given in Table 1. As compared with untreated MCF-7/0 cells, untreated MCF-7/OAP cells and catechol-treated MCF-7/0 cells expressed elevated ALDH-3, pan-GST, DT-D and UDP-GT activities, but not elevated CYP 1A1 activity, whereas 3-methylcholanthrene-treated MCF-7/0 cells expressed elevated activities of all five enzymes. Similarly, compared with untreated MCF-7/OAP cells, those cultured in the presence of catechol expressed elevated ALDH-3, pan-GST, DT-D, and UDP-GT activities, but not elevated CYP 1A1 activity, whereas those cultured in the presence of 3-methylcholanthrene expressed elevated activities of all five enzymes. These findings were entirely as expected given our previous findings and that catechol is a monofunctional inducer and 3-methylcholanthrene is a bifunctional inducer (reviewed in reference 45).

At least three GST isoenzymes, namely α, μ and π, are present in the cytosol of human cells. Each has been shown to be induced in various models by both bifunctional (e.g. 3-methylcholanthrene) and monofunctional (e.g. butylated hydroxyanisole) inducers (reviewed in reference 23). Consistent with these reports, the levels of all three isoenzymes were increased when either MCF-7/0 or MCF-7/OAP cells were cultured in the presence of either 3-methylcholanthrene or catechol for 5 days. Constitutive levels of all three were higher in MCF-7/OAP cells than in MCF-7/0 cells (Table 1). Interestingly, induction of GST μ in MCF-7/0 cells by 3-methylcholanthrene or catechol was substantially greater than induction of GST α and π. Similarly, expression of GST μ in MCF-7/OAP cells, relative to that in MCF-7/0 cells, was greater than the relative expression of GST α and π in these two cell lines.

The sensitivities of untreated, and 3-methylcholanthrene- and catechol-treated, MCF-7/0 and MCF-7/OAP cells to several anticancer drugs are given in Table 2. These findings were essentially also entirely as expected given the results presented in Table 1, and also that ALDH-3, the GSTs and UDP-GT catalyze the detoxification of mafosfamide, melphalan and mitoxantrone,

Table 2 Sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells to antineoplastic drugs before and after growing them in the presence of methylcholanthrene or catechol. Human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells were cultured in the presence of vehicle (MCF-7/0, MCF-7/OAP), 3 μ*M* 3-methylcholanthrene (MCF-7/0/MC, MCF-7/OAP/MC) or 30 μ*M* catechol (MCF-7/0/CAT, MCF-7/OAP/CAT) for 5 days. At the end of this time, cells were harvested, washed, and resuspended in drug-exposure medium. The cells were then incubated with vehicle or various concentrations of mafosfamide (*MAF*), melphalan (*MEL*), EO9, mitoxantrone (*MIT*), ellipticine (*ELP*), doxorubicin (*DXR*) or vincristine (*VCR*) for 30 (all but ellipticine) or 180 (ellipticine) min at 37 °C as described in Materials and methods. The colony-forming assay described in Materials and methods was used to determine surviving fractions. LC_{90} values were obtained from plots of log surviving fractions versus concentrations ($n = 4$–6) of drug. Values are means of two or three separate experiments

Cells	LC_{90} (μ*M*)						
	MAF	MEL	EO9	MIT	ELP	DXR	VCR
MCF-7/0	60[a]	6	2.1	4	185	2.6	14
MCF-7/0/MC	>2000[a]	15	0.9	75	50	4.0	15
MCF-7/0/CAT	>2000[a]	22	0.5	7	190	5.2	13
MCF-7/OAP	>2000[a]	14	1.2	12	175	4.6	12
MCF-7/OAP/MC	>2000	23	0.1	100	30	5.8	11
MCF-7/OAP/CAT	>2000	20	0.2	17	180	7.0	14

[a]Values are from previous publications [46–48, 51]; they are included here for comparative purposes

respectively (reviewed in references 23 and 45; 57) and that DT-D and CYP 1A1 catalyze the toxification of EO9 and ellipticine, respectively (reviewed in references 34 and 58). Moreover, untreated MCF-7/OAP cells were, relative to untreated MCF-7/0 cells, equisensitive to vincristine and < twofold less sensitive to doxorubicin, and MCF-7/0 and MCF-7/OAP cells treated with 3-methylcholanthrene or catechol were, relative to their untreated counterparts, each equisensitive to vincristine and ≤ twofold less sensitive to doxorubicin (Table 2). Thus, the relative insensitivity to mafosfamide, melphalan and mitoxantrone exhibited by untreated MCF-7/OAP cells, and 3-methylcholanthrene- and catechol-treated MCF-7/0 and MCF-7/OAP cells, cannot have been caused by the well-known P-glycoprotein-based, or the recently identified MRP-based, multidrug resistance mechanisms since a substantial decrease in cellular sensitivity to vincristine and doxorubicin would be expected if one of these mechanisms was operative. The small decreases in sensitivity to doxorubicin are probably the consequence of increased GST-catalyzed detoxification of this agent (reviewed in reference 23).

Proportionately, increases in DT-D and CYP 1A1 levels were substantially greater than the increases in tumor cell sensitivity to EO9 and ellipticine, respectively. Underlying these discrepancies may be that the cofactor, NAD(P)H in each case, becomes rate limiting in intact cells or that, while the parent compound is known to be less active than the metabolite generated by the catalytic activity of the enzymes under investigation, it need not be totally without intrinsic activity and/or unable to give rise to an active metabolite in a reaction catalyzed by another enzyme, the cellular level of which was not increased by the manipulations used in this investigation. Xanthine oxidase/dehydrogenase, NADPH:cytochrome P450 reductase and cytochrome b_5 reductase are known to also catalyze the activation of EO9 (reviewed in reference 58). These enzymes are not known to be induced by bifunctional or monofunctional inducers. Cytochrome P450s other than CYP 1A1 are known to catalyze the oxidation, thereby activation, of ellipticine. These enzymes, too, are not known to be induced by bifunctional or monofunctional inducers. Moreover, although much less potent than the metabolite 9-hydroxyellipticine generated by these enzymes, ellipticine itself is cytotoxic (reviewed in reference 34).

3-Methylcholanthrene- and catechol-induced increases in cellular enzyme levels, as well as changes in sensitivity to the drugs of interest, were transient; all values returned to control levels shortly (within 2 weeks) after the inducing agent was removed from the culture medium (data not presented [45, 48, 51]). On the other hand, enzyme overexpression was stable in MCF-7/OAP cells, as were the changes in sensitivity to the drugs of interest, i.e. each was retained seemingly indefinitely when the selecting agent was removed from the culture medium (data not presented [45, 46]).

Inhibitors or alternative substrates of/for these enzymes were used to provide additional support for the notion that changes in the catalytic activities indicated did indeed account for the altered sensitivites to these drugs (comparing values presented in Table 3 with those presented in Table 2). No attempt was made to use maximally tolerated substrate/inhibitor concentrations or substrate/inhibitor concentrations that would give maximal restoration of sensitivity.

Ostensibly, ALDH-3 catalyzes the detoxification of mafosfamide by catalyzing the irreversible oxidation of its pivotal intermediary aldehyde metabolite aldophosphamide to the corresponding acid carboxyphosphamide [8, 9, 37, 42, 46–49, 51]. Benzaldehyde is a relatively good substrate for ALDH-3 [46, 48]. Thus, acting as an alternative substrate, when included in the drug-exposure medium, it should prevent the decreased

Table 3 Influence of selected enzyme inhibitors/substrates on the sensitivity of human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells to antineoplastic drugs before and after growing them in the presence of methylcholanthrene or catechol. Human breast adenocarcinoma MCF-7/0 and MCF-7/OAP cells were cultured in the presence of vehicle (MCF-7/0, MCF-7/OAP), 3 μ*M* 3-methylcholanthrene (MCF-7/0/MC, MCF-7/OAP/MC) or 30 μ*M* catechol (MCF-7/0/CAT, MCF-7/OAP/CAT) for 5 days. At the end of this time, cells were harvested, washed, and resuspended in drug-exposure medium. The cells were then incubated with benzaldehyde (*BENZ*, 5 m*M*), ethacrynic acid (*EA*, 25 μ*M*), dicumarol (*DIC*, 25 μ*M*), α-naphthol (*NAP*, 1 m*M*), α-naphthaflavone (α*NF*, 50 μ*M*) or vehicle for 5 (all but α-naphthol) or 30 (α-naphthol) min at 37 °C after which time various concentrations of mafosfamide (*MAF*), melphalan (*MEL*), EO9, mitoxantrone (*MIT*), ellipticine (*ELP*) or vehicle were added and incubation was continued as before for 30 (all but ellipticine) or 180 (ellipticine) min at 37 °C as described in Materials and methods. The colony-forming assay described in Materials and methods was used to determine surviving fractions. LC_{90} values were obtained from plots of log surviving fractions versus concentrations ($n = 4$–6) of drug. Control LC_{90} values (those obtained in the absence of inhibitor) are given in Table 2 (*ND* not determined)

Cell line	LC_{90} (μ*M*)				
	MAF/BENZ	MEL/EA	EO9/DIC	MIT/NAP	ELP/αNF
MCF-7/0	54[a]	5	2.3	4	200
MCF-7/0/MC	175[a]	9	2.2	15	145
MCF-7/0/CAT	170[a]	11	2.0	3	190
MCF-7/OAP	180[a]	7	2.4	5	175
MCF-7/OAP/MC	220	ND	1.8	20	135
MCF-7/OAP/CAT	200	ND	1.8	6	ND

[a]Values are from previous publications [45–48, 51]; they are included here for comparative purposes

sensitivity to mafosfamide observed in cells expressing increased amounts of ALDH-3 if decreased sensitivity to mafosfamide is the consequence of ALDH-3-catalyzed detoxification of this agent. This is precisely what was observed (Tables 2 and 3). As expected, given that phosphoramide mustard, the cytotoxic metabolite of mafosfamide (reviewed in reference 43), is not a substrate for ALDH-3, inclusion of benzaldehyde in the drug-exposure medium did not alter the sensitivity of any of the cells used in this investigation to phosphoramide mustard (unpublished observations; [37, 45–48]). Benzaldehyde did not inhibit pan-GST-, DT-D-, UDP-GT- or CYP 1A1-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to melphalan, EO9, mitoxantrone or ellipticine (data not shown).

GST catalyzes the conjugation of a number of anticancer drugs, e.g. melphalan, to glutathione, thereby detoxifying them (reviewed in reference 23). Which of the GST isozymes is/are operative in the case of any given agent is not clear, but GST α is almost certainly a major contributor in the case of melphalan (reviewed in reference 23). Conjugation of the type under consideration can also occur without the benefit of enzymatic intervention. Ethacrynic acid is known to inhibit GST-catalyzed conjugation reactions, perhaps by serving as an alternative substrate, though irreversible, as well as reversible, binding of this agent to GSTs has been reported (reviewed in reference 36). In any case, given that reduced glutathione levels in the six cell types are virtually identical [45], inclusion of ethacrynic acid in the drug-exposure medium should prevent the decreased sensitivity to melphalan observed in cells expressing increased amounts of GST if decreased sensitivity to melphalan is the consequence of GST-catalyzed detoxification of this agent. This expectation was at least partially realized (Tables 2 and 3). Ethacrynic acid did not inhibit ALDH-3-, DT-D-, UDP-GT- or CYP 1A1-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to mafosfamide, EO9, mitoxantrone or ellipticine (data not shown).

DT-D, an obligate two-electron donating enzyme, catalyzes the bioreductive activation of EO9 (reviewed in reference 58). Dicumarol is known to be a potent inhibitor of DT-D-catalyzed reactions [16]. Thus, its inclusion in the drug-exposure medium should prevent the increased sensitivity to EO9 observed in cells expressing increased amounts of DT-D if increased sensitivity to EO9 is the consequence of DT-D catalyzed activation of this agent. This expectation was fully realized (Fig. 1, Tables 2 and 3). Dicumarol did not inhibit ALDH-3-, pan-GST-, UDP-GT- or CYP 1A1-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to mafosfamide, melphalan, mitoxantrone or ellipticine (data not shown).

A rat liver 3-methylcholanthrene-inducible UDP-GT has been shown to catalyze the glucuronidation of mitoxantrone [57], and the human counterpart of this isoenzyme confers a tenfold decrease in sensitivity to mitoxantrone when it is transfected into NIH 3T3 cells

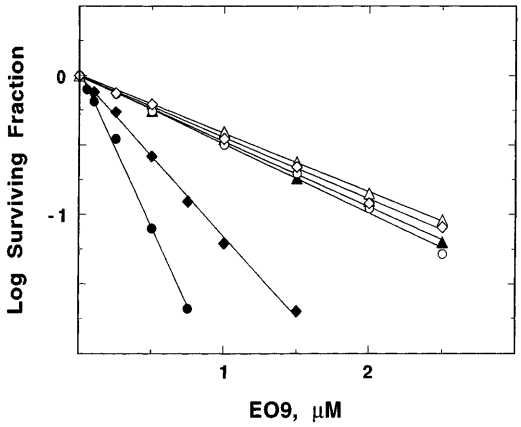

Fig. 1 Influence of dicumarol on the sensitivity of human breast adenocarcinoma MCF-7/0 cells to EO9 before and after growing them in the presence of methylcholanthrene (MCF-7/0/MC) or catechol (MCF-7/0/CAT). MCF-7/0 cells were grown in the absence (▲, △) or presence of 3 μ*M* 3-methylcholanthrene (◆, ◇) or 30 μ*M* catechol (●, ○) for 5 days, after which time they were harvested and preincubated with vehicle (▲, ◆, ●) or 25 μ*M* dicumarol (△, ◇, ○) for 5 min at 37 °C. EO9 was then added and incubation was continued for an additional 30 min after which time the cells were harvested and grown in drug-free growth medium for 15–30 days. The colony-forming assay described in Materials and methods was used to determine surviving fractions. Each point is the mean of measurements on triplicate cultures. DT-diaphorase activities (40 μ*M* dichlorophenol-indophenol; 160 μ*M* NADH) in Lubrol-treated whole homogenates of these cells were 87 (MCF-7/0), 512 (MCF-7/0/MC) and 6308 (MCF-7/0/CAT) mIU/10^7 cells

[10]. These observations are consistent with the notion that the decreased sensitivity to mitoxantrone observed in cells expressing increased amounts of UDP-GT is a consequence of UDP-GT-catalyzed detoxification of this agent. Another possibility, GST-catalyzed detoxification of electrophilic metabolites generated in a CYP 1A1-catalyzed reaction [35, 57], can be ruled out because neither ethacrynic acid nor α-naphthaflavone, an inhibitor of CYP 1A1-catalyzed reactions, altered the sensitivities of untreated and treated cells to mitoxantrone (data not shown). Inclusion of α-naphthol, a substrate for UDP-GT [5], in the drug-exposure medium should prevent the decreased sensitivity to mitoxantrone observed in cells expressing increased amounts of this enzyme if decreased sensitivity to mitoxantrone is the consequence of UDP-GT-catalyzed detoxification of this agent. This expectation was largely realized (Tables 2 and 3). α-Naphthol did not inhibit ALDH-3-, pan-GST-, DT-D-, or CYP 1A1-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to mafosfamide, melphalan, EO9 or ellipticine (data not shown).

The 9-hydroxy derivative of ellipticine is known to exert much greater antitumor activity than the parent compound. Hydroxylation of ellipticine to 9-hydroxyellipticine is known to be catalyzed by CYP 1A1 (reviewed in reference 34). α-Naphthaflavone is known to inhibit CYP 1A1-catalyzed reactions (reviewed in reference 53). Thus, its inclusion in the drug-exposure medium should prevent the increased sensitivity to

ellipticine observed in cells expressing large amounts of CYP 1A1 if increased sensitivity to ellipticine is the consequence of CYP 1A1-catalyzed activation of this agent. This expectation was largely realized (Tables 2 and 3). α-Naphthaflavone did not inhibit ALDH-3-, pan-GST-, or UDP-GT-mediated catalysis, nor did it alter the sensitivities of untreated or treated cells to mafosfamide, melphalan or mitoxantrone (data not shown), but it did inhibit (90% inhibition at 50 μM) DT-D-catalyzed reduction of 2,6-dichlorophenol-indophenol, and, when included in the drug-exposure medium, largely prevented the increased sensitivity to EO9 otherwise exhibited by cells expressing increased amounts of DT-D. However, the possibility that DT-D catalyzes the bioactivation of ellipticine and that α-naphthaflavone inhibits this reaction can be discarded since dicumarol did not prevent the increased sensitivity of these cells to ellipticine (data not shown) and sensitivity to ellipticine was not increased in MCF-7/OAP or catechol-treated MCF-7/0 and MCF-7/OAP cells (Tables 2 and 3).

As expected, given that ALDH-3, GST, DT-D, UDP-GT and CYP 1A1 levels are relatively low in MCF-7/0 cells (Table 1), inclusion of an inhibitor or alternative substrate of/for each of these enzymes (i.e. benzaldehyde, ethacrynic acid, dicumarol, α-naphthol and α-naphthaflavone, respectively) in the drug-exposure medium only marginally altered the sensitivity of these cells to mafosfamide, melphalan, EO9, mitoxantrone and ellipticine, respectively (Tables 2 and 3). Similarly, given that CYP 1A1 levels are also very low in catechol-treated MCF-7/0 and in MCF-7/OAP cells, inclusion of α-naphthaflavone only marginally decreased the sensitivity of these cells to ellipticine.

Discussion

Development of simultaneous resistance to a wide range of drugs is a major impediment to the successful chemotherapy of human tumors.

Multidrug resistance caused by a 170,000 Da cell surface multidrug transporter (P-glycoprotein, P-170) is a well-known experimental phenomenon and appears to be of clinical relevance [reviewed in references 12, 15 and 20]. Recent studies indicate that multidrug resistance may also be caused by another ABC (ATP-binding cassette) transporter, namely, MRP [11, 14, 17, 21], and by LRP (lung resistance-related protein, p110 major vault protein) [25, 29, 40]. Our findings indicate that simultaneous resistance can also be brought about by the coordinated induction of relevant detoxifying enzymes in target tumor cells, and that multidrug resistance of this origin is to a group of anticancer drugs largely different from those that are ineffective because of elevated transporter levels.

Like transporter-mediated multidrug resistance, multienzyme-mediated multidrug resistance can be intrinsic (constitutive) and indefinite as in the case of human colon C carcinoma cells (unpublished observations; [37]) or slowly acquired and stable as in the case of MCF-7/OAP cells [18, 45, 46]. Unlike transporter-mediated multidrug resistance, multienzyme-mediated multidrug resistance can also be rapidly acquired/induced and transient as in the case of 3-methylcholanthrene- or catechol-treated MCF-7/0 cells, and is accompanied by multienzyme-mediated multidrug collateral sensitivity, i.e. increased sensitivity to anticancer drugs that are activated by relevant enzymes. Moreover, it can be readily acquired/induced in the absence of exposure to a selecting agent, i.e. to one of the anticancer drugs to which resistance is induced.

Importantly, as appears to be the case in acquired transporter-mediated multidrug resistance, multienzyme-mediated multidrug resistance/collateral sensitivity is probably the consequence of a single though different event, i.e. a mutation in the case of stable resistance/collateral sensitivity (discussed below), and activation of a promoter element common to the enzymes of interest in the case of transient resistance/collateral sensitivity (reviewed in reference 45).

The molecular alteration(s) underlying the constitutive expression of elevated levels of ALDH-3, GST, DT-D and UDP-GT in MCF-7/OAP cells is unknown. MCF-7/0 cells were cultured in the presence of gradually increasing concentrations of 4-hydroperoxycyclophosphamide for several months to generate the stable oxazaphosphorine-resistant subline termed MCF-7/OAP [18]. A mutation giving rise to some permanent change in the signaling pathway in which the ARE participates to regulate gene transcription would seem likely. This assertion is supported by the following findings: (1) 4-hydroperoxycyclophosphamide is a mutagen [22]; (2) ALDH-3, GST, DT-D and UDP-GT levels were elevated in MCF-7/OAP cells, whereas the level of cytochrome P450 1A1 was not; (3) enzymes coordinately induced by monofunctional inducers include ALDH-3, GST, DT-D and UDP-GT, but not CYP 1A1; (4) common to all but CYP 1A1 appears to be an ARE in the 5′-upstream regions of the genes that code for them [1, 26, 38]; and (5) AREs appear to be essential components of the signaling pathway by which monofunctional inducers such as catechol coordinately induce transcriptional activation (reviewed in reference 26).

Multienzyme-mediated multidrug resistance/collateral sensitivity would be to all anticancer drugs that are converted to pharmacologically less/more active metabolites by enzymes, the expression of which is coordinately elevated whether by exposure to bifunctional or monofunctional inducers or as a consequence of a relevant mutation. Indeed, the therapeutic/toxic potential of all drugs/chemicals that are converted to biologically less/more active metabolites by these enzymes would be altered upon their elevation. Thus, for example, elevated cellular levels of these enzymes may be of significance in carcinogenesis and cancer chemoprevention since they are known to catalyze the biotransformation (bioactivation in some cases and bioinactivation in others) of

(pro)carcinogens (reviewed in references 4, 23, 28, 52, 55 and 56].

Whether multienzyme-mediated multidrug resistance/collateral sensitivity of the type described here is of clinical significance is not known, but it is likely that it is for several reasons. First, simultaneous resistance to several structurally diverse anticancer drugs that effect their cytotoxication by different mechanisms and that are not known to be removed from cells by multidrug transporters is often encountered clinically [19, 25, 32]. Second, agents known to induce the relevant enzymes in model systems are abundant in the diet/environment; for example, commonly and frequently ingested dietary constituents such as coffee and broccoli as well as brussels sprouts and other members of the *Cruciferae* family of vegetables contain such inducers (reviewed in [7, 50]). Third, certain food additives, e.g. butylhydroxyanisole, and pharmaceuticals, e.g. oltipraz, are also known to act as inducers of these enzymes in model systems (reviewed in [3]). Fourth, induction of the relevant enzymes in model systems are induced by relatively low doses/concentrations of the inducing agent [48, 50, 51]. Fifth, there is evidence indicating that the induction of the relevant enzymes by bifunctional and monofunctional inducers occurs in humans; for example, salivary levels of ALDH-3, GSTs or DT-D are coordinately increased by drinking coffee and eating broccoli [50]. Finally, relatively large amounts of the relevant enzymes are sometimes found in primary and metastatic human cancer cells, e.g. breast cancer [2, 27, 31, 44]. Interestingly, cellular levels of these enzymes are, on average, apparently constitutively higher in a number of neoplastic tissues than cellular levels in corresponding normal tissues [2, 27, 31], suggesting that permanent upregulation of their expression often occurs during oncogenesis.

The clinical ramifications of multienzyme-mediated multidrug resistance/collateral sensitivity induced by pharmacological and/or dietary/environmental agents are potentially substantial, especially with regard to therapeutic strategies. For example, given that maximum induction of the relevant enzymes is achieved rapidly (only a few days after first introducing the inducer) and that enzyme levels return to basal levels rapidly (again, within a matter of a few days after the inducer is removed [45, 48, 50, 51]), certain anticancer drugs may be effective (ineffective) at one point in time, and following a relevant change in diet may be ineffective (effective) a few months or even weeks later, i.e. tumor sensitivity (resistance) to such drugs would (appear to) be transient. At least one pharmaceutical agent, oltipraz, is known to induce the relevant enzymes (reviewed in reference 3); it is likely that there are others. Thus, there is the potential for numerous, as yet unrecognized, favorable and unfavorable drug interactions. In some scenarios, deliberate induction of the relevant enzymes prior to chemotherapy could be of therapeutic benefit; in other scenarios, it would be desirable to keep cellular levels of these enzymes at a minimum.

Finally, since the therapeutic effectiveness of certain anticancer drugs would be affected by certain dietary constituents/other pharmaceutical agents, our findings indicate that both must be taken into consideration when using such drugs. In most cases, the choice of an appropriate diet/pharmaceutical agent would be secondary to the choice of an appropriate anticancer drug, but in certain scenarios the reverse could be the case. Considerations of this type may be especially critical when very high-dose chemotherapy followed by hematopoietic stem cell rescue is to be used, since the desire is to give the maximally tolerated dose and the margin of safety is small. Salivary levels of ALDH-3, GST and DT-D may reflect tissue levels of these and other relevant enzymes [50]. Quantification of the salivary enzymes prior to initiating chemotherapy may be of value with regard to optimization of the chemotherapeutic protocol.

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